

Regulation of immunity and autoimmunity by B cells

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Compelling evidence has demonstrated that IL-10-producing regulatory B cells (B_{regs}) are specialized to suppress immune responses and control various immunopathologies. Several reports have provided information on the phenotype, generation, and mechanism of action of B_{regs} . Recent work has also identified B_{regs} in humans and has begun to unravel their phenotype and mode of suppression. A complete analysis of their function in immune-mediated diseases is required for possible future use of B_{regs} as a part of new clinical strategies for treating autoimmune diseases and in the induction of transplant tolerance.

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Introduction

B cells play a multifunctional role in the immune system, and similar to other lymphocytes, can circumstantially either provoke or protect against disease development and severity [1]. In autoimmunity, B cells are generally considered pathogenic since they release autoantibodies, which can cause damage to target tissues [2]. However, autoantibodies can also exert a protective effect by helping to clear apoptotic cells and reduce autoantigen load [3]. B cells are also specialized antigen-presenting cells that contribute to activation/amplification of naïve, activated and autoreactive T-cell responses [4–6]. However, antigen presentation by resting B cells has been previously shown to induce the differentiation of tolerogenic $CD4^+$ T cells [7,8]. B cells can produce different proinflammatory cytokines, including $TNF\alpha$, $IFN\gamma$, and IL12, that are characteristic of responses of B effector 1 cells. Furthermore, a separate B cell subset, B effector type 2 cells, produces IL-4 and IL-13, cytokines associated with Th2-responses [9•]. These features have generated interest in the pleiotropic function that B cells have on maintenance or regulation of immune responses. This

review will focus on novel findings, showing how regulatory B cells (B_{regs}) can both constrain and promote immunopathologies.

Endogenous B_{regs} in inflammatory-driven diseases

Lessons learned from B cell deficient mice: Several studies have shown that the absence of B cells can lead to exacerbated pathological inflammatory responses in autoimmune diseases [10,11]. B cell deficient (μ MT) mice lack the capacity to resolve inflammation in Experimental Autoimmune Encephalomyelitis (EAE), a murine model of multiple sclerosis (MS) [12•], and in antigen-induced arthritis (AIA), a model of rheumatoid arthritis (RA). In both models, sustained inflammatory responses were accompanied by increased antigen-dependent Th1 responses (Carter N, *et al.* unpublished results). However, not all B cells can regulate inflammation. An elegant study by Fillatreau *et al.* showed that only those B cells producing IL-10 helped to resolve EAE [13•]. B cells can also control the severity of the chronic colitis that spontaneously develops in $TCR\alpha^{-/-}$ mice. Exacerbated disease was observed in $TCR\alpha^{-/-}$ mice backcrossed with μ MT mice. Double KO mice had an increase in apoptotic cells, in the colon, mesenteric lymph nodes and in the spleens, compared to those detected in $TCR\alpha^{-/-}$ mice, findings attributed to the lack of antibodies that cleared apoptotic cells [14].

Lesson learned from adoptive transfer experiments: The existence of endogenous B_{regs} and their mechanisms of action were further confirmed by studies involving the adoptive transfer of naïve B cells to recipient mice with autoimmune diseases. We have shown that naïve $CD19^+CD21^hiCD23^hiCD1d^hi$ transitional 2 marginal zone precursor (T2-MZP) B_{regs} can prevent induction of arthritis, although less efficiently than T2-MZP B_{regs} isolated during the resolution phase of the disease [15•]. The suppression of arthritis development was demonstrated to be a consequence of the production of IL-10 by a proportion of the T2-MZP B cells. The finding that T2-MZP B cells isolated from the remission phase of arthritis were more powerful suppressors of disease than their naïve counterparts suggests that inflammation may provide the appropriate stimuli for further activation and possibly expansion of B_{regs} . Whether B_{regs} differentiating in the remission phase are generated *de novo* from newly emigrated transitional B cells or represent an expansion of cells from within pre-existing T2-MZP B_{regs} remains to be ascertained. In this context, B cell depletion treatment in human CD20 transgenic NOD mice resulted in reduced and delayed onset of diabetes. Interestingly, analysis of

repopulated B cells after treatment, revealed an increased frequency of T2-MZP B cells compared to the control group. Although it remains to be formally proven, it is feasible to speculate that the emergence of newly differentiated B_{regs} directly contributes to the clinical efficacy of B cells depletion therapy [16].

Mizoguchi *et al.* have also shown that B_{regs} expressing high levels of CD1d arise in response to inflammation in a colitis model. This B_{reg} subset produces IL-10 and down-regulates the inflammatory response by inhibiting IL-1 upregulation and STAT3 activation [17^{••}]. Interestingly, depletion of B cells with rituximab, a depleting anti-CD20 mAb, exacerbated rather than ameliorated ulcerative colitis, and the disease flare was associated with ablation of IL-10-producing B cells [18].

In addition to T2-MZP B_{regs}, IL-10⁺CD19⁺CD5⁺CD1d^{hi} B cells, designated as B10 B_{regs}, possess regulatory activity and inhibit inflammation [19,20]. Similar to the colitis model, the number of B10 cells increased following contact hypersensitivity sensitization, and adoptive transfer of these cells prevented activation of antigen-primed T cells in this model [19,20]. The importance of B10 cells in autoimmune disease was demonstrated using New Zealand Black/White (NZB/W) mice, which develop spontaneous lupus-like disease. CD19^{-/-}NZB/W mice, which lack B10 B cells, developed exacerbated glomerulonephritis and experienced increased mortality rates compared to CD19^{+/+}NZB/W mice in spite of a lack of autoantibody production [21]. In addition, the number of B10 cells in wild type NZB/W mice increased over the course of disease. However in the alternative MRL/lpr lupus model, the number of T2-MZP B_{regs} is inversely correlated with disease progression and severity of kidney damage [22]. Transfer of unmanipulated T2-MZP B_{regs} failed to protect recipient mice with established lupus, suggesting a dysfunction in B_{regs} as a consequence of the disease. There could be several explanations for these differences in responses. First, disease development is much faster and second, disease is more severe in the MRL/lpr mice compared to that in the NZB/W mice. Therefore, inflammation established in the MRL/lpr mice may be 'too strong' and may overpower the capacity of B_{regs} to expand.

Endogenous B_{regs} in infections and cancer

In contrast to the protective effect exerted in autoimmune disease, endogenous B_{regs} may be exploited by pathogens to subvert CD4⁺Th1 and CD8⁺CTL host protective responses. Increased IL-10-producing B cells were detected after *Leishmania major* infection in BALB/c susceptible mice, but not in C57BL/6 resistant mice. BALB/c mice develop permissive Th2 responses to *L. major* infection instead of protective Th1 responses [23]. Transfer of wild-type naïve B cells, but not of IL-10^{-/-} B cells, was demonstrated to promote Th2 development

and reverse resistance to infection in μ MT mice. This was achieved by inhibition of IL-12 released by DC [24]. The significance of B_{regs} in preventing the clearance of infectious agents by impairing optimal Th1 responses has been extended to several other parasitic infections. B cells from mice infected with *Schistosoma mansoni* or with *Brugia pahangi* (L3) proliferate, secrete IL-10, and promote the development of Th2 immune responses [25[•],26,27]. B cells can also inhibit the immune response to viral infections; mice lacking IL-10 producing B cells, display an enhanced virus-specific CD8⁺ T-cell responses to murine cytomegalovirus and an increase in plasma cell expansion, compared to wild type mice [28]. B_{regs}, however, contribute to the safe clearance of certain pathogens. μ MT mice infected with *Chlamydomydia abortus* died of an acute disease characterized by a disseminated intravascular coagulation syndrome. This is associated with increased serum levels of proinflammatory cytokines along with significantly low levels of TGF- β production [29]. This result suggests that B cells can regulate an early immune response to *C. abortus*, allowing clearance of the infection but preventing the development of immune pathology.

Less information is known about B_{regs} role in cancer [30]. Diminished growth of B16 melanoma, EL4 thymoma, and MC38 colon carcinoma cancer has been reported in μ MT mice compared to wild-type mice, and was attributed to the lack of IL-10-producing B cells [31,32]. Nevertheless, the exact mechanisms by which B_{regs} interfere with protective immune responses against infectious agents or tumor growth remain unclear.

Overview of activating signals

In vitro B cells can be stimulated to produce IL-10 and acquire regulatory properties by a number of mechanisms. We originally demonstrated that stimulation of arthritogenic B cells with antigen (collagen type II) and agonistic anti-CD40 led to the generation of IL-10-producing B_{regs}. On adoptive transfer, these *in vitro* generated B_{regs} protected mice from developing arthritis and ameliorated established disease [33^{••}]. Anti-CD40 stimulation *in vitro* restored the otherwise lost functionality of T2-MZP B_{regs} isolated from MRL/lpr mice at the peak of the disease [22]. Although CD40 engagement has repeatedly been shown to be the primary stimulus for B_{reg} activation and the enrichment of IL-10⁺CD19⁺B cells, which implies a crucial role for activated CD4⁺CD154⁺ T cells in B_{reg} activation, controversy remains regarding the requirement of co-stimulatory stimuli provided by activated CD4⁺T cells in the development of B_{regs}. For instance, B10 development in T cell-deficient mice is amplified compared to control mice suggesting no requirement for T cell costimulation [20]. However, a lack of CD40 expression on B cells leads to the generation of B_{regs} that are unable to control EAE development as their wild-type counterparts are [13^{••}]. It is plausible to

speculate that in spite of their continuing capacity to produce IL-10, B10 cells isolated from T cell deficient mice have lost their suppressive activity due to lack of additional signals provided after interaction with CD4⁺ T cells.

Activation via TLRs with CpG, LPS, or stimulation with granulocyte-macrophage colony-stimulating factor and interleukin-15 fusokine has been shown to promote B_{reg} differentiation and expansion *in vitro*. These *in vitro* expanded B_{regs} can then be used to suppress various immunopathologies *in vivo* after adoptive transfer [34–36]. The importance of TLR stimulation for B_{reg} activation has been further confirmed using chimeric mice reconstituted with B cells that are MyD88 or TLR2/4 deficient. These mice develop exacerbated EAE compared to wild-type mice [37]. B cell-activating factor, a member of the TNF cytokine family (BAFF), is a key regulator of B-cell maturation and survival but also expands IL-10-producing B_{regs} *in vitro*. Transfer of BAFF-induced CD1d⁺CD5⁺ B_{regs} suppresses arthritis development via the release of IL-10 and inhibition of Th1-mediated response [38]. It remains to be ascertained whether administration of BAFF *in vivo* expands IL-10 producing B_{regs}, similarly to the *in vitro* results (the hypothetical route for the generation of regulatory B cells is depicted in Figure 1).

Overview of the mechanisms of suppression by B_{regs}

The discovery of cell surface markers associated with IL-10-producing B cells (B10: CD19⁺, CD5⁺, and CD1d^{hi}) (T2-MZP: CD23⁺CD21⁺CD1d^{hi}) has allowed the enrichment of B_{regs} and permitted a more rigorous study of B cell-mediated suppression. T2-MZP and B10 B_{regs} produce IL-10 and inhibit proliferative responses and cytokine production by effector CD4⁺ T cells [15[•],19]. Findings showing that the transfer of relatively small number of B_{regs} bestows long-lasting protection against several immune-related diseases, suggest that B_{regs} can either proliferate *in vivo* and/or that they initiate an efficient immunosuppressive cascade involving other suppressive cells (Figure 2). B_{regs} not only restrain Th1 responses but also convey a suppressive effect to activated CD4⁺T cells. Indeed, B_{regs} via the provision of IL-10, convert effector T cells into T cells producing IL-10 (regulatory Tr1) [39,22]. Whether suppression by B_{regs} is also mediated by thymically derived T_{regs} remains to be further investigated [40]. We have demonstrated that depletion of T_{regs} in Collagen Induced Arthritis (CIA) prior to the adoptive transfer of B_{regs} did not impair or alter the capacity of B_{regs} to suppress the disease, suggesting that inhibition of disease by B_{reg} cells is independent of thymically derived T_{regs} [15[•],41]. B cells can mediate suppression by the release of TGF-β1, TGF-β3 (cytokines promoting the differentiation of adaptive T_{regs}), and IL-12 [34,42]. B cell-deficient dnTGF-βRII

mice display more liver inflammation and bile duct damage than B cell-sufficient dnTGF-βRII mice. The exacerbated disease in B cell-deficient dnTGF-βRII mice was characterized by an increase in activated CD4⁺ T cells and a decrease in FoxP3⁺ T_{regs} [43]. However, the interaction between peripherally induced T_{regs} and B_{regs} and its potential outcome are not known.

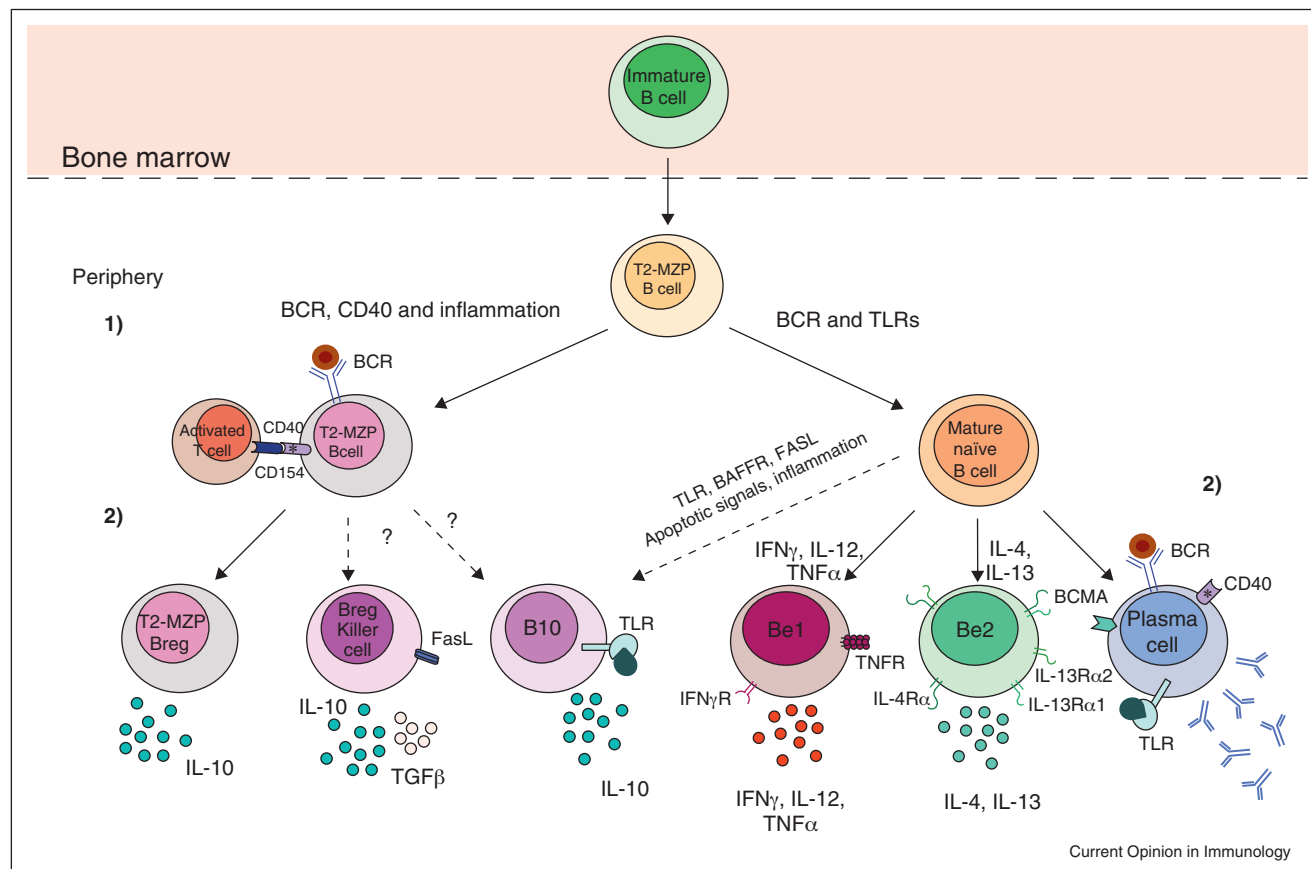
We and others have shown that the suppressive function of both T2-MZP and B10 B_{regs} is antigen dependent. Transfer of B_{regs} isolated from mice immunized with one antigen, to mice immunized with a different antigen, failed to inhibit Th1 responses in the recipients [15[•],19]. Signaling through CD80 and CD86 has been proposed as an additional effector mechanism of suppression [44,26]. Whether the suppressive effect is mediated by counter-activation of CTLA-4 or CD28 pathway is unknown. B_{regs} may also suppress disease by killing target cells. CD5⁺IL-10⁺ B cells have been shown to express elevated levels of FASL *in vivo* and induce apoptosis of arthritogenic T cells. Injection of apoptotic cells alleviates arthritis by generating B_{reg}-inducing Tr1 [45]. Whether the increase in apoptotic cells that is found in autoimmune diseases like systemic lupus erythematosus (SLE) induces the differentiation of B_{regs} with 'killer' activity is yet to be established. These mechanisms may operate synergistically or complementarily.

From experimental models to humans

Human regulatory B cells

Experiments conducted in mice have been instrumental in the identification of an equivalent B_{reg} population in humans. We have recently described a B cell subset which, in response to CD40 engagement, suppresses the differentiation of Th1 cells. This inhibitory effect is IL-10, but not TGF-β dependent, and requires signaling via CD80 and CD86 [46[•]]. IL-10-producing B cells in humans are mainly, although not exclusively, contained within the CD19⁺CD24^{hi}CD38^{hi} immature B cell subset (which is equivalent to transitional B cells in mice) [47]. In the same study, we also demonstrated that B cells isolated from SLE patients with active disease do not respond to CD40 stimulation in the same way as B cells from healthy individuals, and fail to upregulate IL-10 production. In addition, depletion of B_{regs} from healthy PBMCs, but not from SLE patients, leads to increased frequencies of CD4⁺IFNγ, and CD4⁺TNFα T cells. B_{regs} of healthy individuals efficiently suppressed responder CD4⁺T cells isolated from SLE patients, suggesting that CD24^{hi}CD38^{hi} B_{regs} isolated from active SLE patients are incompetent rather than that the effector T cells are refractory to suppression. Human CD19⁺CD24^{hi}CD38^{hi} B_{regs} also express high levels of CD1d and are CD5⁺, suggesting that, at least in humans there may be some concordance between transitional B_{regs} and B10 cells previously described in experimental models [46[•]]. Our data are corroborated by several studies showing that a

Figure 1



Hypothetical route for the generation of regulatory B cells. (1) Stimulation with TLRs agonists and antigens, induces the differentiation of transitional marginal zone precursor (T2-MZP) B cells into mature naïve B cells. However, upon encountering an activated CD4⁺T cells expressing CD154, with the same antigen specificity as the B cell, in the presence of pro-inflammatory stimuli, T2-MZP B cells differentiate into regulatory B cells. (2) In the presence of Th1-like cytokines, mature naïve B cells can further develop into B effector (Be)1 cells, producing IFN γ , IL-12 and TNF α ; if triggered by Th2-like cells they differentiate into Be2 cells, producing IL-4 and IL-13; upon antigen, TLR and CD40 stimulation they develop into antibody producing plasma cells. Based on evidence from the literature we hypothesize the existence of three different B_{regs}: T2-MZP B_{regs} producing IL-10, derived directly from T2-MZP B cells, B10 producing IL-10 and B_{regs} expressing FASL and producing TGF β derived from both T2-MZP or from mature naïve B cells. TLR, Toll-like receptor; BCMA, B cell maturation protein of the TNF α family, receptor.

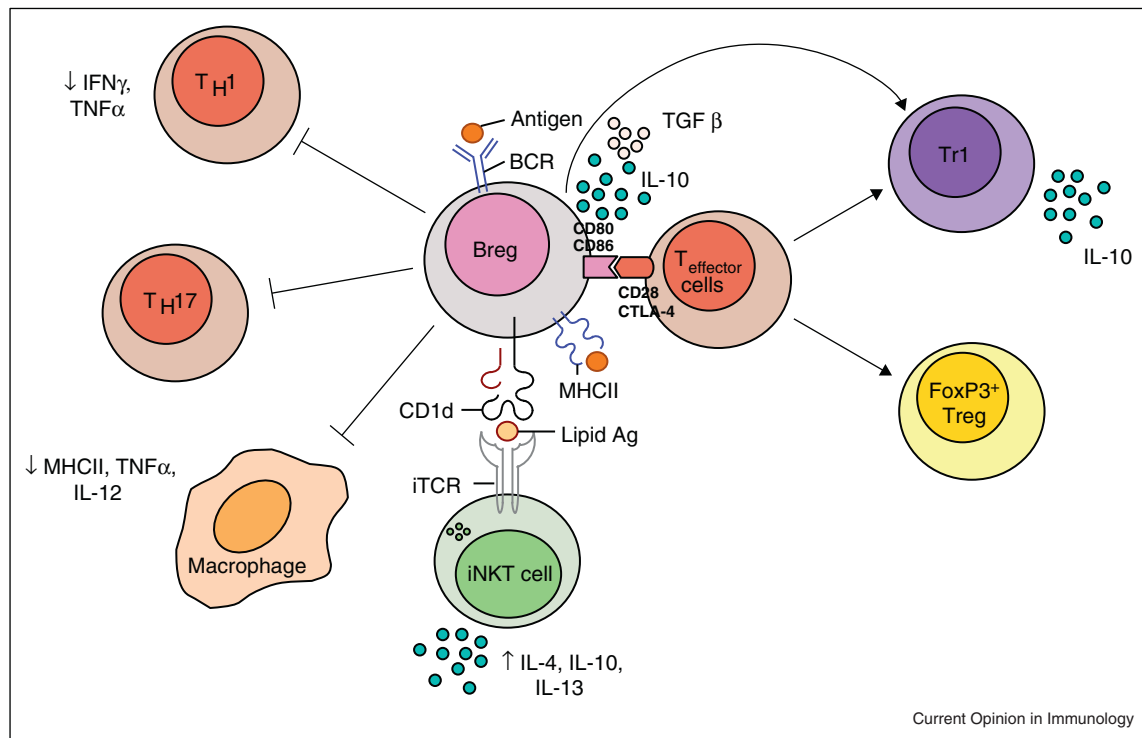
favorable outcome of immuno-mediated diseases positively correlated with the number of transitional B cells. For example, an increase in IL-10⁺CD24^{hi}CD38^{hi} immature B cell frequencies has been observed in a set of tolerant renal transplant recipients who did not require continuous immunosuppressive therapy [48]. The other evidence of the potential importance of these cells in tolerance is provided by studies aimed at understanding the effect of rituximab therapy in patients with rheumatoid arthritis and SLE. These studies show a positive correlation between long-term clinical remission from disease and a B-cell reconstitution profile characterized by a higher ratio of transitional B cells over memory B cells [49–51].

IL-10-producing B cells with a similar phenotype (CD1d^{high}) were also isolated in a group of MS patients

infected with helminths [52]. In addition to IL-10, CD1d⁺B cells of MS patients infected with helminths produced higher levels of a brain-derived neurotrophic factor and nerve growth factors (NGF) than those of MS patients infected with other parasites or non-infected MS patients. However, whether these IL-10 producing B cells were suppressive were not formally tested and therefore whether other soluble disease-specific factors, in addition to IL-10, are involved in the suppressive effect of B_{regs} in this context remains to be investigated.

Several reports have demonstrated the possibility of manipulating 'whole' B cells *in vitro* with the aim of harnessing a subset with suppressive potential. Activation of B cells with *Staphylococcus aureus* and IL-2 increases CD25⁺CD38^{high} B cells, which induce

Figure 2



Effector mechanism of regulatory B cell. Regulatory B cells inhibit Th1 cells differentiation (a suppressive effect on Th17 cells remains to be formally proven) and activation of macrophages. Inhibition is mediated via the release of soluble factors (IL-10 and TGF β), but also requires antigen recognition and engagement of co-stimulatory molecules including CD80/CD86. Interactions between B_{regs} and T effector cells can result in the induction of both FoxP3⁺Tregs (remains to be formally proven *in vivo*) and regulatory CD4⁺T cells producing IL-10 (Tr1). Lipid-antigen presentation via CD1d on B_{regs} may leads to activation of invariant Natural Killer T cell with regulatory function (not yet investigated). iNKT, invariant Natural Killer T cell; Tr1 Type 1 regulatory cell.

CD4⁺ T cell anergy and render the T cells more prone to apoptosis [53]. Allospecific T_{regs} with a potent suppressive activity were induced after interaction with anti-CD40-activated naïve B cells [54,55]. Similar to our human study, engagement of CD80 and CD86 was responsible for the conversion of effector T cells into T_{regs}. Thus, in *sensu lato* these studies have reported a function of B cells, which could be described as regulatory, although IL-10 production was not attributed to these cells.

Conclusion

Since the first description of B cells with suppressive capacity in the early 1970s [56] good progress has been made in characterizing their phenotype and functions. B_{regs} promote protective immune responses in autoimmunity, transplantation, whereas they interfere with protective immune responses in infection and cancer. Although B_{regs} act mainly, but not exclusively, via the release of IL-10, the requirement for other potential soluble factors and co-stimulatory molecules needs to be further investigated. Outstanding questions (Box 1) remaining to be addressed are how and where B_{regs} exert suppression, and whether there are multiple subsets of

B_{regs}, similar to the multiple T_{reg} subsets. A better understanding of all these biological parameters is essential prior to the potential application of B_{reg}-based therapy to a wide range of immunological diseases.

Box 1 Decalogue:

- What are the respective roles of naïve and activated CD4⁺ T cells in the maintenance of B_{regs}?
- Is there more than one B_{reg} subset?
- Do *in vitro* suppression assays directly reflect the suppressive capacity of B_{regs} *in vivo*?
- What is the main molecular mechanism (or mechanisms) of B_{reg}-mediated suppression in mouse?
- Is it the same in humans?
- How stable is the B_{reg} phenotype throughout life?
- Where do B_{regs} exert their inhibitory function?
- How variable is the B-cell receptor repertoire of B_{regs} throughout life?
- Do B_{regs} transform into pathogenic B cells during chronic inflammation?
- A highly specific B_{reg}-surface marker needs to be identified to distinguish pathogenic B cells from protective B cells.

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