Phenotypes, Origins and Functions of Regulatory B Cells in Autoimmune and Inflammatory Diseases

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Appendix A

Phenotypic identification of murine regulatory B cell populations

In mice, B cells with regulatory properties, including splenic CD1d^{hi}CD5^{+} 'B10' cells [1], have most commonly been identified within conventional splenic bone marrow–derived B2 MZ [2-4] and T2–MZP [5-7] B cell lineages. Regulatory B cell populations that share a partial CD19^{+}CD21^{hi}CD23^{hi} T2–MZP immunophenotype [8-10] have additionally been reported in different pathological models. As discussed in the main body of this review, phenotypic variation might be consequential to the adaptation of B cells to sudden environmental changes rather than the existence of multiple regulatory B cell progenitors. As a pertinent example, the recent cell–surface characterization of GIFT15–induced regulatory B cells (GIFT15–iBregs), powerful immune–suppressive B cells that share phenotypic characteristics with T2–MZP regulatory B cells including CD21, CD23, CD24, CD1d, IgM and IgD, revealed that these cells have lost CD19 and gained CD138 expression, mirroring the phenotype of plasma cells [11]. Last but not least, other phenotypes that have been described for murine regulatory B cell subsets include CD138^{+}IgM^{+}CD1d^{hi}CXCR4^{+}MHCII^{lo} plasma cells [12], CD138^{+}CD44^{+} plasmablasts [13], and B220^{+}CD19^{+}PD–L1^{hi} B cells [14]. Importantly, while that the majority of regulatory B cell subtypes reported so far are derived from conventional B2 cells, numerous reports have have also identified regulatory B cells within the B1 lineage [15]. **Table 1** summarizes the foremost different regulatory B cell subsets that have been reported in mice.

Table 1 Mouse regulatory B cell subsets (Adapted from von De Veen, 2016 [16])

Designation	Phenotype	Key feature	Target of suppression	References
B1a–like phenotype (CD5^{+})				
B10 cells	CD19^{+}CD5^{+}CD1d^{hi}	IL-10	CD4^{+} T cells, monocytes	1,17-23
B1a cells	CD19^{+}CD5^{+}	IL-10	pDCs, DCs	24-26
Killer B1a cells	IgM^{hi}CD5^{+}CD1d^{hi}CD178^{+}	FasL	CD4^{+} T cells	27,28
TolBC	CD19^{+}CD5^{+}CX3CR1^{+}	TGF-β	CD4^{+} T cells	29
CD73^{+} B cells	B220^{+}CD39^{+}CD73^{+}	Adenosine	CD4^{+} T cells	30
Immature/transitional B–cell phenotype				
T2-MZP B cells	CD19^{+}CD21^{hi}CD23^{hi}CD24^{hi}IgM^{hi}IgD^{hi}CD1d^{hi}	IL-10, -	DCs, CD4^{+} T cells	5-7
MZ–like phenotype				
MZP B cells	CD19^{+}CD21^{hi}CD23^{-}	IL-10	CD4^{+} T cells	31
MZ B cells	CD24^{hi}IgM^{hi}IgD^{lo}CD1d^{hi}	IL-10	CD4^{+} T cells	2-4
GIFT–15 B cells	B220^{+}CD21^{+}CD22^{+}CD23^{+}CD24^{+}CD1d^{+} CD138^{+}IgD^{+}IgM^{+}	IL-10	DCs, CD4^{+} T cells	11
Plasma cell / Plasmablast phenotype				
Plasma cells	IgM^{+}CD138^{+}TACI^{+}CXCR4^{+}CD1d^{+}TIM-1^{int}	IL-10, IL-35	CD4^{+} T cells	12,32
	CD138^{+}PD-L1^{+}B220^{lo}IgA^{+}	IL-10, PD-L1	NK cells, neutrophils	
Plasmablasts	CD138^{+}CD44^{hi}	IL-10	DCs	13
Undefined subset				
I35–Bregs	B220^{lo}CD19^{+}CD5^{+}	IL-35	-	33,34

Phenotypic identification of human regulatory B cell populations

In humans, several putative regulatory B cell populations have been described. Although almost all human B cells have the capacity to produce IL-10, the available evidence indicates that the 'most efficient' IL-10–producing B cells are predominantly found to be enriched within $CD19^+CD24^{hi}CD27^+$ memory B10 B cells [17], $CD19^+CD27^-CD24^{hi}CD38^{hi}$ 'classical' immature transitional B cells [35-39], or both $CD27^+$ memory and $CD38^{hi}$ transitional B cell subsets together [40,41]. Data from Iwata et al., have helped to partially reconcile these data by showing that B10 B cells and their progenitors predominantly belong to a $CD24^{hi}CD27^+$ B cell population [17]. The various additional described phenotypic variants of regulatory B cells in humans that share the ability to produce IL-10 upon *ex vivo* stimulation include, among others, 'Br1' B cells [42], TIM-1^+ B cells [43,44], plasmablasts [13], plasmablast–like B cells [45,46], circulating $CD19^+CD25^{hi}$IL-10^{hi}TGF-β^{hi} B cells [47], GrB^+ B cell subsets [48,49], and $CD39^{hi}CD73^+$ adenosine–producing B cells [50,51]. It is worth noting here that, in contrast to other reported human regulatory B cells, the 'orchestrator' $CD11b^+$ B1 cells (B1orc), a still ill–defined regulatory B cell–like population, spontaneously secrete IL-10 [52]. A summary of known human regulatory B cell subsets, their phenotype, key features, and target of suppression is reviewed in **Table 2**.

Table 2 Human regulatory B cell subsets (Adapted from von Mauri and Menon, 2017 [53])

Designation	Phenotype	Key feature	Target of suppression	References
Immature B cells (transitional)	$CD19^+CD24^{hi}CD27^-CD38^{hi}$	IL-10, PD-L1, CD80, CD86, CD1d	$CD4^+$ T cells, $CD8^+$ T cells, pDCs, iNKt cells	35-37,39,40,54,55
B10 cells	$CD19^+CD24^{hi}CD148^{hi}CD48^{hi}$	IL-10	Monocytes	17
GrB^+ B cells	$CD19^+CD38^+CD1^+IgM^+CD147^+$	GrB, IL-10, IDO	$CD4^+$ T cells	48
	$CD138^+CD27^+CD5^+CD38^+IgD^-$	GrB, IL-10		49
Br1 cells	$CD19^+CD25^{hi}CD71^{hi}CD73^{lo}$	IL-10, IgG4	$CD4^+$ T cells	42
Plasmablasts	$CD19^+CD24^{hi}CD27^{int}CD38^{hi}$	IL-10	DCs, $CD4^+$ T cells	13
Plasmablast-like B cells	$CD20^{lo}CD24^-CD27^{hi}CD38^{hi}$	IL-10	-	46
iBregs	-	TGF-β, IDO	$CD4^+$ T cells	56
Memory B cells	$CD19^+CD27^+$	IL-10	$CD4^+$ T cells	17,40
$CD73^+$ B cells	$CD20^+CD24^+CD25^{hi}CD38^{hi}CD39^{hi}CD73^+$	Adenosine	$CD4^+$ & $CD8^+$ T cells	50,51
TIM-1^+ B cells	$CD19^+$TIM-1^+	IL-10	$CD4^+$ & $CD8^+$ T cells	43,44,57
Blood $CD19^+CD25^{hi}$	$CD19^+CD25^{hi}CD27^{hi}CD1d^{hi}$	TGF-β	$CD4^+$ T cells	47
$CD19^+$PD-$L1^{hi}$ B cells	$CD19^+CD10^-CD21^+CD27^-$BAFF-R^{hi}	PD-L1	$CD4^+$ T cells	14

Appendix B

Antigen–BCR–derived signals

B cell receptor (BCR) signaling has been postulated to be one of the pathways for regulatory B cells to detect an inflammatory signal. In particular BCR diversity was found to be essential for B10 cell development *in vivo*, with B10 cell numbers reduced by 90% in transgenic mice expressing a fixed Ag receptor [18]. The study of genetic alterations involved in BCR signaling further reveals that pathways that modify intrinsic BCR signals drive IL-10 competence and B10 cell differentiation. In mice, CD19 deficiency, which results in a markedly elevated BCR signaling threshold, is associated with exaggerated T cell–mediated inflammatory responses [1,58,59] and a significant drop in the percentage of B10 cells [1]. On the contrary, transgenic overexpression of CD19 is correlated with the expansion of regulatory B10 cells and substantially reduced inflammation [1]. In a similar manner, absence of CD22, a negative regulator of calcium signaling during stimulation of BCR, is associated with an increased number of B10 cells [60]. Additionally, constitutive CD40 signaling combined with CD22 deficiency is associated in mice with a dramatic expansion in regulatory B10 cells [61]. The importance of BCR–related signals is further accentuated by the demonstration that ablation of the endoplasmic reticulum calcium sensors stromal interaction molecules 1 (Stim1) and Stim2 in B cells caused defects in B cell IL-10 production and suppression of autoimmune neuroinflammation [62]. Remarkably, Ab responses and B cell development were found to be essentially undamaged in these mice, indicating that BCR–induced calcium flux represents one crucial element for B cell–mediated immune regulation but is dispensable for B cell effector functions. Data also indicate that deregulated signaling pathways intrinsic to $lyn^{-/-}$ B cells may favor IL-10–producing B cell development [63]. Finally, it was recently discovered that TIM-1 signaling in regulatory B cells is required for optimal production of IL-10 [64,65]. These observations taken together are consistent with a model in which $IL\text{-}10^{+}$ B cell development or function is determined by the strength, nature, or timing of BCR–generated signals [18,66]. As these signals may be specifically regulated *in vivo*, it is conceivable that chronic BCR ligation by low–affinity self–Ags may program IL-10 competence *in vivo*, while strong BCR signals, such as those elicited by simultaneous engagement of multiple BCRs by potent foreign Ags, may prevent B10 cell clonal expansion or divert B10 progenitor cells along a different functional pathway [18,67]. Consistent with this notion, mitogenic B cell stimulation with a cross–linking anti–IgM Ab was found *in vitro* to restrain B10pro cell acquisition of IL-10 competence [17-19]. Thereby, TLR ligands or other signals such as CD40–CD40L interaction may optimally trigger IL-10 by B cells after Ag selection.

CD40–CD40L interaction

Although the evidence is somewhat mixed, research suggests that CD40, a major co–stimulatory molecule for B cells, contributes to the generation and/or suppressive function of regulatory B cells. Whereas some data indicate that CD40 ligation *in vitro* induces cytoplasmic, but not secreted, IL-10 production by murine B cells [18], *in vitro* stimulation of murine arthritogenic splenocytes with collagen in combination with agonistic CD40 Abs was reported in another study to

generate a subset of B cells that produce high levels of IL-10 and prevent arthritis upon adoptive transfer [68]. On an interesting note, while naive B cells can produce IL-10 upon activation by LPS, co–stimulation with LPS plus anti–CD40 was shown to switch off IL-10 production while inducing that of IL-35 [12]. In *vivo*, while data indicate that CD40 deficiency did not change the frequencies or numbers of $CD1d^{hi}CD5^{+}$ B and IL-10–producing B cells, transgenic ectopic CD40L expression by B cells increased B10 cell numbers [61,69]. Likewise, CD22–deficient mice that also ectopically express CD40L display significantly augmented numbers of both $CD1d^{hi}CD5^{+}$ B cells and B10 cells [18,61]. Based on these results, the authors of these excellent studies have suggested that CD40–CD40L inetractions are not necessary for murine B10 cell acquisition of IL-10 competence in *vivo* but may assist B10 cell maturation under certain conditions [18,61]. In sharp contrast, CD40–dependent cognate interactions between B10 cells and $CD4^{+}$ T cells was found to be necessary for B10 effector cell function, at least in a model of T cell–mediated autoimmunity [70].

In humans, it is still unclear to what extent CD40–CD40L interactions contribute to regulatory B cell functionality, as many of reports provide diverse results that are difficult to unify in a coherent model. It was reported that CD40 stimulation of B cells via CD40 engagement significantly activates the production of IL-10 [71,72]. Additional data indicate that such CD40–mediated increase could be dramatically attenuated by dual CD40– and BCR–mediated stimulation [71]. Contrasting results have been reported by other research groups; IL-10 was found in one previous report to be efficiently induced by B cells through combined BCR– and CD40–mediated stimulation [73], while any significant IL-10 production by naive or memory B cells stimulated with either CD40L alone or BCR and CD40L stimulation in combination was found in different studies [74,75]. These discrepancies may likely be attributed to differences in culturing systems. Yet, a remarkable, more recent, observation about the role for CD40 interaction is that it appears to contribute to the selective induction of IL-10–producing immature transitional human regulatory B cells and not in the induction of regulatory B cells in general [76]. This B cell–help mechanism is thought to be mediated in particular by type 3 innate lymphoid cells (ILC3s), a subset of lymphoid immune cells found in secondary lymphoid organs that play a major role in the regulation of adaptive immune responses [77].

TLR signaling

Both murine and human B cells express several different types of TLRs[78], a group of pattern recognition receptors that recognizes exogenous pathogen–associated molecular patterns, endogenous damage–associated molecular patterns, and microbe–associated molecular patterns that come from pathogens and commensal bacteria. Different B cell subsets or B cells at distinct developmental stages [79] exhibit variations in TLR expression patterns, and signaling via TLRs can modify various B cell effector functions. Consequently, individual TLR expression patterns permit various effector B cell subpopulations to manifest distinct response profiles, including quantitatively and qualitatively unique cytokine secretion, following engagement of different TLR ligands.

Although B cell–intrinsic TLR signals could potentially contribute to the break in peripheral tolerance to self–Ags and cause inflammatory tissue destruction, several publications also emphasize a role for the same signals, in combination with other pathways, in shaping optimal B cell–mediated regulation [80-82]. In a seminal study, Tian and colleagues have shown that transfusion of LPS–activated TLR4–expressing B cells down–regulates Th1 immunity and prevents autoimmune diabetes in nonobese diabetic mice [83]. A few years later, it was reported that TLR4– but not TLR9–activated B cells are tolerogenic in an adoptive transfer model of suppression [84], suggesting that only specific TLR agonists can elicit a suppressive function in B cells. Similarly, using mice carrying B cell–restricted deficiencies in myeloid differentiation factor 88 (MyD88) or in distinct TLRs, Lampropoulo and co–workers have, quite elegantly, established that TLR2/4–, but not TLR9–, activated B cells suppress inflammatory T cell responses and stimulate recovery from EAE [85]. Remarkably, mice with MyD88–deficient B cells were found in this excellent study to form germinal center and to mount normal primary Ab responses after immunization, indicating a major function for MyD88 signaling in B cells may be initiation of their regulatory abilities [85]. This study further highlighted the non–redundant role of TLRs in the regulatory activities of B cells, as mice with a B cell–restricted deficiency in either the signaling adaptor MyD88 or TLR2/4, were shown to develop a chronic form of EAE reminiscent of chimeric mice with B cells lacking IL-10 [86]. Whereas TLR2 and TLR4 signaling definitely shapes the regulatory functions of B cells in certain models, additional evidence also indicates an important role for distinct TLR family members or signaling in B cell regulatory functions. TLR9 activation of MZ B cells in lupus mice was found for instance to regulate T cell–mediated inflammatory responses through increased IL-10 production [87]. Consistent with previous reports documenting the ability of $CD5^+$ B cells to produce IL-10 [24], innate activation of neonatal $CD5^+$ B cells by cytosine-phosphate-guanine (CpG) was also reported to play in an IL-10–dependent manner an active role in the control of neonatal inflammation [25,88]. Remarkably, data suggest that TLR9–mediated recognition of apoptotic cells can also drastically expand *in vivo* B10 and B10 effector cell numbers [89]. In the same vein, TLR7 stimulation was equally found to expand a population of IL-10–producing $CD19^+CD1d^{hi}$ B cells, which can suppress allergic lung inflammation via a Treg–dependent mechanism [90]. Of most interest, whereas MyD88 was reported to be essential for optimal IL-10 production and secretion following LPS stimulation, this critical TLR signaling adaptor was found not necessary for normal B10 cell generation *in vivo* [18]. Equally remarkable is the observation that Freund's adjuvants, one of the most commonly used adjuvants in EAE research, did not drive B10 cell expansion [91].

Human regulatory B cell defects have been reported in allergic and autoimmune diseases [17,35,39,82,92-95], where they reflected, at least in some of the aforementioned cases, reduced suppressive functions or responses to TLR ligands. For instance, TLR9–mediated IL-10 production by B cells was significantly decreased in MS patients compared to healthy controls, which was likely due to decreased TLR9 expression in memory B cells [96]. Likewise, data indicate that IL-10 production levels by total peripheral B cells, including $CD19^+CD24^{hi}CD38^{hi}$ immature B cells, in response to

CpG stimulation were significantly less in patients with autoimmune thrombocytopenia than in healthy controls [97]. Interestingly, $CD24^{hi}CD27^{+}$ B cells, which includes B10 cells, from allergic asthma patients were documented to produce less IL-10 than those from healthy subjects in response to LPS leading to a weaker IL-10 induction in T cells in response to an allergic Ag [93]. It should be emphasized here that while LPS is a powerful mitogen for murine B cells, data however indicate that human B cells produce only modest amounts of IL-10 upon *in vitro* LPS stimulation due to a relatively low level of TLR4 expression compared to that of TLR2 and TLR9 [17,93,98-100]. Nevertheless, coherent with some observations reporting elevated TLR4 expression and function in B cells from patients with inflammatory diseases [101], TLR4–dependent signaling pathways have been documented in some studies to modulate B cell responses [100-103], thereby opening new avenues of B cell–directed treatments to reestablish immune tolerance that were not fully considered previously. Finally, on a personal note, one must keep in mind that although TLR activation likely represents a prime force driving IL-10 production in human regulatory B cells, evidence that co–stimulation mediated by BCR [41], CD40 [17], and cytokine receptor [104], or combinations of them [105] provides a synergistic effect for IL-10 secretion in regulatory B cells.

Postulated development pathways of regulatory B cells

Different activation modes can drive the development of distinct subsets of IL-10–producing regulatory B cells, which have been reviewed herein. It is clear from these data that multiple pathways in the inflammatory microenvironment are necessary for the generation and/or activation of regulatory B cells. It remains, however, not very clear how exactly B cells adopt regulatory functions in response to certain physiologic triggers to control local inflammation. For an example, it is not known whether or not all B cells can acquire regulatory properties following TLR activation.

A first model proposed in 2002 by Mizoguchi and colleagues states that different regulatory B cell populations are produced from already existing B cell subsets depending on distinct activation signals [106]. According to this proposition, the generation of 'innate' type regulatory B cells requires polyclonal activation involving TLR ligands, apoptotic signals, or B cell activating factor (BAFF) stimulation, whereas the differentiation of 'acquired' regulatory B cells involve CD40 ligation induced by cognate T helper–B cell interaction and long–term BCR engagement with self–Ags. Thus, to the question raised above about the importance of TLR signaling in driving regulatory B cell functions, part of the answer is that only certain 'innate' B cell subsets may develop into regulatory B cells following TLR ligation. A second model put forward in 2010 by Fillatreau and co–workers states that B cells acquire their regulatory properties in a two–step activation process via sequential integration of signals provided by TLR engagement to initiate the process and dual BCR/CD40 triggering to strengthen this differentiation. According to this model, all activated B cells are predicted to have the capability to differentiate into regulatory B cells after activation [81]. However, it must be emphasized that the justifications for such classifications are not exceedingly persuasive. The group led by Tedder shows, for example, no reason to partition B10 cells into adaptive or innate subsets because

they respond like most other B cells to all types of signals [67]. A third hypothesis presented by Tedder, therefore proposed that all immature progenitor cells have the potential to progress into mature IL-10–producing B cells after ligation by TLRs and CD40 directly [107]. Finally, in an effort to present a unified model that incorporates all types of regulatory B cells, a novel classification of regulatory B cells into three main types has been recently proposed on the basis of their responding properties: 'adaptive' regulatory B cells with Ag specificity that are generated during the adaptive immune response via BCR and CD40 and possibly TLR signaling; 'immature' regulatory B cells that are developed from a population of immature B cells following direct CD40 engagement; and 'innate' regulatory B cells that are derived from 'innate–like' B cells that are activated upon TLR or innate microbial stimulation[108]. Alternative models of regulatory B cell development likely exists; depending when and where B cells are recruited into inflamed tissues, it is conceivable that they may be exposed to different microenvironmental signals that can drive them to perform specialized functions.

Appendix C

Additional mechanisms of regulatory B cell–mediated suppression

In recent years convincing evidence has emerged to indicate that a variety of mechanisms supports the regulatory functions of B cells in inflammation and autoimmunity [109]. For instance, studies in different experimental models of autoimmunity have provided evidence that 'B killer B lymphocytes', which are defined by the expression of Fas ligand (FasL or CD95L) and other death–inducing ligands, such as tumor necrosis factor related apoptosis inducing ligand (TRAIL), can mediate effector T cell death under many circumstances [83,110-112]. Granzyme B–expressing human regulatory B cells with cytotoxic potential have been also identified in SLE patients and from subjects recently vaccinated [113]. Consistent with the relevance of killer B lymphocytes to normal immune regulation, disease pathogenesis, and inflammation [111], B cells have been further reported to induce peripheral tolerance by sensitizing T cells to activation–induced cell death via the inhibitory inhibitors programmed death 1 (PD–1) and CTLA4 upon Ag re–encounter [114]. In EAE, a fascinating study by Khan and colleagues revealed that B cells expressing high levels of PD–L1 (PD–L1^{hi} B cells) can resolve disease upon adoptive transfer, without a concomitant increased proportion of Tregs, by negatively regulating proinflammatory T cell differentiation in an IL-10–independent manner [14]. Most importantly, other data from this study demonstrate that *in situ* 'naturally' occurring PD–L1^{hi} B cells were primarily responsible for homoeostatic regulation of T helper follicular (T_{FH}) cells and the subsequent humoral response [14]. Intriguingly, PD–L1^{hi} B cells were found to be refractory to B–cell depletion therapy, suggesting thereby that this regulatory B cell population may account for some of the rapid onset of beneficial effects of anti–CD20 treatment in autoimmunity.

Of specifc interest, data also indicate that regulatory B cells play a major role in maintaining iNKT cell (glycolipid-specific, CD1d–restricted innate lymphocytes) homeostasis in humans via lipid–Ag presentation in the context of CD1d [55]. Interestingly, defective B cell–mediated stimulation of iNKT cells in SLE patients was found to be associated with altered CD1d recycling. Finally,

converging lines of evidence also suggests that regulatory Abs may be implicated in the suppression of immune responses. DC activation can be restrained via the binding of IgG to FcγRIIB, as well as IgG–mediated clearance of potentially pathogenic host apoptotic cells [115]. In addition, 'natural' or 'non–immune' Abs have also been shown to possess regulatory functions [116]. In particular, data indicate that IgM production by peritoneal B1 cells may be involved in the suppression of colitis induced by non–hygienic conditions [26]. In a fascinating study, Kirkland and co–workers moreover demonstrated the importance of IgM production and complement in conferring protection against acute colitis [117].

These selected examples illustrate the improvement in the understanding of the multifaceted regulatory properties of B cells in modulating immune responses, given the ability of regulatory B cells to target many components of both the innate and adaptive responses to ensure efficient suppression. This diversity in functional regulatory capacity of B cells is conceivable attributable to the adaptation of B cells to the diversity of functional demands in different tissues in the context of inflammatory and autoimmune diseases.

Cognate interactions control regulatory B cell effector function *in vivo*

The role of cognate B cell–T cell interactions in regulatory B cell development and function during autoimmunity has been investigated most thoroughly on B10 cells. In 2002, Mizogushi and colleagues reported the requirement for B cell class II MHC expression for suppression in IBD, but the role of this pathway was demonstrated to expand the initial population of IL-10–producing B cells rather than to induce B cell for IL-10 production [118]. In latter studies, consistent with the observations in nude mice that B10 cell development is T cell–independent, B cell expression of MHC class I and class II molecules as well as CD40 or IL-10 was reported to be dispensable for normal B10 cell development [18]. Yet, maturation of B10 cells into functional IL-10–producing effector B10 cells that restrain *in vivo* autoimmune reactions was shown to require IL-21– and CD40–dependent cognate interactions with $CD4^+$ T cells [70], implying thereby that B10 cells and $CD4^+$ T cells may necessitate intimate interactions during reciprocal IL-10 and IL-21 production to optimally regulate Ag–specific T cell–mediated immune responses [70]. Importantly, whereas data indicate that maturation of B10 cells is MHC class II–independent, B10 cell expression of MHC class II molecules was documented to be necessary to regulate Ag–specific T cell responses *in vivo* [70]. In summary, the necessity of B10 cells to establish cognate interactions with $CD4^+$ T cells to drive B10 cell development and effector function points to a regulatory–feedback loop whereby the local delivery of B10 cell–derived IL-10 preferentially retrains Ag–specific T cell responses during direct B10–T cell interactions, even though, indirectly, IL-10–mediated regulation of Ag presentation by innate cells, such as DCs or macrophages, is also conceivable. In either possibility, Ag specificity eventually controls the effector function of B10 cells [67]. Quite interestingly, cognate interaction with iNKT cells was recently reported to expand Ag–specific IL-10–producing regulatory B cells [119].

BCR specificity regulates suppressive B cell function

As discussed in Appendix A, data from mice with an altered BCR repertoire [18,66], transduction cascade [62,63] or co–receptors [1,60], suggest that BCR signaling leads to B10 cell acquisition of effector capacities, thereby positively selecting for Ag–specific regulatory B cells *in vivo.* While *in vitro* regulatory B cells can be activated in an Ag–nonspecific manner by many kinds of stimuli, including TLR ligands [18,85,89], CD40 [6,18,68] and cytokines [70], alone or in combination [18,61,70], to promote IL-10 production and effector suppressive functions, other data, however, indicate that regulatory B cells that are *in vivo* activated by one Ag do not confer protection in T–dependent inflammatory models induced by another Ag [1,5,120]. Consistent with the notion that regulatory B cell function is Ag–specific, autoreactive B cell IL-10 provision during EAE necessitates simultaneous auto–Ag and CD40 *in vitro* stimulation [86]. Notably, the potent efficiency of Ag processing and subsequent presentation by B cells requires Ag–induced BCR signaling and that BCR–initiated signaling and T cell help acquired through Ag presentation provide the two decisive signals required for B cell activation and subsequent effector responses [121]. Thus, BCR specificity and signaling intensity are likely developmental checkpoints that allow regulatory B cells to react promptly to self– or foreign Ags as a prime line of defense to defend against vigorous immune responses that could lead to tissue pathology.

Location of regulatory B cell development

These findings call into question the location of regulatory B cell development during disease immunopathogenesis. It is worth noting in this context that most reports so far, as detailed herein, have documented spleen populations of regulatory B cells. Studies of B10 cells in particular have provided valuable insights relevant to this question. Despite the fact that B10 cells represent only 1–2% of spleen cells, these cells have been shown in multiple models to dramatically inhibit the induction of Ag–specific inflammatory reactions and autoimmunity [1,122,123]. In mice, while B10 cells are mainly detected in the spleen [18], they have also been identified in GALTs, such as the mesenteric LNs and peritoneal cavity, as well as in the peripheral blood and LNs [18,19,118]. It is conceivable that certain IL-10$^+$ regulatory B cell subsets are induced in the spleen [124], but then may circulate to other sites. Whether once induced, migrating regulatory B cells can function outside of the spleen is still unclear. In an excellent study exploring the involvement of LN B cells in EAE suppression, data from Matsumoto and colleagues suggest that splenic B cells can suppress autoimmune neuroinflammation in an adoptive transfer setting but that their plasmablast differentiation in the dLNs might be required [13]. Another interesting observation reported in that study was that regulatory B cells can be newly generated in extrafollicular foci where they acquire suppressive effector functions, implying a negative regulation dictated by inflammatory environment to protect excessive inflammation [13].

Location of regulatory B cell effector function

One important question that arises from these observations is whether the control of immune responses by regulatory B cells takes place centrally in secondary lymphoid organs or locally in non–lymphoid tissues where inflammation occurs. Numerous studies suggest that regulatory B cells predominantly control inflammatory responses centrally rather than at the site of inflammation [125]. As for examples, in experimental contact hypersensitivity (CHS), a model for type 1–mediated inflammation, B cells were shown to suppress CHS reaction by influencing effector T cell activation and/or differentiation in the peripheral tissues considering that B cells were absent in the lesion and that B cell infiltration following adoptive transfer of splenic B cells was not observed in the challenged ears during CHS responses [59]. Similarly, while regulatory IL-10–secreting B cells were found upon adoptive transfer to limit CNS inflammation in murine experimental stroke by inhibiting peripheral T cell inflammatory cytokine production and infiltration of inflammatory T cells into lesions, adoptively transferred B cells in the affected brain were not detected [126]. Mechanistically, results from a number of studies indicate that regulatory B cells exert their effect within LNs draining the inflamed areas. In 2002, Mizoguchi and colleagues established that IL-10 produced by mesenteric LN B cells regulate the progression of intestinal inflammation by directly down–regulating T cell activation within intestinal tissue [118]. Consistent with the notion that dLNs are an elective site for tissue immune–surveillance, for the induction of adaptive immune responses and a candidate compartment for the maintenance of peripheral tolerance [127,128], adoptive transfer of IL-10–deficient B cells were reported in a model of arthritis to lead to the accumulation of Th1 and Th17 cells in the LN draining the site of inflammation [10]. Interestingly, adoptive transfer of regulatory spleen B cells has been shown in a model of allergic airway inflammation to expand Tregs to the site of inflammation, including in lungs and lung draining mediastinal LNs, while no specific Treg expansion was detected in the spleen of recipient mice [129]. These findings indicate that regulatory B cells preferentially modulate T cell responses in regional LNs.

Research in the EAE has provided some interesting findings and added to the debate in this area. In a fascinating study, Tedder and co–workers established that B10 cell regulatory function is critical during disease initiation, but not after disease onset [123]. In particular, B10 cell numbers were found to expand quickly within the spleen, but not CNS, following myelin oligodendrocyte glycoprotein (MOG) immunization, which paralleled B10 cell regulation of disease initiation [123]. Similar results were also described for inguinal and auxiliary LNs draining the site of MOG immunization [123]. Data from a recent study led by the group of Bettelli also support, without completely ruling out regulatory B cell activity in the CNS, that an important part of the immunosuppressive effects of regulatory B cells during the course of MOG–induced EAE takes place in both spleen and LNs [130]. In two recent sophisticated studies, data also indicate that upon MOG immunization, a significant proportion of regulatory B cells that express CD138 are present in the spleen and LNs where they can limit T cell responses [12,13]. According to data from Matsumoto and colleagues, it should be, however, emphazised here that plasmablasts in the dLNs, but not splenic

B lineage cells, predominantly expressed IL-10 during EAE [13]. In another study, adoptively transferred PD–L1^{hi} B cells were found to suppress MOG–induced neuroinflammation independently of IL-10 by restricting T cell differentiation in dLNs and CNS cell infiltration but were not observed in the spinal cord, indicating a systemic role, rather than effects at the inflamed site [14]. Different studies have, however, yielded different conclusions. In an interesting recent EAE study led by the group of Zamvil, data raised the possibility that CNS regulatory B10 cells contribute to immune modulation *in situ* in the inflamed tissue [131]. Consistent with these conclusions, data also suggest in a model of viral brain infection, that infiltrating regulatory B10 cells control neuroinflammation *in situ* [132]. These findings altogether indicate that the presence of IL-10–competent B cells in the CNS might be important for the control of pathogenic T cell responses in the target tissue.

Few other studies have also documented in other models the ability of B cells to locally regulate immune responses within extralymphoid tissues. In one study, a subset of regulatory B cells that are abundant within adipose tissue and which potently restrained adipose tissue inflammation were identified to maintain metabolic homeostasis though constitutive IL-10 production [133]. In another study, IL-10^{+} regulatory B cells with known potential to suppress T cell–driven skin inflammation have been reported to preferentially migrate into the inflamed skin of mice to limit cutaneous inflammation [134]. Finally, data indicate that hilar LNs of mice during the resolution phase of the pulmonary allergic airway disease responses are enriched in a population of TGF-β^{+} B cells that had the ability to return to active pulmonary sites of inflammation to regulate immune responses [120].

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