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Regulatory B cells and transplantation: almost prime time?

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Abstract

Purpose of review—Regulatory B cells (Bregs) are potent inhibitors of the immune system with the capacity to suppress autoimmune and alloimmune responses. Murine transplant models showing that Bregs can promote allograft tolerance are now supported by clinical data showing that patients who develop operational tolerance have higher frequency of Bregs. Breg function has been widely studied resulting in improved understanding of their biology and effector mechanisms. However, our overall understanding of Bregs remains poor due the lack of specific marker, limited knowledge of how and where they act *in vivo*, and whether different Breg subpopulations exhibit different functions.

Recent findings—In this review we detail murine and human phenotypic markers used to identify Bregs, their induction, maintenance, and mechanisms of immune suppression. We highlight recent advances in the field including their use as biomarkers to predict allograft rejection, in-vitro expansion of Bregs, and the effects of commonly used immunosuppressive drugs on their induction and frequency.

Summary—Clinical data continue to emerge in support of Bregs playing an important role in preventing transplant rejection. Hence, it is necessary for the transplant field to better comprehend the mechanisms of Breg induction and approaches to preserve or even enhance their activity to improve long-term transplant outcomes.

Keywords

IL-10; phenotype; regulatory B cells; transplant tolerance

INTRODUCTION

Beyond their unique role in humoral immunity, B cells can impact the immune response through antigen presentation, costimulation, and production of an array of cytokines [1–9]. In this regard, B cells contain polarized subsets that exhibit either regulatory activity (Bregs) expressing anti-inflammatory cytokines such as interleukin (IL-10) and IL-35, and effector

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Conflicts of interest

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B cells (Beff) expressing pro-inflammatory cytokines such as IL-6, IL-12, IL-17, TNF-α, and IFNγ [7,10,11] Accordingly, Beff and Bregs profoundly influence the severity of autoimmunity, transplant rejection, and infection [1,10,11,12–17]. Although this review focuses on Bregs, the influence of B cells on the immune response is likely a summation of the opposing activity of both Bregs and Beff cells.

Bregs are potent regulators of the immune response in a variety of inflammatory settings [1,12–17]. Although initially identified in mice, evidence now suggests that Bregs also play a significant role in human disease and transplantation [18–27]. Bregs have been shown to modulate the effector function of both innate and adaptive immune responses [8,12,13,18,28–31]. Although their suppressive function is mainly attributed to their expression of IL-10, Bregs using various other cytokines and mechanisms have been described, including IL-35, Fas ligand, PD-L1, TGF-β, and granzyme B [8,32–35]. However, the relationship of such cells to the more well-studied IL-10^{+} Bregs is unclear. Despite significant advances in our understanding of Bregs, a number of fundamental questions remain that have hindered progress in the field. These include lack of a specific phenotype, a general lack of understanding of their development (distinct lineage vs. stochastic induction), how Bregs actually exert their effector function *in vivo*, and whether or not various ‘types’ of Bregs described in the literature are distinct or interrelated. These issues, along with the role of Bregs in transplantation will be the focus of this review.

THE PROBLEM WITH “REGULATORY B CELL PHENOTYPE”: FREQUENCY VS. QUANTITY

Currently, there are no phenotypic markers or transcription factors that specifically identify Bregs. IL-10 is expressed at very low frequency (~1%) in the overall B-cell population [1,12–14]. As a result, B-cell phenotypes that enrich for IL-10-expressing B cells have been used to elucidate Breg biology and function. For example, in mice, CD19^{+}CD1d+ B cells in the gut were initially shown to transfer IL-10-dependent inhibition of intestinal inflammation [1]. Subsequent studies showed that CD1d^{hi}CD5^{+} and CD21^{hi}CD23^{hi}CD24^{hi} [transitional 2-marginal zone precursor] B cells were enriched for IL-10 expression and again transferred IL-10-dependent amelioration of murine experimental autoimmune encephalomyelitis (EAE) and systemic lupus erythematosus (SLE) [14,31]. Overall, multiple B cell subsets and even plasma cells, have been shown to exhibit Breg activity in various models [8,10,11]. Importantly, while enriched, IL-10^{+} B cells still comprise a minority of cells (e.g., 15%) within each of these B-cell subsets. Moreover, the IL-10^{+} B cells in each of these small phenotypic subsets comprise only 10–20% of all IL-10^{+} B cells in secondary lymphoid organs [12]. Even though the frequency of IL-10^{+} expression is low in the remaining 80–90% of B cells, these make up the large majority of IL-10^{+} B cells. However, their frequency among such B cells is too low to demonstrate activity in B-cell transfer models. Unfortunately, IL-10 expression is usually only observed after *in vitro* stimulation of B cells [1,12–14], precluding transfer of IL-10^{+} cells. Thus, current Breg phenotypes actually represent the subsets most enriched for IL-10 expression in any given disease model, but they are neither specific nor necessarily representative of most IL-10^{+} B cells.

More recently, T cell immunoglobulin and mucin (TIM-1$^+$) and CD9$^+$ B cells were found to be more inclusive markers for Bregs [12,36]. Although still not specific (IL-10$^+$ B cells comprising only ~15–20% of TIM-1$^+$ or CD9$^+$ populations), each of these subsets encompass ~75–85% of all IL-10$^+$ B cells [12,36]. Notably, TIM-1 and CD9 have functional roles, as they have been shown to positively or negatively regulate IL-10 expression, respectively (see below) [12,36–39]. Both TIM-1$^+$ and CD9$^+$ populations contain IL-10$^+$ B cells belonging to each of the various canonical B-cell subsets including transitional 1 and 2, marginal zone (MZ), marginal zone precursor (MZP), follicular (FO), and plasma cells (PCs) [12,36]. The frequency of IL-10 expression varies significantly as does the size of each subset [8,12,29,36]. Our recent findings show that PCs, FO, and MZ B cells each contain 25–30% of IL-10$^+$ B cells [40]. It will be important to determine whether IL-10$^+$ B cells belonging to different subsets exhibit similar, or distinct functions.

As in murine models, the hallmark of human Bregs is their ability to secrete their ‘signature cytokine’ IL-10 [41]. Similar to murine Bregs, only a small proportion of B cells within each of these subpopulations actually express IL-10, and IL-10 is expressed by multiple B-cell subsets. Diverse phenotypes have been reported to enrich for IL-10$^+$ human B cells, including CD24^{hi}CD38^{hi} transitional [transitional B cells (TrB)], CD24^{hi}CD27$^+$ memory B cells, tumor necrosis factor receptor (TNFR2$^+$) B cells, CD25^{hi}CD71^{hi}CD73$^-$ B cells, TIM1$^+$ B cells, and CD27^{int}CD38^{hi} PCs, and all have been shown to suppress pro-inflammatory responses *in vitro* [18,27,29,42,43,44■]. Of note, there is a significant overlap among some of these subsets. For example, TNFR2$^+$ and TIM1$^+$ B cells are both enriched in the IgM$^+$ memory and TrB populations [43,44■]. Similar to mice, several major subsets (e.g., TrB, memory, and naïve) express IL-10 at relatively high frequency (10–15%) [21]. However, these same subsets also express TNFα, and the ratio of IL-10/TNFα was predictive of *in vitro* regulatory activity. However, even naïve B cells (low IL-10/TNFα) were suppressive *in vitro* when TNFα was neutralized. Finally, the IL-10/TNFα ratio falls with acute renal allograft rejection. These data highlight the limitations of current markers, demonstrate the importance of measuring cytokines rather than just phenotype, and suggest that Breg and Beff cells may both contribute to outcomes.

In summary, none of the current phenotypic markers for murine or human Breg populations are specific. They identify subpopulations enriched for IL-10 but not necessarily representative of the majority of IL-10$^+$ B cells that might also exhibit potent Breg activity. Moreover, even more poorly defined Beff cells, present in the same canonical B-cell subsets, may counteract the activity of Bregs and influence the outcomes observed [12,37]. Direct identification of IL-10 in B cells using reporter mice may allow us to better define the role of Bregs and their various “subsets.”

REGULATORY B CELL DEVELOPMENT, INDUCTION, AND REGULATION

It is unknown whether Bregs represent a distinct lineage of B cells similar to regulatory T cells (Tregs) or whether they are stochastically induced in response to localized activation signals. As noted above, Bregs cannot directly be identified by a specific surface marker or transcription factor and attempts to find a master regulator that controls their development using gene arrays have been unsuccessful [8,36]. However, IL-10$^+$ B cells can be detected in

neonatal spleen and found at every stage of B-cell development from pro-B cells to terminally differentiated PCs [6,8,12,36,45]. The fact that IL-10-expressing B cells are present at all B-cell developmental stages suggests that there may be a gene module that is specifically present in only a small fraction of B cells. The best evidence for a Breg lineage comes from studies showing that transfer of Pro-B cells isolated from Cytosine (phosphophodiester) Guanine (CpG)-treated bone marrow give rise to exquisitely potent progeny that exhibit different functions in different tissues to prevent EAE and type 1 diabetes in non obese diabetic mice [45,46]. Whether a lineage marker exists for these cells or whether this study represents a specific (and nonphysiological) case, remains unknown.

Bregs can be detected in naïve mice but expand substantially following autoimmunity, transplantation, or infection. This indicates that Bregs respond to inflammatory signals that lead to their induction and proliferation. Initial studies showed that naïve B cells cannot inhibit inflammatory responses and that Bregs are antigen specific, suggesting that B cell receptor (BCR) stimulation is required for Breg function [12,13,16]. Additionally, Breg development maybe dependent on antigen receptor diversity since BCR transgenic mice (expressing a single BCR) are deficient in IL-10^+ Bregs [6]. Beyond antigen recognition, CD19, a component of the BCR complex, also plays a pivotal role in IL-10 expression by B cells [14]. Absence of CD19 (defective BCR signaling) results in complete lack of IL-10+ Bregs, and conversely, CD19 overexpression leads to a significant increase in IL-10+ Bregs [14]. In line with these findings, B cells deficient in endoplasmic reticulum calcium sensors stromal interaction molecule (STIM)-1 and STIM-2 (found downstream of BCR signaling) are unable to express IL-10 [47]. Toll-like receptor (TLR) recognition of pathogen-associated molecular patterns (PAMPs) is also major contributor to Breg induction. TLR 2, 4, and 9 all have been shown to strongly induce IL-10+ Bregs [6,8,14,15,48–50]. Accordingly, MyD88-deficient B cells are unable to produce IL-10 in response to TLR ligands [15,49]. Therefore, antigen recognition through the BCR and TLR signaling both play vital roles in Breg induction.

Although Breg induction appears to be antigen-dependent, subsequent Breg expansion can be enhanced by several different ligands. CD40-CD40L signaling was shown to result in a major expansion of Bregs both *in vivo* and *in vitro* [16,48,51–53]. TIM-1, mentioned above, also positively regulates IL-10 expression by Bregs and treatment of immunized mice with an anti-TIM-1 antibody (RMT1–10) results in two-fold to four-fold expansion of Bregs and markedly prolongs allograft survival [12,37–39,54]. Notably, anti-TIM-1 treatment maintains TIM-1^+ B-cell expansion for a prolonged period of time [55]. Moreover, a loss-of-function TIM-1 mutation decreases both basal and induced Breg levels and promotes allograft rejection [38,39]. In addition, various cytokines induced in inflammatory settings, including IL-1β, IL-4, IL-6, IL-21, IL-35, and IFN-α can expand Bregs *in vitro* or *in vivo* [12,24,51,56,57]. Remarkably, in-vitro culture using IL-4 and IL-21 along with CD40L/B cell activating factor (BAFF)-expressing feeder cells was shown to expand murine Bregs up to a million-fold. Transfer of these ex-vivo expanded Bregs ameliorated EAE, proving that their regulatory activity was retained [51]. Similarly, TrB from healthy human donors can be expanded with IFN-α and CpG-C *ex vivo* and retain their IL-10 expression, phenotype, and *in vitro* Breg activity [24]. Importantly, this same approach was unsuccessful using B cells from SLE patients. In an in-vivo setting, Bregs may interact with other cell types that could

provide cytokines that promote their expansion, such as macrophages (IL-1-β and IL-6), T follicular helper cell (Tfh) (IL-21), B cells and Tregs (IL-35), and plasmacytoid dendritic cells (IFN-α) [24,51].

Other factors can negatively regulate Breg expansion. For example, CD9-deficient mice have almost double the number of IL-10$^+$ B cells, indicating an inhibitory effect on Breg expansion [36]. Similarly, CD22 negatively regulates BCR signaling and inhibits IL-10 expression [6]. In addition, Breg frequency is reduced in the presence of inflammatory cytokines such as IFNγ and TNF-α [21,37]. Though the mechanisms are unclear, this suggests a reciprocal relationship between Bregs and Beff.

In summary, major gaps remain in our understanding of the processes involved in Breg development, induction, and maintenance. These are critically important if we are to envision harnessing Bregs for therapeutic use. Accumulating evidence suggests that multiple intercellular interactions and soluble factors are involved. Importantly, following their initial induction, Breg expansion and persistence may be dependent on a balance of positive and negative factors that may differ from those required for their induction.

REGULATORY B CELL SUPPRESSIVE FUNCTION

Bregs appear to modulate the effector function of both adoptive and innate immune cells. The impact of Bregs on CD4 T-cell responses has been studied extensively. Numerous studies show that Bregs skew cytokine production from Th1 and Th17 responses toward Th2 and Treg responses [Forkhead Box P3 (Foxp3$^+$) or IL-10$^+$ Tr1] [13,27,28,31,37,56,58,59]. Breg-mediated regulation of CD4$^+$ T cells appears to be both antigen specific and require cognate Breg: T cell interactions [12,13,16,51,60]. We have shown that TIM-1$^+$ Bregs generated in response to a specific major histocompatibility complex (MHC) haplotype will not protect islet allografts from an unrelated ‘third-party’ MHC from rejection [12]. Intriguingly, in a rat heart transplant model, Durand *et al.* [60] showed that B cells isolated from long-term tolerant vs. rejecting rats could recognize distinct peptides from the β−1 region of MHC II. However, they did not demonstrate that peptide-specific B cells from tolerant animals were actually regulatory. Bregs can also inhibit CD8+ T-cell responses, but the studies are limited in nature and do not clearly establish whether regulation is antigen specific [30,61].

Exactly how IL-10$^+$ Bregs actually mediate antigen (Ag)-specific suppression of T cells remains unknown. Evidence suggests that Bregs secrete IL-10 in response to specific signals. For example, CD40 ligation results in cytoplasmic IL-10 accumulation but not IL-10 secretion. However, CD40 signaling in combination with lipopolysaccharide stimulation results in significant secretion of IL-10 *in vitro* [6,51]. Although not tested, the combination of antigen presentation by Bregs and provision of CD40L by T cells could lead to directed cytokine release during cognate B:T synapse formation [62]. In addition, numerous studies have shown that Bregs are involved in Treg expansion and maintenance [28,31,37,56,58,59]. Given that Tregs also act in an antigen-specific manner, Bregs could indirectly inhibit antigen-specific T-effector cells through Tregs.

Bregs also appear to inhibit the activity of various innate immune cells. Several studies have shown that in the presence of Bregs, DC maturation and IL-12 production are inhibited, reducing their ability to prime T cells [29,63]. Similarly, Bregs can inhibit cytokine production by macrophages, monocytes, and neutrophils including IFN-γ, TNF-α, and nitric oxide [15,17,18,61]. Furthermore, neutrophils and natural killer (NK) cells expand significantly more in response to *Salmonella* infection in the absence of IL-10-producing B cells [15].

Thus, taken at face value, Bregs can inhibit multiple arms of innate and adaptive immunity through both cognate and bystander mechanisms. However, it should be noted that in-vivo studies of Breg function involve Breg transfers or studies in B-cell cytokine-knockout mice, where it is difficult to establish which effects are direct vs. indirect. Thus, it is possible that T-cell inhibition is indirectly-mediated by inhibition of antigen presenting cells (APCs). In this scenario, antigen may be required for Breg activation, IL-10 expression, and homing. Once localized, activated Bregs could then suppress APCs via localized IL-10 secretion. The finding that MHC expression and CD40 expression are required for Breg activity could be explained if T:B interactions are required for normal Breg development, activation, or function *in vivo* [51]. Alternatively, inhibition of innate cells by Bregs could be mediated in part by suppression of T-cell inflammation. In the case of Tregs, the *in vivo* mechanism of action remained unclear until two-photon imaging showed that Ag-specific Tregs interact solely with DCs, which subsequently interact less frequently with conventional T cells [64,65].

As alluded to above, it is also possible that different Breg subsets localized in different niches(i.e., PCs in red pulp vs. FO B cells in follicles) could interact with different immune cells and utilize different immune-suppressive mechanisms. Indeed, PCs significantly downregulate their antigen presentation machinery, but regulatory PCs could primarily act by inhibiting surrounding macrophages and DCs in the red pulp. On the other hand, newly activated FO B cells migrate to the T:B cell border and make cognate interactions with T cells. Follicular Bregs might inhibit Tfh generation, germinal centers, and antibody responses [56,57,66,67]. Recently, transitional 2-MZP Bregs cells expressing high levels of CD1d were shown to present lipid antigen to invariant NK T cells and reduce disease severity in a murine arthritis model [68]. Additional, cellular interactions may also be vital for Bregs themselves. Schuetz *et al.* [69■] showed that Breg expansion and subsequent ability to promote allograft survival was dependent on the presence of NK cells.

Thus, Bregs appear to inhibit a variety of innate and adaptive effector cells. This could be because different Breg subsets act in different locations in the secondary lymphoid organs to regulate different arms of the immune response. Interestingly, Bregs may depend on specific cellular interactions with innate and adaptive immune cells to carry out their function, as they are dependent on NK cells for their expansion and Tregs to enhance their Ag-specific suppressive effect. This combination of attributes may imbue Bregs with potent immune regulatory capacity despite their small numbers.

REGULATORY B CELLS AND CLINICAL TRANSPLANTATION

Both indirect and direct evidence points toward an immunomodulatory role for Bregs in clinical transplantation. Various studies have shown an increase in TrBs and/or IL-10$^+$ B cells in peripheral blood of operationally tolerant renal transplant patients when compared to patients with stable function on immunosuppression or those with chronic rejection [19,70,71■,72]. A similar B cell “signature” was also noted in patients rendered tolerant via induction of mixed chimerism [73]. It is however important to note that in these studies, the number of Bregs in tolerant patients was comparable to healthy volunteers and subsequent studies have shown the differences seen in tolerant vs. stable patients may be because of immunosuppression [74,75]. Moreover, this ‘B-cell signature’ is specific to tolerant kidney transplant recipients alone [19,70–72]. However, B cells that secrete granzyme B and regulate T-cell responses *in vitro* were specifically increased in tolerant patients when compared with both patients on maintenance immunosuppression and healthy volunteers [22].

In the much more common nontolerant setting, Bregs appear to play a role inhibiting rejection. B-cell depletion in the peritransplant period can markedly increase acute renal allograft rejection and promote cardiac transplant vasculopathy [76,77■]. TrB, which are enriched for IL-10$^+$ Bregs, have been examined in the context of allograft survival. A higher number of TrB in peripheral blood was independently associated with protection from rejection (hazard ratio 0.6, 95% confidence interval 0.37–0.95, $P = 0.03$) and positively correlated with estimated glomerular filtration rate and superior graft survival in kidney transplantation [78]. This general conclusion has been supported by other studies [79–81]. In a small prospective longitudinal study, renal allograft rejection was associated with an increase in CD86$^+$ activated B cells and PCs and a significant reduction in TrBs and granzyme B-expressing B cells [82■]. However, in this study, the changes in B-cell compartment were not detected prior to rejection, limiting their value in predicting rejection. In this regard, our group showed that TrB were not only reduced in number but also exhibited a decreased ratio of IL-10:TNFα expression in patients with rejection when compared with stable patients or those with graft dysfunction but no rejection. Moreover, TrB from rejecting patients specifically lost their in-vitro regulatory activity. Importantly, at the time of the late for-cause biopsy the transitional B IL-10:TNFα ratio (but not IL-10 alone) could strongly predict the presence of rejection [receiver operating curve (ROC) area under the curve (AUC), 0.82, $P < 0.0001$] and importantly predicted subsequent doubling of serum creatinine or graft loss over a 3-year follow-up [21]. We have further shown that the ratio of transitional 1/transitional 2 B cells closely reflects the changes in the transitional B cell IL-10:TNFα ratio and might serve as a simpler marker of the Breg activity. Importantly, a low transitional 1/transitional 2 ratio in stable patients 2-years posttransplant was independently associated with and strongly predicted graft outcomes over a 5-year follow-up (ROC AUC >0.8, $P < 0.0005$), whereas clinical parameters including delayed graft function, creatinine, and donor-specific antibody were not predictive (ROC AUC range 0.56–0.66) [83■]. In our studies, the ratio of IL-10/TNFα is a far better predictor of renal allograft survival than IL-10 alone – again implicating the balance between Bregs and Beff.

TrBs constitute a majority of B cells in the cord blood and are enriched for IL-10^+ B cells that suppress *in vitro* T cell-proliferation and proinflammatory cytokine expression. Importantly, in patients who received cord blood stem cell transplants, development of GVHD was associated with a sharp decline in IL-10^+ Bregs among the reconstituting B cells and such B cells lose their ability to suppress allogenic T cells *in vitro* [84]. Taken together, these studies suggest that Bregs may help establish an important "immunological set-point." Moreover, they might potentially serve as strong biomarkers that can aid clinical decisions in transplantation.

EFFECTS OF THERAPEUTIC AGENTS ON REGULATORY B CELLS

Given the importance of Bregs in promoting allograft tolerance, we must also consider the impact of various routinely used drugs used to treat transplant patients on Breg development, function, and ultimate ability to promote allograft survival. As such, both immunosuppressive and immune-tolerizing agents may have specific effects on Bregs. A variety of immunosuppressive drugs initially developed to target T-cell signaling/growth, in fact, also inhibit B cells and potentially alter the number of Bregs. For example, studies have shown that many commonly used therapeutic agents including immunosuppressives such as cyclosporine, tacrolimus, prednisolone, azathioprine, and mycophenolate mofetil appear to reduce Breg numbers [74,85,86] (Table 1). Although, belatacept therapy is associated with a persistent elevation in the number of TrB along with a suppression of BAFF levels in the peripheral blood of renal transplant patients, the cytokine expression profile of these cells was not studied [87]. In this regard, a recently published study demonstrated that addition of belimumab to an immunosupression regimen comprised of basiliximab induction and maintenance therapy with tacrolimus, mycophenolate and prednisolone, led to a significant increase in the ratio of B cell IL-10/IL-6 in renal transplant patients for the first 3 months post-transplant. However, the clinical implications of this finding remain to be studied [88■■]. As noted above, a recent analysis of operationally tolerant vs. stable kidney transplant recipients reported that TrB number in the peripheral blood of stable renal transplant recipients was decreased in those treated with either prednisolone or azathioprine and conversely, withdrawal of steroids was associated with a significant increase in their number [74].

A number of experimental tolerogenic agents in mice including anti-TIM-1, anti-TIM-4 and anti-CD45 all require B cells for tolerance induction *in vivo* [12,37,54]. Although only anti-TIM-1 has been conclusively shown to directly induce Bregs, these data again suggest that Bregs may help establish an immunological set-point, and their presence is important for tolerance.

Given evidence for their salutary role in murine and human transplantation, expansion of Bregs would be desirable. Studies noted above demonstrate that expansion of both human and murine Bregs is possible *in vitro*. Anti-TIM-1 and apoptotic cells promote tolerance by *in vivo* expansion of TIM-1^+ Bregs in murine transplant and autoimmune models [39,59]. Interestingly, mammalian target of rapamycin (mTOR) inhibitors have been shown to increase IL-10^+ B cells *in vitro* and in fact may require Bregs to expand Foxp3^{++} Tregs [89]. In human study participants, a variety of agents in including Interferon-β, fingolimod,

liquinimod, tocilizumab, and infliximab that are used to treat autoimmune disorders like multiple sclerosis and rheumatoid arthritis, have been shown to enhance Breg numbers and activity [90–97]. Additionally, other agents such as vitamin D, retinoic acid, and sotrastaurin can induce ex-vivo B-cell proliferation, and thus, IL-10+ Breg expansion might contribute to their therapeutic efficacy [85,98,99]. Many such drugs have not been extensively used in the transplant arena.

CONCLUSION

Bregs have a profound influence on autoimmune, alloimmune, and antimicrobial immune responses. Their ability to inhibit both innate and adaptive immune response while expanding Tregs makes them powerful immune regulators. Accumulating evidence suggests that increased Breg frequency correlates with reduced rejection episodes and long-term allograft survival. Therefore, strategies that specifically aim to expand Bregs *in vivo* or *ex vivo* represent a promising therapeutic means to improve transplant outcomes. However, our understanding of Breg biology remains poor because of the lack of a specific marker that would allow us to specifically isolate them and better understand their development, induction, and in-vivo effector function. The use of current (nonspecific) phenotypic markers to identify Bregs is unlikely to prove acceptable for cellular therapy as only a minority of the cells within these B cells populations actually produce IL-10. Hence, an immediate focus in the Breg field should center on identifying more specific transcriptional and surface markers that can facilitate direct identification. Alternatively, protocols that can expand B cells (*in vivo* or *in vitro*) that are highly enriched for IL-10 or other anti-inflammatory cytokines, while expressing low levels of pro-inflammatory cytokines might suffice. Finally, Bregs (defined by their cytokine expression) may serve as a highly predictive biomarker for subsequent clinical course allowing one to identify high and low-risk patients for preemptive changes in immunosuppression.

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■ of special interest

■■ of outstanding interest

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KEY POINTS

- Bregs are identified by the expression of their “signature cytokine” – IL-10.
- TIM-1 and CD9 are the most inclusive phenotypic markers for Bregs in mice.
- Relative expression of IL-10 to TNF-α is a strong marker for human B regulatory activity.
- Immunosuppressive agents can affect the number of Bregs, and therefore must be taken into account when examining Bregs as biomarkers for graft survivalor tolerance.
- Novel immunosuppressive agents able to potentiate Bregs might have therapeutic potential in clinical transplantation.

Table 1.

Reported effects of various potential therapeutic agents on regulatory B cells

Drug/molecule	Potential effect on Bregs	Reference(s)
Vitamin D	Stimulation of B cell IL-10 secretion *in vitro*	[98]
Retinoic acid	Proliferation and differentiation of $CD24^{hi}CD38^{hi}$ TrB mediated by retinoic acid from tolerogenic DCs	[99]
Sotrastaurin	Increased CD86 expression on B cells and increased B cell IL-10 secretion	[85]
Interferon-β	Increase absolute number of IL-10^{+} $CD24^{hi}CD38^{hi}$ TrB	[21]
Liquinimod	Increases B cell IL-10, CD25, CD86 expression	[91]
Fingolimod	Increase frequency of IL-10 $CD24^{hi}CD38^{hi}$ TrB	[92]
Tocilizumab	Increases TGF-β and CD25 expression on B cells	[93,94]
Infliximab and other TNF-α blockade agents	Increase B cell IL-10, increased $CD24^{hi}CD38^{hi}$ TrB while suppressing memory B cells and plasmablasts	[95■,96,97]
Mycophenolate mofetil	Dose-dependent reduction in B cell IL-10 and decreased expression of CD80 and CD86	[85,86]
Sirolimus (mTOR inhibitors)	Reduced $CD24^{hi}CD38^{hi}$ TrB and increase in $CD27^{+}$ memory B cells. Feedback between Breg and Treg that stabilizes both populations	[79,89,100–102]
Calcineurin inhibitors	No effect or reduced frequency of $CD24^{hi}CD38^{hi}$ TrB	[79,100]
Alemtuzumab	Increased number of $CD24^{hi}CD38^{hi}$ TrB, prolonged suppression of $CD27^{+}$ memory B cells	[81,103]
Azathioprine	Reduced number of $CD24^{hi}CD38^{hi}$ TrB	[74]
Prednisolone	Reduced number of $CD24^{hi}CD38^{hi}$ TrB	[74]

Bregs, regulatory B cells; mTOR, mammalian target of rapamycin; TrB, transitional B cells; Treg, regulatory T cells.

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