

REVIEW

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# Regulatory lymphocytes: the dice that resolve the tumor endgame

Subhadip Pati, Anandi Chowdhury, Sumon Mukherjee, Aharna Guin, Shravanti Mukherjee and Gaurisankar Sa\* 

## Abstract

A large number of cancer patients relapse after chemotherapeutic treatment. The immune system is capable of identifying and destroying cancer cells, so recent studies have highlighted the growing importance of using combinatorial chemotherapy and immunotherapy. However, many patients have innate or acquired resistance to immunotherapies. Long-term follow-up in a pooled meta-analysis exhibited long-term survival in approximately 20% of patients treated with immune checkpoint inhibitors or the adoptive transfer of chimeric T cells. It has been reported that high levels of immunoregulatory cells in cancer patients contribute to immunotherapy resistance via immunosuppression. Among the most important regulatory cell subtypes are the CD4<sup>+</sup> T-regulatory cells (Tregs), identified by their expression of the well-characterized, lineage-specific transcription factor FOXP3. In addition to CD4<sup>+</sup> Tregs, other regulatory cells present in the tumor microenvironment, namely CD8<sup>+</sup> Tregs and IL10-producing B-regulatory cells (Bregs) that also modulate the immune response in solid and lymphoid tumors. These cells together have detrimental effects on tumor immune surveillance and anti-tumor immunity. Therefore, targeting these regulatory lymphocytes will be crucial in improving treatment outcomes for immunotherapy.

**Keywords:** Breg, CTLA4, FOXP3, Immunotherapy, IL10, PDL1, Treg

## Background

The suppression of anti-tumor immune responses and augmentation of tumor-promoting immune responses both contribute to tumor progression [1]. The pioneering work of Sakaguchi et al. revealed the existence of T-regulatory cells (Tregs) that facilitate the development and maintenance of an immunosuppressive environment in the host [2]. The presence of these Tregs in the tumor microenvironment consistently maintains 'pro-tumor' conditions essential for the advancement of cancer [3]. Most conventional cancer immunotherapies involve the blockade of immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein-4 (CTLA4) and programmed death-1 and provide remarkable clinical efficacy across several cancers [4]. The clinical efficacy of immune checkpoint blockade therapy is visible in patients with tumors harbouring somatic mutation-derived

neo-antigens, which are not present in non-cancerous cells [5]. These neo-antigens offer a clinical advantage since the tumor cells expressing neo-antigens are then recognized as 'non-self' by the immune system, which then specifically targets and eliminates them while sparing the non-cancerous cells [6]. It is predicted that reducing the suppressive activity of Tregs has broad potential for cancer immunotherapy in patients with a low number of neo-antigens [7]. Most of the research in this field has concentrated on the immune-suppressive activity of CD4<sup>+</sup> Tregs [3]. However, unique combinations of cytokines and chemokines in the tumor microenvironment can influence the development of new and uncharacterized populations of immune cells [8].

FOXP3-expressing CD8<sup>+</sup> Tregs have also started to gain importance in this regard. Data suggest that the presence of these cells might be a marker of positive clinical outcome for cancer patients [9]. These CD8<sup>+</sup> Tregs also have immunosuppressive activity, like their CD4<sup>+</sup> counterparts, through various mechanisms including cell to cell contact, secretion of cytokines (such as

\* Correspondence: [gauri@jcbosc.ac.in](mailto:gauri@jcbosc.ac.in)

Division of Molecular Medicine, Bose Institute, P1/12, C.I.T Scheme VII M, Kolkata 700054, India



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TGF $\beta$ , IL16, IL10, CCL4), and initiating tolerogenic phenotype in antigen-presenting cells [10, 11].

Recent research in B-cell biology has found a novel IL10-producing subset of B cells that modulates tumor immune responses. This population has been designated as B-regulatory cells (Bregs), which play a significant role in the harmonization of immune responses involved in autoimmunity, inflammation, and cancer [12]. Bregs generally suppress other cells, including T cells through secretion of the anti-inflammatory cytokine IL10, and they can also facilitate the conversion of T-effector cells into Tregs, thus attenuating anti-tumor immune responses. Bregs have also been found to be recruited to the tumor microenvironment to acquire immunosuppressive properties and thereby mitigate anti-tumor immune responses [13]. This review emphasizes regulatory cell-mediated immunosuppressive mechanisms in cancer and the possibility for Treg-/Breg-targeted cancer immunotherapy, leading to a promising clinical efficacy as demonstrated in several preclinical studies as well as in early-phase clinical studies.

#### CD4<sup>+</sup> Tregs

The most common and well-known regulatory lymphocyte is the CD4<sup>+</sup> Treg [14]. There are two forms of CD4<sup>+</sup> Tregs, both expressing the transcription factor fork-head box-p3 (FOXP3): naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> Tregs (nTregs) in the thymus constitutively express FOXP3 while induced Tregs (iTregs) in the periphery are induced to express FOXP3 [14]. iTregs can be further divided into two subtypes depending on the expression of immunosuppressive molecules, namely the IL10-producing Treg type-1 (Tr1) cells and the TGF $\beta$ -producing Th3 cells [15]. nTregs actively participate in the maintenance of immunological self-tolerance and immune homeostasis [16]. Both nTregs and iTregs are categorized by increased levels of CD25, FOXP3, CTLA4, and glucocorticoid-induced tumor necrosis factor-related receptor (GITR). But unlike nTregs, iTregs show lower expression of CD73 and programmed cell death protein-1 (PD), and higher expression of the transcription factor Helios and the surface antigen neuropilin-1 (Nrp1) [17].

Expression of the immunoglobulin-like transmembrane protein lymphocyte-activation gene 3 together with ATP-degrading enzymes CD39 and CD73 on the surface of Tregs might help to enhance the suppressive activity of Tregs (Fig. 1). These ATP-degrading enzymes control the pro-inflammatory state of the tumor microenvironment by a negative-feedback mechanism [18, 19]. Lymphocyte-activation gene 3 expression leads to reduced calcium signaling and thus reduced cytokine production, preventing immune responses [18], whereas enhanced ATP generation in the pathological state

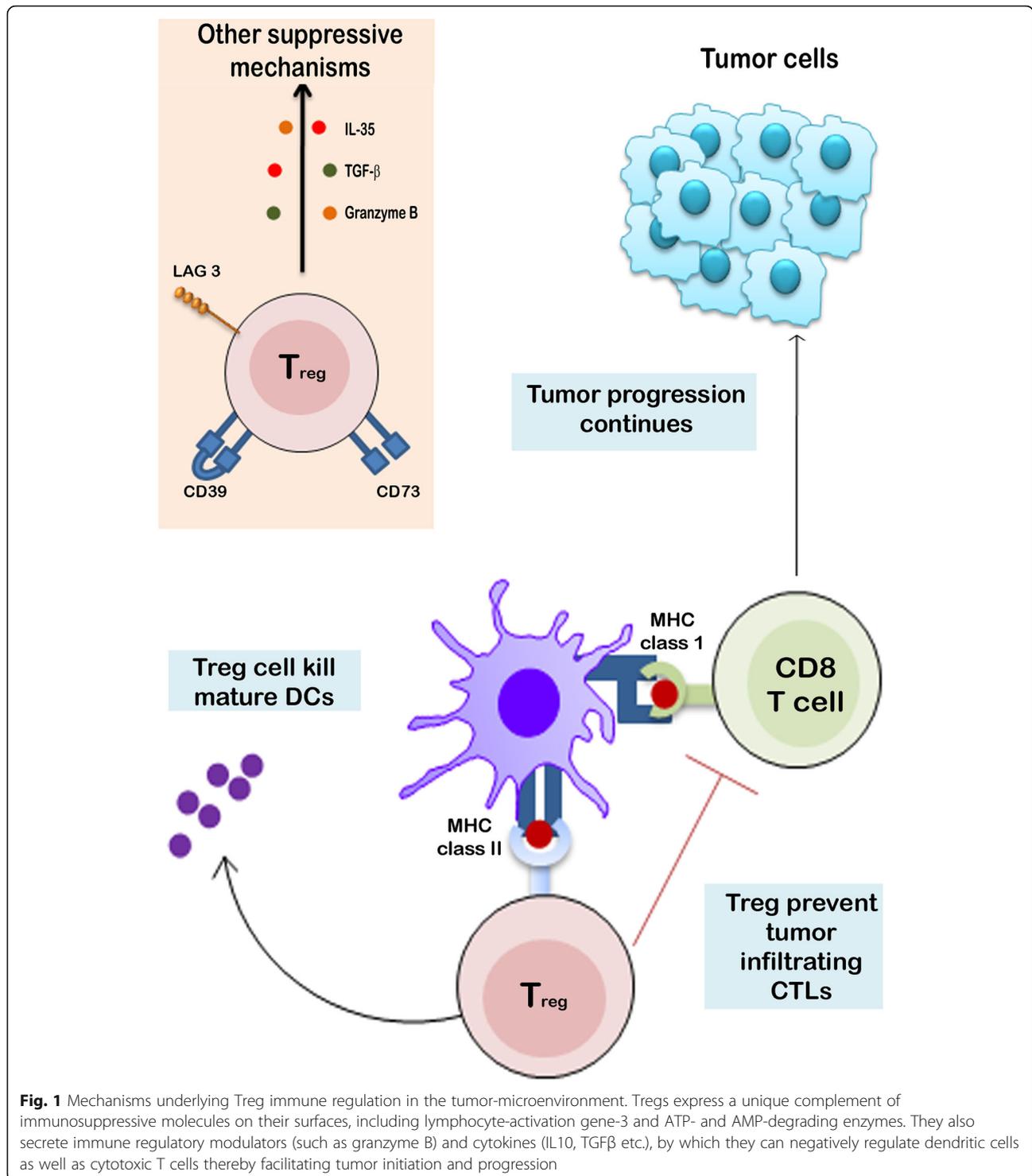
results in high levels of AMP and adenosine and an anti-inflammatory state [19]. Granzyme B-producing tumor-induced Tregs are among the major players underlying the suppression of tumor clearance by blocking natural killer and CD8<sup>+</sup> T cell activity [20].

In most human cancers, IL10 secreted from Tregs in the tumor microenvironment is controlled by the transcription factor FOXP3 in association with signal transducer and activator of transcription-3 [21]. These immunosuppressive cells have been associated with poor cancer prognosis [22]. Besides IL10, Tregs are also known to secrete TGF $\beta$ , which inhibits effector T cells and creates an immunosuppressive environment [14]. There is ample evidence on the impact of Treg depletion on human tumor progression. One study demonstrated that patients with hepatocellular carcinoma contain a higher number of suppressive CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs, and depleting this population by blocking surface receptors like CTLA4, CD25, or various chemokine receptors could enhance anti-tumor immunity [23, 24].

Interestingly, some chemotherapeutic agents (including aromatase inhibitors, cyclophosphamide, fludarabine, gemcitabine, mitoxantrone, anti-GITR, or anti-OX40 antibodies) also reduce Tregs in addition to their well-known tumor-regressing effects [25, 26]. Another study identified mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK)-signaling as a potential target for reversing Treg augmentation in breast cancer patients [27]. Moreover, many reports have shown that blockade of chemokine signaling can reduce the migration of Tregs to the tumor site; hence, targeting chemokine ligands with antibodies could significantly deplete Tregs and produce an antitumor response [28]. The anti-receptor activator of nuclear factor-kappa B ligand (RANKL) antibody denosumab may be useful in inhibiting Treg-mediated metastasis of various tumors via inhibition of RANK signaling [29]. Similarly, increased COX-2 expression is a distinctive feature of the tumor environment, and many COX-2 inhibitors have been shown to reduce Treg numbers [30]. Further in-depth studies are needed to identify a potent, targetable CD4<sup>+</sup> Treg marker to modulate their tumor-promoting role in a clinical scenario.

#### CD8<sup>+</sup> Tregs

In contrast to conventional CD8<sup>+</sup> T lymphocytes that impart a cytotoxic mode of action in the body by acting against various antigens without clonal exhaustion, CD8<sup>+</sup> Tregs play a role in suppressing this immune response to prevent an aggravated reaction while establishing homeostasis of the body's immune system [31]. They also help to protect the body from autoimmune diseases, inflammatory diseases, and transplantation [32]. The



**Fig. 1** Mechanisms underlying Treg immune regulation in the tumor-microenvironment. Tregs express a unique complement of immunosuppressive molecules on their surfaces, including lymphocyte-activation gene-3 and ATP- and AMP-degrading enzymes. They also secrete immune regulatory modulators (such as granzyme B) and cytokines (IL10, TGF $\beta$  etc.), by which they can negatively regulate dendritic cells as well as cytotoxic T cells thereby facilitating tumor initiation and progression

year 1970 was marked by the discovery of regulatory CD8<sup>+</sup> T cells by Gershon and Kondo [33].

Research has identified FOXP3 expression as the most profound marker of T-regulatory lymphocytes [34]. FOXP3 (also known as scurfin) is a member of the fork-like transcription factor family identified in 2001 by

Brunkow et al. as the master regulator for T-cell differentiation and function [35]. In humans, FOXP3 has been observed to be expressed in CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD8<sup>+</sup> T lymphocytes; in mice, FOXP3 expression in CD8<sup>+</sup> T lymphocytes is limited [36]. Though there has long been a dearth of reliable markers to correctly

identify CD8<sup>+</sup> Tregs from regular CD8<sup>+</sup> T lymphocytes, FOXP3 proves to be the most promising [37]. CD8<sup>+</sup> Tregs could also be characterized by levels of CD25 and CD127 [38]. Other phenotypic and functional markers of CD8<sup>+</sup> Tregs include elevated expression of CD94, NKG2a, Ki-67, CTLA4, and ICOS as compared to conventional CD8<sup>+</sup> T cells [39, 40]. Markers such as CD45RA and CD62L with minor or negative expression of CD45RO, CD27, CD28, and CCR7 can also be used for better characterization of CD8<sup>+</sup> Tregs [39]. Studies of CD8<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Tregs in humans with IL2 stimulation *in vitro* and *in vivo* have presented promising results in support of the high suppressive property of these cells [40].

A prevalence of a distinct subset of CD8<sup>+</sup> Tregs has been reported in colon cancer (CD8<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> cells), ovarian cancer (CD8<sup>+</sup>CCR7<sup>+</sup>IL10<sup>+</sup>), prostate cancer (CD8<sup>+</sup>CD28<sup>-</sup>), and non-small cell lung cancer (CD8<sup>+</sup>CD28<sup>-</sup>) [41–45]. Neutralization and proliferation studies in ovarian cancer suggest that *in-vitro* induction of CD8<sup>+</sup> Tregs depends partially on TGFβ1 activation of the p38MAPK-signaling pathway, suggesting that p38MAPK could be targeted by anti-cancer immunotherapy to treat ovarian cancer [44].

Previous studies have shown that an expansion of CD8<sup>+</sup>CD28<sup>-</sup> Treg lymphocytes in cancer patients may be associated with disease advancement and poor survival. CCR7<sup>+</sup>CD45RO<sup>+</sup>CD8<sup>+</sup> Tregs down-regulate the functionality of T-effector cells that act against tumor antigens in the body via IL10 secretion [46]. Thus, therapeutic strategies to alter tumor Treg infiltration could be beneficial for patient survival [47]. It has been observed that decreasing CD8<sup>+</sup>FOXP3<sup>+</sup> Treg levels in the micro-environment of prostate cancer may increase the efficiency of immunotherapy [34]. Research on ovarian cancer shows that while the cancer progresses from benign to malignant form, levels of CD8<sup>+</sup> Tregs increase along with expression of CD25, FOXP3, and CTLA4, and CD28 expression is reduced, in both peripheral circulation and the intra-tumoral microenvironment [42]. CD8<sup>+</sup>CD28<sup>-</sup> T lymphocytes have been known to inhibit CD4<sup>+</sup> T-helper cell functionality via secretion of various inhibitory cytokines, including IL10, IL16, and TGFβ or cell-to-cell contact [10, 11, 48].

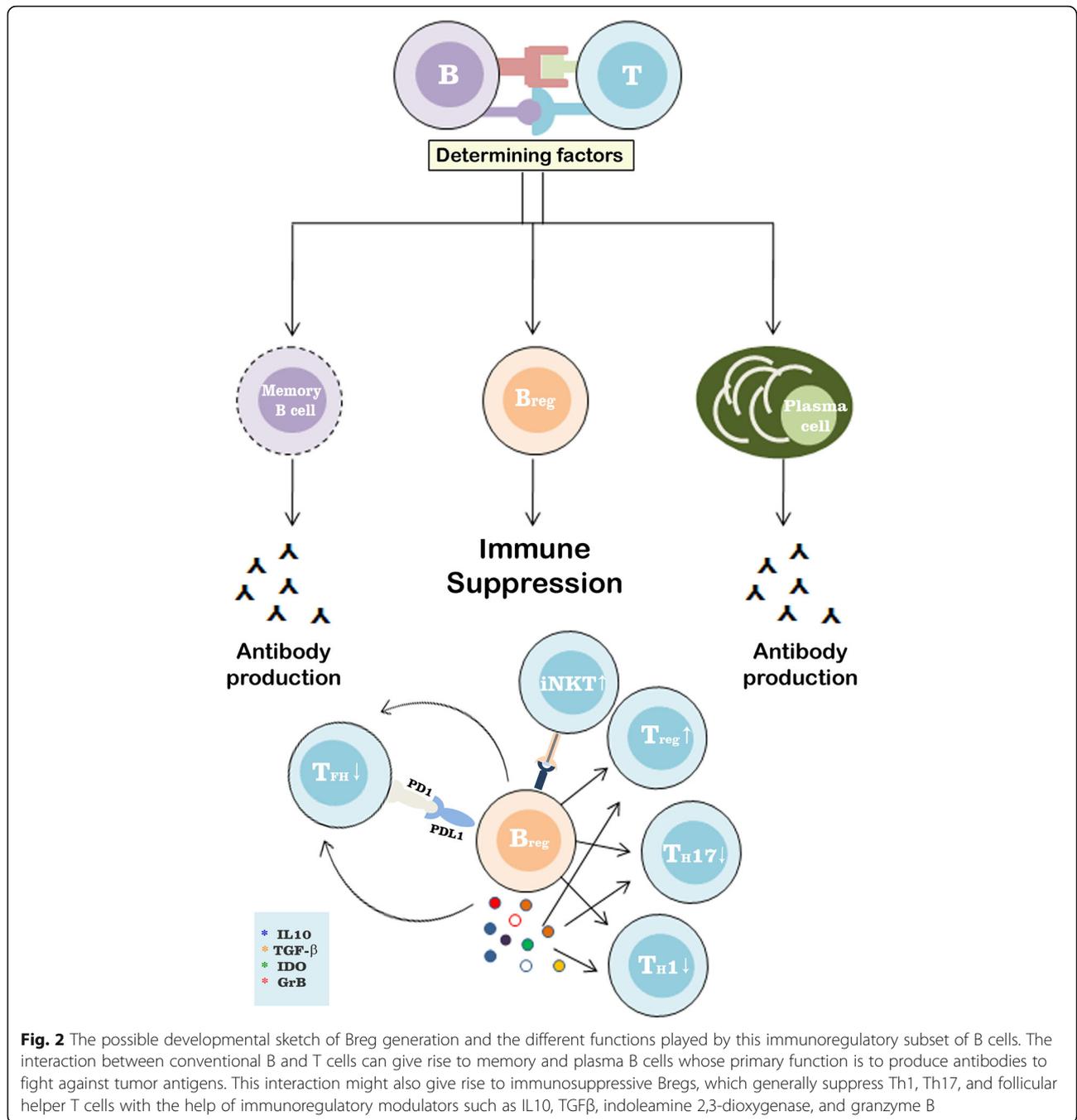
According to antigen stimulation studies, CD8<sup>+</sup> Tregs can be classified into various subtypes [49]. First, alloantigen-specific CD8<sup>+</sup>CD28<sup>-</sup> Treg lymphocytes are generated by MHC class-I stimulation of a peptide expressing a different isoform of FOXP3 (FOXP3α). This subset increases expression of the negative costimulatory receptors immunoglobulin-like transcripts-3 and -4 and downregulates expression of the positive costimulatory molecules CD80 and CD86 on antigen presenting cells, thus supporting a tolerogenic phenotype [34, 50, 51].

Second, Qa-1-specific CD8<sup>+</sup> Tregs are uniquely formed by the induction of Qa-1/peptide complexes that are expressed on CD4<sup>+</sup> T cells after vaccination with myelin basic protein. They characteristically recognize activated CD4<sup>+</sup> T cells expressing Qa-1 molecules, thereby sustaining immune tolerance via eradication of these auto-recognizing cells [52, 53]. Third, CD8<sup>+</sup>CD25<sup>+</sup> Tregs express markers such as FOXP3, GITR, and CD122, and are similar CD4<sup>+</sup> Tregs in that they can inhibit the expansion of naïve CD4<sup>+</sup>, CD8<sup>+</sup>, and effector T lymphocytes [54, 55]. Finally, γδ-T cell receptor-expressing Tregs recognize a few peptide antigens unaccompanied by MHC class-I and -II molecules and thus play an important role in immunosurveillance during cancer progression [56]. These cells are known to down-regulate conventional T-lymphocyte proliferation and dendritic cell maturation [57].

### B-regulatory cells

B lymphocytes represent an essential component of adaptive immunity as they positively regulate immune responses through the production of immunoglobulins and also act as immune modulators by presenting antigen to T cells through the secretion of cytokines. In the context of a tumor, B cells act as a double-edged sword: the conventional B cell facilitates tumor regression, whereas Bregs aid tumor progression [58]. The role of B cells in immune-specific suppression was first described in 1974 in the context of delayed-type hypersensitivity reactions. Later on, the suppressive activity of B cells was also demonstrated in a model of experimental autoimmune encephalomyelitis [59]. Mizoguchi and colleagues were the first to use the term regulatory B cell to designate those having an immunosuppressive nature [60]. The main characteristic that differentiates Bregs is the production of IL10. In addition to expressing IL10, Bregs also express other immune-regulatory cytokines such as TGFβ and IL35 [61]. Currently, multiple Breg cell subsets with many similarities in phenotype and effector functions have been described: CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> transitional B cells, CD35<sup>+</sup>CD80<sup>-</sup> B cells, transitional-2 marginal-zone precursor (T2-MZP) cells, CD5<sup>+</sup>CD1d<sup>hi</sup> B (B10) cells, marginal zone (MZ) B cells, and Tim1<sup>+</sup> B cells [58, 62].

However, the precise stage of Breg differentiation at which they acquire their regulatory capacity remains unknown. B cells mature and differentiate into antibody-producing memory and plasma cells after encountering conventional T cells or cytokines secreted by T cells. This interaction may occasionally give rise to a generation of highly-suppressive Bregs [58] due to influence of an environmental factor that has yet to be identified (Fig. 2). Various Breg subtypes have been found in high numbers in several types of tumors, in which they



attenuate local anti-tumor immune responses through several mechanisms and hence promote tumor progression [63, 64]. Bregs reduce anti-tumor responses by inhibiting the proliferation and function of conventional T cells, especially T helper (Th) 1, Th17 (Fig. 2), and follicular helper T cells [59] as well as macrophages, DCs, natural killer cells, and natural killer T cells by contact-dependent or independent mechanisms [65–67]. Transitional Bregs (CD24<sup>hi</sup>CD38<sup>hi</sup>) stimulated with CD40 have

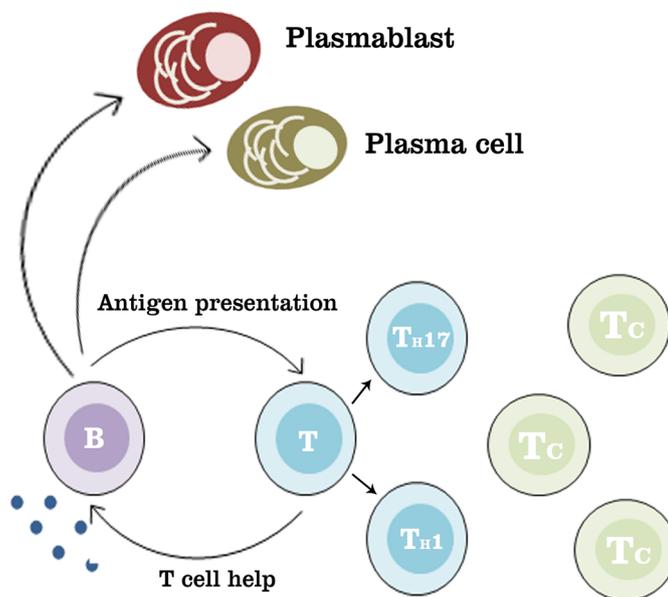
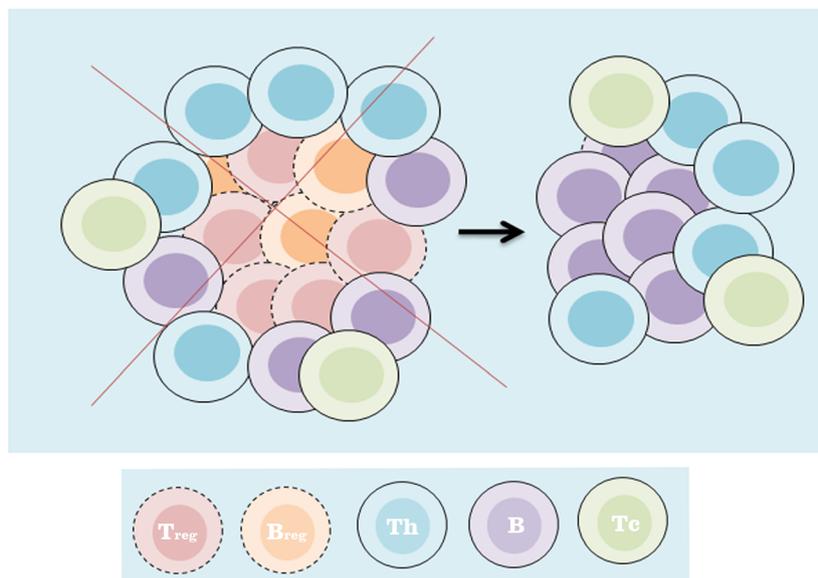
been shown to produce higher levels of IL10, thus reducing the expression of CD86. As a result, the proliferation of T cells and production of tumor necrosis factor-α decrease [68]. Similar to Tregs, Bregs can suppress follicular helper T cells through the expression of programmed death ligand-1 (PDL1) in a contact-dependent manner, which leads to indirect inhibition of humoral responses [69]. Another population of Bregs identified in patients with solid tumors produce

granzyme B when stimulated with IL21 and can suppress CD4<sup>+</sup> T cell proliferation through degradation of the T cell receptor ζ-chain (Fig. 2) [70].

Olkhanud et al. demonstrated that tumor-evoked Bregs play a significant role in tumor growth and

metastasis by converting T-effector cells into Tregs [71]. Human glioma cells secrete phosphatidylinositol-glycan biosynthesis class F protein, which may play a role in mediating conversion of B cells into a regulatory phenotype and increasing differentiation of Bregs [72]. Studies

### Regulatory lymphocytes depletion from total lymphocyte pool



### Sketch of newly reformed lymphocytes after the depletion therapy

**Fig. 3** Proposed mechanism of regulatory lymphocyte depletion therapy in the tumor. The model for depletion therapy suggests that by reducing Breg and Treg populations in the tumor microenvironment, B cells would be allowed to branch into plasmablast and plasma cells while T cells would differentiate into Th1 and Th17 cells. These differentiated B and T cells would, in turn, inhibit tumor cell growth and ultimately disrupt cancer progression

show that during the progression of a tumor, Breg numbers increase, leading to secretion of large amounts of IL10, TGF $\beta$ , indoleamine 2,3-dioxygenase, and induction of PDL1 expression which together lead to complete immune disruption (Fig. 2) [73]. Some reports suggest that CD20<sup>low</sup>4-1BBL<sup>low</sup> immune-suppressive Breg is responsible for the failure of the immunotherapeutic drug rituximab, and IgA<sup>+</sup>CD138<sup>+</sup>PDL1<sup>+</sup>IL10<sup>+</sup> Breg for the failure of the chemotherapeutic drug oxaliplatin [63]. The depletion of CD20<sup>+</sup> B cells with anti-CD20 antibodies results in higher numbers of Bregs in tumor-bearing mice [74].

## Conclusions

After considering the evidence of many studies, this review summarizes how regulatory lymphocytes hamper the host immune response against various tumor antigens. These tumor-specific immunosuppressive cells also increase the metastatic progression of tumors by enhancing T cell-mediated suppression. They mask anti-tumor immune responses and are more effective in the absence of tumor-infiltrating conventional T and B cells. Although the potential immunosuppressive properties of these cells have not been adequately studied, significant advances in the understanding of regulatory cell biology have been made in recent years that have provided valuable insight to this relatively less explored area of tumor immunotherapy. Successful depletion of CD4<sup>+</sup> Treg, CD8<sup>+</sup> Treg, and CD19<sup>+</sup> Breg populations from the tumor microenvironment may lead to the branching of effector T and B cells into Th1 and Th17 lymphocytes, plasmablasts and plasma cells (Fig. 3). These differentiated B and T cells would then in turn inhibit tumor growth. Thus, better characterization of T- and B- regulatory immune subsets and detailed mapping of their immune inhibitory properties is of utmost importance. A better understanding of their complex fundamental mechanisms can pave the way for better targeting of these cells for more efficient and successful tumor immunotherapy.

## Abbreviations

Breg: B-regulatory cell; CCR: Chemokine receptor; COX2: Cyclooxygenase-2; CTLA4: Cytotoxic T lymphocyte-associated antigen-4; FOXP3: Fork-head box P3; G1TR: Glucocorticoid-induced tumor necrosis factor receptor-related protein; IL: Interleukin; PDL1: Programmed death-ligand-1; TGF $\beta$ : Transforming growth factor- $\beta$ ; Th: T helper; Treg: T-regulatory cell

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## Authors' contributions

SP undertook the background literature study and prepared the initial draft of the manuscript and the figures; AC contributed in literature study; SM helped in making the figures and arranging the references; AG contributed in extending the initial draft. SM helped in compiling the manuscript technical corrections to the draft; GS supervised the entire project and made

final corrections to the draft. The author(s) read and approved the final manuscript.

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## Availability of data and materials

Not applicable.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

All authors read and approved the final manuscript and concur with the submission for the publication.

## Competing interests

The authors declare that they have no competing interests.

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