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Brief Reviews

The Journal of Immunology

Regulatory B Cells in Autoimmune Diabetes

Moufida Ben Nasr,[*,†,‡,1] Vera Usuelli,[†,1] Andy Joe Seelam,[†] Francesca D'Addio,[†] Reza Abdi,[‡] James F. Markmann,[§] and Paolo Fiorina[*,†,¶]

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Since they were discovered almost three decades ago, a subset of B cells denoted as regulatory B cells (Bregs) have elicited interest throughout the immunology community. Many investigators have sought to characterize their phenotype and to understand their function and immunosuppressive mechanisms. Indeed, studies in murine models have demonstrated that Bregs possess varied phenotypic markers and could be classified into different subsets whose action and pivotal role depend on the pathological condition or stimuli. Similar conclusions were drawn in clinical settings delineating an analogous Breg population phenotypically resembling the murine Bregs that ultimately may be associated with a state of tolerance. Recent studies suggested that Bregs may play a role in the onset of autoimmune diabetes. This review will focus on deciphering the different subclasses of Bregs, their emerging role in autoimmune diabetes, and their potential use as a cell-based therapeutic. ***The Journal of Immunology*, 2021, 206: 1117–1125.**

An abundance of evidence supports the existence of a population of B lymphocytes with regulatory ability (regulatory B cells [Bregs]). In this study, we revisit the function, mechanism of action, and characteristics of Bregs with the ultimate goal of understanding the role of Bregs in autoimmunity, specifically in type 1 diabetes (T1D), and whether a Breg-based strategy to treat T1D is feasible and clinically relevant (1–4). Various phenotypes of Bregs have been described thus far (i.e., marginal zone [MZ], transitional 2 [T2]–MZ precursors [MZP], CD1d, T cell Ig and mucin domain-1 [TIM-1], and GIFT15 Bregs) in preclinical models of numerous autoimmune diseases (5–9); however, to date, few data in NOD mice and in patients with T1D have been published, and the main regulatory mechanism of Bregs and their relevance in the modulation of immune tolerance in T1D pathogenesis require further investigation (10). The Breg mechanism of action remains incompletely understood but has been shown to include downregulation of effector T cell function and APCs via soluble factors, primarily but not exclusively IL-10 (11–13). Work from our group and others have demonstrated that Bregs and particularly their role in T1D can be successfully discerned in humans by sustaining tolerance to islet autoantigens, thereby providing a basis for the potential use of Bregs as a therapy in T1D in the future (4, 10). However, cellular therapy is currently limited by imprecise phenotypic identification, which prevents specific isolation of Bregs, although attempts to expand Bregs ex vivo using nonspecific stimuli may expand non-Bregs (14).

Identification of Bregs

The potential regulatory role of B cells was first hypothesized by Salvin (15) in 1974, and the term "regulatory B cells" was coined by Mizoguchi and Bhan (16) in 1997. Since then, many different phenotypic characterizations of Bregs have been described (Table I) (17, 18). A primary hurdle in identifying Bregs is the lack of a unique marker, such as exists for regulatory T cells (Tregs) in the form of Foxp3, which would allow for more definitive identification. Bregs were initially observed in diseased mice (19), and it was unclear whether they were physiologically present in nondiseased mice. IL-10 expression is generally considered the hallmark of Bregs (20); however, IL-10 expression is low in resting B cells but increases only after stimulation in vitro. Thus, identification of other Breg markers would represent a major advance for the field. The difficulty in identifying such markers is compounded by the fact that the B cell phenotype changes after activation in vitro. Although IL-10^+ B cells represent $<1\%$ of total B cells, they are enriched (e.g., 15–30%) in B cell subpopulations exhibiting Breg activity (20). It remains unclear whether the regulatory properties of different B cell

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Abbreviations used in this article: AhR, aryl hydrocarbon; Breg, regulatory B cell; CHS, contact hypersensitivity; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; MZ, marginal zone; MZP, MZ precursor; SLE, systemic lupus erythematosus; T2, transitional 2; T1D, type 1 diabetes; T2D, type 2 diabetes; TIM-1, T cell Ig and mucin domain-1; Treg, regulatory T cell.

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Table I. Breg subsets described in literature

Surface Markers	Activity	Stimulus	Tissue	Context
Murine				
MZ Bregs ($CD21^{hi}CD23^{lo}$ cells)	IL-10 production	CpG-TLR9 (in vitro)	Spleen	CHS; SLE
T2-MZP Bregs ($CD1d^{hi}CD21^{hi}CD23^{+}CD24^{hi}$ cells)	IL-10 secretion, T cell suppression, Treg increase	CD40 (in vitro)	Splenic MZ	CIA
CD1d Bregs ($CD1d^{hi}CD5^{+}CD19^{+}$ cells)	IL-10 secretion, Ab production	LPS plus PIM CD40 BCR (in vitro)	Spleen, peritoneal cavity	CHS
TIM-1 Bregs ($CD19^{+}TIM\text{-}1^{+}IL\text{-}10^{+}$ cells)	IL-10 production, IL-4 production	Anti–TIM-1 (in vivo)	All lymphoid tissues	Islet Tx
GIFT15 Bregs ($CD21^{+}CD23^{+}IgM^{+}CD1d^{+}IgD^{+}CD138^{+}$)	IL-10 production	GIFT15 (in vitro)	In vitro generation	EAE
$CD73^{hi}CD39^{+}$ Bregs (B220 + CD23-CD39 + CD73hi cells; $B220^{+}CD5^{+}CD1d^{hi}IL\text{-}10^{+}$)	IL-10 production			DSS-induced colitis
$CD19^{+}CD138^{+}MyD88^{+}IL\text{-}10^{+}$ plasmocytes	IL-10 production	MyD88 TLR2 TLR4	Spleen	*Salmonella typhimurium* infection
$LAG\text{-}3^{+}CD138^{+}IL\text{-}10^{+}$ plasmocytes	IL-10 production		Spleen and lymph nodes	*S. typhimurium* infection
$CD19^{+}CD44^{hi}IL\text{-}10^{+}$ plasmocytes	IL-10 production		Lymph nodes	EAE
$IL\text{-}35^{+}$ B cells	IL-35 production	TLR4 CD40		EAE, *S. typhimurium* infection
Human				
$CD19^{+}CD24^{hi}CD38^{hi}$ cells	IL-10 production, CD80/CD86 pathway	In vitro	Human blood	Human SLE
$CD19^{+}CD24^{hi}CD27^{+}CD48^{hi}CD148^{hi}$ cells	IL-10 production	LPS plus PIB (in vitro)	Human blood	AID
$CD19^{+}IgD^{lo}CD38^{+}$ $CD24^{lo}CD27^{-}$ cells	DC inhibition	CD40L, CpG (in vitro)	Human blood	SLE, RA

AID, autoimmune disease; CIA, collagen-induced arthritis; DSS, dextran sodium sulfate; PIB, PMA plus ionomycin plus brefeldin A; PIM, PMA plus ionomycin plus monensin; RA, rheumatoid arthritis; Tx, transplant.

subpopulations actually result from the relatively high frequency of IL-10–producing cells or whether regulatory activity is a distinct property of cells within a given subset. The main murine and Bregs described so far in the literature, their related mechanism, and sources of their production have been summarized in Table I. Below, we will describe some of the best-characterized murine Breg phenotypes. Mizoguchi et al. (7) first showed that $CD1d^{hi}$ B cells from mesenteric lymph nodes of $TCR\alpha^{-/-}$ mice with spontaneous colitis expressed IL-10 and could inhibit progression of colitis when transferred into naive $TCR\alpha^{-/-}$ mice. CD1d is also expressed on MZ B cells and their precursors, and subsequent studies have indeed identified Bregs within these narrower subsets as detailed below.

MZ Bregs ($CD21^{hi}CD23^{lo}$ phenotype). B cells from the MZ of the spleen, which may constitute an early line of defense against bacteria that enter the bloodstream, have been shown to exert immunoregulatory function (21, 22). MZ B cells from sensitized wild-type mice can normalize the vigorous contact sensitivity response observed in $CD19^{-/-}$ mice and are involved in generation of Tr1 cells mediating tolerance induced in the anterior chamber (21, 23). Moreover, Sonoda et al. (23) showed that wild-type MZ B cells promote generation of Tregs in the anterior chamber of the eye.

T2-MZP Bregs ($CD1d^{hi}CD21^{hi}CD23^{+}IgM^{hi}$ phenotype). Mauri and colleagues (6) identified IL-10 expression in relatively immature MZP B cells from naive wild-type DBA/1 mice. These T2–-MZP partially prevented the induction of collagen-induced arthritis when transferred into recipient mice (40% of adoptively transferred developed arthritis), including those specifically lacking IL-10–expressing B cells ($IL\text{-}10^{-/-}$ B cells) (6, 11). Interestingly, the number of IL-10–expressing T2-MZP cells increased during the remission phase of arthritis, and these cells were more efficient than naive cells at preventing arthritis, which was shown to be mediated by IL-10 (6). The authors have further supported the hypothesis that Bregs arise from a common progenitor identified as T2-MZP B cells, which will trigger their activation through TLRs in response to an initial pathogen stimulation (24). Following the progressive inflammation, this Breg progenitor receives more activation through signaling via CD40, BCR, CD80-CD86, enhancing further their IL-10 production and assuring in parallel their feedback to $CD4^{+}$ T cells to initiate their conversion into $Foxp3^{+}$ Tregs (25).

$CD1d^{hi}CD5^{+}$ Bregs. Tedder and colleagues (12) identified a small subpopulation of splenic $CD1d^{hi}CD5^{+}$ B cells in wild-type C57BL/6 mice that is highly enriched for IL-10. When transferred, these cells can inhibit contact hypersensitivity (CHS), experimental autoimmune encephalomyelitis (EAE), and systemic lupus erythematosus (SLE) in an Ag-specific– and IL-10–dependent manner (12, 13, 26). However, the ontogeny of these cells is unclear. $CD1d^{hi}$ expression is found on MZ and T2-MZP cells, whereas CD5 is expressed on IL-10–producing B cells. Although highly enriched (~15–17%) for IL-10 expression, this small population (1–2%) can only account for ~25% of all IL-10–expressing cells in the spleen (8, 12), and additional markers are required for their efficient identification. Importantly, IL-10 expression in B cells can be detected after brief exposure to PMA, ionomycin, and LPS for 5 h without altering B cell phenotype (12, 20), which has been a drawback of many earlier studies. Nevertheless, the proportion of $CD1d^{hi}CD5^{+}$ B cells expressing IL-10 increases after 48 h of stimulation with CD40 mAb and TLR ligands, and it has been proposed that this increase represents maturation of Breg progenitor cells (20, 27). However, it remains uncertain whether such cells arise from precursors of a distinct lineage or from non-Bregs in a stochastic manner.

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TIM-1⁺ Bregs. Recently, it was reported that TIM-1, previously identified on T cells, was relatively highly expressed on B cells (8). Moreover, TIM-1^+ B cells are 20- to 30-fold enriched (compared with TIM-1^- B cells) for IL-10 expression. Importantly, TIM-1 expression is concomitant with IL-10 expression across all B cell subsets. As a result, TIM-1 identifies over 70% of all IL-10–expressing B cells, making it the most inclusive marker of Bregs to be identified (8). Transfer of TIM-1^+ B cells promotes allograft tolerance and suppresses allergic airway disease in an Ag-specific– and IL-10–dependent manner. Moreover, TIM-1 ligation using a low-affinity mAb (RMT1-10) actually increases the percentage and number of TIM-1^+ Bregs, pointing to the possibility of in vivo expansion of Bregs.

GIFT15 Bregs (CD19-CD21⁺CD23⁺IgM⁺CD1d⁺IgD⁺CD138⁺ phenotype). Ex vivo treatment of murine B cells for 6 d with a unique "GIFT15" fusion protein (generated by fusion of GM-CSF and IL-15) generates Bregs (GIFT15 Breg cells) with an unusual phenotype, uniquely coexpressing markers of both B cells (e.g., IgM, IgD, CD21, CD31) and plasma cells (e.g., CD138) (9). GIFT15 Breg cells induce remission of EAE, and this activity depends on intact STAT6 signaling, suggesting involvement of IL-10 and/or Th2 cytokines.

CD73^hi^CD39⁺ B cells. A recent report described a novel population of B1 cells endowed with immunoregulatory properties that highly express the two ectoenzymes CD39 and CD73, known for their abilities to catalyze the extracellular phosphorylation of adenine nucleotides to adenosine (28). CD73 expression was mostly confined to a B1 cell subpopulation being described to be of $B220^+CD23^-$ phenotype and having a protective role in a model of colitis. B10 cells, defined as $B220^+$ $CD5^+CD1d^{hi}IL\text{-}10^+$, seem to express as well CD73. Functional in vivo studies using a CD73 conditional knockout model on B cells found they developed exacerbated DSS-induced colitis that was ameliorated upon adoptive transfer with $CD73^{hi}$ B1 cells, thus delineating a new mechanism of colitis suppression in an IL-10–independent manner. The same conclusion was reported by Teichmann et al. (29), who observed that conditional deletion of IL-10 on B cells does not affect disease progression in a murine model of lupus and stated that another B cell population, which they identified as $CD73^+$ B1 cells, as the main population responsible for the overseen immunosuppressive effect in an IL-10–independent mechanism.

IL-10–producing plasmocytes ($CD19^+CD138^+MyD88^+IL\text{-}10^+$, $CD138^+CD44^{hi}IL\text{-}10^+$, and $LAG\text{-}3^+CD138^{hi}CD22^-$ $IL\text{-}10^+$). Data from the literature delineated the presence of IL-10–producing immunoregulatory plasmocytes following bacterial infection and during autoimmunity. A subset of plasmocytes identified as $CD19^+CD138^+MyD88^+IL\text{-}10^+$ has been demonstrated to be able to impair bacterial infection and resistance, thereby suppressing NK cells, neutrophils, and T cells activity (30). Another subset of IL-10–producing plasmocytes, identified phenotypically as $CD138^+CD44^{hi}IL\text{-}10^+$, were found in the draining lymph nodes of EAE mice that were demonstrated to be endowed with immunoregulatory abilities (31). In vivo genetic ablation of these regulatory plasmocytes, thereby deleting Blimp1 or IFN regulatory factor 4, rendered mice devoid of $CD138^+CD44^{hi}$ plasmocytes within their draining lymph nodes, and they became more susceptible to EAE (31). Another group has delineated a naturally occurring regulatory plasma cell subset identified by the expression of LAG-3, revealed as the major source of IL-10 and denoted as $LAG\text{-}3^+CD138^{hi}CD22^-IL\text{-}10^+$ (32) and maintained in a quiescent and nonproliferative status; they further express particular genes that drive and control their LAG-3 and IL-10 expressions.

IL-35⁺ Bregs. Shen et al. (33) have identified a novel subset of naturally occurring Bregs that express IL-35 and designated as IL-35^+ Bregs. This subset is mainly confined to plasma cells that were demonstrated to operate in parallel with IL-10–producing plasmocytes and exert immunosuppressive functions during bacterial infection as well as during inflammatory autoimmunity such as the case of EAE (33).

Mechanism of action of Bregs

There is a significant knowledge gap regarding how Bregs suppress autoimmunity. Where they have been examined, Bregs have been shown to act in an Ag-specific manner. For example, TIM-1^+ B cells transferred from mice immunized with alloantigen into a recently transplanted recipient simultaneously prevent rejection of allogeneic islets transplanted from the immunizing strain but allow for rejection of islets from a third-party strain (8). These data are supported by the general finding that Bregs transferred from naive mice are ineffective or less potent than those transferred from immunized mice (6, 8, 12). The most studied and possibly most relevant mechanism of Bregs action is IL-10 production. As noted above, in many murine models of autoimmunity and transplantation, Breg activity in vivo has been shown to be IL-10–dependent (see Table I) (11, 17, 27). This role has been most definitively shown using mice that specifically lack B cell IL-10 expression and exhibit increased severity of collagen-induced arthritis (20). However, in in vitro studies with human Bregs, IL-10 dependence was not universally observed (20). In this regard, other cytokines, such as TGF-β, may also be involved in Breg activity, and various studies have demonstrated a requirement for cell-to-cell contact dependence in vitro (Fig. 1, Table I) (2, 25). In terms of cytokines involved in regulation of diabetes in NOD mice, immature B cells reemerging after B cell depletion can suppress diabetogenic T cells in an IL-10–dependent manner (1, 34). Moreover, Tian and colleagues (2) demonstrated that LPS could protect from T1D development in NOD mice by induction of TGF-β–producing B cells. A recent study presented another mechanism by which Bregs work through their expression of CD1d that interact with invariant NKT cells, thus inducing immunoregulation (35). With regard to targets, IL-10 and TGF-β exert pleiotropic effects that serve to regulate the immune system. IL-10 and TGF-β derived from Bregs have been shown to promote the differentiation and/or expansion of Tr1 and $Foxp3^+$ Tregs in a variety of models, including islet transplantation, SLE, collagen-induced arthritis, and in the anterior chamber of the eye (6, 8, 11, 13, 23, 36). In a tumor model, Bregs are induced and were shown to convert naive $CD4^+$ T cells into Tregs via a TGF-β–dependent mechanism (37). However, Evans et al. (6) showed that Bregs contained within the T2-MZP subset can inhibit arthritis, even in the absence of Tregs, suggesting that Bregs and Tregs may act in concert as well as independently. In terms of

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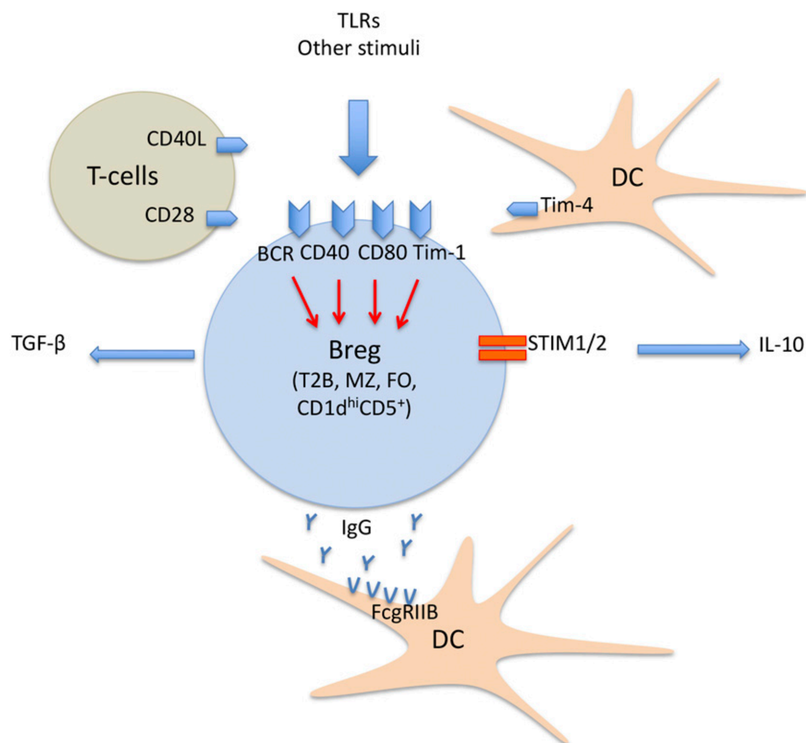

FIGURE 1. Putative mechanisms of action for Bregs. Breg subtypes, such as T2B or $CD1d^{hi}CD5^{+}$, generated through engagement of BCR or upon stimulation with CD40, CD80, or TLR4 exert their immunoregulatory effect directly by dampening inflammation through IL-10 or TGF- β production. Bregs may also indirectly suppress DC Ag–presentation function by downregulating the expression of MHC class II or through IgG secretion that binds FcγRIIB receptor on DCs.

contact-dependent mechanisms, suppression of Th1 differentiation by human Bregs in vitro was found to require intact CD80/CD86 signaling (38). Whether this suppression is mediated through CTLA-4 or PD-1 ligation on T cells or whether these molecules signal through Bregs themselves is not yet clear. A recent publication demonstrated that B cell expression of GITR-L promoted Treg expansion and led to amelioration of EAE in an IL-10–independent fashion (39). Finally, Bregs may also express high levels of Fas-L and induce apoptosis of effector T cells in vivo in an arthritis model (40). Other studies have shown that Bregs can act on APCs. For example, IL-10 production can dampen APC function by downregulating the expression of MHC class II, and TGF-β also inhibits dendritic cell (DC) maturation (41, 42). $CD24^{lo}CD27^{-}$ human B cells can inhibit LPS-stimulated TNF-α expression by monocytes in an IL-10–dependent manner (20). By secreting IgG, Bregs could also suppress DC function through binding to the inhibitory FcγRIIB receptor, which comprises over 75% of total FcR expression on murine DCs (43). Finally, Bregs themselves can act as APCs, thereby contributing to immune regulation; indeed, it has been shown that acting as secondary APCs, Bregs can control T cell responses initiated by DCs (44). After 5 d stimulation with CpG and CD40L, human B cell cultures enriched for the $IgD^{lo}CD38^{+}CD24^{lo}CD27^{-}$ phenotype acquired the ability to inhibit DC maturation by reducing HLA-DR, CD80, and CD86 expression as a consequence of cell-to-cell contact (45). Recent work confirmed that a transcription factor, aryl hydrocarbon (AhR) constitute the hallmark contributor of IL-10 production by B cells (46). The authors reported that AhR was the main regulator of IL-10–producing Bregs and was the only factor that delimited their ultimate fate into their differentiation toward inflammatory B cells. Evidence confirming the potential role of AhR came from studies on chromatin profiling and transcriptomic analysis performed on AhR-deficient B cells as compared with AhR-sufficient B cells and under Breg-polarizing conditions, showing that the former being devoid of AhR displayed a significant reduction in IL-10 expression, skewing their profile into proinflammatory B cells. Further observations in vivo confirmed the ex vivo results, demonstrating that conditional AhR knockout mice on B cells developed exacerbated arthritis while showing a significant decrease in Bregs but also in Tregs counterbalanced by an expansion of Th1 and Th17 inflammatory cells.

Several studies have pointed out the existence of an IL-10–independent mechanism of Bregs that is mainly confined to a distinct population that express IL-35 (33). Like IL-10–producing Bregs, IL-35 Bregs are produced upon TLR4 and CD40 engagement. In further in vivo studies using a model of inflammatory autoimmunity (EAE), IL-35 Bregs were able to limit the burden of EAE pathogenesis mainly by limiting the accumulation of inflammatory pathogenic Th1 and Th17 cells within the target organs (33). Authors also described another population of Bregs characterized by the coexpression of the ectoenzymes CD39 and CD73, which cooperate to convert proinflammatory ATP to the anti-inflammatory adenosine (47).

Bregs in autoimmune diseases

Bregs have been observed in various models of autoimmune disease and transplantation (Table II). Both $CD19^{-/-}$ mice and anti-CD20 mAb-treated B cell–depleted mice display enhanced CHS (21). Adoptive transfer of either MZ B cells or $CD1d^{hi}CD5^{+}$ B cells from sensitized wild-type but not $CD19^{-/-}$ or $IL\text{-}10^{-/-}$ mice normalizes CHS. Accordingly, B cells from $CD19^{-/-}$ mice produce less IL-10 because of deficiency in the $CD1d^{hi}CD5^{+}$ subset (12). Bregs have also been shown to play an important role in EAE, a murine model for multiple sclerosis in humans (48). B cell–deficient and B cell–depleted mice display increased EAE severity due

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Table II. Bregs in preclinical models

Model	Human Disease	Treatment and Effect
CHS	Allergic contact dermatitis	$CD1d^{hi}CD5^{+}$ transfer reduces ear swelling
EAE	Multiple sclerosis	B cell deficiency increases EAE severity; $CD1d^{hi}CD5^{+}$ transfer reduces symptoms
Collagen-induced arthritis	Rheumatoid arthritis	Activated T2-MZP B cell transfer reduces joint inflammation; apoptotic thymocytes induce IL-10 secretion
SLE	Systemic lupus	B cell depletion at 4 wk enhances disease; $CD1d^{hi}CD5^{+}$ transfer reduces kidney disease
Inflammatory bowel disease	Chronic colitis	$CD1d^{+}$ phenotype; IL-10–competent cell transfer reduces inflammation
Experimental autoimmune diabetes (NOD)	T1D	Transfer of BCR-stimulated splenic B cells prevents insulitis; LPS-activated B cells prevent diabetes
Islet transplantation	Transplantation	TIM-1 identifies most IL-10^{+} B cells in mice; B cell depletion inhibits anti–TIM-1–mediated tolerance; TIM-1^{+} but not TIM-1^{-} B cells transfer IL-10–dependent tolerance to otherwise untreated recipients

to the depletion of ($CD1d^{hi}CD5^{+}$) Bregs (3). However, results were shown to be dependent upon timing, given that late depletion of B cells ameliorated disease in an IL-10–dependent fashion. These data indicate that Bregs may play a dominant role during disease initiation, whereas B cells primarily exert effector/APC function during disease progression. In collagen-induced arthritis, a murine model of human rheumatoid arthritis, B cell depletion before immunization has been shown to delay and reduce joint inflammation (49). However, the specific transfer of T2-MZP cells inhibited disease in an IL-10–dependent manner (6). Similar to EAE, B cells play a dual role in the NZB/W F1 murine model of SLE; B cell depletion with anti-CD20 performed after 12 wk of age prolongs survival, whereas earlier B cell depletion accelerates kidney disease and worsens survival (50). Furthermore, in $CD19^{-/-}$ NZB/W F1 mice (deficient in Bregs), disease is accelerated, and transfer of IL-10–enriched $CD1d^{Hi}CD5^{+}$ B cells ameliorates disease (13). In the mrl/lpr lupus model, adoptive transfer of anti-CD40–treated Bregs improved renal disease and survival in an IL-10–dependent fashion (36). $TCR\alpha^{-/-}$ mice spontaneously develop chronic colitis, and disease severity is worsened in the absence of B cells, thereby implicating Bregs (7). Indeed, the transfer of $CD1d^{+}$ IL-10–producing B cells into $TCR\alpha^{-/-}$ mice was shown to improve colitis (7).

Bregs in autoimmune diabetes

Murine Bregs. Recent findings have revealed a novel and more complex role for B cells in diabetes (1, 34). We have identified that the population of Bregs is relatively deficient in hyperglycemic NOD mice and in patients with established T1D as well as those with autoantibodies but devoid of disease onset as compared with long-term normoglycemic and healthy controls (4). A few recent studies suggest that Bregs may play a protective role in the onset of T1D. For example, in a stringent model of recurrent autoimmune diabetes, in which a strong autoimmune response makes the animal resistant to most therapies, B cell depletion could no longer induce tolerance to autoantigens in NOD mice, potentially because of Breg depletion (51). Tian et al. (2) demonstrated a therapeutic role for LPS-activated Bregs that reduced Th1-mediated autoimmunity and appeared to be mediated by enhanced Fas-L expression and TGF-β secretion. Hussain and colleagues (52) showed that adoptive transfer of ex vivo BCR-stimulated B cells obtained from the spleens of NOD mice into young NOD mice lowers incidence and delays onset of diabetes. This effect was IL-10–dependent, given that transfer of IL-$10^{-/-}$ B cells failed to reduce insulitis (52). We demonstrated that naturally occurring IL-10^{+} Bregs were constitutively present in healthy individuals and in long-term normoglycemic NOD mice and were the main factor responsible for maintaining self-tolerance to islet autoantigen insult (4). Interestingly, evidence of the immunosuppressive properties of IL-10^{+} B cells were demonstrated ex vivo in an autoimmune setting and in vivo in a stringent model of autoimmune diabetes. IL-10^{+} B cells but not IL-10^{-} B cells were able to inhibit T cell–mediated autoimmune response when challenged in a coculture with autoreactive T cells in the presence of islet autoantigen BDC2.5. Paralleling the in vitro findings, IL-10^{+} B cells but not IL-10^{-} B cells successfully dampened the autoimmune destruction of pancreatic islets and converted hyperglycemic NOD.scid to normoglycemia (4). Ratiu et al. (47) demonstrated a protection from T1D in NOD mice during pharmacological/genetic modulation of the AID/RAD51 axis that induces an expansion of $CD73^{+}$ Bregs in treated mice and exert their regulatory activity by suppressing diabetogenic T cell–mediated response or by limiting or eliminating self-reactive B lymphocytes. Indeed, investigators reported that $NOD^{-Aicdanull}$ mice showed an expansion of $CD73^{+}$ Bregs that exert their regulatory activity by suppressing diabetogenic T cell–mediated response and protect them from the development of T1D (47). An initial expansion of $CD73^{+}$ Bregs procured a resistance to T1D development following transient treatment of NOD mice with BAFFR-Fc monotherapy but revealed sensitivity to CD20 cotherapy, thus stating the important role of these newly delineated $CD73^{+}$ Bregs in suppressing autoimmune diabetes, which may act in a distinct manner from that of IL-10 (33). In a recent study, authors described another potential mechanism for the suppression of autoimmune diabetes in NOD mice involving cell-to-cell interaction between DCs and Bregs by which IL-10–producing Bregs isolated from LPS-stimulated NOD splenocytes inhibited/deactivated bone marrow–derived DCs that in turn acquired a tolerogenic state, allowing them to suppress insulin-specific autoreactive CD8 T cells in an IL-10–dependent manner (53).

Human Bregs. Human studies clearly substantiate the evidence for immunoregulatory properties of Bregs, as IL-10^{+} B cells were significantly reduced in diabetic individuals. Further ex vivo assays demonstrated that diabetic individuals displayed a

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reduced capacity in generating Bregs. IL-10^+ B cells isolated from T1D individuals were endowed with enhanced immunosuppressive abilities, as they were able to suppress the autoimmune response exerted by autoreactive T cells in the presence of islet Ag mimotope IA-2 (4). Deng et al. (54) further confirmed the scarcity of B10 cells (IL-10–producing $CD19^+CD5^+CD1d^{hi}$ cells) in T1D as compared with patients with latent autoimmune diabetes in Adults and patients with type 2 diabetes (T2D). They further reported that the defect of B10 frequency was found positively correlated with fasting C-peptide and negatively correlated with hemoglobin A_{1c} (54). More recently, the role of Bregs in autoimmune diabetes has gained more attention from the diabetologist, and several studies have emerged in which Bregs were further described in patients with diabetes (10). Studying the immune profiling of B cells in T1D patients, authors found another population of IL-10–producing Bregs to belong to a $CD24^{hi}CD38^{hi}$ B cell subset that were found to be endowed with regulatory properties and found to be defective in patients with T1D (10). A head-to-head comparison of this transitional B cell subset or $CD24^{hi}CD38^{hi}$ B cells with the murine B10-equivalent $CD24^{hi}CD27^+$ B cells confirmed both of them had robust production of IL-10, whereas $CD24^{hi}CD27^+$ B cells expressed increased levels of CD39 and of several integrins, suggesting their distinctive mechanisms of action (55). Although little is known regarding the role of $CD24^{hi}CD27^+$ in T1D, authors have reported recently that $CD24^{hi}CD38^{hi}$ Bregs might play a determinant role in T1D pathogenesis. Importantly, $CD24^{hi}CD38^{hi}$ Bregs isolated from T1D were found numerically and functionally defective as compared with those isolated from healthy controls. They further reported that this defect in IL-10–producing $CD24^{hi}CD38^{hi}$ Bregs negatively correlates with HbA1c levels in T1D patients. These assumptions were confirmed by the reduced IL-10 production by $CD24^{hi}CD38^{hi}$ extracted from T1D, which were found unable to suppress Th1 and Th17 response, neither able to convert naive T cells into Tregs. On the contrary, $CD24^{hi}CD38^{hi}$ isolated from healthy controls were endowed with a robust immunosuppressive capacity maintained by IL-10 production that allowed them to suppresses Th1 and Th17 inflammatory cells and procured for them the ability to convert naive T cells into Th2 cells and Tregs (10). Finally, Deng et al. (56) reported in their study a link between an imbalanced proinflammatory B cell subset in T2D patients, as they reported a positive correlation between glycemia and lipidemia and the elevated frequencies of proinflammatory $CD19^+CD23^+$ B2 cells as compared with subjects in the early stage of the disease with impaired glucose regulation or with normal glucose tolerance. Nevertheless, no alterations in IL-10–producing Bregs ($CD19^+CD5^+CD1d^{hi}$ B10) was observed either in T2D subjects or in impaired glucose regulation or normal glucose tolerance as compared with healthy control subjects (56).

Bregs in humans and potential clinical use

The notion that Bregs may also be present in humans is now supported by studies, paralleling those in mice, showing that B cell depletion using anti-CD20 (rituximab) can lead to exacerbations or de novo manifestations of autoimmune disease (reviewed in Ref. 18, 57) and in certain settings may increase the incidence of acute cellular rejection in renal transplant recipients (58). Recent studies provide new insight into the human Breg phenotype and IL-10 expression (20, 59, 60). In contrast to several earlier studies (see Ref. 27), spontaneous IL-10 expression at the protein level was not observed in healthy subjects or in patients with autoimmunity. In contrast, these studies confirmed previous findings that a proportion of Bregs (e.g., 5%) express IL-10 after stimulation for 2–3 d with CD40 or TLR ligands (particularly LPS or CpG), and this effect is increased up to 2-fold with combined stimulation through both pathways (20, 59, 60). BCR cross-linking has either no effect or markedly inhibits IL-10 expression on human B cells stimulated with CD40L and CpG (20, 60). A notable exception was demonstrated by Bouaziz et al. (59), who found that B cells stimulated with CpG combined with anti-Ig expressed the highest levels of IL-10. Paralleling their murine studies, Iwata et al. (20) showed that PMA and ionomycin stimulation for 5 h in vitro was sufficient to reliably detect IL-10 expression in a small percentage of human B cells (e.g., 0.5%). Such B cells, already primed to express IL-10, were deemed to be "functional Bregs." When B cells were stimulated for 48 h with TLR ligands (with or without CD40 ligation), a much greater proportion expressed IL-10 (e.g., 5–10%). These cells, dubbed "Breg progenitors," gained the capacity to secrete IL-10 upon activation and maturation. Although the concept of progenitor cells may be important, it remains unclear, in both murine and human settings, whether B cells with a capacity for IL-10 expression originate from a distinct subset of IL-10^- progenitors or whether induction of IL-10 expression is stochastic. Distinguishing between these possibilities will require identification of more specific Breg lineage markers, if they exist. Another key point highlighted by the comparison of 5- and 48-h stimulation is that 48-h stimulation dramatically alters the B cell phenotype (20). Based on studies showing that Bregs were present in immature (T2-MZP) murine B cells, Mauri and colleagues (6) examined IL-10 expression in human B cells according to maturation status (60). CD24 and CD38 expression were used to sort PBMC from healthy human subjects into immature ($CD24^{hi}CD38^{hi}$), mature ($CD24^{int}CD38^{int}$), or memory ($CD24^+CD38^-$) B cells (60). Sorted subpopulations were then stimulated through CD40 for 72 h, followed by brief stimulation with PMA and ionomycin. Nearly 15% of immature B cells expressed IL-10 compared with ~4% of mature or memory B cells. Moreover, most $CD1d^{hi}CD5^+$ B cells were contained within the immature population (12, 60). However, because the immature subset comprised only ~10% of B cells, back-calculation would suggest that perhaps only 30% of all IL-10^+ B cells originate in the $CD24^{hi}CD38^{hi}$ subset. In contrast, after stimulation, ~70% of all IL-10^+ B cells were $CD24^{hi}CD38^{hi}$. The gain in IL-10 expression in this subset suggests that 72-h culture results in selective survival and/or acquisition of this phenotype by B cells. Iwata et al. (20) took an unbiased approach by directly comparing the phenotypes of IL-10^+ and IL-10^- cells after 5-h stimulation (when the B cell phenotype is unchanged compared with unstimulated cells) (12). Based on this comparison, it was demonstrated that sorted $CD24^{hi}CD27^+$ B cells (24% of the B cell population) were enriched 10-fold for IL-10 expression compared

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Table III. Open issues for Bregs

Open Problems	Specific Questions	Recommended Studies
Breg-specific marker	Is there a specific transcription factor for Bregs ("The quest for Breg Foxp3")?	Breg transcriptome profiling
Breg expansion protocol	What is the more reproducible and reliable Breg expansion protocol?	Breg ex vivo expansion from human and murine B cells
B cell depletion protocol that spares Bregs	Is there any protocol that depletes effector B cells or abrogates their activation while sparing Bregs?	Murine studies aiming at partially depleting or inactivating B cells
Ag specificity and clonality of Bregs	Do Bregs recognize a particular autoantigen? Are Bregs monoclonal or polyclonal?	Single-cell level cloning of Breg BCR; spectrotyping of the BCR
Breg lineage and *trans*-differentiation	Can Bregs *trans*-differentiate into effector B cells and vice versa?	In vitro *trans*-differentiation studies; analysis of cytokine/costimulatory molecule knockdown mice
Breg mechanisms of action	Where do Bregs act? How do Bregs act in vivo?	Cytokines versus cell contact; trafficking; blocking Abs
Breg and Treg relationship	Are Breg effects dependent upon or independent of Tregs?	Analysis of the Breg population in Treg-deficient models or during anti-Treg treatments

with the $CD24^{lo}CD27^{-}$ population. This study suggests that, before stimulation, the $CD24^{hi}CD27^{+}$ phenotype may better identify the Breg population capable of expressing IL-10 compared with other markers. Importantly, after 48-h stimulation in vitro, both $IL\text{-}10^{+}$ and $IL\text{-}10^{-}$ B cell populations underwent a significant change in their phenotype (12, 20). Although $IL\text{-}10^{+}$ and $IL\text{-}10^{-}$ B cells were more difficult to distinguish after 48-h stimulation, CD24, CD27, and CD38 still appeared to be most highly expressed on $IL\text{-}10^{+}$ B cells. Thus, the finding that CD24 and CD38 identify 70% of $IL\text{-}10^{+}$ cells after in vitro activation appears to be consistent. In contrast, freshly isolated $CD24^{hi}CD38^{hi}$ B cells were essentially $CD27^{-}$ (60). Moreover, Duddy et al. (61) showed that CD40-mediated IL-10 was primarily expressed by B cells that were in the $CD27^{-}$ "naive" rather than the $CD27^{+}$ subset and B cell depletion with rituximab-treated multiple sclerosis patients was associated with increased IL-10 expression by reemergent $CD27^{-}$ B cells compared with pretreatment baseline B cells. Thus, additional studies will be needed to further clarify and extend the phenotype of Bregs in humans. Only recently has the regulatory function of human B cells been examined in in vitro assays. When coincubated with anti-CD3–stimulated $CD4^{+}$ cells, immature ($CD24^{hi}CD38^{hi}$) but not mature or memory B cells significantly inhibited expression of TNF-α and IFN-γ (60). Inhibition was dependent upon CD40 signaling provided by CD154 expressed on activated T cells. The lack of inhibitory activity of other B cell subsets in these assays, even when added in excess, may be due to the dilution of the frequency of $IL\text{-}10^{+}$ Bregs by other nonregulatory or effector B cells in these subpopulations. In this regard, inhibition of $CD4^{+}$ cytokine production by $CD24^{hi}CD38^{hi}$ B cells was IL-10– and also CD80/CD86–dependent (60). In contrast, both IL-10–enriched $CD24^{hi}CD27^{+}$ and IL-10–deficient $CD24^{lo}CD27^{-}$ B cell subsets inhibited $CD4^{+}$ cytokine production, an effect that could not be blocked with addition of anti–IL-10 (20). Finally, recent studies have examined Bregs in patients with autoimmune disease and demonstrated negative correlations between Bregs ratio and their prompt immunosuppressive function along disease activity. Iwata et al. (20) found that the average frequency of $IL\text{-}10^{+}$ B cells after stimulation through CD40 combined with LPS or CpG was significantly increased in 91 patients being treated for a variety of autoimmune diseases compared with normal subjects. However, two studies show that B cells from SLE and multiple sclerosis patients exhibit a specific defect in CD40-induced IL-10 expression whereas responding normally to stimulation with CpG (60, 61). In other studies, a defect in the potential to differentiate from immature $CD19^{+}CD24^{hi}CD38^{hi}$ B cells into mature Bregs was observed in SLE patients (62). Apart from this defect in Bregs, they displayed impaired IL-10 production in response to ex vivo stimuli accompanied by their inability to halt Th1 response. They further confirmed that this substantial loss of Breg function observed in SLE might be due to the increased levels of IFN-α and/or to alterations in phosphorylation of STAT1 and STAT3 signals downstream of IFN-α, which sustainably inhibits Bregs expansion (62). Likewise, in SLE, individuals with rheumatoid arthritis displayed numerically and functionally impaired Bregs in both $IL\text{-}10^{+}TIM1^{+}$ B cells and $IL\text{-}10^{+}CD5^{+}CD1d^{hi}$ B cells that failed to suppress IL-17 inflammatory response and Tregs expansion (63). A similar pattern was observed in patients displaying another autoimmune disorder named pemphigus, categorized as an organ-specific autoimmune bullous disease, also possessing numerically reduced Bregs associated with impaired functionality owing to their inability to suppress Th1 response, found restored in those responders to rituximab (64). The role of Bregs was also valuable in the two forms of inflammatory bowel disease, Crohn disease, and ulcerative colitis, in which reduced numbers and functionalities of Bregs were detrimental to the disease progression and related severe outcomes (65). These findings were not limited to the aforementioned autoimmune diseases, but were also found in other forms of autoimmune disorders, such as psoriasis and systemic sclerosis, in which defects in Bregs numbers and function were concomitant with disease progression, suggesting a possible link between Bregs and the maintenance of self-immune tolerance in autoimmunity (14).

Conclusions

The cumulative data that we have summarized underscore the role of Bregs in autoimmune diseases. This body of work will undoubtedly form the basis for further studies addressing the role of Bregs as clinical tools in human autoimmune diseases. Clinically it will be important to 1) identify more specific markers for Bregs (until such identification is achieved, it will be impossible to expand Bregs in vitro, as neither a starting population can be discerned nor can an expanded population be purified), 2) establish reliable and consistent protocols for Breg expansion in vivo and in vitro, 3) identify ways to deplete

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B cells that spare Bregs [(Table III); an extended list of scientifically engaging but less clinically relevant issues still require clarification and are included in Table III], 4) understand Breg Ag specificity and clonality, 5) clarify whether a Breg lineage exists and whether Bregs and B effector cells can "*trans*-differentiate" into one another, 6) refine Breg mechanisms of action, and finally 7) clarify the relationship between Bregs and Tregs. In conclusion, Bregs are a potentially important and novel tool to add to our therapeutic armamentarium for treating T1D. However, full realization of this potential will require deeper understanding of a number of aspects of Breg biology.

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Disclosures

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