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Non-Antibody Mediated Roles of B Cells in Allograft Survival

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Abstract Antibody production is unquestionably a key effector function of B cells that remains a formidable barrier against long-term graft survival. However, emerging evidence indicates that B cells play a key role in shaping the effector responses by mechanisms that extend beyond their function as antibody producing cells. B cell depletion in transplant recipients has resulted in paradoxical outcomes of increased graft rejection versus improved graft function, implying that B cells function as both enhancers and regulators of the alloimmune response. Based on findings from animal and human studies, we address mechanisms by which B cells modulate the immune response and highlight their role in promoting allograft rejection or tolerance.

Keywords Effector B cells · Antibody-independent · Antigen presentation · Transplant rejection · Regulatory B cells · TNFα · IL-10 · TIM-1 · Transplant tolerance · Allograft survival

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Introduction

Classically, T cells mediate the adaptive cellular immune response and provide helper function to B cells, allowing them to mount a humoral response. In addition to their critical function in host defense, cytotoxic antibodies can play a pathological role targeting parenchymal tissues in autoimmune disease and organ transplantation [1–4]. Based on this paradigm, T cell-directed therapies have been the mainstay of immunosuppressive therapy in transplantation, aiming to prevent both cellular and antibody-mediated rejection. The role of B cells and antibodies has received renewed interest as improved detection and diagnosis reveal that donor-specific antibodies significantly contribute to both acute and chronic allograft loss despite potent immunosuppression [5, 6].

Over two decades ago, B cells were shown to also be able to directly influence T cell responses through their ability to present antigen, provide co-stimulation and produce cytokines [7–11]. However, a major role for B cells in the T cell response was not widely accepted in the field, because such a small fraction of B cells express cytokines and initial studies relied heavily on congenitally B-deficient mice whose defects in lymphoid architecture could directly affect T cell responses. Nonetheless, recent findings in both humans and rodent models have re-energized the field and now provide strong evidence that B cells and their cytokines play a significant role in modulating cellular immune responses. First, the use of bone marrow chimeras and mice carrying specific gene knockouts in B cells definitively demonstrates that B cells can either augment or inhibit T cell-mediated immune responses [11–13, 14••]. Second, with the advent of Rituximab (anti-CD20), efficient and specific B cell depletion in humans has become a reality. Studies using Rituximab show that B cell depletion is effective in treating autoimmune diseases, including multiple sclerosis (MS,) rheumatoid arthritis (RA), and type I diabetes (T1D), that are either T cell-mediated or

demonstrate that treatment is effective without any measurable decrease in antibody levels [11, 14••, 15, 16]. Acute antibody-mediated depletion of B cells in wild-type mice corroborates the ability of B cells to either augment or inhibit T cell responses in various inflammatory settings [1, 12, 17, 18••, 19–21]. While progress has been made identifying their role, our inability to specifically identify rare proinflammatory "Beff" cells exhibiting effector function, or equally rare regulatory B cells (Bregs), remains a major barrier (Fig 1). In the face of therapeutic agents that target B cells, identification of pro- versus anti-inflammatory subsets of B cells and their exact roles, gains even more importance. Here we will review the evidence in mice and humans, for Beff and Breg cells, and their role in modulating the immune response, with a focus on recent findings pertaining to transplantation.

Antibody-Independent Functions of B Cells in Driving T Cell Responses and Graft Rejection

The mature B cell population consists of innate-like B cells (B1 and Marginal Zone, MZ) and follicular (FO) B cells that have diverse functions in immune responses [22]. Innate-like B cells produce natural antibodies to carbohydrate and phospholipid antigens independent of T-cell help (Thymus-independent antigens, e.g., pneumococcal capsular polysaccharides, blood group antigens, phosphatidylcholine, etc.) [22]. These IgM antibodies form immune complexes that modulate DC maturation and contribute to self-tolerance, anti-microbial immunity, and autoimmunity [23, 24]. Follicular B cells respond to protein antigens and depend on T-cell help to mediate IgG responses (Thymus-dependent antigens, e.g., HLA, ovalbumin, etc) [25]. In addition to BCRs, B cells express multiple TLRs and integrate signals derived from PAMPs/endogenous TLR ligands and antigen recognition [26–29]. TLR ligands (e.g., LPS, CpG DNA) either alone (innate-like B cells) or in concert with antigen (FO B cells) can activate B cells leading to rapid antibody responses and upregulated expression of molecules such as MHC II, CD40, CD80, CD86, CD70, OX40L, and ICOSL that facilitate interactions with T cells [22, 28, 30, 31]. Also, TLR- and/or BCR-derived signals induce B cell expression of cytokines such as IL-10, IL-6 and IFNγ that can influence differentiation of T cells [31].

Alloantibodies produced by B cells function as mediators of graft injury via ADCC, endothelial injury, and activating NK cells [32–34]. In addition, alloantibodies contribute to effective T cell priming by APCs, presumably through Fc receptor-mediated pathways, against alloantigens and other linked antigens expressed on the graft [35]. As detailed below, recent studies have shown that B cells also enhance T cell responses in the context of transplantation via mechanisms other than antibody production.

Antigen Presentation by B Cells in Transplant Rejection

B cells are most efficient at presenting antigen taken up via the BCR, while internalized non-specific antigen bound to complement and Fc receptors can also be presented, albeit less efficiently [36–39]. The role of B cells in T cell priming has been controversial and was thought to be limited to reactivation of memory T cells [40, 41]. Initial reports of impaired CD4 T cell responses in B cell-deficient mice were

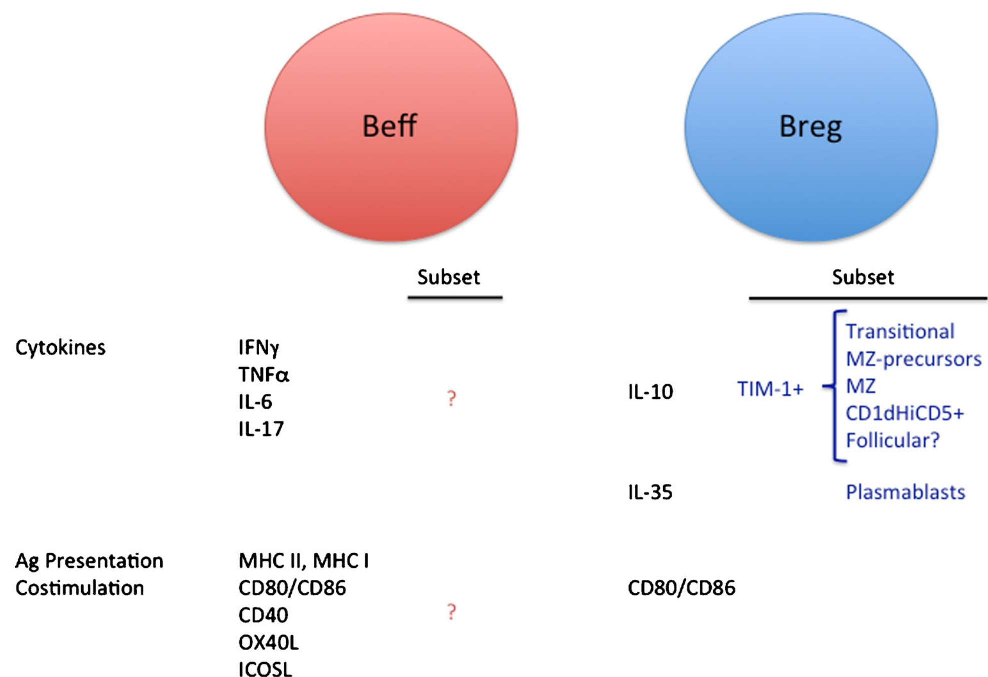

Fig. 1 Properties of Effector (Beff) and Regulatory (Breg) B cells. This representation lists key cytokines, costimulatory molecules, and antigen-presenting functions shown to play an important role in the function of Beff and Bregs. While identification of phenotype of Beff cells remains unclear, B cell subsets contributing to Breg function are listed. *MZ* marginal zone, *TIM-1* (T cell Ig and Mucin domain-1 molecule)

confounded by inherent disruption of lymphoid architecture, loss of follicular DCs, and decreased T cell numbers [42–45]. However, recent studies in multiple-model antigen systems with intact B cell compartment and/or preserved lymphoid architecture, ranging from infections (bacterial, fungal, parasitic, and viral) to autoimmunity, have now established B cells as critical APCs for CD4 and CD8 T cell responses [46–51] (Fig. 1).

It was recognized from earlier studies that heart allograft rejection was delayed in B cell-deficient mice, although this was largely attributed to the lack of antibodies since antibody transfer experiments restored rejection [45,52]. However, subsequent studies from our and other groups have shown that alloreactive T cell memory is impaired in B cell-deficient mice, suggesting a role for B cells beyond antibody production [35, 53, 54]. Alloreactive effector T cells developing in the absence of B cells contained fewer IL7Rα − expressing memory precursors, resulting in decreased memory T cell numbers and impaired memory recall [54]. In B cell–T cell cotransfer experiments, adoptive transfer of primed B cells enhanced proliferation of activated CD4 T cells and upregulated expression of anti-apoptotic Bcl2 in CD8 T cells, resulting in increased memory T cell generation [54]. These findings are consistent with observed results in LCMV and *L. monocytogenes* infections, which showed exaggerated contraction of effector T cells and attenuated T cell memory in the absence of B cells [46, 51].

Noorchashm et al. were the first to demonstrate the role of B cells as APCs in allograft rejection [55]. Mice lacking the ability of B cells to present antigen to CD4 T cells (B cell-specific impairment in either MHC II expression or MHC II peptide loading) showed attenuated donor-reactive CD4 T cell proliferation and IgG antibodies, significantly delaying acute rejection of heart allografts [55]. Despite these compelling results, antibody responses were also attenuated in the absence of B-APC function. Thus, it remained unresolved whether B cells provided requisite help for T cell responses in transplantation independent of antibody production. We addressed this question in a recent study using murine models of chronic allograft vasculopathy since antibodies are important for pathogenesis of chronic rejection [56•]. Chronic rejection was attenuated in mice that lacked both B cells and antibodies (μMT [57]). However, mice containing B cells that expressed surface BCR but could not secrete antibodies (AID/μsKO [58]) developed chronic rejection comparable to wild-type controls. Adoptive transfer of B cells from AID/μsKO into μMT mice recapitulated chronic rejection in the complete absence of circulating antibodies. This conclusively demonstrated that B cells were sufficient and secreted antibodies were not necessary for pathogenesis of chronic rejection. Moreover, cytokine-producing alloreactive T cells and T cell infiltration of allograft vessels were also diminished in the absence of B cells, but were restored by antibody-deficient B cells [56•]. Thus, B cells influence T cell responses via antibody-independent mechanisms promoting chronic rejection.

We next examined whether antigen recognition by B cells (cognate) vs. antigen-independent (non-cognate) mechanisms were providing help for T cells. Using BCR-tg mice, we found that non-cognate B cells could only partially restore chronic rejection and alloreactive T cell responses despite normal lymphoid architecture, suggesting that cognate functions of B cells were also required [56•]. The cognate role of B cells as APCs was further tested in mice containing B cells that lacked MHC I and II expression, and could not present antigen to CD4 and CD8 T cells, respectively. Despite preserved lymphoid architecture, alloreactive CD4 and CD8 T cell cytokine production and chronic rejection were attenuated in the absence of antigen presentation by B cells. Thus, B cells function as key APCs to present cognate antigen, and this is required to drive T cell responses causing chronic rejection.

In conjunction with B – APC function, CD40 and CD80/86 expression on B cells contributes to anti-pneumocystis and arthritogenic CD4 T cell responses [47, 59]. In addition to cognate antigen presentation, the ability of B cells to present non-cognate antigen internalized via Fc or complement receptor-mediated uptake, overcomes the limitation of low frequencies of antigen-specific B cells [38, 39]. B1 and MZ B cells constitutively express CD80 and CD86, and present antigen efficiently to T cells [60–62] and it remains to be tested if these B cells exhibit superior APC function than FO B cells in alloimmune responses. Also, determining whether B cells are influencing CD8 T cell responses in transplantation by enabling CD4 T cell help or by cross-presenting antigen to CD8 T cells needs to be explored in future studies.

Tertiary Lymphoid Organs in Transplant Rejection

B cells influence the formation of tertiary lymphoid structures found at sites of inflammation that facilitate in-situ antibody and T cell responses [63]. Tertiary lymphoid organs are found in grafts undergoing chronic rejection and support the development of alloantibodies, and of alloreactive effector and memory T cells [64–66]. Indeed, abrogation of tertiary lymphoid organ formation by LTβR-Ig treatment after transplantation inhibits B cell responses and prevents chronic rejection [67].

Additional mechanisms by which B cells contribute to immune responses that are yet to be explored in transplantation include costimulation, effector cytokine production, and modulation of innate responses. B cells express several costimulatory ligands such as ICOSL, OX40L, 41BBL, and CD70 that provide late costimulatory signals important for T cell differentiation to memory cells, and can function as potent costimulators of T cells [30, 68, 69]. Indeed, B cell-specific expression of OX40L was shown to be important for effector

differentiation and memory development of OT-II TCR-tg T cells [68]. Cognate and non-cognate B cells also influence the generation and expansion of T_{FH} cells by providing costimulation via ICOSL [70••, 71•, 72•]. In addition to antigen presentation and costimulation, effector cytokines secreted by B cells play an important role in shaping T cell responses in infections and autoimmune diseases [11]. IL-2 from B cells was essential for protective Th2 memory T cells in *H. polygyrus* infection [13]. B cell TNFα was important for protective antibodies in *H. polygyrus* and the expansion of Th1 cells in *T. gondii* infections [13, 73]. IL-6 from B cells following *Salmonella* infection and EAE induction stimulated CD4 T cell IL-17 production [14••, 50]. Beyond shaping adaptive T cell responses, B cells also play an important role in modulating innate immune responses by mechanisms other than antibody production. GM-CSF secreted by B cells was shown to be essential for neutrophil phagocytosis, bacterial clearance, and survival in sepsis [74••]. IL-12 production by DCs is enhanced in the absence of B cells shifting the balance toward Th1 responses due to lack of B cell IL-10 [75]. CCL7 from B cells guides monocyte infiltration into myocardium after myocardial infarction, exacerbating injury that is attenuated with B cell depletion [76••]. How these costimulatory molecules and cytokines from B cells shape the alloimmune response and influence graft survival remains to be explored. Future studies need to understand how and when to intervene to selectively target effector B cells and alter the course of alloimmune response to prevent rejection.

Regulatory B Cells: Lessons from Murine Models

Reports that B cells can inhibit T cell responses date back four decades [77]. However, a regulatory role for B cells did not begin gaining traction until the early 2000s when more definitive studies were performed. Mizoguchi and Bhan first coined the term “regulatory B cells” when they noted that spontaneous colitis developing in dysregulated TCRα-/- mice was significantly worse when crossed onto a B–deficient (μMT) background [78]. They subsequently demonstrated that colitis in TCRα-/- mice led to an increase in CD1d + (marginal zone) B cells expressing IL-10, which could inhibit colitis when transferred into B-deficient μMT.TCRα-/- mice, and this inhibition was IL-10-dependent [79] (Fig 1). Around the same time, Fillatreau explained earlier observations that B-deficient (μMT) mice developed aggressive EAE (a murine model of MS) by showing that B cells ameliorated the disease by producing IL-10 [12, 80]. This was done by generating mixed bone marrow chimeras in μMT mice so that only the B cells lacked IL-10. In contrast to control chimeras with wild-type B cells, mice whose B cells lacked IL-10 exhibited aggressive, non-remitting EAE, as seen in B-deficient mice. These experiments clearly attributed Breg activity to IL-10 expression, and moreover, the latter study addressed the role of Bregs in intact mice whose splenic architecture and B cell compartment were restored by bone marrow transplantation.

An immunoregulatory role for B cells has since been supported by the demonstration that acute depletion or deficiency of B cells can worsen disease, while transfer of B cells (or subpopulations) from diseased or immunized mice can suppress inflammatory responses in a variety of experimental models including rheumatoid arthritis (collagen-induced arthritis), contact hypersensitivity, type-1 diabetes, SLE, allergic airway disease, and allogeneic transplantation [18••, 21, 81–85]. In almost every case, Breg activity is IL-10 dependent, although this does not preclude a role for other mechanisms [10, 18••, 83, 86]. In EAE and SLE, B cell depletion before disease onset worsens disease, whereas depletion once disease has been induced ameliorates disease [1, 87]. These studies suggest that Bregs may play a dominant role during disease initiation, while B cells have a predominant effector/APC function during disease progression.

Phenotypic Identification of Bregs Remains a Major Problem in the Field:

The studies above demonstrate that Bregs inhibit disease in an IL-10-dependent manner in multiple disease models. Thus, expression of IL-10 has become the sine qua non for identification of Bregs. Despite this, for reasons detailed below, there remains no way to specifically identify Bregs based on phenotype, and this may be the single largest barrier to further understanding Breg immunobiology. Unfortunately, IL-10 is not observed in situ, or in freshly isolated B cells. Rather, IL-10 expression is induced after activation of B cells in vitro with potent mitogenic stimuli. Thus, identification of other markers is essential, but thus far, none are specific. In earlier studies, B cells were stimulated in culture for up to 72 h with CD40-ligation and/or TLR-ligands, and such conditions alter phenotype. Refinements in cytokine detection by flow cytometry and in in-vitro stimulation protocols now allow reproducible detection of IL-10 expression by B cells after only five hours—greatly improving accuracy of phenotyping [83]. After such stimulation, IL-10+ B cells typically represent ~1 % of total splenic B cells, although they are enriched (e.g., 10–25 %) in certain B cell subpopulations. Yet, it remains uncertain how stimulation of B cells with phorbol esters, ionomycin, and LPS relate to what actually occurs in vivo. Moreover, the number of B cells expressing IL-10 increases 3–5 fold if cells are first cultured for 48 hours with CD40-ligation followed by the above cocktail for five hours [10]. It has been proposed that cells capable of IL-10 expression in five hours are functional Bregs, whereas those that become IL-10-competent after 48 hours are Breg progenitors [10]. However, direct evidence that Bregs really belong to a specific

lineage is lacking because there is no definitive marker or transcriptional signature. Rather, IL-10 expression may represent a state activation that is directed or stochastic.

Using such stimulatory conditions, various B cell subsets were shown to be enriched for IL-10 and such subsets exhibit Breg activity upon adoptive transfer. Thus, splenic marginal zone (MZ) [88–90], less-mature MZ-precursor (MZ-P) or Transitional 2 (T2) [79, 81, 91], follicular (FO) [81, 90, 92] B cells, and those expressing a CD1dhiCD5+ phenotype that partially overlaps with MZ and B1 B cells [83] have all been described as regulatory. However, these appear to contain the highest proportion of IL-10+ B cells in particular models, rather than representing a true "Breg phenotype". This is based in the following two issues. First, despite being enriched, IL-10+ B cells remain a minority of the B cells in these subsets. Those subsets with the highest frequency of IL-10+ cells (and likely, with the lowest frequency of pro-inflammatory B cells) will appear to be regulatory compared to other subsets where the frequency of IL-10+ B cells is lower. (The ratio of pro and anti-inflammatory B cells in a given subset is particularly evident in humans, as detailed below). Second, many of these B cell subsets are small and while enriched, they contain only a fraction of total IL-10 expressing B cells. For example, a B cell subset may be highly enriched for IL-10+ B cells (25 %) but may comprise only 2 % of the total B cell population. If 1 % of the remaining 98 % of the B cell population express IL-10, this will contain twice as many IL-10+ B cells as the smaller population. While the larger subset may not exhibit regulatory activity due to the low frequency of IL-10+ B cells, referring to the small, enriched subset as "the Breg population" is misleading. At present, it is really not known whether IL-10+ B cells belonging to various B cell subpopulations have similar or distinct activities. Nor is it known whether IL-10- cells within certain subpopulations can contribute to the regulatory activity of the IL-10+ subpopulation. Recent advances (such as selection of TIM-1+ B cells discussed below, or in-vitro expansion) may allow enrichment of IL-10+ B cells belonging to different subsets to be compared directly. As an additional source of confusion, the small CD1dhiCD5+ population was initially dubbed "B10" because it was highly enriched for IL-10 expression. While this subset actually contains only 20–25 % of IL-10+ B cells [18••], others have used "B10" to describe any IL-10+ B cells.

TIM-1 is an Inclusive Marker for Bregs That Have Activity in Allograft Models:

TIM-1 is a member of the T immunoglobulin and mucin domain family of costimulatory molecules. We discovered that TIM-1 is constitutively expressed on 6–8 % of B cells, and increases after immunization [18••]. Importantly, TIM-1+ B cells are 10–30 fold enriched for cells expressing IL-10 compared to their TIM-1- counterparts, in every B cell subset examined (including CD1dHiCD5+ and FO B cells). Thus, as a single marker, TIM-1 identifies ~70 % of all IL-10+ B cells, making it the most inclusive marker of IL-10 expressing B cells [18••].

The regulatory role of TIM-1+ B cells was demonstrated by showing that TIM-1+, but not TIM-1-, B cells from alloimmunized mice could transfer long-term islet allograft survival into otherwise untreated B-deficient recipients. IL-10 expression was essential for graft prolongation. Since TIM-1+ B cells are enriched for IL-10+ cells from various B cell subpopulations, our findings suggest that IL-10+ cells in different B cell subsets are likely to exhibit Breg activity, and TIM-1 may provide sufficient enrichment to directly test this.

We found that TIM-1+ Bregs transferred from naïve mice were ineffective at prolonging allograft survival, while Bregs transferred from alloimmunized hosts prolonged allograft survival in an antigen-specific manner [18••]. This is in agreement with the general finding that Bregs transferred from naïve mice are ineffective, or less potent than those from immunized mice [18••, 81, 83] and suggests that control of aggressive immune responses requires prior expansion of antigen-specific Breg clones [18••]. In collagen-induced arthritis, Bregs were less potent when transferred from mice during the acute phase of the disease, and more potent after disease resolution [81]. Nonetheless, TIM-1+ B cells from acutely allo-immunized mice could transfer tolerance to B-deficient allograft recipients [18••]. Lee et al. extended these findings by showing that whole B cells from tolerant mice (treated with anti-TIM-1 and anti-CD45RB) could transfer tolerance to wild-type allograft recipients. Whether cells from tolerant mice are more potent than those from acutely immunized mice was not examined. An unresolved question is whether B cells from mice that are tolerant (or after disease resolution), express more IL-10+ Bregs, or have less proinflammatory B cells mixed into the transferred population.

The Role of TIM-1 and Bregs in Allograft Models

The studies above show that TIM-1 is an inclusive marker for IL-10-expressing B cells that can transfer allospecific tolerance. To gain further insight into the role of Bregs in allograft models we examined a tolerogenic low-affinity anti-TIM-1 mAb (RMT1-10) [93, 94]. This mAb had been previously shown to prolong allograft survival (and inhibit EAE) by skewing the immune response from a Th1 to a Th2 response and promoting Tregs [93, 94]. We found that in the absence of B cells, this same anti-TIM-1 mAb actually accelerated rejection, suggesting a costimulatory effect on T cells similar to higher-affinity anti-TIM-1 mAbs [94, 95]. Prolonged graft survival could be restored by reconstituting anti-Tim-1-treated B-deficient mice with wild-type but not IL-10-deficient B cells. Moreover, the salutary CD4 responses induced by anti-

TIM-1 were all B cell-dependent. Thus, in the absence of B cells, anti-TIM-1 enhanced IFNγ expression and no longer increased IL-4, IL-10, or Treg number. Breg transfer had similar effects on CD4 cells as anti-TIM-1 treatment. These data suggest that in allograft settings, Bregs promote less injurious Th2 responses, reduce Th1 responses, and enhance Tregs. Bregs have been shown to enhance Tregs in several studies [18••, 96, 97], likely through their expression of IL-10 and TGFβ [98–101]. However, Breg activity was found to be either distinct from, or independent of, Tregs in collagen-induced arthritis and EAE models [81, 102]. In allograft models, it is uncertain whether the reported requirement for Tregs in the presence of Bregs is due to independent or interdependent effects [103].

Given that anti-TIM-1 only prolongs allograft survival in the presence of B cells and that TIM-1 is expressed by Bregs, we examined the effect of this mAb on B cells. We found that anti-TIM-1 enhances TIM-1 and IL-10 expression, giving rise to a five-fold induction of IL-10+ B cells [18••]. Taken together, our data indicate for the first time that Bregs can be directly targeted for expansion in vivo, and moreover, that anti-TIM-1 promotes tolerance through a Breg-dependent mechanism.

Breg Expansion for Therapeutic Purposes:

The finding that anti-TIM-1 can induce Bregs indicates that TIM-1 is more than a marker for Bregs and TIM-1 signaling is involved in Breg induction. In this regard, it was previously demonstrated that administration of apoptotic cells induces B cell IL-10 expression, and treated mice are resistant to EAE and collagen–induced arthritis [90, 104•]. This appears to involve antigen-binding by self-reactive BCRs (enriched on natural IgM-expressing MZ B cells) and TLR-9 signals. However, TIM-1 is expressed by B cells enriched for IL-10 expression and is a known phosphatidylserine (PS) receptor [105]. This raises the question as to whether TIM-1 is also involved in AC binding and IL-10 production by B cells. It should be noted that anti-CD40 stimulation of B cells ex vivo has been shown to enhance activity of transferred Bregs [91]. Finally, Tedder and colleagues recently shown that Bregs can be dramatically expanded in vitro through CD40 ligation and provision of IL-21 [106••]. Thus, there are various ways that Bregs might be manipulated for therapeutic applications.

The Broader Role of Bregs in Allograft Models:

The loss of ability of anti-TIM-1 to prolong allograft survival in the absence of B cells is reminiscent of the previously unexplained B cell-dependence of anti-CD45RB, a therapy that we previously showed can induce Tregs [107–109]. Preliminary findings of ours and others suggest that anti-CD154+ DST and anti-TIM-4 also require B cells for allograft tolerance ([110] and DR unpublished data). While anti-TIM-1 is unique in that allograft survival primarily occurs through Breg induction, these studies suggest that Bregs may be essential to establish a set-point for allograft tolerance induced by various agents that do not necessarily directly target Bregs. In this regard, the absence of Bregs may raise the threshold for tolerance by augmenting immune responses, much in the same way as Tregs.

A new Subset of Regulatory B Cells: IL-35 Producing Plasmablasts:

A recent report reveals that B cells can also secrete the suppressive cytokine IL-35, and mice with B cells deficient in IL-35 are more susceptible to EAE but exhibit enhanced resistance to salmonella compared to those with wild-type B cells [111••]. IL-35 and IL-10 were expressed by distinct B cells—suggesting that both may operate in different fashions to suppress EAE. Surprisingly, most IL-10 and IL-35 expression in mice after salmonella infection or induction of EAE came from IgM+CD138+ plasmablasts, challenging the dogma that plasma cells' sole function is to produce antibodies and also raising questions about the degree to which Bregs exert their activity as B cells versus plasma cells. However, it is uncertain whether immature B cells (or transitional or MZ precursors), found to transfer Breg activity in many models, must fully mature into plasma cells before exertive suppressive activity.

Bregs in Humans

Role of B Cells in Allograft Survival and Tolerance:

B cell depletion with Rituximab is effective in treating several human autoimmune diseases by disrupting proinflammatory and costimulatory functions of B cells. However, B cell depletion can also lead to exacerbations or de-novo manifestations of autoimmune disease (reviewed in [112, 113]). Importantly, at least in certain settings, B cell depletion prior to transplantation may markedly increase the incidence of acute cellular rejection [114]. These studies lend support to the presence of Bregs and to the importance of trying to better identify inflammatory and regulatory subsets for more selective targeting. However, recent data detailed below suggest that this may not be straightforward.

Identification of Human Bregs:

Recent studies provide new insight into human regulatory B cell phenotype and IL-10 expression. As in mice, IL-10 protein expression is not observed in human B cells unless they are activated in vitro [115, 116•, 117].

In humans, transitional B cells (TrB; CD24^{hi}CD38^{hi}) from peripheral blood of healthy volunteers were shown to be enriched for IL-10 expression and capable of suppressing inflammatory cytokine expression by autologous CD4^{+} T cells in vitro [117]. In this regard, tolerant renal allograft patients were shown to express higher numbers of B cells, TrB, and more IL-10+ TrB than immunosuppressed patients with stable renal allograft function [118, 119]. Moreover, TrB cells from SLE patients were shown to express less IL-10 in response to CD40 ligation than those from healthy controls and lacked in-vitro regulatory activity [117]. In contrast, Iwata et al. showed that IL-10 is highest in human CD24^{hi}CD27^{+} memory B cells (MemB) and that IL-10 expression in patients with various autoimmune diseases including SLE and RA is actually higher than in healthy controls [116•]. Thus, there is a discrepancy in the current literature on the identity and role of human Bregs.

To readdress this issue, we directly compared B cell subpopulations from healthy controls. Given that cells within these subpopulations are not uniform, and IL-10 is expressed by only a fraction of these B cells, we asked whether the concomitant expression of pro-inflammatory cytokines influenced the regulatory activity observed. We examined B cell expression of TNFα based on its importance in both antibody-mediated responses in mice and in promoting T cell responsiveness in MS [13, 120••, 121]. After stimulation (CPG and CD40L) for 48 h, we found that IL-10 expression by B cells in isolated healthy volunteers (HV) was similarly enriched amongst Memory (MemB), and Transitional B (TrB) compared to naïve B cells [120••]. However, a significant fraction of B cells within each subset co-expressed TNFα, and in fact, many B cells simultaneously expressed both cytokines. Compared to MemB, TrB expressed similar IL-10, but much lower TNFα, resulting in a higher IL-10:TNFα ratio (~1.5 vs. 0.6). Similar results were obtained when B cells were sorted into subsets prior to in-vitro stimulation. The IL-10:TNFα ratio correlated with potent suppression of Th1 cytokines in vitro by TrB, but not MemB or naïve B subsets. Neutralization of IL-10 blocked TrB regulatory function, while neutralization of TNFα uncovered regulatory function of MemB, showing that these cytokines are involved in in-vitro suppressive activity.

We then examined the utility of the TrB IL-10/TNF-α ratio in a cross-sectional study of 88 renal transplant recipients 2-15 years post-transplant [120]. Patients with stable allograft function and those with graft dysfunction but no rejection had TrB IL-10:TNFα ratios comparable to HV. In contrast, TrB from patients with graft dysfunction and rejection were not only reduced in number, but exhibited a more inflammatory cytokine profile with a significant fall in IL-10:TNFα ratio and they lost their in-vitro regulatory activity. Neither TrB IL-10 alone, nor IL-10:TNFα ratio of total B cells correlated with rejection. Thus, human TrB may exhibit more suppressive or inflammatory activity depending on the subject's immunological status.

Importantly, the TrB IL-10/TNF-α ratio at the time of for-cause biopsy was a predictor of graft outcomes over a three-year follow-up [120]. Amongst 47 patients with for-cause biopsies, those with a high ratio (above the overall group mean) had stable graft function, whereas 40 % of those with low ratios had a two-fold decrease in GFR or graft loss over three years. Amongst the relatively small number with rejection, there was a strong trend toward poor outcome that did not quite reach statistical significance ($p=0.056$). Of note, in this study, rejection was primarily due to chronic, antibody-mediated rejection (AMR), while 25 % had superimposed, acute cellular rejection (ACR).

These findings suggest that standard human B cell subsets are insufficient in separating potentially suppressive and inflammatory B cells. Additionally, current in-vitro simulation protocols induce both pro- and anti-inflammatory cytokines by the same cells. The IL-10/TNF-α ratio not only correlated with in-vitro suppressive function, but both the ratio and in-vitro activity were altered in the presence of an active immune (rejection) response. Taken together, our results imply that the cytokine ratio within the TrB subset is a much more accurate way to gauge suppressive versus effector function. This is important because this ratio changes in the presence of immunological activity. While these findings clearly require confirmation in a larger cohort, they suggest that at the time of biopsy, the IL-10/TNF-α ratio may be predictive of outcome. In turn, these findings amplify the notion that both B cell inflammatory and anti-inflammatory cytokines play an important role in immune response. It remains to be determined whether such changes can be used prospectively to guide therapy for patients at risk.

Conclusion

It is now evident that in addition to their role in humoral immunity, B cells play an important role in modulating T cell-mediated immune responses. Studies in mice with specific B cell defects demonstrate key roles for B cells in enhancing effector T cell and memory T cell responses on the one hand, and inhibiting effector T cell responses and promoting tolerance on the other. In allograft models, effector B cells play an important role as APCs that promote T cell activation and chronic rejection. Production of the inflammatory cytokine, TNFα, by B cells also augments production of antibodies by B cells and may play a role in promoting humoral immunity in humans. Alternatively, regulatory B cells help establish a set-point for allograft tolerance in murine models and may promote immune quiescence in both tolerant and immunosuppressed human renal allograft recipients. While targeting CD20+ B cells is of unclear benefit in treating antibody-mediated rejection, this approach inhibits ongoing T cell

responses in autoimmune disease, and could have a role in treating acute rejection and/or inhibiting chronic rejection. On the other hand, non-selective depletion of all B cells has the potential to dysregulate the immune system and even precipitate acute rejection. If confirmed, recent findings that plasmablasts may be potent immune regulators only complicates new approaches aimed at specifically targeting these antibody-producing cells. Thus, in the allograft setting we need to identify approaches to selectively deplete or inhibit effector B cells while enhancing regulatory B cells. The finding that the same B cells can secrete both regulatory and inflammatory cytokines further complicates this task. If these in-vitro findings correspond to their capacity in vivo, simple depletion based on phenotype may not be possible. Clearly, we have much to learn about the immunobiology of effector and regulatory B cells, starting with more accurate identification, understanding their plasticity. In this regard little is known about where or how these cells function in vivo. Understanding this, may help us gain insight into selective inhibition or enhancement of their function. In the meantime, if the utility of the ratio of B cell inflammatory to inhibitory cytokines as a marker for subsequent clinical course is confirmed, it may identify patients whose clinical course can be modified by more intensive immunosuppression.

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Compliance with Ethics Guidelines

Conflict of Interest Geetha Chalasani and David Rothstein declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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