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

Molnarfi, Nicolas

UNIVERSITÉ DE GENÈVE
FACULTÉ DE MÉDECINE

Division of Clinical Pathology

Department of Pathology and Immunology

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

Thesis submitted to the Faculty of Medicine of
the University of Geneva

for the degree of Privat-Docent

by

Nicolas MOLNARFI

Geneva

2018

Table of Contents

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

Abstract

In recent years, an impressive body of evidence has accumulated demonstrating that B cells fulfill a number of immunological functions beyond eliciting humoral immune responses. The ability of B cells to stimulate T cell–mediated immune responses through antigen presentation, co–stimulation and cytokine production has attracted great attention from researchers and clinicians due to its significance for therapeutic intervention in a series of inflammatory and autoimmune disorders. Nowadays, observations from a number of experimental approaches as well as in patients with inflammatory and autoimmune diseases have equally emphasized a significant function for B cells in maintaining immune homeostasis through maintenance of tolerance and prevention of unrestrained inflammation. Whereas studies by many investigators have started to identify the key mechanisms and molecules that support these processes, further effort is necessary to comprehend the diversity of regulatory B cell subsets and the cues that prompt their fate as regulatory versus proinflammatory. In this review I summarize the latest knowledge on the phenotypic and functional characteristics of different regulatory B cell populations, the cues that orchestrate their induction, and their potential therapeutic relevance in controlling both human inflammatory and autoimmune disorders and also report in this context the insights of my personal research.

Summary

B cells, also known as B lymphocytes, are an integral part of the adaptive immune response. B cells have been traditionally reported to function as effector cells in both physiological immunity and pathological autoimmunity via multiple pathways that include production of antibodies (Abs), secondary and/or ectopic lymphoid tissue formation, secretion of cytokines, and presentation of antigen (Ag) to T cells . These observations have paved the way for testing B cell–directed therapies in the treatment of immune–mediated inflammatory diseases such as multiple sclerosis (MS). While the mechanisms underlying the benefit of B cell–depletion in chronic inflammatory disorders remain to be fully elucidated, B cell–targeted therapy has unquestionably renewed the interest in the role of B cells in many immunopathologies. In several collaborative research projects in experimental autoimmune encephalomyelitis (EAE), a prototype of organ–specific $CD4^+$ T cell–mediated autoimmune disease, my work has adressed *in vivo* the different functions of B cells during autoimmune neuroinflammation, findings which may be relevant to therapies targeting B cells [1-3].

Remarkably, parallel to the growing interest to use new B cell–targeted therapeutic options for the treatment of inflammatory and autoimmune conditions, a role, quite paradoxical for B cells with immunosuppressive and/or immunoregulatory properties, in restraining inflammatory reactions and maintaining tolerance has been also progressively appreciated over the last twenty years. The current literature suggests that a broad variety of phenotypically distinct subtypes of regulatory B

cells exerts the ability to restrict potentially damaging inflammatory reactions both in animal models and human subjects [4,5]. These findings suggest that B cell–mediated immune regulation could be translated therapeutically in patients with immune–driven inflammatory disorders. In this context, my recent research results in EAE, a widely–accepted preclinical model of MS, have documented *in vivo* the importance of transforming growth factor-β (TGF-β)–producing B cells in regulating the initiation of autoimmune neuroinflammation [6]. By using *in vitro*–activated human naive B cells, my research has further documented evidence that B cell activation may contribute to immunological abnormalities seen in MS by reducing the spontaneous production of TGF-β by B cells, consequently favoring the overall proinflammatory properties of activated human B cells [7].

Successful translation requires a critical understanding of the mechanisms of action of the different reported regulatory B cell subsets, their relationship, plasticity and the conditions that orchestrate their generation. In this review, I discuss current hypotheses and points of polemic associated with the phenotype, origin, mode of action of regulatory B cells in the control of several autoimmune and inflammatory reactions and also discusses their potential application as cellular therapeutic agents for treating immune–mediated inflammatory diseases in the future. While underappreciated for years, it is now very likely, as the field constantly grows with an exponential number of studies, that regulatory B cells will not only be seen as another major brake for T–cell immunity, but also that regulatory B cell immunotherapy will become a bedside reality in clinical immunology.

Introduction

The concept of suppression mediated by B cells is as old as the discovery of suppressive T cells, when the capacity of B cells was reported more than forty years ago to suppress the development of delayed–type hypersensitivity reactions in guinea pigs [8,9]. Despite the enormous potential of these early observations, this field of investigation was however largely ignored by the scientific community, due in part to a relatively poor understanding of the biochemical and molecular mechanisms of action responsible for this phenomenon. It was not until the 80's that these initial results were again supported by *in vivo* studies reporting that activated splenic B cells could adoptively transfer tolerance to naive recipient mice via differentiation of T lymphocytes into suppressor T cells [10,11]. In 1996, Wolf and colleagues were the first to genuinely re–awaken the interest in the suppressive activities of B cells when they revealed that spontaneous recovery from EAE is impaired in the congenital absence of B cells [12]. One–year latter, Mizoguchi et al. demonstrated a suppressive role for B cells in the pathogenesis of spontaneous chronic colitis in T–cell receptor α mutant ($TCR\alpha^{-/-}$) mice [13]. Consistent with a suppressive role of B cells in inflammatory diseases mediated by helper T (Th) cells, B cells were subsequently shown in 1998 to down–regulate the late stage of $CD4^+$ T cell–mediated granulomatous pathology in *Schistoma mansoni* infection [14]. A few years later, several studies expanded on these findings and described that B cells exerted part of their suppressive effects via the release of interleukin-10 (IL-10) in EAE [15], chronic colitis [16] and collagen–induced arthritis (CIA) [17]. Remarkably, 'regulatory B cells', a term coined by Mizoguchi

and Bhan [16] that describes heterogeneous B cell subtypes with regulatory properties, analogous to regulatory T cells (Tregs), were rapidly reported in several other studies in the early 2000's to employ a number of different mechanisms to restrain both effector $CD4^+$ and $CD8^+$ T cells [4]. Research over the past 15 years has provided a more comprehensive understanding of the mechanisms and molecular pathways by which regulatory B cells mediate immune suppression *in vivo* and has provided preliminary empirical evidence for their potential therapeutic efficacy in immune–mediated inflammatory diseases. It appears now clear that regulatory B cell functions are associated with a battery of immunomodulatory mechanisms that affect all facets of the immune response, which makes regulatory B cells attractive therapeutic candidates for many conditions, including infections, inflammatory diseases and autoimmune diseases.

Phenotypic identification of regulatory B cell populations

Detailed phenotypical and functional characterization of regulatory B cells *in vivo* is essential for future therapeutic strategies for inflammatory and autoimmune diseases. It embraces in particular the development of novel selective agents that spare or enhance regulatory B cells in order to greatly optimize current B cell–targeting therapies. Thus far, numerous subtypes of regulatory B cells with distinct and/or partially overlapping molecular and cellular features, mechanisms of suppression, and tissue distribution have been described both in mice and human subjects depending on the disease entities and physiopathological states [5]. Due to space constraints, however, only a sampling of this large body of work is discussed in this review. Detailed phenotypic analyses of both murine and human regulatory B cell populations is provided in the Appendix A (optional). Here, I will discuss briefly some of the salient observations of these studies.

Significant differences have been reported with regard to the phenotypic identification of both murine and human regulatory B cells. Phenotypic inconsistencies reported across early studies could be, in part, attributed to the use of different *ex vivo* induction methods to appreciate their effector function. In particular, as spontaneous *ex vivo* IL-10 production by B cells rarely occurs, identification of regulatory B cells based on their competency to produce and secrete IL-10 following *in vitro* appropriate stimulation has led the researchers to draw inaccurate conclusions, leading to a great deal of confusion for some time regarding the characterization of regulatory B cells. It is also important to recognize that a lack of ‘standard gold’ validated phenotypic cell–surface markers or set of markers to define and isolate regulatory B cells has long been, and partially continues to be, a major road block for studying their ontology, development, relationships, and immunological roles. Nowadays, recent advances in instrumentation, primarily in the realm of multiparametric flow cytometry, transcriptomic analyses and reporter mice, have however been made to overcome some of these limitations and are currently revealing new insights into regulatory B cell developmental pathways and phenotype and also have greatly improved our understanding of the phenotypic diversity of regulatory B cells. The results of these researches, as summarized herein, have undoubtedly reconciled at least some contrasting findings and conclusions across studies [18].

Phenotypic identification of murine regulatory B cell populations

In mice, a common feature of the vast majority of all reported regulatory B cell subsets is their ability to produce IL-10 in a broad spectrum of immune pathological conditions [16,18-20]. From these observations, it was surmised that most, if not all, multiple phenotypically defined murine IL-10–competent B cell subsets only contain the highest frequency of IL-10–producing B cells rather than representing a true 'regulatory B cell phenotype' [21]. Consistent with this view, it is generally acknowledged that B cell–derived IL-10 identifies a population of cells with a fairly homogenous cell surface phenotype [22]. Murine B10 cells [20,23-25], the first regulatory B cells that were recognized, share overlapping phenotypic markers with $CD5^+$ B1a cells, $CD1d^{hi}CD21^+CD23^-$ marginal zone (MZ) B cells, and less–mature transitional 2 MZ pecursor (T2–MZP) $CD1d^{hi}CD21^+CD23^+$ B cells [20,24-31] but do not exclusively belong to either subset. The characterization of additional identified IL-10–producer regulatory B cells, such as for example IL-33–induced/expanded regulatory B cells ($Breg^{IL-33}$) [32], indicates similar conclusions.

From these observations, B cell–derived IL-10 production might conceivably not be therefore considered as a single marker for B10 cells or other $IL\text{-}10^+$ B cells but as a hallmark feature of an inflammatory microenvironment, which critically depends upon the different physiopathological models. This hypothesis provided the impetus for the search of a unique marker that would exclusively and exhaustively identify regulatory B cells. In this context, T cell immunoglobulin mucin domain-1 (TIM-1), a co–stimulatory molecule, was found in a study to be expressed by most murine IL-10–competent B cells in all major B cell compartments, including transitional, MZ, follicular (FO) B cells, and $CD1d^{hi}CD5^+$ B10 cells [33]. Other data however indicate that $IL\text{-}10^+$ B cells could also be found in the $TIM\text{-}1^-$ compartment, and that $TIM\text{-}1^+$ B cells could be detected in the non–$CD1d^{hi}CD5^+$ B10 compartment [34]. Additional data also revealed CD9 as a surface marker for a variety of murine IL-10–competent B cells and their progenitors [35]. Besides their role as reasonable inclusive surface markers for $IL\text{-}10^+$ B cells, both TIM-1 and CD9 play a major role in the immunosuppressive activity of regulatory B cells [33,35]. Newer observations by Matsushita and colleagues also put forward the coexpression CD9 and CD80 as a novel phenotypic parameter for both MZ–B10 and B1–B10 cells [36]. Higher expression of CD86 was also noted in $IL\text{-}10^+$ B cells relative than in $IL\text{-}10^-$ B cells, although the difference was not as distinct as that observed for CD80 [36]. The identification of these diverse cell–surface molecules is undoubtedly a first, but critical, step in the process of defining the ontology of $IL\text{-}10^+$ B cells as it allows the identification of $IL\text{-}10^+$ B cells without exogenous stimulation of IL-10 production and thereby assists in the identification of transcription factors driving regulatory B cell induction. This is a critical aspect as external stimuli, commonly used to elicit IL-10 production, would otherwise alter regulatory B cell transcriptional networks and activity.

In summary, mouse regulatory B cells remain challenging to identify, as distinct subpopulations based on differential expression of cell–surface markers have been documented to produce IL-10 and B cells exert regulatory abilities through various mechanisms. Interestingly, alike $IL\text{-}10^+$ B cells, program death–ligand (PD–L)1^{hi} B cells, which are considered to be critical regulators

of humoral immunity, also appear to be diffusely scattered throughout the B cell lineage, rather than confined to a specific B cell compartment. Further work is therefore needed to determine their ontogeny and relationship to previously defined regulatory B cells. On a personal note, I should emphasized here that only a rare number of murine regulatory B cell subsets could possibly exist and therefore that the genuine variety of regulatory B cell populations may be smaller than currently reported. Conceivably, IL-10 production for instance may identify B cells that have rapidly adapted to different environmental conditions and gained distinct functional programs in response to signals of various nature. Pertinent to this notion, studies using IL-10 reporter mice revealed that B10 effector cells are hardly detected in naive mice [19,23,25,29,37] and that a modest increase in B10 cell frequencies is observed upon *in vivo* lipopolysaccharide (LPS) stimulation [25,37] or following intestinal injury [19,23]. This reveals that murine B10 effector cells, against all odds, are detected at low frequencies in models of infection or intestinal injury where they are commonly acknowledged to potently control disease severity [34]. One possible reason, among many, for this singular observation, is that B10 cells only transiently produce IL-10 prior to their progressive terminal maturation into Ab–producing plasmablasts and plasma cells that contribute to humoral immunity [25].

Phenotypic identification of human regulatory B cell populations

Phenotypic characterization of human immune–regulating B cells faces numerous problems including in particular a restricted access to lymphoid tissues; mouse studies indeed indicate that the vast majority of these cells belongs to non–migratory populations residing in secondary lymphoid tisssues [38]. Despite this limitation, several putative phenotypically distinct human regulatory B cell populations, primarily identified by their capability to express IL-10, have been described [39]. Findings from numerous studies further collectively suggest that human regulatory B cells, akin to their murine counterparts, can adopt a wide range of phenotypes. From these obserbations, it appears thus justifiable to assume that human IL-10–competent B cells may not be restricted to a single clearly defined subpopulation, identified by a unique phenotype, but that human IL-10–secreting B cells are likely enriched in different B cell pools that adapt to their microenvironment. Future studies will undoubtedly lead to the documentation of additional immunoregulatory B cell populations and mechanisms of actions, which is expected to uncover a substantial level of functional diversity within the B cell lineage.

Further research is likely also to facilitate the resolution of discrepant phenotypic findings. Some discrepant observations may indeed relate to the different approaches that have been used to characterize the various regulatory human B cell populations. Primarily, as the vast majority of freshly isolated human B cells do not spontaneously express detectable IL-10 levels [7], detection of IL-10 expression following *ex vivo* stimulation does not allow, as aforementioned, the proper *in vivo* identification and isolation of regulatory B cells. It is also conceivable that diverse types of stimulation induce IL-10 production via distinct pathways and in different B cell populations. Pertinent to this point, only a minute proportion (3–4%) of $CD24^{hi}CD27^{+}$ B cells are producing IL-10 after succinct stimulation [40], while merely 10–15% of $CD24^{hi}CD38^{hi}$ B cells express IL-10 after 72 hours stimulation

[41], suggesting the existence of a certain degree of plasticity in regard to IL-10 production in B cells. An additional issue is that membrane–bound molecules associated so far with human regulatory B cell phenotypes do not identify unique IL-10–competent B cell populations. Some recent data suggest that TIM-1 is a viable, but not exclusive, marker for IL-10$^+$ regulatory B cells in humans [42]. TIM-1 was reported to be expressed in the majority of transitional IL-10$^+$ B cells, while naive, memory, and plasmablast TIM-1$^+$ B cells did not show major IL-10 expression [42]. Improved phenotypic, but also transcriptomic, characterization of regulatory B cells is needed to facilitate the preclinical testing of novel B cell–targeted immunotherapies in several inflammatory and autoimmune disorders and could also be potentially helpful in immunomonitoring these various diseases.

Ultimately, it is also important to recognize that a thriving program of cell therapy would rely decisively on precise evaluation of regulatory B cell suppressive function instantaneously prior administration into patients. In this view, assessment of anti–inflammatory cytokine production and/or immunomodulatory cell–surface molecule expression by B cells should not be considered alone to ascertain the true functional status of regulatory B cells. Among others, cellular response measurements of responding T cell activation (e.g. CD69 and CD154) in short–term regulatory B–T cell co–cultures can act, for instance, as an early readout for regulatory B cell–mediated suppression. This is an important aspect that should deserve more attention in future research.

Origin and development of regulatory B cells

The origin of regulatory B cells is not yet clear. Three different hypothetical theories explaining regulatory B cell subtypes have been built on the basis of their considerable phenotypic and functional diversity and inducing factors [5]. These three hypotheses are not mutually exclusive and all three may contribute to the observed co–occurrence of multiple regulatory B cell subsets.

A first hypothesis, a ‘single–lineage model’, assumes that regulatory B cells, similar to thymus–derived Tregs, derive from a single progenitor, therefore representing a dedicated functional lineage. This assumption infers that a specific transcription factor controls the expression of genes responsible for their regulatory and/or suppressive properties and thus that at least two progenitors (B1 and B2 progenitors) give rise to various regulatory B cell subsets. Although, to the best of the authors' knowledge, no study that has conducted expression arrays on murine or human regulatory B cells has convincingly identified a major lineage–specific transcription factor equivalent to FoxP3 [43,44], it still remains possible that the different reported regulatory B cell subsets can be developmentally related. For example, expression of the surface markers CD5, CD1d, CD21, CD9, CD80 and TIM-1 alone or in combination have been shown to identify most IL-10–competent murine B cells [33,35,43]. It is thus conceivable that these phenotypic surface markers simply characterize regulatory B cells at a particular stage of development and that the functional immunophenotype of these cells adjusts in response to specific environmental cues and stimuli that surround them.

Based on shared phenotypic surface marker overlap between most described IL-10–competent B cell subsets, a second hypothesis, a ‘multiple–lineage model’, proposed by DiLillo and colleagues in 2010 claims that all different B cell subsets contain regulatory B cell precursors that

have the capacity to mature to IL-10–producing B cells upon activation [18]. Supporting this notion, B10 cells, which may constitute the major 'natural' subset of splenic IL-10–producing B cells [18,20], can develop from a progenitor population (B10Pro). It remains hence possible that phenotypically distinct regulatory B cell subpopulations expressing overlapping cell–surface markers, such as TIM-1, shared by different regulatory B cell subsets may evolve from distinct lineages but share the common functional trait of being IL-10 competent. Nowadays, 'naturally' occurring regulatory B cells, which arise from specific regulatory B cell progenitors, with different phenotypes have been identified within at least three main subsets of B lymphocytes: the B1a, B2 MZ and T2–MZP cell populations.

As regulatory B cells embrace many phenotypically separate B cell lineages and are for the most part identified by the production of immunosuppressive cytokines, it could be argued that regulatory B cells do not truly represent distinct lineages or subsets but rather possess or acquire common effector function capabilities that can be elicited by any B cells. In this regard, a third hypothesis, an 'activation–induced model' suggests that the generation of regulatory B cells depends on the right mix of environmental stimuli and that any B cell can obtain regulatory properties when immunosuppression is required. Therefore, unlike natural Tregs, B cells with regulatory properties may not be lineage–specific but rather 'reactive'. There are actually strong reasons, described in the next sections, for considering the role of microenvironmental factors in the induction of regulatory B cells [4,45-47]. Consistent with this model is the recent demonstrations that regulatory B cells can arise at different stages of development.

Until recently, regulatory B cell subsets were thought to exclusively develop at a stage of development preceding terminally differentiated plasma cells. A study using IL-10 reporter mice has however recently demonstrated that IL-10–producing plasmablasts, generated only during EAE inflammation, could exert regulatory function in autoimmune neuroinflammation [48]. Likewise, a study conducted by Shen and co–workers has shown that IL-10 and IL-35 expression acquired during plasma cell maturation accounts for the activities of Ab–secreting 'regulatory plasma cells' during EAE and *Salmonella typhimurium* infection [43]. It is worth pointing out that the population of IL-35–producing B cells (i35–Breg) has been alleged by some to represent a distinct B cell lineage [49] as IL-35 production, which is restricted only to $CD138^+$ plasma cells, appears to be stringently regulated by this B cell type [43,46]. Yet, other data suggest that IL-35 expression may not be a characteristic feature of $CD138^+$ plasma cells during inflammation [48]. This suggests that the i35–Breg population is not unique but rather reactive to specific inflammatory stimuli. Additional evidence indicate that human B cells also differentiate into IL-10–competent plasmablasts [25] and that human plasmablasts are enriched in IL-10–producing B cells [50]. The concept that Ab–producing B cells also take part to the limitation of immune responses is at odds with their traditional role in supporting chronic inflammatory processes in autoimmune diseases as professional pathogenic autoAb factories. Due to their extreme specialization, it had indeed been generally presumed that they were devoid of any other relevant immune function. In an attempt to reconcile this picture, some have postulated that there could be a minority of plasmablasts that maintain their ability to regulate inflammatory

responses while secreting Ab [4]. For example, naive (IgM), but not memory (IgG), B cell–derived human plasmablasts were reported to secrete IL-10 [48], suggesting, at least, that a subset of plasmablasts contained within the IgM^+ plasmablast pool of B cells may exert regulatory functions. Other studies also support the conclusion that only a restricted number of B cell subsets at later stages of development could produce regulatory factors and exhibit suppressive capacity. In particular, data indicate that while B10 cells maintain a capacity for plasma cell differentiation, their ability to produce IL-10 expression stops during B10 cell differentiation into Ab–secreting cells [25].

Signals/activation modes involved in regulatory B cell function and development

Exploring and exploiting the therapeutic potential of regulatory B cells would require the identification of the factors responsible for their development and activity. Regulatory B cells have been documented in various models of inflammation, autoimmunity, allergy, transplantation reactions, infection, and in anti–tumor immunity. It is hence likely that B cells are highly susceptible to immune microenvironmental cues, which may ensure a fine–tuning of their *in vivo* behavior via the induction of specialized activation programs. Regulatory B cells generated under different conditions might also conceivably display distinct activation requirements. To date, several factors have been shown to confer a regulatory function on B cells. Although the majority of evidence demonstrating the importance of these signals derives from murine studies, several of those have also been reported to be of relevance for the development of regulatory B cells in humans. In addition to signaling pathways from Toll–like receptors (TLRs), CD40, and/or B cell receptor (BCR), which are known to play dominant roles in regulatory B cell activation and function, there is emerging evidence that specific inflammatory cytokine receptor signaling pathways may also be essential to the induction of immunosuppressive regulatory B cells [39]. I will specifically discuss in the next section some of the salient features of this emerging theme. Due to space limitations, the importance of TLR, CD40, and BCR pathways will not be discussed further here. Complementary knowledge regarding the integrative cellular and molecular signals controlling the development and activity of regulatory B lineage cells is provided in the Appendix B (optional).

Inflammatory signals

Increasing evidence points to inflammation as a requisite for the development and function of regulatory B cells. While under normal non–inflammatory conditions, data indicate that regulatory B cells are present at relatively low levels to maintain adequate immunological self–tolerance [4], the frequency and immunosuppressive capacity of regulatory B cells were reported, by most studies, to augment during the progression of inflammation in various models of T cell–mediated inflammatory and autoimmune diseases [15,16,20,27,48,51-53].

Although most of the current findings indicate the spleen as the primary site for regulatory B cell development , these observations nevertheless imply that the signals that initiate autoimmune and/or inflammatory disease could also control regulatory B cell induction. To date, only a few of studies have identified a direct causal link between inflammation and the generation of regulatory B

cells. More than 15 years ago, Mizoguchi and colleagues established in a outstanding study that induction of a subset of IL-10–producing regulatory B cells, which were found in the gut–associated lymphoid tissues (GALTs), but not spleen, was associated with the development of intestinal inflammation [16]. More recently, and in a similar vein, Maseda and colleagues have established that gut–associated inflammation induced B10 effector cell–derived IL-10 production in both the peritoneal cavity and mesenteric lymph nodes (MLNs) [23]. A fascinating study conducted by Matsumoto and colleagues identified plasmablasts generated specifically in the draining LNs (dLNs) after EAE induction as the dominant IL-10–producing regulatory B cells during autoimmune neuroinflammation [48]. More importantly, this study revealed that plasmablast differentiation in the dLNs was not affected by splenectomy. Whereas the specific inflammatory signals involved in the generation of regulatory B cells in these experimental models remains unresolved, it is quite clear that regulatory B cells can develop and obtain their suppressive properties outside the spleen, in the LNs draining the site of inflammation after the development of the disease, supporting together the idea that the induction of regulatory B cells is dictated by inflammatory environment. This notion has received strong support from the recent research results of Rosser and colleagues who showed unambiguously in an experimental model of inflammatory arthritis that regulatory B cells are induced by gut microbiota–driven IL-1β and IL-6 production [53]. The authors of this excellent study further showed that microbiota changes after antibiotic intervention or by modifications in the sterility of housing conditions decreased both the number and activity of regulatory B cells and severity of arthritis [53]. These observations clearly suggest that B cells could sense inflammatory cytokines as 'endogenous danger signals' and, in turn, react by restraining the inflammatory response that would otherwise propagate. To further support this view, other proinflammatory cytokines have also been found to be important in regulatory B cell expansion and differentiation. In particular, it was reported that the adoptive transfer of IL-21–receptor deficient B cells before EAE induction are unable to normalize EAE course, documenting the role of IL-21 signaling in driving regulatory B cell expansion and effector cell generation [45]. Other data also indicate that granulocyte–macrophage colony–stimulating factor (GM–CSF), as a fusion cytokine with the immune stimulatory protein IL-15 or monocyte chemotactic protein-3 (MCP3), can convert *ex vivo* naive $CD19^+$ B cells to suppressive IL-10–competent regulatory B cells [54,55].

Of special note is also the finding that robust inflammation or immune suppression by B cells depends on the family member and the exact combination of heterodimeric cytokine subunit proteins. Recent data indeed indicate that IL-12p35, a common subunit to IL-35 and IL-12, which have opposing effects on inflammation, induces regulatory B cells and can be used therapeutically to limit autoimmune uveitis in mice [56]. Therefore, altering the balance between the different subunits may be considered as a potential strategy to regulate inflammatory responses. Importantly, as B cell fate decisions critically further depend on the precise combination and strength of contributing signals [57], distinct proinflammatory cytokine concentrations have, conversely, also the potential to give rise to B cells with inflammatory rather than regulatory functions. Evidence in favor of this notion

was recently obtained in an elegant study by Menon and co–workers that showed that the level of exposure to type–I interferon (IFN)-α from plasmacytoid dendritic cells (pDCs) determines whether an immature B cell develops into a regulatory B cell or a plasmablast; lower concentrations promoted both regulatory B cell and plasmablast generation and higher ones skewed differentiation towards plasmablasts but failed to expand regulatory B cells [58]. These data were further consolidated by assessment of *in vivo* frequency and function of regulatory B cells in patients with systemic lupus erythematosus (SLE), raising the possibility that *in vivo* exposure of B cells to high levels of IFN-α released by chronically activated pDCs could be responsible for the reduced frequency of IL-10$^+$ B cells and preferential accumulation of autoAb–producing plasma cells seen in these patients [58]. Conceivably, insufficient suppression of inflammation in autoimmune diseases may thus result from impaired immune–regulatory responses of B cells and/or altered generation of functional regulatory B cells as a consequence of chronic inflammation [39].

Anti–inflammatory cytokines may also be important in the generation of regulatory B cells. In a mouse model of experimental autoimmune uveitis, the anti–inflammatory cytokine IL-35 has been shown to induce the conversion of conventional B cells or B10 cells into IL–35-producing B cells (i35–Breg), a population of potent regulatory B cells that suppresses the development and progression of the disease [46]. In another study, B cells were reported to mediate the anti–inflammatory effects of IFN-β therapy in an experimental model of MS [47]. Data also suggest that IL-10 autocrine and paracrine signaling *in vivo* supports the induction of an immunoregulatory phenotype in B cells that exerts substantial anti–inflammatory and immunosuppressive functions [59,60]. Finally, a few 'dual cytokines' with potential pro– or anti–inflammatory functions depending on specific local microenvironments, have been reported to control regulatory B cell development. IL-33, a member of the IL-1 superfamily of cytokines [61], was found to induce a subset of IL-10–producing B cells, which, upon adoptive transfer, can potently block the development of spontaneous colitis [32]. Similarly, data indicate that the cytokine B cell activation factor of the TNF family (BAFF) induces *in vitro* the differentiation of CD1d$^+$CD5$^+$ IL-10–producing regulatory B cells and expand *in vivo* the number of MZ regulatory B cells in an animal model of arthritis [62]. Interestingly, high concentrations of BAFF was found to significantly decrease IL-10$^+$ B cells *in vitro*, therefore reversing the effect that BAFF showed in lower concentrations [62].

As these and other inflammatory signaling pathways are very common in most B cell subpopulations, these data taken together emphasize the considerable difficulty in characterizing cues that are restricted to regulatory B cell function versus overall B cell differentiation, maturation, survival and tissue localization.

Mechanisms of regulatory B cell–mediated suppression

As a heterogeneous population, results summarized from multiple studies indicate that regulatory B cells are multifaceted immune suppressors with diverse roles in health and disease [63]. While the precise mechanisms underlying the immunosuppressive functions of regulatory B cells is still the focus of intense research scrutiny, suppression by regulatory B cells, as recently reviewed

by Rosser and colleagues, may be cognate, cell–contact–mediated, bystander or indirect [64], which reveals the multiplicity and complexity of the regulatory functions of B cells. Briefly, four major actions, which are assumed to orchestrate the suppressive or regulatory properties of B cells, have been emphasized so far: (i) secretion of an array of soluble factors that exert specific immune suppression activity, (ii) expression of multiple immune–inhibitory molecules, (iii) immunomodulation of T cell responses by manipulation of Ag–presenting cells (APCs) in cell contact– or proximity–dependent manner, and (iv) targeted cell death. Additional strategies are likely to contribute to immune regulation by B cells but remain to be further documented. Complementary mechanisms of action of regulatory B cells are provided in the Appendix C (optional).

Role of IL-10 in regulatory B cell function

While it has long been known that B cells can secrete cytokines [65], the notion that the cytokines produced by B cells are implicated in regulating the activation or function of T cells is relatively recent [66]. The soluble factor IL-10, and to some extent IL-35 and TGF–β, has garnered significant interest in the regulatory B cell literature as the primary cytokine by which immune regulation is orchestrated. However, due to space limitations, its importance in regulation of immune response will be not covered in details here. For further information on the role of IL-10 in regulatory B cell function I refer the readers to the review of Mauri and Menon [39]. In this section, I will outline two key aspects of such observation.

Given its role in preserving the delicate equilibrium between effective immunity and tissue protection, it is obvious that IL-10 expression requires to be tightly regulated. Consistent with this notion, data indicate that development and effector function of IL-10–producing B cells are stringently controlled *in vivo* through molecularly distinct mechanisms in order to avoid general immunosuppression due to indiscriminant IL-10 production [45,67]. In this context, an excellent study by Madan and colleagues using mice containing selective B cell IL-10–deficiency reveals a non–redundant role for B cell–derived IL-10 during infection, a more modest role in restraining immune activation after anti–IgD challenge, and no evident role during endotoxemia, even though B cells are a main source of IL-10 in all three models [37]. B cell–specific IL-10 knockout mice used in this study also fail to develop inflammatory bowel disease, indicating that B cell–derived IL-10 may not be absolutely required for protection against pathological inflammation of the gut [37]. Possible reasons could include differences in the location, timing, and overall vigor of the resulting immune responses in these models. In the same vein, B10 cells in EAE were found to predominantly control disease initiation but not disease progression [26], suggesting that in addition to their pre–programmed capacity to promptly proliferate in response to exogenous stimuli and secrete IL-10, the size of the endogenous B10 and progenitor cell pool is a crucial parameter for controlling the magnitude of acute inflammation and the induction of autoimmunity.

It should be also re-emphasized here that given that IL-10 controls multiple B cell functions, whether B cell–derived IL-10 acts exclusively on target cells, such as APCs or T cells, or whether the absence of this factor in B cells also causes defects in other immunosuppressive B cell functions

remains to be also completely elucidated. For an example, it was reported that the loss of IL-10 in B cells resulted in decreased immune CD73–mediated suppressive function of B cells, via the release of adenosine, as a result of diminished cell–surface expression of CD73 [68].

IL-35–producing B cells as critical regulators of immunity

The addition of IL-35 to the regulatory B cell arsenal in 2014 [43,46] sparked tremendous enthusiasm in the regulatory B cell field as there has been unceasing search for new mechanisms of regulatory B cell suppression that can be optimally controlled to either improve or constrain regulatory B cell activity. Two milestone studies implicate IL-35 as a cytokine necessary to restrain excessive inflammation during autoimmune diseases [43,46]. IL-35, a member of the IL-12 family of heterodimeric cytokines, is a powerful immunosuppressive factor produced by natural Tregs that is able to differentiate naive or human or mouse T cells into suppressor T cells, coined 'iT(R)35 cells', that mediate suppression via IL-35 but not via IL-10 or TGF-β [69]. IL-35 also suppresses both T and B cell proliferation [46,69] and, most importantly, induces the expansion of IL-10–producing regulatory B cells and conversion of the latter cells into a regulatory B cell subpopulation that produces IL-35 [46]. In their very interesting study, Egwuagu and co–workers established that both IL-10 and IL-35 signaling are required for suppressive functions of IL-35$^+$ regulatory B cells [46]. Treatment of mice with IL-35 was found to repress experimental autoimmune uveitis by inhibiting Th1 and Th17 pathogenic T cells while promoting the expansion of regulatory IL-10$^+$/IL-35$^+$ regulatory B cells and Tregs [46]. In another excellent study led by the group of Fillatreau, mice lacking IL-35 production by B cells, as well as mice in which IL-10 deficiency was restricted to B cells, lost their ability to recover from EAE [15,43] and demonstrated elevated CD4$^+$ T cell central nervous system (CNS) infiltration and a higher activation of pathogenic T cells [43]. Interestingly, data from this study further showed that increased T cell responses were not due to a defect in Tregs, as B cell–derived IL-35 had no effect on Treg frequencies or suppressive activities. B cells isolated from mice with a B cell–specific IL-35 deficiency were, however, shown to be more potent Ag–presenting cells (APCs) than control B cells, stimulating higher proliferation and production of inflammatory cytokines (IL-17 and GM–CSF) by encephalitogenic CD4$^+$ T cells [43]. Most importantly, plasma cells were identified as the main B cell population expressing IL-10 and IL-35 subunits during EAE [43]. Results from Egwuagu and co–workers further suggest that regulatory IL-10$^+$ B cells and regulatory IL-35$^+$ B cells are at distinct stages of regulatory B cell development and that exposure of regulatory IL-10$^+$ B cells to IL-35 *in vivo* may prompt their terminal maturation into regulatory IL-35$^+$ plasma cells [46]. In any event, these two studies have raised the notion that Ab–secreting cells are, in some cases at least, important contributors to what has been traditionally regarded as regulatory B cell activity. This view has been confirmed by Matsumoto and colleagues that showed that mice lacking plasmablasts developed an exacerbated EAE [48].

TGF-β–mediated mechanisms of suppression by regulatory B cells

The immunoregulatory role of TGF-β, a multifunctional cytokine with at least three isoforms (TGF-β1, β2, and β3), is only beginning to become recognized. Much less is known about specific subset phenotypes of this pleiotropic cytokine relating to B cell production. Tian and colleagues (2001) were the first to suggest a potential role for B cell–derived TGF-β (with no indication of the actual isoform) in immune down–modulation by showing that transfusion of activated B cells secreting TGF-β restricts the activity of APCs and Th1 responses and in turn insulin–dependent diabetes mellitus in non–obese diabetic mice [70]. In recent years, several *in vitro* assays or experimental models whereby cells were adoptively transferred have further revealed that B cell subpopulations expressing TGF-β can control Treg induction, immune tolerance promotion, and/or innate and adaptive immune response suppression [71-80]. But, none of these reports, except for mine, have demonstrated a direct role of TGF-β in mediating *in vivo* the regulatory functions of B cells [6].

Some studies have pointed to a role for particular TGF-β isoforms in supporting regulatory B cell functions. Cell surface–associated TGF-β1, the most abundant isoform of TGF-β found in lymphoid organs, on activated murine B cells was shown to exert potent *in vitro* inhibitory effects on $CD8^+$ T cells [81]. B cell intrinsic TGF-β1 production was latter shown to play a major role in controlling B cell immunobiology in an autonomous manner, including B cell responses, isotype switching, and homeostasis of peripheral B1 and splenic B cells, but no evident role in regulating Treg cell homeostasis [82]. This latter finding was confirmed by my own research results using different genetically modified mice [6]. Consistent with decreased percentages of peripheral Tregs in B cell–deficient mice, proliferation of Tregs was shown in *in vitro* experiments to preferentially depend on the production of TGF-β3 by resting B cells [72]. Of note, these experiments established that decreased Treg expansion upon B cell activation was paralleled by lower expression of TGF-β3 by B cells and conversely increased levels of TGF-β1 [72]. Other findings suggest the possibility that B cells might promote the *de novo* conversion of naive T cells to induced Tregs. In a transplant setting, activated, but not resting, regulatory B cells were reported to promote Treg conversion [83]. In a model of allergic airway disease (AAD), a Th2–driven inflammatory reaction against harmless inhaled Ags, TGF-β1 expression was suggested to mediate *de novo* conversion of iTregs by hilar LN, but not spleen, B cells [71]. In a different study, LN B cells were further established during AAD to reside in a $CD5^+$ TGF-β1–producing subpopulation that co–localize with Tregs [74].

Other data indicate that B cells expressing TGF-β1 also efficiently down–regulate Th1 immunity to β cell self–Ags and inhibit diabetes progression in prediabetic NOD mice [70]. Mechanistically, the regulatory actions of TGF-β1–expressing B cells in prediabetic NOD mice appear to predominantly take place in the peripheral lymphoid tissues rather than in the pancreatic islets, and to be mediated through the down–regulation of Ag–presenting activity of APCs [70]. Consistent with data showing that TGF-β1 dampens self–reactive T cell responses in autoimmunity through DCs [84], the results of my personal research has demonstrated that B cell–derived TGF-β1

production could limit EAE induction by restraining Th1 and Th17 responses, at least in part, through modulation of DC functions and frequencies [6].

Whether TGF-β–producing B cells are restricted to a unique subset of regulatory B cells or, alternatively, if they represent a hallmark of an inflammatory microenvironment is not yet known. So far, only limited phenotypic characterization of TGF-β–producing B cell has been done. Consistent with *in vitro* data indicating that TGF-β may counterbalance B10 cell expansion [45], phenotypic characterization of TGF-β1–producing B cells during EAE, as assessed in my recent work, reveals that these cells do not belong to the $CD5^+CD1d^{hi}$ B10 subset or $CD138^+$ plasma cells, which were reported to be the main source of B cell–derived IL-10 and IL-35 during CNS autoimmunity [43,48]. It was also recently reported in a mouse islet allograft model of C57BL/6 diabetic recipients and islets from BALB/C that tolerance induction could depend on the enrichment of splenic $TIM\text{-}1^+$ $IL\text{-}10^+$ B co–expressing the TGF-β associated latency–associated peptide (LAP) [83]. In a model of allergic airway inflammation, $CD5^+$ B cells were found to suppress allergic asthma via TGF-β secretion [74]. Other data have documented the potential functional relevance of regulatory TGF-β–producing B1a–like cells with gut–homing potential ($CD5^+CX3CR1^+$), as well as regulatory TGF-β–producing B cells expressing thrombospondin 1 and CD35, in suppressing allergic intestinal inflammation [75,85]. Together, these studies suggest that several regulatory subpopulations of TGF-β–producing B cells may exist, and that their development may depend on the nature of the stimuli and the anatomical sites of Ag presentation. The results of my recent research further indicate that inflammatory signals may be essential for the *in vivo* generation of TGF-β1–producing B cells, as no modulation of DC frequencies and functions were observed in healthy unimmunized EAE mice with selective deletion of TGF-β1 in B cells [6]. It is worth noting in that context that B cells produce relatively low amounts of pro– and active TGF-β in physiological conditions [86,87], whereas they express high levels of active TGF-β in response to 'innate' LPS stimulation [70,81]. Most importantly, LPS–activated B cells were reported to express comparably much higher amounts of TGF-β1 than B cells stimulated with by co–engagement of CD40 and BCR [81], revealing that TGF-β1 may specifically or preferentially mediate innate regulatory response of B cells.

In human subjects, similar to IL-10–producing B cells, TGF-β–producing $CD19^+CD5^+$ B cells have the capacity to regulate cow's milk allergic responses [88,89]. Besides reducing proliferation of autologous stimulated $CD4^+$ T cells, human $CD25^+CD27^+CD86^+CD1d^+IL\text{-}10^+TGF\text{-}\beta^+$ regulatory B cells can increase FoxP3 and CTLA4 expression in Tregs mostly by a direct contact–mediated mechanism but also through TGF-β, but not IL-10 [73]. Other data indicate that activated human B cells regulate $CD4^+$ T cell proliferation through production of TGF-β and indoleamine 2, 3-dioxygenase (IDO). In turn, CTLA4 by Tregs can induce B cells to produce IDO and to become effective inducible regulatory B cells [80]. In stark contrast, my research has documented evidence that reduced TGF-β1 production by human B cells was found upon dual BCR and TLR9 engagement [7], which appears to govern the functional B cell responses, directly linking cell–intrinsic innate and adaptive immune programs. Based on these observations, it might therefore seem that B cell

activation could under certain conditions accelerate the development of excessive immune responses and autoimmunity by limiting TGF-β1 production by resting B cells. Another point worth mentioning is that in this study, resting TGF-β1–producing human B cells fall within both the naive ($CD27^-$) and memory ($CD27^+$) B cell compartments [7].

Induction of regulatory T cell populations

The first evidence that B cells plays a role in maintaining the Treg cell compartment has come from early studies showing that congenital B cell–deficient mice have fewer numbers of Tregs [90], but research in subsequent years in mice congenitally deficient for B cells or depleted of $CD20^+$ B cells has pointed to a more complex picture, with mixed results, as detailed below, showing that B cells can positively or negatively impact the size and activity of the Treg cell population [91,92]. The reasons for such discrepancies are not clear, although they could be the result of genetic differences between mouse strains, or differences between colonies, experimental models, stimulations of inflammatory environments, and/or whether thymic and peripheral Tregs were considered.

A number of preclinical studies in wild–type mice have reported that B cell–depletion using anti–CD20 Abs, similar to prevention of B cell development beyond the transitional stage by treatment with recombinant Fc-BAFF, enhances Treg activity, which helps limit the development of organ–specific autoimmune diseases [91,93-99]. This has led to an assumption that activation of Tregs in the relative absence of B cells could contribute to the beneficial effects of B cell–targeted therapies in autoimmune diseases [92]. These findings, although convincing, have not been observed in all models. For instance, whereas depletion of B cells in non–obese diabetic–H-2h4 (NOD–H-2h4) by anti–CD20 Abs, which inhibited spontaneous autoimmune thyroiditis, was associated with a two–fold increased numbers of splenic Tregs [96], congenital lack of B cells in NOD–H-2h4 had any effects on the absolute numbers of peripheral Tregs [100]. Of particular interest is the observation by Fillatreau and co–workers that B cells are also dispensable for Treg activation in EAE [101]. Additional pre–clinical results from mouse models of infection, tolerance and autoimmunity, indicate that B cells, including intrathymic B cells, are required for robust classical Treg activation [90,102-105] as well as for induction of functionally and phenotypically distinct Treg populations that are dissimilar to the conventional $FoxP3^+$ Tregs or Tr1 cells [106-108]. Finally, there is also strong evidence, including data from my research studies, that B cell–deficient mice present a significant reduction in the number of Tregs in peripheral lymphoid tissues [1,91,102,109]. Based on the expression of the integrin CD103, which allows subdividing the peripheral Treg cell compartment into two different subsets, congenital lack of B cells appears to selectively affect $CD103^-$, but not $CD103^+$ Tregs in secondary lymphoid organs [101]. Remarkably, diminution of peripheral Treg numbers could be rescued to a significant extent by adoptive transfers of B cells [102,108].

Many data now show that intimate cognate molecular interactions between regulatory B cells and T cells constitute a major mechanism controlling B cell–mediated Treg homeostasis. Early evidence from Mann and colleagues indicates that B cells regulate the emergence of Tregs in the

CNS in a B7 (CD80/CD86)–dependent manner [110]. The role of B7 expression by B cells on peripheral Treg populations was however not tested at that time. More recent EAE data indicate that CD86, but not CD80, plays a minor role in Treg homeostasis and that B cell expression of major histocompatibility complex (MHC) class II, akin to IL-10, is not required for the maintenance of Tregs, indicating thereby that regulation of autoimmune responses by regulatory B cells could occur independently of Ag presentation [102]. On a personal note, by using novel transgenic mice selectively deficient in MHC class II on B cells [2], my research results have also established that B cell MHC class II expression was dispensable for Treg homeostatsis (unpublished data). It should be noted, however, that similar to IL-10– or IL-21R–deficient B cells, B10 cells lacking selective expression of CD40 or MHC class II molecules do not exert regulatory function during EAE [45], strongly suggesting that Ag presentation is a key molecular checkpoint for the regulatory function of B10 cells during CNS autoimmunity [20]. Consistent with the demonstration that transgenic mice overexpressing glucocorticoid–induced receptor superfamily member 18 ligands (GITRL) in B cells exhibit increased numbers of peripheral Tregs [111], B cells were found to regulate natural Treg homeostasis in EAE by inducing their proliferation via GITRL [102]. Remarkably, it appears that this B cell property is constitutive [108]. Plausibly, such interactions could play some role in the protective effects of B cells in colitis [112].

Consistent with the notion that B cells could positively control Treg responses to autoimmune diseases, a few early case reports have noticed individuals in whom B cell–depletion exacerbated some pathologies [113-116]. Supporting these observations, human regulatory B cells were shown to induce upregulation of FoxP3 and CTLA4 in Tregs [73]. Other, more robust data in clinical settings, however indicate that Treg numbers are either unaltered [117] or increased after B cell–depletion induced by anti–B cell therapy in various immunopathologies [91,118]. These results may suggest that self–reactive $CD4^+$ T cells are preferentially skewed toward regulatory subsets when auto–Ags are initially presented by non–B cell APCs [119]. It is of note that DCs are more potent activators of Treg cells than B cells *in vitro* [120]. Apart from conventional Tregs, additional observations also suggest that regulatory B cells can also potently induce IL-10–producing Tr1 populations [28,121]. Remarkably, adoptively transferred GIFT15–induced regulatory B cells (GIFT15-iBregs) [54] into mice with EAE were reported to migrate to the spleen and mesenteric LNs, leading to an expansion of Tregs and Tr1 in an Ag–specific manner [122]. Similar findings have been reported by others [123]. Most importantly, GIFT15-iBregs were found to secrete both IL-10, a signal critical for Tr1 cell function *in vivo* [124], and IL-27, a cytokine that is essential for the induction of Tr1s [125,126].

Regulatory B cells modulate innate immunity

A wealth of studies indicates that regulatory B cells control DC functions [127]. In 2000, Moulin and colleagues were the first to report a role for B cells in regulating DC function *in vivo* [128]. Remarkably, the authors revealed that B cell–produced IL-10 potently reduced the secretion by DCs of IL-12, while increasing that of IL-4, and thereby influenced the Th1/Th2 balance. Subsequent studies have documented a function for IL-10 from TLR– or UVB–activated B cells in limiting Th1

responses or immunity by inhibition of DC production of IL-12 [129-131]. Further data in EAE also established the importance of B cell–derived IL-10 in decreasing DC production of IL-23 and IL-6 and consequential suppression of Th17 responses [130]. Consistent with these results, the suppressive effects of IL-10–producing plasmablasts on CNS inflammation was recently reported to be mediated, at least in part, via inhibition of DC functions leading to reduction in autoreactive T cell differentiation [132]. In *vitro*, B10 cells were further found to downregulate the ability of DCs to act as APCs and thereby to indirectly modulate T cell proliferation [133]. My own personal reserch results also indicate that B cells may prevent EAE development and Th1/Th17 responses with a mechanism that possibly involves modulation of distinct DC populations by TGF-β1 [6]. Remarkably, there is some evidence, including data from my research studies, to indicate that B cell–depletion in this autoimmune model results in an augmention of the capability of residual APCs to activate encephalitogenic T cells [1,118]. Part of these results may further be potentially extended to parasite–induced regulatory B cells as data indicate that *Leishmania major*–exposed B cells could abolish DC production of IL-12 in an IL-10–dependent manner [134].

In humans, data indicate that activated B cells restrain the development of monocytes into immature DCs and their differentiation into mature DCs as well as inhibit DC–induced T cell proliferation [135]. Additional findings suggest that $CD24^{hi}CD27^{+}$ B10 cells regulate monocyte tumor necrosis factor (TNF) production via IL-10 [40]. Correspondingly, anti–CD20 treatment was reported to enhance monocyte expression of TNF and signaling lymphocytic activation molecule (SLAM) in neuroimmunological patients [118]. Collectively, these findings suggest that inefficient B cell regulation may shift the balance between effector and protective T cells in favor of an augmented immune response.

Regulatory B cell–based therapies for autoimmunity

As outlined above, regulatory B cells are potent immune response suppressors that promote self–immunological tolerance and maintain immune homeostasis in many ways and as such could feasibly be exploited as a form of cell immunotherapy for certain conditions such as autoimmune disorders and transplantation. Alternatively, as regulatory B cell–mediated immune suppression may neutralize anti–tumor immunity or host defense against infection, therapeutic inhibition of regulatory B cells can have favorable outcomes in the control of cancers and chronic infection. This section offers a brief summary of the different therapeutic approaches targeting regulatory B cells for better management of several immune–mediated disorders and addresses some questions that are of particular importance and relevance to the development of regulatory B cell–targeted therapies. For a complete overview on the role of regulatory B cells in different disease settings, the readers are referred to the extensive reviews by Miyagaki and colleagues [136] and Mauri and Menon [39].

Manipulating regulatory B cells: an exciting immunotherapeutic strategy

Manipulation of regulatory B cells, specifically auto–Ag–specific regulatory B cells is regarded as an exciting immunotherapeutic strategy for several immune–mediated chronic inflammatory

disorders, autoimmune diseases, allergy and transplantation, since regulatory B cells may offer the advantage of Ag specificity without broad immunosuppression [39,55,67]. As reviewed herein, adoptive transfer of regulatory B cells to restore or induce tolerance toward self–Ags or allo–Ags undeniably represents an effective method in preclinical murine models to prevent or alleviate several T cell–mediated inflammatory conditions [17,21,27,32,33,48,49,52,54,55,62,83,133,137]. In addition, there is also indication that adoptively transferred regulatory B cells have the capacity to migrate to local inflammatory sites [71,138] and, further, possibly to reside in the inflamed tissue for several weeks [138]. Consequently, regulatory B cells may conceivably exert suppressive functions in local inflammatory sites depending on their homing properties and the provision of survival signals in the local environment.

While providing some proof–of–concept that adoptive transfer or regulatory B cells could be an efficient therapeutic opportunity for various human immune–mediated diseases, it should not be overlooked that preclinical studies have not been systematically performed in spontaneous models of human disorders, but in models of diseases induced by active immunization with Ags. In this sense, the conclusion of some preclinical research was conceivably entirely to be anticipated on the basis of previous work on Ag–specific tolerance [139]. Most importantly, even though clinical benefit of adoptive regulatory B cell transfer has been obtained in treatment of spontaneous pathological process, which clearly represents an important step toward the potential application of regulatory B cell–based immunotherapy in humans, it is not known whether the efficacy of human regulatory B cell–based therapy may mirror the information collected from the various preclinical findings. As summarized in this review, regulatory B cells differ from one pathological condition to another. In mice, regulatory B cells present different phenotypes, require different stimulatory signals to promote their induction, expansion or effector function, and are able to modulate the immune system, including Ag–specific autoreactive T cells, inflammatory innate cells and/or distinct regulatory cells, by different molecular mechanisms. It is conceivable therefore that the definitive outcome of regulatory B cell–based therapy, which cannot be predicted by any model, will be an inhibition of disease development, a diminution of disease severity and/or a complete recovery.

One of the greatest difficulties to overcome in establishing an effective adoptive cell–based therapy in humans is the need to transfuse large numbers of cells into patients. In this context, an advantageous facet of regulatory B cells is that these cells are inducible *ex vivo*, therefore, regulatory B cells of the desired Ag–specificity can be easily generated and expanded *in vitro*. Effective *ex vivo* expansion of functional regulatory B cells was recently documented in different studies. In one study, IL-10–competent B cells were shown to be very efficiently generated and expanded *in vitro* from purified spleen B cells stimulated with CD40L and IL-4, followed by IL-21 [45]. Regulatory effector B cells expanded with these conditions were primarily $CD5^+$ and could inhibit the initiation and progression of 'active' EAE. Whether self–Ag specificity was required for their therapeutic effect was not directly addressed in this study. Additional data nevertheless show that MHC class II expression was essential [45]. Likewise, exposure of murine naive splenocytes to the GM-CSF– and IL-15–derived fusokine, GIFT15, was reported to lead to the generation of IL-10–competent regulatory B cells,

which completely reversed inflammation–induced paralysis in 'active' EAE mice [54]. MHC class II–deficient GIFT15–iBregs did however not suppress the development of EAE. Another study reported that anti–CD40 stimulation could specifically expand *ex vivo* IL-10–producing T2-like B cells isolated from autoimmune lupus–prone mice and empowered them with adequate regulatory capacity to reverse spontaneous autoimmunity in mice [28]. Such *ex vivo* approaches could potentially be extended to human patients to develop targeted therapeutic options for treating chronic inflammatory and autoimmune disorders.

While Ag–specific strategies have the greatest therapeutic potential [139], their successful applications require an in–depth understanding of the Ags implicated in the perpetuation and diversification of the ongoing immune response. It is of note that the Ags recognized by the T cells in immune–mediated diseases are often unknown. Moreover, this potential clinical therapeutic strategy is also hampered by the problematic isolation of rare specificities from the natural B cell repertoire. These difficulties may be alleviated by the direct infusion of Ag–non–specific suppressor B cells. As a pertinent example, *in vitro* treatment of unfractionated splenic lymphocytes with a synthetic cytokine linking GM-CSF to MCP3 was recently found to convert naive B cells to a suppressor phenotype (B_{GMME3}) that were capable to modulate the progression of EAE upon adoptive transfer [55]. Mechanistically, *in vitro*–generated IL-10–producing B_{GMME3} cells were found to mediate their inhibitory functions in an indirect manner via repression of MHC class II–specific Ag presentation by APCs. Similarly, adoptively transferred GIFT15–iBregs were shown to suppress EAE despite their lack of exposure to myelin Ags *in vitro* [54]. The observations of Ag–non–specific suppressor function of B cells open hence new avenues for the development of biological agents for cellular immunotherapy of inflammatory and autoimmune diseases.

Nevertheless, there are some important questions that must be answered before the therapeutic application of regulatory B cells can be considered. One of the key challenges is determining the functional stability of donor regulatory B cells *in vivo*. Would *ex vivo* manipulated or expanded regulatory B cells remain stable after infusion into patients, or would they become pathogenic under the influence of various inflammatory factors? There is indeed evidence that depending on the immunological requirements, any B cell can adapt and adopt new immune regulatory properties or effector functions, or concurrently display both activities, as recently illustrated in mice and in humans with IL-10–secreting regulatory plasmablasts and Br1 cells [44,48].

Most importantly, as B cells conditioned in an inflammatory milieu can acquire immunosuppressive properties [51,121], it could be conjectured that for B cell–based adoptive approaches to be of clinical benefit, regulatory B cells must become activated *in vivo* against an Ag expressed within the autoinflammatory environment. As such, agents inducing or expanding regulatory B cells *in vivo* by modulating the environmental milieu may represent a more effective therapeutic strategy than manipulating B cells *ex vivo*. Pertinent to this point, *in vivo* administration of anti–CD40 monoclonal Abs was demonstrated to reverse nephritis and increased survival in mice by enhancing the number of regulatory T2 B cells [28]. Likewise, low dose of GM-CSF was reported

to expand B10 cells *in vivo* and to supress experimental autoimmune myasthenia gravis [140]. However, caution should be taken to ensure that the potential concomitant stimulation of effector B cells may not counter the favorable effects of induced regulatory B cells. As an alternative to proinflammatory regimens, IL-35 treatment in mice was shown in an fascinating study to confer protection from uveitis [46]. Mechanistically, data from this study indicate that mice lacking IL-35 expression or defective in IL-35-signaling pathway produced less regulatory B cells and developed severe uveitis.

In summary, although tremendous progress has been made from preclinical studies in understanding the mechanisms of suppression by regulatory B cells, it remains to be elucidated whether these various suppressive mechanisms play a role in mediating tolerance and maintaining immune homeostasis in humans and whether they are specific to a given disease state or tissue location. These questions are central to determine how to exploit or control the suppressive capacity of regulatory B cells for targeted therapy against a broad spectrum of diseases.

Regulatory B cell–depletion

B cell–depletion has been used for the management of several autoimmune disorders. There is some evidence from preclinical and clinical research indicating that the re–emerging B cells in patients after B cell–depletion therapy exhibit a regulatory phenotype that may directly contribute to the long–term clinical and immunological outcome of this therapeutic approach [94,141]. While such therapies have been effective in some patients, data also indicate that removal of B cells may precipitate T cell–mediated autoimmune reactions in some cases [113-116].

In recent years, it has become increasingly clear that regulatory B cells might be inducible from different stages of development, from immature cells to terminally differentiated plasma cells, demonstrating that regulatory B cells are not locked in a terminally differentiated stage [43]. Conceivably, depletion of certain B cell subsets might induce unexpected results and therefore may worsen certain autoimmune conditions by altering the relative balance between protective and pathogenic B cell populations.

Achieving more effective strategies will require more in–depth phenotypic characterization of regulatory B cells. Importantly, identification of cell–surface markers that can specifically define human regulatory B cells would enable the development of novel strategies of B cell–depletion that selectively target either regulatory B cells or effector B cells. As such, novel therapies targeting regulatory B cell function could be a promising strategy for treating chronic infections and cancer where regulatory B cell activities are deleterious.

Conclusion

The assumption that chronic inflammatory diseases originate from a failure of T cell regulation has been challenged in recent years by compelling evidence that B cells also exert effective immunoregulatory capacities that may contribute to the inhibition of inflammatory and autoimmune responses. Understanding the multiple immune functions of B cells – Ab production,

cytokine secretion, Ag presentation and formation of ectopic germinal centers – in the pathophysiology of inflammation and autoimmunity and the subtle equilibrium between different B cell subtypes may accelerate the development of B cell–targeted therapies.

Although a great deal of progress has been made in understanding the mechanisms of regulation by B cells, more attention is needed to elucidate the origin, phenotype, function and fate of regulatory B cells. Collectively, the diversity of phenotypic variants and their mechanisms of action suggest that regulatory B cells do not represent a distinct lineage within the B cell repertoire but rather acquire, transiently or permanently, unique functional suppressive phenotypes in response to their environmental cues. It is conceivable that regulatory B cells are short–lived effector cells that are especially generated when immunosuppression is most needed. Alternatively, all neo-generated inducible regulatory B cells may terminally differentiate into Ab–secreting plasma cells after the resolution of inflammation. In conclusion, while the field of regulatory B cells is evolving at an exhilarating rate, whereby new biological process that control regulatory B cell development and functions have been identified, many questions remained to be answered to truly understand what is their life cycle and 'lifestyle'.

References

1 Weber, M. S. *et al.* B-cell activation influences T-cell polarization and outcome of anti-CD20 B-cell depletion in central nervous system autoimmunity. *Ann Neurol* **68**, 369-383, doi:10.1002/ana.22081 (2010).
2 Molnarfi, N. *et al.* MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J Exp Med* **210**, 2921-2937, doi:10.1084/jem.20130699 (2013).
3 Lalive, P. H., Molnarfi, N., Benkhoucha, M., Weber, M. S. & Santiago-Raber, M. L. Antibody response in MOG(35-55) induced EAE. *J Neuroimmunol* **240-241**, 28-33, doi:10.1016/j.jneuroim.2011.09.005 (2011).
4 Rosser, E. C. & Mauri, C. Regulatory B cells: origin, phenotype, and function. *Immunity* **42**, 607-612, doi:10.1016/j.immuni.2015.04.005 (2015).
5 Mauri, C. & Menon, M. The expanding family of regulatory B cells. *Int Immunol* **27**, 479-486, doi:10.1093/intimm/dxv038 (2015).
6 Bjarnadottir, K. *et al.* B cell-derived transforming growth factor-beta1 expression limits the induction phase of autoimmune neuroinflammation. *Scientific reports* **6**, 34594, doi:10.1038/srep34594 (2016).
7 Molnarfi, N., Bjarnadottir, K., Benkhoucha, M., Juillard, C. & Lalive, P. H. Activation of human B cells negatively regulates TGF-beta1 production. *J Neuroinflammation* **14**, 13, doi:10.1186/s12974-017-0798-5 (2017).
8 Katz, S. I., Parker, D. & Turk, J. L. B-cell suppression of delayed hypersensitivity reactions. *Nature* **251**, 550-551 (1974).
9 Neta, R. & Salvin, S. B. Specific suppression of delayed hypersensitivity: the possible presence of a suppressor B cell in the regulation of delayed hypersensitivity. *J Immunol* **113**, 1716-1725 (1974).
10 Shimamura, T., Hashimoto, K. & Sasaki, S. Feedback suppression of the immune response in vivo. I. Immune B cells induce antigen-specific suppressor T cells. *Cell Immunol* **68**, 104-113 (1982).
11 Shimamura, T., Habu, S., Hashimoto, K. & Sasaki, S. Feedback suppression of the immune response in vivo. III. Lyt-1+ B cells are suppressor-inducer cells. *Cell Immunol* **83**, 221-224 (1984).
12 Wolf, S. D., Dittel, B. N., Hardardottir, F. & Janeway, C. A., Jr. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med* **184**, 2271-2278 (1996).
13 Mizoguchi, A., Mizoguchi, E., Smith, R. N., Preffer, F. I. & Bhan, A. K. Suppressive role of B cells in chronic colitis of T cell receptor alpha mutant mice. *J Exp Med* **186**, 1749-1756 (1997).
14 Jankovic, D. *et al.* CD4+ T cell-mediated granulomatous pathology in schistosomiasis is downregulated by a B cell-dependent mechanism requiring Fc receptor signaling. *J Exp Med* **187**, 619-629 (1998).
15 Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D. & Anderton, S. M. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* **3**, 944-950 (2002).
16 Mizoguchi, A., Mizoguchi, E., Takedatsu, H., Blumberg, R. S. & Bhan, A. K. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* **16**, 219-230 (2002).
17 Mauri, C., Gray, D., Mushtaq, N. & Londei, M. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med* **197**, 489-501 (2003).
18 DiLillo, D. J., Matsushita, T. & Tedder, T. F. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. *Ann N Y Acad Sci* **1183**, 38-57, doi:10.1111/j.1749-6632.2009.05137.x (2010).
19 Yanaba, K. *et al.* IL-10-producing regulatory B10 cells inhibit intestinal injury in a mouse model. *Am J Pathol* **178**, 735-743, doi:10.1016/j.ajpath.2010.10.022 (2011).
20 Yanaba, K. *et al.* A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity* **28**, 639-650, doi:S1074-7613(08)00193-3 [pii] 10.1016/j.immuni.2008.03.017 (2008).
21 Yeung, M. Y. *et al.* TIM-1 signaling is required for maintenance and induction of regulatory B cells. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* **15**, 942-953, doi:10.1111/ajt.13087 (2015).
22 Tedder, T. F. & Matsushita, T. Regulatory B cells that produce IL-10: a breath of fresh air in allergic airway disease. *J Allergy Clin Immunol* **125**, 1125-1127, doi:10.1016/j.jaci.2010.03.024 (2010).
23 Maseda, D. *et al.* Peritoneal cavity regulatory B cells (B10 cells) modulate IFN-gamma+CD4+ T cell numbers during colitis development in mice. *J Immunol* **191**, 2780-2795, doi:10.4049/jimmunol.1300649 (2013).
24 Haas, K. M. *et al.* Protective and pathogenic roles for B cells during systemic autoimmunity in NZB/W F1 mice. *J Immunol* **184**, 4789-4800, doi:10.4049/jimmunol.0902391 (2010).
25 Maseda, D. *et al.* Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. *J Immunol* **188**, 1036-1048, doi:10.4049/jimmunol.1102500 (2012).
26 Matsushita, T., Yanaba, K., Bouaziz, J. D., Fujimoto, M. & Tedder, T. F. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest* **118**, 3420-3430, doi:10.1172/JCI36030 (2008).
27 Evans, J. G. *et al.* Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol* **178**, 7868-7878, doi:178/12/7868 [pii] (2007).
28 Blair, P. A. *et al.* Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice. *J Immunol* **182**, 3492-3502, doi:10.4049/jimmunol.0803052 (2009).
29 Neves, P. *et al.* Signaling via the MyD88 adaptor protein in B cells suppresses protective immunity during Salmonella typhimurium infection. *Immunity* **33**, 777-790, doi:10.1016/j.immuni.2010.10.016 (2010).
30 Spencer, N. F. & Daynes, R. A. IL-12 directly stimulates expression of IL-10 by CD5+ B cells and IL-6 by both CD5+ and CD5- B cells: possible involvement in age-associated cytokine dysregulation. *Int Immunol* **9**, 745-754 (1997).
31 Brummel, R. & Lenert, P. Activation of marginal zone B cells from lupus mice with type A(D) CpG-oligodeoxynucleotides. *J Immunol* **174**, 2429-2434 (2005).
32 Sattler, S. *et al.* IL-10-producing regulatory B cells induced by IL-33 (Breg(IL-33)) effectively attenuate mucosal inflammatory responses in the gut. *J Autoimmun* **50**, 107-122, doi:10.1016/j.jaut.2014.01.032 (2014).
33 Ding, Q. *et al.* Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. *J Clin Invest* **121**, 3645-3656, doi:10.1172/JCI46274 (2011).
34 Tedder, T. F. B10 cells: a functionally defined regulatory B cell subset. *J Immunol* **194**, 1395-1401, doi:10.4049/jimmunol.1401329 (2015).
35 Sun, J. *et al.* Transcriptomics Identify CD9 as a Marker of Murine IL-10-Competent Regulatory B Cells. *Cell reports* **13**, 1110-1117, doi:10.1016/j.celrep.2015.09.070 (2015).
36 Matsushita, T. *et al.* A novel splenic B1 regulatory cell subset suppresses allergic disease through phosphatidylinositol 3-kinase-Akt pathway activation. *J Allergy Clin Immunol* **138**, 1170-1182 e1179, doi:10.1016/j.jaci.2015.12.1319 (2016).

37 Madan, R. *et al.* Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. *J Immunol* **183**, 2312-2320, doi:10.4049/jimmunol.0900185 (2009).
38 Stolp, J., Turka, L. A. & Wood, K. J. B cells with immune-regulating function in transplantation. *Nat Rev Nephrol* **10**, 389-397, doi:10.1038/nrneph.2014.80 (2014).
39 Mauri, C. & Menon, M. Human regulatory B cells in health and disease: therapeutic potential. *J Clin Invest* **127**, 772-779, doi:10.1172/JCI85113 (2017).
40 Iwata, Y. *et al.* Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood* **117**, 530-541, doi:10.1182/blood-2010-07-294249 (2011).
41 Blair, P. A. *et al.* CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* **32**, 129-140, doi:10.1016/j.immuni.2009.11.009 (2010).
42 Aravena, O. *et al.* TIM-1 defines a human regulatory B cell population that is altered in frequency and function in systemic sclerosis patients. *Arthritis Res Ther* **19**, 8, doi:10.1186/s13075-016-1213-9 (2017).
43 Shen, P. *et al.* IL-35-producing B cells are critical regulators of immunity during autoimmune and infectious diseases. *Nature* **507**, 366-370, doi:10.1038/nature12979 (2014).
44 van de Veen, W. *et al.* IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol* **131**, 1204-1212, doi:10.1016/j.jaci.2013.01.014 (2013).
45 Yoshizaki, A. *et al.* Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* **491**, 264-268, doi:10.1038/nature11501 (2012).
46 Wang, R. X. *et al.* Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* **20**, 633-641, doi:10.1038/nm.3554 (2014).
47 Schubert, R. D. *et al.* IFN-beta treatment requires B cells for efficacy in neuroautoimmunity. *J Immunol* **194**, 2110-2116, doi:10.4049/jimmunol.1402029 (2015).
48 Matsumoto, M. *et al.* Interleukin-10-Producing Plasmablasts Exert Regulatory Function in Autoimmune Inflammation. *Immunity*, doi:10.1016/j.immuni.2014.10.016 (2014).
49 Egwuagu, C. E. & Yu, C. R. Interleukin 35-Producing B Cells (i35-Breg): A New Mediator of Regulatory B-Cell Functions in CNS Autoimmune Diseases. *Crit Rev Immunol* **35**, 49-57 (2015).
50 de Masson, A. *et al.* CD24(hi)CD27(+) and plasmablast-like regulatory B cells in human chronic graft-versus-host disease. *Blood* **125**, 1830-1839, doi:10.1182/blood-2014-09-599159 (2015).
51 Yanaba, K., Bouaziz, J.-D., Matsushita, T., Tsubata, T. & Tedder, T. F. The Development and Function of Regulatory B Cells Expressing IL-10 (B10 Cells) Requires Antigen Receptor Diversity and TLR Signals. *J Immunol* **182**, 7459-7472, doi:10.4049/jimmunol.0900270 (2009).
52 Carter, N. A. *et al.* Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *J Immunol* **186**, 5569-5579, doi:10.4049/jimmunol.1100284 (2011).
53 Rosser, E. C. *et al*. Regulatory B cells are induced by gut microbiota-driven interleukin-1beta and interleukin-6 production. *Nat Med* **20**, 1334-1339, doi:10.1038/nm.3680 (2014).
54 Rafei, M. *et al.* A granulocyte-macrophage colony-stimulating factor and interleukin-15 fusokine induces a regulatory B cell population with immune suppressive properties. *Nat Med* **15**, 1038-1045, doi:10.1038/nm.2003 (2009).
55 Hsieh, J. *et al.* Inducible IL10(+) suppressor B cells inhibit CNS inflammation and T helper 17 polarization. *Molecular therapy : the journal of the American Society of Gene Therapy* **20**, 1767-1777, doi:10.1038/mt.2012.127 (2012).
56 Dambuza, I. M. *et al.* IL-12p35 induces expansion of IL-10 and IL-35-expressing regulatory B cells and ameliorates autoimmune disease. *Nature communications* **8**, 719, doi:10.1038/s41467-017-00838-4 (2017).
57 Duddy, M. E., Alter, A. & Bar-Or, A. Distinct profiles of human B cell effector cytokines: a role in immune regulation? *J Immunol* **172**, 3422-3427 (2004).
58 Menon, M., Blair, P. A., Isenberg, D. A. & Mauri, C. A Regulatory Feedback between Plasmacytoid Dendritic Cells and Regulatory B Cells Is Aberrant in Systemic Lupus Erythematosus. *Immunity* **44**, 683-697, doi:10.1016/j.immuni.2016.02.012 (2016).
59 Kim, H. S. *et al.* Autocrine stimulation of IL-10 is critical to the enrichment of IL-10-producing CD40(hi)CD5(+) regulatory B cells in vitro and in vivo. *BMB Rep* **48**, 54-59 (2015).
60 Stanic, B. *et al.* IL-10-overexpressing B cells regulate innate and adaptive immune responses. *J Allergy Clin Immunol* **135**, 771-780 e778, doi:10.1016/j.jaci.2014.07.041 (2015).
61 Pei, C. *et al.* Emerging role of interleukin-33 in autoimmune diseases. *Immunology* **141**, 9-17, doi:10.1111/imm.12174 (2014).
62 Yang, M. *et al.* Novel function of B cell-activating factor in the induction of IL-10-producing regulatory B cells. *J Immunol* **184**, 3321-3325, doi:10.4049/jimmunol.0902551 (2010).
63 Klinker, M. W. & Lundy, S. K. Multiple mechanisms of immune suppression by B lymphocytes. *Mol Med* **18**, 123-137, doi:10.2119/molmed.2011.00333 (2012).
64 Rosser, E. C., Blair, P. A. & Mauri, C. Cellular targets of regulatory B cell-mediated suppression. *Mol Immunol* **62**, 296-304, doi:10.1016/j.molimm.2014.01.014 (2014).
65 Pistoia, V. Production of cytokines by human B cells in health and disease. *Immunol Today* **18**, 343-350 (1997).
66 Lund, F. E., Garvy, B. A., Randall, T. D. & Harris, D. P. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. *Current directions in autoimmunity* **8**, 25-54, doi:10.1159/000082086 (2005).
67 Lykken, J. M., Candando, K. M. & Tedder, T. F. Regulatory B10 cell development and function. *Int Immunol* **27**, 471-477, doi:10.1093/intimm/dxv046 (2015).
68 Kaku, H., Cheng, K. F., Al-Abed, Y. & Rothstein, T. L. A novel mechanism of B cell-mediated immune suppression through CD73 expression and adenosine production. *J Immunol* **193**, 5904-5913, doi:10.4049/jimmunol.1400336 (2014).
69 Collison, L. W. *et al.* The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **450**, 566-569, doi:10.1038/nature06306 (2007).
70 Tian, J. *et al.* Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. *J Immunol* **167**, 1081-1089 (2001).
71 Singh, A. *et al.* Regulatory role of B cells in a murine model of allergic airway disease. *J Immunol* **180**, 7318-7326 (2008).
72 Shah, S. & Qiao, L. Resting B cells expand a CD4+CD25+Foxp3+ Treg population via TGF-beta3. *Eur J Immunol* **38**, 2488-2498, doi:10.1002/eji.200838201 (2008).
73 Kessel, A. *et al.* Human CD19(+)CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. *Autoimmunity reviews* **11**, 670-677, doi:10.1016/j.autrev.2011.11.018 (2012).

74 Natarajan, P. *et al.* Regulatory B cells from hilar lymph nodes of tolerant mice in a murine model of allergic airway disease are CD5+, express TGF-beta, and co-localize with CD4+Foxp3+ T cells. *Mucosal immunology* **5**, 691-701, doi:10.1038/mi.2012.42 (2012).
75 Liu, Z. Q. *et al.* Tolerogenic CX3CR1+ B cells suppress food allergy-induced intestinal inflammation in mice. *Allergy* **68**, 1241-1248, doi:10.1111/all.12218 (2013).
76 Gonnella, P. A., Chen, Y. H., Waldner, H. & Weiner, H. L. Induction of oral tolerization in CD86 deficient mice: a role for CD86 and B cells in the up-regulation of TGF-beta. *J Autoimmun* **26**, 73-81, doi:10.1016/j.jaut.2005.10.003 (2006).
77 Lee, J. H. *et al.* Allergen-Specific Transforming Growth Factor-beta-Producing CD19(+)CD5(+) Regulatory B-Cell (Br3) Responses in Human Late Eczematous Allergic Reactions to Cow's Milk. *J Interferon Cytokine Res*, doi:10.1089/jir.2010.0020 (2011).
78 Mishima, Y. *et al.* Decreased production of interleukin-10 and transforming growth factor-beta in Toll-like receptor-activated intestinal B cells in SAMP1/Yit mice. *Immunology* **131**, 473-487, doi:10.1111/j.1365-2567.2010.03318.x (2010).
79 Guo, Y., Zhang, X., Qin, M. & Wang, X. Changes in peripheral CD19(+)Foxp3(+) and CD19(+)TGFbeta(+) regulatory B cell populations in rheumatoid arthritis patients with interstitial lung disease. *Journal of thoracic disease* **7**, 471-477, doi:10.3978/j.issn.2072-1439.2015.02.11 (2015).
80 Nouel, A. *et al.* B-Cells induce regulatory T cells through TGF-beta/IDO production in A CTLA-4 dependent manner. *J Autoimmun* **59**, 53-60, doi:10.1016/j.jaut.2015.02.004 (2015).
81 Parekh, V. V. *et al.* B cells activated by lipopolysaccharide, but not by anti-Ig and anti-CD40 antibody, induce anergy in CD8+ T cells: role of TGF-beta 1. *J Immunol* **170**, 5897-5911 (2003).
82 Gros, M. J., Naquet, P. & Guinamard, R. R. Cell intrinsic TGF-beta 1 regulation of B cells. *J Immunol* **180**, 8153-8158, doi:180/12/8153 [pii] (2008).
83 Lee, K. M. *et al.* TGF-beta-producing regulatory B cells induce regulatory T cells and promote transplantation tolerance. *Eur J Immunol* **44**, 1728-1736, doi:10.1002/eji.201344062 (2014).
84 Laouar, Y. *et al.* TGF-beta signaling in dendritic cells is a prerequisite for the control of autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* **105**, 10865-10870, doi:0805058105 [pii]
10.1073/pnas.0805058105 (2008).
85 Zhang, H. P. *et al.* TSP1-producing B cells show immune regulatory property and suppress allergy-related mucosal inflammation. *Scientific reports* **3**, 3345, doi:10.1038/srep03345 (2013).
86 Kehrl, J. H. *et al.* Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. *J Immunol* **137**, 3855-3860 (1986).
87 Zan, H., Cerutti, A., Dramitinos, P., Schaffer, A. & Casali, P. CD40 engagement triggers switching to IgA1 and IgA2 in human B cells through induction of endogenous TGF-beta: evidence for TGF-beta but not IL-10-dependent direct S mu-->S alpha and sequential S mu-->S gamma, S gamma-->S alpha DNA recombination. *J Immunol* **161**, 5217-5225 (1998).
88 Noh, J. *et al.* Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br1) in Cow Milk Allergy. *Cell Immunol* **264**, 143-149, doi:10.1016/j.cellimm.2010.05.013 (2010).
89 Lee, J. H. *et al.* Allergen-specific transforming growth factor-beta-producing CD19+CD5+ regulatory B-cell (Br3) responses in human late eczematous allergic reactions to cow's milk. *J Interferon Cytokine Res* **31**, 441-449, doi:10.1089/jir.2010.0020 (2011).
90 Sun, J. B., Flach, C. F., Czerkinsky, C. & Holmgren, J. B lymphocytes promote expansion of regulatory T cells in oral tolerance: powerful induction by antigen coupled to cholera toxin B subunit. *J Immunol* **181**, 8278-8287 (2008).
91 Lund, F. E. & Randall, T. D. Effector and regulatory B cells: modulators of CD4+ T cell immunity. *Nat Rev Immunol* **10**, 236-247, doi:10.1038/nri2729 (2010).
92 Ellis, J. S. & Braley-Mullen, H. Mechanisms by Which B Cells and Regulatory T Cells Influence Development of Murine Organ-Specific Autoimmune Diseases. *J Clin Med* **6**, doi:10.3390/jcm6020013 (2017).
93 Hamel, K. M. *et al.* B cell depletion enhances T regulatory cell activity essential in the suppression of arthritis. *J Immunol* **187**, 4900-4906, doi:10.4049/jimmunol.1101844 (2011).
94 Hu, C. Y. *et al.* Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. *J Clin Invest* **117**, 3857-3867 (2007).
95 Serreze, D. V. *et al.* Loss of intra-islet CD20 expression may complicate efficacy of B-cell-directed type 1 diabetes therapies. *Diabetes* **60**, 2914-2921, doi:10.2337/db11-0705 (2011).
96 Yu, S., Ellis, J. S., Dunn, R., Kehry, M. R. & Braley-Mullen, H. Transient depletion of B cells in young mice results in activation of regulatory T cells that inhibit development of autoimmune disease in adults. *Int Immunol* **24**, 233-242, doi:10.1093/intimm/dxs003 (2012).
97 Marino, E. *et al.* CD4(+)CD25(+) T-cells control autoimmunity in the absence of B-cells. *Diabetes* **58**, 1568-1577, doi:10.2337/db08-1504 (2009).
98 Olalekan, S. A., Cao, Y., Hamel, K. M. & Finnegan, A. B cells expressing IFN-gamma suppress Treg-cell differentiation and promote autoimmune experimental arthritis. *Eur J Immunol* **45**, 988-998, doi:10.1002/eji.201445036 (2015).
99 Feldman, A. G., Tucker, R. M., Fenner, E. K., Pelanda, R. & Mack, C. L. B cell deficient mice are protected from biliary obstruction in the rotavirus-induced mouse model of biliary atresia. *PLoS One* **8**, e73644, doi:10.1371/journal.pone.0073644 (2013).
100 Yu, S., Maiti, P. K., Dyson, M., Jain, R. & Braley-Mullen, H. B cell-deficient NOD.H-2h4 mice have CD4+CD25+ T regulatory cells that inhibit the development of spontaneous autoimmune thyroiditis. *J Exp Med* **203**, 349-358, doi:10.1084/jem.20051438 (2006).
101 Hoehlig, K. *et al.* Activation of CD4(+) Foxp3(+) regulatory T cells proceeds normally in the absence of B cells during EAE. *Eur J Immunol* **42**, 1164-1173, doi:10.1002/eji.201142242 (2012).
102 Ray, A., Basu, S., Williams, C. B., Salzman, N. H. & Dittel, B. N. A Novel IL-10-Independent Regulatory Role for B Cells in Suppressing Autoimmunity by Maintenance of Regulatory T Cells via GITR Ligand. *J Immunol* **188**, 3188-3198, doi:10.4049/jimmunol.1103354 (2012).
103 Chen, X. & Jensen, P. E. Cutting edge: primary B lymphocytes preferentially expand allogeneic FoxP3+ CD4 T cells. *J Immunol* **179**, 2046-2050, doi:179/4/2046 [pii] (2007).
104 Walters, S. N., Webster, K. E., Daley, S. & Grey, S. T. A role for intrathymic B cells in the generation of natural regulatory T cells. *J Immunol* **193**, 170-176, doi:10.4049/jimmunol.1302519 (2014).
105 Lu, F. T. *et al.* Thymic B cells promote thymus-derived regulatory T cell development and proliferation. *J Autoimmun* **61**, 62-72, doi:10.1016/j.jaut.2015.05.008 (2015).

106 Shao, T. Y., Hsu, L. H., Chien, C. H. & Chiang, B. L. Novel Foxp3(-) IL-10(-) Regulatory T-cells Induced by B-Cells Alleviate Intestinal Inflammation in Vivo. *Scientific reports* **6**, 32415, doi:10.1038/srep32415 (2016).
107 Hsu, L. H., Li, K. P., Chu, K. H. & Chiang, B. L. A B-1a cell subset induces Foxp3(-) T cells with regulatory activity through an IL-10-independent pathway. *Cellular & molecular immunology* **12**, 354-365, doi:10.1038/cmi.2014.56 (2015).
108 Moore, T. C., Gonzaga, L. M., Mather, J. M., Messer, R. J. & Hasenkrug, K. J. B Cell Requirement for Robust Regulatory T Cell Responses to Friend Retrovirus Infection. *MBio* **8**, doi:10.1128/mBio.01122-17 (2017).
109 Xiang, Y. *et al.* The dual effects of B cell depletion on antigen-specific T cells in BDC2.5NOD mice. *J Immunol* **188**, 4747-4758, doi:10.4049/jimmunol.1103055 (2012).
110 Mann, M. K., Maresz, K., Shriver, L. P., Tan, Y. & Dittel, B. N. B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. *J Immunol* **178**, 3447-3456 (2007).
111 van Olffen, R. W. *et al.* GITR triggering induces expansion of both effector and regulatory CD4+ T cells in vivo. *J Immunol* **182**, 7490-7500, doi:10.4049/jimmunol.0802751 (2009).
112 Wang, L. *et al.* T regulatory cells and B cells cooperate to form a regulatory loop that maintains gut homeostasis and suppresses dextran sulfate sodium-induced colitis. *Mucosal immunology* **8**, 1297-1312, doi:10.1038/mi.2015.20 (2015).
113 Dass, S., Vital, E. M. & Emery, P. Development of psoriasis after B cell depletion with rituximab. *Arthritis Rheum* **56**, 2715-2718, doi:10.1002/art.22811 (2007).
114 El Fassi, D., Nielsen, C. H., Kjeldsen, J., Clemmensen, O. & Hegedus, L. Ulcerative colitis following B lymphocyte depletion with rituximab in a patient with Graves' disease. *Gut* **57**, 714-715, doi:10.1136/gut.2007.138305 (2008).
115 Ray, A., Mann, M. K., Basu, S. & Dittel, B. N. A case for regulatory B cells in controlling the severity of autoimmune-mediated inflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. *J Neuroimmunol* **230**, 1-9, doi:S0165-5728(10)00488-1 [pii]
10.1016/j.jneuroim.2010.10.037 (2011).
116 Goetz, M., Atreya, R., Ghalibafian, M., Galle, P. R. & Neurath, M. F. Exacerbation of ulcerative colitis after rituximab salvage therapy. *Inflamm Bowel Dis* **13**, 1365-1368, doi:10.1002/ibd.20215 (2007).
117 Chamberlain, N. *et al.* Rituximab does not reset defective early B cell tolerance checkpoints. *J Clin Invest* **126**, 282-287, doi:10.1172/JCI83840 (2016).
118 Lehmann-Horn, K. *et al.* Anti-CD20 B-cell depletion enhances monocyte reactivity in neuroimmunological disorders. *J Neuroinflammation* **8**, 146, doi:10.1186/1742-2094-8-146 (2011).
119 Ellis, J. S., Wan, X. & Braley-Mullen, H. Transient depletion of CD4+ CD25+ regulatory T cells results in multiple autoimmune diseases in wild-type and B-cell-deficient NOD mice. *Immunology* **139**, 179-186, doi:10.1111/imm.12065 (2013).
120 Yamazaki, S. *et al.* Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* **198**, 235-247, doi:10.1084/jem.20030422 (2003).
121 Gray, M., Miles, K., Salter, D., Gray, D. & Savill, J. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A* **104**, 14080-14085, doi:10.1073/pnas.0700326104 (2007).
122 Pennati, A. *et al.* Regulatory B Cells Induce Formation of IL-10-Expressing T Cells in Mice with Autoimmune Neuroinflammation. *J Neurosci* **36**, 12598-12610, doi:10.1523/JNEUROSCI.1994-16.2016 (2016).
123 Reichardt, P. *et al.* Naive B cells generate regulatory T cells in the presence of a mature immunologic synapse. *Blood* **110**, 1519-1529 (2007).
124 Brockmann, L. *et al.* IL-10 Receptor Signaling Is Essential for TR1 Cell Function In Vivo. *J Immunol* **198**, 1130-1141, doi:10.4049/jimmunol.1601045 (2017).
125 Awasthi, A. *et al.* A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* **8**, 1380-1389 (2007).
126 Murugaiyan, G. *et al.* IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells. *J Immunol* **183**, 2435-2443, doi:10.4049/jimmunol.0900568 (2009).
127 Lo-Man, R. Regulatory B cells control dendritic cell functions. *Immunotherapy* **3**, 19-20, doi:10.2217/imt.11.34 (2011).
128 Moulin, V. *et al.* B lymphocytes regulate dendritic cell (DC) function in vivo: increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. *J Exp Med* **192**, 475-482 (2000).
129 Sun, C. M., Deriaud, E., Leclerc, C. & Lo-Man, R. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* **22**, 467-477, doi:10.1016/j.immuni.2005.02.008 (2005).
130 Lampropoulou, V. *et al.* TLR-Activated B Cells Suppress T Cell-Mediated Autoimmunity. *J Immunol* **180**, 4763-4773 (2008).
131 Byrne, S. N. & Halliday, G. M. B cells activated in lymph nodes in response to ultraviolet irradiation or by interleukin-10 inhibit dendritic cell induction of immunity. *J Invest Dermatol* **124**, 570-578, doi:JID23615 [pii] 10.1111/j.0022-202X.2005.23615.x (2005).
132 Matsumoto, M. *et al.* Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. *Immunity* **41**, 1040-1051, doi:10.1016/j.immuni.2014.10.016 (2014).
133 Matsushita, T., Horikawa, M., Iwata, Y. & Tedder, T. F. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. *J Immunol* **185**, 2240-2252, doi:10.4049/jimmunol.1001307 (2010).
134 Ronet, C. *et al.* Regulatory B cells shape the development of Th2 immune responses in BALB/c mice infected with Leishmania major through IL-10 production. *J Immunol* **184**, 886-894, doi:10.4049/jimmunol.0901114 (2010).
135 Morva, A. *et al.* Maturation and function of human dendritic cells are regulated by B lymphocytes. *Blood* **119**, 106-114, doi:10.1182/blood-2011-06-360768 (2012).
136 Miyagaki, T., Fujimoto, M. & Sato, S. Regulatory B cells in human inflammatory and autoimmune diseases: from mouse models to clinical research. *Int Immunol* **27**, 495-504, doi:10.1093/intimm/dxv026 (2015).
137 Khan, A. R. *et al.* PD-L1hi B cells are critical regulators of humoral immunity. *Nature communications* **6**, 5997, doi:10.1038/ncomms6997 (2015).
138 Yang, M. *et al.* IL-10-producing regulatory B10 cells ameliorate collagen-induced arthritis via suppressing Th17 cell generation. *Am J Pathol* **180**, 2375-2385, doi:10.1016/j.ajpath.2012.03.010 (2012).
139 Turley, D. M. & Miller, S. D. Prospects for antigen-specific tolerance based therapies for the treatment of multiple sclerosis. *Results Probl Cell Differ* **51**, 217-235, doi:10.1007/400_2008_13 (2010).
140 Sheng, J. R., Quan, S. & Soliven, B. CD1d(hi)CD5+ B cells expanded by GM-CSF in vivo suppress experimental autoimmune myasthenia gravis. *J Immunol* **193**, 2669-2677, doi:10.4049/jimmunol.1303397 (2014).
141 Fiorina, P. *et al.* Targeting CD22 reprograms B-cells and reverses autoimmune diabetes. *Diabetes* **57**, 3013-3024, doi:10.2337/db08-0420 (2008).