

# T-Regulatory Cells In Tumor Progression And Therapy

This article was published in the following Dove Press journal:  
*Cancer Management and Research*

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**Abstract:** Regulatory T cells (Tregs) are important members of the immune system regulating the host responses to infection and neoplasms. Tregs prevent autoimmune disorders by protecting the host-cells from an immune response, related to the peripheral tolerance. However, tumor cells use Tregs as a shield to protect themselves against anti-tumor immune response. Thus, Tregs are a hurdle in achieving the complete potential of anti-cancer therapies including immunotherapy. This has prompted the development of novel adjuvant therapies that obviate their negative effects thereby enhancing the therapeutic efficacy. Our earlier studies have shown the efficacy of the glycolytic inhibitor, 2-deoxy-D-glucose (2-DG) by reducing the induced Tregs pool and enhance immune stimulation as well as local tumor control. These findings have suggested its potential for enhancing the efficacy of immunotherapy, besides radiotherapy and chemotherapy. This review provides a brief account of the current status of Tregs as a component of the immune-biology of tumors and various preclinical and clinical strategies pursued to obviate the limitations imposed by them in achieving therapeutic efficacy.

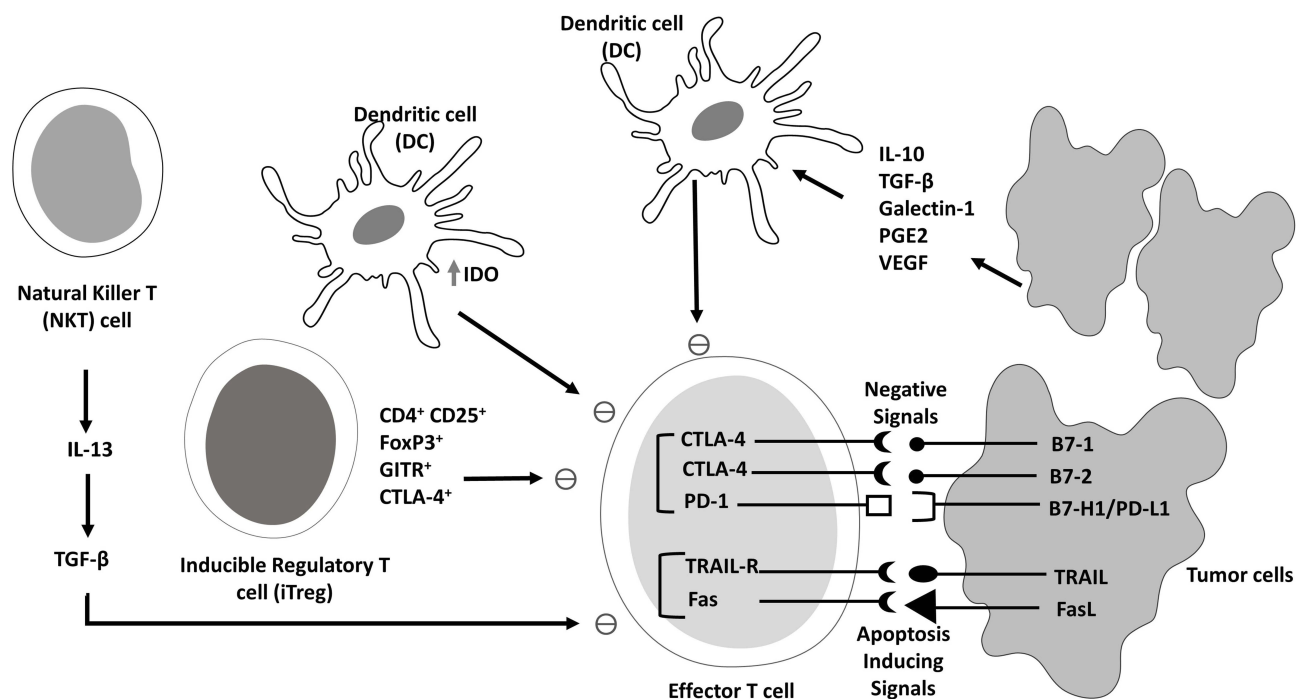
**Keywords:** T-regulatory cells, cyclophosphamide, dendritic cells, immune enhancement, targeted cancer therapy, 2-deoxy-D-glucose, metabolic inhibitor

## Introduction

Cancer accounts for the major cause of death after cardiovascular disorders worldwide.<sup>1</sup> Cancer primarily is a disease that arises due to the deregulation of the growth of functionally matured (somatic) cells leading to a state of “malignant” behavior, which is reflected in the well-established hallmarks of the disease as described by Hanahan and Weinberg.<sup>2</sup> Several pioneering studies over the past few years have established immune evasion as one of the key events for the successful establishment of tumors.<sup>3</sup> Cancer cells modulate several pathways leading defective antigen presentation, secretion of immunosuppressive mediators, tolerance and immune deviation, apoptosis and release of immunosuppressive cells to evade immune responses<sup>4</sup> (Figure 1). Recruitment of immunosuppressive cells like myeloid-derived suppressor cells (MDSCs), tumor-derived macrophages, modulated dendritic cells (DCs) and T-regulatory cells (Tregs), are important mechanisms underlying the immune evasion achieved by cancer cells. Among these immunosuppressive cells, the master regulatory cells, Tregs not only secrete molecules that promote initiation and progression of tumors, but also induce neoangiogenesis facilitating metastasis.<sup>5-7</sup> The role of Tregs has also been well established in pathogenic infections and allergic response.<sup>8,9</sup> Despite more than 20 years of their

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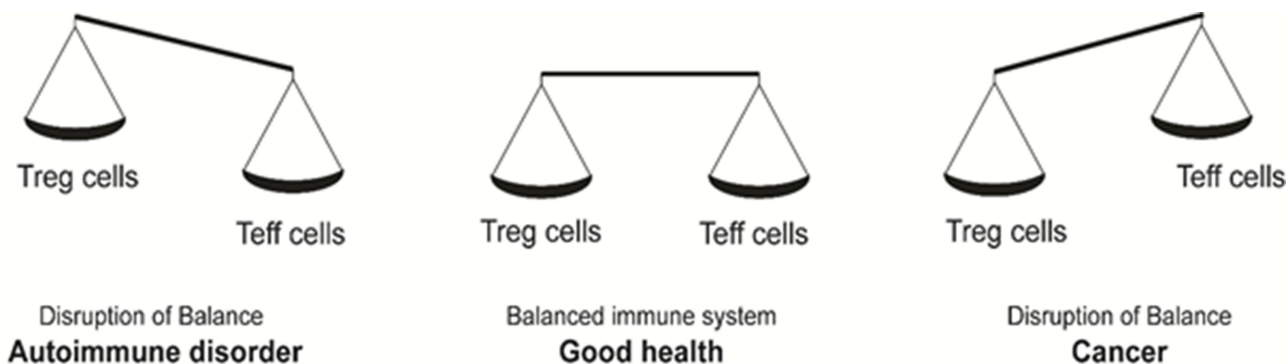
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**Figure 1** Model of immune evasion by tumor cells. Cancer cells modulate several pathways leading to defective antigen presentation, secretion of immunosuppressive mediators [immunosuppressive cytokines like IL-10, vascular endothelial growth factor (VEGF), transforming growth factor (TGF-β), immunosuppressive enzymes like indoleamine 2,3 dioxygenase (IDO), etc], tolerance and immune deviation, apoptosis and release of immunosuppressive cells (Treg cells), which evade immune responses by induction of immune checkpoints like PD-1 and CTLA-4, absence of co-stimulatory molecules like GITR and OX40. These are some of the primary mechanisms involved in tumor cells mediated immune evasion.

identification, unraveling of their role in many disease states, the precise mechanisms underlying their suppressive function remains to be completely understood.<sup>10</sup> In a disease state such as cancer, Tregs become an impediment as they compromise the anti-tumor response of the host by dampening the efficiency of T-effector (Teff) cells. Therefore, maintaining an optimum balance between Treg and Teff cells is vital in not only avoiding autoimmunity, but also keeping in check the progression of

malignancy, avoiding therapeutic resistance, leading to better prognosis of patients (Figure 2). Emerging evidences suggest that the avoidance of tumor cell death from therapeutic agents is linked to the up-regulation of the Treg pool and escape from immune response.<sup>11–18</sup> Therefore, therapeutic approaches, which also modify Tregs appear to be successful in the management of tumors. Several mechanisms appear to be involved in Treg-mediated immunosuppression including the down-



**Figure 2** Imbalances in the immune system homeostasis results in a disease state. A balance in the levels of Treg and T effector cells maintains the homeostatic and disease-free state. A shift in the balance towards Tregs causes a decrease in anti-cancer immunity, resulting in cancer. Contrarily, a shift in the balance towards T effector cells causes a decrease in Treg levels and T effector cells hyperactivation leading to auto-immune disorders.

regulation of MHC complexes, shedding of antigens, induction of immune checkpoints like programmed death protein 1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), reduction in co-stimulatory molecules (GITR and OX40), release of various cytokines and factors such as IL-10, VEGF, TGF- $\beta$ , indoleamine 2,3 dioxygenase (IDO).<sup>19</sup> Hence, targeting Tregs associated mechanisms have been considered a major strategy in immunotherapy. Many agents such as ipilimumab (anti-CTLA-4 antibody, brand name: Yervoy), which are in different phases of pre-clinical and clinical trials for metastatic renal cell carcinoma and other cancers, are also known to target Tregs.<sup>20</sup>

We present here an overview on the existing knowledge about the role of Tregs in tumorigenesis as well as merits and limitations of approaches using conventional chemotherapeutic agents that target Tregs for improving therapeutic gain. Some new agents that also target Tregs and show negligible or absence of any side effects on normal cells are also discussed (Table 1).

## Tregs Characterization And Immune Evasion

Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) belong to the family of CD4<sup>+</sup> T cells. These cells have a high expression of CD25 (IL-2 receptor) and transcription factor Foxp3 (Forkhead box P3). FoxP3 plays an important role in the generation and production of Tregs, including the maintenance of their functionality; loss of which is associated with immune dysregulation and lymphoproliferative diseases both in mice and humans.<sup>21</sup> Tregs have two subtypes on the basis of their origin: naturally occurring Tregs (n-Tregs) and inducible Tregs (i-Tregs). n-Tregs are mainly formed in thymus and need costimulatory molecules for their development and lineage commitment.<sup>18</sup> n-Tregs suppress immune effector lymphocytes like T helper cells (Th cells), Th1, Th2, Th17 and follicular Th cells (Tfh cells) in a contact-dependent manner that requires Granzymes B/perforin and Fas/FasL pathways.<sup>12</sup> i-Tregs are generated in the periphery and do not require costimulatory molecules for activation, but at least require T cell receptor (TCR), TGF- $\beta$  and IL-2 under a variety of pathological conditions.<sup>8</sup> i-Tregs mediate immunosuppression in multiple ways that involve the secretion of immunosuppressive cytokines (interleukins, IL-10, IL-35, and TGF- $\beta$ ), granzymes-induced cytolysis, metabolic reprogramming of effector cells, and DC-mediated suppression.<sup>22,23</sup>

In vivo studies in mice models indicate that Tregs regulate concomitant immunity and cross-reactive anti-tumor immunity.<sup>24,25</sup> Tregs not only suppress the natural killer (NK) cell-mediated cytotoxicity but also check the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and inhibit the interferon (IFN)- $\gamma$  secretion by immune cells, thereby leading to the impairment of effective anti-tumor immune response.<sup>20</sup> Indeed, higher Tregs activity has been related to decreased survival and poor prognosis in patients of breast cancer, gastric carcinoma, non-small cell lung cancer (NSCLC), squamous cell carcinoma of head and neck (SCCHN), pancreatic cancer and ovarian cancer.<sup>17,26–30</sup>

Anti-tumor immunity can be reinforced by the reduction of Treg cells, which are responsible for suppressing immune-response against syngeneic tumors in-vivo and in-vitro.<sup>31,32</sup> Several therapeutic agents besides having a direct anti-tumor function also leads to a reduction in Treg numbers by inhibiting the immune checkpoints, CD25 and several chemokine receptors (e.g. CCR5), which induce intra-tumoral recruitment of Tregs.<sup>31,33–35</sup> Similarly, the administration of anti-folate receptor monoclonal antibodies (mAbs) also effectively reduces the number of Tregs.<sup>36</sup> Anti-GITR or anti-OX40 agonist antibodies based immunotherapy also stimulates anti-tumor immune response via blocking Treg-induced immuno-suppression, resulting in the eradication of established tumors.<sup>37,38</sup> Moreover, many chemotherapeutic agents have also been found to deplete Tregs (aromatase inhibitor, cyclophosphamide, mitoxantrone, etc).<sup>39–41</sup> Recent studies from our laboratory have demonstrated that glycolytic inhibitor, 2-deoxy-D-glucose (2-DG), which inhibits metabolic modulation in tumors, reduces the i-Tregs frequency. Therefore, 2-DG can be used in combination with immunotherapy in addition to its recognized role as an adjuvant for chemo- and radiotherapies.<sup>42,43</sup> Hence, these studies reveal Tregs as a potential target for restoring anti-tumor immunity, thereby improving the anti-tumor response.

## Factors Influencing Treg Level, Activity, And Intra-Tumoral Migration

Identification of factors that affect the Treg level, their activity, and migration is essential for developing strategies that target them. Expression of Foxp3 is indispensable for Treg development. Foxp3 when expressed ectopically in conventional T cells can generate a suppressive phenotype and Foxp3 gene mutation results in Treg cells deficiency that

Table 1 Current Status Of Anti-Cancer Therapies Influencing Treg Levels

Target	Agents	Disease/system tested	Outcome of the study	Reference number
<b>Antibody</b>				
<b>CTLA-4</b>	Tremelimumab	Advanced colorectal cancer/ Human/Phase II	Tremelimumab is not effective clinically as a single-agent in this patient cohort. However, higher number of patients survived at 6 months and one patient with confirmed partial response.	169
		Advanced gastric and esophageal adenocarcinoma /Human/Phase II	Tremelimumab treatment did not confer advantage to the patients due to increased Tregs except one patient had a remarkably durable benefit.	170
		Advanced Melanoma /Human/Phase II	Tolerance to Tremelimumab demonstrated and single-agent anti-tumor activity established in patients with melanoma.	171, 172
	Ipilimumab (Yervoy, Bristol Myers-Squibb)	Approved for melanoma/Phase III for prostate cancer/Human	Ipilimumab in randomized, double-blind phase III trial demonstrates a benefit in overall survival (OS) in the treated population.	34
<b>CD-25</b>	Daclizumab /Human	Advanced breast cancer	Tregs are depleted following a single intravenous infusion of daclizumab in patients with metastatic breast cancer.	173
	Denileukin diftitox (cancer vaccine)	Cancer/ Human/Phase I	Denileukin diftitox significantly depletes Tregs, in melanoma patients resulting in enhanced anti-tumor immunity specifically antigen-specific CD8 <sup>+</sup> T cells in vaccinated individuals.	174
	Immunotoxin LMB-2	Advanced Melanoma /Human	LMB2 selectively mediates a transient partial reduction in circulating and tumor-infiltrating Treg cells in metastatic melanoma patients, bolstering anti-tumor immunity.	175
	RFT5-SMPT-dgA	Advanced Melanoma/ Human	RFT5-SMPT-dgA shows partial reduction in Treg-cell frequency with no objective anti-tumor responses.	70
<b>PD-1</b>	PC61	Syngeneic intracranial glioblastoma Human glioblastoma/ (GBM) in mouse	PC61 in combination with immunotherapy, inhibits clonal expansion of tumor antigen-specific T cells thereby enhancing antitumor immunity.	176
	Anti-PD-1 mAb (Pembrolizumab)	Ipilimumab resistant Metastatic melanoma/ Human	Pembrolizumab was well tolerated with dose dependent ORR, with <3% adverse events like fatigue.	177
		Non-small cell lung cancer (NSCLC)/ Human	ORR of 19.4% with acceptable side effects. PD-L1 expression in at least 50% of tumor cells correlated with improved efficacy.	82
<b>OX40</b>	OX40 ligand IgG4P Fc fusion protein	Human T-cells and tumor cells admix mouse/ <i>in-vivo</i>	Enhanced cytolytic ability of tumor reactive T-cells leads to reduced tumor burden. Also induces Th1 response resistant to Treg-mediated suppression.	178
<b>GITR</b>	Anti-GITR mAb (DTA1)	Mouse/ <i>in-vivo</i>	GITR-GITR-ligand interactions co-stimulate both responder T-cell functions and the suppressive functions of Treg cells.	87
<b>FR4</b>	Anti-folate	Mouse/ <i>in-vivo</i>	FR4 mAb decreases Treg cells, enhances anti-tumor immunity in tumor-bearing animals. An autoimmune disease was elicited in young mice with similar treatment.	104
	Farletuzumab	Ovarian cancer/ Human Phase III	Farletuzumab in combination with platinum and taxane in platinum-sensitive ovarian cancer patients showed a 7% complete response, 63% partial response and 89% of the patients achieved normal CA-125 levels.	179

<b>IL-2</b>	Anti-IL-2 + CTLA-4 mAb	Advanced Melanoma/ Human phase II	No demonstrated evidence of synergistic effect of CTLA-4 blockade and IL-2 administration. However tumor regression evident in patients treated with the combination.	180
<b>Chemotherapeutic Agents</b>				
<b>Estrogen</b>	Aromatase Inhibitors	Breast cancer/ Human Phase II	Significant reduction in Tregs following letrozole and letrozole-cyclophosphamide treatments. Indirect anti-tumor mechanism of action of aromatase inhibitors through reduction of Tregs in breast tumors has been suggested	127
<b>Glycolytic inhibitor</b>	2-deoxy-D-glucose, 2-DG	Glioblastoma Multiforme as an adjuvant to radiotherapy	Phase-III clinical trials of 2-DG with hypofractionated radiotherapy in cerebral glioma patients show negligible normal tissue toxicity, enhanced survival and significant improvement in the quality of life. Preliminary, preclinical studies suggest 2-DG directly sensitizes tumor cells and reduces immune tolerance by reducing Tregs.	42,43,117,131,136,137
<b>Ionizing radiation</b>				
<b>γ-ray irradiation</b>	Anti-CD25 mAb or anti-CTLA-4 or Stat3 inhibitor + γ-ray irradiation	Head and Neck Cancer/ mice	CTLA-4 mAb did not produce any enhanced tumor reduction but anti-CD25 mAb or STAT3 inhibition resulted in reduction of Tregs and enhanced tumor eradication.	148

leads to severe autoimmune disorders in mice and humans.<sup>44</sup> Foxp3 gene locus contains several conserved noncoding sequences (CNS), which are involved with different signaling pathways.<sup>45</sup> CNS0 is involved in the activation of Treg-specific super-enhancers involved in Foxp3 expression. CNS1 regulates TGF-β-mediated Foxp3 expression in the periphery. It has a binding site for a nuclear factor of activated T cells and activator protein 1 that regulates TGF-β signaling. CNS2 has binding sites for transcription factors like STAT5, RUNX, cAMP response element-binding protein and is stimulated by TCR expression and IL-2, maintaining a high stability and activity of Foxp3 in Tregs.<sup>44,45</sup> CNS3 has binding sites for c-Rel and other transcription factors that mediate expression of Foxp3 during Treg differentiation. Epigenetic regulation of these CNS, mainly demethylation, is important for Foxp3 expression during Treg differentiation.<sup>46-53</sup> Thus, genetic and epigenetic factors regulate the Foxp3 level thereby play an important role in Treg levels and activity. Further, a highly dynamic T cell metabolism has a tremendous impact on the ability of T cells to grow, activate and differentiate. Recent observations suggest that Teff cells and Tregs require completely distinct metabolic pathways for their proliferation and activity.<sup>54</sup> T effector cells are highly glycolytic and have high Glut-1 levels on their surface.<sup>55</sup> In mice and humans, the high glycolytic rate is due to hyper-activation of the mTOR signaling pathway and is operative during nutrient-deprived conditions.<sup>54,56</sup> Contrarily, Tregs have higher metabolic flexibility compared to CD4<sup>+</sup> Teff cells. During Treg proliferation enhanced glycolysis not only provides energy in the form of ATP and NADH but also relays its metabolic intermediates to nucleotide biosynthetic pathways.<sup>54</sup> Also, during low energy conditions, Tregs rely on fatty acid oxidation (FAO), due to the increased level of carnitine palmitoyltransferase 1a (CPT1a), which is critical for FAO as a rate-limiting enzyme. This facilitates the acyl groups to enter into the mitochondria thereby suggesting that Tregs use multiple metabolic pathways needed for extensive proliferation. Apart from development and proliferation, the intra-tumoral migration of Tregs also contributes to its immunosuppressive activity. Tumors secrete several chemokines like C-C motif chemokine ligand 22 (CCL22), which is a ligand to C-C chemokine receptor (CCR) 4 present on Tregs, thereby attracting them near to the tumor tissue as observed in human ovarian cancers.<sup>52,57-60</sup> Several of these factors have been taken into consideration to develop approaches that

target the immunosuppressive Tregs. We present here the existing approaches for reducing Treg activity as well as their numbers (Figure 3).

## Approaches For Regulating Treg-Mediated Tumor Control

### Antibody-Based Treg Modulation

#### Antibody Against CD25

The alpha chain of heterotrimeric IL-2 receptor complex, CD25 has an inevitable role in both the Treg development and their activity and can, therefore, be exploited as a potential target against Tregs.<sup>22</sup> IL-2 is generally essential for proliferation of the activated T cells. Due to the upregulated expression of CD25 on Tregs, most of the IL-2 present in the milieu is utilized by the Tregs present in the vicinity, thereby preventing Teff cells from proliferation and sustained survival.<sup>61</sup> The depletion of CD25 positive Treg population via targeted antibody therapy enhanced the anti-tumor immunity that correlated well with the progressive reduction of tumor volume in murine cancer models.<sup>62,63</sup> A phase-I/II study with daclizumab (that blocks CD25) in combination with the DC vaccine has shown a transient but complete depletion of CD25<sup>high</sup> cells in melanoma patients.<sup>64</sup> Similarly, in metastatic melanoma patients (with lymphopenia induced via temozolomide), anti-CD25 antibody depleted Tregs significantly without impairing Teff cell functions, thereby augmenting the anti-tumor immune response.<sup>65</sup> Administration of daclizumab has also been found to decrease Tregs in the peripheral blood mononuclear cells (PBMCs) of patients with melanoma.<sup>66</sup> Since, CD25 is also present on the Teff cells, targeting Tregs with CD25 blockade is also expected to collaterally deplete Teff cells due to IL-2 deprivation, which may lead to opportunistic infections in patients receiving the treatment.<sup>67</sup> Furthermore, daclizumab has a short half-life of 20 days and therefore has a transient effect, which is reversible under in-vitro conditions.<sup>61</sup> Other limitations of CD25 blockade include side effects such as severe acute hypersensitivity reactions, cytokine release syndrome, infections, and local skin reactions.<sup>68</sup> Daclizumab marketed for relapsed multiple sclerosis has been withdrawn from the market due to deadly episodes of encephalitis and meningoencephalitis.<sup>69</sup> Interestingly, it has been reported that treating metastatic melanoma patients with anti-CD25 RFT5-SMPT-dgA (IMTOX25) leads to a significant but transient decrease in Treg numbers. However, the desired anti-tumor activity was not

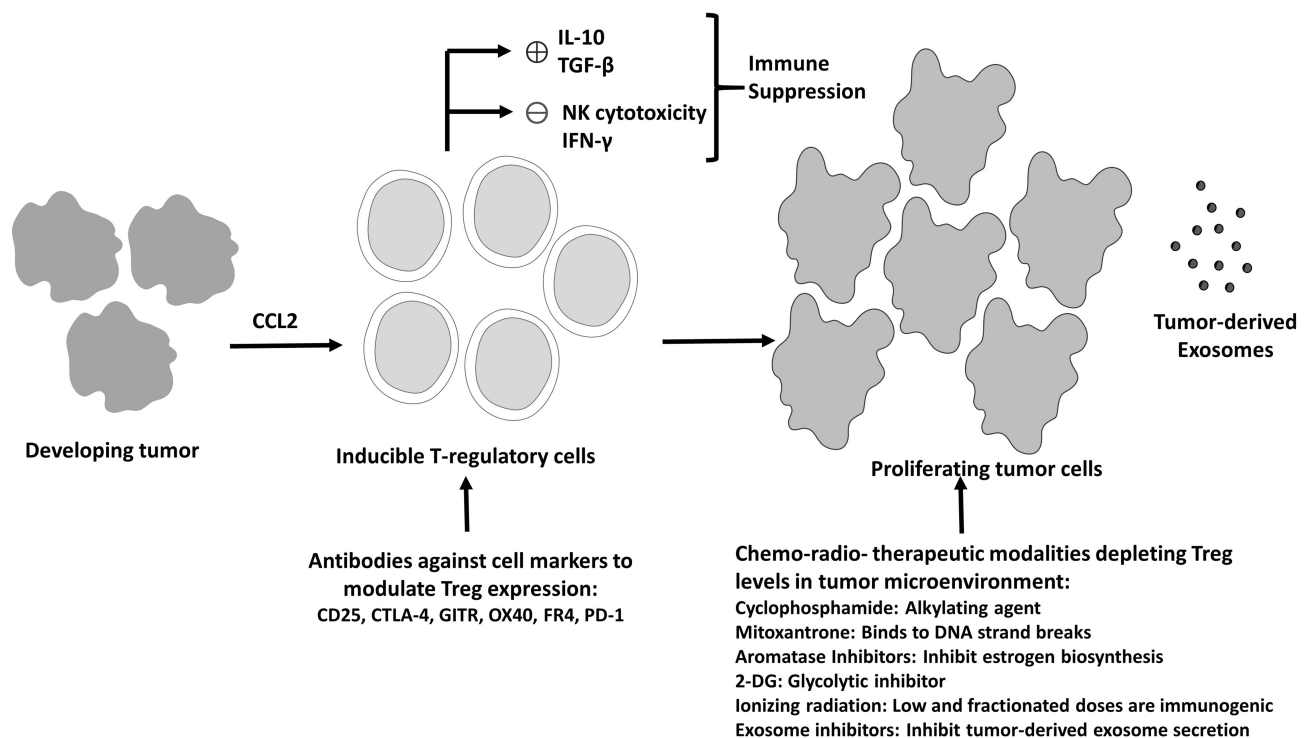
observed.<sup>70</sup> Despite the preliminary success, many questions still need to be addressed before developing a successful therapeutic strategy exploiting CD25 as a potential target for Tregs depletion.

#### Antibody Against CTLA-4

CTLA-4 is constitutively expressed in Treg cells but only upregulated in conventional T cells after activation, a phenomenon which is particularly notable in cancers.<sup>71</sup> CTLA-4 plays an indispensable function in blocking CD28 (a co-stimulatory receptor on Teff cells) interaction with B7 that is present on antigen-presenting cells (APCs).<sup>72,73</sup> The expression of CTLA-4 increases after its binding with B7, delivering a signal that suppresses the proliferation of T-cells.<sup>74</sup> Another aspect of CTLA-4 function, which hinders effective anti-tumor immunity is an increase in the levels of IDO in DCs. This consequently leads to the metabolism of tryptophan, producing kynurenines and picolinic acid, thereby abrogating the proliferation and function of Teff cells.<sup>75</sup> Possibly Tregs exploit this property of CTLA-4 for implementing immunosuppression as they constitutively express CTLA-4.<sup>76,77</sup> Synergistic effects of antibodies against CTLA-4 in combination with other anti-tumor therapeutics are well documented in the recent years.<sup>78,79</sup> CTLA-4 deficiency results in lymphoproliferation followed by splenomegaly, development of autoimmune diseases and increased IgE secretion in Treg-specific Foxp3<sup>+</sup> CTLA-4 deficient mice, similar to Foxp3 deficient mice.<sup>33</sup> Thus, inhibition of CTLA-4 has been found to increase the immune response against pre-established tumors as well as to effectively suppress a secondary tumor challenge i.e. to enhance memory response.<sup>80</sup> Co-culturing Tregs with T cells that lack CTLA-4 shows that the absence of CTLA-4 abrogates the immunosuppressive activity of Tregs.<sup>72</sup> Treatment of patients having metastatic melanoma with anti-CTLA-4 in a Phase-III clinical trial has shown improved overall survival (OS) with ipilimumab (3 mg/kg), although adverse immune effects were observed at higher doses.<sup>34</sup> Similarly, in another clinical trial, targeting CTLA-4 was found to improve the OS but was associated with undesirable toxicity such as autoimmune response (mostly inflammatory bowel disease).<sup>81</sup> At present, it is unclear if anti-CTLA-4 treatment enhances anti-tumor immunity by inhibiting the functions of the Tregs or by increasing Teff cells activity and needs further investigations.

#### Antibody Against Programmed Death Protein 1 (PD-1)

PD-1, a coinhibitory receptor is present on CD4<sup>+</sup>, CD8<sup>+</sup> T cells, and B cells, activated by PDL-1 and PDL-2 ligands. Blocking anti-PD1 antibodies have durable anti-cancer



**Figure 3** Current Treg therapeutics targeting cancer. Treg cells mediated immunosuppression orchestrated by immunosuppressive mediators like IL-10 and TGF- $\beta$  leads to reduction in antitumor immunity (IFN- $\gamma$  production and NK cells induced tumor cell cytotoxicity), resulting in excessive tumor cells proliferation. Several strategies such as antibodies targeting the important molecules for Treg activity or small molecule inhibitors, ionizing radiation and exosome inhibitors, which reduce the Treg cell number and activity represent the currently available modalities for Treg depletion and enhanced anti-tumor activity.

effects and been approved for ipilimumab resistant metastatic melanoma, non-small cell lung cancer (NSCLC), metastatic renal cell carcinoma (RCC), Hodgkin's Lymphoma (HL), metastatic urothelial carcinoma, metastatic colorectal cancer.<sup>82</sup> High PD-1 expression in Tregs is of paramount importance due to their role in blocking CD8<sup>+</sup> mediated anti-tumor immunity.<sup>21,83</sup> High PD-1 expression on Tregs resulted in the suppression of CD8<sup>+</sup> T cell function in various organs like lung, spleen and draining lymph nodes of prostate tumor-bearing mice.<sup>84</sup> Further, PD-1 increases the affinity of Tregs to TGF- $\beta$ -mediated signals and induces the differentiation of naive T cells into Treg cells.<sup>85</sup> However, the relationship between PD-1 expression and Treg is still not well understood as a large subset of patients in clinical trials did not respond to PD-1 blockade. The anti-PD-1 antibody (nivolumab) showed an excellent 18–28% objective therapeutic response in patients with advanced non-small-cell lung carcinoma, melanoma, and renal cell carcinoma.<sup>86</sup> Interestingly, patients that were cured and showed no signs and symptoms of the disease exhibited high Treg levels after nivolumab therapy while relapsing patients did not.<sup>21,87</sup> The differential response was attributed to high

pSTAT3 (phospho-signal transducer and activator of transcription) level and IL-10 production following nivolumab.<sup>87</sup> Thus, the relationship between anti-PD-1 blockade based immunotherapy and Treg levels need extensive investigation in cancer patients including their response to combinational therapies involving inhibitors of immune checkpoint (agents that inhibit the activity of proteins involved in the suppression of anti-tumor immunity like PD-1 and CTLA-4).<sup>88</sup>

### Antibody Against GITR

GITR, (glucocorticoid-induced TNFR family related gene) is a member of the TNFR superfamily (TNFRSF) and a co-stimulatory molecule that is expressed in resting CD4<sup>+</sup> and CD8<sup>+</sup> population and increases in expression upon T-cell activation.<sup>89</sup> It has also been shown that GITR is expressed constitutively at a high level in Tregs<sup>36,90</sup> and treatment with anti-GITR agonistic mAb reduces the suppressive activity of Tregs.<sup>91</sup> A study in GITR knockout mice, which lack GITR signaling, reported that the responder CD4<sup>+</sup>CD25<sup>-</sup> population becomes resistant to the suppressive CD4<sup>+</sup>CD25<sup>+</sup> population.<sup>91</sup> Furthermore, the use of anti-GITR mAb has been shown to elevate CD4<sup>+</sup> and CD8<sup>+</sup> T cell response against tumor cells, an effect

especially seen in large tumors.<sup>88,92</sup> A combination treatment with anti-GITR antibody and tumor antigen stimulation augments the proliferation of “antigen-primed” Teff cells and furthermore makes them resistant to CD4<sup>+</sup>CD25<sup>+</sup> Tregs mediated-suppression.<sup>38</sup> A humanized anti-GITR mAb, TRX518, which is under clinical trials, has shown encouraging results by augmenting immune responses against tumors in murine cancer models.<sup>93</sup> Due to a relatively ubiquitous prevalence of GITR on T lymphocyte population including on Teff cells, a local administration of anti-GITR mAb to target Tregs at the site of tumor may be the preferred method of treatment since an intravenous administration may lead to increased autoimmune response.<sup>94</sup> In total, anti-GITR therapy is a promising candidate for cancer immunotherapy through depleting Tregs but further investigations for clinical response, dosage, and use in combination therapy as well as normal tissue toxicity are needed.

### Antibody Against OX40

OX40 (CD134), is a co-stimulatory molecule that is expressed transiently on activated T-cells and constitutively on CD4<sup>+</sup>CD25<sup>+</sup> Tregs.<sup>95–98</sup> Agonistic mAb against OX40 has been shown to reduce CD4<sup>+</sup>CD25<sup>+</sup> Tregs-mediated immunosuppression.<sup>37,99</sup> An intra-tumoral injection of anti-OX40 mAb induces tumor rejection in mice, an effect abrogated by CD8<sup>+</sup> T cell depletion.<sup>99</sup> Further, the activity of CD25<sup>+</sup>CD4<sup>+</sup> Tregs and CD25<sup>-</sup>CD4<sup>+</sup> cells is altered in OX40 deficient conditions. Moreover, intra-tumoral anti-OX40 injection induces the migration of infiltrating DCs to draining lymph nodes and deploys a population of newly-formed cytotoxic T-lymphocytes (CTLs) that are tumor-specific.<sup>99</sup> Recently, an intra-tumoral injection of agonist OX86 mAb that stimulates OX40 significantly reduced the immunosuppression mediated by the Tregs thereby increasing an activation of Teff cells in a murine cancer model.<sup>100</sup> Moreover, the tumor-infiltrating Tregs produced less IL-10 in response to the treatment, besides resulting in DC maturation (in-vitro) and DC migration (in-vivo), leading to augmented tumor immunity. Administration of anti-OX40 fusion protein OX40-Fc has been found to result in the regression of murine sarcoma, supporting this proposition.<sup>101</sup> The combined use of cyclophosphamide and anti-OX40 mAb has shown a significant increase in immune responses against tumors by depleting Tregs in tumor vicinity and simultaneously inducing an influx of CD8<sup>+</sup> Teff cells in B16 melanoma murine models.<sup>37</sup> Interestingly, combination therapy increased the number of

peripheral Tregs but depleted intra-tumoral Tregs, thereby suggesting that tumor regression largely relies on the depletion of Tregs present in the tumor microenvironment (TME).<sup>37</sup> However, another study using a combination therapy with checkpoint inhibitor antibodies (Abs) like PD-1 showed a negative effect on the anti-tumor activity of OX40 agonist Ab, emphasizing the need for the appropriate design of immunotherapy combinations.<sup>102</sup> Thus, anti-OX40 therapy enhances the anti-tumor immunity by altering the suppressive function of CD25<sup>+</sup>CD4<sup>+</sup> Tregs although further investigations to establish it as an immunotherapeutic modality are still needed.

### Antibody Against FR4

Folate receptor4 or FR4 is a characteristic marker of Rodent Tregs, which express a high level of this receptor. Naïve T cells require folate for protein and nucleic acid synthesis.<sup>103</sup> T cell receptor (TCR) stimulation upregulate the expression of FR4 by several folds in Foxp3<sup>+</sup> Tregs as compared to Foxp3<sup>-</sup> T cells.<sup>104</sup> Consequently, FR4 molecule can be targeted in anti-FR4 mAb based therapy for specifically depleting suppressive Treg population to promote tumor immunity. However, folate is an important ingredient for proliferation of cells and is, therefore, an essential nutrient.<sup>105</sup> Therefore, research on the human counterpart of rodent FR4 is necessary to determine its potential as a target for cancer immunotherapy.

### Chemotherapeutic Agents

Several small-molecule chemotherapeutic agents and adjuvant have been found to exert an off-target effect on Tregs that correlate well with their overall anti-cancer efficacy. These include both DNA targeting drugs as well as those that target the biosynthetic pathways or metabolic reprogramming of tumors. In the case of some drugs, the effects on Tregs have been observed at doses significantly lower than the doses used for affecting the primary targets.

### Cyclophosphamide

Cyclophosphamide is a DNA crosslinking agent and a widely used anti-cancer drug.<sup>106,107</sup> Cyclophosphamide decreases the immune system response and is also used in the treatment of autoimmune disorders such as granulomatosis with polyangiitis.<sup>108</sup> Cyclophosphamide shows a differential dose-response concerning immune response, where a high dose required for effective anti-tumor chemotherapy may be associated with immunosuppression while a low dose has been demonstrated to show increased



anti-tumor immunity in mice models and metastatic melanoma patients.<sup>109,110</sup> The low dose immune-stimulatory property of cyclophosphamide was first realized when the regression was observed in a cyclophosphamide-resistant lymphoma (L5178Y) in mice.<sup>111,112</sup> Many studies have demonstrated that cyclophosphamide depletes Tregs in normal<sup>109</sup> and tumor-bearing mice as well as in rats-bearing chemically induced colon cancer (PROb).<sup>25,106,113</sup> However, cyclophosphamide does not specifically deplete Tregs (CD4<sup>+</sup>CD25<sup>+</sup>), since it also reduces T cells (CD4<sup>+</sup>CD25<sup>-</sup>).<sup>114</sup> Interestingly, continuous administration of cyclophosphamide at low doses has been shown to inhibit the renewal of Tregs in multiple myeloma-bearing mice and restore the anti-tumor immunity thereby prolonging the overall survival and preventing the tumor-recurrence.<sup>115</sup> We demonstrated that lower doses of cyclophosphamide enhanced radiosensitization by a glycolytic inhibitor 2-DG that could be linked to its ability to deplete iTregs.<sup>116,117</sup> Reduced Treg infiltration with an increase in CD8<sup>+</sup> T cells has been detected in patients with metastatic carcinoma that were treated with cyclophosphamide and immunotherapy (intra-tumor BCG).<sup>118</sup> Recent studies have shown that it is the CD8<sup>+</sup> resident DCs that activate Tregs, and the anti-tumor response triggered by cyclophosphamide is due to selective depletion of the CD8<sup>+</sup> resident DCs.<sup>119</sup> However, selective depletion of CD8<sup>+</sup> resident DCs by cyclophosphamide might compromise the immunity of host when exposed to an infectious pathogen as CD8<sup>+</sup> resident DCs serve as APCs for exogenous antigens. In a phase-II trial, progression-free survival was shown in more than 50% of the ovarian cancer patients treated with a combination of bevacizumab and low dose cyclophosphamide (50 mg/day), while trials in hepatocellular carcinoma patients using a low dose of cyclophosphamide impaired Treg suppression and unmasked the Teff cell response against  $\alpha$ -fetoprotein in 6 out of 13 patients.<sup>120,121</sup> The capability of cyclophosphamide to inhibit the suppressive functions of Treg cells, leading to enhanced tumor immunity, entitles it to be an effective chemotherapeutic agent or an adjuvant.<sup>107</sup>

### Mitoxantrone

Mitoxantrone is a DNA binding anthracenedione that binds to deoxyribose sugar leading to a strand break.<sup>122</sup> Another marked effect of mitoxantrone, which augments anti-cancer immunity, is through cell surface expression of calreticulin in cancer cells, which in turn promotes phagocytosis of dying cancer cells through DCs.<sup>123</sup> Treatment of

breast cancer patients with mitoxantrone does not seem to significantly change the CD4<sup>+</sup>/CD8<sup>+</sup> ratio, but lead to depletion of B lymphocytes and Treg population.<sup>40</sup> Therefore, mitoxantrone can be combined with other modalities to formulate an effective immunotherapeutic regimen for cancer treatment. However, it should be noted that these effects of mitoxantrone could be non-specific and therefore requires further investigations before it can be used for cancer immunotherapy.

### Aromatase Inhibitors

The aromatase enzyme plays a key role in estrogen biosynthesis. Estrogen has been attributed to the promotion of immune tolerance by augmenting Treg proliferation in humans and mice.<sup>124,125</sup> It is well established that Tregs infiltration in tumors results in a shorter survival in breast cancer patients.<sup>126</sup> Therefore, the blocking of estrogen receptor  $\alpha$ -signaling by aromatase inhibitors can be exploited as a therapeutic strategy, especially in estrogen-dependent breast cancer.<sup>126</sup> In a phase-II trial, 83 patients with breast cancer were treated with letrozole, an aromatase inhibitor. The results showed that reduced infiltrating Treg numbers in biopsies correlate well with higher immune response in patients.<sup>127</sup> Furthermore, in a controlled phase-II trial, letrozole-treated patients with breast cancer showed a marked reduction of tumor-infiltrating Tregs in the primary tumor post-therapy. However, combining cyclophosphamide with letrozole did not further affect the frequency of Tregs in the tumor.<sup>127</sup> Furthermore, adverse effects on cognitive functioning have been observed in patients administered with letrozole.<sup>128</sup> Thus, these evidences suggest that aromatase inhibitors cause immunomodulatory events resulting in a targeted decrease in proliferation of tumor infiltrating Tregs and therefore have immunotherapeutic potential but their toxic effects need further consideration.

### 2-Deoxy-D-Glucose (2-DG)

Metabolic reprogramming (MRP) has emerged as one of the vital features of tumors, wherein the tumor cells reprogram their metabolism by switching to glycolysis despite adequate oxygen rather than the much more efficient oxidative phosphorylation pathway, which is known as the Warburg effect.<sup>129</sup> MRP purportedly helps in the survival and growth of tumor cells by augmenting energy production, macromolecular (DNA, protein etc.) synthesis and defense against oxidative stress. By targeting this phenotype, among the various glycolytic inhibitors, 2-DG is well established for its cytotoxic and

radio/chemo-sensitizing effects on the cancer cells, including limited clinical trials.<sup>130,131</sup> Multiple mechanisms underlying this sensitization by 2-DG have been elucidated, which include energy (mainly ATP) crunch, altered anti-oxidant defense, enhanced unfolded protein response (UPR), inhibition of DNA repair, impaired cell cycle regulation, altered calcium influx, and apoptosis.<sup>132</sup> Studies in murine tumors have shown enhanced tumor regression and tumor-free survival following treatment with 2-DG and radiation.<sup>133,134</sup> Moreover, in phase-I-III clinical trials, negligible normal tissue toxicity, enhanced survival and significant improvement in the quality of life has been observed in cerebral glioma patients following administration of 2-DG with hypofractionated radiotherapy.<sup>40,133–137</sup>

Results from murine studies have shown that the response of the tumors to the combined treatment of 2-DG and irradiation is heterogeneous with complete (cure; tumor-free survival) and partial (growth delay) response, which correlates well with the immune stimulation, switch of Th2 cells to Th1 cells and a significant reduction in Treg numbers in spleen, peripheral blood, lymph node and tumor.<sup>133,134,138</sup> However, significant effects on Treg cells was not observed in the non-tumor bearing mice, suggesting a differential effect of 2-DG on Tregs in normal and tumor-bearing mice. Recently, glycolysis has been shown to regulate the induction of Tregs (iTregs) by modulating the expression of FOXP3 exon2 splice variants.<sup>139</sup> Moreover, several studies have shown that 2-DG protects the normal cells from radiation- and chemotherapy-induced damage.<sup>140</sup> Optimal TCR stimulation of CD4<sup>+</sup> T cells in the presence of glycolysis results in the autocrine secretion of IL-2 and generation of a limited number of Tregs in healthy conditions.<sup>141</sup> On the other hand, suboptimal stimulation of TCR in CD4<sup>+</sup> T cells results in an enhanced Treg activity via the generation of Foxp3-E2 isoform.<sup>140</sup> Treatment with 2-DG or impaired glycolytic conditions such as relapsed, remitting multiple sclerosis reduces the abundance of Foxp3-E2 isoform, necessary for the Treg function.<sup>139</sup> Since suboptimal TCR engagement occurs in most tumors, 2-DG appears to be a good component for the combination therapy. Thus 2-DG directly sensitizes tumor cells by compromising the energy dependent repair and recovery processes, and also reduces immune tolerance by reducing Tregs. Although, 2-DG is a well-known adjuvant for radio- and chemotherapy of cancer, further studies are required to assess its combination with immunotherapies.<sup>132</sup>

It is likely that other anti-cancer therapeutics, besides the one discussed above may also have effects on Tregs, which may partly contribute to their overall efficacy. However, considering them as immune targeting agents is less realistic compared to other immune modulators like checkpoint inhibitors such as anti-PD-1 and anti-CTLA-4 antibodies. Besides, several such agents have shown non-specific effects on Tregs and need further investigations before they can be used to target Tregs.

## Effects Of Ionizing Radiation On Tregs

Radiation enhances anti-tumor immunity which is associated with alterations in tumor cell phenotype and increased activity of immune cells like CD4<sup>+</sup>, CD8<sup>+</sup> T cells, macrophages, APCs, NK cells, etc. Irradiation of tumors releases tumor-specific antigens leading to the immune recognition of tumor-related new peptides by increasing major histocompatibility complex-I molecules (MHC-I), besides up-regulating the expression of Fas antigen, to activate the T-cell mediated cytotoxicity.<sup>12,142,143</sup> Radiation up-regulates the expression of various adhesion molecules like vascular cell adhesion protein 1 (VCAM-1), E-selectin, and intercellular adhesion molecule 1 (ICAM-1) on endothelial cells, enhancing the leukocyte adhesion and migration, leading to alterations in the TME.<sup>144</sup> Contrarily, radiation enhances the proliferative capacity and activity of Tregs at therapeutic doses leading to immunosuppression and tumor relapse. Recent studies suggest that the major mechanism involved in radio-resistance of Tregs is intrinsically through the high expression of GITR and extrinsically through radiation-induced production of TGF- $\beta$ ; an important cytokine needed for Treg proliferation and activity.<sup>145,146</sup> Stereotactic radiotherapy has been suggested to enhance the functionality of Treg cells in the TME, which is independent of TGF- $\beta$  and IL33 thereby indicating the involvement of multiple mechanisms in Tregs radioresistance.<sup>147</sup> Moreover, Tregs show a differential response to a low and high dose of radiation. CTLA4 was found to be upregulated at a low dose (1.8 Gy) and decreased at high dose (30 Gy) suggesting low dose enhanced Treg cell proliferation and activity whereas high dose abolished the suppressive capacity of Tregs attributed to increased apoptosis and pro-apoptotic protein Bax.<sup>12,146</sup> Tregs have been considered as a major hurdle in realizing the efficacy of radiotherapy and therefore Treg cell-based immunotherapy is recommended in combination with radiation to achieve maximum therapeutic gain. STAT3 inhibition in combination with radiation has been

recently shown to enhance delay in the tumor growth, which is accompanied by a decrease in Tregs, MDSCs and M2 macrophages while an increase in the effector T cells and M1 macrophages in an orthotopic mouse model of Head and Neck Cancer.<sup>147</sup> Blockade of CD25 results in better local tumor control with enhanced T cell-mediated immune responses against the tumor, whereas blockade of CTLA-4 in combination with radiation does not appear to be effective.<sup>148</sup> Taken together, a greater caution must be exercised during planning strategies aimed at combining radiation with Treg-targeting agents.

## Other Approaches

Exosomes secreted by several cell types contain many inter-cellular communication-related molecules, including components of the immune network, facilitating the communication among various immune cells.<sup>149,150</sup> Tumor-derived exosomes contribute to increased status of Tregs through their surface-bound TGF- $\beta$ 1 that mediates Foxp3 expression and the associated suppressive functions in the malignant effusions.<sup>151,152</sup> Attenuating the impact of the tumor-derived exosome or controlling their release has been suggested as an immunotherapy strategy in the management of advanced cancer with malignant effusions.<sup>152</sup> Since exosomes can contain part of the overall genomic DNA and proteins, targeting them or their effects may have far-reaching consequences, besides Treg related effects.

## Limitations Of Approaches Targeting Tregs

Impact of Tregs on anti-tumor immunity can be reduced by their depletion, interference in their trafficking into tumors, or attenuating their differentiation thereby provoking or enhancing anti-tumor immunity, through the development of tumor-specific effector cells. However, the complete ablation of CD25<sup>+</sup>CD4<sup>+</sup> Treg population disturbs natural self-tolerance and leads to several chronic and destructive autoimmune disorders.<sup>146–161</sup> Freshly isolated or ex-vivo expanded donor-derived Tregs have been shown to delay or even prevent graft-versus-host disease (GVHD) in murine models of allogeneic bone marrow transplantation, whereas the depletion of Tregs in the transplant results in increased severity of acute GVHD.<sup>162–165</sup> Depletion of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells boosts anti-tumor immunity,<sup>30,62,63,166</sup> enhances immune responses against invading pathogens, triggers allergic responses to environmental substances and causes autoimmune disorders or uncontrolled pathological immune

responses and breaches fetomaternal tolerance during pregnancy.<sup>167</sup> More recently apoptotic death of Tregs has been shown to enhance immune suppression through the A<sub>2A</sub> pathway that is triggered by adenosine (generated from the ATP released by apoptotic cells).<sup>168</sup> This suggests that approaches that lead to functional inactivation of Tregs may be more promising in achieving better tumor control and therapeutic gain since eliminating or depleting Tregs by inducing apoptosis may be counterproductive.

## Conclusions

Taken together, existing and emerging evidence suggests that the depletion of Treg cells via targeted therapy as well as by chemo-radio-therapeutic modalities result in enhanced anti-tumor immunity and therapeutic outcome. However, these modalities also have normal tissue toxicity as the same target protein or receptor is also shared by other effector T cells. Moreover, their depletion below a particular level may result in the generation of autoimmunity. Therefore, targeting of Tregs induced by tumors should be thoroughly investigated and the threshold of depletion should be carefully taken into consideration. Our recent results suggest that metabolic inhibitors like 2-DG, which can reduce the impact of Tregs with negligible effects on the normal cells, may demonstrate higher success as adjuvants to immunotherapy/radiotherapy to overcome immune suppression by Tregs. Thus, the Treg chariot that the tumor cells ride is a pertinent cellular target in the battlefield of cancer but needs to be cautiously maneuvered.

## Abbreviations

2-DG, 2-deoxy-D-glucose; APCs, antigen-presenting cells; CD, cluster of differentiation; CTLs, cytotoxic T lymphocytes; DC, Dendritic cell; FAO, fatty acid oxidation; FR4, folate Receptor 4; Foxp3, Forkhead box P3; GVHD, graft-versus-host disease; IL, Interleukin; IDO, indoleamine 2,3 dioxygenase; i-Tregs, inducible Tregs; mAb, monoclonal antibody; NKT, natural killer T cells; MRP, metabolic reprogramming; n-Tregs, naturally occurring Tregs; PBMC, peripheral blood mononuclear cells; SCCHN, squamous cell carcinoma of head and neck; TCR, T cell receptor; Teff, T-effector; TLRs, toll-like receptors; TGF- $\beta$ , transforming growth factor-beta; Tregs, T regulatory cells; VEGF, vascular endothelial growth factor.

## Acknowledgments

This work is supported by the grants INM-301 and INM-311 from DRDO, Government of India. BSD thanks Dr. Jiade Lu, and Dr. Xiaodong Wu, Shanghai Proton and Heavy Ion

Center, Shanghai, China for their encouragement and support. All authors thank Dr. Abdullah Farooque, who met with an untimely death, for his help and support during the initial conception and drafting of the review.

## Author Contributions

All authors contributed to conceptualization of the review, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of this review.

## Disclosure

The authors report no conflicts of interest in this work.

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