

## Meddling with meddlers: curbing regulatory T cells and augmenting antitumor immunity

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### ABSTRACT

CD4<sup>+</sup> regulatory T cells (Tregs) expressing the transcription factor forkhead box P3 (FoxP3) play an important role in self-tolerance and immune homeostasis. Tregs have evolved to protect the host from aberrant immune responses against self-components and collateral damages occurring in the process of defense against invading pathogens by softening immune responses. However, they turned to be a scourge in malignant tumors by not only allowing and promoting tumor growth but also suppressing effective antitumor actions, both inherent (host's immune surveillance) and extrinsic (anticancer therapy). An increase in the number of Tregs infiltrating into tumor sites and a concomitant decrease in the number of CD8<sup>+</sup> cytotoxic T lymphocytes are associated with a poor prognosis for various types of cancers, marking Tregs as notorious meddlers with an effective antitumor response. Various cancer immunotherapy approaches are often dampened by meddling Tregs, making them one of the major targets in the treatment of cancer. The recent success of immune checkpoint inhibitors (ICIs) that target immune checkpoint molecules expressed by Tregs or effector T cells implies, that “meddling with meddlers” represents an effective strategy in cancer immunotherapy. However, clinical responses to ICIs are effective and durable only in some patients with cancer, whereas more than half of them do not show significant clinical improvement. This implies that a therapeutic approach based on the use of a single ICI, or targeting Tregs alone, is insufficient, highlighting the need for combinatorial approaches. With regard to antitumor immune stimulation, several approaches, such as vaccination with peptides (or the corresponding DNA) to stimulate antigen-presenting CD8<sup>+</sup> T cells with tumor-specific neoantigens, cancer/testis antigens, or cancer stem cell antigens, that eventually boost effective cytotoxic antitumor responses are being tested. This review describes the immunosuppressive physiology of Tregs and their meddling with the host's antitumor immunity; current and prospective approaches to curb Tregs; and approaches to augment antitumor immunity.

Keywords: regulatory T cells, Foxp3, immune suppression, tumor antigens, immune checkpoints

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### INTRODUCTION

In the struggle for existence and evolution, the immune system of multicellular organisms has evolved the ability to discriminate nonself/foreign antigens (pathogens) from self/own tissue

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Received: June 19, 2018; accepted: July 2, 2018

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antigens as a defense strategy. For example, evolutionarily ancient toll-like receptors on leukocytes evolved to nonspecifically bind common bacterial, viral, and fungal molecules, leading to the activation of the host's innate and (later in evolution) adaptive immune responses. However, successful survival and biological prosperity required defense mechanisms with higher precision and resulted in the emergence of the adaptive immune system with its diverse T-cell receptors (TCRs), major histocompatibility complexes (MHCs), and antibodies, which allowed for a more precise distinction between friend (self/own tissue) and foe (nonself/foreign antigens). As a counterweight to such evolutionary advances, multicellular organisms have co-evolved safety mechanisms that would aim at minimizing aberrant immune responses against self-components and collateral damages occurring in the process of defense against invading pathogens by softening immune responses.

Similar to pathogens, the presence of tumor cells in an organism is tightly controlled by the immune system, both innate and adaptive. Immune cells continuously survey MHC/peptide complexes on all nucleated cells to detect and eliminate potentially dangerous cells. Reportedly, in addition to tumor-specific antigens (TSAs), which are either oncogenic viral or abnormal proteins resulting from gene alterations (neoantigens), some tumor-associated antigens (TAAs) derived from self/own normal tissues are, in fact, recognized by the host's tumor-reactive lymphocytes.<sup>1-3</sup> This implies that tumor immunity is, to a certain degree, a type of autoimmunity, which results in triggering safety mechanisms that maintain immunological self-tolerance and downplay effective antitumor immunity.<sup>4</sup>

## REGULATORY T CELLS

Regulatory T cells (Tregs) are a subset of CD4<sup>+</sup> thymic lymphocytes that play a central role in maintaining self-tolerance and immune homeostasis by suppressing a wide range of physiological and pathological immune responses against self and foreign antigens, including those derived from tumors.<sup>5,6</sup> Tregs were originally identified as CD4<sup>+</sup> T lymphocytes expressing high levels of CD25 (interleukin-2 [IL-2] receptor  $\alpha$ -chain)<sup>7</sup> and were shown to possess potent immune suppressive properties.<sup>8,9</sup> CD25<sup>+</sup>CD4<sup>+</sup> Tregs are characterized by expression of the master regulatory transcription factor Foxp3 (forkhead box P3), which is an absolute requirement for their differentiation in the thymus.<sup>10-12</sup> Tregs expressing Foxp3 are essential for self-tolerance, demonstrated by the fact that Foxp3 deficiency results in fatal autoimmune disorders.<sup>13-16</sup> A continuous high level of Foxp3 expression is important for Treg lineage stability and functionality; it maintains the transcriptional and functional program established during Treg development and represses the production of proinflammatory cytokines by Tregs. The perturbation of *Foxp3* gene expression in mature Tregs results in an autoimmune pathology and an enhanced production of cytokines that are characteristic of proinflammatory T helper-2 (Th2) effector cells.<sup>17-19</sup> Tregs maintain self-tolerance in healthy individuals, protecting them from developing autoimmune diseases or allergies, whereas in malignancy, they often suppress effective antitumor immunity, inadvertently allowing tumor evasion and progression.<sup>20</sup>

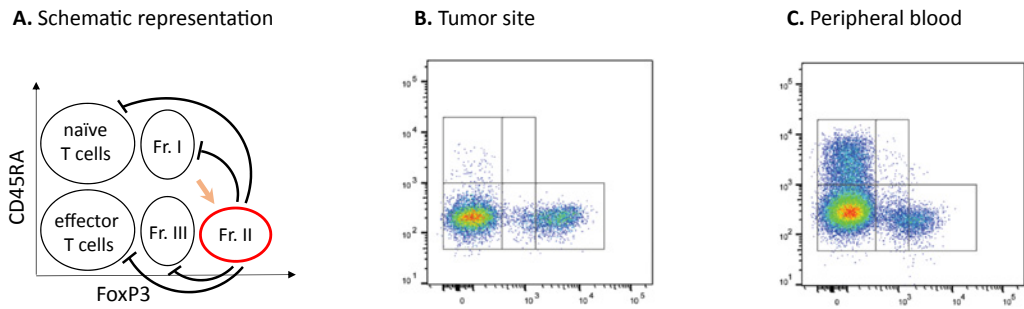
Tregs are subdivided into natural/thymic Tregs (tTreg) and induced/peripheral Tregs (iTreg) depending on their site of origin.<sup>21</sup> As their name implies, tTregs originate in the thymus, where self-antigen-primed autoreactive T cells that have a high-affinity TCR acquire expression of CD25, through which IL-2 transmits signals via STAT5 to stimulate Foxp3 expression. This spares CD25<sup>+</sup>CD4<sup>+</sup> cells from clonal deletion. Foxp3 seems to confer a survival advantage, while cells that have equivalent TCR signaling but lack Foxp3 expression are deleted.<sup>17,22-26</sup> tTregs migrate to inflammatory sites and suppress various immune cells, especially CD4<sup>+</sup> helper T cells, CD8<sup>+</sup>

cytotoxic T cells (CTLs), and CD11c<sup>+</sup> (integrin alpha L<sup>+</sup>) dendritic cells (DCs).<sup>27</sup> There are gene expression markers associated with tTregs: the transcription factors Helios, encoded by the *IKZF2* gene, and neuropilin-1, encoded by the *NRP1* gene.<sup>28-30</sup> Conversely, peripheral iTregs lack or express low levels of *IKZF2* and *NRP1* gene transcripts. The differentiation of iTregs likely occurs from conventional T cells (Tconvs) in response to nonself-antigens like allergens, food, and commensal bacteria. For example, immune tolerance to a food allergen can be induced in neonatal mice upon maternal sensitization with ovalbumin. Maternal IgG/ovalbumin immune complexes can be transferred in breast milk and presented by CD11c<sup>+</sup> DCs in the offspring, inducing ovalbumin-specific iTregs, thereby preventing food anaphylaxis, OVA-specific IgE production, and intestinal mast cell expansion.<sup>31</sup> Transforming growth factor- $\beta$  receptor (TGF- $\beta$ R) signaling appears to be necessary for Foxp3 activation in CD25<sup>+</sup>CD4<sup>+</sup> T cells.<sup>17,32,33</sup> Naturally occurring intestinal helminths of rodents and ruminant animals exploit the generation of iTregs to inhibit host immunity during a chronic infection. For example, the roundworm *Heligmosomoides polygyrus* lives in the intestine of rodents and secretes proteins (HES antigens) that bind to TGF- $\beta$ R, activating downstream signaling and inducing *de novo* Foxp3 expression in Foxp3<sup>-</sup>splenocytes. HES-induced Tregs suppress both *in vitro* effector cell proliferation and *in vivo* allergic airway inflammation.<sup>34</sup> However, interestingly, Tregs can also be converted back to proinflammatory effector Th2 cells; during *H. polygyrus* infection, a significant proportion of Th2 cells are derived from Foxp3<sup>+</sup> T cells. Such ex-Foxp3 Th2 cells exhibit characteristic Th2 effector functions and provide immunity to *H. polygyrus*, concomitantly limiting Treg-mediated suppression.<sup>35</sup>

Although FoxP3 is a master regulatory element in Tregs, it is also expressed in other cells, particularly in humans. For example, FoxP3 is upregulated following TCR stimulation in naive T cells in humans.<sup>36</sup> In addition to the thymic and peripheral Treg classification, a more functional subdivision is required, and we have proposed a classification of FoxP3<sup>+</sup> T cells based on CD45RA and FoxP3 expression that reflects the pathophysiology of autoimmune and inflammatory diseases in humans<sup>26,37</sup> (Fig. 1). CD45RA is a protein tyrosine phosphatase receptor type C (CD45), encoded by the *PTPRC* gene. Its RA isoform is located on naive T cells, making it a T-cell “naivety” marker. FoxP3<sup>+</sup>CD4<sup>+</sup> T cells can thus be divided into three groups:

- *Fraction I*: resting, naive Tregs (CD45RA<sup>+</sup>FoxP3<sup>low</sup>CD4<sup>+</sup>). These express low levels of CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and IL-7 receptor  $\alpha$ -chain (CD127) and are negative for the nuclear protein Ki-67, a cellular marker of proliferation. Naive Tregs have recently egressed from the thymus, have not yet been activated in the periphery, and possess weak immunosuppressive activity. Upon TCR stimulation, they proliferate and differentiate into effector Tregs.
- *Fraction II*: activated, **effector Tregs** (CD45RA<sup>-</sup>FoxP3<sup>high</sup>CD4<sup>+</sup>). These express high levels of CD25, CTLA-4, Ki-67, programmed cell death protein 1 (PD-1), T-cell immunoglobulin-3 (TIM-3), glucocorticoid-induced TNF receptor (GITR), the first apoptosis signal receptor (FAS), IL-10, TGF- $\beta$ , and chemokine receptor 4 (CCR4), possess strong immunosuppressive activity, and are susceptible to apoptosis. They tend to increase in peripheral blood with age.
- *Fraction III*: non-Tregs (CD45RA<sup>-</sup>FoxP3<sup>low</sup>CD4<sup>+</sup>). These produce inflammatory cytokines, such as IL-2, interferon- $\gamma$  (IFN- $\gamma$ ), and/or IL-17, and are proinflammatory cells without any immune suppressive activity.

While abundant Treg infiltration into tumor microenvironment (TME) is associated with poor clinical outcomes in various types of cancers, FoxP3<sup>+</sup> T-cell infiltration in some colorectal cancers (CRCs) is indicative of a better prognosis. The aforementioned functional classification of Tregs can explain this apparent contradiction: CRCs, which are commonly infiltrated by suppression-competent fraction II effector Tregs, also contain varying numbers of fraction III non-Tregs. The latter secrete inflammatory cytokines and are associated with a better prognosis for patients with



**Fig. 1 Classification of human Tregs based on CD45RA and FoxP3 expression (modified from ref. 26)**  
 CD4<sup>+</sup> T cells (A) are separated depending on the expression of CD45RA (T-cell naïvity marker) and FoxP3. FoxP3<sup>+</sup> T cells are further subdivided into Fraction I (CD45RA<sup>+</sup>FoxP3<sup>low</sup>CD4<sup>+</sup>): resting, naïve Tregs; Fraction II (CD45RA<sup>-</sup>FoxP3<sup>high</sup>CD4<sup>+</sup>): activated, effector Tregs; and Fraction III (CD45RA<sup>-</sup>FoxP3<sup>low</sup>CD4<sup>+</sup>): non-Tregs. Upon activation, naïve Tregs (Fr. I) differentiate into effector Tregs (Fr. II) that express high levels of CTLA-4, PD-1, Tim-3, and CCR4. Effector Tregs migrate to tumor sites (B), where they suppress other T cells (A). Unlike in tumor sites, effector Tregs are not dominant in the peripheral blood (C). Frequency of non-Tregs (Fr. III), which do not possess suppressive function varies depending on the type of cancer.

CRCs that are abundantly infiltrated with CD45RA-FoxP3<sup>low</sup>CD4<sup>+</sup> T cells.<sup>38</sup>

## MECHANISMS OF T REG-MEDIATED SUPPRESSION

Tregs mediate immune suppression by employing several mechanisms:

- Contact-dependent suppression of antigen-presenting cell (APC) maturation and as a consequence, loss of priming of CD4<sup>+</sup> helper and CD8<sup>+</sup> CTLs. CTLA-4 molecules are constitutively expressed by Tregs and compete with costimulatory CD28 molecules on CD4<sup>+</sup> helper and CD8<sup>+</sup> CTLs for binding to B7 molecules (B7-1 and B7-2, also known as CD80 and CD86) on APCs, such as DCs. Sequestration of B7 molecules by CTLA-4 inhibits APC maturation. Due to a higher affinity of the binding of CTLA-4 to B7 than of CD28 to B7, Tregs outcompete the priming, activation, and proliferation of effector T cells.<sup>27,39,40</sup>
- Secretion of inhibitory cytokines (IL-10, TGF- $\beta$ , and IL-35) that target effector T cells, macrophages, and cancer-associated fibroblasts.
- Constitutive expression of CD25 molecules on Tregs enables them to bind to and consume IL-2, which is required for effector T-cell differentiation and survival.
- Expression of granzyme and perforin, which directly kill effector T cells.<sup>20</sup>
- Adenosine immunosuppressive signaling via A<sub>2A</sub> receptors on the surface of tumor-infiltrating effector lymphocytes and APCs. Tregs express the ectonucleotidases CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5'-nucleotidase), which hydrolyze ATP, ADP, and AMP to yield adenosine. In the TME, Tregs undergo apoptosis as a result of oxidative stress generated mainly by neutrophils. Dying Tregs release large amounts of ATP into the extracellular space, where CD39 and CD73 then mediate the conversion of ATP to immunoinhibitory adenosine.<sup>41-43</sup>

## TRAFFICKING AND FUNCTIONING OF TREGS

Tregs are attracted to and accumulate in the sites of inflammation, such as the TME, by means of chemo-attraction, with the combination of chemokine ligands and their receptors varying depending on the type of cancer.<sup>26</sup> Chemokines that attract Tregs are generated by tumor cells and tumor-associated macrophages in the TME. For example, ovarian cancer cells produce the Treg-recruiting chemokine ligand 28 (CCL28) under hypoxic conditions<sup>44</sup> and CD8<sup>+</sup> tumor-infiltrating lymphocytes (TILs) produce the CCR4-binding chemokine CCL22 that attracts Tregs to the TME. Moreover, inflammation-mediated IFN- $\gamma$  production by TILs upregulates the expression of indoleamine-2,3-dioxygenase (IDO) and programmed death-ligand 1 (PD-L1) in tumor cells, reinforcing their immunosuppressive effect.<sup>45</sup>

In the TME of mammary carcinoma FoxP3<sup>+</sup>CD25<sup>+</sup>CD4<sup>+</sup>, Tregs are a major source of receptor activator of nuclear factor- $\kappa$ B (RANK) ligand (RANKL), which stimulates RANK<sup>+</sup> breast cancer cells to metastasize to the lung.<sup>46</sup> The presence of tumor-infiltrating Tregs is associated with invasion, metastasis, and a poor prognosis. The recruitment of Tregs to a tumor is partly dependent on CCL5 (also known as RANTES), a chemokine from mesenchymal stem cells, which acts in a paracrine manner on cancer cells. The production of CCL5 is associated with higher grades of human breast cancer and metastasis. Thus, RANKL–RANK antagonists could block the pro-metastatic effect of tumor-infiltrating Tregs.<sup>46–48</sup> Tumor-infiltrating Tregs seem to be principally activated by self-antigens released by proliferating and dying tumor cells and presented to Tregs by immature myeloid DCs in a TGF- $\beta$  dependent manner.<sup>49,50</sup> Indeed, a number of Tregs specific for self-antigens (such as NY-ESO-1, TRAG-3, LAGE-1, gp100, TRP1, survivin, mucin-1, HER2/neu, CEA, EGFR, telomerase, and WT1) have been detected in various types of cancer in humans.<sup>26</sup>

## EFFECTIVE ANTITUMOR IMMUNITY

Malignant cells harboring genetic alterations translate these into polypeptides with corresponding amino acid mutations. Such mutated proteins, whether they are drivers of the malignancy or passengers, are eventually degraded to peptides and presented by the antigen-processing machinery on the surface of cancer cells via MHC class I complexes, which serve as fingerprints that permit the detection and elimination of cancer cells by the immune system. The major effectors in cancer cell eradication CD8<sup>+</sup> CTLs with cognate TCRs recognize these fingerprints when they encounter them and kill the cells. For T-cell activation, APCs in the lymph nodes must properly present the tumor antigens via MHC class I and II molecules to CD8<sup>+</sup> and CD4<sup>+</sup> lymphocytes, respectively, permitting fingerprint-specific (peptide-specific) T-cell activation, proliferation, and memory T-cell formation.<sup>51</sup> In contrast to TSAs (oncogenic viral proteins, neoantigens), which are exclusively encoded by the genome of tumor cells, TAAs are derived from wild-type self/own tissue antigens, which are subject to central tolerance. They are tolerated in most instances due to deletion of CD8<sup>+</sup> T cells with self-reacting TCRs during their early development in the thymus. Thus, lymphocytes with weak affinity for self-peptide are spared. The low avidity of self-peptide/MHC complexes for TCRs of CD8<sup>+</sup> T cells,<sup>52</sup> as well as the instability of complexes of self-peptide with MHC on APCs prior to their exposure to T cells,<sup>53</sup> could partially explain the limited clinical responses to TAA peptide vaccination. To overcome this low avidity/immunogenicity of self-antigens, genetically engineered affinity-enhanced TCRs have been developed, in which TCR affinity is modulated using mutations in specific residues within the complementary-determining regions (CDRs).<sup>54</sup> The substitution of just one or two

amino acids within native CDRs can substantially enhance the affinity of such TCRs for peptide/MHC complexes.<sup>55</sup> CD8<sup>+</sup> and CD4<sup>+</sup> T cells from the patient's peripheral blood are retrovirally transduced with the modified TCR, stimulated using anti-CD3 antibody and IL-2, and expanded *ex vivo* prior to adoptive cell transfer (ACT). In patients with synovial cell sarcomas and malignant melanomas, such T lymphocytes effectively recognize and kill cancer cells bearing the NY-ESO-1 peptide in the context of HLA-A\*0201,<sup>56</sup> however, unexpected cross-reactivity of the engineered TCRs against self-peptides can sometimes occur. For example, a high-affinity TCR directed to an HLA-A\*01-restricted MAGE-A3 peptide off-targeted an unrelated peptide derived from titin, which is expressed in striated muscles, causing cardiac muscle damage and death.<sup>57,58</sup>

Cancer cells are often characterized by epigenetic re-activation of cancer/testis (CT) genes,<sup>59</sup> the expression of which is normally limited to immune privileged sites, mostly immature germ cells of the testis (spermatogonia), fetal ovary (oogonia), and placenta (trophoblasts). To date, over 150 CT protein antigens have been identified (<http://www.cta.lncc.br>). Both mesenchymal stem cells (MSCs) and primordial germ cells are derived from embryonic epiblast and have activated CT genes, which are downregulated later in development as MSCs differentiate (into osteo-, chondro-, myo-, and adipocytes). Thus, CT genes are essential for embryonic development and multipotent stromal cell maintenance. CT genes are expressed in MSCs from various tissues,<sup>60</sup> also suggesting that cancer stem-like cells (CSCs) in tumors would express them.<sup>61,62</sup> CSCs possess stem cell properties that favor tumor maintenance, proliferation, and metastasis.<sup>63-65</sup> They represent a minor subpopulation with greater tumor-initiating, self-renewal, and differentiation abilities. CSCs are resistant to standard chemo-, radio-, and molecular targeting therapies, making their eradication critical for the cancer to be cured. CTLs can recognize CSCs in an antigen-specific manner because they express various TAAs and CT antigens.<sup>66-68</sup>

Interestingly, CT genes and other TAAs are expressed in medullary thymic epithelial cells for the induction of central tolerance.<sup>69,70</sup> Nonetheless, peptides derived from CT genes, including NY-ESO-1 and MAGE family genes, are able to elicit spontaneous humoral and cellular immune responses in patients with cancer.<sup>71,72</sup> CT antigens specifically expressed in CSCs (BORIS and DNAJB8) and their MHC-restricted epitopes have been identified and shown to induce CD8<sup>+</sup> T-cell responses,<sup>73-75</sup> but non-CT TAAs that are exclusively expressed in CSCs, have also been identified. For example, ankyrin repeat and SOCS box protein 4 (ASB4), a CSC-specific TAA, elicited CTL responses to CSCs, but not to non-CSCs in CRC. Moreover, adoptively transferred CTLs specific for the HLA-A\*2402-restricted ASB4 epitope infiltrated implanted tumors and efficiently prevented tumor growth in a mouse model. CD8<sup>+</sup> T cells of patients with CRC are not immunologically tolerant to ASB4 antigen because stimulation with the peptide elicited specific T-cell responses.<sup>76</sup> Another CSC-specific antigen, CD133, which is also used as a marker for CSC isolation, was shown to be efficiently recognized by CTLs in an HLA-A\*0201-restricted manner in glioblastoma multiforme. Finally, studies on mouse models have demonstrated immunogenicity in the absence of autoimmune damage, supporting the use of CD133-epitope vaccines to target CSCs.<sup>77</sup>

Various CT antigens, as well as self-peptides derived from TAAs, have been extensively used in cancer vaccine formulations in numerous clinical trials.<sup>78-81</sup> A recent proof-of-principle study showed that tumor-reactive CD8<sup>+</sup> effector T cells can be generated *in vitro* by exposure to CT antigens induced by DNA demethylation in autologous CD4<sup>+</sup> T helper cells. Upon treatment with a DNA-demethylating agent, CD4<sup>+</sup> T-cells express various CT antigens and function as APCs for autologous CTLs. In a phase I trial, 3 of the 25 patients with glioblastoma multiforme showed stable tumor regression.<sup>82</sup> However, in general, despite the reliable induction of T-cell responses, vaccination approaches, so far, have had limited success in inducing objective tumor regression, with only some of the treated patients experiencing clinical benefits from vaccination.<sup>83,84</sup> In

addition to the approaches briefly described above, a number of vaccination immunotherapy strategies and vaccine delivery methods have been explored for their cancer targeting ability, including short/long peptide and protein vaccines, DNA and RNA vaccines, DC vaccines, numerous adjuvants, autologous/heterologous tumor cell lysate vaccines, and ACT of *ex vivo* expanded TAA-specific CTLs or T cells expressing chimeric antigen receptor. These approaches have been described in detail elsewhere.<sup>85,86</sup>

Conversely, peptide antigens bearing somatic mutations or novel, previously untranslated amino acid sequences (neoantigens) have been considered very promising targets because they are specific to tumor cells. During the development of central tolerance in the thymus, neoantigens are absent and therefore remain “unknown” to the immune system. This renders them highly immunogenic when they are encountered by T cells bearing cognate TCRs.<sup>87</sup> However, neoantigens are highly patient-specific and are not shared among different tumors. Furthermore, the methods for their systematic discovery and evaluation are rather cumbersome; therefore, this approach is far from routine at present.

## SUPPRESSION OF EFFECTIVE ANTITUMOR IMMUNITY BY TREGS

To avoid immune attacks, cancer cells acquire resistance and escape mechanisms for the immune system by selecting less immunogenic cells and establishing an immunosuppressive environment, permitting them to eventually develop into clinically apparent cancers. In cancer tissues, the immunosuppressive network, comprising immune suppressive cytokines, molecules, and cells, including Tregs, inhibits effective antitumor immunity, including that induced by the abovementioned vaccination approaches, which allows the cancer to progress.<sup>26,88,89</sup> In a subset of patients, an active immune response, including the infiltration of tumor sites with CD8<sup>+</sup> T cells, is detected. Nevertheless, such tumors often remain protected from immunological attacks, suggesting they can restrain an effective antitumor immune response, and Tregs seem to significantly suppress effective antitumor immune responses.<sup>89</sup> Analogous to the Treg-mediated suppression of immune responses to pathogens, in which the ratio of Treg and effector T cells can determine the outcome of an infection,<sup>35</sup> the number of Tregs infiltrating a tumor and the concomitant decrease in the CTL/Treg ratio are associated with a poor prognosis in multiple cancers.<sup>90</sup> Unlike in conventional effector or memory CD4<sup>+</sup> T cells, the TCR repertoire of thymus-derived Tregs is skewed toward self-antigens, implying that Tregs *in vivo* are constantly being activated and easily recognize TAAs, including those used for vaccination.<sup>91,92</sup> In fact, MAGE-A3 peptide vaccination results in the induction of Tregs, which suppress effector T-cell responses.<sup>93</sup> Conversely, the depletion of CD25<sup>+</sup>CD4<sup>+</sup> Tregs derived from patients' peripheral blood results in the induction of NY-ESO-1-specific effector CD4<sup>+</sup> Th1 responses.<sup>94</sup> This implies that the activation of T cells, which possess TCRs that are capable of reacting with self-TAAs, is strictly controlled by Tregs. Thus, Tregs have been the focus of extensive research aimed at curbing their effects on antitumor immunity and immunotherapy.

## APPROACHES TO OVERCOME TREG-MEDIATED SUPPRESSION

The blockade of immune checkpoint molecules such as CTLA-4 and PD-1, using monoclonal antibodies has proved to be effective even in patients with advanced cancers.<sup>26</sup> CTLA-4 is regarded as a leading checkpoint inhibitor because it restrains autoreactive T cells at an earlier stage than PD-1. Unlike Tregs, which constitutively express CTLA-4 in their resting state,

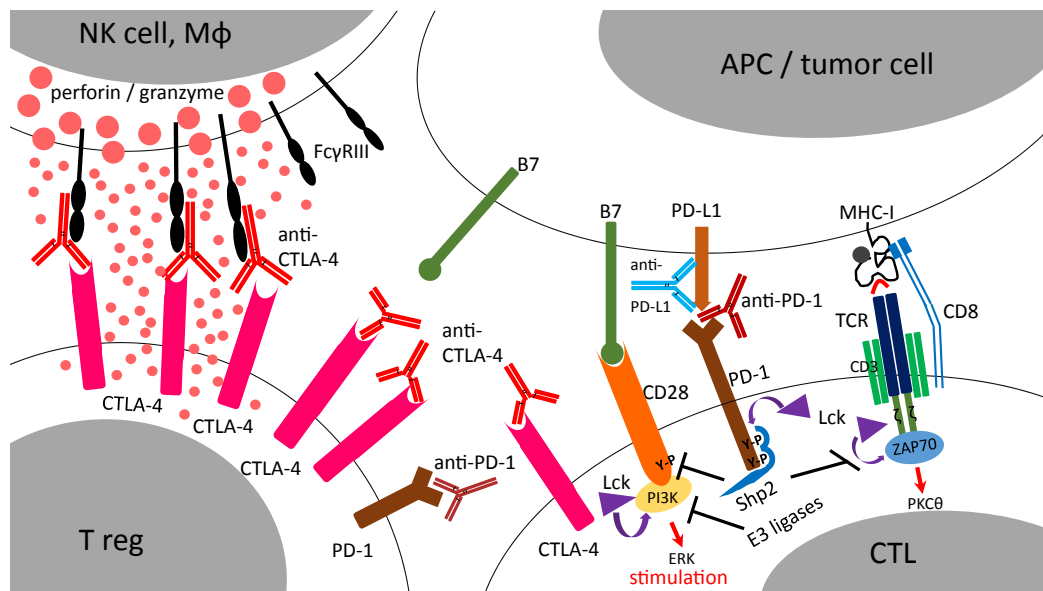
conventional T cells begin expressing CTLA-4 once they are activated.<sup>95</sup> The administration of the anti-CTLA-4 mAb ipilimumab causes therapeutic tumor rejection,<sup>96</sup> possibly mediated through the inhibition of signaling between B7 and CTLA-4, thereby releasing brakes on conventional T cells<sup>97</sup> (Fig. 2). The crystal structure analysis of the anti-CTLA-4 mAbs ipilimumab and tremelimumab and B7 showed that they all share the same binding site on CTLA-4.<sup>98</sup> However, recent animal studies using an anti-CTLA-4 mAb lacking antibody-dependent cell-mediated cytotoxicity (ADCC) activity because of an altered Fc receptor function have shown that the antitumor activity of the mAbs is mainly due to the depletion of Tregs in TME, rather than because of the disinhibition of conventional T cells.<sup>99-102</sup> Furthermore, Du, et al reported that anti-B7 antibodies that block CD4<sup>+</sup> T-cell activation and *de novo* CD8<sup>+</sup> T-cell priming in lymphoid organs do not have a negative impact on the immunotherapeutic effect of ipilimumab, possibly because such tumor rejection is independent of co-inhibitory signal blockade and is caused by Fc receptor-dependent Treg depletion.<sup>103</sup>

Another frequently targeted checkpoint molecule PD-1, is mainly expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes and B cells and interacts with the B7-family member ligands PD-L1 and PD-L2. PD-L1 is expressed by APCs and tumor cells, as well as by T and B cells, upon the activation of TCR and BCR signaling, respectively, whereas PD-L2 expression is restricted to APCs, such as DCs and certain tumors. The engagement of PD-1 on T cells by PD-L1 considerably inhibits TCR-mediated proliferation and cytokine production by CD4<sup>+</sup> T cells.<sup>104</sup> In T cells, the PD-1/PD-L1 complex can directly recruit the SH1 and SH2-domain-containing protein tyrosine phosphatases Shp1 and Shp2 to PD-1's immunoreceptor tyrosine-based inhibitory (ITIM) and immunoreceptor tyrosine-based switch (ITSM) motifs. These phosphatases, particularly Shp2, inhibit ZAP70 and PI3K signaling, terminating the downstream ERK and PKC $\theta$  pathways.<sup>105,106</sup> Indirectly, the interaction of PD-1 and PD-L1 induces the transcription of E3-ubiquitin ligases (c-CBL, CBL-b, and ITCH), which cause the polyubiquitination of CD3 $\zeta$ -chains, impairing their phosphorylation and association with ZAP70.<sup>107</sup> PI3K is ubiquitinated by E3-ligases and is also degraded.<sup>108</sup> All this leads to the removal of TCRs from the cell surface, preventing TAA recognition, and results in T-cell dysfunction.<sup>109-111</sup> Another mechanism of PD-1/PD-L-mediated suppression of T-cell activity has also been recently elucidated. The inhibition of T-cell activity is achieved by interfering with the CD28/B7-costimulatory signaling pathway, similar to CTLA-4. While CTLA-4 competitively deprives CD28 of its ligand by mechanistically binding B7 molecules (extracellular sequestration), the interaction between PD-1 and PD-L1 causes the inhibition of a series of intracellular signaling pathways in the effector T cells, leading to the downregulation of CD28 signaling (Fig. 2). Specifically, the PD-1/PD-L1 interaction was shown to induce Lck-mediated tyrosine phosphorylation on the PD-1 cytoplasmic tail, followed by the binding of Shp2, but not Shp1, to these phosphoresidues. Shp2 then interacts with CD28 and rapidly dephosphorylates it, thereby inactivating CD28 signaling.<sup>112,113</sup> Therefore, PD-1 is a potent inhibitor of T-cell activation. Monoclonal antibodies against PD-1 (nivolumab and pembrolizumab) and anti-PD-L1 mAbs (atezolizumab, avelumab, and durvalumab) have been approved and are currently in clinical use for the treatment of various types of cancer.<sup>114</sup>

Although both immune checkpoints were shown to converge in their control of the CD28/B7 interaction,<sup>113</sup> CTLA-4 and PD-1 inhibition results in distinct responses; while PD-1 blockade predominantly induces the expansion of tumor-specific CD8<sup>+</sup> TILs, CTLA-4 inhibition induces the expansion of an ICOS<sup>+</sup> (inducible T-cell costimulatory, or CD278) Th1-like CD4<sup>+</sup> effector and CD8<sup>+</sup> T cells.<sup>115</sup>

Other molecules predominantly expressed by Tregs that affect their functions and activities are also the subject of extensive research as possible therapeutic targets.





**Fig. 2 Overcoming Treg-mediated suppression. Proposed molecular mechanisms of immune checkpoint blockades**

An anti-CTLA-4 antibody prevents interaction between CTLA-4 and B7, permitting the CD28/B7 interaction and CTL activation. At the same time, Tregs in tumor tissues are depleted by ADCC: Fc-receptor (FcγRIII) on natural killer (NK) cells and/or macrophages (Mφ) binds to the Fc portion of the anti-CTLA-4 antibody, which results in the release of cytotoxic factors (perforin, granzyme) and cellular lysis. Contrary to Tregs, which constitutively express CTLA-4, effector T cells begin expressing CTLA-4 upon activation, thereby preparing to dampen its own activity in order to diminish the damage to self-components. Thus, administered anti-CTLA-4 antibody would meddle with this natural precaution mechanism and sustain the activated status of CTLs. Needless to say, such sustained activation comes at the expense of autoimmune adverse effects. Anti-PD-1 and anti-PD-L1 antibodies prevent the PD-1/PD-L1 interaction and disturb its intracellular signaling, which eventually leads to the preservation of TCR and CD28 signaling pathways crucial for the effective CTL activation (see details in the text).

**OX40 (CD134)** is a member of the tumor necrosis factor (TNF) receptor superfamily and is mainly expressed by activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In humans, tumor-infiltrating Tregs show higher OX40 expression than peripheral Tregs. Upon the activation of TCRs on TILs, OX40 is transiently expressed to transmit a potent costimulatory signal when it is bound to OX40L. Thus, OX40 blockade would inhibit undesirable inflammation and autoimmunity, whereas OX40 agonists would augment antitumor immunity.<sup>116</sup> The activation of OX40 reduces the suppressive effects of Tregs, while simultaneously, the presence of OX40<sup>+</sup> TILs is indicative of a favorable prognosis.<sup>117,118</sup> Further analyses in humans are warranted to determine the optimal method for its clinical applications.

**Glucocorticoid-induced TNF receptor (GITR)** is an immune costimulatory molecule that is upregulated in lymphocytes upon T-cell activation. GITR is constitutively expressed by Tregs; however, TILs show higher expression of GITR than peripheral lymphocytes. The activation of GITR signaling by its ligand, which is expressed by APCs and endothelial cells, reveals its role as an immune activator.<sup>119</sup> GITRL directly activates effector CD8<sup>+</sup> T cells in the TME, resulting in tumor rejection. Furthermore, when GITR stimulation was combined with TAA DNA vaccination, antigen-specific CD8<sup>+</sup> T cells became refractory to suppression by Tregs, in contrast to CD8<sup>+</sup> T cells that were induced without GITR signaling.<sup>120</sup> Thus, anti-GITR agonistic mAbs would

function by increasing the effector CTL/Treg ratio.<sup>26</sup>

**T-cell immunoreceptor with Ig and ITIM domains (TIGIT)** is a member of the CD28 family of receptors and is a novel immune checkpoint molecule. CD8<sup>+</sup> TILs often co-express TIGIT and PD-1. TIGIT was first identified and characterized as a suppressor of CD4<sup>+</sup> T-cell priming and autoimmunity. It suppresses T-cell activation by promoting the generation of mature immunoregulatory DCs, limiting natural killer (NK)-cell cytotoxicity, and enhancing the suppressive activity of Tregs. In a mouse model, the co-inhibition of TIGIT and PD-L1 was necessary to cause the rejection of large established tumors in a CD8<sup>+</sup> T-cell-dependent manner and to confer antigen-specific immunity against tumor re-challenge. Along with PD-1/PD-L1, TIGIT suppresses the effector function of CD8<sup>+</sup> TILs within the TME.<sup>121</sup> Tregs and NK cells demonstrated high levels of TIGIT expression,<sup>122</sup> and CD112 and CD155 are the TIGIT ligands expressed by APCs, T lymphocytes, and tumor cells.<sup>123</sup> TIGIT<sup>+</sup> Tregs have been shown to be more suppressive and to inhibit the differentiation and responses of Th1 and Th17 lymphocytes.<sup>95,124</sup> Thus, TIGIT is another potential candidate for use in cancer immunotherapy, alone or in combination with PD-1/PD-L1 inhibition.

**Lymphocyte activation gene (LAG-3)** is a CD4 homologue in terms of both structure and function; however, its affinity for MHC class II molecules is even higher than that of CD4 and it is upregulated after T-cell activation.<sup>125</sup> LAG-3 inhibition on Tregs abrogates their suppressive function<sup>126</sup> and CD25<sup>high</sup>FoxP3<sup>+</sup>CD4<sup>+</sup> Tregs with high LAG-3 expression accumulate at tumor sites and in the peripheral blood of patients with cancer patients.<sup>127</sup> In advanced cancer, intratumoral Tregs express even higher levels of LAG-3, along with CTLA-4 and PD-1, than Tregs in adjacent tissues and peripheral blood.<sup>128</sup> Thus, LAG-3 seems to define the activation status of Tregs with greater immunosuppressive functions. LAG-3 blockade by mAbs would thus interfere with MHC class II interaction and impair Treg functionality.<sup>129</sup>

**T cell immunoglobulin-3 (Tim-3)**, initially identified as a cell surface molecule on IFN- $\gamma$ -producing CD4<sup>+</sup> Th1 and CD8<sup>+</sup> CTLs, has also been detected on Tregs and on innate immune cells (DCs, NK cells, myeloid-derived suppressor cells, and monocytes),<sup>130</sup> in all of which it negatively regulates immune responses and promotes tolerance. Studies on Tim-3-deficient mice have shown that Tim-3 is required for the induction of antigen-specific tolerance and that its blockade results in the development of autoimmunity.<sup>131,132</sup> In chronic viral infections and cancer, Tim-3 and PD-1 are markers of dysfunctional, so-called exhausted antigen-specific CD8<sup>+</sup> T cells with severely impaired proinflammatory cytokine (IL-2, TNF- $\alpha$ , and IFN- $\gamma$ ) production. FoxP3<sup>+</sup>CD4<sup>+</sup>Tim-3<sup>+</sup> Tregs, which are enriched in inflammatory sites, have greater suppressive properties than their Tim-3<sup>-</sup> counterparts. They also co-express higher levels of other suppressive components as well (LAG-3, CTLA-4, PD-1, IL-10, FoxP3, granzyme, and perforin).<sup>133</sup> Thus, Tim-3 blockade with mAbs would affect multiple cell types, resulting in nonspecific immune responses.<sup>130</sup>

**Chemokine receptor CCR4** has been shown to be specifically expressed by a subset of terminally differentiated, predominantly tumor-infiltrating, and highly immunosuppressive CD45RA<sup>-</sup>FoxP3<sup>high</sup>CD4<sup>+</sup> Tregs in patients with malignant melanoma. Mogamulizumab is a humanized IgG1 mAb that targets CCR4 and efficiently depletes CCR4<sup>+</sup> Tregs in adult T-cell leukemia-lymphoma patients, resulting in the induction of CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells specific for the CT antigen NY-ESO-1. Currently, multiple clinical trials are underway, either using mogamulizumab as a monotherapy or in combination with PD-1/PD-L1 inhibition or the costimulatory agonistic anti-4-1-BB mAb.<sup>26,134,135</sup>

**Interleukin-2 receptor (IL-2R)** binds and responds to IL-2 binding. Low affinity dimeric IL-2R comprises two subunits, IL-2R $\beta$  (CD122) and IL-2R $\gamma$ c (CD132), whereas high-affinity trimeric IL-2R also includes an IL-2R $\alpha$  (CD25) subunit. High-affinity IL-2R is constitutively expressed on

the cell surface of Tregs. IL-2R signaling involves the activation of the intracellular JAK-STAT, MAPK, and PI3K pathways, resulting in functional T-cell activation. IL-2R agonists have been developed to potentiate IL-2 antitumor effects in malignant melanomas and breast and lung cancers,<sup>119</sup> but these would also activate Tregs. As an opposite approach, the specific depletion of Tregs using mAbs targeting the IL-2R $\alpha$  (CD25) subunit has also been used. Daclizumab is a humanized IgG1 mAb that targets CD25, but owing to its dual CTL and Treg-depleting effects, its use in anticancer therapy have yielded mixed results.<sup>26</sup> In March 2018, it was withdrawn from the market after reports that it caused encephalitis. However, another anti-CD25 mAb, basiliximab, is employed as an immunosuppressant to prevent immediate transplant rejection.

**Cyclophosphamide** is an alkylating chemotherapeutic agent used to treat cancers and autoimmune diseases. Its metabolite causes inter- and intrastrand crosslinks to form in cellular DNA, leading to apoptosis.<sup>136</sup> In clinical trials, it has been used in low doses<sup>81</sup> in combination with a multipptide cancer vaccine to deplete Tregs in patients with advanced renal cancer<sup>78</sup> and in combination with the receptor tyrosine kinase inhibitor sunitinib.<sup>26</sup>

**Adenosine A2A receptor (A2AR)** is one of the four subtypes of adenosine receptors (A1R, A2AR, A2BR, and A3R) that belong to the G protein-coupled receptor family. Adenosine signaling via A2AR activates adenylate cyclase, which uses intracellular ATP to synthesize cAMP. In the TME, higher concentrations of adenosine positively affect the recruitment, presence, and immunosuppressive function of Tregs, whereas the function of effector cells (CTLs, macrophages, APCs, NK, and neutrophils) is reduced. CD73 is widely expressed in tissues and significantly contributes to free adenosine production. Thus, both A2AR and CD73 antagonists have been developed to inhibit the adenosine signaling pathway in the TME.<sup>43,119</sup>

**Indoleamine 2,3-dioxygenase (IDO)** is an intracellular tryptophan-catabolizing enzyme that initiates the degradation of tryptophan to kynurenine metabolites. IDO is expressed in normal cells (endothelial cells in the placenta, lung, and female genital tract; mesenchymal stromal cells; fibroblasts; myeloid-derived APCs; mature DCs; and macrophages) and in various tumor cells.<sup>137</sup> IDO was originally described as contributing to peripheral tolerance and immunosuppression in pregnancy; in mice, the inhibition of IDO with 1-methyl-tryptophan causes the T-cell-mediated rejection of embryos in early pregnancy.<sup>138</sup> Through the action of kynurenine metabolites, IDO promotes the differentiation and activity of Tregs, whereas tryptophan depletion suppresses CD8<sup>+</sup> T-cell and NK-cell proliferation and induces their apoptosis. Along with PD-1/PD-L1 immunosuppression, IDO is one of the endogenous pathways used by cancer cells to induce tolerance to tumor antigens, which significantly contributes to an immunosuppressive TME.<sup>137</sup> Thus, IDO inhibitors are being investigated as potential therapeutic targets for the restoration of antitumor immunity. In clinical trials, at least two IDO inhibitors, indoximod (D-isomer of 1-methyl-tryptophan) and epacadostat, were reportedly effective when used in combination with various immune checkpoint inhibitors (ICIs) and are being clinically tested.<sup>119</sup>

## CONCLUSION

Considering that the Treg-mediated suppression of immune responses against antigens derived from ubiquitously expressed proteins results in an irreversible anergic state in CTLs but not in nonself-reactive T cells,<sup>139</sup> it may be necessary to combine both Treg attenuation and effector T-cell activation strategies for the successful immunotherapy of cancers with low levels of neoantigen. Therefore, while patients with highly immunogenic tumors harboring a large number of neoantigens can respond to monotherapy with ICIs, those with tumors harboring few neoantigens would benefit from combination therapy. Moreover, effector T-cell activation approaches should

be mechanistically optimized in the light of cancer vaccine experience. For example, vaccination with peptide epitopes of an exact length, which does not require intracellular processing by APCs, induces a temporary CTL response. Such peptides would externally bind to the corresponding MHC class I molecules on all nucleated cells, and at a high concentration, the injected peptides would occupy the corresponding MHC molecules on cells other than APCs (such as fibroblasts). In the absence of costimulatory signals, this would tolerize T-cell induction.<sup>86,140-142</sup>

Although neoantigens are very patient-specific, the use of personal neoantigen vaccines is one of the promising immunotherapeutic approaches. Recent advances and the availability of massively parallel sequencing for the detection of coding mutations, as well as advances in bioinformatic approaches for the prediction of the binding of mutated peptides to a specific HLA type, have made the discovery and evaluation of neoantigens in cancer cells more feasible and systematic. Ott, et al tested a cancer vaccine that targets up to 20 neoantigens predicted from a patient's whole-exome and RNA-sequence data. The administration of long synthetic peptides (15–30 amino acids) in combination with adjuvants successfully cured four of the six patients with malignant melanoma during a median follow-up of 25 months. The two patients who had experienced recurrence at 25 months were treated with an anti-PD-1 antibody, and they experienced complete tumor regression.<sup>143</sup> Similar results were achieved with patient-specific neoantigens encoded in RNA that were administered with or without PD-1-inhibiting therapy.<sup>144</sup>

Likewise, a combination of anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab and pembrolizumab) antibodies had a synergistic effect in the treatment of malignant melanoma, such that it was more effective than monotherapy.<sup>145</sup> In clinical trials, dual immune checkpoint blockade in patients with malignant melanoma and non-small cell lung cancer was associated with better response rates; however, this was at the expense of significant immune-related adverse effects in most of the patients.<sup>146-148</sup>

As the mechanisms of antitumor responses and their suppressions are further elucidated, molecular and functional technologies will be integrated to develop novel approaches with higher precision to manipulate the immunosuppressive TME in each patient and to augment specific antitumor responses.

## CONFLICT OF INTEREST

H.N. has received honoraria and research funding from Ono Pharmaceutical Co. Ltd., Bristol-Myers Squibb Co Ltd. and Chugai Pharmaceutical Co. Ltd., as well as research funding from Taiho Pharmaceutical Co. Ltd., Daiichi-Sankyo Co. Ltd., Kyowa-Hakko Kirin Co. Ltd., Zenyaku Kogyo Co. Ltd., Astellas Pharmaceutical Co. Ltd., Sysmex Co. Ltd., and BD Japan Co. Ltd., outside this study.

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