

Regulatory B Cells: Dark Horse in Pregnancy Immunotherapy?

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Abstract

There are many unanswered questions surrounding the function of immune cells and how they interact with the reproductive system to support successful pregnancy or contribute to pregnancy pathologies. While the role of immune cells such as uterine natural killer and dendritic cells, and more recently regulatory T cells has been established, the role of another major immune cell population, the B cell, and particularly the regulatory B cells, is relatively poorly understood. This review outlines what is known about B-cell subsets in the context of pregnancy, what constitutes a regulatory B cell and what role they may play, particularly during early pregnancy. Lastly, we discuss why immunotherapies for the treatment of pregnancy disorders is not widely progressed clinically and speculate on the potential of functional regulatory B cells as the basis of novel immunotherapeutic approaches for the treatment of immune-based pregnancy pathologies.

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Introduction

In pregnancy, the maternal immune system is required to perform a paradox of functions; it must be immunologically tolerant to the growing semi-allogeneic fetus while maintaining the capacity to respond with effector immunity upon recognition of foreign pathogenic antigens. A healthy pregnancy thus requires complex interactions between fetal and maternal cells and tissues, to ensure appropriate immunological recognition and response. Ongoing efforts to define the immune interactions during pregnancy are focused on understanding the functional role of the various immune, endometrial, and fetal cells, as well as elucidating the complex cross talk between them. Knowledge of these underlying mechanisms is critical to the design of therapies to treat pregnancy pathologies, particularly since many studies have repeatedly shown that immune dysfunction contributes to pregnancy disorders, such as recurrent pregnancy loss, preeclampsia, and immune-based infertility [1–4].

Changes in the immune profile necessary to support a healthy gestation have been well documented for some innate and adaptive immune cell types and include suppression of T effector cells specific for foreign fetal antigens, generation of regulatory T cells (Tregs), and modulation of natural killer (NK), macrophage, monocyte, and dendritic cell function [5,6]. Historically, the other major immune cell involved in adaptive immunity, the B cell, has not been given equal attention with regard to its role in pregnancy as early studies primarily explored the traditional function of B cells as producers of antibodies, and only hinted at the fact that B cells may participate in other multi-faceted ways during pregnancy. Studies of other diseases have expanded our view of B-cell function and it is now widely accepted that B cells exhibit functional promiscuity, from operating as agents of adaptive immunity to performing innate-like suppressive functions [7,8].

In recent years, a subgroup of B cells, the regulatory B cells or Bregs, have emerged as potential key players in immunological tolerance. Bregs, so named due to their capacity to negatively

regulate other immune cell types, have been extensively studied in the fields of autoimmunity, transplantation tolerance, and cancer, with the consensus that despite their low numbers, Bregs can potentially modulate immune responses [9]. While knowledge of the nature and mechanism of action of Bregs in the context of pregnancy remains limited, their importance has been revealed by seminal studies that showed that Bregs can restore maternal immune tolerance and therefore support healthy pregnancies in mouse models of early abortion [10–13]. Furthermore, clinical studies have established a correlation between Breg impairment and poor pregnancy outcomes such as repeated implantation failure (RIF) and recurrent pregnancy loss [3,14–16]. Thus far, there is mounting evidence suggestive of a probable pivotal role of Bregs within the pregnancy immune network, which can potentially be harnessed into a form of immunotherapy to treat pathologies of pregnancy. Current treatment modalities addressing unexplained repeated pregnancy loss such as lymphocyte immunization, trophoblast membrane immunization, and intravenous immunoglobulin (IVIg) immunization have reportedly failed to improve obstetric outcomes [17]. Therefore, the development of an effective targeted immunotherapy for the improvement of pregnancy outcome is an unexplored avenue of research.

In this review, we will highlight the latest research exploring Breg cell contribution to modulation of maternal immune tolerance during pregnancy. Furthermore, we will speculate on the potential capacity of Bregs to be used as an immunotherapeutic agent for averting or treating pregnancy pathologies and complications.

B cells and pregnancy

A successful pregnancy is governed by a maternal immune system that can effectively maintain an appropriate immune response across all stages of conceptus development. Compared to other immune cell subsets, little is known about the role of B cells in pregnancy. However, what studies have been done collectively point toward an active engagement of B cells during gestation, especially where abnormalities in B-cell subsets were related to infertility and poor obstetric outcomes, such as repeated pregnancy loss and preterm labor [2,3,11]. Moreover, recent studies using mouse models of pregnancy have shown that alterations in B-cell surface marker expression as well as differences in cellular function can affect the health and well-being of pregnancy [18–20].

Humoral immunity in pregnancy

Traditionally, B-cell function is primarily associated with humoral immunity and as such B cells were

thought to play a role in pregnancy by virtue of antibody production. Experiments conducted during the 1970s elegantly showed that the immunoglobulin G (IgG) fraction of maternal serum prevented maternal effector T cells from delivering a functional cytotoxic response against allogeneic trophoblast tissue [21]. Moreover, activation of spontaneous humoral responsiveness during pregnancy as demonstrated by an increase in the levels of both IgG- and IgM-secreting cells in the uterine draining lymph nodes was reported early on as one of the possible suppressive mechanisms in place during pregnancy [22]. More recently, murine and clinical studies provided supporting evidence, featuring significantly enhanced serum levels of IgM and IgA in pregnant individuals compared to non-pregnant controls [12,16,23]. An elegant study expounding the human memory B-cell phenotype during pregnancy has likewise shown that the level of expression of the memory marker CD27, which markedly increases during gestation, has a direct impact on the profile of immunoglobulin isotypes being produced, with upregulated CD27 expression associated with increased IgG and IgA isotype production [24]. Lastly, splenic B cells from a murine model of pregnancy with poor gestational outcome have been reported to deliver a significantly higher titer of IgG isotypes, as well as IgA and IgM, relative to titers obtained from mice with good gestational outcome [20]. Thus far, activation of the B-cell humoral response leading to antibody production is thought to be a necessary step toward achieving a healthy pregnancy.

The 1970s was also the period during which a subset of IgG called ‘asymmetric antibodies’ was discovered. Asymmetric antibodies are IgG possessing a mannose-rich oligosaccharide residue bound to one Fab region, thus rendering them incapable of activating immune effector functions such as complement fixation, phagocytosis, and cytotoxicity [25]. In the context of pregnancy, serum from pregnant women displayed higher titers of asymmetric antibodies compared to non-pregnant women, most with paternal antigen specificity [26,27]. Conversely, women experiencing recurrent spontaneous abortions had significantly lower proportions of asymmetric antibodies in serum compared to healthy pregnant women [27,28]. Asymmetric antibodies were therefore suggested to have a protective role during pregnancy and referred to as protective maternal antibodies; however, the mechanisms underpinning this response have not been pursued in subsequent preclinical nor clinical studies.

At the other end of the spectrum, a group of antibodies called autoantibodies have been implicated in pregnancy failure. The majority of these studies have investigated the impact of antiphospholipid (aPL) antibodies and agonistic autoantibodies against the angiotensin II type I receptor [29–31]. High levels of aPL have been implicated in the etiology of

antiphospholipid syndrome, an autoimmune disease characterized by a hypercoagulable state that induces systemic blood clotting (thrombosis). In pregnancy, antiphospholipid syndrome leads to compromised reproductive outcomes such as miscarriages, fetal loss, and severe preeclampsia [29,32,33]. In addition, angiotensin II type I receptor autoantibodies have also been reported to have a significant role in the pathology and possible initiation of preeclampsia. These agonistic autoantibodies mimic the natural ligand of the angiotensin type I receptor and induce downstream production of anti-angiogenic factors s-Flt1 and Endoglin, which lead to the onset of pre-eclamptic symptoms such as hypertension, proteinuria, glomerular endotheliosis, resulting in placental abnormalities, and embryonic defects [34,35].

Spatiotemporal dynamics of B-cell subsets during pregnancy

Variations in the proportions of maternal B cells and changes in B-cell subset distribution have been shown to occur during pregnancy. The earliest studies describe a general diminution of B-cell numbers during gestation and the conditional responsiveness of B cells toward endocrine factors such as estrogen and human chorionic somatomammotropin [36,37]. The depression in B-cell frequency was investigated further in murine models of allogeneic pregnancy where studies have shown that numbers of pre/pro and immature B cells in the bone marrow, as well as splenic follicular B cells, are significantly reduced mid-gestation resulting in systemic B-cell lymphopenia during the latter half of pregnancy [12,23,38]. Clinically, percentages of circulating CD19-positive B cells in low-risk pregnant women are significantly lower during the second and third trimesters, and on delivery day, compared to non-pregnant women [39–42]. Further characterization identified the specific B-cell subsets undergoing lymphopenia during human pregnancy; these included IgD⁺CD38^{hi} transitional B cells, CD24^{hi}CD38^{hi} transitional B cells, IgD⁺CD38[–] unswitched memory B cells, IgD[–]CD38[–] resting memory B cells, IgD[–]CD38^{hi} plasmablasts, and CD5⁺ B cells [16,40,43,44]. Aside from the general repression of lymphopoiesis mid-gestation, the decrease in circulating B cells from mid-pregnancy onwards has been reported to be caused in part by the recruitment to and retention of B cells in the growing placenta. Histological examination of the immune infiltrate found in first trimester placenta shows that B cells are extant in the decidua of normal pregnancies and significantly increased in decidual tissue exhibiting hydatidiform moles [45]. A recent clinical study has also shown that placental intervillous blood is rich in the chemokine ligand CCL20, which attracts mature naïve B cells that express the non-promiscuous receptor CCR6 [46].

It is speculated that increasing B cells within the placental tissue may contribute to the safe-guarding of term pregnancies *via* production of progesterone-induced blocking factor 1 (PIBF1) [39,46,47].

In contrast, certain B-cell subsets are reported to expand, at least during murine gestation, and include splenic marginal zone B cells, peritoneal B1 and B2 cells, B cells located within the uterine-draining lymph nodes, and B cells within the uterus itself [12,38,48]. Likewise, percentages of IgD⁺CD38⁺ naïve B cells in peripheral blood were expanded during late human pregnancy [40]. Moreover, an increase in the production of natural antibodies IgM, IgA, IgG3, and IgG4, as measured in the serum of pregnant individuals compared to non-pregnant controls, was reported in both murine models and human samples [16,23].

Alterations in the normal distribution of B cells have been associated with infertility and recurrent pregnancy loss. Analysis of the uterine immunophenotype in patients with poor reproductive histories reveal significantly higher percentages of CD20⁺ B cells in the endometrium of those that exhibited recurrent miscarriages, compared to control patients [2,49]. Likewise, increased peripheral blood CD19⁺ B cells with low levels of switched (CD27⁺IgD[–]) and non-switched (CD27⁺IgD⁺) memory B cells were observed in a patient with a history of recurrent pregnancy loss and obstetric complications [3]. Conversely, a significant decrease in the percentage of peripheral CD19⁺IL-10⁺ B cells, as well as in the mRNA levels of IL-10⁺ B-cell related genes (IL-10 and PD-L1), was seen in patients with RIF [14]. Thus far, studies on B cells and their causal or contributing relationship to pregnancy pathologies are limited and warrant further investigation.

Bregs: emerging role in pregnancy

Bregs are currently defined as any B-cell subset that exhibits immunosuppressive function. As negative regulators of immune cells, Breg-targeted or -based cell therapies are being explored for mediation of autoimmune diseases, induction of transplantation tolerance, and induction of allergy tolerance, where the underlying pathology is attributed to inadequate control of an overactive immune response [50–54]. At the other end of the spectrum, expanded populations of Bregs have been correlated with poor prognosis in cancer and infectious disease outcomes, as too much suppression can tip the immune response in favor of tumorigenesis or failed pathogen control [51,55,56]. Hypothetically, in the context of pregnancy, Bregs are uniquely positioned to perform the dichotomy of function required for a successful gestation. Mechanisms for the induction of successful maternal immune tolerance may parallel that already known about the role

Table 1. Regulatory B-cell subsets identified in mice and/or humans during pregnancy

Species	Phenotype	Mechanism	Reference
Mouse/human	IL-10 ⁺ B cells	IL-10-mediated protection	[10,13]
Mouse	CD5 ⁺ CD1d ⁺ B10 cells	IL-10-mediated protection	[11]
Human	CD24 ^{hi} CD27 ⁺ B10 cells	IL-10-mediated protection	[15,58,59]
Mouse	CD80 ⁺ CD86 ⁺ B cells	Co-stimulatory effect of CD80 and CD86	[18,38]
Mouse	CD80 ⁺ CD86 ⁺ CD27 ⁺ IL-10 ⁺ B cells	IL-10-mediated protection	[38]
Mouse/Human	IL-35 ⁺ Bregs	IL-35-mediated protection <i>via</i> activation of STAT family	[13,58]
Mouse	PIBF1 ⁺ choriodecidual B cells	IL-33-mediated protection against pre-term labor	[47]
Human	CD24 ^{hi} CD38 ^{hi} transitional B cells	Reduction of pro-inflammatory cytokines production;	[40,58,60]
	CD27 ⁺ IgM ⁺ memory B cells	IL-10 production; counteract detrimental T-cell-mediated inflammation	
	CD38 ^{hi} CD27 ^{hi} plasmablasts		

of Bregs in various autoimmune diseases and transplantation tolerance. Likewise, mechanisms that accommodate the growing semi-allogenic fetus may be similar to the pro-tumor/pro-pathogen mechanism of Bregs in cancer and infectious diseases [57]. Therefore, in principle, Bregs are likely to have a major role in orchestrating the immune interactions required for a successful pregnancy.

Breg phenotypes associated with pregnancy

Unlike Tregs, which are uniquely identified by expression of the key transcription factor Foxp3, Bregs do not have an equivalent unifying 'marker' common to all B-cell subsets of varying phenotypes that exhibit regulatory properties. This may be attributed to the evolutionary development of Bregs as well as the plasticity of B-cell function. The molecular architecture and the differential function of B-cell phenotypes are heavily influenced by the external milieu and the presence of foreign antigens [7]. Therefore, in the dynamic scenario of pregnancy, where expression of paternal antigens increases over time, it is reasonable to expect corresponding responsive changes in the B-cell architecture, to support adaptation of the maternal immune response to pregnancy.

B-cell subsets that have been recognized as Bregs in murine pregnancy include IL-10⁺ B cells, CD5⁺CD1d⁺ B cells, CD80⁺CD86⁺ B cells, CD80⁺CD86⁺CD27⁺IL-10⁺ B cells, IL-35⁺ B cells, and PIBF1⁺ choriodecidual B cells. In humans, identified Breg subsets in pregnancy are IL-10⁺ B cells, CD24^{hi}CD27⁺ B cells, CD24^{hi}CD38^{hi} transitional B cells, CD27⁺IgM⁺ memory B cells, CD38^{hi}CD27^{hi} plasmablasts, marginal zone B cells, and IL-35⁺ B cells (Table 1). In both mice and humans, the capacity for IL-10 production, and to a lesser extent TGFβ, remains a hallmark for Breg identification, although additional markers such as production of cytokine IL-35 and protein PIBF1 have been reported to instill a regulatory capacity in B cells during pregnancy [13,47,58]. The inherent difficulty of precise identification of Bregs by cytokine production has led to the use of surrogate markers and include CD5⁺CD1d⁺ for IL-

10-producing B cells (B10 cells) in mice, and CD24^{hi}CD27⁺ for the human equivalent [61,62].

Marginal zone (MZ) B cells that are expanded in the spleen of normal pregnant mice were found lacking in mice with pregnancy complications, whereas successful pregnancy correlated with the capacity of MZ B cells to produce enhanced levels of immunoglobulin (IgM and IgA), which likely alters the immune response from a Th1-like to a Th2-like profile. Recently, IL-10-producing uterine B cells were shown to exhibit upregulated expression of the co-stimulatory molecules CD80 and CD86 during murine pregnancy [38]. Moreover, splenic CD19-positive B cells in normal pregnant mice exhibited significantly upregulated expression of co-stimulatory molecule CD86 suggesting that B cells have the capacity to modulate Treg abundance and cytokine production, factors that could be crucial in determining pregnancy outcomes [18,63,64]. Interestingly, the increase in CD80 and CD86 expression in both the uterine B cells and splenic B cells takes place during the pre-implantation period of pregnancy, suggesting their potential involvement in establishing the immune conditions for successful implantation. IL-35-producing Bregs in the spleen, uterine-draining lymph nodes, and peripheral blood were demonstrated recently as making a vital contribution to establishing maternal/fetal tolerance and maintaining a healthy pregnancy [13,58]. Lastly, choriodecidual B cells have also been identified as possessing regulatory qualities *via* IL-33-mediated PIBF1 production. These cells showed an ability to protect against pre-term labor, by production of PIBF1 in late gestation, which was boosted by therapeutic delivery of IL-33 [47]. Moreover, they displayed higher expression of the activated memory B-cell or plasma cell-associated molecules: CD11c, CD27, CD38, CD70, CD80, CD86, CD95, CD138, and B-cell maturation antigen, suggesting that choriodecidual B cells exhibit a higher activation status, class switching, memory, and plasmacytoid differentiation.

In humans, IL-10-producing B-cell populations express increased levels of CD24, CD38, IgM, and intermediate expression of CD27, for which combinations of these surface markers identify a range of

B-cell subsets including the CD24^{hi}CD27⁺ B10 cells, CD24^{hi}CD38^{hi} transitional B cells, CD27⁺IgM⁺ memory B cells, and CD38^{hi}CD27^{hi} plasmablasts [15,58,60].

Endocrine and molecular factors associated with Breg function in pregnancy

Endocrine stimulation through the primary sex hormones estrogen and progesterone has been shown to cause immune cells to adopt phenotypes that support fetal tolerance. Pioneer studies demonstrated that B lymphopoiesis is subject to negative regulation by pregnancy-related hormones estrogen and progesterone [23,36,57,65,66]. However, the relationship between these hormones and Breg function in pregnancy remains an active research pursuit. Estrogen has been demonstrated to confer protection against autoimmune diseases by expanding the population of splenic B10 cells *via* the PD-1/PD-L1 pathway [67–69]. Further, estrogen-receptor positive B cells were shown to respond well to estrogen stimulation and perform Breg functions such as upregulation of Treg number and function in experimental autoimmune encephalomyelitis [68]. An analogous mechanism for estrogen-mediated activation of Breg function may be in place during pregnancy. Progesterone, on the other hand, has been implicated in the promotion of a Th2-dominant immune profile by suppressing B-cell antigen expression and increasing production of protective asymmetric antibodies during pregnancy [70]. A close link between progesterone and Breg function is suggested in parallel studies in cancer and autoimmune diseases where it was demonstrated that progesterone treatment leads to inhibition of T-cell proliferation, production bias of anti-inflammatory cytokines over pro-inflammatory cytokines, and increase in IL-10 production concurrent with expansion of B cells [71–73]. Interestingly, a recent study featured progesterone in the induction of higher CD83 expression in B cells, a proposed novel pathway for establishing fetal tolerance [74]. Collectively, these studies suggest involvement of progesterone in the regulatory mechanisms activated during pregnancy.

The blastocyst is a rich source of hormones, cytokines, chemokines, and other soluble factors that can heavily influence the immune profiles within the pregnancy microenvironment. Cross-talk between the outer trophoblast cells and immune cells has been shown to result in the recruitment of immune cells, education of the immune cells to a specific phenotype, and the subsequent modification of immune cell function [75]. Furthermore, soluble factors produced by trophoblast cells can educate human B cells toward the acquisition of regulatory properties that protected against the detrimental effect of excessive T-cell inflammation on trophoblast cell migration, an essential process for subse-

quent implantation [60]. While the exact molecular factors influencing this phenomenon remain unknown, it is likely that hormones and cytokine signaling play a crucial role.

At the onset of pregnancy, trophoblast cells secrete copious amounts of chorionic gonadotrophic hormone (hCG) to aid growth and development of the embryo. Formerly only recognized as the stimulant to progesterone production in early pregnancy, the hCG functional repertoire has now been expanded to include angiogenic and vasculogenic roles as well as being a potent activator of multiple immune cell types, including Bregs [76,77]. Seminal murine studies have demonstrated that hCG can suppress the proliferation of splenic B cells, while simultaneously inducing the generation and expansion of IL-10-producing B cells, and promoting the production of pregnancy-protective asymmetric antibodies [59]. These studies indicate that hCG provides endocrinal support for the induction and expansion of Breg subsets during pregnancy.

The cytokine IL-35 is a member of the IL-12 family that participates in downstream signaling pathways that positively or negatively regulate the immune system. Studies of autoimmune diseases have established that Breg cells can produce IL-35 and that recombinant IL-35 (rIL-35) can induce Breg cells to secrete IL-10 and IL-35 [78,79]. In pregnancy, there is an emerging view of a similar regulatory role, as IL-35 has been shown to induce the expansion of IL-10⁺ B cells as well as convert splenic CD19⁺ B cells into IL-35⁺ Bregs in both humans and mouse models [13,58], with diminished levels of circulating IL-35-producing B cells associated with spontaneous abortion [13]. Like hCG, human placental trophoblast cells are a potent source of IL-35 during pregnancy [80].

In the same vein, the pro-inflammatory cytokine IL-21 has been examined as a mediator for Breg activation and induction across different fields of research. Recent reports on autoimmune diseases, organ transplantation, and cancer collectively demonstrate the ability of IL-21 to induce the differentiation and expansion of IL-10⁺ or Granzyme-B⁺ Breg populations capable of suppressing effector T-cell function [81–83]. In pregnancy, IL-21 responsiveness is shown to influence B-cell function, thus contributing in the determination of pregnancy outcome. Splenic B cells in a normal murine pregnancy have downregulated expression of the IL-21 receptor, favoring induction of a Th2 milieu and protective phenotype. In contrast, splenic B cells from mice with disturbed pregnancies did not downregulate IL-21 receptor expression, and upon inflammatory challenge exhibited an increase in cell death and apoptosis, decreased IL-10 expression, and higher proportions of T follicular helper cells that contributed to the generation of a Th1 type milieu and impact on pregnancy outcome [20].

CD83 is a type I transmembrane glycoprotein that was initially described as a highly specific maturation

marker for activated human dendritic cells, but is now identified on many activated immune cells and thought to play a role in controlling active immune responses [84]. Assessment of CD83 expression on B cells have previously revealed that overexpression induced an increased MHCII and CD86 expression as well as augmented IL-10 production upon activation, alluding to a regulatory phenotype [85]. The regulatory role of CD83⁺ B cells is currently being investigated as a novel pathway of fetal tolerance and protection during pregnancy. In a murine model of allogeneic pregnancy, membrane-bound CD83 on B cells has been shown to be a contributing factor for the establishment of immune tolerance, as evidenced by its upregulated expression in major splenic B-cell populations and in the uterus-draining lymph nodes during pregnancy. Further, these CD83-expressing B cells subsequently release soluble CD83 upon activation that leads to the inhibition of T-cell cytokine production [74]. Interestingly, a disturbed pregnancy with prevailing pro-inflammatory conditions has been shown to exhibit an altered level of expression of membrane bound and soluble CD83 on B cells. Poor pregnancy outcome was correlated to reduced serum levels of soluble CD83 and higher membrane-bound CD83 expression on splenic B cells compared to female mice with good reproductive outcome [19]. These studies suggest that CD83 expression has a direct impact on the regulatory capacity of B cells during pregnancy and may be one of the mechanisms in place to ensure B cells develop a fetal-tolerant phenotype.

An elegant study investigating molecular signaling during pregnancy has shown that adaptive immune cell subsets, including B cells, are specifically enhanced during gestation and have a higher response to stimulation from a combination of extracellular cytokines to elicit receptor-specific intracellular response (IL-2, IL-6, GM-CSF, and IFN- α 2A) [86]. For B cells in particular, this may be due to higher transcriptional activation resultant of elevated levels of transcription factors phosphorylated signal transducer and activator of transcription 3 (p-STAT3) and p-STAT5 [86]. Interestingly, therapeutic delivery of hCG and/or IL-35 leads to an expansion of Breg cells, which in turn has also been attributed to increased transcriptional activation of the STAT protein family. Treatment with hCG led to the phosphorylation of STAT3 while treatment with IL-35 activated both STAT1 and STAT3 in splenic B cells [58]. The activation of STAT family transcription factors has been previously demonstrated to be associated with IL-10 production in B cells [87]; therefore, it is likely that molecular influences that are geared toward converting B cells into Bregs would affect this signaling pathway. However, the molecular mechanisms underpinning Breg generation and expansion remain poorly understood and warrant further investigation.

Breg function in pregnancy

The regulatory role of Bregs during pregnancy has yet to be fully explored. However, in studies performed to date, several recurring themes are apparent; (1) reduced levels and/or dysfunction of circulating Bregs may indicate poor obstetric outcomes, (2) Bregs may be involved in fostering the appropriate conditions necessary for implantation, and (3) Bregs can restore fetal tolerance in conditions afflicted with immune-mediated pregnancy complications.

Pioneering studies in mice have definitively shown that reduced frequencies of Breg cells during early pregnancy are associated with spontaneous abortion [11,15]. Repopulation of this cell subset by passive transfer of splenic CD5⁺CD1d^{hi} B10 cells rescues these pregnancies from failure by inhibition of dendritic cell maturation and expansion of Treg cell populations [11]. Similarly, transfer of B10 cells, but not IL-10-negative B effector cells or B cells from IL-10-deficient mice, prevented fetal loss induced by LPS-driven inflammation, by reducing IL-17A and IL-6 production by T cells and expanding the Treg population [10]. In humans, IL-10-producing CD19⁺CD24^{hi}CD27⁺ B cells in peripheral blood are significantly higher in women undergoing normal pregnancies as opposed to non-pregnant women, or women who suffer spontaneous abortions [15,58]. Furthermore, CD19⁺CD24^{hi}CD27⁺ Bregs isolated from peripheral blood taken from women in their first trimester of pregnancy could successfully inhibit TNF secretion by activated T effector cells *ex vivo* [15].

Recent studies in mice have demonstrated the participation of Bregs during the peri-implantation period of pregnancy. Breg-associated markers TLR9 and CD86 are upregulated on splenic B cells 3.5 days post-copulation, while significant expansion of uterine B cells and alterations in the B-cell phenotype were observed from 2.5 to 8.5 days post-copulation [18,38]. Moreover, these uterine B cells, enriched with subsets of CD80⁺CD86⁺ B cells and IL-10⁺ B cells, were effective in suppressing the proliferation and activation of syngeneic CD4⁺ effector T cells [38]. The modifications in B-cell phenotype may well be a result of trophoblast-education, presumably in the earliest phase of pregnancy [60]. Lastly, impairment in IL-10 production by B cells has been reported as one of the key pathological mechanisms in RIF in humans, as peripheral B cells from women who have experienced RIF possessed lower IL-10 mRNA levels and secreted significantly lower levels of IL-10 upon *ex vivo* restimulation, relative to healthy controls [14]. These studies suggest a beneficial role for Bregs in appropriate embryo implantation during early pregnancy.

Although limited in number, these studies highlight the important regulatory role that Bregs play in pregnancy, and parallel that observed in autoimmune

disease, graft tolerance, and cancer. In all conditions, Bregs aid in control of the tissue environment *via* induction and maintenance of Tregs, modification of the helper T-cell response, and inhibition of effector cell responses, including by cytolytic T cells, NK cells, and dendritic cells.

Immunotherapy and pregnancy

Treatments for many pregnancy pathologies are often broad in nature and limited due to insufficient knowledge of the immune mechanisms at play, as well as the complex interactions between the circulatory, endocrine, and immune systems. For example, prescription for treatment of recurrent miscarriage relies solely on the general immunomodulatory effects of certain substances; aspirin for its anti-inflammatory effect, anticoagulants for their anti-inflammatory and anticoagulant effect, and steroids for their anti-inflammatory effect [88]. Early attempts at personalized immunotherapy for treatment of recurrent pregnancy loss include paternal leukocyte immunization wherein the paternal parent's mononuclear cells are injected into the mother in an attempt to change the balance of Th1 and Th2 cytokines to suppress any maternal T-cell activity against paternal antigens. Other forms of immunotherapy that have been tested in clinical trials including donor white cell immunization, trophoblast membrane immunization, and IVIg immunization. However, the latest analysis has shown that none of these immunotherapies lower the risk of future miscarriage in women who have undergone multiple pregnancy losses [17]. The failure of these treatment modalities only highlights the need to further understand the specific mechanisms of the underlying pathophysiology, as it is only when a thorough understanding of the intricate cellular interplay with associated networks that dictate the health and well-being of pregnancy is known that next generation therapies can be developed and tested.

Many studies within the Breg field usually present their finding *vis a vis* the more studied subset of regulatory cells, the Tregs. In the context of pregnancy, it is generally well accepted that Tregs are master regulators and essential in facilitating uterine receptivity and embryo implantation; a significant expansion in Tregs commences during estrus and continues during early pregnancy [89]. The pathophysiology of certain unexplained pregnancy disorders such as primary infertility, idiopathic recurrent spontaneous abortion, and preeclampsia has been linked to inadequate numbers of Tregs, an imbalance of Tregs and effector T cells, or an impairment of Treg function [90–93]. Therefore, immunotherapeutic targeting of Tregs to address these obstetric complications is an appealing prospect.

With respect to Tregs, a plethora of studies, especially in the fields of autoimmunity and transplantation, provide evidence of a functional interplay between the two regulatory lymphocyte populations, with many revealing the ability of Bregs to facilitate the primary induction of Tregs. *In vitro* co-culture studies of CD4⁺CD25[−] effector T cells and IL-10⁺ Breg cells resulted in an increased contact time and suppression of pro-inflammatory cytokine production, enhancement of Treg activity, and conversion of effector T cells into Treg by upregulation of Foxp3 expression [94,95]. *In vivo*, adoptive transfer of different subtypes of Bregs such as transitional 2 marginal zone precursor Bregs and IL-10-competent CD19⁺Foxp3⁺ Bregs similarly increased the number of Foxp3⁺ Tregs [94,96]. Clinically, IL-10 secretion and upregulated expression of co-stimulatory molecules CD80 and CD86 by B cells have been shown to drive naïve T-cell differentiation toward CD4⁺Foxp3⁺ Tregs and CD4⁺IL-10⁺ Tr1 cells [97]. With Bregs intrinsically linked to Treg expansion in autoimmunity and transplantation, our own investigations sought to determine percentages of CD4⁺CD25⁺Foxp3⁺ Tregs and CD19⁺IL-10⁺ Bregs throughout gestation. The relatively high percentages of both sets of regulatory cells in the uterus in early pregnancy suggested their joint involvement in the processes pertinent to embryo implantation [38], and continue to provide us with the motivation to definitively ascertain whether Bregs can induce expansion of Tregs during pregnancy.

Clinical translational perspectives

Approximately half of early pregnancy pathologies that result in recurrent pregnancy loss are classified as idiopathic, not attributed to fetal chromosome abnormalities, infections, endocrinological causes, uterine abnormalities, aPL syndrome, or other autoimmune disease [98]. In these cases, immunological dysregulation remains the likely etiological candidate as evidenced by accompanying abnormal immune profiles such as elevated NK cell levels, elevated NK activity in peripheral blood or intrauterine environment, dysregulated cytokines, imbalanced Th1 and Th2 cell reactions, elevated Th1/Th2 cell ratio, and sharing of human leukocyte antigen alleles between partners [98,99]. Within this context, immunotherapy directed toward improving uterine receptivity for optimal implantation is likely the most effective treatment intervention to improve pregnancy outcomes. However, the effectiveness (or even the clinical attractiveness) of immunotherapeutic interventions in pregnancies remains contentious, predominately because the actual benefit of preventing early miscarriages has not been clinically demonstrated beyond doubt.

Current immunotherapies include the use of IVIg, lymphocyte immunotherapy, intrauterine infusion of

granulocyte colony-stimulating factor and PBMCs, subcutaneous administration of TNF inhibitors, leukemia inhibitory factor, and oral administration of glucocorticoids [100]. All these methods use a blanket approach to treatment, hence generating a multitude of questions about the underlying mechanisms and their possible side effects, such as genetic abnormalities. More targeted approaches such as adoptive transfer of uterus-like NK cells or Treg cells to modify the local pregnancy microenvironment are gathering momentum in terms of support from the scientific research community but remain in the early stages of clinical development [101–105]. We propose that regulated Breg function should also be considered as a potential immunotherapeutic agent for reducing the risk of pregnancy complications. Additionally, the rationale for this is made stronger by the scientific indications here and elsewhere that Bregs have an earlier involvement in immune processes compared to Tregs. Thus, the therapeutic effect of Bregs may be broader than that of Tregs.

From a clinical perspective, the role of Bregs as vital contributors to clinical immune homeostasis has been acknowledged and appreciated, especially in the field of autoimmunity where it was first demonstrated that pan-B-cell depletion by rituximab had limited effectiveness due to the concomitant depletion of beneficial Bregs [106]. However, as the Breg field remains relatively new, innovative clinical translational studies have been few and far between. Clinical trials involving Bregs over the past 10 years have focused primarily on determining Bregs' potential as a prognostic or diagnostic marker of disease. Moreover, these trials have been limited to autoimmune diseases such as chronic immune thrombocytopenia, inflammatory rheumatism, and systemic lupus erythematosus (clinicaltrials.gov). Intellectual property-wise, a wide-ranging patent covering IL-10-producing B cells as tools for manipulating autoimmune or inflammatory diseases and conditions, and treatment for infectious disease and/or cancer was granted in 2018 (Tedder, T. *et al.*, US Patent 10611999B2, 2018). Also patented is a method of generating IL-10-producing B cells (Tedder, T. *et al.*, US Patent 10611999B2, 2018), but other than that no significant clinical applications for a Breg-based therapy used as an intervention for any kind of condition has commenced.

In the context of pregnancy, for now we can only speculate on the possible details of a Breg-based therapy. Who would be the possible target population? What would be the best clinical regimen? What are the possible modalities of treatment: personalized or 'off-the shelf' therapies?

The plasticity of B-cell populations at the peri-implantation period in murine pregnancy has recently been documented [38,60]. The increase in maternal Bregs during this time and their corre-

sponding functionality establish their underlying importance at this pregnancy phase. Therefore, logically a Breg-mediated immunotherapy applied early in pregnancy might have a reasonable chance of inhibiting the etiology of many pregnancy pathologies. Perhaps one of the most studied immune cells that have been investigated for clinical application against gynecologic malignancy are NK cells. A recent study [101] demonstrated that adoptive transfer of uterus-like NK cells on day 6.5 of gestation reversed impaired fetal growth and rebuilt the appropriate local environment to foster a healthy pregnancy. Similar to Bregs, uterine NK cells peak during early pregnancy and fetal cells such as trophoblasts orchestrate NK cells with desirable functional capabilities, such as promotion of placental vascular growth, facilitation of decidualization and trophoblast invasion, and appropriate immune balance [107–110]. Thus, the period of administration during pregnancy that would be ideal for Breg immunotherapy is during the peri-implantation period, making it a potential interventional strategy for addressing RIF, as well as other recurrent pregnancy pathologies.

Pregnancy pathologies with a known immune origin may well benefit from intravenous infusion of Bregs. An increase in circulating Bregs to maintain or develop the appropriate local uterine microenvironment in early pregnancy is therefore a primary goal. In the clinical setting, the strategy by which this could be effectively done has yet to be established. Hypothetically, expanding Breg populations for therapeutic application in humans could take one of two approaches, each with corresponding merits and drawbacks: (1) *In vivo* generation of Bregs by injection of drugs that can induce the activation signals required for Breg cell differentiation and expansion, or (2) Adoptive Breg cell therapy wherein Breg cells or Breg progenitors are isolated and expanded and/or activated *ex vivo* and thereafter, these autologous cells are reinfused into the recipient to stimulate efficient regulatory function (Figure 1).

In vivo generation and expansion of naturally occurring Bregs using drugs offers ease and convenience. However, this simplistic approach would require extensive safety studies and innovative targeting solutions to ensure that the drug of choice does not elicit undesirable side effects on other cells and tissues. At this point, therapeutic agents that have correlated with Breg expansion *in vivo* include the cytokine IL-35, which promoted the generation of a unique IL-35-producing Breg subset in mouse models, and the hormone hCG during pregnancy in mice and humans [15,58,79]. Therefore, it is paramount to first define the optimum signals that act as Breg drivers, to allow effective design and fine-tuned *in vivo* application, particularly with regard to the complex biological phenomenon that is pregnancy.

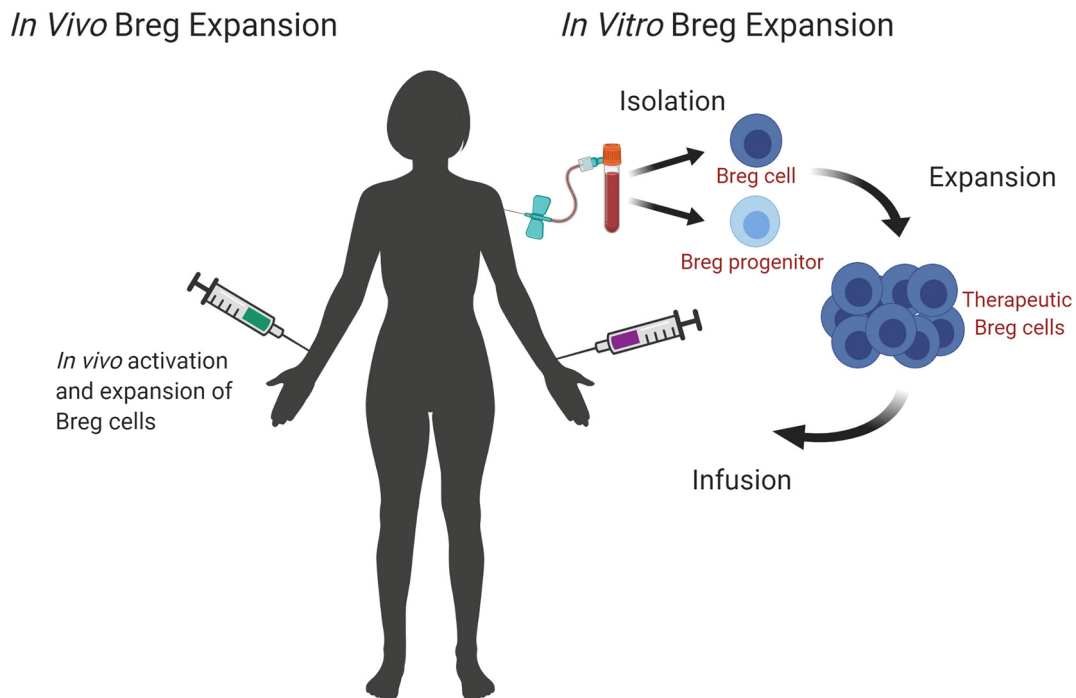


Figure 1. Expansion of Breg cells for therapeutic use may be induced *in vivo* or *in vitro*. *In vitro* expansion of Breg cells employs the use of drugs or reagents previously established as Breg inducers, to be administered to the patient for systemic activation and expansion. *In vitro* expansion involves the isolation of Breg cells or progenitors from the patient, expansion of the Breg population, and infusion of the resultant enriched cell culture for therapy.

The second method involving *ex vivo* manipulations to generate Bregs has been widely investigated in murine models across different fields. This method uses a three-step approach: isolation, expansion, then infusion (Figure 2). B cells positively isolated from peripheral blood apheresis units are incubated with reagents known to induce and expand the Breg population. A multitude of studies have demonstrated several extrinsic stimulators for inducing and expanding Breg cells, by modifying B cells to express regulatory characteristics. Engagement by agonistic CD40 mAb or CD40 ligand, TLR9 ligand CpG, BAFF, and the GM-CSF and IL-15-derived fusokine GIFT15 has all been shown to promote Breg differentiation and expansion *in vitro* [111].

Genetic reprogramming aimed at producing Bregs has also been explored. Transduction using a retroviral vector encoding an antigen fused to an immunoglobulin heavy-chain molecule or a lentiviral vector expressing IL-10 has been shown to enhance B-cell inhibitory activity on antigen-specific CD4⁺ T cells, CD8⁺ T cells, and B cells, and increase their capacity to induce immune tolerance [112,113]. Breakthroughs in genetic engineering and gene editing also pose an exciting and attractive strategy for generating therapeutic Breg cells. Using CRISPR-Cas9 genome editing technology, isolated B cells can be engineered to undergo genome alterations that mediate their functional properties. Reprogramming

parts of the B-cell receptor, thereby modifying antigen/antibody specificity and function, has been recently demonstrated [114,115]. Thus, it is now conceivable to 'make' Bregs from B cells and direct their functionality toward a specific antigen, particularly relevant for a pregnancy-focused immunotherapy. The last step in this method is infusion of the therapeutic immune cells into the patient. A key advantage of an *ex vivo*

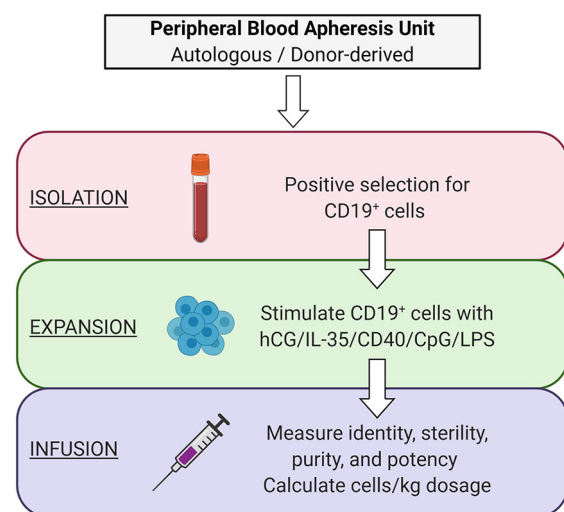


Figure 2. Three-step approach of a Breg-based immunotherapy utilizing the *in vitro* method of Breg expansion.

expansion strategy includes the ability to perform cellular phenotyping of the resultant population after expansion, to purify the desired Breg population, and govern and track the dose of administered cells.

Lastly, we explore the possibility of an 'off-the-shelf' Breg-based therapy. Cell therapy can be autologous, where it is derived from a patient's own cells, or allogeneic, where it is derived from donor cells. Moreover, allogeneic therapy enables large numbers of cells to be generated from just one donor to treat multiple patients. Autologous therapy can be time-intensive and expensive due to the complicated process of a personalized treatment, whereas the allogeneic approach could offer greater convenience and lower costs, and make treatment available to more patients [116]. Considering that the cost burden of pregnancy difficulties and complications is generally high, an allogeneic therapy offering the benefits mentioned is a favorable option. Additionally, any type of therapy that employs manipulation of the maternal immune system for ensuring the viability of pregnancy may only be effective within a critical time-frame, i.e. the pre-implantation period, thus an allogeneic therapy that requires less time for preparation and administration is advantageous. Perhaps the biggest challenge associated with allogeneic therapy is the concomitant immune response mounted by the recipient's immune system to non-self-donor cells that would result in rejection. However, with the advent of genetic editing, this challenge can be met by engineering cells to be hypoimmunogenic thus eliminating the risk of rejection [116]. An outstanding example of a CRISPR-Cas9 engineered allogeneic therapy with clinical trials underway is CTX110, a healthy donor-derived gene-edited allogeneic chimeric antigen receptor (CAR)-T therapy targeting CD19 for the treatment of relapsed or refractory B-cell malignancies (clinicaltrials.gov). Thus, the possibility of a Breg-based therapy that is directed to increase Breg numbers in an antigen-nonspecific and tissue-targeted manner is entirely plausible.

Concluding remarks

As it stands, research on Bregs and their role in healthy pregnancies remains in its infancy. Although progress has been achieved, many questions remain unanswered, particularly surrounding the interactions that this population of immune cells has with other more well-studied populations, and how together they orchestrate the induction and maintenance of maternal immune tolerance. As many pregnancy pathologies have their basis in sub-optimal implantation, a critical phase in gestation and one that is highly immune-regulated, insufficient knowledge of these events remain an impediment to translational clinical developments.

We predict, however, that further discoveries of the fundamental immune mechanisms behind maternal tolerance, combined with the rapid and exciting advances in cell-based immunotherapies in the cancer domain can be harnessed, and innovative immunotherapeutic interventions designed and applied to many pregnancy pathologies. Certainly, Bregs have the desirable attributes to be the basis of such a therapeutic intervention and one that we at least are currently pursuing.

CRedit authorship contribution statement

Ruth Marian Guzman-Genuino: Conceptualization, Writing - original draft. **John D. Hayball:** Conceptualization, Writing - review & editing, Supervision. **Kerrilyn R. Diener:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision.

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Tregs, regulatory T cells; NK, natural killer; Bregs, regulatory B cells; IVIg, intravenous immunoglobulin; IgG, immunoglobulin G; aPL, antiphospholipid; hCG, chorionic gonadotrophic hormone; RIF, repeated implantation failure.

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