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New Insights into IL-10 Dependent and IL-10 Independent Mechanisms of Regulatory B Cell Immune Suppression

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Abstract Regulatory B (Breg) cells are important regulators of immune responses and in recent years our understanding of their phenotypical and functional characteristics has improved considerably. Initially all suppressive capabilities of Breg cells were attributed to the actions of the anti-inflammatory cytokine interleukin (IL)-10 secreted by Breg cells. Recent studies however, highlight additional and novel mechanisms that influence both the expansion of Breg cells and their capacity to suppress immunity. Here we provide an overview of the complexity of Breg cell populations and address the newly discovered IL-10 independent mechanisms of Breg cell expansion and immune-suppression.

Keywords B cells · regulatory B cells · inflammation · IL-10

Introduction

B cell depletion therapy was originally developed for the treatment of malignant B cell lymphomas, but was subsequently implemented in the treatment of multiple autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS) and systemic lupus erythematosus (SLE). The rationale for such therapies was the hypothesis that depletion of B cells will lead to reduction in the levels of autoreactive antibodies. However, several examples exist were B cell depletion therapy improved the patients clinical scores without a significant decrease in autoantibody levels [1, 2].

These observations refocused attention on the role of B cells as being only antibody producers and address potential additional functions as cytokine producing cells and antigen presenting cells (APC). Indeed, B cells can act as immune-modulators either directly by presenting antigen in complex with MHC-II molecules or indirectly by producing cytokines that modulate T cell responses. B cells can enhance or inhibit immune responses through their secretion of pro-inflammatory cytokines such as IL-6 [3] or through their secretion of the anti-inflammatory cytokine IL-10 [4].

The existence of B cell populations that were capable of inhibiting immune responses was initially reported in the mid-1970s in studies performed in guinea pigs. Adoptive transfer of B-cell depleted splenocytes was unable to inhibit delayed-type hypersensitivity skin reactions [5]. Since these original studies, inferring the existence of B cells with regulatory activity, it is now established that regulatory B (Breg) cells, both in mice and humans, are a potent regulatory component of the immune system. In this review we will provide a brief overview of phenotypical characteristics of Breg cell populations in humans and mice and highlight newly described mechanisms of Breg cell expansion and immune-suppression with special emphasis given on non-IL-10 mediated immune-regulation.

Murine Regulatory B Cells

Murine Breg cells have been defined phenotypically and functionally by their ability to produce IL-10 and therefore down-regulate inflammation in multiple disease models [4, 6, 7]. B

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cells with regulatory capacity are present in very low frequencies in healthy naive mice (1–5 % of spleen or lymph node B cells, and up to 10 % of peritoneal cavity B cells) but expand following Toll-like receptor (TLR) [8–10], B cell receptor (BCR) [11] or CD40 [4] stimulation in vitro. Breg cells expand in vivo during inflammatory conditions such as Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of multiple sclerosis [7], Collagen Induced Arthritis (CIA) [4] or during helminth infections [12, 13]. Furthermore, in mice and humans infected with Schistosoma parasites a distinct Breg cell population has been described [14, 15].

Murine Breg cell subsets are conventionally defined by phenotypical characteristics based on surface marker expression as $CD19^{+}CD1d^{high}CD5^{+}$ B cells [16], transitional type 2-marginal zone precursors (T2-MZP) $CD19^{+}CD21^{high}CD23^{high}IgM^{high}$ [17], or $Tim1^{+}$ B cells [18]. In these B cell subsets not all cells produce IL-10, rather IL-10 producing Breg cells are enriched in these populations. Murine Breg cells share their phenotype with marginal zone B cells ($CD19^{+}CD21^{+}CD23^{-}CD1d^{high}$), transitional B cells ($CD19^{+}CD23^{-/+}CD24^{+}CD21^{-}$) and B1a B cells ($CD19^{+}CD5^{+}$), in addition they are predominantly $IgD^{low}IgM^{high}$ expressing cells [19–21]. In recent years it was demonstrated that Breg cells, both under in vitro and in vivo conditions, can differentiate into plasma cells [21]. In EAE, antigen specific IL-10 expressing plasmablasts have been identified in the draining lymph nodes of mice [22], adding to the B cell subsets with IL-10 dependent regulatory function. Despite the extensive phenotypical characterization of these cells, a single marker defining this subset in mice is yet to be identified, making the cytokine IL-10 a key protein when identifying Breg cells, hence the description of such cells as B10 cells [23].

Unlike T regulatory (reg) cells, which can be defined by the expression of the transcription factor FoxP3 in combination with surface expression of CD25, there is no specific transcription factor identified for murine Breg cells. However, it has been demonstrated that the transcription factor interferon regulatory factor-4 (IRF4) is important for the promotion of *Il10* transcription in Breg cells as IRF4 binds to a promoter region of the IL-10 encoding gene [22], making it a candidate for Breg cell identification. A summary of known murine Breg cell subsets, their phenotype and function is reviewed in Table 1.

Human Regulatory B Cells

Human IL-10 producing B cells, as currently described, represent less than 1–2 % of peripheral blood B cells. This subset of B cells is enriched in the $CD19^{+}CD24^{high}CD27^{-}CD38^{high}CD1d^{high}CD5^{+}$ transitional B cell subset [24]. In addition $CD24^{+}CD27^{+}$ B cells are highly enriched in IL-10 expressing B (B10) cells [25]. Human IL-10 expressing B cells have also been shown to express CD48 (an activation marker) and CD148 (considered to be a marker of memory B cells) [25]. In addition, culture of human naive immature B cells ($CD24^{high}CD27^{-}CD38^{low}$) resulted in the induction of $CD27^{int}$ plasmablasts with the ability to produce IL-10 [22], indicating that IL-10 producing plasmablasts may play a role during disease.

Similar to murine Breg cells, the currently known human IL-10 expressing B cells share their phenotypical characteristics with other B cell subsets, transitional $CD24^{+}CD38^{+}$, naive $CD27^{-}$ and memory $CD27^{+}$ B cells. However, a specific marker to identify Breg cells in humans is not currently available. Increased frequencies of $CD19^{+}CD24^{high}CD38^{high}$ Breg cells have been identified in the blood of patients with autoimmune diseases including RA and SLE [24]. The Breg cells from RA patients produce high levels of IL-10 after CD40 stimulation and have the ability to suppress type-1 T helper cell (Th1) responses and type-17 T helper cell (Th17) differentiation [26]. However, the same $CD19^{+}CD24^{high}CD38^{high}$ Breg cell population in SLE patients have impaired IL-10 production after CD40 stimulation and fail to suppress Th1 responses [24]. Although the presence and suppressive capacity of Breg cells seems desired in autoimmune diseases they may contribute to immune dysfunction during infectious diseases such as HIV infection. In patients with HIV-1, higher number of IL-10 producing $CD19^{+}CD24^{high}CD38^{high}$ Breg cells positively correlate with serum viral load and suppressed HIV-1 specific CD8 T cell responses [27]. A summary of known human Breg cell subsets, their phenotype and function is reviewed in Table 1.

Breg Cell-Derived IL-10

IL-10 can inhibit the production of chemokines, pro-inflammatory cytokines and additionally result in the down-regulation of co-stimulatory molecules by APC [28]. The cytokine IL-10 mediates its effects by multiple mechanisms involving both the innate and adaptive immune system. IL-10 producing B cells have been shown to negatively regulate the ability of dendritic cells to present antigen [7], suppress Th1 and Th17 cell differentiation [29] while enhancing Th2 polarization [30]. When stimulated through CD40, transitional B cells ($CD24^{high}CD38^{high}$) have been shown to produce more IL-10 and downregulate the expression of CD86, resulting in decreased T cell proliferation and TNFα production [31].

It has previously been reported that $IL\text{-}10^{-/-}$ mice develop an exacerbated form of CIA [32]. Interestingly, Carter et al. have shown that mice with an IL-10 deficiency restricted to the B cell compartment develop antigen-induced arthritis with

Table 1 Murine and human Breg cell populations and their phenotypical and functional characteristics

Breg cells, Name used in the literature	Phenotype Mouse	Phenotype Human	Function	Properties	References
B1a cells	$CD5^{+}$	–	IL-10	Produce IL-10	[60]
MZ B cells	$CD21^{high}CD23^{-}$	–	IL-10	Inhibit Ag-specific effector T cells	[61]
T2-MZP B cells	$CD21^{high}CD23^{high}CD24^{high}$ $IgM^{high}IgD^{high}CD1d^{high}$	–	IL-10	Inhibit Ag-specific T cells and Th1-type cells	[17]
GIFT15 driven B cells	$B220^{+}CD1d^{+}CD21^{+}CD22^{+}$ $CD23^{+}CD24^{+}CD27^{-}CD43^{+}$ $CD79b^{+}CD138^{+}IgD^{+}IgM^{+}$	–	IL-10	Inhibit IFNγ producing cells	[62]
Killer B cells	$CD5^{+}CD95L^{+}$	–	FasL	Induce T cell death	[63]
B10 cells	$CD1d^{high}CD5^{+}$	$CD24^{high}CD27^{+}$	IL-10	Inhibit $CD4^{+}$ T cells, monocytes	[25, 64]
Immature/transitional	–	$CD24^{high}CD38^{high}IgM^{high}IgD^{high}$ $CD5^{+}CD27^{-}CD1d^{high}$	IL-10, CD80, CD86	Suppress Th1 cells	[24]
Reg B cell	$CD19^{+}IL\text{-}10^{+}CD1d^{high}CD5^{+}$ $CD21^{high}CD23^{+}IgD^{+}IgM^{high}$	$CD19^{+}CD1d^{high}$	IL10	Helminth-induced. Suppress Th2 cells, Induce Tregs	[34]
TIM-1 B cells	$Tim\text{-}1^{+}$	–	IL-10	Suppress effector CD4 T cells	[18]
Granzyme B^{+} B cells	–	$CD38^{+}CD1d^{+}IgM^{+}CD147^{+}$	GrB, IL-10, IDO	Suppress T cells	[65]
BR1 cells	–	$CD25^{high}CD71^{high}CD73^{low}$	IL-10, IgG4	Suppress Ag-specific T cells	[66]
CD73 expressing B cells	$CD39^{+}CD73^{+}$	$CD39^{+}CD73^{+}$	Adenosine	Inhibit T cell proliferation and cytokine production	[45, 67]
Plasmablasts	$CD138^{+}CD44^{high}$	$CD27^{int}CD38^{high}$	IL-10	Inhibit DCs and effector CD4 T cells	[22]
Plasma cells	$IgM^{+}CD138^{high}TACI^{+}CXCR4^{+}$ $CD1d^{int}Tim^{int}$	–	IL-10, IL-35	Suppress effector CD4 T cells	[68]
iBreg	–	–	TGF-β, IDO	Induce Treg cells	[69]
$CD9^{+}$ B cells	$CD9^{+}$		IL-10, cell contact	Inhibit T cells	[70]
PD-$L1^{high}$ B cells	PD-$L1^{high}$	–	PD-L1	Regulate T_{FH} cell activity	[48]

a reduction in numbers of FoxP3$^+$ Treg cells [33], indicating a specific role for B cell derived IL-10 in suppressing the severity of arthritis in this model. The aforementioned observations highlight the importance of B cell derived IL-10 in immune regulation and Treg cell maintenance. Infection of mice with the human parasitic helminth *Schistosoma mansoni* has been shown to have a protective effect against subsequent ovalbumin-mediated allergic airway inflammation [34]. This protective effect is mediated by splenic CD1d^{high} B cells with protection from allergic airway inflammation being IL-10 dependent and involving FoxP3$^+$ Treg cells [34]. It has recently been proposed that B cell derived IL-10 in addition to being important in Treg cell maintenance also results in the enrichment of IL-10 producing Breg cells due to autocrine stimulation [35]. Furthermore, the effect of B cell derived IL-10 in controlling inflammation has been demonstrated in Lyn deficient mice [36]. Lyn is a tyrosine kinase expressed by B cells and myeloid cells, Lyn deficient mice have been reported to develop an autoimmune disease that demonstrates symptoms resembling lupus erythematosus [36]. Mice deficient in both Lyn$^{-/-}$ and IL-10$^{-/-}$ develop an exacerbated autoinflammatory response that cannot be controlled by the adoptive transfer of wild type IL-10 competent T cells, it can however be controlled by adoptive transfer of IL-10 competent B cells [36]. Transfer of IL-10 competent B cells positively correlates with increased FoxP3 expression by the Lyn$^{-/-}$IL-10$^{-/-}$ recipient's Treg cells [36].

B cells make a qualitative as well as quantitative contribution to IL-10 production in vivo. Using an IL-10 eGFP reporter mouse Madan et al., were able to demonstrate that following challenge with LPS, B cells were the dominant IL-10 producers in the spleen and peritoneal cavity but not in the liver [37]. Additionally, CD19-Cre/IL-10 loxp mice, deficient only in B cell-derived IL-10, had increased numbers of antigen specific CD8$^+$ T cells following infection with mouse cytomegalovirus (MCMV) [37].

While the role of Breg cells as a source of IL-10 is well established, recently new stimulators of the expansion of distinct Breg subpopulations are being identified (Fig. 1). Sattler et al., described a novel IL-10 producing B cell population, identified as CD19$^+$CD25$^+$CD1d^{high}IgM^{high}CD5$^-$CD23$^-$Tim-1$^-$, which exhibit a protective function in gut inflammation [38]. Interestingly, CD25$^+$ Breg cells are induced in vivo by the alarmin cytokine IL-33 [38]. The ability to respond to IL-33 points towards a "quick reaction" mechanism at the gut barrier that could allow regulatory B cells to respond to early "danger" signals and regulate ensuing inflammatory immune responses.

IL-33 is not the only cytokine that has been reported to induce Breg cells. The cytokine B cell activating factor (BAFF), a key modulator of B cell maturation and survival, can induce IL-10 expressing CD1d^{high}CD5$^+$ B cells both in vivo and in vitro [39]. Interestingly high concentrations of BAFF resulted in significant reduction in IL-10$^+$ B cells in vitro, therefore reversing the effect that BAFF showed in lower concentrations. This highlights that BAFF has a dual function in modulating B cell mediated anti-inflammatory properties [39]. IL-35, a member of the IL-12 family of cytokines, has also been shown to induce Breg cell mediated immunesuppression [40]. Recombinant IL-35 inhibited B and T cell proliferation by inducing Breg cells, while blockade of IL-10 abrogated the effect of IL-35. Interestingly, a population of IL-35-induced B cells that expressed IL-35 were described that could amplify the expansion of Breg cells [40]. Using the EAE model, Yoshizaki et al. showed that maturation of Breg cells into IL-10 producing effector cells requires IL-21 and CD40/CD40L interactions. Transfer of Breg cells generated in vitro in the presence of IL-21 and CD40L were able to inhibit established EAE in the recipient mice [30]. Additionally, IL-6 and IL-1β may induce IL-10 producing Breg cells, as mice lacking IL-6R or IL-1R1 selectively on B cells have reduced numbers of IL-10 producing Breg cells [41]. This indicates that different cytokines may affect the Breg cells function and overall suppressive capacity therefore tipping the balance towards inflammation.

In addition to cytokines recent studies have implicated steroids, such as estrogen, in Breg cell induction. Women diagnosed with MS show increased disease remission during pregnancy [42]. Bodhankar et al. showed that treatment of mice with E2 (17β-estradiol), a steroid and estrogen sex hormone, selectively increased CD1d^{high}CD5$^+$ IL-10 producing B cells and upregulated PD-L1 expression on B cells. In this study PD-L1 was critical for the E2 mediated protection, as PD-L1$^{-/-}$ mice, treated with E2 did not show any protection from EAE induction [42]. A more recent study showed that E2 treatment together with IL-10 producing B cells expressing PD-L1 induced protection in EAE mice [43]. While IL-10 is a major "weapon" in the arsenal of a Breg cell, there are other mechanisms of Breg cell immunosuppression that are IL-10 independent.

IL-10-Independent Breg Cell Immune-Suppression Mechanisms

In addition to the compelling evidence regarding the immunoregulatory role of B cell derived IL-10, a number of recent studies have shown B cells can exert immunosuppressive action independent of the production of IL-10 (Fig. 2). Following depletion of murine B cells, using an anti-CD20 antibody, there is a reduction in FoxP3$^+$ Treg cells, with similar effects observed in B cell deficient μMT mice [44]. Depletion of B cells in transgenic mice expressing a myelin basic protein (MBP) specific T cell receptor (TCR) induces an accelerated form of spontaneous EAE [44]. Adoptive transfer of normal or IL-10 deficient B cells induced comparable increase in the levels of FoxP3$^+$ Treg cells, thus illustrating that

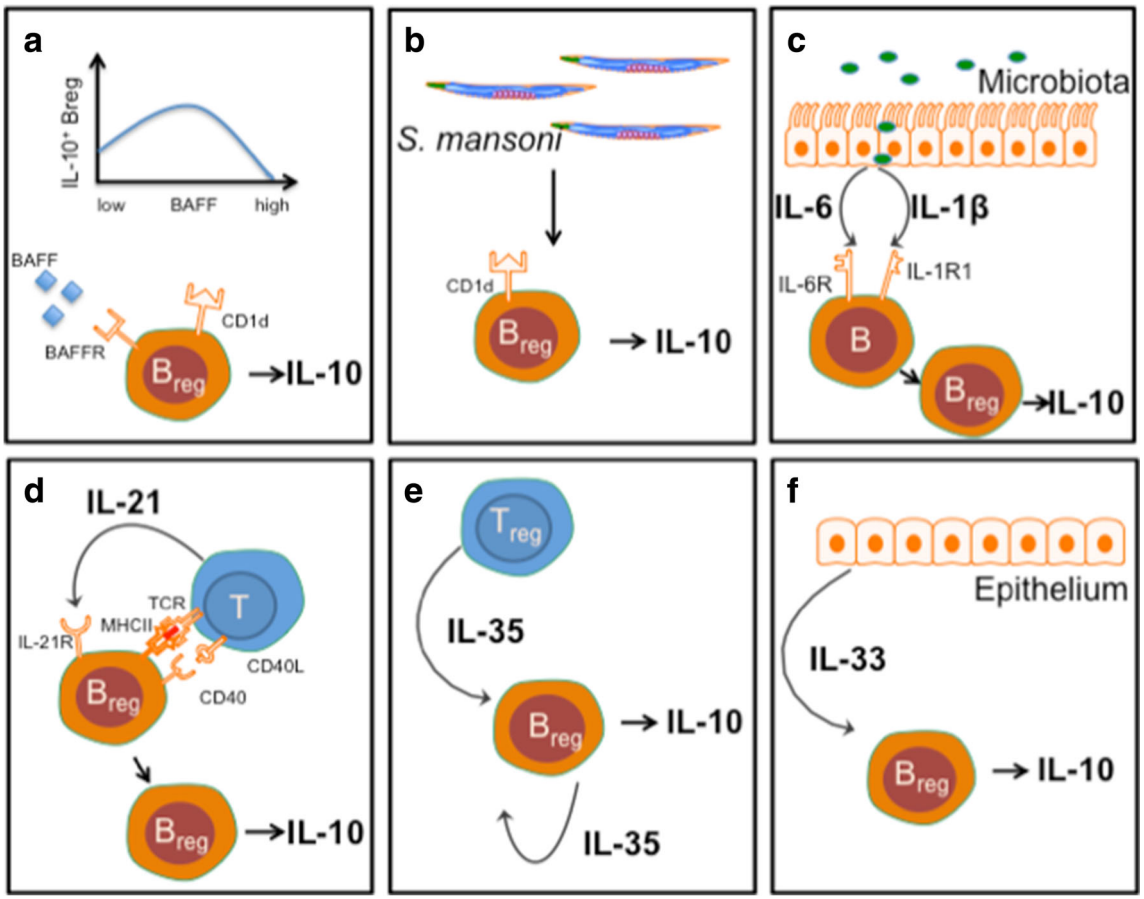

Fig. 1 New mechanisms of Breg cell expansion. Several cytokines have recently been shown to induce Breg cell expansion in addition to enhancing their suppressive function through the up-regulation of IL-10 production. **a**. BAFF mediated increase in the frequency of IL-10 producing $CD1d^{high}CD5^{+}$ Breg cells. **b**. *S. mansoni* infection results in the expansion of $CD1d^{high}$ Breg cells that can ameliorate established allergic airway inflammation in an IL-10 dependent manner. **c**. Gut microbiota driven IL-6 and IL-1β production enhance Breg cell differentiation and IL-10 production, limiting excessive immune responses. **d**. T cell derived IL-21, in combination with CD40/CD40L contact-dependent interactions, drive Breg cell development leading to inhibition of inflammation in a murine model of multiple sclerosis. **e**. *IL*-35 promotes the conversion of B cells to IL-35/IL-10 producing Breg cells with the ability to suppress Th1 and Th17 responses effectively inhibiting autoimmune inflammation in mice. **f**. Epithelial cell derived IL-33 leads to Breg cell expansion and enhanced IL-10 production

B cell IL-10 is not a prerequisite for B cell mediated maintenance of Treg cells [44]. However, transfer of B cells following an antibody-mediated block of the B cell expressed glucocorticoid-induced TNFR ligand (GITRL) prevented the upregulation of $FoxP3^{+}$ T cells [44]. Additionally, a population of human peripheral blood B cells that express the ectonucleidases CD73 and CD39 has been identified [45]. The aforementioned B cells can convert ATP to 5′-AMP and eventually ADO, with the latter previously shown to exert anti-proliferative effects on T cells [45]. Co-culture of activated T cells with $CD39^{+}CD73^{+}$ B cells in the absence of T regulatory cells resulted in significant inhibition of T and B cell proliferation [45].

A recent study described the identification of mouse $CD35^{+}CD80^{-}$ B cells that express high levels of thrombospondin 1(TSP1) [46]. When these B cells were co-cultured with dendritic cells (DC), a reduction in DC surface expression of CD80 and CD86 was noted [46]. Additionally, $CD35^{+}CD80^{-}$ sorted B cells co-cultured with T cells led to an increase in $FoxP3^{+}$ T cells that was abolished by the addition of an anti-TGF-β neutralizing antibody but not by the addition of an anti-IL-10 neutralizing antibody [46]. More recently, B cell derived TSP1 has also been implicated in the maintenance of TGF-β^{+} tolerogenic DC (TolDC) [47]. LPS-activated B cells from donor wild-type or TSP1 deficient mice were cultured with bone marrow derived DC that showed expression of the TSP1 receptor CD36. Even though TGF-β^{+} TolDC were induced in a manner depended on B cell frequency under these experimental conditions, no TGF-β^{+} TolDC were identified when DC were co-cultured with pre-activated TSP1 deficient B cells. Interestingly, IL-13 suppressed B cell TSP1 production indicating that IL-13 can inhibit B cell mediated generation of TolDC in vivo and in vitro [47].

Breg cells also modulate T cell responses in a contact dependent manner. Recently, a population of Breg cells expressing high levels of PD-L1 was identified [48]. $PD\text{-}L1^{high}$ Breg

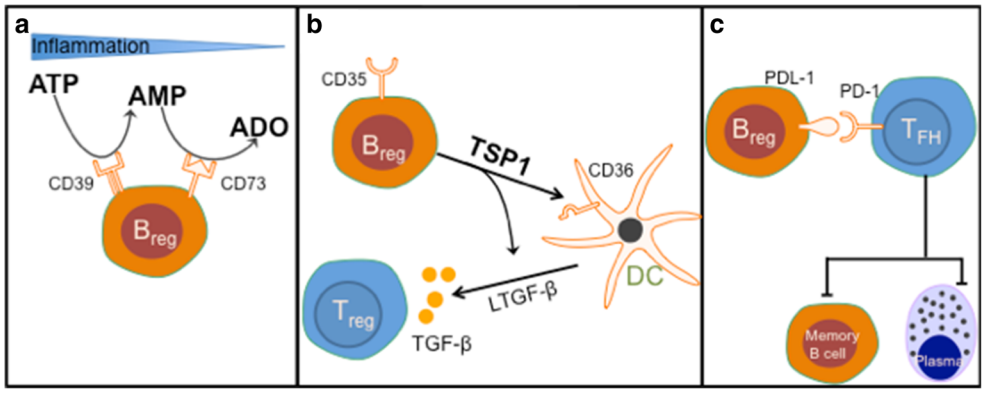

Fig. 2 IL-10-independent Breg cell-mediated immune-suppression. Even though IL-10 is a key anti-inflammatory cytokine and contributor to Breg cell-dependent immune suppression, non-IL-10-mediated mechanisms of Breg cell immune-suppression have been described recently. **a**. Breg cells co-expressing the ectoenzymes CD39 and CD73 can catalyse the deposphorylation of adenine (ATP) to immunosuppressive adenosine (ADO). **b**. Breg cells expressing the enzyme thrombospondin (TSP1) are required for the maintenance of tolerogenic DC. TSP1 signals DC directly through the TSP1 receptor CD36, while additionally converting latent TGFβ (LTGF-β) to active TGF-β. **c**. PD-L1 expressing Breg cells suppress T_{FH} cell development and expansion, in a contact dependent manner, subsequently limiting plasma cell differentiation and memory B cell development

cells suppress T follicular helper cells (T_{FH}) cells, which leads to indirect suppression of humoral responses. Interestingly, these PD-L1^{high} Breg cells are resistant to anti-CD20 antibody mediated B cell depletion primarily due to their high levels of B-cell activating factor receptor (BAFF-R) expression [48].

These studies demonstrate that B cells have the capacity to perform immunosuppressive function in an IL-10 dependent manner, a Treg cell dependent manner, a Treg and T_{FH} cell and IL-10 independent manner. The ability of B cells to suppress T cell responses by several different mechanisms and in a variety of inflammatory scenarios highlights their importance in immunoregulation of inflammatory and autoimmune diseases. Therefore in order to achieve the maximum desired outcome, better understanding of B cell depletion and other B cell targeted therapies in the context of B cell mediated immunoregulation is required.

B Cell Depletion Therapy and Re-Population Studies

B cell depletion therapy is not the only B cell targeted therapy under development, however, it is widely tested and has emerged as an effective approach in controlling multiple autoimmune diseases in humans [49–51]. B cell depletion therapies include treatment with the chimeric anti-CD20 antibody (Rituximab), the humanized B cell activating factor (BAFF) targeting monoclonal antibody (Belimumab) [52] or the humanized anti-CD22 antibody (Epratuzumab) [53]. These antibodies act by inducing complement mediated cell cytotoxicity (Rituximab), antibody-dependent cell mediated cytotoxicity (Rituximab and Epratuzumab) [53], or by prohibiting the delivery of survival signals to B cells (Belimumab) [54]. Second generation antibodies/molecules aimed to improve efficacy of B cell depletion are currently under development including additional CD20 targeting antibodies such as humanized Rituximab alternatives that recognize different epitopes with reduced off-rate and increased complement dependent cytotoxicity [55, 56].

The peripheral blood B cell pool of RA patients treated with Rituximab does not become repopulated for at least 16 weeks, while the first of the returning B cells exhibit a transitional B cell phenotype characterized as $CD24^{+}CD5^{+}CD38^{high}IgD^{+}$ followed by the return of naïve $CD19^{+}CD27^{-}$ B cells [57]. The naïve peripheral blood B cell population reached baseline levels approximately 12 months following Rituximab treatment, while the memory B cell compartment reached only 50 % of the baseline levels 24 months following treatment [57]. Comparable peripheral blood B cell repopulation has been seen with MS patients following treatment with Rituximab. Interestingly, repopulation of naïve B cells correlated with increased levels of IL-10 in the serum [58].

A recent study identified increased frequency of pro-inflammatory granulocyte macrophage colony stimulating factor (GM-CSF) producing memory B cells, which additionally expressed IL-6 and TNFα, and induced pro-inflammatory myeloid cell activation in MS patients [59]. Elevated STAT5 and STAT6 signaling was required for induction of B cell GM-CSF production, with inhibition of both STAT5 and STAT6 resulting in reduced GM-CSF and increased IL-10 production. Following B cell depletion in MS patients, emerging B cells showed reduced STAT5/6 signaling that resulted in normalization of GM-CSF and IL-10 B cell production and diminished frequency of pro-inflammatory myeloid cells [59].

These studies highlight that repopulating B cells, including Breg cells, following B cell depletion are of an anti-inflammatory disposition; however, a clear phenotypical characterization of returning Breg cells is still required.

Concluding Remarks

Breg cells are important regulators of immune responses. This is highlighted by the variety and complexity of the different IL-10 dependent and IL-10 independent mechanisms that they can employ in order to exert their suppressive functions. Their high versatility however has hindered the identification and characterization of these cells since their importance as immune-regulators is reflected by their complex and variable phenotype. Improved phenotypical resolution and further investigation of the mechanisms that influence Breg cell expansion and non-IL-10 mediated suppression, will greatly improve our capacity to manipulate Breg cell responses in disease.

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