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Reprogramming of B cells into regulatory cells with engineered fusokines

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Abstract

B cells play a pivotal role in host adaptive immunity against pathogenic microorganisms, but may also maladaptively contribute to the pathogenesis of autoimmune diseases. In contrast, distinct B cell subsets have the capacity to regulate host immune response, and suppress inflammation. B regulatory cells are a rare population of endogenous B-lymphocytes defined in part by production of the anti-inflammatory cytokine IL-10. Although “natural” B regulatory cells exist *in vivo*, the low frequency of B regulatory cells may be a limiting factor on their impact in autoimmune ailments. In answer to this unmet need, we have developed a novel strategy for alternate lymphoid activation: fusokines. These wholly engineered chimeric leukines fuse two functionally unrelated cytokines for the purpose of alternate immune modulation. The GM-CSF- and IL-15-derived fusokine: GIFT15, possesses entirely novel and unheralded immune modulating properties mediated through the IL15 receptor which reprograms naïve B cells into B regulatory cells (Bregs). In this article, we review the current approaches to generate Bregs *in vitro*, and highlight gain-of-function mechanisms by which GIFT15-induced Bregs abrogate pathogenic autoimmunity in mice. We also demonstrate that the human equivalent of inducible Bregs may also serve as a new potent therapeutic tool for treatment of autoimmune disease.

Keywords

Fusokine; GM-CSF; IL-10; IL-15; Bregs; inflammation; multiple sclerosis

Two Timing B cells

B-cell and T-cell immune responses are two arms of host adaptive immunity against pathogenic microorganismal assault. Being a heterogeneous population of lymphatic cells, B cells possess multiple immune functions. Since their description by Max Cooper [1], B-cells and their progeny, have been defined as the stalwarts of humoral immunity [2]. It is now well known B cells also prime host immunity through specific antigen presentation to T cells [3], and promote immune response via secretion of a variety of cytokines upon infectious stimuli [4]. Regrettably, B cells also contribute to the pathogenesis of autoimmune diseases via maladapted autoantibody-mediated pathology as exemplified by autoimmune response to red blood cells and neuronal synapses [5].

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Notwithstanding their ill-behaved brethren, accumulating evidence from both murine models of autoimmune disease and human subjects reveals the existence of a “regulatory” B-cell subset – Bregs akin to Tregs [6, 7, 8]. Native Bregs produce the anti-inflammatory cytokine IL-10 and TGF- β , negatively modulate T effectors or antigen-presenting cells, and suppress chronic inflammatory response [9]. The existence of Bregs was first inferred in mice with B-cell deficiency (μ MT). Mice lacking B cells could not constrain the inflammatory response to myelin basic protein peptide Ac1–11, suffering an unusual severe and chronic form of experimental autoimmune encephalomyelitis [10, 11, 12], suggesting the existence of a benevolent Breg subset. Complementary evidence suggested the existence of Bregs. Indeed, pharmacological therapeutic depletion of B cells in human subjects exacerbated ulcerative colitis [13] or psoriasis [14]. Moreover, B cells are seemingly involved in human renal allograft transplant tolerance [15].

As a minority B-cell population that has shared features with CD5⁺ B1 and CD1d⁺ B2 cells [16], IL-10 producing Bregs are reported to express a pattern of unique surface markers. In murine spleen, IL-10 positive B10 cells express CD19, CD1d and CD5 [17]. Marginal zone IL-10-producing Bregs are CD1d⁺CD21⁺CD23^{low}IgM⁺ [18, 19]. Additionally, IL10-secreting transitional T2-MZ precursor B cells bear CD1d, CD21, CD23, CD24, and IgM [6, 16]. In humans, Bregs in peripheral blood are CD19⁺CD27⁺ [20] or CD19⁺CD24⁺CD38⁺ [6, 21]. Recently, a low frequency population of human IL-10⁺ Bregs was found to co-express CD24 and CD27 [22]. Bregs are detectable within most lymphoid tissues, spleen and bone marrow, with frequencies of 1.4% Bregs in the spleen [23], 0.4–0.8% in blood, and 2–3% in bone marrow [24]. Due to their low abundance *in vivo*, harvest of autologous Bregs for clinical use is limited. Unlike T-cells, there are no defined clinical methods to expand the number of Bregs for adoptive cell therapy strategies. Thus, it would be of great use to develop efficient methods for generating Bregs *in vitro* that could be utilized for treatment of patients with immune diseases.

In this review, we will review potential extrinsic stimulators for Bregs *in vitro* and will specifically describe how the novel fusion cytokine GIFT15 reprograms naïve B cells to *inducible* Bregs (iBregs). We will highlight the phenotype, function and immune suppressive properties of iBregs, and discuss the challenge ahead generating iBregs for first-in-human clinical use. We propose that iBregs can serve as a potent therapeutic tool for personalized cell therapy for human autoimmune diseases.

Extrinsic stimulators for Breg induction

B cells express a panel of surface molecules for cell interaction and activation. B-cell receptors (BCR), B cell-activating factor [25], CD40 and Toll-like receptors (TLR) [26] are the receptors expressed on B cell surface that are essential for B cell maturation, antigen presentation, T cell interaction and pathogen sensing [25, 27, 28]. However, activation of B cells through BCR pathway, CD40 ligation, TLR or BAFF signaling can also induce distinct populations of regulatory B cells [29, 30, 31].

CD40 - a costimulatory molecule [32] is required for the generation and suppressive function of Bregs. Treatment of CD40 with agonistic CD40 monoclonal antibody or CD40 ligand *in vitro* activates B cells to secrete the anti-inflammatory cytokine IL-10 [29, 33]. Activation of naïve B cells isolated from untreated patients by CD40 engagement results in a significant increase of IL-10 production in the B cells [34]. CD40 ligation on arthritic-genic B cells generated a subset of IL-10-secreting B cells that prevent arthritis [35]. It is interesting that IL-10 production was found to be restricted to the subpopulation of CD40L⁺ B cells [36], pinpointing the essential role of the CD40 molecule and its ligand CD154 for the induction of Bregs.

TLRs are pathogenic pattern recognition receptors that sense the presence of a variety of microbes [37] or sterile self-products [38]. Activation of TLRs such as TLR4, and TLR9 via MyD88 signaling pathway can induce immune-suppressive murine regulatory B cells *in vitro* secreting IL-10 [39]. TLR4 ligand LPS or TLR9 ligand CpG, have been shown to promote Breg differentiation and expansion *in vitro*. Those TLR-activated murine Bregs could suppress inflammation *in vivo* after adoptive transfer in the mouse model of EAE. However, human B cells do not express TLR4. Therefore, LPS stimulation cannot induce human Bregs [26]. B cells without TLR4 or MyD88 also cannot suppress chronic EAE [39]. TLR9 stimulation with CpG induces murine MZ B cells to secrete IL-10 [40], and inhibits Th1 pro-inflammatory immune responses [41]. It was also reported TLR9 activation *ex vivo* is involved in the production of IL-10 by human CD27⁺ B cells or CD38^{high} transitional B-cells [42]. Based on the important roles of TLR, BCR, and CD40 on the generation of Bregs, a two-step model of Breg induction has been proposed [30]: an initial step by TLR priming, followed by BCR recognition and CD40 engagement.

As a cytokine of the TNF family that regulates B cell maturation and survival [25], BAFF triggers B cell activation, and induces the differentiation of CD1d⁺CD5⁺ IL-10-producing B regulatory cells *in vitro* [31]. Moreover, BAFF treatment expands the number of marginal zone Bregs *in vivo*. Consequently, BAFF-induced CD1d⁺CD5⁺ Bregs can suppress autoimmune arthritis development in mice through the release of IL-10.

A gene therapy strategy for production of Bregs has also been tested. B cells were transduced with a retroviral vector encoding an antigen fused to an immunoglobulin heavy chain molecule [43]; or transduced with a lentiviral vector expressing IL-10 or/and myelin oligodendrocyte glycoprotein [10]. Those gene-enhanced B cells were shown to inhibit antigen-specific T and B cells, or induce immune tolerance [43]. The adoptive transfer of those modified B cells before or after the induction of EAE significantly inhibited the chronic inflammation in the murine model [10], due to the suppressive function of those B cells on Th1 and Th17 T cell responses.

In summary, it is possible to generate a Breg-like phenotype through activation by TLR ligands or CD40 ligation, or genetic modification with an antigen-encoded viral vector. However, technical and regulatory limitations make these strategies challenging to translate in to practical pharmaceutical strategies. Hence, the potential of Bregs is constrained by these limitations and defines an unmet technological need. The development of fusokines and in particular GIFT15 addresses this issue.

Reprogramming of naïve B cells to iBregs by GIFT15

In recent years, we have developed a new family of fusion cytokines for cell-based immune modulation to treat cancer or autoimmune diseases. Those fusokines are derived from parental molecules GM-CSF and interleukins (aka GIFTs) [44, 45, 46]. GIFT15 is one member of this family of fusokines, generated from the partnering of GM-CSF and IL-15 [45] (Figure 1). GM-CSF has been routinely used in clinic due to its immune-promoting function to enhance adaptive immunity [47]. IL-15, one of the common γ -chain cytokine family members besides IL-2, IL-4, IL-7, IL-9 and IL-21 [48], plays an essential role in maintaining immune responses [49], but has a very short half-time in comparison with GM-CSF [50]. Pairing GM-CSF and IL-15 together likely increases the longevity of IL-15, and lengthens the exposure of immune system to IL-15 signaling. More importantly, GIFT15 possesses novel biochemical properties that alter its affinities to the IL-15R with remarkable gain-of-function features [45]. Our seminal discovery was that the coupling of two functionally distinct cytokines into a bi-functional hybrid allows for unheralded and novel immune activity that could not seen or predicted by simply combined use of the parental

components. GIFTs essentially behave as entirely new cytokines that hijack normal receptor signaling in responsive lymphoid cells in a manner not seen in nature. The effect of GIFT15 on B-cells was such an event and the genesis of GIFT15-induced Bregs an unexpected and fortuitous outcome.

GIFT15 and the genesis of *inducible* Bregs (iBregs)

GIFT15 was originally designed with the objective of enhancing pro-inflammatory effects that link innate immune response with adaptive immunity. However, to our surprise, GIFT15 displayed an unexpected immune suppressive function manifest by the conversion of naïve B cells into a unique population of IL-10 producing iBregs [51]. This feature allowed us to rapidly generate a large number of iBregs *in vitro* from peripherally available naïve B cells.

B-cell development and differentiation are strictly controlled by intrinsic signaling and transcription factors [52]. GIFT15 stimulation activates asymmetrical signaling downstream of the IL15 receptor (R) β and γ chains in B cells, causing hypophosphorylation of STAT5 and hyper-phosphorylation of STAT3 [45]. STAT3 has suppressive, proliferative, anti-apoptotic, and pro-survival properties [53]. Consequently, aberrant signaling downstream of the IL15R complex ultimately induces the differentiation of suppressive Bregs from naïve B cells [51]. We discovered that GIFT15 treatment of mice could induce tolerance to allogeneic and xenogeneic tumor transplant in mice [45]. We have also shown that adoptive transfer of syngeneic GIFT15-iBregs reverses symptomatic neuroinflammation in a mouse model of EAE [51].

What are iBregs? GIFT15-generated iBregs express an array of surface molecules [51], which share some of the typical markers within a panel of inducible Bregs [17, 19, 40]. Flow cytometry profiles murine GIFT15-iBreg as B220⁺, CD1d⁺, CD21⁺, CD22⁺, CD23⁺, CD24⁺, CD43⁺, CD79B⁺, CD138⁺, IgD⁺, IgM⁺, and MHCII⁺ (Figure 2). Those iBreg also express the trimeric IL-15 receptor that is consistent of IL-15R α , IL-2R β and the common γ chain. However, GIFT15-iBregs cells do not express CD131 (a component of GM-CSF receptor) and CD27 that is phenotypically expressed on human memory B cells. Cell surface markers are differentially expressed during B-cell activation and differentiation. CD1d, an MHCII-like molecule, is now commonly accepted as a surface marker for IL10-secreting Bregs [18, 54]. CD1d is also required for natural Breg's suppressive function [55]. CD1d up-regulation on GIFT15-reprogrammed naïve B cells indicates GIFT15-iBreg formation possess some commonality with other types of inducible Bregs. The positive express of CD1d, together with CD21, CD23, IgM and IgD classify GIFT15-iBreg with characteristics of the transitional two-marginal zone B cells [40]. CD138 is a typical surface marker for plasma cells. GIFT15 treatment of naïve B cells induces CD138 expression, indicating GIFT15-iBreg is unique in comparison with other forms of inducible Bregs. Overall, GIFT15-iBregs have a combined phenotype that is partially aligned with regulatory B10 cells, MZ-T2 regulatory B cells and plasma cells.

Immune suppressive mechanisms of GIFT15-iBreg

It has been suggested that the immune suppressive properties of Bregs arise from the secretion of soluble inhibitory factor IL-10 or TGF- β , as recently described in murine models of human autoimmune diseases [55, 56, 57]. We have demonstrated that GIFT15-iBreg acquire immune suppressor function via IL-10 [51] (Figure 3), but not TGF- β (data not shown). Indeed, GIFT15-iBregs derived from IL-10^{-/-} mice are incapable of suppressing inflammation *in vivo*. As an anti-inflammatory cytokine, IL-10 inhibits T cell polarization and proliferation [42, 56, 58], blocks antigen presentation by antigen presenting cells, and inhibits inflammatory cytokine production by immune effector cells [39]. It was

reported that IL-10 promotes the differentiation and expansion of regulatory T cells [59, 60]. It is therefore possible that IL-10 secreted by GIFT15-iBreg may also promote the expansion and development of T regulatory cells *in vivo*.

Notwithstanding the requirement of IL-10 for iBreg suppressor function, we've also observed that suppressor function also arises through an IL-10-independent mechanism that does not involve soluble factors. Indeed, regulatory functions of endogenous Bregs are apparently dependent on cell-cell contact through major histocompatibility complex (MHC), B7 co-stimulatory molecules or Fas [61, 62, 63]. We have found this to be operative in GIFT15-iBregs as well; we also observed that MHCII is up regulated on the surface of GIFT15-iBregs and that GIFT15-iBregs derived from MHCII^{-/-} mice are devoid of suppressor *in vitro* and *in vivo* [51], despite their abundant production of IL10. These observations confirm the pivotal role of MHCII expression as well as IL-10 for the suppressor function of GIFT15-iBregs. Intriguingly, GIFT15-iBregs can suppress EAE despite their lack of exposure to MOG antigen *in vitro*. Hence, whether and how GIFT15-iBregs interact in a MHCII-driven synapse with MOG-specific T cell subsets remains unclear.

GIFT15-iBreg for cell immune therapy

Amongst the first use of B-cell based cell therapy for autoimmune disorders was the adoptive transfer of activated B cells for induction of immune tolerance in mice [64, 65]. In μ MT B-cell deficient mice, absence of B cells did not prevent symptomatic EAE but instead worsened disease outcome after immunization with MOG peptides. Adoptive transfer of normal B cells, but not IL-10^{-/-} B cells, ameliorated EAE disease severity in the μ MT mice. In our hands, GIFT15-iBregs as an immune therapeutic tool were tested in an established mouse model of EAE. Adoptive transfer of 2 million syngeneic GIFT15-iBregs efficiently suppressed MOG-specific activated T cells, and stopped inflammation in spinal cords, leading to complete recovery of EAE mice. Control mice treated with naïve B-cells or GIFT15 iBregs derived from IL-10^{-/-} or MHCII^{-/-} mice did not improve their disease score [51]. Interestingly, EAE mice (on a C57BL/6 background) treated with MHC mismatched GIFT15-iBregs (derived from Balb/c mice) did not improve as well, suggesting that a functional MHCII synapse is required or that GIFT15-Bregs are immune rejected in mismatched recipients. Therefore, from a translation perspective, “universal donor” GIFT15-iBregs would be predictably of no use to treat an immune ailment in recipients with a normal immune system. The sum of these observations support the notion that autologous blood-derived naïve B-cells collected from a routine leukapheresis procedure would be amenable to *ex vivo* iBreg conversion and subsequently utilized as a personalized adoptive cell therapy. Transplanted cell death may be a limiting factor on the overall effectiveness of cell therapies such as iBregs-based treatment. How iBregs are recognized by the host immune system in patients will determine whether an innate or adaptive immune response is evoked. Being derived from self, autologous GIFT15-iBregs will not be immune rejected by patients with auto-inflammatory diseases.

We recently explored the possibility whether we could convert B cells from peripheral blood of patients with multiple sclerosis (MS) to iBreg by GIFT15. Indeed, a population of IL-10-producing GIFT15-iBregs was reprogrammed from naïve B cells in the circulation of MS patients after six days of GIFT15 treatment (Figure 4). Harvesting and *ex vivo* manipulation of autologous naïve B cells from peripheral blood of patients is an entirely feasible procedure in MS. The successful rapid generating of human iBregs with GIFT15 provides the rationale for developing personalized GIFT15-iBregs-based adoptive therapy for MS patients.

GIFT15-iBregs could also be a therapeutic option for other autoimmune and inflammatory diseases, such as graft-versus-host disease (GvHD), inflammatory arthritis and inflammatory bowel disease (IBD). IL-10-producing B cells have a protective effect on acute GvHD in mice, attenuating CD4⁺ T-cell proliferation and suppressing Th1 differentiation [66]. A high content of B cell progenitors in stem cell graft in human is associated with a significantly lower rate of acute GvHD [67]. Adoptive transfer of TLR4-activated B cells that express Fas ligand and secrete TGF- β 1 prevents the development of diabetes in NOD mice [56]. Administration of B220⁺CD1d⁺CD21⁺CD23⁺IgM⁺ Bregs improved collagen-induced arthritis in mouse model [35]. IL-10⁺ regulatory B10 cells inhibited intestinal injury in a mouse model of dextran sulfate sodium-induced inflammatory bowel disease [68]. The potential role of endogenous Bregs in those diseases, and the proven therapeutic property of GIFT15-iBreg for EAE strongly suggest that GIFT15-iBregs may be a very promising approach for the treatment of an array of alloimmune and autoimmune ailments.

Conclusion

Utilization of inducible Breg provides a new strategy for cell therapy for autoimmune diseases. A bioengineered chimeric fusokine GIFT15 displays remarkable and unheralded immune-modulatory properties and robustly reprogram naïve B cells, even pathogenic B cells from diseased subjects, to inducible B regulatory cells. GIFT15-iBregs require both IL-10- and MHCII-dependent molecular mechanisms for suppressive action, although how GIFT15-iBregs specifically interact with pathogenic T cells is incompletely understood. We predict that reprogrammed autologous GIFT15-iBregs may be of benefit for treatment of human auto-inflammatory disease.

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Abbreviations used in this article

BAFF	B cell-activating factor
BCR	B-cell receptors
EAE	experimental autoimmune encephalomyelitis
GIFT15	GM-CSF and IL-15 derived fusokine
GIFT15-iBregs	GIFT15 induced B regulatory cells
GM-CSF	granulocyte macrophage colony-stimulating factor
GvHD	graft-versus-host disease
IBD	inflammatory bowel disease
iBreg	inducible B regulatory cells
MHCII	major histocompatibility complex class II
MS	multiple sclerosis
MOG	myelin oligodendrocyte glycoprotein peptide
STAT	Signal Transducers and Activators of Transcription protein
TGF-β	Transforming growth factor beta

TLR

Toll-like receptors

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Figure 1. Structure of fusokine Gift15 protein

A: amino acid sequence. **B:** three dimension structure, modified from the reference 45 (*Blood*, 109: 2234-42, 2007).

Figure 2. Surface markers of Gift15-iBregs
Blue line is primary antibody. Red line is antibody isotype control.

Figure 3. IL-10 production by Gift15-iBregs

Naïve B cells from mouse spleen (5×10^5 cells/ml) were treated with Gift15 protein in RPMI-1640 medium for 5 days. IL-10 production by Gift15 induced Bregs was quantified with mouse IL-10 ELISA kit.

Figure 4. Converting naïve B cells from MS patients to Gift15-iBregs

Circulating naïve B cells (5×10^5 cells/ml) from patients were treated with or without Gift15 protein for 5 days. IL-10 secretion by Gift15-converted Bregs (**middle**), or control B cells (**left**) was quantified with human IL-10 ELISA kit (**right**).