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Review Article

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Regulatory T Cells in Tumor Microenvironment and Approach for Anticancer Immunotherapy

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ABSTRACT

Tregs have a role in immunological tolerance and immune homeostasis by suppressing immune reactions, and its therapeutic potential is critical in autoimmune diseases and cancers. There have been multiple studies conducted on Tregs because of their roles in immune suppression and therapeutic potential. In tumor immunity, Tregs can promote the development and progression of tumors by preventing effective anti-tumor immune responses in tumor-bearing hosts. High infiltration of Tregs into tumor tissue results in poor survival in various types of cancer patients. Identifying factors specifically expressed in Tregs that affect the maintenance of stability and function of Tregs is important for understanding cancer pathogenesis and identifying therapeutic targets. Thus, manipulation of Tregs is a promising anticancer strategy, but finding markers for Treg-specific depletion and controlling these cells require fine-tuning and further research. Here, we discuss the role of Tregs in cancer and the development of Treg-targeted therapies to promote cancer immunotherapy.

Keywords: T-lymphocytes, regulatory (Treg cells); Tumor microenvironment; Immunotherapy

INTRODUCTION

Tregs have been known to function as suppressors of immune responses to self- or foreign-Ags in order to maintain immune homeostasis (1). Tregs are characterized by the expression of a master transcription factor, forkhead box P3 (FOXP3), which is critical for Treg differentiation and function, including secretion of suppressive cytokines and expression of inhibitory surface molecules (1-3). Severe autoimmune-related diseases leading to scurfy phenotype develop in mice that have the transcription factor *FOXP3* gene deleted, and humans with impaired FOXP3 suffer from immune-dysregulation, poly-endocrinopathy, enteropathy, and X-linked syndrome (IPEX), which is characterized by the development of multiple autoimmune disorders (4). Therefore, $FOXP3^+$ Tregs have attracted tremendous interest because of their essential role in maintaining immune tolerance and their therapeutic potential.

In cancer, a large population of $CD4^+FOXP3^+$ T cells infiltrates into several tumor tissues to suppress the effector functions of tumor-specific T cells (5). Therefore, the depletion of Tregs

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Abbreviations

A2AR, A2A receptor; APC, Ag-presenting cell; BACH2, broad complex-tramtrack-bric a brac and Cap'n'collar homology 2; CCR, C-C motif chemokine receptor; CNS1, conserved non-coding sequence 1; CRC, colorectal cancer; DC, dendritic cell; eTreg, effector Treg; FOXP3, Forkhead box p 3; FR, folate receptor; GITR, glucocorticoid-induced TNFR-related protein; ICI, immune checkpoint inhibitor; ICOS, inducible T-cell costimulatory; IDO, indoleamine 2,3-dioxygenase; IPEX, immune-dysregulation, poly-endocrinopathy, enteropathy, and X-linked syndrome; iTreg, induced Treg; LAG3, lymphocyte-activation gene 3 protein; LAG-3, lymphocyte activation gene-3; PTEN, phosphatase and tensin homolog; pTreg Peripheral Treg; Teff, effector T; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIL, tumor-infiltrating lymphocyte; TIM3, T cell immunoglobulin mucin receptor 3; TME, tumor microenvironment; tTreg, thymus-derived Treg; VEGFR, VEGF receptor

Author Contributions

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in the tumor microenvironment (TME) leads to anti-tumor effects via the reactivation of effector T (Teff) cells (6). Indeed, in cancer patients, FOXP3$^+$ Tregs migrate into the TME and suppress various types of effector lymphocytes, including CD4$^+$ Th cells and CD8$^+$ CTLs (7,8).

Anticancer immunotherapy, especially immune checkpoint inhibitors (ICIs), can reverse the effects of immunosuppression and revitalize dysfunctional or "exhausted" CTLs, enabling them to attack cancer cells (9,10). mAbs targeting PD-1, PD-L1, and CTLA-4 have exceptional clinical efficacy against various types of cancer (11-13). However, the efficacy of ICIs proved to be unsatisfactory in most patients, and more effective therapies are required, including combination immunotherapy.

Here, we discuss the roles Tregs play in cancer and how cancer immunotherapy can be developed by targeting Tregs for immune precision medicine.

ONTOGENIC CLASSIFICATION AND DEVELOPMENT OF TREGS

Tregs can be classified into 2 subtypes depending on the site of development (14,15). Thymus-derived Tregs (tTregs) comprise the immunosuppressive subpopulation that originates from the thymus. tTregs develop by strong interactions between the TCR of CD4/CD8 double-positive or CD4 single-positive thymocytes and self-peptide–MHC complexes in the thymus, resulting in the suppression of autoimmune reactions directed against self-Ags (16,17). Whereas thymic selection leads to differentiation of self-Ag-specific tTregs, peripheral Tregs (pTregs) induced in peripheral tissues mediate tolerance to innocuous foreign Ags not encountered in the thymus (18). Consequently, pTregs prevent inflammation directed against innocuous Ags, which are expressed by commensal microflora or dietary components. In certain environments, such as a TME, some Teff cells turn into FOXP3$^+$ Tregs in the periphery, which are termed induced Tregs (iTregs). These different subtypes of Tregs share significant similarities, such as their dependence on the activity of the transcription factors FOXP3 and broad complex-tramtrack-bric a brac and Cap'n'collar homology 2 (BACH2); however, some distinguishable features exist (19-22). tTregs overexpress helios (a member of the Ikaros family of transcription factors) and neurophilin1 (a type 1 transmembrane protein), which are involved in the immunosuppressive activity and dominant Ag recognition, whereas iTregs frequently lack or express less of these proteins(23-25). On the other hand, an intronic *FOXP3* cis-regulatory element, conserved non-coding sequence 1, harboring SMAD3 binding sites, is necessary for pTreg differentiation but is dispensable for tTreg differentiation (26). Additionally, the TCR specificity of tTregs and pTregs is distinct in many ways (18,27).

THE SUBTYPE OF TREGS CLASSIFIED BY SUPPRESSIVE FUNCTION

Tregs were initially defined as CD4$^+$ T cells with high expression of CD25, an α-subunit of IL-2 receptor. However, CD25 is a general marker of T cell activation and not exclusive to Tregs, thus emphasizing the need for additional Treg-specific markers. Although FOXP3 expression is mostly restricted to the Treg population in mice, FOXP3$^+$ T cells in humans possess heterogeneous properties in terms of their phenotype and immunosuppressive

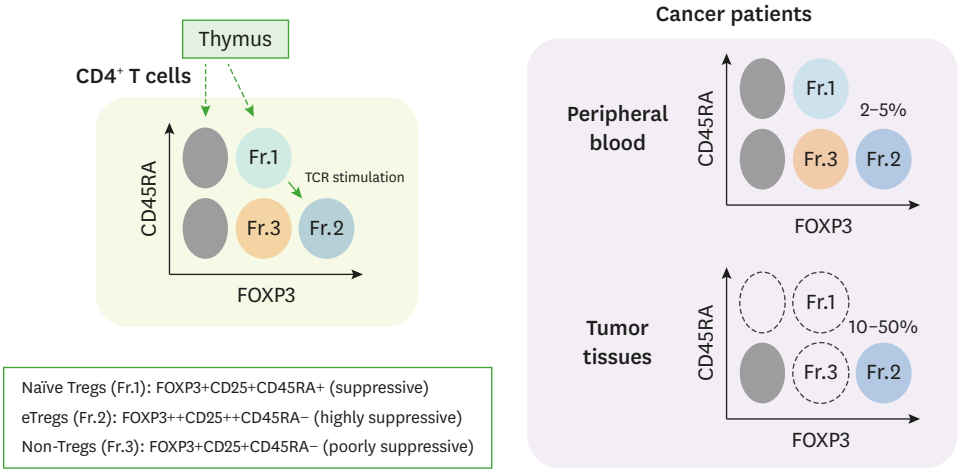

Figure 1. Classification of human CD4⁺FOXP3⁺ T cells. In humans, CD4⁺FOXP3⁺ T cells can be classified into three subsets: naïve Tregs (Fr.1), eTregs (Fr.2), and non-Tregs (Fr.3). These three fractions can be distinguished based on the expression of CD45RA, cell surface markers of naive T cells, and the transcription factor FOXP3. Moreover, these subpopulations are functionally different in terms of their suppressive activity. Effector Tregs harbor strong immune suppressive activity, but non-Tregs do not possess immune suppressive activity. In the majority of cancer, eTregs predominantly infiltrate into tumor tissues. In general, the frequency of eTregs in cancer patients is 2~5% in peripheral blood but approximately 10~50% in the tumor tissues. In contrast, naïve Tregs and FOXP3⁺ non-Tregs are insufficient or absent altogether.

functions, despite the high expression level of FOXP3 upon TCR stimulation of Teff cells (28). CD4⁺CD25⁺ Tregs expressing low levels of CD127 (the α-chain of the IL-7 receptor) are regarded as functional Tregs with suppressive activities (29,30). However, TCR stimulation of naïve T cells transiently induces FOXP3 expression along with the downregulation of CD127. Given this fact, CD4⁺CD25⁺CD127^{lo} T cells may contain some activated non-Tregs in their population. Therefore, the expression levels of CD45RA, a marker of naïve T cells, have been previously proposed as a complementary marker, as well as CD25 and FOXP3, for alternative classification of Tregs (14,15,31). According to this classification, CD4⁺CD25⁺FOXP3⁺ T cells can be categorized into three fractions: naïve Tregs (CD4⁺CD25^{lo}FOXP3^{lo}CD45RA⁺); effector Tregs (eTregs) (CD4⁺CD25^{hi}FOXP3^{hi}CD45RA⁻); and non-Tregs (CD4⁺CD25^{lo}FOXP3^{lo}CD45RA⁻) (**Figure 1**). Naïve Tregs are separated from the thymus but have not yet been stimulated in the periphery, and barely possess any immunosuppressive function. After TCR stimulation, naïve Tregs differentiate into eTregs and thus display highly immunosuppressive activities. However, FOXP3⁺ non-Tregs are not immunosuppressive but rather are immunostimulatory, providing inflammatory cytokines, such as IFN-γ and IL-17 (31). Therefore, the features of these types of CD4⁺FOXP3⁺ T cells are closely connected to human autoimmune and inflammatory diseases. Specifically, eTregs have been referred to as the dominant CD4⁺FOXP3⁺ T cell subpopulation in patients with inflammatory diseases (including sarcoidosis), whereas FOXP3⁺ non-Tregs have been implicated as the predominant subpopulation for those with autoimmune diseases, such as lupus erythematosus (31).

MECHANISMS OF IMMUNOSUPPRESSION FOR TREGS

Tregs exert their immunosuppressive function through various modes of action. The first suppressive mechanism is associated with cytokines, and include the expenditure of IL-2 by Tregs with high levels of CD25 expression (32,33), and suppression by inhibitory cytokines (such as TGF-β, IL-10, and IL-35) (34-37). Metabolite-related suppressive mechanisms include

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conversion of ATP into adenosine that can prevent optimal T cell activation (38,39), as well as the expression of indoleamine 2,3-dioxygenase (IDO) in dendritic cells (DCs), which results in T cell exhaustion by depleting amino acids essential for survival (40). Other important suppressive mechanisms involving immune checkpoint-related pathways include the disruption of Teff cells by the lymphocyte activation gene-3 (LAG-3)-MHC class II interaction, the inducible T-cell costimulator (ICOS)-ICOS ligand (ICOSL)-mediated T cell activation, and the interaction between PD-1/PD-L1 (41). Impairment of Ag-presenting cell (APC) maturation is considered as a crucial mode of action for immune suppression through the binding of CTLA-4 expressed in eTregs, which causes downregulation of CD80/86 expression. Moreover, APCs are directly eliminated by Fas/Fas ligand, perforin, and granzyme B signaling (42). The majority of observations seem to indicate that, CTLA-4-dependent and/or high-affinity IL-2R-dependent suppression of T cell activity is an especially crucial process for immunosuppression by Tregs: mice specifically lacking CTLA-4 in Tregs have impaired Treg-mediated immunosuppression (43); heterozygous *CTLA4* mutations have been described in patients with multiple autoimmune symptoms, and are associated with impairments in the immunosuppressive activity of Tregs (44,45); treating CTLA-4-immunoglobulin fusion protein leads to the conversion of Teff cells into an anergic state (46); high-dose IL-2 neutralizes Treg-mediated suppression of T cell activation and proliferation *in vitro* (32,33). Through these mechanisms, Tregs can suppress Ag-specific Teff cells.

Treg AND CANCER

Tregs in the TME

The association between Tregs and tumors in the TME has been studied for decades. The involvement of Tregs in anti-tumor immunity was initially reported in 1999 (47,48). It is demonstrated that anti-CD25 Ab depleting $CD4^+CD25^+$ Tregs retarded tumor growth in T cell-deficient mice transplanted with $CD25^+$ cell-depleted splenocytes. Tregs accumulate at tumor sites and in the peripheral circulation of patients with cancer, and their immunosuppressive function, as well as their number, are increased compared to those found in healthy donors (47,48). Tregs that have infiltrated into human tumors account for 10%–50% of $CD4^+$ T cells in tumors, which is more abundant relative to the 2%–5% of $CD4^+$ T cells found in the peripheral blood of individuals without cancer. Furthermore, higher levels of tumor-infiltrating Tregs and Treg/Teff cell ratio indicate poor prognosis in patients with various types of cancers, such as non-small cell lung carcinoma (NSCLC), melanoma, and gastric cancer (49,50). The accumulation of Tregs in tumors are well-studied in the previous reports, elucidating their ability to effectively migrate into tissue sites depending on the expression of multiple chemokine receptors; for example, CXCR5 in Tregs from the lymph node of patients with lung cancer (51). C-C motif chemokine receptor (CCR) 4 with CCL12, CCR4 with CCL17, CCR10 with CCL28, and CXCR4 with CXCL1 have been reported as other chemokine receptors on Tregs with their partner chemokines (51-56). Treg infiltration into tumor tissues has been extensively investigated in the context of recent "immune-oncology" researches (57,58). These studies confirmed the conspicuous presence of Tregs among tumor-infiltrating lymphocytes, especially in tumors harboring large immune cell infiltrates (59). Also, based on numerous articles, it is demonstrated that Tregs block antitumor immunity, and thus enhance tumor progression, and their presence in the TME is profoundly linked with unfavorable prognosis, resulting in short OS (60,61). Notably, Tregs that directly interact with the tumor are more essential for the study of immune evasion by the tumor, because the peripheral Tregs do not always represent immune-tolerant TME (49). Recently,

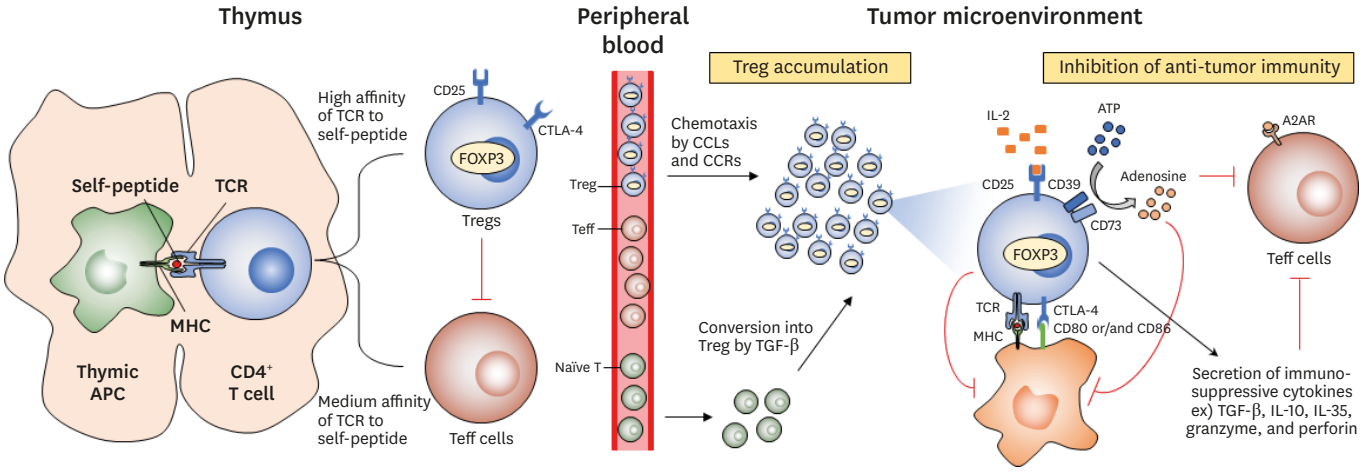

Figure 2. Role of Tregs in immune-evasion of cancer after differentiation from the thymus. Natural Tregs, generated in the thymus, are initially differentiated from the thymocytes by using thymic "positive selection" based on the binding affinity of TCR to the self- peptides-MHC complexes expressed on thymic APCs. The $CD4^+$ T cells which bind to self-peptide-MHC complexes with the highest affinity are removed through apoptosis, and those that cannot bind at all with the complexes will also be removed because of the absence of TCR stimulation. After strong TCR stimulation, these immature precursor cells undergo IL-2-mediated signaling, thus expressing the master transcription factor FOXP3, which orchestrates the differentiation of these cells into Tregs. By contrast, immature T cells with lower affinity for self-peptide–MHC complexes are also positively selected but differentiate into Teff cells. Even though some Teff cells are auto-reactive, Tregs can block the autoimmunity of Teff cells owing to their higher affinity. These immune cells that have departed from the thymus travel through the blood vessels and move wherever they are needed. In the tumor microenvironment, especially, Tregs expressing the chemokine receptors, such as CCR4, CCR5, CCR8, and CCR10, are recruited to and around the tumors by binding to chemokines including CCL1, CCL5, CCL22, and CCL28 that are secreted from various kinds of tumors. Moreover, Tregs constitutively express the IL-2 receptor subunit-α (also known as CD25) that binds to IL-2 with higher affinity, resulting in the depletion of IL-2 from their surroundings. This leads to the reduction of the availability of this cytokine to Teff cells. Tregs also constitutively express CTLA-4, a checkpoint protein suppressing the immune response, which binds to CD80 and CD86 on APC, thereby transmitting suppressive signals to Teff cells. In addition, Tregs secrete cytokines, such as IL-10, IL-35, and TGF-β, which can decrease the activity of APCs and Teff cells and secrete granzymes and perforins that can directly kill these cells. Moreover, abundant adenosine is produced by Tregs via nucleotidase activity of CD39 and CD73, which provides immunosuppressive signals to Teff cells and APCs through the engagement of adenosine A2AR.

compelling evidence suggests that colorectal cancer (CRC) abundantly infiltrated with the $FOXP3^{hi}$ subset of suppression-competent eTregs lead to poor prognosis, while the presence of pro-inflammatory cytokine-secreting $CD4^+CD45RA^{lo}FOXP3^{lo}$ T cells (non-Tregs) in tumor tissues is associated with favorable outcomes (50). Therefore, especially in cancer patients with high numbers of tumor-infiltrating Tregs, further analysis needs to be conducted in order to distinguish $FOXP3^+$ non-Tregs from $FOXP3^{hi}$ eTregs in tumors. This will help evaluate the clinical importance of $FOXP3^+$ cells in tumor tissues. In summary, Tregs, particularly in the TME, are a key factor of hindrance in anti-tumor immunity in various types of cancer patients, resulting in the initiation of tumor progression or resistance against cancer immunotherapy (**Figure 2**).

Molecular and cellular characteristics of Tregs in the tumor

On the basis of the functional classification of Tregs described above, Tregs in the TME is mostly composed of bona fide Treg (eTreg) cells that overexpress immunosuppressive molecules including CTLA-4 and T cell immunoreceptor with Ig and ITIM domains (TIGIT), which are not expressed much in naïve Tregs (14,62,63). Also, transcriptome analysis on human cancer specimens shows that tumor-infiltrating Tregs have high expression levels of Treg-activation surface markers, such as glucocorticoid-induced TNFR-related protein (GITR; also known as TNFRSF18), lymphocyte-activation gene 3 protein (LAG3), T cell immunoglobulin mucin receptor 3 (TIM3; also known as HAVCR2), OX40 (also known as TNFRSF4), and ICOS (64). These phenotypes, distinct from peripheral Tregs, indicate that Tregs in the TME show potent immunosuppressive activities in terms of function and number. One possible mechanism that has been suggested is that proliferating and dying

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tumor cells produce a large number of self-Ags, which are recognized by Tregs, thereby inducing the activation of Tregs in the TME (65). As part of the mechanism mentioned above, whether Tregs recognize Ags exclusively or share Ags with Th cells remains unclear at this stage (65,66). Nevertheless, Tregs usually possess a higher binding affinity to TCRs than does Teff cells, resulting in the predominant activation of Tregs in the TME, even in the presence of competition with Teff cells. Furthermore, tumors can harbor some immature dendritic cells, which drive the activation and/or proliferation of Tregs in a TGF-β-dependent manner in animal models. In contrast to the abundant animal studies regarding iTregs, the existence of TGF-β-iTreg cells in humans have not been elucidated clearly; accordingly, investigations on human tumor specimens are crucial to understanding the phenotypes and origins of tumor-infiltrating Tregs.

Regulation of tumor Ag-specific T cells by Tregs

Generally, 2 different types of Ags can exist in tumor cells. First, 'neoantigens' are non-self-Ags derived from either oncogenic viral proteins or abnormal self- proteins caused by somatic mutations. Second, self-Ags that arise from the aberrant overexpression of endogenous proteins are categorized as 'shared antigens.' How $CD8^+$ T cells distinguish each of these 2 types of Ag for anti-tumor immunity remains unclear. Therefore, the different immunosuppressive mechanisms of Tregs against $CD8^+$ T cells specific for shared Ags versus neoantigens need to be resolved through further research. Interestingly, in some animal models, it is suggested that Tregs select for non-self-Ag specific $CD8^+$ T cells harboring high-affinity TCRs by manipulating co-stimulatory signaling (67). In particular, CD8+ T cells targeting self-Ags are more susceptible to Tregs due to the APCs that provide limited co-stimulatory signals (68). By contrast, non-self-specific $CD8^+$ T cells are resistant to suppression by Tregs in humans (68). These results demonstrate that $CD8^+$ T cells specific for neoantigens are more resistant to Treg-mediated immunosuppression, and given this fact, tumors that express shared Ags can serve as more vulnerable targets for cancer immunotherapy.

TARGETING TREGS FOR CANCER IMMUNOTHERAPY

Tregs, which express the transcription factor FOXP3, are indispensable for immunological self-tolerance and immune homeostasis. They also disturb tumor immunity and can, therefore, be targeted to elicit an anti-tumor immune response by depleting them or diminishing their suppressive capabilities (69).

FOXP3, a well-characterized Treg-specific marker and the key phenotype of Tregs to function as suppressive cells, is a transcription factor expressed in the nucleus and is therefore hard to detect for clinical use.

Therapies targeting Tregs are not likely to be effective against all types of tumors. For example, Treg depletion in animal models led to the regression of tumors from certain cell lines, such as RL-male1 or MethA cells, but did not in other cell lines like AKSL2 or RL-female8 cells (70).

Thus, the identification of novel and specific biomarkers that distinguish Tregs from other cells in the TME is essential for increasing the possibility of successfully developing effective cancer therapies targeting Tregs.

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Specific surface molecules on Tregs

Depletion of Tregs or attenuation of their suppressive activity can enhance tumor immunity. Tregs in the TME reveal several cell surface markers, including CD25, CTLA-4, GITR, OX40, ICOS, PD-1, LAG3, TIM3, TIGIT, CCR4, folate receptor (FR) 4 (71) and CD15s (72), and specific mAbs for these cell surface marker can be used to deplete Tregs or hinder their function (**Table 1**).

CD25

Several studies show that the removal of $CD25^+CD4^+$Tregs by anti-CD25 mAb or toxin-conjugated anti-IL-2 (Denileukin diftitox) facilitates the activation of Teff cells, which greatly inhibit tumor growth in rodents (47,48,73,74). Treg depletion using an anti-CD25 mAb has been evaluated in clinical trials. When patients with breast cancer were vaccinated with various tumor-associated peptides followed by a treatment with daclizumab—an anti-CD25 mAb—to deplete Tregs, there was robust T cell priming with prolonged stable disease for 6 out of 10 patients and a median progression-free survival of 4.8 months (75). By contrast, another study showed that the administration of daclizumab depleted Teff cells as well as Tregs in patients with melanoma, but neither an antitumor immune response nor Ab production was observed (76). Because activation of Teff cells induces CD25 expression, Treg depletion by targeting CD25 can be accompanied by a deficiency in Teff cells. Thus, anti-CD25 mAb administration may lead to limited efficacy in increasing antitumor T cell responses.

CTLA-4

CTLA-4, an immune-checkpoint molecule, is expressed by tumor-infiltrating $CD4^+$ and $CD8^+$ Teff cells and $FOXP3^+CD4^+$ Tregs (77). The anti-tumor activity of anti-CTLA-4 mAb was originally thought to be dependent on the reinvigoration of exhausted Teff cells expressing CTLA-4 (78). However, several preclinical studies indicate that the anti-tumor activity of anti-CTLA-4 mAb is instead dependent on the depletion of CTLA-4-expressing Tregs in the TME through Ab-dependent cellular cytotoxicity, thereby increasing the Teff cell to Treg ratio. Consequently, disrupting the function of the Fc portion of the Ab completely abrogated the anti-tumor activity of the anti-CTLA-4 mAb (79-82). Therefore, further research to address the relative roles of CTLA-4 in Teff cells and Tregs in the TME of various cancers is needed.

Co-stimulatory molecules (GITR, OX40, and ICOS)

Co-stimulatory receptors, such as GITR, OX40, and ICOS, highly expressed by Tregs can be candidates for Treg depletion and functional modulation.

GITR is expressed at a high level by Tregs but at a low level by resting $CD4^+$ and $CD8^+$ T cells, and they play an important role in Treg expansion (83). Activation of GITR signaling

Table 1. Ab-drug development status of Treg-targeting therapy

Treg markers	Representative drugs	Function of Ab	Tumor types	Development stage
CD25	Daclizumab	Depletion	Leukemia/lymphoma	Phase 2
CTLA-4	Ipilimumab	Antagonist	Melanoma	Commercialized
PD-1	Nivolumab	Antagonist	Melanoma, lymphoma	Commercialized
GITR	TRX518	Agonist	Melanoma	Phase 1
CCR4	Mogamulizumab	Antagonist	$CCR4^+$ adult T-cell leukemia/lymphoma	Commercialized
OX40	PF-04518600	Agonist	Advanced malignant cancer	Phase 2
ICOS	JTX-2011	Agonist	Advanced/refractory solid cancer	Phase 1/2
LAG3	Sym-2011	Antagonist	Solid tumor, lymphoma	Phase 1
TIM-3	Sym-023	Antagonist	Solid tumor, lymphoma	Phase 1
TIGIT	BMS-986207	Antagonist	Multiple myeloma	Phase 1/2

through an agonistic anti-GITR mAb inhibits the suppression activity of Tregs and induces Treg-resistant Teff cells (84). The GITR agonists are now being investigated in patients with advanced solid cancer.

OX40 is constitutively expressed by a subset of Tregs, but is also found on Teff cells (85). Although OX40-agonists are used to stimulate anti-tumor responses of Teff cells, the effect on Tregs in cancer is not well understood. OX40 agonists are being investigated alone or in combination with other immunotherapies in patients with solid cancer or melanoma (86).

ICOS is important in Treg function and homeostasis (87,88), and is highly expressed by activated Tregs in tumor-infiltrating lymphocyte (TIL) of gastric cancer patients (89). Agonistic anti-ICOS mAbs, like OX40 and GITR agonists, are expected to have a dual-mode of action involving activation of Ag-specific $CD4^+$ Teff cells and selective depletion of Tregs (90).

Co-inhibitory molecules (TIGIT, LAG3, and TIM3)

Immune co-inhibitory receptors predominantly expressed by Tregs are also being explored as Treg-targeted immunotherapies. TIGIT marks a population of Tregs with an enhanced suppressive capacity in the TME (91,92). $TIGIT^+$Tregs have a highly suppressive activity and they express more co-inhibitory molecules, such as LAG3, TIM3, and PD-1 compared to TIGIT-Tregs (91). In contrast, another study showed that TIGIT expression correlated with $CD8^+$ Teff cell exhaustion, and TIGIT blockade increased the production of effector cytokines, such as IFN-γ and TNF-α, by $CD8^+$ Teff cells in a Treg-independent manner (93). Thus, TIGIT blockade may promote anti-tumor immunity through both Treg dependent and independent mechanisms. LAG3 is expressed on TILs, especially on Tregs. Interestingly, $CD4^+CD25^+FOXP3^-LAG3^+$ T cell population from colorectal cancer patients produce immunosuppressive cytokines, such as IL-10 and TGF-β, and show 50% more suppressive activity than $FOXP3^+$ Tregs (94). The humanized LAG3 Ab is under phase I and phase II clinical trials in patients with various solid cancers. TIM3 is expressed on activated T cells and certain subsets of Tregs and binds to several identified ligands (i.e. galectin-9, HMGB1, caecam, phosphatidyl serine) (95,96). The co-inhibitory function of TIM3 is implicated in tumor evasion and $TIM3^+$ Tregs have an increased suppressive function (97,98). Co-inhibitory receptors such as LAG3, TIM3, and TIGIT seem to offer an advantage as they are dominantly overexpressed on tumor-infiltrating Tregs. However, broader studies need to be conducted in order to determine their safety and efficacy.

CCR4

Chemokine receptors, which allow Tregs to migrate to the TME site, can be a candidate molecule for Treg depletion (99). Tumor-infiltrating macrophages and tumor cells produce the CCL22, which chemoattracts Tregs expressing CCR4 (52,100,101). CCR4 is highly expressed by eTregs but not by naive Tregs or most Teff cells, except for some Th2 and Th17 cells in peripheral blood (102). *In vitro* or *in vivo* anti-CCR4 mAb treatment selectively depleted eTregs and efficiently induced tumor-specific effector $CD4^+$ and $CD8^+$ T cells (63). Additionally, the administration of an anti-CCR4 mAb (mogamulizumab) on advanced solid cancer patients significantly reduces eTregs in peripheral blood (70). Additional clinical trials are underway with immune checkpoint blockades.

Treg and Immune checkpoint inhibition

Immune checkpoint molecules, including CTLA-4 and PD-1, are highly expressed by activated Tregs and Teff cells (49,77). The role of CTLA-4 in Tregs is mentioned above. The role of the

inhibitory receptor PD-1 on Teff cells is well established, but its function in Tregs is less clear. Tregs in the TME show comparable levels of PD-1 expression with that of Teff cells. Because PD-1 signaling in Treg reduces its immunosuppressive activity, PD-1-deficient Tregs might potentiate the activation and immunosuppressive function of Tregs (103). Various studies reported that the anti-PD-1 mAb, nivolumab, reduced the immune-suppressive activity of Tregs (104). However, another research maintains that PD-1 inhibition induced the immune-suppressive activity mediated by Tregs in some cancer patients (105). Therefore, more research is needed to investigate the role of PD-1 in Teff cells and the role of Tregs in the TME.

Treg modulation factor in the TME

Cytokines

The TGF-β and IL-2 signaling pathways are essential to maintain the differentiation and survival of Tregs in the thymus and peripheral tissues. The effect of cancer therapy by IL-2 blockade is still unclear. In particular, hyperactivation of the TGF-β pathway in the TME enhances tumor progression by stimulating angiogenesis and inhibiting innate and adaptive anti-tumor immune responses (106). A type I TGF-β receptor serine/threonine kinase inhibitor (galunisertib) increased the ratio of $CD8^+$ T cells to Tregs in melanoma animal models in a combination treatment with an anti-CTLA-4 mAb (107). In addition, a combination therapy of galunisertib with an anti-PD-1 or anti-PD-L1 mAb is currently underway in clinical trials (108). Thus, the regulation of TGF-β signaling pathways can be a noteworthy candidate for Treg control.

Targeting intracellular signaling in Tregs

PI3K signaling pathway, which is crucial for Treg maintenance and function, is a promising target for Treg-directed therapy (109). Inhibitors of PI3K effectively reduced immune suppression by Tregs in mouse models. In particular, selective inactivation of PI3Kδ in Tregs increases the activity of $CD8^+$ T cells, preventing or slowing tumor development, progression, and metastasis (110). Specific ablation of the PI3K-phosphatase and tensin homolog (PTEN)-mTOR pathway in Tregs impairs mitochondrial fitness, upregulates glycolysis, leads to the loss of FOXP3 expression in Tregs, and induces Teff cell activity (111,112). Combination treatment of pembrolizumab and PI3Kδ inhibitors is currently being explored at an early stage of phase I trial in patients with advanced solid tumors. Also, tyrosine kinase inhibitors, including imatinib and dasatinib, which are known to target specific TCR signaling molecules, have been shown to reduce Treg survival and function through off-target effects (113,114). In the discontinued clinical trial for dasatinib, Treg reduction was observed and showed favorable clinical outcomes in patients with chronic myeloid leukemia (113).

CD39 and CD73

Tregs produce extracellular adenosine by the activity of CD39 and CD73 on their cell surface. Tregs express high levels of CD39 and CD73 and directly inhibit T cell activation via interaction with adenosine A2A receptor (A2AR). Moreover, adenosine increases tolerogenic APCs and enhances the immunosuppressive activity of Tregs (115). Therefore, CD39 and CD73, which are important for adenosine metabolism, can be promising therapeutic targets.

VEGF signaling

VEGF receptor (VEGFR) 2 plays an important role in tumor angiogenesis, and this signaling pathway has been shown to increase the infiltration of Tregs into tumors in animal models (116,117). In addition, blockade of VEGF-VEGFR2 signaling has been reported to inhibit tumor growth by reducing the accumulation of immunosuppressive cells, including Tregs, myeloid-

derived suppressor cells, and M2 macrophages in the TME (118). Furthermore, researchers have established that treatment of a humanized anti-VEGFR2 mAb, ramucirumab, led to a decrease in PD-1 expression in $CD8^+$ T cells and a reduction in eTreg infiltration into the TME (49,119). Thus, targeting VEGFR2 molecules expressed by activated Tregs or blocking the VEGF-VEGFR2 signaling may contribute to cancer therapy through Treg inhibition.

CONCLUSION

Tregs serve as a specialized cell lineage that plays an essential role in the immunological tolerance of immune homeostasis through their immune suppressive activity. High levels of Treg infiltration in the TME lead to an undesirable prognosis in patients with various types of cancers. Depleting Tregs and regulating their function in the TME may be potential strategies for cancer therapy. Several Treg-targeted therapies are under investigation, but the lack of specific markers for Tregs has limited their clinical application. Since drugs that selectively deplete Tregs in the TME of cancer patients have not been developed at present, identification of specific targets for disrupting and depleting Tregs is important for the success of cancer immunotherapy. In the future, the development of Treg-targeted therapies based on the TME's comprehensive immune profiling may lead to new therapies and immune precision for individual cancer patients.

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