Expansion of B10 cells *in vitro*: Pathways, techniques and applications in transplantation (Review)

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Abstract. Cellular immunotherapy represents a pivotal treatment modality in clinical practice. Regulatory B cells (Bregs), a key subset of B lymphocytes, hold promise in the management of autoimmune diseases, cancer and transplantation immunity. The expansion of Bregs for cell therapy is a promising strategy to alleviate inflammation and promote immune tolerance. Achieving immune tolerance relies on balance between regulatory and effector cells. One primary objective of cellular therapy is to shift this balance towards Bregs, fostering a more tolerant immune microenvironment. The adoptive transfer of Bregs not only increases their quantity but also modulates the number and function of other immune cells. Maximizing *in vitro* expansion of Bregs and enhancing their regulatory functions are key focuses in transplant immunology. However, the precise mechanisms underlying the *in vitro* expansion of IL-10-secreting B cells (B10) remain inadequately understood. The present review aims to provide a comprehensive overview of the signaling pathways involved in B10 activation and expansion, as well as to highlight the techniques for *in vitro* amplification and development of adoptive B10 therapy in transplantation, which aims to advance the field of cellular therapy targeting Bregs.

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1. Introduction

Regulatory B cells (Bregs) represent a distinct subset within the B lymphocyte population and serve a key role in maintaining immune homeostasis, modulating intensity of immune response and suppressing excessive inflammatory reactions. Unlike regulatory T cells (Tregs), which are defined by specific transcription factors such as Foxp3, Bregs are primarily characterized by the regulatory elements they secrete (1). Bregs exert their immunomodulatory functions mainly through the secretion of inhibitory cytokines, including IL-10, transforming growth factor (TGF)-β and IL-35 (2-5). Among these, IL-10-secreting Bregs (B10 cells) suppress autoimmune and inflammatory responses (6). Yanaba (7) identified the cellular phenotype of Bregs as expressing $CD1d^{high}CD5^{+}CD19^{+}$. Secretion of IL-10 inhibits the activity of co-stimulatory molecules such as B7, thereby impeding T cell activation, which underscores the intricate mechanisms underlying their function (7).

Bregs are primarily categorized by their cytokine profiles, as specific phenotypes and transcription factors have yet to be established (8). This lack of distinct phenotypes presents a challenge for the expansion of specific Bregs. Moreover, Bregs play a critical role in various immune-associated diseases, including autoimmune and inflammatory conditions, tumor immune escape and organ transplantation (9-11). However, the *in vivo* numbers of B10 cells are often insufficient to harness their therapeutic potential.

Numerous signaling pathways and transcription factors have been shown to regulate the development and function of B10 cells (12). Key pathways include toll-like receptor

(TLR) signaling pathway, B cell receptor (BCR) signaling, CD40-CD40L interactions and B lymphocyte-activating factor (BAFF), alongside critical transcription factors such as signal transducer and activator of transcription 3 (STAT3), nuclear factor-κB (NF-κB) and interferon-regulatory factors (IRFs) (13). Under appropriate stimuli, such as cytokines IL-10 and TGF-β, pathogen-associated molecular patterns (PAMPs) cytosine-phosphate-guanine (CpG)-containing oligodeoxynucleotide (ODN) and lipopolysaccharide (LPS), immune checkpoint signals CD40 activation and PD-1/PD-L1 interactions, and cytokines like IL-21, B cells differentiate into Bregs, facilitated by these pathways and factors that govern their development and immunomodulatory functions (8,14).

Furthermore, the *ex vivo* expansion of B10 cells presents a broader array of cellular therapeutic options in immune transplantation. The adoptive transfer of B10 cells can exert immunomodulatory functions, suppress transplant rejection, promote transplant tolerance and decrease the reliance on immunosuppressive agents and their associated side effects. Additionally, B10 cells can be employed to investigate cellular immune regulatory mechanisms (15). While Tregs have been the primary focus of adoptive cell therapies due to their established expansion protocols and widespread clinical applications in transplant tolerance research (16), Bregs are gaining recognition for their ability to enhance regulatory functions of various immune cells, including Tregs (17).

Although both Bregs and Tregs contribute to promoting transplantation tolerance (18), Bregs possess advantages in terms of antigen recognition. Bregs are involved in the immune response at an earlier stage and respond more rapidly than Tregs; B cells can directly recognize naïve antigens via BCR, while T cells require antigen processing before responding (8). Despite ongoing debates regarding the role of IL-10 in transplant immunization, induction of graft tolerance with anti-CD45RB antibodies depends solely on the presence of B cells, independent of IL-10 (19-21). Regardless of the role of IL-10, the contribution of B10 cells in establishing a tolerogenic environment is key (22).

The present review discusses the significance of the TLR, BCR and CD40-CD40L signaling pathways in the generation of B10 cells, focusing on their expansion capacity and inhibitory functions. Given the lack of comprehensive literature addressing the mechanisms involved in B cell differentiation into B10 cells *in vitro*, the present review aims to elucidate the mechanisms underlying their expansion to facilitate advances in *in vitro* expansion technologies and cellular therapies.

2. Mechanisms involved in the classical signaling pathways for B10 expansion *in vitro*

TLR signaling pathways. At present, the prevailing method of expansion entails *in vitro* activation of naive B cells, with the TLR signaling pathway serving a crucial role in their secretion of cytokines such as IL-10 (23,24). TLRs are highly conserved pattern recognition receptors, primarily responsible for recognizing PAMPs and initiating associated signaling pathways (25,26). Consequently, TLRs elicit the inflammatory immune response, activate immune cells and exert anti-infection functions (27-29). In B cells, the TLR/myeloid differentiation primary response 88 (MyD88) pathway governs the production of immunosuppressive factors such as IL-10 (30-33) and represents a pivotal pathway for *in vitro* expansion. TLRs are type I transmembrane glycoproteins with a transmembrane helix (34). The extracellular region features a leucine-rich repeat sequence that binds to PAMPs (34). TLR signaling occurs when the toll/IL-1 receptor (TIR) structural domain interacts with MyD88, an adaptor protein located within the TIR receptor region (34). Typically, stimulation of TLR is followed by activation of B cells and production of antibodies (35). Nevertheless, previous experiments have demonstrated that B10 cells are produced (32,36-38) upon appropriate stimulation such as LPS, a component of the outer membrane of Gram-negative bacteria, and CpG ODN, which are synthetic DNA sequences containing unmethylated CpG motifs that can stimulate the immune system via Toll-like receptor 9 (TLR9).

Following TLR stimulation, signaling pathways, such as NF-κB, MAPK and PI-3K/AKT pathways, are activated in B cells in response to inflammatory factors (39). Downstream, STAT3 and ERK are key for B cells to produce IL-10 after TLR agonist stimulation (33,40-42).

TLR9 in endosomes: Ongoing signaling. Currently, *in vitro* expansion of Bregs is primarily achieved through TLR9 stimulation. TLR9 recognizes sequences in bacterial and viral double-stranded DNA that encompass CpG sequences (43). As a non-ligand crystal, TLR9 can bind CpG-DNA (44). Clinical trials have shown that CpG ODN activates B cells via TLR9, thereby establishing *the in vitro* activation and generation of Bregs (45,46). This recognition initiates the downstream signaling pathway and activates B cells.

TLR9 is primarily located in the endoplasmic reticulum, endosome and lysosomes. Following uptake by lyosomal vesicles, CpG ODN binds to TLR9 and give rise to TLR9-CpG complexes. In general, agonist CpG-DNA binds to TLR9 in a 2:2 ratio, forming a symmetrical TLR9-CpG-DNA complex (47). Unlike natural TLR9 agonists, artificially designed agonists possess a structure that resists nuclease degradation and leads to more robust activation (48). Due to differences in TLR9 expression across species, immune stimulation in humans and large animals is less pronounced, and higher doses of CpG ODN are required to achieve an effect in larger species. Additionally, to ensure the effective translation of mouse experimental results to human clinical applications, it is essential to design TLR9 ligands tailored to the immune responses of each species. In addition, to fulfill the research requirements of TLR9 agonists, it is necessary to design separate TLR9 agonists for humans and mice (49).

The formation of the TLR9-CpG complex triggers TLR9 oligomerization, entailing the binding of multiple TLR9 molecules. Following TLR9 oligomerization, the ligand-bound oligomers recruit the appropriate adaptor protein to form the TLR9-MyD88 complex (50). TLR9 interacts with MyD88, forming the TLR9/MyD88 pathway which activates downstream signaling molecules through TIR structural domain-mediated signaling (51). The activation involves proteins from the IL-1 receptor-associated kinase (IRAK) family, as well as TNF receptor-associated factor (TRAF) and TGF-β-activated kinase 1 (TAK1)-binding protein (52). IRAK1 and IRAK4 are key components of the TLR9 signaling

pathway (51). These are phosphorylated and activated to form a complex that activates TAK1 (52). The activated TAK1 triggers a series of signaling molecules, including MAPK, IκB kinase (IKK) and NF-κB (53).

The MAPK pathway encompasses downstream molecules such as ERK, JNK and p38. The activated MAPK has the potential to activate the transcription factor activator protein (AP)-1 (54), a key effector molecule downstream of the JNK pathway. AP-1 may bind to the IL-10 gene promoter, participating in transcriptional regulation of this gene. Microarray analysis demonstrated BAFF operates through AP-1, binding to the IL-10 promoter region, thereby enhancing IL-10 secretion (55). This suggests that the products of the MAPK pathway influence the differentiation and proliferation of B cells into Bregs.

By contrast, the activation of IKK leads to the phosphorylation of IκB (an intracellular inhibitory factor), resulting in the degradation of IκB and release of NF-κB. NF-κB in B cells then enters the nucleus and binds to specific DNA sequences (56), facilitating the transcription of Breg-related genes.

Recent study have indicated an upregulation in the expression of tyrosine hydroxylase (TH), a key enzyme for catecholamine production, following TLR9 activation by CpG ODN (57). This upregulation is associated with increased production of IL-10 (58). Additionally, B cells produce catecholamines in a time- and stimulus-dependent manner, further inducing the production of IL-10 (57). To the best of our knowledge, non-classical pathways of TLR9-induced Breg production have not been identified.

In general, TLR9 is a pathway that requires further investigation to facilitate development of more effective agonists. By studying the structure and stability of TLR9 agonists to resist nuclease degradation, while also exploring other signaling axes, a deeper understanding of the impact mechanisms of TLR9 agonists on B10 *ex vivo* expansion can be achieved, optimizing the expansion outcomes.

TLR4 on the membrane: Key signaling in B10 expansion. TLR4 is primarily located on the cell membrane surface and activates B cells by recognizing ligands such as LPS. Inflammatory stimuli activate the TLR4-MyD88 axis of B cells, which ultimately indirectly phosphorylates STAT3 (31), regulating key cellular processes such as cell differentiation, proliferation and survival (59-62). STAT3 interacts with JAK to promote formation of phosphorylated (p)STAT3 dimers (59-61) and the translocation of pSTAT3 protein from the cytoplasm to the nucleus. In the nucleus, STAT3 binds specific DNA sequences to promote transcription of target genes and the differentiation of B cells to Bregs (63). Furthermore, STAT3 can regulate the transcription of key genes, such as B lymphocyte-induced differentiation protein (63,64), which is essential for driving B cell differentiation and modulating immune responses. This regulation initiates a cascade of responses in downstream signaling molecules, including MAPK and NF-κB pathways (40).

Resveratrol inhibits phosphorylation of STAT3, thereby reducing the production of Breg-derived TGF-β and decreasing Treg formation (65). Similarly, in $CD1d^{hi}CD5^{+}$ B10 cells, activation of TLR4 by LPS leads to the interaction of pB cell linker protein with Bruton's tyrosine kinase (Btk), resulting in phosphorylation and subsequent nuclear translocation of STAT3 to transduce IL-10 gene (66). Similar to the TLR9-MyD88 axis, this process ultimately leads to the activation of ERK, JNK, p38 and NF-κB (54). Activated NF-κB enters the nucleus and binds to the promoter regions of Breg-related genes, facilitating the transcription and expression of these genes (40).

In the TLR pathway, the activation of NF-κB is essential for inducing IL-10 production (67). Blocking experiments have shown that NF-κB serves a vital role in Breg formation (66,68). Lee *et al* (69), using bromodomain and extra-terminal domain protein blocker JQ1, demonstrated that bromodomain protein 4 (BRD4), which produces the promoter of the IL-10 gene, promotes IL-10 production in Bregs when BRD4 interacts with NF-κB. Further research is still needed to determine the role NF-κB plays and how it can be utilized for *in vitro* amplification.

TLR3, TLR4 and TLR5 activate the Myd88-independent pathway, with TLR4 recruiting TIR domain-containing adaptor inducing IFN-β (TRIF), which recruits TRAF3 and the kinases TBK (TANK-binding kinase 1) and IKKε/IKKi. TBK, which plays a key role in regulating immune responses, leads to the activation of NF-κB and the phosphorylation of IRF-3, which undergoes nuclear translocation and induces expression of type I interferons. Previous study of B cell dephosphorylation using small molecule inhibitor ezrin have shown that in the TLR-Myd88-independent pathway, a key TRIF-TBK1-IKK-IRF3 axis, as well as a robust NF-κB signaling, leads to higher levels of IL-10 secretion (70). Current studies have focused on the Myd88-dependent pathway (30,71); the Myd88-independent pathway may be an alternative mode of action for *in vitro* expansion of Bregs (Fig. 1).

Together, the aforementioned mechanisms lead to *in vitro* expansion of Bregs. Further research is still needed on the biological properties of Bregs, the TLR pathways involved in their expansion and transcription factors such as STAT3, IRF7 and NF-κB.

BCR signaling pathway. BCR is an antigen recognition receptor on the surface of B cells (72). Each B cell expresses a specific BCR, consisting of two primary components: A membrane-anchored immunoglobulin (mIg) and a signal molecule tightly binding to it (Fig. 2). The Ig portion of the BCR comprises two heavy and two light chains, linked together by disulfide bonds to form an antigen-binding unit. Signaling transduction molecules heterodimers associated with BCR Igs through noncovalent bonds, including Igα (CD79a) and Igβ (CD79b), which contain immunoreceptor tyrosine-activated motifs (ITAM) (73-76). ITAM binds to SRC kinases such as LYN, FYN kinase and B-lymphoid tyrosine kinase. Cross-linking of BCR to specific antigens induces phosphorylation of ITAM tyrosine by these SRC kinases (77). CD19, which acts as a co-receptor, synergistically lowers the threshold for B cell activation; tyrosines in CD19 structural domains are phosphorylated by SRC kinases, serving as docking sites for SH2-containing proteins (Src Homology 2), such as PI3K (78).

BCR serves an essential role in B cell differentiation. When activated, BCR can generate a prolonged activation signal within the B cell (79-81), thereby prompting Bregs to

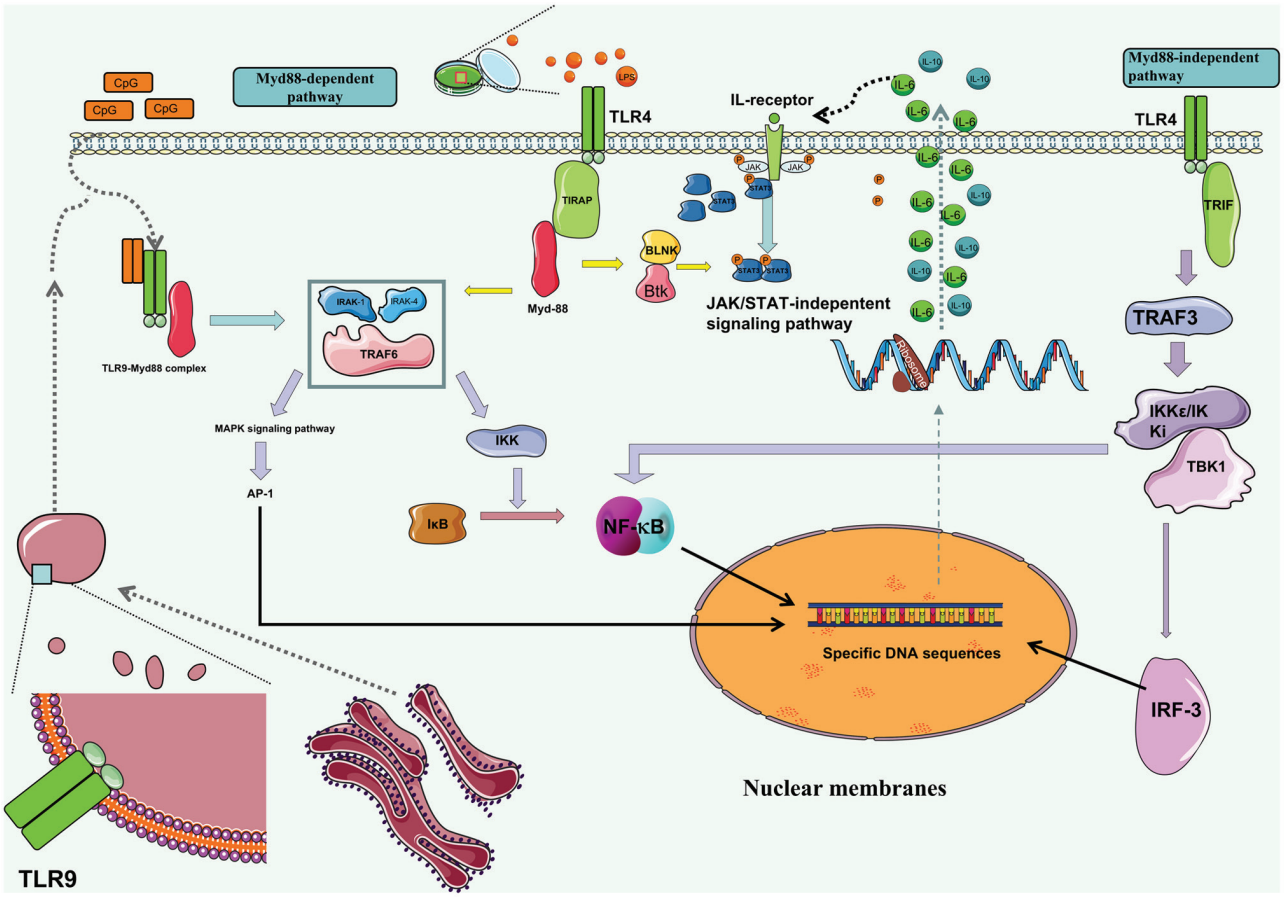

Figure 1. Generation of B10 cells via *in vitro* stimulation of TLR pathways. LPS, CpG ODN and other stimulants added *in vitro* activate the TLR/MyD88 signaling pathway through TLR4 and TLR9 receptors, promoting the activation of MAPK pathway, NF-κB and STAT3 phosphorylation, causing transcription factors to enter the nucleus. After binding promoter sequences of suppressor genes such as IL-10, molecules such as NF-κB and AP-1, can promote their expression and exert immunosuppressive function. Cytokines such as IL-6, which are expressed as a result of TLR activation, cause STAT3 phosphorylation through the JAK/STAT pathway. Phosphorylated STAT3 promotes the expression of Blimp-1 and other factors, leading to the differentiation of B cells into Bregs. In addition, TLR3, 4 and 5 activate the Myd88-independent pathway and cause B cell differentiation toward Bregs. Futher, BCR and CD19 signaling pathways work together to promote production of IL-10. LPS, lipopolysaccharide; CpG ODN, Cytosine-phosphate-Guanine oligodeoxynucleotide; TLR, toll-like receptor; Blimp, B lymphocyte-induced maturation protein; Breg, regulatory B cell; BCR, B cell receptor; IRAK, Interleukin-1 receptor-associated kinase; TRAF, TNF receptor-associated factor; BLNK, B cell linker protein; Btk, Bruton's tyrosine kinase; TRIF, TIR-domain-containing adapter-inducing interferon-β; AP, activator protein; IRF, interferon regulatory factor; IKK, IκB kinase; TBK, TANK-binding kinase.

proliferate and transmit signals to the cell via signal molecules. These signals will trigger cellular responses, enabling B cells to differentiate into regulatory or antibody-producing cells (82,83). B cells activated by BCR protect NOD mice from type 1 diabetes in an IL-10-dependent manner (84). LPS-induced Breg proliferation is reduced in inhibitor of κB, NF-κB essential modulator)-deficient mice (85). However, BCR stimulation compensates for this reduction by promoting Breg expansion (85). This further suggests that the BCR complex promotes Breg proliferation and differentiation.

Although antigen and BCR signaling serve a vital role in the early development of Bregs, the expansion of Bregs can be further optimized by activation of TLRs (8,36,86,87). BCR and TLR (88,89) activate the PI3K/Akt/ glycogen synthase kinase (GSK)-3 pathway. Following activation, PI3K is recruited to the membrane by binding to sequences containing p-tyrosine on membrane-associated scaffold proteins (such as CD19) through its Src homology 2 domain (90-93). Once at the membrane, PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) by phosphorylating membrane phosphatidylinositol 4,5-bisphosphate (94,95). PIP3 is dephosphorylated to produce PI(3,4)P2. Acting as anchors, PIP3 and PI(3,4)P2 recruit proteins containing PH homology domains to the membrane (96). This leads to recruitment and activation of signaling enzymes containing the PH domain (such as Btk, phospholipase C-2 and Akt) at the membrane. Akt is the primary mediator of the anti-apoptotic signal produced by PI3K (97), and phosphorylates proteins that regulate cell survival. Additionally, Akt can phosphorylate serine/threonine (Ser/Thr) kinase GSK-3α and GSK-3β (98). GSK-3 is a multifunctional kinase involved in regulation of various physiological and pathological processes (99-101), such as glycogen synthesis, cell death and survival. These negative regulatory sites on GSK-3α and GSK-3β are phosphorylated by Akt. GSK-3β is a multifunctional Ser/Thr protein kinase that plays a role in B cell metabolic activity and proliferation. During differentiation of B cells into Bregs, phosphorylation of the Ser residues of GSK-3 leads to its inactivation (99), resulting in phosphorylation of the negative regulatory sites on GSK by BCR, inhibiting GSK-3 activity (102). *In vitro* use of GSK-3 inhibitors significantly increases its phosphorylation, thereby inhibiting GSK-3 activity and resulting in a significant increase in the common

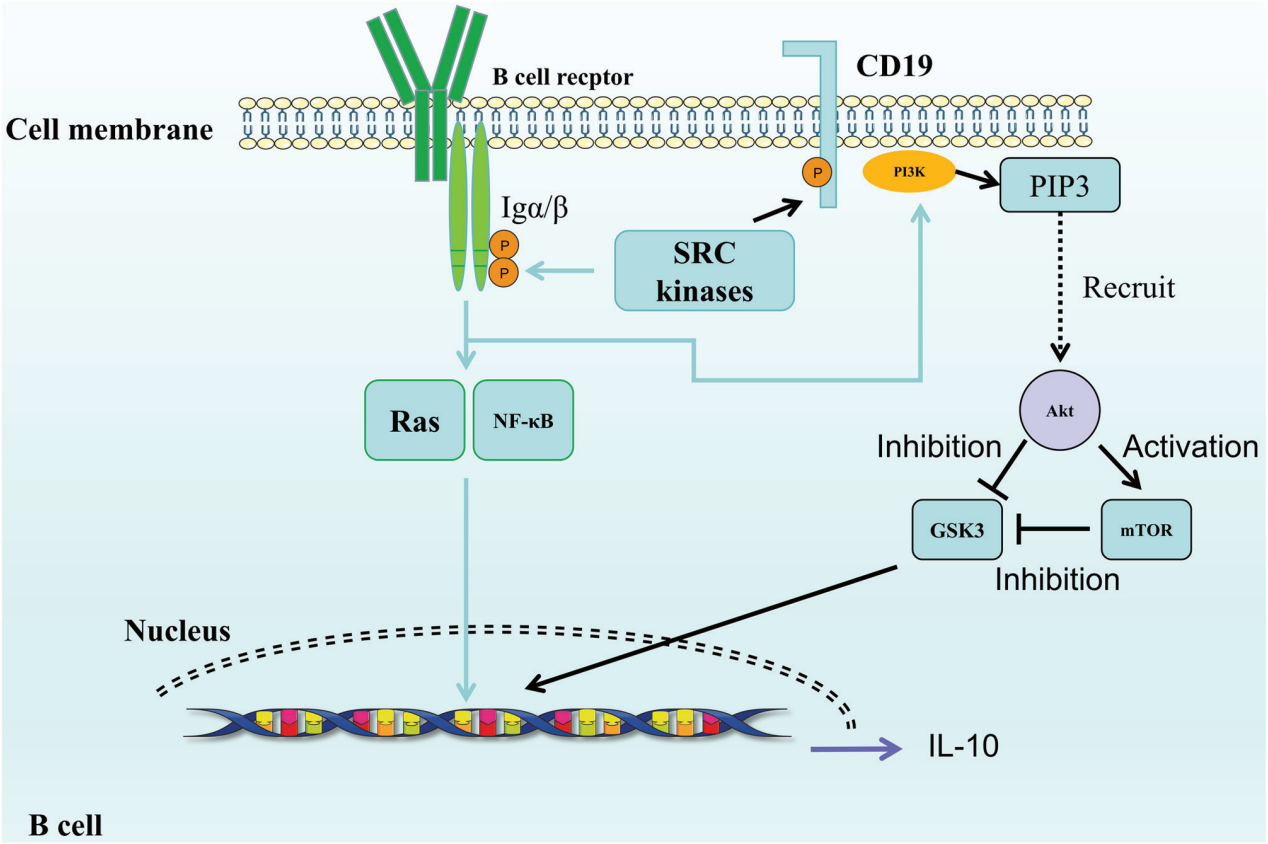

Figure 2. BCR and CD19 signaling pathways work together to promote production of IL-10. Following antigen stimulation of BCR, intracellular SRC kinases bind to specific motifs and domains on both the BCR and CD19, leading to their phosphorylation. Phosphorylation of the CD19 domain allows it to interact with the recruited PI3K, which subsequently generates PIP3. This process recruits and activates intracellular Akt, ultimately promoting the expression of IL-10 by facilitating the binding of transcription factors to specific regions in the nucleus. PIP3, phosphatidylinositol (3,4,5)-trisphosphate; BCR, B cell receptor.

Breg subtypes ($CD19^+CD24^+CD27^+$, $CD19^+CD24^+CD38^{high}$, $CD19^+CD27^{int}CD38^{hi}$, $CD19^+CD39^+CD73^+$). Expression of mBreg ($CD19^+CD24^{hi}CD27^+$) is most significantly increased, and subsequent adoptive transfer experiment, where mBreg cells were transferred into murine GVHD model, have confirmed the enhancement of its inhibitory function (99). Following mTOR activation, GSK-3 is inhibited, specifically leading to secretion of IL-10 by B cells and driving the generation of Tregs (103). This can also account for the reduction in regulatory cells observed after use of the mTOR inhibitor rapamycin (104). GSK-3, downstream of mTORC1, serves as a key metabolic regulatory factor for IL-10 secretion in B cells (104).

The activation of BCR triggers signaling pathways, including PI3K/Akt, Ras/MAPK and NF-κB pathways (79). Since Bregs control inflammation and regulate immunity, their amplified signaling pathways partially overlap with pathways involved in the immune response of B cells (84). Under specific conditions, such as mTOR agonist and GSK-3 inhibitor (104), these signaling pathways promote proliferation of Bregs, so *in vitro* expansion methods mimic the stimulation of inflammation.

CD40 signaling: Working with TLRs and BCR. CD40 is a membrane-bound molecule on the surface of B cells and is part of the TNF receptor family. This molecule is activated by membrane-bound ligands through direct cell-to-cell contact or by soluble ligands in solution (105).

During the adaptive immune response, CD40 interacts with CD40 ligand (L) on the surface of antigen-activated $CD4^+$ T cells, stimulating B cell proliferation and activation (106,107). CD40 agonist antibody mimics CD40L binding to CD40, thus simulating T cell-dependent signaling that activates B cells. CD40 signaling pathway can activate various downstream signaling molecules, such as TRAF, NF-κB and MAPK, which influence B cell proliferation and function. As a co-stimulatory receptor, CD40 can work synergistically with cytokines, BCR and TLRs to elevate STAT3 phosphorylation levels (108). Moreover, CD40 promotes intracellular signal by recruiting TRAF in intracellular membranes, activating different signaling pathways, including the classical and non-classical NF-κB, MAPK, PI3K and PLCγ pathways (109).

The aforementioned findings illustrate the importance of the CD40 pathway in efficient amplification of Bregs. Previous study have found that *in vitro* stimulation of CD40 and IL-21 receptor signaling can drive the development and proliferation of B10 cells four million-fold and that the IL-10 produced is effective in suppressing the inflammatory response *in vivo* (36). Splenic $CD1d^{hi}CD5^+$ Bregs are significantly amplified *in vivo* through an intrinsic genetic mechanism (involving highly reactive CD40) that can suppress autoantibody and T-dependent antibody responses and inhibit germinal centers (110). In addition, recent tumor study have found that PPARδ in Bregs may

bind agonistic anti-CD40 antibodies, thereby stimulating B10 production (111). Advances in *in vitro* amplication technology may be facilitated by simulating changes in Bregs within tumors *in vitro*.

In summary, inflammatory factors are responsible for generation of the immune response and regulatory cells. In addition, in the absence of TLR agonists, stimulation of BCR and CD40 only marginally increases the expression of IL-10 (33). This suggests that effective stimulation of TLRs is more key for IL-10 production than BCR and CD40. However, it has also been shown that by blocking CD40, more granzyme (GZM)B is produced, serving a regulatory role in transplantation (112). Hence, the effects of stimulation or blocking of the CD40 signaling pathway on Bregs are complex and varied.

3. B10 *in vitro* amplification technology

Immune activation is required for Bregs to exhibit suppressive functions. Bregs are present in B cell populations at different stages of maturation and differentiation (8), suggesting several lineages can be induced to acquire regulatory capacity in response to environmental factors (113). When exposed to specific antigens or appropriate stimuli, subpopulations of B cells, such as those triggered by TLR ligands and anti-CD40 stimulators, have the capacity to differentiate into regulatory B cells (8,14). *In vitro,* specifically act on TLRs, CD40 and BCR to promote activation and expansion of B10 cells. In addition to the addition of stimulus, the use of drugs and cell coculture used to study the expansion of Bregs. Selecting appropriate stimulants and culture conditions is necessary according to the specific experimental design and research purpose. At the same time, care should be taken to control the concentration of the stimuli and treatment time to avoid excessive or incomplete activation affecting generation and function of Bregs (68,104).

Breg expansion induced by stimulants. Currently, Bregs are primarily expanded *in vitro* by stimulating TLR4, TLR9 and CD40 (68,83,114). The chronology and location of the aforementioned events *in vivo* remain to be clarified. TLR agonists, such as LPS from gram-negative bacteria, peptidoglycan from gram-positive bacteria or CpG-containing oligonucleotides that mimic bacterial DNA (115), are potent factors in inducing IL-10 production by naïve B cells (32,37). In addition, IFN-α, an important cytokine that plays a crucial role in the regulation of the immune system and antiviral responses, also increases TLR7- or TLR8-induced IL-10 production in B cells (33). The differentiation and activation of Bregs necessitate the engagement of various molecules for binding, such as TLR-2, TLR-4, TLR-9, BCR signaling, and co-stimulatory factors CD40, CD80/CD86, BAFF, as well as cytokines (IL-1β, IL-2, IL-6, IL-21, IL-35, IFN-α) (36,116-119) (Table I).

Stimulation of signaling pathways. LPS stimulates the production of B10 cells. B10 cells exert immunomodulatory functions that rely not only on secretion of cytokines such as IL-10 but also on the involvement of surface ligands such as FasL. In mice, LPS was found to rapidly increase FasL expression in $CD1d^{hi}CD5^{+}$ B cells; this subpopulation was able to inhibit $CD4^{+}$ T cell proliferation (68). Therefore, when inducing Breg generation, it is insufficient to solely focus on the ability of cells to secrete IL-10. Furthermore, ultraviolet irradiation induces upregulation of TLR4 on the surface of B cells, enhancing regulatory capacity in contact hypersensitivity reactions (120). Earlier study found that splenic $CD1d^{hi}CD5^{+}$ Bregs producing IL-10 are notably induced in mice by LPS and anti-CD40 antibodies (83). The use of radiation in combination with stimulants can be considered for *in vitro* expansion.

Sole stimulation of TLR9 is insufficient and typically necessitates a combination of other agonists for optimal efficacy. In experimental autoimmune encephalomyelitis (EAE), use of the TLR9 agonist ODN 1826 in conjunction with irradiation results in the enhanced generation of B10 cells (10). The potency of the TLR9 stimulator ODN is augmented through various means, such as co-formulation with deposit-forming adjuvants and use of polyphosphate polyelectrolyte analogs to enhance the immunoreactivity of the CpG ODN (49).

The upregulation of IL-10 expression in B cells is evident upon stimulation with CD40 monoclonal antibodies (83), thus affirming an association between CD40 activation and B10 amplification. B cells derived from $CD22^{-/-}$ mice exhibit a noteworthy degree of hyperresponsiveness to CD40 signaling (121). In subsequent study, CD40 stimulation led to a 16-fold increase in the population of B10 cells compared with wild-type mice (110). The existing methods for activating CD40 signaling have evolved beyond the sole reliance on antibodies and use foster cells for coculture-based stimulation (36).

Previous studies have reported IL-10 production and regulatory functions when human B cells or Breg subpopulations are activated by CpG and anti-CD40 antibodies (122-124). Previous experiment have reinforced these findings, noting increased expression of IL-10 compared with that induced by the stimulation of TLR4 alone (68). These observations support the potential for a more robust immunosuppressive effect of Bregs, particularly via enhanced IL-10 secretion, which plays a critical role in dampening inflammatory responses and promoting immune tolerance, warranting further exploration.

The immunosuppressive effect of cells obtained by stimulating TLR9 with CpG alone is inferior to that of cells obtained following combined stimulation with TLR4, TLR9 and PMA (Phorbol 12-myristate-13-acetate (PMA), a synthetic compound that activates protein kinase C (PKC) (114,125). PMA serves as a mimetic of diacylglycerol (DAG), while ionomycin acts as a Ca^{2+} transporter, facilitating the transfer of Ca^{2+} from organelles to the cytosol. PKC can be activated by the concerted action of DAG and Ca^{2+}. Intracellularly, activation of PKC instigates the phosphorylation of numerous downstream protein kinases, initiating a cascade reaction that leads to the expression of multiple proteins, such as PI3K, NF-κB, thereby inducing cellular activation. Consequently, the combined influence of PMA and ionomycin activates PKC, thereby triggering downstream responses (83,126). The principal outcome of this activation is stimulation of cytokine production, providing a plausible explanation for the observed increase in IL-10 production in response to TLR agonists and PMA activation in B cells. In periodontitis, $CD25^{+}$ B cells induced *in vitro* with Breg functionality exhibit augmented production of IL-10, IL-35 and TGF-β when subjected to co-stimulation with both LPS and CpG compared with stimulation with LPS or CpG alone (127). Moreover, the stimulation of TLR4 and TLR9, combined with the addition of PMA and ionomycin,

Table I. Methods of IL-10-secreting B cell stimulation *in vitro*.

Species	Stimulus	Phenotype	Disease	Effect	(Refs.)
C57BL/6 mice	Astilbin + LPS	$CD19^{+}CD1d^{hi}$ and $CD19^{+}TIM\text{-}1^{+}$	Colonitis		(31)
	LPS	$CD5^{+}CD1d^{hi}FasL^{+}$	Contact hypersensitivity to oxazolone; autoimmune disease	Pro-inflammatory factors increased	(36,68)
	IL-21	$CD1d^{hi}CD5^{+}$	Autoimmune disease	Effect on B cells is poor	(36,117)
	NIH-3T3-CD154/BLyS cells + IL-21	$CD1d^{hi}CD5^{+}$ and $CD1d^{lo}CD5^{-}$	Autoimmune disease	Amplifcation of B10 cells ~4000000-fold	(36)
	CpG ODN + β-ADR agonist	$CD19^{+}CD1d^{+}$, $TIM\text{-}1^{+}$, $CD5^{+}$ and $CD5^{-}$	Autoimmune disease	Expression of enzyme tyrosine hydroxylase induced in B cells upon activation of TLR9	(57)
	Anti-CD40 mAb + CpG	$CD5^{+}CD1d^{hi}$	Contact hypersensitivity to oxazolone		(68)
	LPS + anti-CD40 mAb	$CD1d^{hi}CD5^{+}CD19^{+}$	Autoimmune disease		(83)
	LPS + CpG ODN + ionomycin	$CD19^{+}CD25^{+}TIM\text{-}1^{+}LAP^{+}PD\text{-}L1^{+}$	Transplantation		(114)
	CpG ODN	$CD19^{+}$	Transplantation		(114)
	LPS + MSC		Inflammatory disease	Greater effect on B cells than that of LPS alone; IL-10 expression increased by 18.4 times and pro-inflammatory factor levels decreased	(139)
Human	$CD154^{+}$ Chinese hamster ovary	$CD73^{-}CD25^{+}CD71^{+}$	Transplantation	Amplified B10 ~900-fold and for >14 days	(42)
	mTOR agonist	$CD19^{+}IL\text{-}10^{+}$	Inflammatory disease		(104)
	GSK-3 inhibitor	$CD19^{+}CD24^{hi}CD2^{7}+$	Inflammatory disease		(104)
	CD40L + CpG + IL-21	$CD19^{+}$	Autoimmune disease		(129)
	TLR7 agonist + Tα-1	$CD19^{+}CD24^{+}CD38^{hi}$ and $CD24^{low/neg}CD38^{hi}$	Multiple sclerosis		(138)
	Sirolimus	$CD19^{+}CD24^{+}CD38^{+}$ and $CD19^{+}CD24^{+}CD38^{+}TGF\text{-}\beta^{+}$	Organ transplantation		(141)
	$CD40L^{+}$ ILCs	$CD19^{+}CD27^{-}$ $IgD^{+}IgM^{+}CD24^{high}CD38^{high}CD1d^{high}$	Allergic disease		(149)
	Abatacept		Rheumatoid arthritis		(155)

Table I. Continued.

Species	Stimulus	Phenotype	Disease	Effect	(Refs.)
Spragye-Dawley rats	LPS + CpG ODN MSC	IL-35$^+$TGF-β$^+$ $CD19^+CD24^{high}CD38^{high}$	Periodontitis Autoimmune disease		(127) (150,151)
BALB/c, C57BL/6, $TLR5^{-/-}$, and $MyD88^{-/-}$ mice	Recombinant fusion protein (rFlaA:Betv1)	$CD19^+CD24^+CD1d^+IgM^+CD38^+$	Allergic disease		(138)

LPS, lipopolysaccharide; hi, high; TIM, T-cell immunoglobulin and Mucin-domain containing molecules; NIH-3T3-CD154/BLyS, NIH-3T3 cells expressing the T-cell ligand CD40 (CD154) and B-lymphocyte stimulator (BLyS); lo, low; LAP, latency-associated peptide; ADR, adrenergic receptor; MSC, mesenchymal stem cell; GSK, glycogen synthase kinase; CD40L, CD40 ligand; ILC, innate lymphoid cell; TLR, toll-like receptor; Tα-1, thymosin alpha-1; neg, negative; rFlaA: Betv1, recombinant Flagellin A: Birch pollen allergen Bet v 1.

significantly upregulates expression of T cell immunoglobulin and mucin domain-1 (TIM-1) in the resulting Breg population. TIM-1 is a protein that plays a crucial regulatory role in the immune system, promoting proliferation and differentiation of Bregs by enhancing the expression of STAT3 (42).

IL-21 is necessary for B10 cells to mature into IL-10-secreting effector cells (36,128), however, IL-21 alone has little effect (36,117). In a study conducted by Zheremyan (129), B cells were isolated from human blood and cultured *in vitro*; CD40L + CpG led to the highest production of IL-10 by Bregs (129). The combination of CD40L + CpG + IL-21 demonstrated the strongest immunosuppressive ability (129). In a previous study by Chesneau (130), the combination of CD40L + CpG + IL-21 + F(ab')2 anti-BCR Abs + IL-2 resulted in generation of Bregs secreting granzyme B (GZMB). GZMB is a cytotoxic protease belonging to the GZM family, primarily produced by cytotoxic T and natural killer cells. GZMB plays a key role in the immune system, particularly in the immune response against viral infections and tumor cells (131). Secreted GZMB promotes the proliferation and expansion of GZMB$^+$ B cells in an ERK1/2-dependent manner, while concurrently inhibiting proliferation of Teff (effector T cells) cells in a contact-dependent manner (130). However, GZMB$^+$ B cells are susceptible to apoptosis, possibly due to relatively high caspase-3 activity, which limits application of GZMB$^+$ B cells (130).

Furthermore, the combination of CD40 with cytokines has been shown to induce generation of B10 cells (129). Yoshizaki *et al* (36) devised a method involving feeder cells (NIH-3T3-CD154/BLyS cells) expressing CD40L and BAFF, which were cocultured with IL-4 and IL-21 cytokines; they observed notable expansion of IL-10$^+$ B cells in mice of up to $4x10^6$ times. These *in vitro* expanded eBregs, were subsequently infused into mice with EAE, resulting in significant alleviation of EAE symptoms (36). This demonstrates that these expanded eBregs retain their regulatory function *in vivo*, representing the highest expansion efficiency documented thus far. Additionally, Menon *et al* (132) conducted a study in which B cells from both healthy individuals and patients with systemic lupus erythematosus (SLE) were expanded *in vitro* through stimulation with IFN-α and CpG-C; there was notable proliferation of B10 cells while maintaining their cellular phenotype and immunomodulatory capabilities in the healthy group. Conversely, no comparable effect was observed in the SLE group (132).

Stimulation of adrenergic receptors on B cells, in conjunction with the activation of TLR9 receptors, induces the generation of B10 cells (57). A total of >80% of IL-10-producing B cells concurrently express the enzyme tyrosine hydroxylase (TH), suggesting TH as a potential biomarker for Bregs generated by TLR9 activation (57). This diverges from the perspective maintained by certain scholars regarding TIM-1 as a biomarker of B10 cells as TIM-1 is involved in the maintenance and induction of B10 cells, identifying over 70% of B10 cells (42,133).

It is unclear which optimal stimulatory signals are required to achieve maximal inhibition of Teff. It is unclear whether TLR-9 or TLR-4 stimuli are superior. Moreover, while CpG ODNs are known to stimulate TLR9, there are multiple types, and it remains unclear which type most effectively stimulates TLR9 to induce the production of Bregs. Additionally, although IL-10$^+$ Bregs are predominantly utilized for cellular therapy, selection of phenotypical markers or Bregs for therapeutic purposes is still undetermined. Overall, none of the aforementioned methods have been proven safe and effective by large-scale clinical trials and the therapeutic mechanism still needs to be clarified, which limits amplification tecniques. Exploration of novel methods and techniques to expand Bregs to serve a broader range of therapies is key.

Combining pharmacotherapy or cell-based approaches in in vitro cultivation. Certain immunosuppressants, such as mTOR inhibitors (e.g., sirolimus), abatacept (134,135), can stimulate the production of B10 cells. Besides the addition of agonists, experiments using drugs for *in vitro* expansion have been conducted (135,136). Sirolimus, an immunosuppressant drug used to prevent organ transplant rejection, amplifies Bregs in peripheral blood mononuclear cell *in vitro*, which secrete IL-10 as well as TGF-β (135). The use of astilbin, a flavonoid compound that has an anti-inflammatory activity, in combination with LPS induces the production of IL-10$^+$ Bregs by promoting STAT3 phosphorylation in TIM-1$^+$ B cells and

they suppresses the development and symptoms of dextran sulphate sodium-induced colitis (31).

GSK-3 inhibition selectively increases IL-10 production by B cells, without increasing TNF-α or IL-6, and can rescue IL-10 production even in the absence of mTOR activity following rapamycin treatment. Additionally, GSK-3 inhibition, selectively enhances IL-10 production, leading to enhanced ability of B cells to induce the differentiation of $CD4^+CD25^-$ T cells into IL-10-producing Type 1 regulatory T cells (Tr1) in coculture experiments, even in the absence of TLR activation (104,137). In addition, a recombinant fusion protein composed of the TLR5 ligand flagellin A from *Listeria monocytogenes* (rFlaA) and the major birch pollen allergen Bet v 1 (rFlaA:Betv1) promotes an increase in B10 cells via a similar mTOR agonist (138).

The addition of TLR stimulants promotes differentiation of Bregs and leads to the production of pro-inflammatory factors, so appropriate drugs are required to increase B10 cells production and functionality. In an *in vitro* experiment stimulating TLR7, the addition of Thymosin-α1 (Tα-1 can promote Breg while decreasing production of pro-inflammatory factors, but its function is dependent on the stimulation of TLR7 (139).

As dendritic cells have a proliferation-promoting effect on Bregs via type I IFNs, exploring the feasibility of their coculture is necessary (33,140). BAFF secreted by immune cells, as well as a proliferation-inducing ligand (APRIL), have a role in promoting Breg production and enhanced function by binding to BAFF receptor on the surface of the B cells, as well as to transmembrane activator and CAML interactor (TACI) (55,141-147). TACI is a receptor protein that serves a key role in the immune system. It is a member of the BAFF receptor family, binding to BAFF and APRIL, and is key in B cell activation and immune responses. Investigating BAFF receptor may provide valuable insights into the interaction between B cells and other immune cell populations. For example, in *in vitro* cell coculture experiments, the high expression of CD40L in palatine tonsil lymphocytes sustains the survival of B cells and facilitates differentiation of B cells into Bregs via BAFF receptor, ultimately leading to an increase in Bregs expressing IL-10 and PD-L1, when ratio of B cells to palatine tonsil lymphocytes is 1:1 (148). As the earliest stem cells used in clinical practice, mesenchymal stem cells (149,150) with immunomodulatory properties promote the production of Tregs and alleviate autoimmune diseases such as SLE by inhibiting the activation, proliferation, and cytokine production of B and T lymphocytes. Mesenchymal stem cells expand Bregs *in vitro* in a dose-dependent manner; this effect is increased by addition of LPS (151).

To the best of our knowledge, the efficacy of *in vitro* amplification of Bregs using novel drugs has not been compared with traditional stimulants, nor has the potential for synergistic stimulation been explored. Additionally, *in vitro* cell co-culture represents another research avenue beyond drug-mediated expansion of Bregs. This includes co-culture with tumor cells, although such approaches must address safety and ethical considerations (152).

Engineering Bregs. The purification and amplification of Bregs face limitations due to their relatively low abundance in human blood and the slow rate of *in vitro* expansion (42). The number and function of specific Bregs isolated and expanded alone may not be sufficient for clinical applications. However, modifying cells may solve this problem (153,154). As a new technological tool, cell engineering promotes the expansion of regulatory B cells through different strategies, enhancing immunosuppressive functions of Bregs more robustly. In a previous study, lentiviral transfection of B cells specifically enhanced expression of their suppressor genes, inducing the generation of cells capable of specifically secreting IL-10 *in vitro* (155). Naïve B cells, as an alternative to activated B cells, do not secrete antibodies, express low levels of co-stimulatory molecules and exhibit poor immunogenicity (155). Alonso-Guallart *et al* engineered CD40L-stimulated B cells (CD40L-sBc) by inducing the CD40L K562 leukemia cell line to produce CD40L-sBc (used as immunostimulatory antigen-presenting cells, for *ex vivo* expansion of Tregs and inhibition of pro-inflammatory factor formation, providing strategies for modulating immune responses in therapeutic applications (156). There is need to modify Bregs to produce cells with enhanced immunosuppressive functions and antigen specificity, which could improve targeted immune regulation. This approach could help treat autoimmune diseases, prevent transplant rejection, and reduce side effects of broad immunosuppressive therapies.

Chimeric antigen receptor (CAR). CAR technology is a cell immunotherapy approach primarily used in cancer treatment. This technique leverages genetic engineering to introduce specific receptor genes into T cells, enabling them to recognize and attack tumor cells (157,158). In recent years, CAR has been applied to treatment of autoimmune diseases (153). For example, CD19 CAR-T cells that specifically eliminate B cells have been studied for their potential to alleviate symptoms of SLE (159). CAR-Tregs enhance the ability of Tregs to modulate immune responses, effectively suppressing excessive immune reactions and autoimmune diseases (154,160,161).

Furthermore, with advancements in CAR-Treg (131), which involves the expression of fragment antigen-binding regions specific to alloantigens or autoantigens, robust suppression of alloimmune or autoimmune responses can be achieved (132-134). Human leukocyte antigen (HLA) is a component of the major histocompatibility complex in humans, responsible for regulating immune system response. Since the primary cause of transplant rejection is incompatibility of HLA receptors between the donor and recipient, these molecules are considered suitable targets for Tregs to enhance protection of transplanted organs (162,163). The mouse models of heart and skin transplantation have revealed that Tregs from mice carrying anti-HLA-A2 CAR significantly suppress proliferation of $CD4^+$ T cells in response to specific allogeneic antigens (160,164). Moreover, combination with immunosuppressants significantly prolongs graft survival time (160,165). In this context, exploration of CAR-Breg holds promising prospects for future research. The integration of CD19, CD40, BCR and TLR components may facilitate the development of an effective CAR-Breg design. By designing CAR-Bregs targeting transplant rejection-related markers such as HLA-G, it may be possible to enhance immunosuppressive functions (166). The design of CAR-Bregs aims to amplify the regulatory functions of B cells, particularly in inhibiting abnormal immune response and promoting immune tolerance.

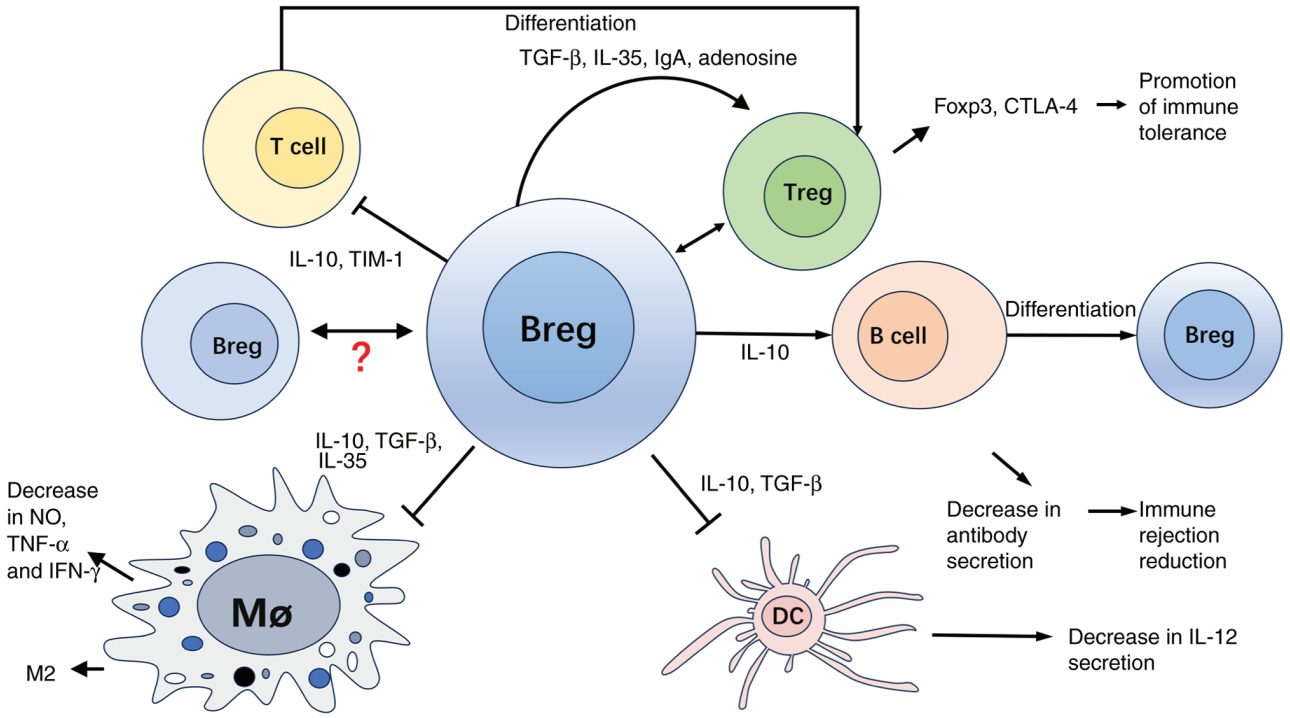

Figure 3. Expanded Bregs *in vitro* may play a role in surrounding immune cells and microenvironment by secreting cytokines and cell-to-cell contact and inducing graft tolerance. Following adoptive transfer, Bregs secreted cytokines such as IL-10 and engaged in intercellular interactions to inhibit pro-inflammatory Teff cells. Additionally, Bregs promoted the differentiation of B cells into Bregs, macrophages (Mø) into the M2 inhibitory phenotype, and T cells into Tregs, leading to increased expression of Foxp3 and CTLA-4. Moreover, TGF-β, an anti-inflammatory cytokine secreted by Bregs, supported the survival of these anti-inflammatory cells. Collectively, these mechanisms contribute to establishment of an immune-tolerant environment in transplant recipients. TIM, T-cell immunoglobulin and Mucin-domain-containing molecule; Breg, regulatory B cell; Treg, regulatory T cell; M2, M2 macrophage; Mø, macrophage; CTLA, cytotoxic T-lymphocyte antigen; DC, dendritic cell; Foxp, forkhead box protein.

This highlights the potential of CAR-Bregs for enhancing immune regulation in clinical settings. Currently, the design of CAR-Bregs remains largely theoretical, and there is need for further breakthroughs regarding potential application in transplantation immunization.

4. Applications of cellular therapy in transplantation

Immunotherapy and *in vitro* expansion techniques are complementary. Cellular therapy, originating in tumor treatment, involves transplanting or infusing normal or bioengineered human cells into a patient, where the newly introduced cells replace damaged ones (167,168). With the continuous development of cellular therapies, adoptive regulatory cell therapy has been recognized as a potential strategy to enhance graft tolerance (169-171). The numbers of Bregs in tolerant patients are similar to those in healthy individuals, whereas Breg populations are impaired in patients with chronic rejection (172,173). Bregs serve an important role in maintaining organ transplantation tolerance. Thus, adoptive Breg therapy may provide a new immunomodulatory therapeutic technique aimed at fostering transplantation tolerance by interacting with other immune cells (Fig. 3). However, research in this area remains largely in the preclinical stage, facing challenges such as insufficient expansion of regulatory cells and low efficiency (168).

Cell therapy in transplantation. B cells are involved in development of immune tolerance in transplant recipients. A previous study have shown that deletion of B cells in transplant patients using CD20 antibodies results in acute rejection, which is most likely due to the elimination of Bregs (174). Although there is evidence that Bregs play a central role in the induction and maintenance of tolerance in various immunopathologies, tolerant kidney transplant patients exhibit an increase in B cell numbers *in vivo* (175). However, due to interspecies differences, the predictive value of animal models is limited, highlighting the need to regulate both the quantity and functional activity of B10 cells. The *in vitro* expansion of human Bregs is challenging due to the rarity of Bregs in peripheral blood, the complex and specific stimulatory signals required for their differentiation, and difficulties in maintaining their functional properties, such as IL-10 production (175). Additionally, certain phenotypes are exclusively expressed in humans, which necessitates targeted strategies for the expansion of specific human Breg phenotypes.

During formation of immune tolerance in transplant recipients, B10 cells serve a major role. In our previous study on an immune tolerance model for islet transplantation in diabetic mice (21), an increase in B10 cell levels *in vivo* was associated with disease alleviation. Similar findings were noted in on graft-vs.-host disease (GVHD) and kidney transplantation (176,177), where increased levels of B10 cells are linked to improved disease outcomes. These results suggest that B10 serve a key role in immunoregulation during transplantation.

Following *in vitro* expansion, B10 cells can be reinfused into transplant recipients to extend graft survival time. Shankar *et al* (42) reported adoptive transfer of human-derived Bregss after *in vitro* expansion into mice,

extending the survival time of skin grafts. By contrast, a previous study focused on murine Breg cell expansion *in vitro* (42). Mouse model studies have demonstrated that the adoptive transfer of B cells with induced regulatory function can effectively mitigate the disease process (11,177-182). For example, in a murine cardiac transplantation model, antibody-induced tolerance is disrupted following B cell depletion and is associated with IL-10^{+} B cells (22,183). In a murine model of GVHD, allogeneic bone marrow transplantation (BMT) followed by adoptive transfer of B10 cells revealed that GVHD is more severe in mice transplanted with CD19$^{-/-}$ donor cells compared with those receiving CD19$^{+/+}$ cells; this is associated with a significant increase in CD8^{+} T cells, as well as TNF-α- and IFN-γ-producing CD4^{+} T cells in the spleen 14 days post-BMT. However, when B cells are cultured *in vitro* with PMA, ionomycin, LPS and brefeldin A to generate B10 cells, adoptive transfer of these cells into CD19$^{-/-}$ donor mice resultes in a significant improvement in GVHD severity, with a marked reduction in skin scores at time of transplantation compared with 14 days post-transplant (176). In a model of allogeneic cardiac transplantation, transplant tolerance induced by anti-CD40L mAb is disrupted following treatment with anti-CD20 mAb. However, adoptive transfer of B10 from IL-10-sufficient littermate mice rescues graft survival. Compared with the adoptive transfer of B cells from IL-10$^{-/-}$ or IL-10-deficient mice, this approach significantly prolongs cardiac graft survival beyond 40 days and notably decreases histopathological rejection scores in allogeneic transplants (22). Further transplantation experiments in small animal models are needed to validate the feasibility and safety of the adoptive transfer of B10 cells in transplantation across different organ sites.

In clinical investigations, it has been hypothesized that B10 cells negatively regulate transplant immunity following solid organ transplantation and adoptive transfer of autologous B10 cells could serve as a novel therapeutic approach for transplant recipients to manage persistent chronic rejection (184). Shankar (42) demonstrated in a humanized mouse model of skin transplantation that *in vitro* stimulation with CD154 and expansion of human IL-10^{+} B cells significantly prolongs graft survival (>80 days). Notably, the frequency of B10 cells in the graft and spleen markedly increase and production of TNF-α and IFN-γ by CD4^{+} T cells is significantly suppressed. These findings indirectly support the potential of adoptive transfer of B10 to improve human solid organ transplantation outcomes, providing a basis for further clinical translation (42).

Adoptively transferred B10 cells alter induction and localization of T follicular helper and regulatory cells *in vivo* and secrete the anti-inflammatory cytokine IL-10. These effects lead to an indirect increase in the number and function of Tregs in the recipient, thereby modulating immune response and inflammation to exert anti-inflammatory effects, which extends graft survival time (183,185). Although B10 exhibits a broad-spectrum effect, in islet transplantation experiment, stimulating B cells *in vitro* with pro-inflammatory factors generates regulatory cells, some of which secrete TGF-β (125). Bregs that secrete TGF-β significantly prolong graft survival compared with B10, suggesting phenotypes of Bregs are adapted to specific transplanted organs (114,125).

Potential problems and improvement strategies. There are numerous issues and challenges of Breg therapy. One issue is the difficulty in expanding Breg cells *in vitro* (42), with improvements in expansion efficiency being a major focus of ongoing research. Addressing this challenge requires continuous exploration of novel stimulation methods, including adjustments to duration and types of stimulants employed (68,114). In transplantation, B10 cell therapy requires identification of the most suitable *in vitro* expansion methods to balance both quantity and quality. Additionally, maintaining purity and activity of Bregs is challenging (42), as prolonged culture periods can result in phenotypical instability, which may compromise therapeutic efficacy. This necessitates the exploration of standardized expansion protocols to achieve a stable B10 cell phenotype. Moreover, there is an increased risk of infections and tumor development associated with cell therapy, particularly in immunosuppressed patients. This underscores the need for large-scale animal studies to ensure the safety and efficacy of cell therapy. Furthermore, to enhance therapeutic effects, there is need for further exploration of effective delivery routes and targeted technologies to enable Bregs to reach disease sites directly; this may be facilitated by CAR technology (125). Based on patient conditions and the organ transplanted, different B10 cell phenotypes may need to be reinfused, allowing development of personalized treatment plans.

In summary, adoptively transferred B10 cells balance Treg/Teff dynamics *in vivo*, resulting in prolonged organ or tissue transplants survival times. Ongoing exploration of endogenous Breg expansion and harvesting, optimization of *in vitro* expansion methods and improvement of *in vivo* injection methods provide a basis for the theoretical and practical use of Breg therapy in the clinical phase.

5. Conclusion

Bregs serve a crucial immunosuppressive role in promoting the long-term survival of grafts (42). Various methods have successfully achieved efficient *in vitro* expansion of certain Breg phenotypes and there is a relatively comprehensive understanding of the signaling pathways involved in *in vivo* expansion mechanisms (36,139). However, the development of Breg therapy is still hindered by several challenges, including lack of clear definitions for specific cell phenotypes, inadequate *in vitro* expansion technologies, the absence of standardized methodology and no definitive conclusions regarding the types of stimulant to be utilized (42,57,133).

Enhancing *in vitro* expansion technologies be facilitated by studying and simulating Breg expansion under *in vivo* inflammatory and tumor conditions (67,119,186). Future advancements in Breg expansion strategies should integrate both *in vitro* expansion and *in vivo* facilitation to maximize cell yield and therapeutic effectiveness for the patient. This may involve the combined action of pharmacological agents or microorganisms (140,187).

Beyond transplantation immunology, expansion technologies hold potential for the treatment of autoimmune diseases and other immune-associated disorders. Individualized treatment approaches could emerge by expanding Bregs tailored to meet specific immunomodulatory needs of patients. Different

stimulation methods yield distinct subtypes of Breg cells, paving the way for their application in a variety of diseases to fulfill specific therapeutic roles. Additionally, these technologies serve as valuable research tools to elucidate the biological properties and functional mechanisms of Bregs.

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Authors' contributions

GZ and DZ conceived the study and wrote the manuscript. GH constructed figures. YuY and YC revised the manuscript. YiY, GH, YuY, GZ, DZ and YC edited the manuscript. Data authentication is not applicable. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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