



FoxP3⁺ T regulatory cells in cancer: Prognostic biomarkers and therapeutic targets

Reem Saleh^a, Eyad Elkord^{a,b,*}

^a Cancer Research Center, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation (QF), Doha, Qatar

^b Biomedical Research Center, School of Science, Engineering and Environment, University of Salford, Manchester, United Kingdom

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ABSTRACT

T Regulatory cells (Tregs) can have both protective and pathological roles. They maintain immune homeostasis and inhibit immune responses in various diseases, including cancer. Proportions of Tregs in the peripheral blood of some cancer patients increase by approximately two-fold, compared to those in healthy individuals. Tregs contribute to cancer development and progression by suppressing T effector cell functions, thereby compromising tumor killing and promoting tumor growth. Highly immunosuppressive Tregs express upregulated levels of the transcription factor, Forkhead box protein P3 (FoxP3). Elevated levels of FoxP3⁺ Tregs within the tumor microenvironment (TME) showed a positive correlation with poor prognosis in various cancer patients. Despite the success of immunotherapy, including the use of immune checkpoint inhibitors, a significant proportion of patients show low response rates as a result of primary or acquired resistance against therapy. Some of the mechanisms which underlie the development of therapy resistance are associated with Treg suppressive function. In this review, we describe Treg contribution to cancer development/progression, and the mechanisms of Treg-mediated immunosuppression. We discuss the prognostic significance of FoxP3⁺ Tregs in different cancers and their potential use as prognostic biomarkers. We also describe potential therapeutic strategies to target Tregs in combination with other types of immunotherapies aiming to overcome tumor resistance and improve clinical outcomes in cancer patients. Overall, understanding the prognostic significance of FoxP3⁺ Tregs in various cancers and their contribution to therapy resistance could help in the development of more effective targeted therapeutic strategies to enhance the clinical outcomes in cancer patients.

1. Introduction

T regulatory cells (Tregs) are immunosuppressive subsets of CD4⁺ T cells, characterized by the expression of the master regulatory transcription factor, Forkhead box protein P3 (FoxP3), which plays an indispensable role in regulating Treg differentiation and development [1–3]. Tregs orchestrate cellular and molecular networks to induce an immunosuppressive environment favoring tumorigenesis [3–5]. Tregs within the tumor microenvironment (TME) are highly activated and immunosuppressive, characterized by upregulated levels of FoxP3 [5], and Helios [6,7]. Tregs exert suppressive activities on effector cells, such as T effector cells (Teffs), natural killer (NK) cells, monocytes/macrophages and antigen-presenting cells (APCs), via various mechanisms leading to the induction of apoptosis and inhibition of effector cell activation/proliferation [8]. These mechanisms include

increased consumption of IL-2 and Treg deprivation [9,10], release of high levels of interleukin (IL)-10, IL-35, transforming growth factor-beta (TGF-β) [11,12], granzyme B and perforin [13], and upregulated levels of inhibitory immune checkpoints (ICs), such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed cell death-1 (PD-1), lymphocyte activation gene-3 (LAG-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) [14].

In healthy individuals, Tregs constitute 5–10% of circulating CD4⁺ T cells, while they represent significant levels of circulating CD4⁺ T cells in the peripheral blood of cancer patients, including those with lung, ovarian and gastric cancers, and melanoma [3,15]. In cancer patients, the frequency of circulating Tregs increases by approximately 2-fold, compared to healthy individuals [16,17]. Additionally, increased tumor-infiltrating Tregs has been positively correlated with poor

* Corresponding author. Cancer Research Center, Qatar Biomedical Research Institute, Hamad Bin Khalifa University, Qatar Foundation, P.O. Box: 34110, Doha, Qatar.

E-mail addresses: eelkord@hbku.edu.qa, e.elkord@salford.ac.uk (E. Elkord).

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prognosis and low survival rates in various cancer patients, including those with lung, ovarian, gastric, breast and pancreatic cancers, head and neck squamous cell carcinoma (HNSCC), and melanoma [14,18,19].

In solid tumors, the prognostic value of tumor-infiltrating lymphocytes (TILs) is one of the most important aspects, which reflects the ability of the existing immune response in eradicating tumor cells, and predicts the clinical response to a particular cancer therapy. The association between patient survival and the presence of TILs has been demonstrated in many tumor types [20–22]. Increased intratumoral CD8⁺ T cell infiltration has been associated with increased anti-tumor responses and better clinical outcomes following therapy [23,24]. However, higher ratios of FoxP3⁺ Tregs:CD8⁺ T cell within the TME is one of the major contributing factors to tumor progression and immunosuppression [25–27]. Additionally, FoxP3⁺ Tregs play a central role, not only in tumor progression, but also in the development of tumor resistance to immunotherapies, such as immune checkpoint inhibitors (ICIs), resulting in tumor progression and poor clinical outcomes [8, 28–32]. Therefore, targeting Tregs alone or in combination with ICIs could be beneficial in treating cancer, overcoming Treg-mediated tumor resistance, and maximizing the therapeutic efficacy and immune

responses in cancer patients.

In this review, we briefly describe Treg contribution to cancer development and progression, and outline the mechanisms of Treg-mediated immunosuppression. We focus on the prognostic significance of FoxP3⁺ Tregs in cancer and their potential use as prognostic biomarkers. We also describe potential therapeutic approaches to target FoxP3⁺ Tregs expressing other Treg-related markers such as CD25, C–C chemokine receptor 4 (CCR4), suppressive cytokines and other markers alone or in combination with other types of immunotherapies aiming to overcome tumor resistance and improve the clinical response in cancer patients.

2. Treg development and trafficking to tumor sites

CD4⁺FoxP3⁺ Tregs are crucial for the maintenance of immunological tolerance; they can develop and differentiate in the thymus (thymus-derived Tregs; tTregs) upon the recognition of self-antigens presented by thymic stromal cells, including epithelial cells and dendritic cells (DCs) [15,33] (Fig. 1). FoxP3⁺ tTregs also express CD25, CTLA-4 along with other immune checkpoints, and produce IL-10 and TGF-β [34].

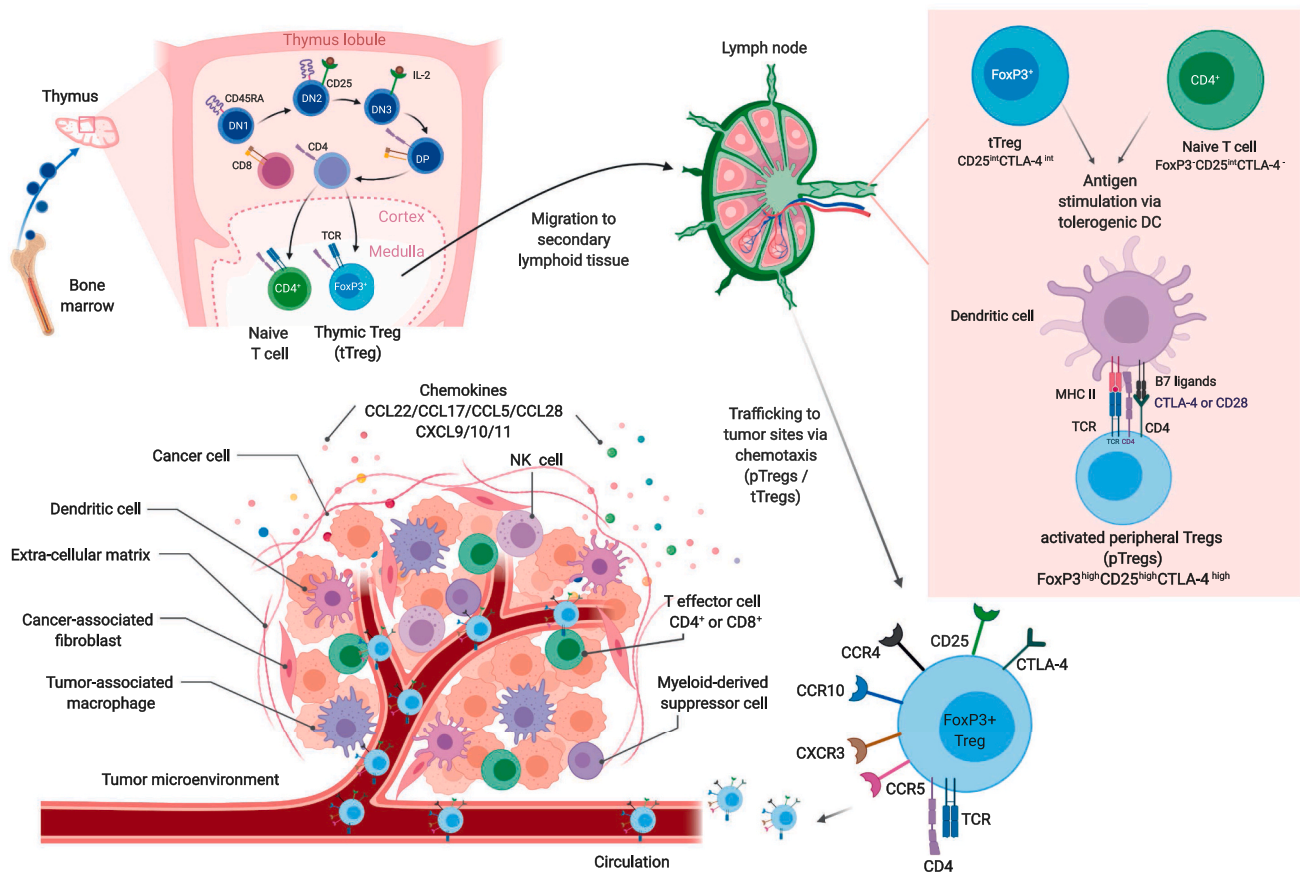


Fig. 1. Treg development, recruitment and trafficking to tumor sites. Thymic-derived Tregs (tTregs) expressing FoxP3 are autoreactive T cells, activated upon their recognition of self-antigens presented by thymic stromal cells in the medulla and cortex compartments of the thymus. Peripheral Tregs (pTregs) are induced from naïve CD4⁺ T cells upon antigen recognition in the secondary lymphoid tissues, such as lymph node or peripheral tissues. In addition to FoxP3⁺ pTregs, there are FoxP3⁻ pTregs, which are composed of two subsets with important roles in immune tolerance: Tr1 and Th3 cells (not shown here). tTregs can be also activated, while pTregs can be induced in response to antigen recognition by tolerogenic DCs in lymph nodes. FoxP3⁺CD25⁺CTLA-4⁺ tTregs and pTregs expressing chemokine receptors including, CCR4, CCR5, CCR10 and CXCR3 are recruited to the TME in a chemotactic gradient induced by chemokines released from tumor cells, TAMs and MDSCs, such as CCL17, CCL22, CCL5, CCL28 and CXCL9–11. Within the TME, tumor-derived factors and the suppressive cytokine milieu, comprising TGF-β and IL-10, favor Treg survival, differentiation and expansion either via the conversion of FoxP3⁻ T cells into FoxP3⁺ Tregs, by enhancing the recruitment of tTregs and pTregs to the TME or supporting the proliferation of existing FoxP3⁺ Tregs within the TME.

Additionally, FoxP3⁺ Tregs can differentiate from naïve CD4⁺ T cells upon antigen recognition or under certain cytokine conditions in peripheral tissues [called peripheral Tregs (pTregs)] (Fig. 1), or can be generated in vitro [called induced Tregs (iTregs)] [35,36]. pTregs consist of other FoxP3⁻ subsets: type 1 T regulatory (Tr1) cells and T helper 3 (Th3) cells, which have also been implicated in immune tolerance [18,34]. Unlike activated Tregs (CD4⁺CD45RA⁻CD25⁺FoxP3⁺), naïve Tregs (CD4⁺CD45RA⁺CD25⁻FoxP3⁺), exhibit a limited immunosuppressive capacity [15,37,38]. As depicted in Fig. 1, tTregs and pTregs expressing chemokine receptors are recruited to tumor sites via chemotaxis induced by a chemokine gradient [39,40]. Tumor cells and tumor-associated immune cells, such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), release chemokines, such as CCL17, CCL22, CCL5, CCL6 or CCL28 depending on the tumor type/site, to attract activated Tregs expressing chemokine receptors, such as CCR4, CCR5, CCR10 and

CXCR3, from secondary lymphoid tissues to the TME [3,8,41] (Fig. 1). From CD4⁺ naïve T cells, Tregs could be also induced and activated within the TME via antigen presentation by tolerogenic DCs and tumor-derived factors, thereby enhancing the suppressive activity of Tregs characterized by upregulated levels of CD25 and FoxP3 [42]. Tumor cells could induce the proliferation and differentiation of Tregs by converting immature DCs in the TME into TGF-β-producing cells [43]. In turn, TGF-β is an essential mediator for Treg differentiation and survival [44]. Collectively, the accumulation of FoxP3⁺ Tregs within the TME can occur by the recruitment of tTregs or pTregs via chemotaxis, support of Treg proliferation, differentiation and survival via tumor-derived factors, such as TGF-β and IL-10, or possibly via the conversion and differentiation of naïve CD4⁺FoxP3⁻ T cells to CD4⁺FoxP3⁺ Tregs [18,45].

Tregs within the TME orchestrate a complex cellular/molecular suppressive network, which involves regulating the activity and

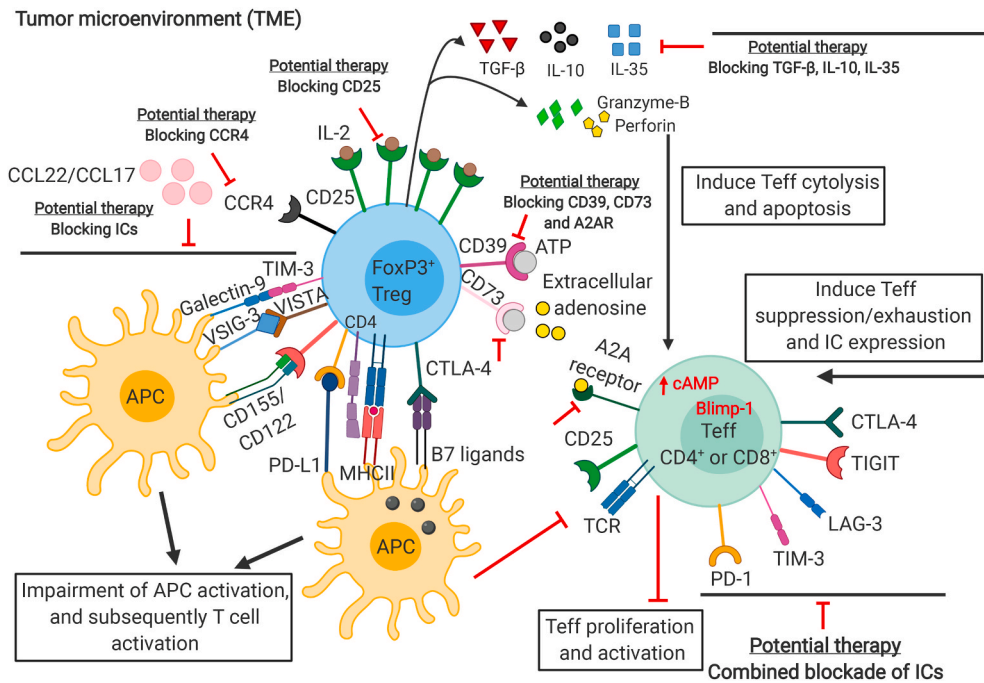


Fig. 2. Treg-mediated immunosuppression mechanisms and potential therapeutic targets to alleviate immunosuppression. A schematic diagram illustrates the mechanisms by which Tregs mediate immunosuppression within the TME and promote tumorigenesis. For example, CCR4⁺ Tregs are recruited to the TME via a chemotactic gradient mediated by chemokines, CCL17 and CCL22. Highly immunosuppressive FoxP3⁺ Tregs release high levels of granzyme B and perforin, which act on Teffs to induce apoptosis and cytolysis; release high levels of TGF-β, IL-10 and IL-35, which suppress Teff function and induce Blimp-1-mediated T cell exhaustion and expression of ICs (PD-1, TIM-3, LAG-3 and TIGIT). Highly immunosuppressive FoxP3⁺ Tregs express high levels of ICs. CTLA-4 binds to B7 ligands resulting in the expression of indoleamine 2,3-dioxygenase (IDO), tryptophan catabolic enzyme, which subsequently suppresses APC function and the generation of activation signals for T cells. Interactions between ICs (on Treg) and their ligands (on APC), PD-1/PD-L1, TIM-3/galectin-9, TIGIT/CD155 and CD122, VISTA/VSIG-3 and LAG-3/MHCII, also lead to the impairment of APC activation, and subsequently inhibit T cell activation. FoxP3⁺ Tregs express high levels of ectoenzymes CD39 and CD73, which catalyze the conversion of extracellular adenosine triphosphate (ATP) to extracellular adenosine. Adenosine binds to its receptor, adenosine 2A (A2A), on Teffs and results in the accumulation of intracellular 3'-5'-cyclic adenosine monophosphate (cAMP), which in turn inhibits the function of Teffs and suppresses their proliferation. Increased expression of CD25 on FoxP3⁺ Tregs increase their consumption of IL-2, and therefore deprive Teffs of IL-2 and inhibit Teff differentiation and proliferation. Targeting Treg-mediated immunosuppression mechanisms, for example blocking CCR4 and Treg chemotaxis; blocking TGF-β, IL-10 and IL-35 signaling; blocking CD39, CD73 and adenosine-mediated suppression pathway, combined blockade of ICs, and blocking CD25, could improve clinical outcomes in cancer patients and could be used in combination with approved ICIs.

differentiation of tumor-associated cells, such as TAMs, MDSCs and cancer-associated fibroblasts (CAFs), thereby contributing to immunosuppression and tumorigenesis via indirect means [8]. Furthermore, Tregs can directly suppress anti-tumor immunity by impairing the function of APCs and NK cells and diminishing the activation and proliferation of cytotoxic CD8⁺ T cells and CD4⁺ T effector cells, leading to tumor growth and progression [3].

3. Mechanisms of Treg-mediated immunosuppression in cancer

There are different mechanisms by which Tregs promote tumorigenesis and immunosuppression (Fig. 2), and may lead to resistance of cancer against approved ICIs targeting CTLA-4 and PD-1/PD-L1, as discussed briefly below. Strategies targeting Treg-mediated immunosuppression mechanisms could improve clinical outcomes in cancer patients (Fig. 2), and could be used in combination with cancer immunotherapies such as ICIs.

3.1. Increased consumption of IL-2

Studies on animals showed that IL-2 deficiency led to the reduction of Tregs, in particular FoxP3⁺ Tregs, suggesting the importance of IL-2 signaling not only for the development of Tregs, but also for their suppressive function (9, 59, 60). IL-2 signalling is important for the survival and proliferation of both Tregs [46] and Teffs [47]. IL-2 is produced by Teffs upon the activation of T cell receptor (TCR) signaling, however, it cannot be produced by Tregs [48–50]. Therefore, Tregs and Teffs present within the same TME compete for IL-2 consumption [51]. Tregs constitutively express high levels CD25, alternatively known as IL-2 receptor- α chain (IL-2R α), which bind with a high affinity to IL-2 produced by activated Teffs [10,46]. Hence, Tregs end up with a higher consumption of IL-2 favoring their own survival and supporting their suppressive function, causing Teff deprivation of IL-2 and suppressing the function of effector immune cells [9,10,46] (Fig. 2).

3.2. Increased production of IL-10, IL-35, TGF- β and cytolytic molecules

Tregs are one of the major cellular sources for IL-10 and IL-35 within the TME [11,52,53]. The importance of IL-10 [54–58] and IL-35 [53,59] in immunosuppression and tumorigenesis has been shown in several preclinical models. Increased expression of IL-10 in a melanoma mouse model is associated with tumor progression, impairment of DC maturation, inactivation of CD8⁺ T cell function and reduced expression of IFN- γ and granzyme B, while its deficiency reduced tumor growth, impaired Treg suppressive function and enhanced cytotoxic CD8⁺ T cell function [57]. IL-35 secreted by Tregs can promote T cell exhaustion by inducing the expression of PD-1, TIM-3 and LAG-3 on Teffs [60], and lead to cell cycle arrest and the inhibition of Teff proliferation [61]. Sawant et al. showed that IL-10 produced by Tregs, in cooperation with IL-35, limits the anti-tumor immune response in mouse tumor models by promoting the exhaustion of tumor-infiltrating CD4⁺ and CD8⁺ T cells through the induction of Blimp-1-mediated pathway and the upregulation of ICs, including PD-1, TIM-3, LAG-3 and TIGIT [62] (Fig. 2). IL-10 and IL-35, in turn, are essential for the induction and suppressive activity of Tregs [12,63,64]. Similarly, TGF- β is a crucial mediator for FoxP3 induction, Treg differentiation, maintenance and suppressive activity [65,66]. Secreting high levels of TGF- β is one of the characteristics of highly immunosuppressive intratumoral Tregs [44,66,67]. There is evidence suggesting that TGF- β can influence the composition and function of TILs, favoring Treg accumulation and Treg-mediated immunosuppression [68–70]. Additionally, Tregs can deplete Teffs in the tumor via the release of granzyme B and perforin and the induction of cytotoxicity [13] (Fig. 2).

3.3. Adenosine-mediated immunosuppression pathway

The hypoxic environment within the tumor could be responsible for the increase of extracellular adenosine, which in turn enhances immunosuppression and promotes tumorigenesis [71]. FoxP3⁺ Tregs express high levels of ectoenzymes, CD39 and CD73, and induce the adenosine-mediated immunosuppression pathway [72–74], thereby inhibiting Teff function and suppressing Teff proliferation [72,75,76] (Fig. 2). Adenosine signaling pathway can affect multiple cell types and leads to the inhibition of NK activation, impairment of APC function and enhancement of Treg function [71].

3.4. Upregulation of inhibitory immune checkpoints

Tregs in the TME express upregulated levels of inhibitory IC receptors, such as TIM-3, TIGIT, LAG-3, CTLA-4, PD-1 and V-domain containing Ig Suppressor of T cell Activation (VISTA) [77,78]. Upregulated levels of these ICs on Tregs can indirectly inhibit the activation of Teffs by negatively influencing the function of APCs (via IC-IC ligand interaction, Fig. 2), which is essential for Teff activation, as we previously reviewed in Ref. [77]. CTLA-4 [79–83], PD-1 [84,85], TIM-3 [86,87], LAG-3 [88–91], TIGIT [92,93] and VISTA [94,95] on Tregs induce inhibitory signals upon their interaction with their respective ligands, leading to the inhibition of APC function, as well as Teff activation and proliferation within tumor sites [84] (Fig. 2). The overexpression of these ICs on Tregs can also positively regulate Treg stability/differentiation and enhance their suppressive activity [77,88,96]. There is evidence suggesting that PD-1 can enhance Treg stability by promoting FoxP3 expression [14], TIM-3 and TIGIT can increase the suppressive activity of Tregs as measured by IL-10, perforin and granzymes production [96,97], and VISTA signaling pathway can act on naïve T cells to induce their differentiation into Tregs [98]. Collectively, these data implicate that the upregulation of ICs on Tregs can increase and support the survival, differentiation and function of Tregs by enhancing the expression of suppressive cytokines and cytolytic molecules. As a result, a positive feedback loop is generated favoring Treg accumulation within the tumor, and promoting the induction of Teff apoptosis or the inhibition of Teff activation. It may also negatively influence Th1 polarization via IL-10 and TGF- β .

4. Prognostic significance of Tregs in cancer

One of the major factors which facilitates immune evasion and tumor growth is the increased ratios of Tregs:CD8⁺ T cells within the TME [25–27]. However, there are some conflicting results in regards to the prognostic significance of Tregs in certain cancer types, which do not support the notion of targeting Tregs for cancer therapy. Table 1 shows some of the key studies on the prognostic impact of FoxP3⁺ Tregs in various cancers. Studies reported that increased frequency of FoxP3⁺ Tregs within tumors is associated with improved prognosis in some cancer types, such as colorectal cancer [99–101], and head and neck cancer [102]. It has been proposed that FoxP3⁺ Treg infiltration is beneficial for suppressing the inflammatory response to gut microbes in early stages of colorectal cancer [103]. Contradictory results rising from the assessment of the prognostic impact of Tregs in tumor tissues of head and neck cancer patients (Table 1) could be attributed to the randomness of Treg quantification; Treg assessment should be subdivided into groups based on their localization in the tumor tissue and the prognostic impact of each group should be individually examined [102].

Increased numbers of FoxP3⁺Helios⁺PD-1⁺CTLA-4⁺CD39⁺ Tregs and exhaustive phenotype of CD4⁺ and CD8⁺ T cells have been detected in tumor tissues of breast and colorectal cancer patients, and proposed to create an immunosuppressive environment, which favors immune evasion and tumor progression [104,105]. Additionally, CTLA-4, LAG-3 and TIM-3 were mainly co-expressed by tumor-infiltrating FoxP3⁺Helios⁺ Tregs, and little was co-expressed by FoxP3⁺Helios⁻

Table 1
Key studies show the prognostic significance of FoxP3⁺ Tregs in cancer.

Prognostic measure	Tumor type	Prognostic indicator/ clinical outcomes	Ref.
High numbers of intratumoral FoxP3 ⁺ Tregs	Colorectal ^a	Improved prognosis associated with high OS rates	[101, 191, 192]
High numbers of intratumoral FoxP3 ⁺ Tregs	Head and neck ^a	Longer RFS and OS rates	[193]
High levels of tumor-infiltrating CD4 ⁺ FoxP3 ⁺ Tregs	Colorectal ^a	Positive correlation with lymph node metastasis	[194]
Increased intratumoral CD8 ⁺ /FoxP3 ⁺ Tregs ratio		Short survival rates	
High levels of tumor-infiltrating CD4 ⁺ FoxP3 ⁺ Tregs	Head and neck ^a	Poor prognosis and short survival rates	[124, 125]
High numbers of tumor-infiltrating FoxP3 ⁺ lymphocytes	Lung	Poor overall survival rates	[195]
Elevated levels of CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs in the circulation and tumor tissue	Renal	Poor disease prognosis	[196]
Elevated levels of CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs in the circulation		Short survival rates	[197]
High levels of tumor-infiltrating CD4 ⁺ FoxP3 ⁺ Tregs	Gastric	Positive correlation with TNM staging	[117]
High levels of tumor-infiltrating FoxP3 ⁺ lymphocytes	Breast	Short RFS and OS rates	[121, 198]
Elevated levels of CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs in tumor tissue	Ovarian	Death hazard and inverse correlation with survival	[110]
High levels of FoxP3 ⁺ Tregs in tumor tissue		Poor prognosis and reduced OS rates	[199]
High levels of tumor-infiltrating CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs	Pancreatic	Positive correlation with advanced tumor stage, distant metastasis and high tumor grade	[200]
High levels of tumor-infiltrating CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs	Prostate	Poor survival rates	[201]

^a Refers to contradictory findings/OS = overall survival; RFS = recurrence-free-survival.

Tregs [106].

Up to date, there are many studies reporting the correlation between intratumoral Treg infiltration and disease prognosis in various tumor types. However, different methodologies were used in these studies to determine the level of Tregs in tumor tissue. Some researchers used immunohistochemistry slides, while others used tissue microarray [19]. Also in some studies, FoxP3 was used as a sole marker for Tregs, while in other studies FoxP3, CD25 and CCR4 were used [27]. Additionally, the prognostic significance of Tregs is influenced by tumor site, molecular subtype and tumor stage [19]. Therefore, the prognostic significance of Tregs in cancer remains controversial for some cancer types.

Meta-analysis of 74 studies encompassing 17 types of cancer (n = 15,512 patients) showed that high intratumoral FoxP3⁺ Treg infiltration is associated with poor overall survival (OS) rates in the majority of solid tumors studied [19]. Increased levels of intratumoral FoxP3⁺ Tregs have been positively correlated with worsened disease outcomes and short OS rates in patients with melanoma, breast, glioblastoma, cervical, renal, gastric and hepatocellular cancers [19,27,107,108]. In contrast to this, high intratumoral FoxP3⁺ Treg infiltration in colorectal, head and neck and esophageal cancers was associated with improved disease prognosis and higher OS rates [19]; however, the prognostic impact of Tregs in colorectal and head/neck cancers remains controversial. A recent meta-analysis showed that increased frequencies of Tregs in the

circulation and tumor tissues of patients with gynecological cancers (GCs) can be used as a biomarker for clinical outcomes [109] (Table 1). Levels of Tregs in the circulation and tumor tissues in GC patients with advanced stages were found to be higher than those with early stages; however, no significant associations were found between Treg levels in the circulation or tumor tissues and the survival of GC patients [109].

Curriel et al. reported elevated numbers of FoxP3⁺CD25⁺ Tregs in human ovarian tumor tissues, compared to normal tissues, and demonstrated *in vivo* that recruitment of CCR4⁺ Tregs into tumor tissue via chemokine gradient, leads to Treg accumulation and impairs anti-tumor immunity in ovarian cancer model [110]. Furthermore, authors showed that accumulation of Tregs within tumor tissue correlated with poor survival rates in ovarian cancer patients, and could be an independent risk factor for death [110] (Table 1). The prognostic significance of FoxP3⁺ Tregs could vary with disease stages. Three studies reported that increased FoxP3⁺ Treg infiltration in ovarian tumors of patients with advanced stages is associated with long OS rates and improved disease prognosis [21,111,112]. On the other hand, other studies showed that high FoxP3⁺ Tregs infiltration in patients with ovarian cancer, regardless of disease stages, is associated with poor disease prognosis and short OS rates [19,110]. For pancreatic cancer, low FoxP3⁺/granzyme B⁺ ratio in tumor tissues has been associated with high recurrence-free survival (RFS) rates in patients with left-side resected pancreatic ductal adenocarcinoma (PDAC) [113]. Another study by Liu et al. showed that Treg/Teff ratios in the circulation of patients with unresectable PDAC could be used as a predictor for OS and chemotherapeutic response [114]. Authors also showed that elevated levels of Tregs in the circulation of patients with unresectable PDAC were associated with poor disease prognosis [114]. Studies on gastric cancer showed that increased tumor infiltration with FoxP3⁺ Tregs is positively correlated with poor prognosis, and short RFS and OS rates in patients, while increased tumor-infiltrating CD8⁺ T cells was associated with improved disease prognosis, including RFS and OS rates [115–117]. Similarly, increased numbers of FoxP3⁺ Tregs in tumor tissues of patients with renal cell carcinoma (RCC) [118], NSCLC [119, 120] have been associated with poor disease outcomes and short survival rates (Table 1). Elevated levels of circulating CD4⁺ Treg subsets expressing TGF-β and IL-10 have been associated with clinical outcomes in untreated NSCLC patients [17]. Moreover, elevated levels of naïve Tregs in the circulation correlated with poor clinical outcomes, while levels of effector Tregs in the circulation correlated with improved prognosis, proposing that targeting Treg recruitment into the TME could be beneficial in enhancing anti-tumor immune responses [17].

For breast cancer, Bates et al. reported that FoxP3⁺ Treg infiltration in invasive breast tumors was positively correlated with high tumor grades and positive lymph node status [121]. Patients with breast tumors (invasive and noninvasive) containing high FoxP3⁺ Treg infiltration had short OS and RFS rates [121]. Furthermore, authors found that FoxP3⁺ Treg infiltration had no impact on the survival rates of breast cancer patients based on estrogen receptor (ER) subtype; both patient subgroups ER⁺ and ER⁻ with high FoxP3⁺ Treg infiltration showed no difference in survival rates [121]. Another study by Kim et al. demonstrated that low numbers of tumor-infiltrating CD8⁺ T cells were significantly associated with advanced stages (stage III), lymph node metastasis and Ki-67 (cell proliferation marker) expression in breast cancer [122]. On the contrary, authors found that high numbers of tumor-infiltrating FoxP3⁺ Tregs were significantly correlated with lymph node metastasis, ER positivity, and Ki-67 and p53 expression [122]. Additionally, disease-free survival (DFS) rates of patients with non-triple negative breast cancer were significantly associated with the number of FoxP3⁺ Tregs [122]. Triple negative breast cancer, on the other hand, was positively correlated with low ratios of tumor-infiltrating FoxP3⁺ Tregs/CD4⁺ T cells [122]. Meta-analysis of 14 studies (n = 10,259 patients) showed that FoxP3⁺ Treg infiltration in breast tumors increased with high histological grade and correlated with the lack of ER expression and the expression of human epidermal growth

factor 2 (HER2) [123]. In addition, it was reported that FoxP3⁺ Treg infiltration in breast tumor tissues was correlated with poor RFS [123] (Table 1). Therefore, based on these findings, the prognostic effect of FoxP3⁺ Tregs on the clinical outcomes of breast cancer patients can vary depending on the histological grade and molecular subtype of tumor.

5. Therapeutic approaches to target Tregs in combination with immune checkpoint inhibitors

For tumors where FoxP3⁺ Treg infiltration is considered to be a prognostic factor, targeting Tregs could be a potential therapeutic strategy. These tumors are from a wide range of human cancers, including HNSCC [124,125] and localized or metastatic hepatocellular [126], breast [121], gastric [127], lung [30], ovarian [110] and cervical [128] cancers. Targeting Tregs in cancer patients could increase intratumoral CD8⁺ cytotoxic T cell infiltration, reduce the infiltration of myeloid suppressive cells within the TME, enhance the function of APCs and suppress tumor growth [8,77]. Although the use of monoclonal antibodies (mAbs) against PD-1, PD-L1 and CTLA-4 for treating solid tumors has shown a therapeutic efficacy associated with improved prognosis and OS rates in patients, tumor recurrence and the emergence of tumor resistance against cancer immunotherapy remain a challenge. There is an accumulating evidence suggesting that increased number of Tregs in tumors is associated with the development of acquired resistance against cancer immunotherapies.

5.1. Targeting CD25

Tregs could be targeted via the use of antibodies against CD25 (Fig. 2). A study using mouse model of head and neck cancer showed that radiotherapy with combined blockade of CD25, PD-L1 and TIM-3 resulted in a prolonged survival response and increased therapeutic efficacy, compared to radiotherapy with anti-PD-L1/TIM-3 alone [129]. Another study by Shimizu et al. showed that targeting CD25 in mouse tumor models increased the number of Tregs within the tumor and the release of IFN- γ [4]. However, it is worth noting that targeting CD25 would not be exclusive to Tregs as non-Tregs also express CD25 [130]. Various drugs have been developed to test the effect of blocking CD25 in hematologic malignancies and going under clinical trials in cancer patients [131]. Alternatively, it was shown in mouse tumor models that intratumoral administration of anti-CD4 mAb in late stages results in the depletion of Tregs, downregulation of IL-10, upregulation of IFN- γ , and tumor rejection [32], suggesting that the majority of intratumoral CD4⁺ T cells in late tumor stages are Tregs. This implicates that the effect of targeting Tregs depends on the timing of treatment application.

5.2. Targeting FoxP3 expression

Another Treg marker which could also be targeted is FoxP3. Multiple lines of evidence from mouse and human studies demonstrate the importance of FoxP3 in Treg function and activity [132–137]. Moreover, reduced expression of FoxP3 and IL-10 in induced Tregs upon anti-PD-1 has been reported, suggesting a potential link between FoxP3 and activity of Tregs [138]. Stability of FoxP3 expression is mediated by selective demethylation with the FoxP3 element, Treg-specific demethylated region (TSDR) [139]. Using transfection and luciferase vector assays, Polansky et al. showed that murine CD4⁺ T cell line carrying methylated construct of TSDR lacks the transcriptional activity of TSDR, compared to those carrying the insert of demethylated TSDR construct [139], suggesting that methylation of TSDR could be used an approach for targeting FoxP3 expression. Wang et al. showed that the binding of methyl-binding domain 2 (Mdb2) protein to TSDR promotes ten-eleven translocation 2 (TET2)-mediated demethylation, which in turn induces FoxP3 expression [140], implicating that inhibiting the activity of TET2 enzyme could diminish Treg stability. In support of this, other studies have shown that using epigenetic modifier (DNA

methylation agent, for example TET inhibitor) to target TSDR, could be beneficial in interrupting the stability of FoxP3 expression in intratumoral Tregs, and therefore diminishing FoxP3⁺ Treg infiltrates in the tumor [140–142]. FoxP3 expression can be transiently expressed by recently activated Tregs; however, its expression is more prolonged and stable in Tregs due to the selective existence of TSDR in natural Tregs, which does not occur in other T cell subsets [143]. Hence, reversing TSDR demethylation is very specific to Tregs, compared to targeting CD25, and may represent a better therapeutic approach for Treg depletion. FoxP3 expression in Tregs could be also targeted via other epigenetic modulation such as the use of histone deacetylation inhibitors and ubiquitylation agents [144]; however, these epigenetic modifiers have multiple target genes and their use could as well have effects on FoxP3⁺ cells.

5.3. Targeting PI3K signaling pathway

Targeting pathways which positively regulate Treg stability, function and survival such as phosphatidylinositol 3-kinase (PI3K) signaling pathway [145,146] could be more selective in diminishing CD4⁺CD25⁺ Treg proliferation, and has shown a therapeutic efficacy in mouse tumor models [145,147,148]. Qin et al. showed that silencing microRNA-126 (miR-126) in a mouse breast cancer model impairs the activation of PI3K pathway, which subsequently reduces the induction and suppressive function of Tregs [149]. Other studies demonstrated that inhibition of PI3K signaling in intratumoral Tregs resulted in tumor regression associated with reduction in Treg proliferation and increase of CD8⁺ T cell activation [145,147,148]. Moreover, the inhibition of PI3K activity in combination with anti-PD-1 and/or anti-CTLA-4 mAbs has enhanced the therapeutic efficacy and clinical response in murine models of melanoma [145] and HNSCC [150]. Together, these findings implicate that inhibition of PI3K combined with the blockade of ICs could selectively target Tregs and alleviate Treg-mediated resistance.

5.4. Targeting chemokine receptors

Tregs are recruited to the TME in response to a chemotactic gradient which is facilitated by chemokine and its receptor; different tumor sites are infiltrated with Tregs that express different chemokine receptors (CCRs). For instance, CCR6⁺ Tregs are highly infiltrated in tumor tissues of patients with breast cancers and hepatocellular carcinoma, while tumor tissues of ovarian and gastric cancers are highly infiltrated with CCR4⁺ Tregs [151]. Therefore, tumor sites and Treg phenotypes should be considered before clinical application of CCR inhibitors. Targeting CCR4 which is associated with Treg chemotaxis to tumor sites of different cancer types (118–120) could have therapeutic benefits. Studies showed that targeting CCR4 via neutralizing antibodies or antagonists could diminish Treg infiltration in tumor sites (118, 121) (Fig. 2). Targeting CCR4 could be more selective in diminishing Tregs (CD4⁺FoxP3⁺), unlike targeting CD25 which results in the depletion of a fraction of non-Tregs (118). It was demonstrated that CCR4 is primarily expressed by Tregs in melanoma, and its depletion in PBMCs isolated from melanoma patients was able to induce the generation of CD4⁺ and CD8⁺ T cells specific for cancer-testis antigens (118). Authors also demonstrated that CCR4 blockade in vivo, using anti-CCR4 mAb (mogamulizumab), led to the reduction of melanoma-infiltrating Tregs (118). Moreover, the blockade of CCR4 in patients with acute T-cell leukemia/lymphoma (ATL), a condition characterized by high numbers CCR4-expressing Tregs, enhanced the anti-tumor immune response mediated by CD4⁺ and CD8⁺ T cells (118), and resulted in the approval of using mogamulizumab to treat ATL in Japan (118). The therapeutic efficacy of mogamulizumab, as a monotherapy, is under investigation in ongoing early phase clinical trials (NCT01929486) (122), or in combination with either anti-PD-1 mAb (NCT02476123 and NCT02705105), anti-PD-L1 mAb (NCT02444793) or anti-CTLA-4 mAb (NCT02301130) in advanced solid tumors.

5.5. Targeting immunosuppressive cytokines

The inhibition of Treg function represents another mean of therapy by targeting immunosuppressive cytokines including TGF- β , IL-10 and IL-35 (Fig. 2). TGF- β signaling could be targeted in cancer to reduce the differentiation/suppressive function of Tregs and their survival within the TME [152,153]. In vitro, Becker et al. showed microRNA (miR-466a-3b) can inhibit the expression of TGF- β 2, which subsequently diminished the differentiation of FoxP3⁺ Tregs [154]. Other in vitro studies by Hou et al. demonstrated the efficacy of TGF- β chimeric antigen receptor (CAR) T cells in alleviating TGF- β -mediated immunosuppression, thereby promoting the anti-tumor immune response mediated by CD8⁺ and CD4⁺ T cells specific for tumor antigens [155]. Additionally, authors showed that TGF- β chimeric antigen receptor (CAR) T cells were able to diminish the induction and differentiation of FoxP3⁺ Tregs [155]. These findings proposed targeting TGF- β as a mean of adoptive T cell therapy and its potential therapeutic efficacy in cancer.

A study by Ravi et al. showed that in vitro stimulation of human PBMC with IL-2, TGF- β and anti-CD3/anti-CD28-coated beads treated with engineered antibodies targeting CTLA4 and TGF β RII simultaneously abolished the TGF- β -mediated FoxP3 expression, while the single blockade of CTLA-4 in stimulated human PBMC did not show such effect [156]. In vivo, authors demonstrated that the simultaneous inhibition of CTLA-4 or PD-L1 with TGF- β signaling, using engineered antibodies, is more effective in reducing tumor-infiltrating Tregs, increasing the number of IFN- γ ⁺CD8⁺ T cells and promoting anti-tumor cytotoxic effects in a mouse melanoma model, compared to the single inhibition of CTLA-4 or PD-1 [156]. These data implicate that simultaneous blockade of ICs and TGF- β signaling may maximize the therapeutic efficacy and synergistically improve the clinical and immune response compared to the blockade of ICs alone. Based on these findings, the dual inhibition of TGF- β and ICs is currently under clinical investigation in various cancer patients [as reviewed in Ref. [153]].

The inhibition of IL-10 could be considered as a potential therapeutic strategy to target Treg suppressive functions within the TME. Increased serum levels of IL-10 have been associated with poor disease prognosis and low DFS rates in many patients with solid tumors or hematologic malignancies [157], including hepatocellular carcinoma, diffuse large B cell lymphoma, melanoma and Hodgkin's lymphoma [158–161]. Elevated levels of IL-10 within the TME have also been associated with poor disease prognosis and clinical outcomes in cancer patients [162]. IL-10 produced by Tregs could be associated with cancer progression, impaired tumor-resident APC activation and suppression of Teff functions and Th17 cells [163]. Sawant et al. showed that IL-10 produced by Tregs, in cooperation with IL-35, limits the anti-tumor immune response in mouse tumor models by promoting tumor-infiltrating CD4⁺ and CD8⁺ T cell exhaustion through the induction of Blimp-1-mediated T cell exhaustion pathway and the upregulation of ICs, including PD-1, TIM-3, LAG-3 and TIGIT [62]. Furthermore, it was demonstrated that IL-10 deficiency in mouse tumor models was able to reduce Treg-mediated immunosuppression by reducing the expression of neuropilin on Tregs within the TME [57]. This in turn resulted in the simultaneous activation of Th1 and Th17 immune response, leading to tumor regression and enhanced anti-tumor immunity [57]. Together, these data implicate the potential therapeutic benefits of blocking IL-10 produced by Tregs in cancer. Naing et al. reported that PEGylated-recombinant human IL-10 (PEG-rhuIL-10) increased serum levels of IFN- γ , IL-18 and reduced levels of TGF- β in cancer patients with advanced solid tumors (Phase I clinical trial, NCT02009449) [164]. In addition, treated patients showed some partial clinical responses [164]. A phase III clinical trial investigating PEG-rhuIL-10 in combination with FOLFOX in metastatic pancreatic cancer patients is currently ongoing (NCT02923921).

The blockade of IL-35 in multiple cancer models has shown to be effective in increasing the number of tumor-infiltrating CD4⁺ and CD8⁺ Teffs and reducing tumor growth, thereby proposing that targeting IL-35

could be beneficial for enhancing the anti-tumor immune response [60].

5.6. Targeting CD39, CD73 and adenosine-mediated signaling pathway

Targeting components of the adenosine signaling pathway in cancer could have clinical implications and therapeutic benefits by reducing the induction and suppressive function of FoxP3⁺ Tregs. FoxP3⁺ Tregs can be induced in vitro upon the stimulation of adenosine receptor (A2AR) [165,166], suggesting the potential role of adenosine in Treg induction. Tregs lacking expression of CD39 and A2AR have shown limited suppressive activity [76,167,168]. Moreover, inhibitors against CD39 and CD73 can reduce the suppressive activity of Tregs [71,169]. CD73-deficient mice and the in vivo blockade of CD73 in mouse melanoma and colorectal cancer models reduced pulmonary metastasis and tumor growth [170]. Results from in vivo models showed the therapeutic efficacy of inhibiting A2AR via pharmacological inhibitors, siRNAs or antibodies, enhancing anti-tumor immunity, tumor regression and improving clinical outcomes [171,172].

Targeting CD39, CD73 and adenosine-mediated immunosuppression pathway could be beneficial in augmenting the anti-tumor immune response against tumor cells, and enhancing the sensitivity to ICIs in the case of acquired resistance [72,75,173–175] (Fig. 2). Using syngeneic and humanized tumor models, inhibition of CD39 was able to activate NLRP3-mediated inflammasome pathway, increase the number of intratumoral Teffs, enhance anti-tumor immune response and alleviate tumor resistance against anti-PD-1 [176]. The combined blockade of ICs and CD73 is under clinical investigation (NCT02503774) [174]. Furthermore, targeting adenosine/A2AR signaling combined with CAR T cell therapy or ICIs has showed therapeutic efficacy in preclinical models [177–179]. Clinical trials to investigate the effect of inhibiting A2AR alone or in combination with anti-PD-1/PD-L1 in cancer patients are ongoing [178].

5.7. Targeting immune checkpoints

The single or combined blockade of CTLA-4 and PD-1/PD-L1 pathway(s) in tumors with high numbers of immunosuppressive Tregs expressing elevated levels of PD-1 and CTLA-4 can have a therapeutic efficacy associated with improved clinical outcomes [180]. However, overtime some tumors become less immunogenic resulting in the production of more self-antigens to activate Tregs and enhance their suppressive function, and the emergence of compensatory mechanisms to escape anti-tumor immunity in response to anti-PD-1/PD-L1 and/or anti-CTLA-4 mAb(s) [8,77,181]. Elevated expression of newly emerged ICs, such as LAG-3, VISTA, TIM-3 and TIGIT, on intratumoral T cells including Tregs act as compensatory pathways to allow immune evasion and promote tumor growth/progression in response to anti-PD-1, PD-L1 or anti-CTLA-4 therapies [77,129,182]. Therefore, it was rationalized that combined blockade of LAG-3, VISTA, TIGIT or TIM-3/TIGIT with anti-PD-1/PD-L1 mAbs or anti-CTLA-4 mAb could provide alternative therapeutic modalities to target Treg-mediated immunosuppression, overcome resistance, and maximize clinical outcomes including survival rates and disease prognosis (Fig. 2). The therapeutic efficacy of blocking TIM-3, TIGIT and LAG-3 in a wide-range of cancer patients is under investigation in ongoing clinical trials (as reviewed in Ref. [14,183]). Despite this, several studies have shown that ICIs can partially deplete Tregs [129,184] or expand them [185–187]. Therefore, other therapeutic means, apart from ICIs, are required to target Treg infiltration within the TME.

6. Conclusions and future perspectives

Although the prognostic significance of Tregs in some cancer types remains controversial, the majority of studies have reported positive correlations between intratumoral FoxP3⁺ Treg infiltration and poor prognosis/clinical outcomes in various tumor types. The

controversiality could be attributed to the different methodologies used to determine the level of Tregs in tumor tissue [19,27]. Additionally, the prognostic significance of Tregs is influenced by tumor site, histological features, molecular subtype and tumor stage [19]. Targeting Tregs could be beneficial for treating certain cancer types with certain tumor stages/molecular type.

Based on the findings discussed in Section 4, it is imperative to consider tumor type, disease stage, histological grade and molecular subtype during the assignment of therapeutic protocols to cancer patients as the prognostic effect of FoxP3⁺ Tregs varied with these parameters, and their targeting could be beneficial, deleterious or negligible. Therefore, understanding the prognostic significance of FoxP3⁺ Tregs in various cancers and their contribution to therapy resistance could help in the development of more effective targeted therapeutic strategies to enhance the clinical outcomes in cancer patients.

Targeting Tregs in combination with ICIs could overcome Treg-mediated resistance and increase the sensitivity of tumor cells to therapy, thereby enhancing tumor regression. Targeting the underlying mechanisms by which Tregs trigger tumor resistance in response to immunotherapies could result in better outcomes associated with improved prognosis and survival rates in cancer patients. Several clinical trials are underway to investigate the effect of small molecule inhibitors, epigenetic modifiers, mAbs targeting CD25 and chemokine receptors, and the combined blockade of multiple ICs [174,183, 188–190].

Different markers have been used to quantify the levels of FoxP3⁺ Tregs; some studies have used FoxP3 as the sole marker, while other studies have used CD25 and/or CCR4 in addition to FoxP3 [19]. Therefore, consistency in markers selection and careful analyses are required to determine the levels of Tregs in tumor tissues, and to characterize their phenotypes and determine their prognostic significance across different tumor types/stages/histological and molecular subtypes. This should help in designing more improved immunotherapeutic protocols and determining whether Treg targeting is a suitable treatment for patients with certain solid tumors based on the clinical and histopathological parameters. Additionally, it is important to monitor the number and characterize the phenotype of Tregs before and after the application of ICIs to identify novel predictive biomarkers which could be associated with Treg-mediated resistance in response to ICIs.

Declaration of competing interest

The authors declare no conflicts of interest.

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