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REVIEW ARTICLE

# Regulatory B cells in transplantation: roadmaps to clinic

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## SUMMARY

Over the last two decades, an additional and important role for B cells has been established in immune regulation. Preclinical studies demonstrate that regulatory B cells (Breg) can prolong allograft survival in animal models and induce regulatory T cells. Operationally tolerant human kidney transplant recipients demonstrate B-cell-associated gene signatures of immune tolerance, and novel therapeutic agents can induce Bregs in phase I clinical trials in transplantation. Our rapidly expanding appreciation of this novel B-cell subtype has made the road to clinical application a reality. Here, we outline several translational pathways by which Bregs could soon be introduced to the transplant clinic.

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**Key words**
B cells, immunosuppression, immunosuppression experimental, macrophages, novel immunosuppressants, T cells, tolerance induction, tolerance strategies and mechanisms

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## Introduction

Several cellular therapies have now been introduced in clinical trials of human kidney transplant recipients. The landmark ONE study explored the protective role of several regulatory cell types including regulatory T cells (Tregs), T regulatory type 1 cells (Tr1), dendritic cells (DC) and regulatory macrophages in transplantation [1,2]. Over the last decade, evidence has emerged of an additional and novel regulatory cell type, regulatory B cells (Bregs), in transplantation. Adoptive transfer studies have demonstrated the protective capacity of mouse Bregs in prolonging allograft survival [3,4]. Operationally tolerant, human kidney transplant recipients exhibit increased frequencies of circulating Breg populations, and B-cell signatures have been shown to be sensitive predictors of a tolerogenic state [5,6]. Multiple Breg populations at several stages of B-cell maturation have been identified, with a range of phenotypes and suppressive mechanisms. Bregs can suppress Th1 and Th17 responses and DCs, whilst promoting regulatory populations such as Tr1 cells and Treg. These subsets and their mechanistic functions have been extensively reviewed elsewhere, and include mouse marginal zone (MZ), transitional-2 marginal zone precursor (T2 MZP) and B10 Bregs, as well as human $CD24^{hi}CD38^{hi}$, CD24hiCD27$^{+}$ and TIM-1$^{+}$ Bregs (Table 1) [7,8]. Whilst much of the initial experimental work to characterize Breg has been in the context of autoimmunity, most Breg populations identified in transplantation are characterized by their ability

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**Table 1.** IL-10$^{+}$ Breg

| Surface markers | Organism | Suppressive mechanism | Suppressive targets | Disease process | Activation protocol | Ref |
|---|---|---|---|---|---|---|
| CD21$^{hi}$CD23$^{hi}$ CD24$^{hi}$IgM$^{hi}$ IgD$^{hi}$CD1d$^{hi}$ ('T2-MZP cells') | Mouse | IL-10 | $T_H17$, $T_H1$, Treg | Autoimmunity: CIA, lupus<br>Allergy: Allergic airway inflammation | Collagen, PMA, iono; anti-CD40; *Schistosoma Mansoni* infection | [40,100,101] |
| CD21$^{hi}$CD23$^{-}$CD24$^{hi}$IgM$^{hi}$ IgD$^{lo}$CD1d$^{hi}$ ('MZ cells') | Mouse | IL-10 | CD4$^{+}$ T cells, CD8$^{+}$ T cells, Tr1 | Autoimmunity: CIA<br>Infection: *Leishmania Donavani* | AC; Anti-IgM & anti-CD21-coated beads; *Leishmania Donavani* infection | [102,103] |
| CD24$^{hi}$CD38$^{hi}$ | Human | IL-10, cell contact, TGF-b | $T_H1$, Treg, $T_H17$, pDC | Transplant: Lung, kidney, cGvHD<br>Autoimmunity: SLE, RA<br>Cancer: Gastric<br>Healthy | PIB, CD40L; CpG, pDC; CpG, LPS, iono, PMA; CD154, PMA, iono; IL-10; Tumour environment; CpG, CD40L; CD40L, CpG, BCR ligation; | [23,29,31-33, 43,54,104-108] |
| CD24$^{hi}$CD27$^{+}$ | Human | IL-10, PD-L1, TGF-b, GrB, IL-6, TNF-a | Monocytes, $T_H1$ | Transplant: Liver, kidney, cGvHD<br>Autoimmunity: SLE, RA, PSS, AVSD, MS, | CpG, PIB; CpG, CD40L; CpG, anti-BCR, CD40L; CpG, CD40L, PIB; | [26,28,30,53,109] |
| CD73$^{-}$CD25$^{+}$ CD71$^{+}$ | Human | IL-10 | CD4$^{+}$ T cells | Allergy: Bee venom allergy<br>Healthy | CpG; PMA, iono | [68,110] |
| CD5$^{+}$ | Human<br>Mouse | IL-10, TGF-b, IL-35 | BMMCs, $T_H1$, $T_H17$, Treg | Autoimmunity: EAU<br>Allergy: IgE-mediated allergic response, Late Eczematous Allergic Reactions to Cow's Milk, AAD, Casein-induced allergic response | IL-35, LPS; OVA; BSA, CD40L; Casein | [65,111-114] |
| CD1d$^{hi}$CD5$^{+}$ ('B10 cells') | Human<br>Mouse | IL-10, IL35 | $T_H17$, $T_H1$, Tfh, Treg | Transplant: Islet<br>Autoimmunity: EAU, EAE, SLE<br>Infection: *Chlamydia muridarum*<br>Allergy: CHS<br>Healthy | Anti-CD45RB/Anti-TIM-1; BAFF; IL-21; LPS, PMA, iono; IRBP, anti-CD40 or PMA, iono; *Chlamydia muridarum;* LPS, PIB, IL-21; | [41,44,115-120] |
| CD138$^{+}$<br>CD138$^{hi}$TACI$^{+}$<br>CXCR4$^{+}$CD1d$^{int}$Tim1$^{int}$ | Human<br>Mouse | GrB, cell contact, IL-35, IL-10 | $T_H1$, $T_H17$, Macrophage, Neutrophil, NK cells | Transplant: Kidney<br>Autoimmunity: EAE, EAU<br>Infection: *Salmonella typhimurium* | CD40L, CpG; TLR-4, CD40L; PMA, iono; IL-2, IL-6, CpG, IFN-a or β; *Salmonella typhimurium;* | [5,57,66,74] |
| TIM-1$^{+}$ | Human<br>Mouse | IL-10, IL-4, TGF-b | $T_H1$, $T_H17$, Tr1, Treg | Transplant: Islet, skin, heart<br>Autoimmunity: EAE, SSc, GD, HT<br>Healthy | Anti-TIM-1 mAB (RMT1-10); AC, anti-IgM, anti-CD40, IL-21; CpG, PMA, iono, anti-IgG and IgM; CD45RB/Anti-TIM-1; PS; TG, CpG, PMA, iono | [4,38,51,60,90, 121-123] |

**Table 1.** Continued.

| Surface markers | Organism | Suppressive mechanism | Suppressive targets | Disease process | Activation protocol | Ref |
|---|---|---|---|---|---|---|
| $CD11b^+$ | Human Mouse | IL-10; CD86 | $CD4^+$ T cells, Treg | Allergy: CHS Healthy | OVA; PMA, iono | [124-126] |

Columns from left to right include the Breg phenotype, the organism in which they were identified, the proposed target of suppression, the disease model (where applicable) from which they were obtained, and Breg activation protocols.

AAD, allergic airway disease; AC, apoptotic cell; AVSD, autoimmune vesiculobullous skin disease; BAFF, B-cell-activating factor; BCR, B-cell receptor; BMMC, bone-marrow-derived mast cells; BSA, bovine serum albumin; cGVHD, chronic graft-versus-host disease; CHS, contact hypersensitivity; CIA, collagen-induced arthritis; CpG, CpG DNA; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; GD, Graves' disease; GrB, granzyme B; HT, Hashimoto's thyroiditis; IFN, interferon; Ig, immunoglobulin; iono, ionomycin; IRBP, interphotoreceptor retinoid-binding protein; LPS, lipopolysaccharide; MS, multiple sclerosis; MZ, marginal zone; NK, natural killer; OVA, ovalbumin; pDC, plasmacytoid dendritic cell; PD-L1, programmed death ligand 1; PIB, phorbol 12-myristate 13-acetate, ionomycin and brefeldin A; PIM, phorbol 12-myristate 13-acetate, ionomycin and monensin; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidyl serine; PSS, primary Sjögren syndrome; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; TG, thyroglobulin; TGF-b, transforming growth factor beta; TIM-1, T-cell immunoglobulin and mucin domain 1; TLR, Toll-like receptor; Tfh, T follicular helper cell; and T2-MZP, transitional 2-marginal zone precursor.

to produce IL-10, and IL-10 itself can be induced in Bregs and their precursors by multiple stimuli (Table 1). In this review, we discuss recent advances in $IL\text{-}10^+$ Bregs and explore how this cell population may be harnessed therapeutically in clinical transplantation.

## Immune monitoring

The majority of transplant recipients require the use of potent immunosuppressants to prevent acute allograft rejection. However, not all recipients who take immunosuppression will need such high dosage regimens. In renal transplantation, patient weight determines calcineurin inhibitor (CNI) and sirolimus doses, whilst azathioprine and mycophenolate doses are dictated largely by population-based data [9]. Many patients may be over-immunosuppressed, accounting for increased mortality from cardiovascular complications, cancer or infection in patients with functioning grafts. However, chronic rejection remains a leading cause of long-term graft loss, demonstrating the importance of adequate immunosuppression over time.

To date, there is no robust definition of immunological tolerance in humans; descriptions of 'clinically operational tolerance' rely on the stability of graft function in the absence of immunosuppression for a minimum period. Accurate identification of such recipients will be critical to guide the tailored reduction of immunosuppression and its associated co-morbidity in this cohort. Whilst blood may not necessarily be the best compartment by which to measure clinical pathology across all organ types in transplantation, disease gene profiles showed good concordance between blood and solid tissues [10]. Evidence is now emerging of potential blood-based biomarkers of operational tolerance and predictors of rejection that focus upon Bregs. These may offer personalized regimens for patients who do not require as potent a regimen as the current standard, as well as for patients who are at risk of developing chronic rejection and losing their grafts (Fig. 1).

### B-cell-related tolerance signatures

The importance of Bregs in transplantation was highlighted by the identification of B-cell signatures of tolerance in human kidney transplant recipients in the European Indices of Tolerance (IoT) and American Immune Tolerance Network (ITN) studies [11,12]. Tolerant kidney transplant recipients ('TOL'), who were defined by stable graft function with no immunosuppression for over 1 year, were compared to control cohorts

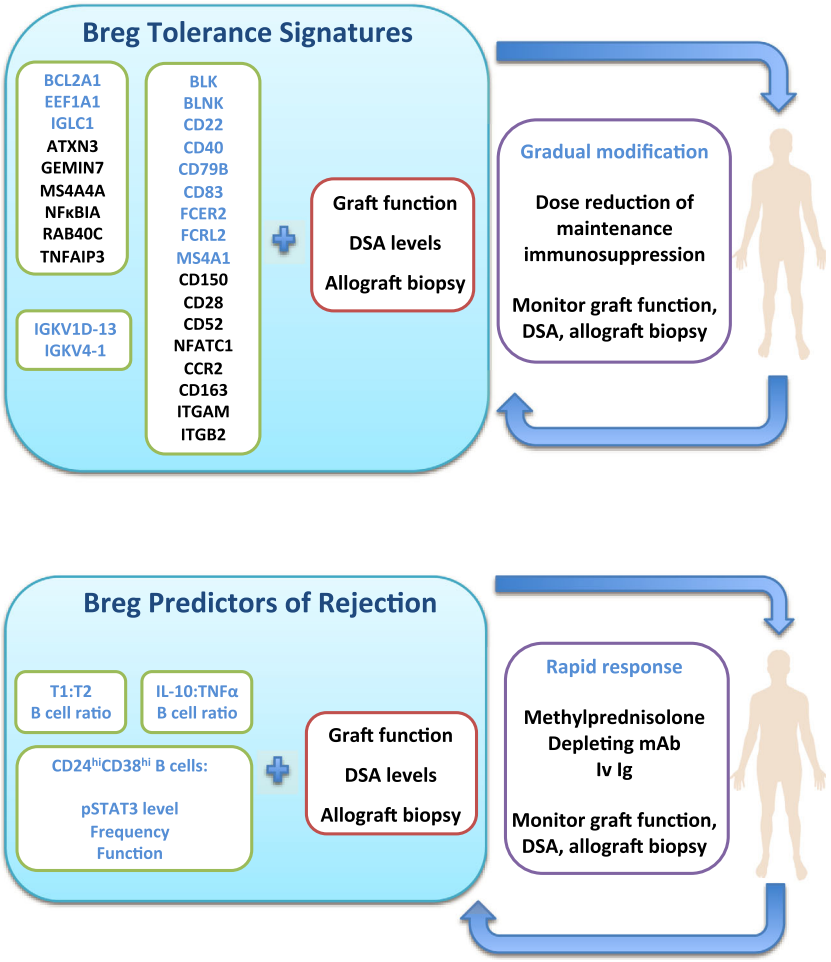


**Figure 1** Breg-focused immune monitoring. Breg signatures can be used in transplantation to improve graft and patient survival. Tolerance signatures have been identified and validated by consortia including the ITN [6,18] and IoT [17]. Meta-analyses of tolerance studies also describe a B-cell-specific signature [20]. Drug minimization trials could be implemented in patients with predicted tolerance, alongside careful and long-term monitoring of graft function, DSA levels and protocol biopsies. Bregs have also been identified in rejection predictors [28,29]. The tolerance signature genes and rejection tools in blue denote those associated with B cells and/or Bregs. Clinical utilization of such tools may pre-empt rejection and facilitate accelerated anti-rejection treatment to improve graft survival and function within the context of dedicated trials.

including recipients with stable graft function who were receiving immunosuppression ('SI'). Tolerant kidney transplant recipients demonstrated increased frequencies of naive $CD19^{+}$ B cells and $CD24^{hi}CD38^{hi}IL\text{-}10^{+}$ B cells in peripheral blood, and reduced donor-specific antibody (DSA) levels when compared to control groups other than healthy subjects, in whom levels were equivalent. Gene microarray analysis revealed a bias towards differential expression of B-cell-related genes. The ITN study identified three B-cell-related genes, *IGKV4-1*, *IGLL1* and *IGKV1D-13,* that were predictive of tolerance. These observations resulted in specific and sensitive cross-platform signatures that could predict tolerance in human kidney transplant recipients. Other groups described similar enrichments of $Il\text{-}10^{+}$ Breg in tolerant kidney transplant recipients, as well as a decrease in differentiated plasma cells when compared to SI cohorts [13,14].

Critically however, tolerance signatures in these studies were compared to those of patients still taking immunosuppression, and so may be confounded by effects of immunosuppressants upon circulating B cells. Moreover, these B-cell signatures were similar to those described in healthy individuals, so may instead represent a signature of health, rather than of tolerance.

The IoT authors repeated their analysis in three independent kidney transplant recipient cohorts [15]: the original IoT data set; CNI-free data set; and data set of kidney transplant recipients being weaned off steroids for clinical reasons. Using this approach, the authors established that previous tolerance signatures were biased by immunosuppression. Prednisolone affected the IoT gene data set [12], whilst prednisolone and azathioprine adversely affected the frequency of $CD24^{hi}CD38^{hi}$ B cells. These findings were reinforced by work within our group, which described a decrease in transitional and naive B-cell numbers in association with azathioprine, within peripheral blood of kidney transplant recipients [16].

However, the IoT re-analysis revealed a novel 9-gene set that was highly predictive of tolerance and which distinguished operationally tolerant kidney transplant recipients from SI recipients [17]. When applied to 173 SI kidney transplant recipients, this new gene set predicted that almost 12% were 'probably tolerant'. The signature importantly discriminated tolerant patients from healthy

controls and B-cell-associated pathways were still prominently represented in 3 of the 9-gene set: BCL2A1, EEF1A1 and IGLC1. The ITN also addressed the immunosuppression question and found that expression of 2 of the 3-gene ITN B-cell tolerance signature [11] increased over time in SI kidney transplant recipients, despite largely unchanged immunosuppression [18]. 14% of 124 recipients on varying immunosuppressive regimens consistently displayed the tolerance signature of IGKV1D-13 and IGKV4-1 over 2 years at multiple timepoints [6]. Despite increased frequencies of all circulating B-cell subsets in peripheral blood, these patients demonstrated a clinically significant improvement in renal function that increased over time, and persistently lower DSA levels when compared to patients who did not express this signature. It seems unlikely that immunosuppressive drugs prevented development of a B-cell tolerance signature; moreover, the absence of immunosuppression was not a prerequisite for developing a B-cell-correlated tolerogenic state. Notably, predicted tolerance was greatest in patients receiving a CNI-based maintenance immunosuppressive regimen and reduced in those receiving corticosteroids, mycophenolate or thymoglobulin induction. Whether these associations reflect direct effects of immunosuppressants on B cells and are independent of a tolerogenic effect of Breg, or whether they enable B-cell changes that promote a reduction in the overall alloimmune response, remains to be determined.

Interestingly, a comparison of tolerant liver transplant recipients to those receiving immunosuppression such as CNIs and azathioprine failed to demonstrate the changes in B-cell subsets and B-cell-related genes that characterized spontaneous tolerance in kidney transplantation, but which have been attributed to immunosuppression [19]. Moreover, the IoT and ITN signatures demonstrate little overlap, and other immune compartments may warrant inclusion as demonstrated by a meta-analysis of tolerance studies which identified a gene signature involving proliferation of B and $CD4^+$ T cells and the inhibition of $CD14^+$ monocyte-related functions [20]. These observations highlight that whilst B-cell signatures of tolerance identified in renal transplantation may not be wholly attributed to confounding immunosuppression, there are likely alternate tolerance mechanisms in different allograft settings.

Two studies have attempted to wean CNIs in patients predicted to be at a low risk of rejection on the basis of clinical characteristics (absence of DSA, stable graft function, biopsies without evidence of inflammation). Both were stopped prematurely due to high rates of rejection and/or the formation of DSA following attempted drug withdrawal [21,22], highlighting current challenges of immunosuppressive drug minimization in kidney transplant recipients with stable function. B-cell-predominant signatures may provide a standardized tool towards personalized medicine for the monitoring of tolerant or low-risk recipients. We are close approaching the time for immunosuppression-minimizing clinical trials based on signatures that include not just clinical parameters, but also immunology-based criteria, alongside careful patient selection and stringent monitoring.

## Predictors of rejection

The discovery of Breg association with tolerance signatures has been complemented by the emergence of B cells as potential predictors of rejection in renal transplantation. Peripheral blood analysis in a tolerant kidney transplant recipients cohort revealed an increase in memory B cells with inhibitory characteristics when compared to recipients undergoing chronic rejection. B cells in the tolerant cohort exhibited a decreased FcγRIIA/FcγRIIB ratio and an increase in BANK1 expression, which serves as a preventative signal of hyperactive B-cell responses [14]. Silva et al. found that operationally tolerant kidney transplant recipients had a diverse BCR repertoire similar to that of healthy controls, unlike recipients undergoing rejection, thus mirroring observations in mouse models [23-25]. Operationally tolerant patients retained the ability to activate CD40 and signal transducer and activator of transcription 3 (STAT3) signalling pathways in the $CD24^{hi}CD38^{hi}$ B-cell subset enriched for human Breg. This ability was decreased in $CD24^{hi}CD38^{hi}$ B cells of patients undergoing chronic rejection or GVHD [23,26].

When appraising such studies however, it is important to note that mechanisms that maintain tolerance are not necessarily the same as those that predict tolerance development. Moreover, the lack of such a signature does not necessarily predict rejection. Studies that follow patient outcomes over time may be more informative. Clatworthy et al. uncovered the involvement of human Bregs in the prevention of acute rejection in transplantation [27]. In this study, the investigators suspended a randomized controlled trial of anti-CD20 mAb rituximab induction therapy versus anti-CD25 mAb daclizumab after recruitment of 13 human kidney transplant recipients: a high incidence of acute cellular rejection occurred in the rituximab group (83% vs. 35% in the daclizumab group). The potential role of Bregs in the prevention of allograft rejection has since been reiterated in renal, liver and lung transplantation [28-32], with reduced frequencies of

circulating IL-10$^+$ Breg-enriched CD24$^{hi}$CD38$^{hi}$ and/or CD24$^{hi}$CD27$^+$ B-cell subsets found in recipients who developed rejection. Similarly, graft-versus-host disease (GvHD) was associated with a decline in frequency and suppressive function of CD24$^{hi}$CD27$^+$IL-10$^+$ Breg in stem cell recipients [33].

Following on from this work, Cherukuri and colleagues found that kidney transplant recipients who underwent rejection demonstrated a decreased IL-10:TNFα ratio expressed by CD24$^{hi}$CD38$^{hi}$ B cells, irrespective of steroid use [28]. CD24$^{hi}$CD38$^{hi}$ B cells from rejecting recipients were functionally deficient *in vitro* when compared to those of control cohorts. Interestingly, the function and frequency of Treg were unchanged across all patient groups. At the time of the late for-cause biopsy, the CD24$^{hi}$CD38$^{hi}$ B-cell IL-10 to TNFα ratio strongly predicted rejection, graft loss and the doubling of serum creatinine over a 3-year follow-up period, highlighting the clinical relevance of such findings. Using the definition of more naïve, CD24$^{hi}$CD38$^{hi}$ B cells as 'T1', and more mature CD24$^{int}$CD38$^{int}$ B cells as 'T2' B cells, the authors identified that the T1/T2 ratio reflected the changes associated with IL-10:TNFα ratio [29]. Low T1/T2 ratios in stable kidney transplant recipients who were 2 years post-transplant were independently associated with and strongly predicted graft outcomes over a 5-year follow-up period in an independent validation set, whereas clinical parameters including delayed graft function, eGFR and DSA levels were not predictive.

The interplay of different B-cell effector and regulatory subsets in chronic rejection beyond DSA production has been elegantly characterized in recent years. In an animal model of heart transplantation, AID/μS knockout mice in which B cells cannot undergo affinity maturation or secrete antibody, underwent chronic allograft vasculopathy (CAV) comparable to that of wild-type mice [34]. However, μMT mice which were wholly deficient in both B cells and antibody were protected from CAV. The use of chimeric mice, in which B cells were present but were unable to present antigen, demonstrated that both alloreactive T-cell responses and CAV were significantly reduced in the absence of B-cell-mediated antigen presentation. CAV was restored following adoptive transfer of polyclonal AID/μS KO B cells. These findings indicate that chronic rejection could occur in the complete absence of antibodies by mechanisms involving B-cell-mediated antigen presentation. This may account for why some patients develop chronic rejection in the absence of detectable alloantibodies.

Work by Shiu and colleagues has further defined the complex interactions of B effector and regulatory subsets in the context of chronic rejection in human renal transplantation [35,36]. The examination of peripheral blood from 65 patients with biopsy-proven AMR, non-immune-related graft dysfunction or stable graft function revealed that approximately half of the biopsy-proven AMR samples did not demonstrate a CD4$^+$ anti-donor IFNγ response unless either CD25$^+$ Treg or CD19$^+$ B cells were depleted. Notably, the depleted CD19 + B cells in these samples were polarized to IL-10 production following polyclonal stimulation with IgM and IgG. In addition, following depletion of Treg or CD19$^+$ B cells, alloresponsiveness in patients with no histological signs of immune-mediated graft dysfunction was restored *in vitro*. Subsequent interrogation of the peripheral blood of renal transplant patients with AMR revealed the association of suppressed CD4$^+$ anti-donor IFNγ production with a sustained improvement in eGFR following the optimization of immunosuppression, rather than anti-CD19 mAb rituximab administration [37]. Those patients whose graft function improved upon immunosuppression optimization demonstrated an increased proportion of T1 B cells in peripheral blood when compared to T2 B cells, and CD19$^+$ B-cell-dependent suppression of CD4$^+$ anti-donor IFNγ production. Conversely, following rituximab administration, there was a reduction in the proportion of donor-reactive responses suppressed by CD19$^+$ B cells, whilst the proportion of samples showing unregulated B-cell-dependent IFNγ production increased. B-cell-mediated suppression of IFNγ production, the associated increase in T1 B cells and slower declines in graft function were abolished by rituximab, which depleted transitional B cells for prolonged periods. Rituximab appeared to disturb the balance of the two opposing roles of B cells, by selectively reducing the relative proportion of transitional regulatory B cells, whilst failing to sustainably deplete B effector cells that support anti-donor responses by antigen presentation.

Thus, simple biomarkers based upon Breg and B-cell effector composition and function may be used in a carefully selected group of transplant recipients to monitor and pre-empt allograft dysfunction and rejection to improve both short- and long-term outcomes.

## Cellular therapy

The use of Bregs as a cellular therapy will require the identification of a stable Breg population with unique phenotypic markers to allow their precise isolation. It remains unclear if the various methods of Breg *in vivo*



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or *in vitro* induction result in a distinct Breg lineage controlled by a specific set of transcription factors, or indeed whether different Breg populations are generated depending on the mode of stimulation and immunological microenvironment. Since IL-10$^{+}$ B cells have been identified at all developmental stages of B cells, but only a fraction of each subset will express IL-10 upon appropriate stimulation, this suggests that a specific gene module within Breg precursors may exist [7]. The uncertainty that surrounds Breg identity and the lack of easily identifiable phenotypic markers beyond IL-10 may continue to hamper the progress of Breg cell therapy. Alternatively, protocols that could *ex vivo* expand B cells that are highly enriched for the expression of IL-10 and phenotypic markers associated with regulatory function, such as TIM-1, CD24 and CD38 [38], might suffice. To embark on such a strategy, the experimental approaches available that could be used clinically to generate human Bregs must be considered.

## Adaptive signalling

As early as 2003, the Londei laboratory demonstrated the induction of mouse IL-10$^{+}$ Bregs by stimulation of the CD154-CD40 pathway [39]. *In vitro* stimulation with an agonistic anti-CD40 monoclonal antibody (mAb) together with antigen (type II collagen) generated B cells capable of inhibiting Th1 differentiation, preventing and ameliorating collagen-induced arthritis (CIA) [39]. Short-term co-culture of agonistic CD40 mAb with mouse splenic B cells generated IL-10-producing Breg with a transitional 2 (T2) phenotype; this B-cell subset could reverse autoimmunity *in vivo* upon injection and suppress Th1 responses in an IL-10-dependent manner [40]. In a study of mouse Breg expansion, the co-culture of mouse splenic B cells with NIH3T3 cells that expressed CD154 and BAFF, together with IL-4 and IL-21, resulted in greater than 4x10$^{6}$ fold expansion of mouse B10 cells *ex vivo*, that were then able to inhibit autoimmune disease *in vivo,* in a model of experimental autoimmune encephalitis (EAE) [41]. Several studies have gone on to exploit this interaction to induce and expand human Bregs. Lemoine and Blair stimulated human B cells with membrane-bound CD154 to induce IL-10 production and Breg-mediated suppression *in vitro* [42,43]. The involvement of the CD154-CD40 pathway highlights the importance of T-cell-derived signals in IL-10$^{+}$ Breg function. CD1d$^{hi}$CD5$^{+}$ Bregs express IL-10 and suppress inflammation in multiple immune-related disorders upon *in vitro* 5-hour stimulation with LPS, phorbol 12--myristate 13-acetate (PMA), ionomycin and monensin, and are known as B10 cells [44,45]. Using *CD40*$^{-/-}$ and *MHC*$^{-/-}$ mice in a model of experimental autoimmune encephalitis (EAE), it has been shown that the maturation of precursors to B10 cells ('B10pro') into functional IL-10-secreting Bregs was critically dependent on CD40 engagement and cognate interactions with T cells [41].

Antigen recognition is also important to Breg development and function. In MD4 mice, where the B-cell receptor (BCR) has been fixed for an irrelevant antigen, Breg activation was impaired and Breg frequency reduced in mice that could not recover from EAE [45,46]. Similar findings were observed in mice with decreased BCR signalling, such as in CD19$^{-/-}$ mice in the context of inflammatory bowel disease (IBD) and contact hypersensitivity, whilst CD19 overexpression expanded B10 cell numbers [44,45,47,48]. Increasing CD40 signalling elevated B10 and B10pro cell numbers by at least 10-fold *in vivo* [49]. Moreover, the calcium sensors, stromal interaction molecule 1 (STIM-1) and STIM-2, which are involved in facilitating intracellular calcium influx following antigen recognition by the BCR, were required for B-cell IL-10 production [50]. BCR signalling pathways may drive the capacity of B cells to express IL-10, thereby selecting for antigen-specific Breg *in vivo*. The requirement for Breg antigen specificity in transplantation was indicated when the adoptive transfer of mouse TIM-1$^{+}$ Bregs from tolerant recipients of H2$^{d}$ islet allografts prolonged survival of H2$^{d}$ grafts but not those of 3$^{rd}$ party H2$^{k}$ grafts [3,51]. In a series of elegant experiments, tolerized C57BL/6 mice with ovalbumin (OVA)-specific BCR were used to demonstrate tolerance in the presence of OVA$^{+}$ skin grafts, but not when the allograft lacked a recognizable antigen (BM12 skin grafts from MHC class II variant C57BL/6 mice). In contrast, wild-type C57BL/6 mice could accept BM12 skin grafts, which survived long term. Antigen specificity and recognition by the BCR therefore appear to be crucial for Breg function.

## Innate signalling

Whilst adaptive signals are important for Breg development and induction, innate signals also induce and expand IL-10$^{+}$ Bregs. CpG oligonucleotides can stimulate mouse and human B10 cells to produce IL-10 [52,53]. LPS can induce mouse B10pro cells to mature and become functional B10 cells [45]. Similarly, CD24$^{hi}$CD38$^{hi}$ B cells can be expanded with IFN$\alpha$ and CpG *ex vivo* and retain IL-10 expression, CD24$^{hi}$CD38$^{hi}$ phenotype, and *in vitro* suppressive function [54]. TLR signals, such as those that stimulate TLRs −2, −4 and

−9, and apoptotic cells can expand B10 cell numbers *in vivo* [24,55,56]. MyD88 is a universal adaptor protein downstream of TLR. In the context of EAE, $CD1d^{hi}CD5^{+}$ B cells from $MyD88^{-/-}$ mice produced lower levels of IL-10 in response to LPS, PMA and ionomycin stimulation when compared to wild-type mice. MyD88-generated signals were critical for normal IL-10 producing, $CD1d^{hi}CD5^{+}$ B10 cell development [45]. Moreover, a selective deficiency of MyD88 in B cells improved control of *Salmonella typhimurium* bacterial replication and prolonged survival of infected mice. B cells of MyD88-sufficient mice expressed IL-10 and could suppress neutrophil, NK cell and effector T-cell activation, leaving mice more susceptible to infection [57]. The release of IL-10 by TLR-stimulated B cells was increased upon CD40 engagement, suggesting that *in vivo,* optimal production of IL-10 occurred after TLR-activated B cells interacted with CD154-expressing $CD4^{+}$ T cells [58,59]. Activation of other molecules such as TIM-1, a phosphatidylserine receptor, resulted in the induction of IL-$10^{+}$ Breg and transplant tolerance *in vivo*. B cells that expressed a mutant form of TIM-1 lacking the mucin domain exhibited decreased phosphatidylserine binding and were unable to produce IL-10 in response to either apoptotic cells or ligation with anti-TIM-1 [60]. It has been hypothesized that both innate and adaptive Breg exist. Alternatively, the same Breg could respond to different stimuli to exert suppressive mechanisms including the secretion of IL-10 [61].

## Cytokine-driven induction

Cytokines are involved in both adaptive and innate immune pathways and have proven critical to the induction and expansion of Breg subsets. The *in vitro* co-culture of mouse B cells with exogenous IL-21 and no other stimulation for 48–72 hours is able to induce up to a x5.3 fold increase in mouse B10 frequencies [41]. Rosser et al. characterized Breg induction by pro-inflammatory cytokines IL-1β and IL-6, as a method of auto-regulation in CIA [62]. In support of this view, Bregs increase in number during the inflammatory phase of several autoimmune disorders; in their absence, mice develop exacerbated disease [39,53,63,64]. However, the immunosuppressive cytokine IL-35 can also induce IL-$10^{+}$ and IL-$35^{+}$ Breg, which profoundly suppresses inflammation in mouse models of autoimmunity [65,66]. Mice in which only B cells did not express IL-35 demonstrated enhanced survival after *Salmonella* infection. IL-10 has been shown as critical to the generation of mouse Breg in early experiments [39], with more recent work also supporting an autocrine role for IL-10 in Breg function [67].

## Clinical protocols

The lessons learnt from experimental models and human studies could be used to generate a GMP-suitable IL-$10^{+}$ Breg expansion or enrichment protocol (Fig. 2). It is of course worth remembering that some of the experimental manipulations in animal models are potentially dependent on undefined and disease-specific factors which may be relevant only to that experimental context. Those mechanisms that appear to be consistent across multiple disease processes are more likely to result in replicable clinical protocols. These strategies may involve a combination of ligand-based stimuli including CD40- and TIM-1-ligation, and innate signals to stimulate the TLR. Soluble factors such as IL-21, IL-6, IL-10 and BAFF could be included as adjuncts to optimize IL-$10^{+}$ Breg and improve efficiency. Following expansion, a standardized approach to detect IL-10-expression should be decided upon prior to *in vivo* administration: whether this is a short, 5-h stimulation using LPS, PMA and ionomycin as advocated when identifying human B10 cells [53], or whether an additional preliminary step such as 48- to 72-h stimulation with LPS, CpG or CD40 is required [43]. Given the phenotypic uncertainties surrounding Breg, it is difficult to draw the line between methods which can induce Breg that express IL-10, and those that can be subsequently used to detect Breg by IL-10 expression. Furthermore, work by Cherukuri and colleagues has demonstrated that a distinguishing feature of human Breg was not only the capacity to express Il-10 upon stimulation, but also to express reduced levels of TNFα [28]. Any manufactured IL-$10^{+}$ Breg therapy will need to be closely examined to ensure the reduction or absence of pro-inflammatory cytokine production.

Additional issues to consider include whether to use a human B-cell population already enriched for IL-$10^{+}$ Bregs, such as $CD24^{hi}CD38^{hi}$ transitional, $CD24^{hi}CD27^{+}$ memory or TIM-$1^{+}$ B cells, or whether to stimulate whole $CD19^{+}$ B cells as the initial substrate (Fig. 2). Whilst the proportional precursor frequencies of IL-$10^{+}$ Breg are greater in circulating B-cell subsets, they are rare in peripheral blood and so absolute starting numbers prior to expansion will be small [38,43,53,68]. Since IL-$10^{+}$ B cells can be generated from multiple B-cell sub-populations by exploiting similar signalling pathways, it may be pragmatic for clinical use to

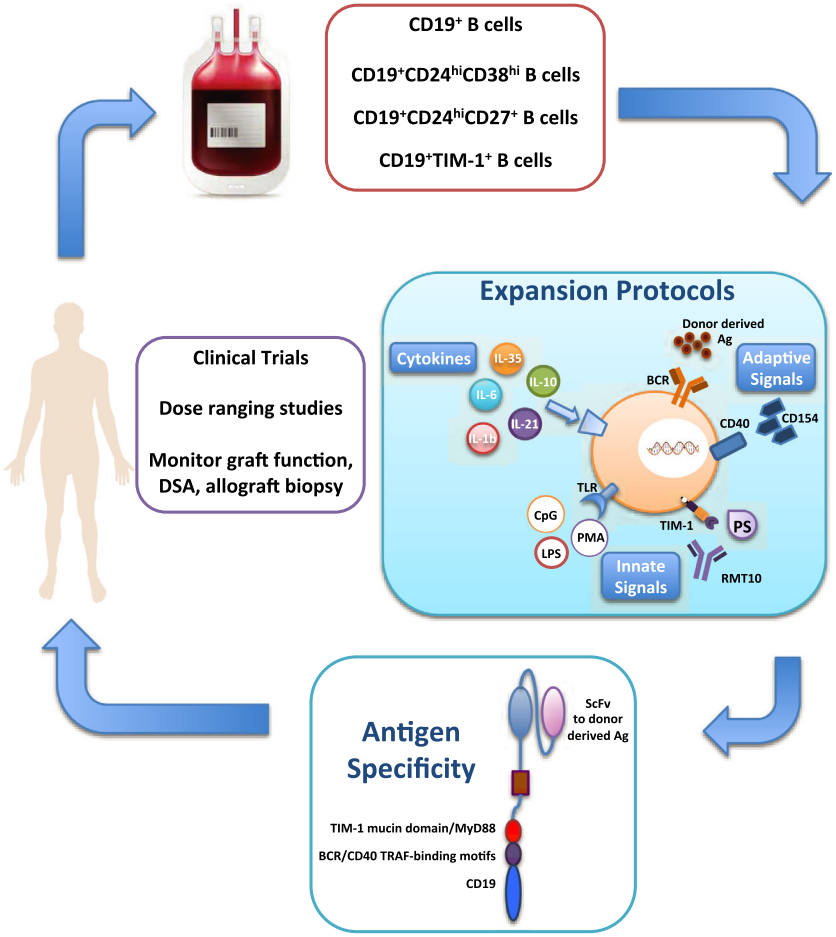


**Figure 2** Autologous Breg cellular therapy. Breg preclinical studies and clinical trials of other cell therapies in transplantation offer a template by which Breg cellular therapy could be introduced to the clinic. Autologous whole CD19$^+$ B cells or B-cell subsets enriched for IL-10$^+$ Breg may be isolated from the potential organ recipient and expanded using known signalling pathways which promote Breg induction and/or generation *in vitro*. Antigen specificity could be introduced by genetic engineering methods including lentiviral or CRISPR transfection. Breg-CAR constructs with intracellular components including those of the BCR, CD40, TIM-1 and TLR could generate antigen-specific, stable Breg cell therapy. Quality control measures should include *in vitro* evaluation of phenotype, IL-10 expression and capacity for STAT3 phosphorylation, prior to re-infusion into the recipient within a clinical trial.

initially isolate larger numbers of whole CD19$^+$ B cells, and then subject the entire population to an optimized protocol for IL-10 + Breg induction and expansion.

Antigen specificity should also be considered. Polyclonal Treg cell therapy is being used in clinical trials with promising effect, but preclinical work has demonstrated the improved efficiency of alloantigen-specific Treg. In a humanized mouse model of alloimmune-mediated injury of human skin grafts, the infusion of alloantigen-specific Tregs resulted in a significant reduction in dermal tissue injury when compared to polyclonal Tregs [69,70]. Antigen-specific B cells have a low precursor frequency and are difficult to isolate and expand in high yields [71]. The advent of chimeric antigen receptors (CAR) is revolutionizing T-cell therapy, and could facilitate development of an antigen-specific IL-10$^+$ Breg therapy [72]. Constructs that include components of CD19, CD40, BCR and even the TLR could be combined for a potent Breg-CAR design (Fig. 2). Antigen-specific cell therapy has obvious advantages, including the reduction in off-target effects of immunosuppression such as opportunistic infection and cancer, which indeed are a source of serious morbidity in the transplant recipient. Increased potency will also encourage lower dose regimens, and the likelihood of an improved safety profile.

Amongst these exciting possibilities, a robust framework is needed to evaluate the functional stability and antibody-producing capacity of any such product prior to administration in the patient. As with mouse models of EAE, models of transplantation tolerance have highlighted the divergent roles for pro-inflammatory and regulatory B cells. For example, B cells can positively or negatively regulate allograft rejection depending on the nature of the allograft and the intensity of the rejection response [73]. Evidence supports the role of regulatory plasma cells [26,66,74], but given the detrimental consequences of ABMR, it will be vital to the safety and success of any Breg cell therapy to confidently interrogate its role in humoral immunity.

## Pharmacological manipulation

The importance of Bregs in transplantation suggests that Breg promotion *in vivo* would benefit long-term

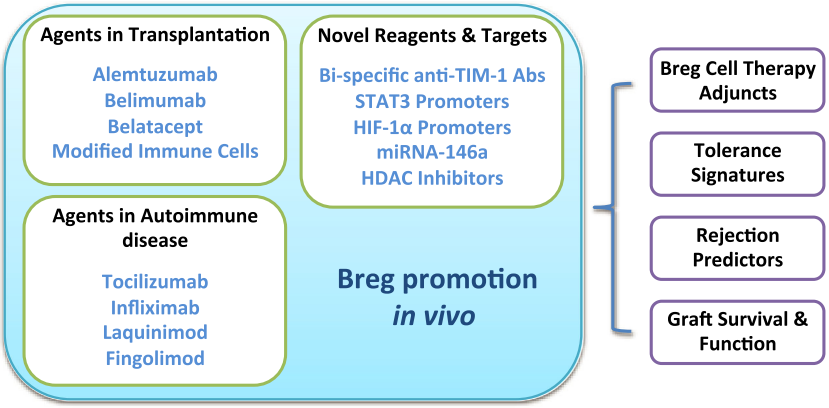


**Figure 3** Promotion of Bregs *in vivo*. Several induction agents and cell therapies have been demonstrated to support Breg survival and induction *in vivo* in transplantation. Multiple immune modulators in the context of autoimmune disease also promote Breg, offering additional therapeutic candidates [97,98,127-130]. Novel reagents in development may be able to soon offer specific Breg targeting. These reagents could be utilized to enhance Breg cell therapies both *in vivo* and during *in vitro* expansion protocols. Drug administration should be followed by careful monitoring of Breg-predominant tolerance signatures and predictors of rejection. Long-term graft outcomes and monitoring of humoral immunity will be critical to appreciating the benefits of such strategies.

allograft outcomes. Whilst we have explored the possibility of *ex vivo*-generated Breg cellular therapy infusion, we will now address those agents that may support *in vivo* expansion (Fig. 3).

## Existing therapies in transplantation

As discussed earlier, maintenance immunosuppressants such as prednisolone and azathioprine may have an effect on circulating IL-10$^{+}$ Breg [16,17]. Sirolimus may also support the *in vivo* and *in vitro* expansion of IL-10$^{+}$ Bregs as well as FoxP3$^{+}$ Tregs in liver transplantation [75].

The administration of several monoclonal antibody induction therapies has also been associated with Breg induction and/or expansion in renal transplantation. Alemtuzumab, a depleting anti-CD52 mAb, reduced the risk of biopsy-proven acute rejection in a broad range of human kidney transplant recipients, when compared to basiliximab induction [76]. Our group also observed a transient increase in CD24$^{hi}$CD38$^{hi}$ Breg in peripheral blood during the first year post-transplant in alemtuzumab-treated human kidney transplant recipients [77]. Similar increases in transitional B-cell frequency have been reported during B-cell repopulation following a single dose of rituximab in the peri-transplant period in human kidney transplant recipients [78]. Intriguingly, a second dose of rituximab within 1–2 weeks of transplantation precipitated T-cell-mediated rejection in kidney transplant recipients, and cardiac allograft vasculopathy in patients receiving a heart transplant [27,79]. The dynamics of Breg repopulation following B-cell depletion may be important in determining long-term allograft outcomes, in particular with regard to ABMR, and warrants further investigation.

A high serum concentration of the B-cell survival factor, BAFF, in kidney transplant recipients is associated with the development of *de novo* and increased preformed DSA levels, and increased ABMR rates [80,81]. Administration of belimumab, an anti-BAFF mAb, to kidney transplant recipients, led to an increase in the B-cell IL-10 to IL-6 ratio for the first 3 months post-transplant [82]. Despite a decrease in the frequency of transitional B cells, the belimumab group demonstrated a skewing of the IL-10 to IL-6 ratio in transitional and memory B-cell subsets, towards a more regulatory profile compared with controls. The belimumab group demonstrated lower frequencies of activated memory B cells, circulating plasmablasts and reduced formation of *de novo* IgG to new antigenic specificities post-transplant. The B-cell compartment may have been diverted from a humoral response to a regulatory one. Longer-term outcomes from such trials will help to inform ABMR rates and the success of such an approach.

Treatment with belatacept, a CTLA-4-Ig fusion protein, is associated with lower BAFF levels and the persistent elevation in circulating transitional B-cell frequencies in kidney transplant recipients [83]. Seven-year follow-up of kidney transplant recipients, comparing belatacept to cyclosporine-based immunosuppression, demonstrated higher patient and graft survival as well as increased mean eGFR in the belatacept group [84]. Ten-year follow-up analysis of transplant biopsies revealed increased frequencies of IL-10$^{+}$ Breg, FoxP3$^{+}$



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Treg and plasmacytoid dendritic regulatory cells in the allografts of belatacept group [85]. *In vitro* experiments have shown that belatacept induces STAT3 activation in stimulated B cells, thus providing a potential mechanistic explanation for the reported *in vivo* effects upon IL-$10^+$ Breg [86]. The promotion of the regulatory immune compartment may contribute to better overall outcomes observed with belatacept, and illustrate the likely important interplay between different regulatory cell networks.

A recent phase I clinical trial of modified immune cells (MIC) in renal transplantation exemplified such *in vivo* regulatory cell interactions [80]. Preclinical studies have demonstrated that monocytes, when treated with mitomycin C and matured into DCs, take on the morphology and phenotype of early immature DCs. This MIC population could strongly suppress T-cell responses and induce donor-specific immunosuppression in experimental models [87,88]. Kidney transplant recipients were infused with donor-derived MIC pretransplant, and a control group retrospectively selected to match maintenance regimens. No DSAs or rejection episodes were noted in the MIC group. Allograft function was preserved and circulating Breg frequencies increased from day 30 post-transplant, despite steroid use. At the last follow-up time-point (median 810 days; range 720-1080 days), Breg frequencies were 24 times higher than levels in transplanted controls. Treg frequencies, however, remained unchanged. Whilst the numbers of patients were small, the differences in Breg frequencies between the MIC and control groups were striking. Analysis of IGKV4-1 and IGKV1D-13, the ITN tolerance signature, revealed increased expression levels in the high-dose MIC group when compared to lower dose groups. Importantly, the patients in the high-dose MIC group demonstrated absent donor-specific T-cell responses at day 360 post-transplant, but preserved third-party responses, unlike control patients.

Longer-term outcomes and larger trials using these approaches will provide further insights as to whether such strategies can offer a Breg-targeted therapy that promotes B-cell-mediated regulation and which reduces donor-specific humoral immunity.

## Novel therapeutic targets

Whilst IL-10 expression is critical to both identification and function of most Breg subsets, no IL-10 therapy has passed clinical phase III studies [89]. A better understanding of Breg intracellular signalling pathways may lead to the development of novel agents that can target Breg function at a molecular level.

The cell surface molecule, TIM-1, plays an important role in IL-$10^+$ Breg function and has been suggested as an almost universal marker of this subset [4]. IL-$10^+$ Bregs have been induced by TIM-1-ligation using the low affinity antibody, RMT10, and TIM-$1^+$IL-$10^+$ Breg could induce tolerance to islet allografts in an IL-10-dependent fashion. Mouse B cells expressing the TIM-$1^{\Delta mucin}$ mutation were also unable to produce IL-10 in response to RMT10-ligation. Furthermore, TIM-$1^{\Delta mucin}$ B cells displayed increased production of pro-inflammatory cytokines IL-1 and IL-6, promoted Th1 and Th17 responses, inhibited the generation of Treg and Tr1 cells and enhanced the severity of EAE [60,90]. TIM-1 signalling may play a pivotal role in Breg function, and so TIM-1 ligation may provide a therapeutic 'B-cell switch'. Given that TIM-1 is also expressed as a stimulatory ligand by activated $CD4^+$ T cells [91], a B-cell-specific approach to TIM-1 ligation would probably be required to prevent off-target effects.

Many studies have demonstrated the importance of STAT3 phosphorylation in IL-$10^+$ Breg function [26,38,41,43,65]. Selective targeting of this pathway may provide a coherent approach to promote Breg function. Hypoxia-inducible factor (HIF)-1α, the oxygen-sensitive subunit of the HIF transcriptional complex, was recently shown to be important for IL-10 production in a STAT3-dependent manner by $CD1d^{hi}CD5^+$ Breg in murine CIA and EAE models [92]. BCR ligation increased the expression of HIF-1α by B cells, which was suppressed in the presence of ERK or STAT3 inhibition. Crucially, pSTAT3 was found to bind the *Hif1α* promoter, which was enhanced with BCR stimulation [92]. B-cell-specific deletion of HIF-1α reduced $CD1d^{hi}CD5^+$ Breg proliferation, decreased IL-10 expression and lowered levels of glucose uptake and lactate secretion, revealing an important role for glycolysis in Breg expansion. Co-immunoprecipitation assays revealed two hypoxia responsive elements within the *il10* promoter to which HIF-1α, HIF-1β and pSTAT3 could bind; HIF-1α and pSTAT3 could bind together upon BCR stimulation. Thus, BCR activation may induce IL-10 production through formation of HIF-1α/pSTAT3 complexes at the *il10* promoter. Activating the HIF-1α axis through pharmacological agents may provide tools to augment Breg function.

Work examining IL-10 signalling within Bregs has revealed roles for the c-Maf transcription factor and miRNA-146a [93,94]. LPS stimulation of $B220^+$ B cells results in increased c-Maf expression in mice, with c-

Maf directly binding a Maf recognition element within the *il10* promoter. c-Maf knockdown results in decreased IL-10 production in collagen-induced arthritis (CIA) mice [94]. miRNA-146a deletion resulted in the development of autoimmune syndrome and a reduced frequency of $CD1d^{hi}CD5^{+}IL\text{-}10^{+}$ Breg in mice. Interestingly, *miRNA-146*$^{-/-}$ B cells exhibited almost total abrogation of TIM-1 expression. Given the role of TIM-1 in IL-10 expression within Breg, miRNA-146 may influence Breg development and function through the regulation of TIM-1 expression [93].

Attention has also turned to negative regulators of IL-10 within Bregs, such as transcription factors including NFATc1 and histone deacetylases (HDAC). B-cell-specific *Nfatc1* knockout resulted in amelioration of murine psoriasis in an IL-10-dependent manner. By binding to HDAC1 and the *Il10* gene, NFATc1 suppressed B-cell IL-10 expression [95]. Shao et al. reported higher levels of HDAC11 and lower levels of IL-10 expression in B cells from allergic rhinitis patients when compared to healthy controls [96]. Introduction of HDAC11 shRNA significantly attenuated murine allergic rhinitis, and HDAC11-mediated suppression of IL-10 expression was elicited through HDAC-mediated deacetylation of c-Maf, which reduced c-Maf binding to the *il10* locus. Small molecule inhibitors of HDACs have received clinical approval for use in a range of diseases and so may present a rapidly translatable avenue to promote Breg function *in vivo*, in transplantation.

The use of mAbs directed against pro-inflammatory cytokines such as TNFα (anti-TNFα therapy) and IL-6 (anti-IL-6R mAb, tocilizumab) in the treatment of autoimmune disease has resulted in an increased frequency of human Breg in peripheral blood in association with improved clinical outcomes within 3-6 months of treatment [97,98]. An observational study of patients with rheumatoid arthritis also reported that a high baseline level of $IL\text{-}10^{+}$ Breg appeared to be predictive of a clinical response at 3 months upon anti-TNFα treatment [99]. These observations reinforce the importance of the expression of pro-inflammatory cytokines relative to that of immunosuppressive IL-10 in the context of Breg function. Work in transplantation has also demonstrated that whilst neutralization of IL-10 inhibited $CD24^{hi}CD38^{hi}$ Breg-mediated suppression of autologous Th1 cytokine expression, TNF-α blockade increased the suppressive capacity of both memory and naïve B-cell subsets [28]. The translation of such clinical observations from autoimmune disease may offer additional strategies to enhance endogenous Breg function and induction/expansion in transplantation.

Our rapidly expanding appreciation of Breg at transcriptional and post-transcriptional levels offers new approaches to target this subset's potent regulatory role. The introduction of novel small molecule agents and mAbs that can redirect B-cell function away from humoral responses and towards regulatory roles may improve long-term clinical outcomes in transplantation.

## Future perspectives

Bregs present an exciting avenue by which to approach long-term allograft tolerance and to switch B-cell responses from the humoral to the immunosuppressive. Evidence of the benefit of Bregs in transplantation has been steadily growing in animal models and human studies. The strategies discussed in this review provide a framework to continue to build on in order to eventually translate existing knowledge to clinical practice. Central to these efforts will be the identification of Breg markers for standardization of generation protocols and monitoring of cellular therapy, as well as a greater appreciation of Breg humoral properties. Breg-specific signatures of tolerance and predictors of rejection could help to dictate immunosuppressant dose reduction, prevent immunological allograft damage and minimize morbidity. Novel and existing reagents may also be prioritized to generate Bregs *in vivo*.

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