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REVIEW

TGF-β-secreting regulatory B cells: unsung players in immune regulation

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Abstract

Regulatory B cells contribute to the regulation of immune responses in cancer, autoimmune disorders, allergic conditions and inflammatory diseases. Although most studies focus on regulatory B lymphocytes expressing interleukin-10, there is growing evidence that B cells producing transforming growth factor β (TGF-β) can also regulate T-cell immunity in inflammatory diseases and promote the emergence of regulatory T cells that contribute to the induction and maintenance of natural and induced immune tolerance. Most research on TGF-β^+ regulatory B cells has been conducted in models of allergy, cancer and autoimmune diseases, but there has, as yet, been limited scrutiny of their role in the transplant setting. Herein, we review recent investigations seeking to understand how TGF-β-producing B cells direct the immune response in various inflammatory diseases and whether these regulatory cells may have a role in fostering tolerance in transplantation.

Keywords: allergy, autoimmune diseases, cancer, TGF-β^+ regulatory B cells, transplantation

INTRODUCTION

The immune system relies on a complex and intimately intertwined network of regulatory cells that restrain the response to self-antigens, prevent autoimmunity and temper physiological activation to promote resolution of immune responses. The regulatory cell population most thoroughly studied to date is the FOXP3-expressing CD4 T cell (Tregs).[1,2] However, regulatory activity has been identified in varied immune cell lineages, including myeloid-derived suppressor cells (MDSCs), DC-regs and monocyte-derived regulatory macrophages, as well as NK and B cells.[3] The anti-inflammatory and immunomodulatory functions of B cells were first described in the 1970s by Katz *et al*. and Neta *et al*., who showed that B cells with suppressive qualities play a role in preventing delayed hypersensitivity.[4,5] In more recent studies, regulatory B cells (Bregs) have been found to modulate the immune response against tumors, autoimmune diseases, graft rejection and inflammatory diseases.[6–8] In contrast to Tregs, which are predominately identified by the transcription factor, FOXP3, there are no lineage-specific markers or transcription factors that clearly define a discrete Breg subset.[9] Therefore, the basic characterisation of Bregs, including identifiable surface markers and the mechanisms of immunosuppression, remains areas of active research.

The regulatory actions of Bregs are exerted primarily via the elaboration of immunoregulatory cytokines, such as interleukin-10 (IL-10). In fact, in many studies, IL-10 expression is used as the best marker to define the subset of B cells with regulatory activity. However, other cytokines can also contribute to Breg function, including TGF-β and IL-35.[10–12] In addition, TGF-β secretion by Bregs has been found to also play an important and independent role in immune regulation in various inflammatory diseases.[13] Studies examining the suppression of IL-17$^+$ and IFN-γ$^+$ T cells by B cells indicate that both IL-10- and TGF-β-dependent processes are involved. In murine studies of Brucella infection, neutralisation of both IL-10 and TGF-β is more efficacious in promoting clearance of infection than either alone.[14] These findings suggest that IL-10 and TGF-β can work in concert to dampen harmful immune responses.[15]

Additional studies have demonstrated the singular importance of TGF-β1 in models of immune regulation. Bjarnadottir *et al*.[16] showed that B-cell-specific deletion of TGF-β1 (B-TGF-β1$^{-/-}$) in mice led to earlier onset of experimental autoimmune encephalitis (EAE), higher cumulative disease burden and higher T-cell production of GM-CSF and IFN-γ. Experiments utilising a co-culture system of human CD19$^+$CD25^{hi} B cells and CD4$^+$ T cells *in vitro* found TGF-β, not IL-10, to be the primary B-cell cytokine fostering the differentiation of Tregs.[17] Furthermore, Bregs isolated from human blood suppressed the proliferation of CD4$^+$ T cells and enhanced the expression of FOXP3 and CTLA-4 of Treg cells in TGF-β alone or with IDO, but not through IL-10-dependent ways.[18] Collectively, these findings suggest that TGF-β secreted by B cells may not only partner with IL-10 but also possess a unique and, in some cases, an independent and dominant role in regulating the immune response.

This review examines the pathways that underlie B-cell production of TGF-β and how its function is both independent of and complementary to other Breg factors. We discuss how TGF-β has emerged as a key mediator in multiple Breg subsets with importance in cancer, allergy and autoimmune diseases. Finally, we examine the potential of TGF-β as an essential component of Breg-dependent transplant tolerance and how this cytokine might be utilised in concert with other immunomodulators to produce safer, more effective tools for clinical use.

TGF-β ACTIVATION, FUNCTION AND SIGNALLING PATHWAY

Transforming growth factor-β has three isoforms (TGF-β1, TGF-β2 and TGF-β3), with TGF-β1 as the prototypical TGF-β family member.[19] TGF-β is secreted in a latent form, non-covalently associated with a homodimer of the latency-associated peptide (LAP).[20] This LAP-TGF-β complex is either secreted or associated with another protein, LAP-TGF-β-binding protein (LTBP), to produce a larger latent form deposited onto the extracellular matrix.[21] TGF-β can only function once separated from LAP and TGF-β is released through interactions between LAP and integrins, although integrin-independent pathways have been described, including alterations in pH, ROS and proteases.[22] Once in its active form, TGF-β exerts its functions by binding to one of its cognate transmembrane signalling receptors, TGF-β type II receptor (TGFβRII), which then phosphorylates the accompanying TGF-β type I receptor (TGFβRI). TGFβRI will then activate S-mothers against decapentaplegic homolog (SMAD)-dependent pathways, which translocate to the nucleus and regulate the expression of several genes.[19] In addition, TGF-β can activate several SMAD-independent signalling pathways, including the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT). These pathways may also contribute to regulating various cellular functions depending on cellular and tissue contexts.[23]

B CELLS PRODUCE TGF-β IN MULTIPLE IMMUNOLOGICAL ENVIRONMENTS

Under physiological, healthy conditions, most B cells produce relatively low levels of both the precursor and active forms of TGF-β.[24,25] Thus, the production and activation of TGF-β by a subset of B cells with suppressive functions mark a significant departure from typical B-cell activity. Given the complexity of Breg activity found in both murine and human systems, it is not surprising that TGF-β regulation is also complex, dependent on multiple signalling pathways and impacted greatly by the local environment. While there is overlap among the various mediators of TGF-β, three broad categories are found in B cells: (1) classic signalling pathway stimulation (e.g. TLR, BCR and CD40/CD40L), (2) growth

factor stimulation and (3) tumor-induced signalling.

Classic signalling pathway stimulation

Established B-cell signalling pathways, such as TLR, BCR and CD40/CD40L signalling, which are known to stimulate IL-10 production, can also regulate the production and activation of TGF-β.[26–28] Mishima *et al*. explored the effect of TLR stimulation on murine B cells by examining the differential production of IL-10 and TGF-β1 when B cells are cultured with lipopolysaccharide (LPS, a TLR-4 agonist) or unmethylated bacterial DNA (CpG, a TLR-9 agonist). The results showed that B cells upregulate the expression of both IL-10 and TGF-β when stimulated by these TLR ligands, with some differences in the degree of TGF-β expression. Of note, the level of TGF-β production in CpG-stimulated B cells was significantly higher than in B cells stimulated with LPS.[29,30] Additionally, Parekh *et al*.[31] have demonstrated that concurrent *in vitro* stimulation through the BCR and CD40 pathways can upregulate TGF-β expression in murine splenic B cells; however, when compared to the LPS-activated B cells, the level of TGF-β in the LPS-activated B cells was significantly higher.

Beyond the impact on gene and protein expression, multiple lines of research have revealed important signalling pathways regulating the activation of TGF-β in B cells. Recently, a type I transmembrane docking receptor, glycoprotein A repetitions predominant (GARP), was identified as the key cellular membrane attachment of the LAP-TGF-β complex and as a mediator of TGF-β activation and availability.[32,33] Additionally, the overexpression of GARP on B lymphocytes was demonstrated to reduce B-cell proliferation, decrease T-cell-independent antibody production and increase class-switch recombination (CSR) to IgA.[34] Stimulation through various Toll-like receptors, including TLR4, TLR7/8 and TLR9, can drive surface GARP expression on Peyer's patch B cells. Using murine splenic B cells and human peripheral B cells, Wallace *et al*. have shown that *in vitro* stimulation of TLRs and the BCR results in upregulation of surface GARP and latent TGF-β. In these studies, stimulation of TLR4, TLR7/8 or TLR9 resulted in higher levels of surface GARP-TGF-β than in anti-IgM stimulation.[34] In earlier studies, Dedobbeleer *et al*. also demonstrated that these pathways can upregulate GARP. In addition to upregulation of surface GARP through TLR and BCR signalling, they demonstrated that CD40L stimulation can induce GARP at a low level on human B cells. In contrast to the results from Wallace *et al*., they found stimulation of the BCR to be more potent than either TLR signalling or CD40 stimulation in the induction of surface GARP. Dedobbeleer *et al*.[35] also demonstrated that signalling through TLR, BCR, CD40L and cytokines together results in even higher levels of surface GARP and TGF-β, than in stimulation through only one pathway. While these two studies demonstrate the potential of these pathways to regulate TGF-β, the differences with regard to the relative potency of TLR and BCR signalling highlight the complexity of TGF-β regulation and the need for further research to fully understand how best to control B-cell-derived TGF-β.

Thrombospondin 1 (TSP1) is another important factor for TGF-β signalling by converting the LAP-TGF-β to active TGF-β. Some studies report that activated B cells, expressing TSP1, can convert DCs to tolerogenic dendritic cells (TolDCs), which can secrete TGF-β.[36] TSP1-producing CD35$^+$ B cells generated after specific immunotherapy (SIT) can attenuate ongoing allergic reactions in the intestine through Treg induction in a TGF-β-dependent fashion.[37] *In vitro* studies using human B cells, stimulation by integrins αvβ6 plus anti-IgM antibody can upregulate TSP1.[38]

Growth factor stimulation

Published data indicate that multiple growth factors can induce immune Bregs and upregulate TGF-β. Under the right stimulatory conditions, glioma cells can produce placenta growth factor (PlGF), a key molecule in angiogenesis and vasculogenesis, which can induce differentiation of naïve B cells into TGF-β-producing Bregs. Therefore, *in vitro* co-culture of B cells with glioma-derived exosomes, anti-CD40 antibody and anti-IgM antibody could promote TGF-β$^+$ Breg development.[39] A study of patients with advanced stages of breast cancer showed that epidermal growth factor receptor 2 (HER2) is amplified as the proportion of circulating Tregs increases, correlating with high expression of TGF-β1 from CD19$^+$CD24^{hi}CD38^{hi} B cells.[40] This correlation suggests that TGF-β from B cells may contribute to Treg induction. Other growth factors, such as insulin-like growth factor-2, have been associated

with the generation of Bregs.[41] The direct impact of such growth factors on TGF-β expression and activation in B cells remains to be fully explored.

Tumor-induced signalling

Several studies suggest that B cells can be converted to TGF-β-producing B cells by exposure to various human cancer cell lines, including breast, ovarian and colon carcinomas. Importantly, the ability of cancer cells to generate Bregs may differ *in vivo* versus *in vitro*. *In vivo* examination of tumor-infiltrating B cells (TIL-B) isolated from tumor tissue from mice injected subcutaneously with EMT-6 tumor cells noted that approximately 30% of recovered TIL-B cells expressed TGF-β and 5% expressed IL-10. The majority (~ 75%) of the IL-10$^+$ TIL-B cells also expressed LAP/TGF-β. *In vitro*, B cells co-cultured with EMT-6 cells showed similarly increased levels of LAP/TGF-β1 to TIL-B. Of note, these Bregs induced by EMT-6 cells require contact between B cells and tumor cells.[42] *In vivo*, CD19$^+$ B cells isolated from mice bearing transplanted 4T1 mammary adenocarcinoma cells or infused with 4T1 cell-conditioned media were found to suppress T-cell proliferation. *In vitro* treatment of B cells with cultured media from the 4T1 cell line results in a specific type of tumor-evoked Bregs (tBregs) that express high levels of TGF-β. These tBregs were found to be functionally and phenotypically different from TLR- or BCR-activated suppressive B cells and other Bregs involved in autoimmune responses.[43] While the underlying signalling that produces tBregs is not fully understood, their existence highlights a treatment strategy to promote TGF-β distinct from the earlier mentioned signalling.

Additional signalling pathways involved TGF-β regulation

While the three categories of stimuli mentioned above constitute the best understood regulators of TGF-β in B cells, several other mechanisms have been shown to be important. These mechanisms include allergen stimulation, cytokines and products from microbes. Among allergens, the mixture of purified a-, b- and k-casein has been shown to stimulate PBMC from milk-tolerant individuals to produce TGF-β-producing CD19$^+$CD5$^+$ Bregs.[44] For cytokines, B-cell-activating factor (BAFF, TNF family) stimulation has been shown to stimulate B-cell production of TGF-β.[43] Also, the cytokine IL-1β combined with the TLR3 immunostimulant, poly(I:C), can induce surface expression of GARP on B cells.[34] Additionally, treatment with IL-4 in conjunction with CpG and CD40L enhances B-cell-dependent induction of Tregs through TGF-β.[45] One final promoter of B cell that has been studied in somewhat limited series is microbial factors, such as phorbol myristate acetate (PMA) from Penicillium and ionomycin from Streptomycetes, which facilitate TGF-β production from B cells.[21] Moreover, stimulation by the bacterium *Staphylococcus aureus* Cowan (SAC) complements CD40 and BCR signalling in B cells, resulting in suppression of T-cell stimulation through and IL-10 and PD-L1, and induction of Tregs by TGF-β.[46]

In sum, several stimuli can act alone or in combination to stimulate high expression levels and activation of TGF-β in B cells. Thus, optimising future production of TGF-β-producing Bregs requires further study and will likely utilise more than one of these various signalling pathways.

TGF-β-DEPENDENT REGULATORY B CELLS FUNCTION IN INFLAMMATORY DISEASES

Several studies have demonstrated that TGF-β-producing B cells can regulate the immune response in various inflammatory diseases,[47] including autoimmune diseases, cancer and allergic reactions (Table 1).[7] From these studies, it is apparent that multiple mechanisms exist whereby TGF-β-producing B cells suppress immune responses that are distinct from IL-10-producing B cells (summarised in Figure 1). This diversity in functional capacity is likely impacted by differences in inflammatory environments and specific stimuli, resulting in TGF-β$^+$ Breg generation.

TGF-β PRODUCING B CELLS IN AUTOIMMUNE DISEASE

Studies in experimental autoimmune disease models reveal that Bregs modulate the course of the disease via the production of suppressive cytokines, principally IL-10, but also through the generation of TGF-β. In fact, the first definitive evidence for the existence of Bregs was found in the observation that mice selectively lacking TGF-β in B cells (B–TGF-β1$^{-/-}$) developed an exacerbated form of EAE, compared to wild-type controls.[16]

Table 1. Different TGF-β-producing B cells regulate the immune response in various inflammatory diseases

Species	Study	Designation/phenotype	Mechanism	Mediator	Induction	Diseases	Refs
Mice	*In vitro/in vivo*	$CD5^+CD19^+CX3CR1^+$ Tol B	Th2↓; Tregs↑; suppress T-cell activation	TGF-β	αvβ6 + anti-IgM	Food allergy	38
	In vitro	LPS-stimulated B	$CD8^+$ T proliferation↓; $CD8^+$ T-cell anergy; IL-2, IFN-γ, TNF-α, IL-6, IL-13↓	TGF-β1/Fas-L	LPS; anti-Ig + anti-CD40	Healthy	31
	In vitro/in vivo	LAP^+GARP^+ B	GARP↑; B-cell proliferation and activation↓; ANA↓; IgM↓; IgG↓; IgA↑	TGF-β1, TGF-β2	Anti-u/anti-CD40L/LPSCPG, R488, LPS,TLR-3 + IL-1β + PolyI:C	SLE; oral-tolerance	34
	In vitro/in vivo	LPS-activated B	T and B apoptosis; Th1↓	TGF-β/Fas-L	LPS	T1DM	50
	In vitro/in vivo	CpG-pulsed B	$CD4^+$ Treg ↑; MDSCs ($CD11b^+Gr\text{-}1^+$) ↑; $CD8^+$ Tregs↑	IL-10/TGF-β	CPG	Lung carcinoma	55
	In vitro/in vivo	$CD19^+CD25^{hi}B7H1^{hi}CD81^{hi}$ $CD86^{hi}CCR6^{hi}CD62L^{lo}$ $IgM^{int/low}$	Tregs↑; T-cell proliferation↓; GrzB-expressing $CD8^+$ T cells ↓; $CD8^+$ INF-y^+↓	TGF-β	CM-4T1 PE	Breast cancer	43
	In vivo	$CD19^+CD44^+TGF\text{-}β1^+$	Th1/Th2/Th17 balance; Tregs↑	TGF-β	—	Allergic rhinitis	59
	In vivo/in vitro	TSP1-producing $CD35^+$	TSP1↑; Tregs↑; CD80/CD86 of DC↓; Th2↓	TGF-β	SIT/TSP1	Food allergy	60
	In vitro	$PD\text{-}L1^{hi}CD86^{hi}I\text{-}Ad^{hi}$ $CD62L^{hi}LAP^+CD44^{lo}$	$CD4^+$ T↓; $CD8^+$ T↓; NK↓; Th1↓	TGF-β/PD-L1	EMT-6	Mammary tumor	42
	In vivo/in vitro	$CD19^+B220^+CD21^{int}IgD^{lo}IgM^{int}$ $CD1d^{hi}CD5^+$	Tregs↑; CXCR4 and CXCR5↑	TGF-β	LIT	Asthma	61
	In vivo	$TIM\text{-}1^+LAP^+$	Tregs↑; CCR6; CXCR3↑;	TGF-β	Anti-CD45RB + anti-Tim-1	Islet Tx	67
	In vivo/in vitro	$Tim\text{-}1^+$Transitional 2 B	Tregs↑; TNF-α↓; CD86/CD80↓$CD4^+$ T-cell activation↓	TGF-β	DST + MRI	Skin Tx	70
	In vivo/in vitro	$CD19^+IgM^{lo}LAP^+CD4^{hi}$ $CD21^+CD23^-$	$CD4^+$ T-cell proliferation↓	TGF-β	Anti-CD45RB	Skin Tx	71
Human	*In vitro*	$CD19^+CD5^+$	B-cell apoptosis	TGF-β	Casein	Allergy	44
	In vitro	$TGF\text{-}β^+$ B	IL-10↑; TGF-β↑; CXCR4↑; CXCL12↑; $CD40^+HLA^-DR^+ICAM\text{-}1^+$ B↓	IL-10/TGF-β	Fingolimod	MS	51,52
	In vitro	$IL\text{-}10^+CD86^+CD25^+$	$CD4^+$ T-cell proliferation↓; $CD4^+$ INF-y^+↓; IL-4↓ IL-10↑; TGF-β↑;	IL-10/TGF-β	Laquinimod	RRMS	53
	In vitro	$CD19^+CD24^{hi}CD38^{hi}$	Tregs↑; TGF-β1↑	TGF-β1	HER2	Breast cancer	40
	In vitro	$CD19^+CD24^{hi}CD38^{hi}$	TBX21 ↓; RORC2 ↓; Tregs ↑	TGF-β1/IL-10/PD-L1	CD40L + anti-Ig + SAC	End plate inflammation/ healthy	46

(Continued)

Table 1. Continued.

Species	Study	Designation/phenotype	Mechanism	Mediator	Induction	Diseases	Refs
	In vitro	$CD19^{+}CD24^{hi}CD38^{hi}$	Tregs↑; TGF-β1↑; $CD4^{+}IFN\text{-}\gamma^{+}$ ↓; $CD4^{+}TNF\text{-}\alpha^{+}$ ↓	TGF-β1/IL-10	—	Gastric cancer	40,54
	In vitro	$CD25^{hi}CD27^{hi}CD86^{hi}$ $CD1d^{hi}IL\text{-}10^{hi}$ $TGF\text{-}\beta^{hi}$	CD4 T-cell proliferation↓; Foxp3↑; CTLA↑	TGF-β	CPG + CD40L + IL-4	Healthy	45
	In vitro	Transitional 2 B	TGF-β-producing B cells↑	—	—	Renal Tx	75
	In vitro	CPG-activated B	Natural Tregs ($CD4^{+}Foxp3^{+}$) ↑; type 1 Tregs (IL-10) ↑; Th3 Tregs (TGF-β) ↑;$CD4^{+}IFN\text{-}\gamma^{+}$ ↓; $CD4^{+}TNF\text{-}\alpha^{+}$ ↓	TGF-β/IDO	CPG	Healthy	18
	In vitro	$CD19^{+}TGF\text{-}\beta^{+}$	PIGF↑; $CD8^{+}$ T proliferation↓; perforin and granzyme B↓	TGF-β	Glioma-derived exosomes + anti-CD40 + anti-IgM	Glioma	39

↑ increase; ↓ decrease.

Also noteworthy is the observation of reduced levels of $CD19^{+}Foxp3^{+}$ and $CD19^{+}TGF\beta^{+}$ Bregs in blood PBMC of patients with a severe, accelerated form of rheumatoid arthritis (RA) associated with the development of interstitial lung disease (ILD). This observation suggests that TGF-β^{+} Bregs may contribute to the prevention of RA and ILD in a manner discrete from IL-10^{+} Bregs.[48] TGF-β-producing B cells were demonstrated that primarily exert their anti-inflammatory effects early in the course of EAE through TGF-β-dependent signaling and that Bregs downregulate the surface expression levels of MHC class II and CD86 molecules of DCs.[49] This may impede the initiation of a replete T-cell response, thus abrogating the encephalitogenic Th1/17 responses and pro-inflammatory cytokines, such as GM-CSF and IFN-γ, suggesting TGF-β may be relevant for B-cell-targeted therapies.[49]

TGF-β-producing B cells can regulate autoimmune responses in both mice and humans. In mice, adoptive transfer of LPS-activated splenic B cells from NOD mice into prediabetic NOD mice inhibited spontaneous autoimmunity. Further experiments in mice suggest that these activated B cells can trigger apoptosis of T and B cells through TGF-β expression and/or by secretion of Fas ligand (Fas-L), preventing immune-mediated tissue destruction.[50] Moreover, TGF-β secreted from LPS-activated B cells can induce $CD8^{+}$ T-cell anergy.[31] In patients with relapsing multiple sclerosis (MS), treatment with the immunomodulatory drug, fingolimod, increases TGF-β expression on Bregs and induces an exhausted-like phenotype in T cells, characterised by the reduction in IL-17 and IFN-γ expression, elevation of IL-10 and TGF-β, and expression of exhaustion markers such as PD-1 and Tim-3.[51,52] Similarly, the MS treatment, laquinimod, is capable of modulating B-cell surface markers and increasing IL-10 and TGF-β in both B and T cells, in a B-cell-mediated manner.[53]

Collectively, these data from multiple experimental settings substantiate a role for TGF-β-producing Bregs in preventing autoimmune disease. Furthermore, the action of some clinically used therapies may involve increasing TGF-β expression from B cells to regulate autoimmunity. Based on *in vitro* experiments showing increased TGF-β expression in B cells under various stimulatory conditions, future efforts may capitalise on this function of B cells to design novel therapies for autoimmune diseases.

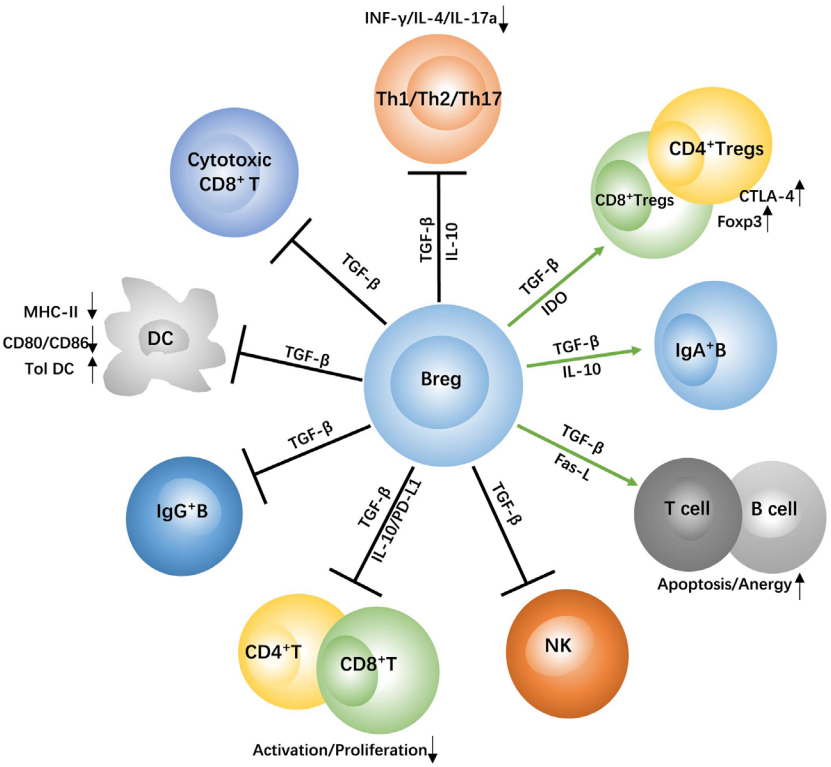

Figure 1. Mechanisms of TGF-β+ Bregs in regulation of the immune response. Transforming growth factor β (TGF-β) produced by B cells has been demonstrated to suppress the differentiation and function of Th1, Th2, Th17 and cytotoxic $CD8^+$ T cells. TGF-β+ Bregs suppress $CD4^+$ T and $CD8^+$ T-cell activation and proliferation through TGF-β, IL-10 and PD-L1. B-cell-specific TGF-β can induce tolerance and/or inhibit DC. TGF-β alone, or with IDO, can enhance the Foxp3 and CTLA-4 expression in $CD4^+$ Tregs, as well as the induction of $CD8^+$ Tregs. Furthermore, TGF-β and/or Fas-L secreted by TGF-β Bregs can induce T- and B-cell apoptosis, and TGF-β has been shown to induce T-cell anergy. Finally, TGF-β produced by Bregs has the ability to inhibit B cells switch to IgG isotypes and accelerate B-cell differentiation into IgA^+ B cells.

TGF-β-PRODUCING B CELLS IN CANCER

It is widely understood that cancer immunosurveillance and immunoediting are complex processes. Numerous regulatory components have been identified to limit an effective antitumor immune response. Regulatory effects of B cells and TGF-β produced by them have been implicated in various neoplastic settings. In B-cell-deficient mice, the growth rate of certain tumors is lower than in wild-type mice, and tumor cell growth increases markedly after the adoptive transfer of B cells, suggesting that the transferred B cells negatively modulate the anticancer immune response underway. This phenomenon has been demonstrated in cancer models using EL-4 thymoma, MC38 colon carcinoma, EMT-6 breast carcinoma and D5 mouse melanoma.[42] These studies also found convincing evidence that Bregs aid in mediating tumor escape.[43] However, the exact mechanism of Breg-mediated immune escape remains elusive, and how different subsets of Bregs, including TGF-β+ Bregs, contribute to protecting cancer cells is yet to be determined.

Multiple studies have demonstrated that TGF-β+ Bregs can be induced *in vitro* and *in vivo* directly by tumor cells or by various pro-tumorigenic growth stimuli, depending on the specific tumor environment. These Bregs express a high level of TGF-β and regulate the tumor immune response through the induction of Tregs.[43] In both breast and gastric cancer, an increase in $CD19^+CD24^{hi}CD38^{hi}$ Bregs correlates with higher levels of $CD4^+FOXP3^+$ Tregs. TGF-β produced by these Bregs plays a significant immunosuppressive role in these cancer settings by inhibiting $CD4^+$ effector T-cell cytokine production and converting $CD4^+CD25^-$ T cells to $CD4^+FOXP3^+$ Tregs, in a TGF-β-dependent fashion.[54] TLR9-activated splenic B cells promote lung tumor growth by producing an increased immune-suppressive environment

because of enhanced recruitment of Tregs, MDSCs and CD8+ Tregs cells together with higher levels of suppressive cytokines such as IL-10 and TGF-β.[55] As mentioned earlier, incubation with 4T1 cancer cell-conditioned media for 2 days induces tBregs *in vitro*. These tBregs can mediate TGF-β-dependent conversion of non-Treg CD4+ T cells into metastasis-promoting FOXP3+ Tregs, which subsequently inactivate antitumor NK cells and effector CD8+ T cells, thereby protecting metastasising cancer cells.[43] Some human cancer cell lines induce the generation of TGF-β+ Bregs and Tregs, which collaborate to increase the tumor's potential to escape immunosurveillance and metastasise. Tregs, induced with B-cell-derived TGF-β, is an attractive therapeutic for future clinical application. For example, the natural-occurring phenol, resveratrol, has been shown, at high doses, to suppress cancer progression in mice via direct induction of apoptosis of malignant cells and indirect blockade of the generation of tumor escape-promoting FOXP3+ Tregs.[56] Interestingly, Bodegai *et al*. found that *in vivo* treatment with CpG delivered via a modified form of CXCL13 could, through inactivating Bregs and bolstering an antitumor T-cell response, block lung metastasis.[57]

These findings collectively demonstrate that TGF-β+ Bregs are present in various tumor environments and can play a role in the growth and metastasis of the tumor. In many of these models, the induced Bregs increase the level of Tregs in a TGF-β-dependent manner, which assists in tumor escape from immunity; however, there are many other mechanisms that could also be B-cell-mediated. Understanding the role of TGF-B and Bregs in tumorigenesis and tumor immune escape will be of great importance for the rapidly advancing field of anticancer immunotherapeutics.

TGF-β-PRODUCING B CELLS IN ALLERGY

A key component of an allergy-prone immune environment is the failure of antigen-specific immune tolerance, this has been linked in some studies to TGF-β+ Bregs-induced regulation. Lee *et al*. studied human eczematous allergic reactions to cow's milk and found that the proportion of TGF-β-producing CD19+CD5+ B cells increased and proliferated in the milk-tolerant group but not in the milk-allergic group.[44] The disruption of TGF-β receptor signalling predisposes patients to develop allergic pathology, including asthma, food allergy, eczema and allergic rhinitis.[58] Therefore, TGF-β-producing Bregs may factor in the generation of antigen-specific immune tolerance.

Some studies have demonstrated that TGF-β+ Bregs can regulate the allergic immune response through various mechanisms such as altering the balance of T-cell subtypes, inducing Treg development and enhancing apoptosis of effector inflammatory cells. In an OVA-based allergic airway inflammation model, the resulting allergic response manifested an increase in Th17 cells and skewed the balance of Th1/Th2 cells towards Th2. Interestingly, when monitored over time, the proportion of TGF-β+ Bregs and Tregs increased, and the expression of Th2-associated cytokines was inhibited. Meanwhile, the ratio of Th1/Th2 and the functioning of Th17 returned to normal.[59] TSP1-producing CD35+ B cells, which increased after immunotherapy, can decrease the levels of CD80/CD86 on dendritic cells, convert naive CD4+ T cells to Tregs and suppress the Th2 response.[60] TGF-β+ Bregs, isolated *in vivo* or produced from *in vitro* stimulation, can suppress the allergic reaction after adoptive transfer to recipients known to be allergic to specific stimuli. B cells isolated from hilar lymph nodes (HLNs) in mice with local inhalational tolerance (LIT) contained fivefold more TGF-β+ cells than IL-10+ cells and could convert naive CD4+CD25− T cells into functionally suppressive CD4+CD25+FOXP3+ Tregs. After adoptive transfer of the LIT HLN CD5+ B cells into OVA-sensitised recipients, TGF-β-expressing CD5+ B cells and Tregs co-localised in B-cell zones, and the chemokines, CXCR4 and CXCR5, were upregulated. In the setting of this local immune-suppressed microenvironment, airway eosinophilia was inhibited.[61] Another study on using a TGF-β-producing suppressive B cells, termed TolBCs (CD5+CD19+CX3CR1+ B), found that these cells can convert Th0 cells to CD4+CD25+FOXP3+ Tregs through TGF-β-dependent but not IL-10-dependent signalling. Adoptive transfer of TolBCs markedly suppressed the food allergy-induced intestinal Th2 inflammation pattern in mice.[38]

These studies solidly establish the participation of TGF-β+ Bregs in the regulation of allergic immunity and that this may be mediated through diverse pathways. Future TGF-β+ Breg-based cell

therapies could be a promising treatment for allergic inflammation.

THE PROSPECT OF TGF-β-PRODUCING B CELLS IN TRANSPLANTATION TOLERANCE

In transplantation, B cells impact the response to a foreign graft in diverse ways, including a role in both the immune system's attack on the graft and, paradoxically, the promotion of tolerance. The former action is tied to anti-allograft antibody generation and B-cell-mediated presentation of allograft antigens, resulting in allo-aggressive responses to organ transplants. The catastrophic impact of anti-allograft antibodies has been well-studied. In contrast, the graft-protective functions of B cells are much less understood and linked to the suppressive effects of a Breg subset that can constrain an immune response and prevent rejection. Bregs may be a novel and untapped candidate as an immunotherapeutic to curtail allo-aggressive T- and B-cell-mediated alloimmunity.[31] As noted above for autoimmunity, cancer and allergy, the regulatory mechanism of Bregs is most often ascribed to IL-10. However, secreted TGF-β has the powerful ability to convert effector T cells to Tregs, which are an attractive weapon in the fight for long-lasting immune tolerance.[62] The mechanisms of Breg-mediated TGF-β in multiple immune-privileged environments provide significant insight into the underpinnings of the role Bregs play in graft acceptance and transplantation tolerance induction.[63]

A murine model of B-cell-dependent transplantation tolerance was first reported in 2007.[64] This first demonstration utilised tolerance induction through *in vivo* treatment of wild-type and B-cell-deficient mice with anti-CD45RB.[65] Mice lacking B cells failed to produce durable tolerance to islet allograft transplants, establishing B cells as a requisite mediator of tolerance in the model. The contribution of IL-10 was explored using the model of anti-CD45RB-induced allograft tolerance, through the administration of anti-IL-10 antibodies or by repopulating B-cell-deficient mice with B cells from IL-$10^{-/-}$ mice. Surprisingly, IL-10 neutralisation or deficiency failed to prevent tolerance and even accelerated rejection in some circumstances.[66] In contrast, antibody neutralisation of TGF-β consistently prevented tolerance development. In additional studies, TGF-$\beta^{-/-}$ B cells were evaluated for their ability to promote tolerance in the same model. Adoptive transfer of B cells from B-cell-specific TGF-$\beta^{-/-}$ mice were unable to support tolerance development, whereas their littermate controls, TGF-$\beta^{+/-}$ heterozygotes, did so, providing strong evidence that TGF-β specifically from B cells is integral in the formation of allograft tolerance.

Parallel studies using a modified tolerance protocol consisting of anti-CD45RB and anti-TIM-1 antibody treatment ('dual antibody treatment') result in 100% long-term islet allograft survival that is dependent on the production of IL-10 by Tim-1^+ Bregs.[67,68] Interestingly, these dual antibody-treated islet transplant animals co-express a significantly higher percentage of TIM-1^+LAP$^+$ B cells versus no treatment, and these B cells can convert CD4$^+$CD25$^-$ T cells to CD4$^+$CD25$^+$FOXP3$^+$ Tregs. As expected by this finding of LAP$^+$ Bregs, tolerance induction using this protocol relies on TGF-β, in addition to the aforementioned IL-10. Tolerance via dual antibody treatment was abrogated when TGF-β activity was neutralised by anti-TGF-β antibody treatment. Tolerance through the adoptive transfer of B cells isolated from long-term survival mice was also abrogated by treatment with anti-TGF-β antibody, resulting promptly in rejection of allo-islets. These data demonstrate that the dual antibody treatment can induce Bregs that rely on the expression of both IL-10 and TGF-β to promote islet transplantation tolerance.[69]

Similar to those experiments demonstrating TGF-β-dependent tolerance, transitional-2 (T2) splenic B cells from mice tolerant to MHC class I-mismatched skin grafts express high levels of Tim-1 and are capable of prolonging skin allograft survival and suppressing T-cell activation. The mechanism of tolerised T2 B cells may rely on direct infiltration of the graft and upregulated expression of TGF-β to alter Treg/T effector ratios.[70] Recently, B cells isolated from OVA-specific B-cell receptor (OB1) mice, which underwent OVA skin graft and anti-CD45RB treatment to induce tolerance, were found to express almost 10 times higher LAP than IL-10. Furthermore, these tolerant mice failed to develop long-term graft survival after receiving the anti-TGF-β antibody. Adoptive transfer of the antigen-specific tolerant OB1 B cells is more potent than wild-type B cells at conferring tolerance to recipients undergoing OVA$^+$ skin grafting. These experiments in mice demonstrate

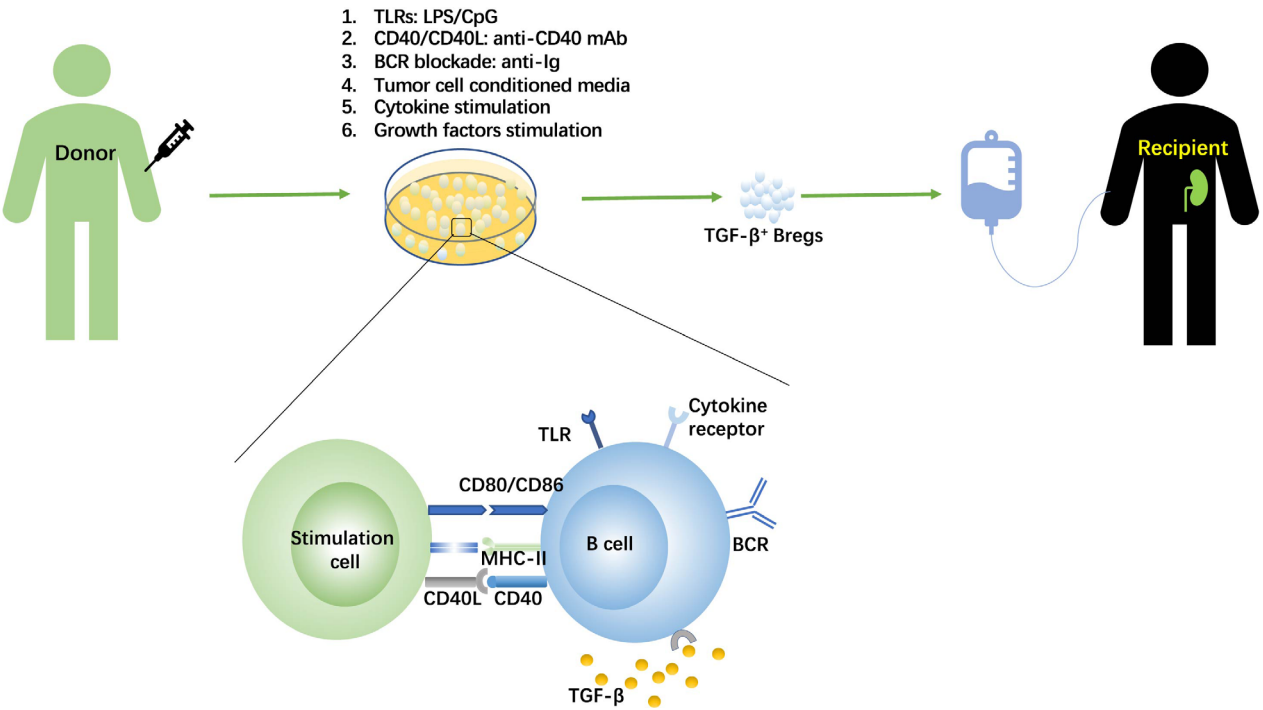

Figure 2. The future application of TGF-β+ Bregs in transplantation tolerance. B cells isolated from donor blood. Purified B cells are then stimulated *in vitro* to secrete transforming growth factor β (TGF-β) through different B-cell signalling pathways, such as BCR, TLR and CD40. Multiple different signalling pathways may function to produce these therapeutic cells: (1) TLR-stimulating agents such as CpG and LPS; (2) anti-CD40 antibody stimulation; (3) anti-Ig to stimulate BCR; (4) co-culture with tumor cell conditioned media such as EMT-6 and 4T1 cell; (5) cytokine stimulation with IL-4, BAFF, IL-1β and so on; (6) growth factors including PlGF. Future therapeutic production strategies may use these pathways alone or in combination to optimally produce TGF-β+ Bregs, which can be adoptive transfer to an organ transplant recipient, to promote durable tolerance.

that Breg-dependent tolerance relies on the function of TGF-β and that this process is, at least in some circumstances, antigen-specific.[71]

In NHP studies and human clinical transplants, there is mounting evidence that Bregs play a key role in transplant outcome.[72] In an interesting clinical trial for kidney transplants using B-cell depletion as the only induction therapy, there appeared to be an increased incidence of early rejection, possibly because of the elimination of the Breg subset[73]. Also, a study of spontaneously tolerant recipients of kidney grafts identified a molecular B-cell signature associated with renal transplant tolerance through comparison of three groups: (1) tolerant patients who had stable graft function at least 1 year off immunosuppression, (2) patients with stable graft function while on immunosuppression and (3) healthy (non-transplanted) control subjects. Unexpectedly, these studies discovered that T1 and T2 transitional B-cell populations were found to express high levels of IL-10 in tolerant patients, but failed to show differences in the number of TGF-β-expressing B cells.[74] However, others have reported that *in vitro* stimulation of B cells, isolated from the peripheral blood of tolerant patients, possess increased expression of TGF-β, but no significant differences in IL-10 production.[75] These observations foster speculation that renal transplant tolerance may be associated with alterations in both T-cell- and B-cell-mediated functions. Whether IL-10 or TGF-β produced by B cells is required for tolerance in these human patients remains an open question.

Collectively, the data from mouse and human investigations indicate both B-cell-derived IL-10 and TGF-β contribute to the regulation of cellular immunity in the transplant setting. Based on established models of autoimmune disease, cancer and allergy, IL-10 and TGF-β can play different and complementary roles in regulation. The relative expression and importance of these two tolerogenic mediators may be dictated by the environment that provokes their expression in B

cells. For example, in the tumor environment, high levels of Tregs are needed for tumor cell growth, migration and immune escape. TGF-β produced by Bregs has the potential to convert the $CD4^+CD25^-$ effector T cells to $CD4^+CD25^+FOXP3^+$ Tregs and, to some extent, can induce $CD8^+$ Tregs and tolerogenic DCs and macrophages, whereas IL-10 can suppress the generation of pro-inflammatory cytokines by T cells. Therefore, TGF-β from Bregs may be readily produced in the tumor environment through different mechanisms compared to those that promote IL-10 production.

Induction of donor-specific Tregs *in vivo* in transplant recipients is a theoretically attractive approach to limit acute and chronic allograft rejection and to avoid the toxicities of prolonged immunosuppressive therapy.[63] Thus, the TGF-β^+ Bregs may possess attributes directly relevant to developing innovative strategies for inducing transplant tolerance. Theoretically, to produce Breg-based cell therapies for clinical use (Figure 2), B cells isolated from donor PBMCs would be stimulated by one or more signalling pathways to generate TGF-β^+ Bregs. Next, these Bregs would be adoptively transferred to a recipient of the donor's organ to promote durable tolerance. As mentioned earlier, the exact combination of stimuli to optimally produce TGF-β^+ Bregs requires further research, but likely include factors that have been previously identified in various Breg environments, such as CpG, PMA, ionomycin, glioma-derived exosomes, anti-CD40, anti-IgM and IL-4. To some extent, the potency of TGF-β^+ Bregs stimulated by a combination of stimuli may be stronger than if stimulated through a single signalling pathway, which would be expected based on the work from Dedobbeleer *et al*.[35] Currently, our team at MGH is investigating the tolerogenic potential of B-cell activation by combination treatment with CpG, LPS, PMA and ionomycin. This protocol is a potent inducer of TGF-β^+ B cells from naïve B cells *in vitro*. Adoptive transfer of B cells stimulated in this manner to murine transplant recipients results in long-term survival in a majority of recipients, in both islet and skin graft models. The results to date show promise in induced TGF-β^+ B cells to suppress the T-cell immune response and prolong allograft survival.

Some key issues have to be addressed in order to establish TGF-β^+ Bregs as a cell-based treatment for clinical transplantation tolerance induction, including (1) safe stimulation that avoids pro-inflammatory B-cell development, (2) understanding long-term *in vivo* survival and stability and (3) appropriate dosing depending on recipient and transplant factors. (1) The stimulation strategy to generate TGF-β^+ Bregs *in vitro* must ensure that a safe product is produced. Multiple stimulation strategies can activate B cells to produce TGF-β in *vitro*, but different combinations of stimuli result in the different functions of TGF-β^+ Bregs in various inflammatory responses and sometimes may lead to the opposite function. For example, after stimulation with BCR and anti-CD40 antibody, B cells showed markedly higher IgG expression than LPS-activated B cells.[31] When B cells are stimulated with CpG and BCR, the result showed low levels of TGF-β1 and high expression of IL-6 and TNF.[11] These data suggest that some stimuli may promote excessive immune responses and favor the pro-inflammatory cytokine secretion from activated B cells. These different findings may be attributed to distinct stimulatory conditions of the B cells. For instance, human B cells activated by T-cell-dependent stimuli such as CD40/CD40L and BCR can confer strong $CD8^+$ T-cell stimulation and response, while stimulation by T-independent pathways such as LPS may induce Breg-mediated anergy of $CD8^+$ T cells, the results owing to distinct levels of surface TGF-β1 produced by B cells.[31] The function of TGF-β from B cells is dependent not just on expression, but also on conversion from its latent to active form. The activation depends on the immunology circumstances and the target cell type. Therefore, identifying and understanding the regulatory function of Bregs from different stimuli are essential to their effective and safe application. (2) The stability of TGF-β^+ Bregs *in vivo* is unclear, and the impact on long-term graft survival is not completely determined. Theoretically, if transferred Bregs could revert from their regulatory function and develop anti-allograft or anti-host properties, the outcome for the patient could be catastrophic. Thus, the plasticity and overall functional stability of Bregs *in vivo* require further investigation. (3) The optimal number of Bregs to be delivered will need to be ascertained to ensure both safety and efficacy of the therapy. Furthermore, the migration of Bregs to the target transplantation site may impact the number of

cells needed to achieve tolerance. How best to target the Bregs will be an important future direction to achieve a functional treatment. To address these important concerns for developing clinically valuable Breg-mediated transplant tolerance, it will be vital to utilise murine models, large animal transplant models and careful clinical observation.

CONCLUSION

B cells exhibit an array of powerful immunological functions including antigen presentation, antibody secretion and pro-inflammatory cytokine production. The nature and scope of B cells as regulators of immune responses in transplant are just now coming into focus. Bregs have demonstrated T regulatory functions through secretion of cytokines including IL-10, TGF-β and IL-35, as well as other immunomodulatory molecules such as granzyme B and the expression of negative co-stimulatory molecules such as PD-L1. IL-10 production is the most commonly studied and best understood suppressive phenotype, yet the role for TGF-β^+ B cells in the regulatory network continues to grow and to be better explicated. Its role is now widely accepted in cancer immunity, allergy, autoimmune disease and other inflammatory diseases. The mechanisms by which TGF-β-producing B cells regulate immunity in the setting of various inflammatory diseases should be thoroughly considered for relevant application to the field of transplantation. A robust, mechanistic understanding of Breg-mediated immune regulation will enable further work to establish TGF-β^+ Bregs as effective mediators of transplantation tolerance. Consequently, work towards the therapeutic administration of TGF-β^+ Bregs may progress quickly given the straightforward manufacturing protocols used to produce large numbers of these cells *in vitro* alone or combined with Tregs. Bregs have enticing therapeutic potential for application in future transplant tolerance-inducing regimens.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Guoli Huai: Data curation; Investigation; Project administration; Writing-original draft; Writing-review & editing. **James F Markmann:** Funding acquisition; Project administration; Supervision; Validation. **Shaoping Deng:** Funding acquisition; Validation. **Charles Gerard Rickert:** Validation; Writing-review & editing.

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