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Regulatory T cells in immune checkpoint blockade antitumor therapy

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Abstract
Regulatory T cells (Tregs), an essential component of the human immune system, are a heterogeneous group of T lymphocytes with the ability to suppress immune responses and maintain immune homeostasis. Recent evidence indicates that Tregs may impair antitumor immunity and facilitate cancer progression by weakening functions of effector T cells (Teffs). Consequently, targeting Tregs to eliminate them from tumor microenvironments to improve Teffs' activity could emerge as an effective strategy for cancer immunotherapy. This review outlines the biology of Tregs, detailing their origins, classification, and crucial markers. Our focus lies on the complex role of Tregs in cancer's development, progression and treatment, particularly on their suppressive role upon antitumor responses via multiple mechanisms. We delve into Tregs' involvement in immune checkpoint blockade (ICB) therapy, their dual effect on cancer immunotherapy and their potential biomarkers for ICB therapy effectiveness. We also summarize advances in the therapies that adjust Tregs to optimize ICB therapy, which may be crucial for devising innovative cancer treatment strategies.

Keywords Regulatory T cells, Tumor microenvironment, Immune checkpoint blockade

Introduction

Regulatory T cells, known as Tregs, are an integral component of the T cell family and play an essential role in sustaining immune equilibrium. In both mice and human peripheral blood, Tregs account for approximately 5–10% of $CD4^+$ T cells [1]. They primarily exert immune-suppressive effect through the secretion of inhibitory cytokines, the inhibition of cytolysis, and by inducing metabolic disruption, further preserving the body's immunological balance [2]. Nevertheless, recent investigations have revealed that Tregs also contribute to tumor progression. Their capacity for immunosuppression is considered a principal element in enabling tumors to evade immune detection, thereby challenging the effectiveness of cancer immunotherapies [3]. Consequently, an in-depth understanding of Tregs' immunosuppressive mechanisms is indispensable for the development of more efficacious, tumor-specific therapies. It has been proved that, compared to non-tumor tissues, Tregs within tumors exhibit higher viability and a more active proliferative ability. They promote tumor progression by suppressing anti-tumor immune responses [4]. Therefore, we need to effectively control Tregs to enhance anti-tumor immunotherapies.

Immune checkpoints, encompassing programmed death receptors and their ligands, constitute key

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elements in maintaining immune system homeostasis. These checkpoint proteins serve as "brakes" on the immune response and are engaged with co-stimulatory molecules to regulate T cell activation and inhibition [5]. Specifically, through the interaction of Tregs with these checkpoints, immune responses may be enhanced or suppressed, reflecting the environmental demands. Tumor cells have demonstrated the ability to evade recognition and clearance by the immune system through bypassing these immune checkpoints [6]. Consequently, blockade strategies targeting immune checkpoint receptors have emerged as a significant approach to cancer immunotherapy, becoming one of the most prominent cancer treatment strategies in recent years [7]. Within the tumor microenvironment, Tregs can effectively suppress anti-tumor immune responses through the expression and amplification of a variety of immune checkpoints. Utilizing immune checkpoint inhibitors (ICIs) can disrupt this interaction and reversely activate the immune components to combat tumors [8]. Recently, ICIs have yielded remarkable results in the treatment of a range of tumors, such as melanoma, hepatocellular carcinoma, lung cancer, gastric cancer, and intestinal cancer, improving the overall survival rate and progression-free survival of patients [9, 10]. A comprehensive review of randomized clinical trials involving ICIs revealed that long-term survivors experienced an approximate 10% increase in survival probability compared to patients not treated with ICIs, highlighting ICIs' significant clinical benefits [11]. For example, data from clinical trials of non-small cell lung cancer (NSCLC) showed that the one-year survival rate of patients treated with pembrolizumab (anti-PD-1 drug) and chemotherapy was 69.2%, significantly higher than the 49.4% observed in patients who received chemotherapy alone [12]. To date, ICB therapy has achieved considerable success. However, in clinical practice, only a small number of patients (approximately 10–30%) demonstrated a lasting treatment response, and varying degrees of immune-related adverse events (irAEs) [13, 14]. In preclinical models of irAEs, a negative correlation between Treg number and irAEs has been reported [14].

In our review, we first introduced the fundamental aspects of Tregs, discussed the anti-tumor mechanisms of Tregs in the TME, as well as their evolving role in ICB therapy. We analyzed the current application value and unique advantages of combining Tregs-related therapies with ICB therapy based on the expression of various markers in Tregs, and finally explored progressions in targeting tumor-infiltrating Tregs (TI-Tregs).

Basics of tregs biology

Origin and classification of regulatory T cells

Tregs, a subset of $CD4^+$ T lymphocytes, are essential for immune system regulation. They develop primarily in the thymus, with their maturation regulated by three key signals: T cell receptor (TCR) recognition of peptide-MHC ligands, co-stimulatory cluster of differentiation 80 (CD80)/cluster of differentiation 86's (CD86) interaction with cluster of differentiation 28 (CD28), and activation by interleukin-2 (IL-2) or interleukin-15 [15]. Tregs are categorized into two main types: thymus-derived Tregs (tTregs) and peripherally induced Tregs (pTregs) (Fig. 1). tTregs, which constitute the majority proportion of Tregs, are capable of responding to autoantigens and play a crucial role in maintaining immune self-tolerance [16]. In contrast, pTregs arise from peripheral $CD4^+$ T cells under antigen stimulation and increased FoxP3 expression, often occurring in tissues like the intestine, where it is rich in TGF-β and retinoic acid [17]. Both tTregs and pTregs require IL-2 for their survival and function, with TGF-β being particularly important for iTregs induction in vitro [18]. Tregs are thus characterized by high expression of the transcription factor FoxP3 and the IL-2 receptor alpha chain (CD25), making the $CD4^+$ $CD25^+$ $FoxP3^+$ phenotype a classic identifier for Tregs [19]. These markers facilitate the study of Tregs functions and offer insights into their role in immune tolerance and potential therapeutic applications [20, 21].

There are differences between human and mouse Tregs. Tregs in both humans and mice exhibit conserved expression of the hallmark transcription factor FoxP3; however, in human Tregs, FoxP3 expression is more sensitive to modulation by the inflammatory milieu. While mouse Tregs primarily develop in the thymus, a substantial number of human Tregs are generated in peripheral tissues [22]. Functionally, both human and mouse Tregs suppress immune responses [23]. Human Tregs often rely on external factors (e.g., IL-2) to potentiate their suppressive capacity, whereas mouse Tregs demonstrate a relatively lower dependence on IL-2 [24].

Regulatory T cells in the tumor microenvironment

To gain deeper insight into the role of Tregs in cancer and to explore their potential anti-tumor effects, researchers are increasingly focusing on the tumor microenvironment (TME), which includes immune cells, blood vessels, stromal cells and many other types of cells [25]. In the TME, Tregs inhibit anti-tumor activity in ICB through various pathways, leading to increased drug resistance. The abundant expression of chemokine ligand C-C motif chemokine ligand 22 (CCL22) attracts activated Tregs that express chemokine receptor C-C motif chemokine receptor 4 (CCR4) [26]. These activated Tregs suppress the antigen-presenting function of dendritic cells and

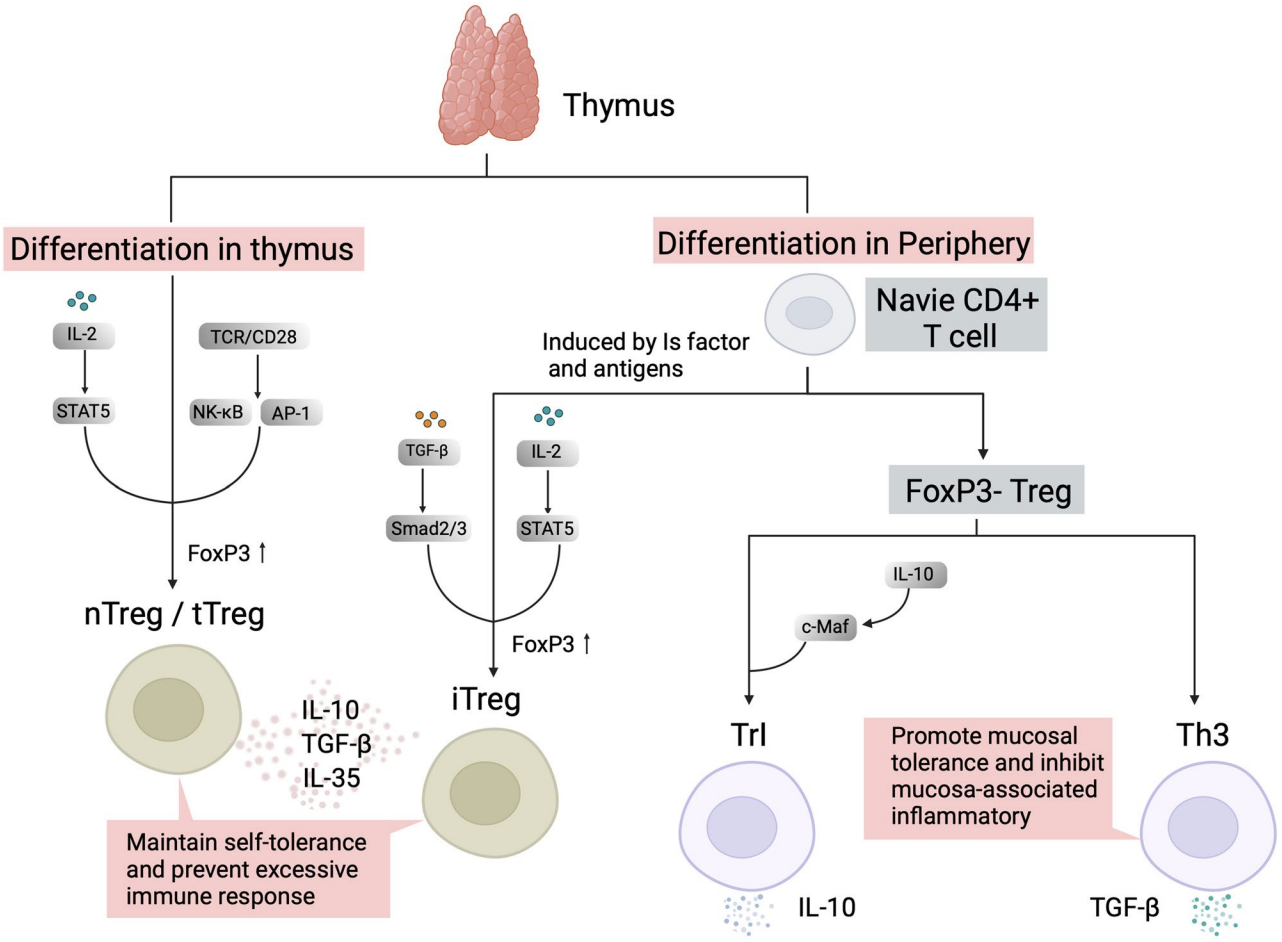

Fig. 1 Generation and classification of tregs. The picture shows two main differentiation routes. The first leads to FoxP3 + nTreg/Tregs, which represent nTregs, also known as thymic Tregs (tTregs). These cells are characterized by the expression of the transcription factor FoxP3 and are crucial for maintaining immune tolerance and preventing autoimmune responses.The second route shows the activation of naïve CD4 + T cells by an immunosuppressive (IS) factor and an antigen, leading to the differentiation into FoxP3 + induced regulatory T cells (iTregs). iTregs are similar to nTregs in their function of immune regulation, but they are induced in the periphery from conventional T cells.There is further differentiation into two other FoxP3- Tregs : Tr1 cells and Th3 cells. Tr1 cells are a type of regulatory T cell that produces high levels of interleukin-10 (IL-10), a cytokine involved in the suppression of inflammatory responses. Th3 cells are another subset of regulatory T cells known for their role in mucosal immunity and their production of transforming growth factor-beta (TGF-β), which also has immunosuppressive properties

induce T cell exhaustion within the tumor by secreting inhibitory cytokines, such as transforming growth factor-β (TGF-β), interleukin-35 (IL-35) and interleukin-10 (IL-10), and by regulating the expression of inhibitory receptors [27]. Moreover, Tregs impair Teffs' activity through metabolic disruption. In the TME, IL-2 acts as a key growth factor. Tregs compete for IL-2 with Teffs and lead to a scarcity of IL-2 by highly expressing cluster of differentiation 25 (CD25), a high-affinity receptor for IL-2 [28]. Simultaneously, Tregs express the co-inhibitory receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) that binds to cluster of CD80 / b7-1 molecule and CD86 / b7-1 molecule on antigen presenting cells (APCs), disrupting co-stimulatory signals and thereby inhibiting effector T cell activity [29]. In the TME, Tregs create tiny pores in the target cells' membrane through perforin, facilitating the entry of granzyme B which subsequently activates caspase to induce apoptosis in the target cell, resulting in the suppression of Teffs, NK cells and other anti-tumor immune cells. Through this cytotoxic effect, Tregs maintain an immunosuppressive state in the TME, promoting immune escape and diminishing the efficacy of ICB [30]. The immunosuppressive mechanism of Tregs in TME is also a key factor causing resistance to immunotherapy [31]. Of course, further research is needed to understand the interactions between the different functions of Tregs. Considering the significant role of Tregs in tumor immunity, researchers have proposed two major strategies to enhance tumor immunity: depleting Tregs and reducing their suppressive function.

Engineering tregs

In addition to conventional Tregs, engineered Tregs have garnered the interest of immunology researchers as an emerging area of study, wherein these cells employ the immunosuppressive functions of their natural counterparts for potential clinical therapeutic applications [32]. Using advanced gene editing tools such as CRISPR/Cas9, researchers conducted a loss-of-function screening on approximately 500 nuclear factors to ascertain which genes enhance or inhibit FoxP3 expression. This screening revealed ubiquitin specific peptidase 22 as a positive regulator of FoxP3 expression, while E3 ubiquitin ligase ring finger protein 20 emerged as a negative regulator. These findings not only unveiled previously unknown FoxP3 regulators but also introduced a novel screening method with broad applicability to Treg-based cancer immunotherapy [33]. Subsequent studies have demonstrated that, based on this screening technique, DNA editing can modify multiple genes to regulate Treg functions. By modifying the stability and function of Tregs, this approach establishes the groundwork for engineered Treg-based cancer therapies [34]. Recent advances in this field also include the development of low-immunogenic pluripotent stem cells that can be induced into Tregs through gene editing, a process with important implications for the advancement of engineered Treg cell therapy [35].

Marker genes for tregs

Tregs' vital role in maintaining immune tolerance has been repeatedly revealed, they suppress autoimmune responses and inhibit tumor-killing immune responses. Researchers have identified a series of marker genes that encode proteins and molecules with specific expression patterns and functions in Tregs [36]. Tregs are notably characterized by the expression of the transcription factor FoxP3, which distinguishes them from other T cell subsets [37]. Although FoxP3 expression is almost exclusive to Tregs in human [38], non-regulatory T cells may also transiently express FoxP3 upon activation in some cases. Therefore, researchers have combined other markers, such as high expression of CD25 and low or no expression of cluster of differentiation 127 (CD127), to more accurately identify Tregs [39]. Helios (IKZF2) and Eos (IKZF4), zinc-finger transcription factors, are also proved to be important marker genes for Tregs and work alongside FoxP3 to regulate key molecules and signaling pathways, maintaining Treg cell development and function [40]. These marker genes can be used as diagnostic tools to identify immune system dysregulation, predict disease progression, and assess therapeutic responses. In addition, a recent study by Michael Delacher et al. employed single-cell gene expression analysis and reported that the transcription factor BATF and chemokine receptor CCR8 expressed by Tregs in mouse and human tissues, are capable of promoting tissue homeostasis and regeneration, indicating that targeting these factors may help balance the immune response and may serve as a potential marker for immunotherapy [41].

The expression of surface molecules on Tregs is critical for their identification, classification and functional studies, showcasing their distinctiveness (Fig. 2). Key molecules include CD4, cluster of differentiation 39 (CD39), cluster of differentiation 73 (CD73), and CD25, with the latter being vital for Tregs survival and function [42]. Inhibitory co-stimulatory molecules such as CTLA-4, programmed death-1 (PD-1), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) and T cell immunoreceptors with IG and ITIM domains (TIGIT) play crucial roles in maintaining immune homeostasis and preventing excessive immune responses, with their interactions with co-inhibitory ligands contributing to tumor immune escape [43, 44]. Additionally, TI-Tregs express tumor necrosis factor receptors like glucocorticoid-induced TNFR-related protein (GITR), inducible T-cell costimulatory (ICOS), tumor necrosis factor receptor superfamily member 4 (OX40) and tumor necrosis factor receptor superfamily member 9 (4-1BB) on their surface [45]. In the tumor microenvironment, the presence of chemokine receptors such as CCR4, C-C motif chemokine receptor 6, C-C motif chemokine receptor 8 (CCR8), and tumor necrosis factor receptor superfamily member 4 is essential for Treg migration and expansion, enabling their localization to specific tissues and sites [46]. These marker genes and molecules collectively define the suppressive nature of Tregs. By studying these markers in depth, we gain insights into the biological functions of Tregs and their micro-mechanisms in the tumor microenvironment, providing a theoretical foundation for the treatment of immune- and tumor-related diseases.

Tregs in antitumor immunity regulation

Cancer remains a significant threat to global health and is a major concern for humanity. According to the American Association for Cancer Research, by 2040, the worldwide cancer patient population is predicted to reach 28 million, with approximately 16.2 million expected to succumb to the disease (AACR Cancer Progress Report 2022). The evolution of cancer treatment has transitioned from traditional modalities such as surgery, radiation, and chemotherapy to contemporary approaches including targeted therapies and immunotherapy [47]. Immunotherapy, which harnesses the body's immune system's ability to identify and attack cancer cells, represents a groundbreaking advancement in anti-cancer research. In particular, ICB therapy has exhibited remarkable clinical efficacy by blocking immunosuppressive signaling

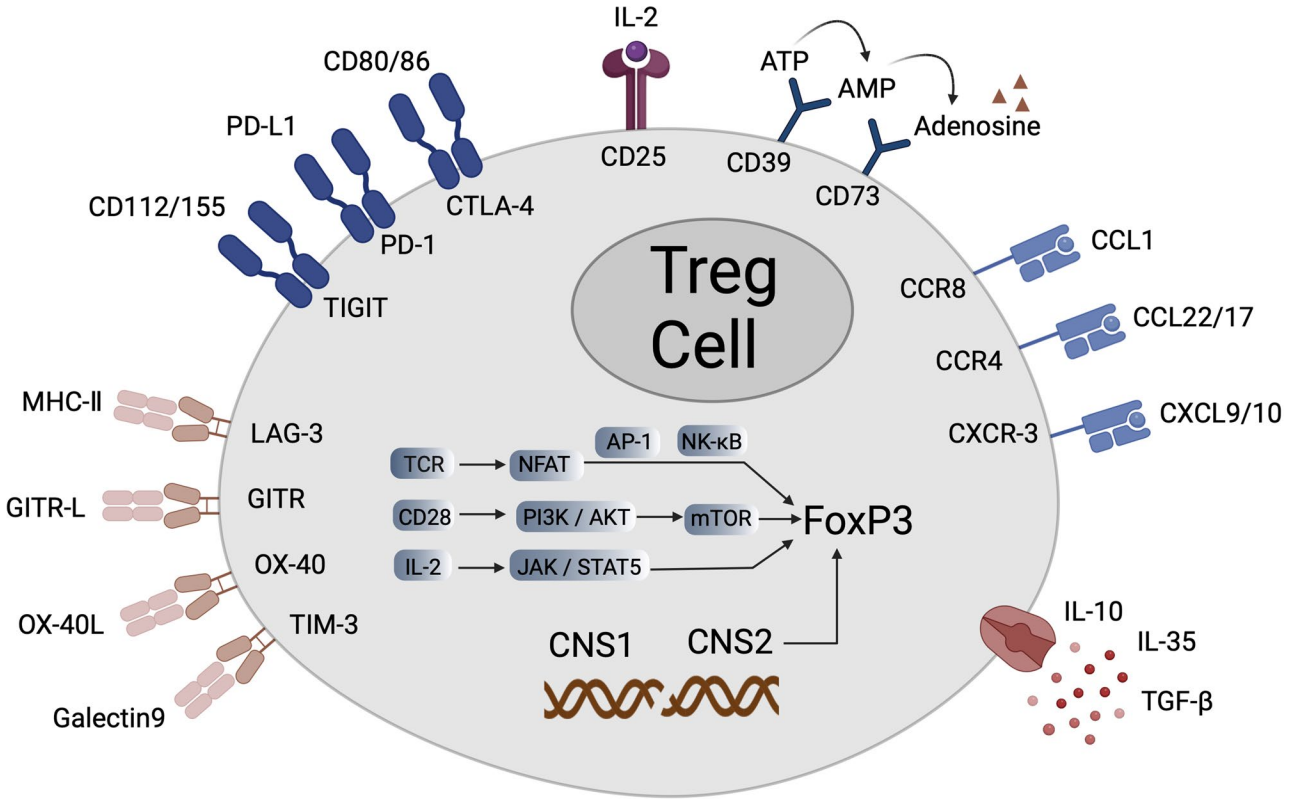

Fig. 2 Various genes expressed by Tregs and their surface molecules. In Tregs, FoxP3 is the key transcription factor that maintains their regulatory function. The expression of FoxP3 is closely related to the function of Tregs. The recognition of antigens by the T-cell receptor (TCR) triggers signal transduction, activating NFAT, which works in conjunction with AP-1 or NF-κB to promote the expression of FoxP3. Concurrently, co-stimulatory molecules like CD28 activate the downstream PI3K/Akt signaling pathway, thereby affecting the mTOR pathway, which is crucial for the stable expression of FoxP3 and the function of Tregs. Additionally, the cytokine signal IL-2 promotes the expression of FoxP3 through the JAK/STAT pathway, particularly STAT5.Other surface molecules also play an important role in the development and function of Tregs

pathways and enhancing the anti-tumor response of T cells [48]. Within this context, Tregs play a pivotal role in tumor therapy. In tumor tissues, the proliferation of Tregs is often markedly increased, which is believed to be a strategy by which tumors evade immune system attacks [49]. Meanwhile, TI-Treg is a key mediator of resistance to cancer immunotherapy. Tregs impair the anti-tumor immune response by inhibiting the activity of other immune cells, particularly $CD8^+$ T cells. As a result, targeted immunotherapy reduce the ratio of Tregs to Teffs in the TME has emerged as a highly promising avenue in cancer therapy via leveraging the immunosuppressive role of Tregs, especially in ICB therapy [50, 51]. To thoroughly understand the role of Tregs in tumor immunity, scientists are actively exploring their subpopulations and functional regulatory mechanisms. This endeavor includes modulating the number and function of Tregs through drug intervention, gene editing, or immunotherapy to bolster the body's immune response to tumors (Fig. 3).

FoxP3+ tregs

The human FoxP3 gene, located on the p-arm of the X chromosome, encodes a transcription factor that leads to the differentiation of T cells into Tregs [52]. FoxP3 plays a crucial role in the development of Tregs, serving as a distinctive marker for their identification and is essential for the establishment and maintenance of gene expression [53]. Benjamin Bilgüvar and Alexander Varki et al. demonstrated the importance of FoxP3 in maintaining immune homeostasis by studying mutations in the FoxP3 gene in mice and human. Mutations of this gene give rise to severe autoimmune diseases in mice and are associated with human immune polymorphic inflammatory syndrome [54]. The transcription of FoxP3 is mainly regulated by five elements, including conserved non-coding sequences located in the FoxP3 locus in Tregs, on which transcription initiation and maintenance of FoxP3 are highly dependent [55]. In particular, the CpG region in CNS2, known as the Treg-specific demethylated region, maintains a highly demethylated state to preserve FoxP3 expression [15, 56].

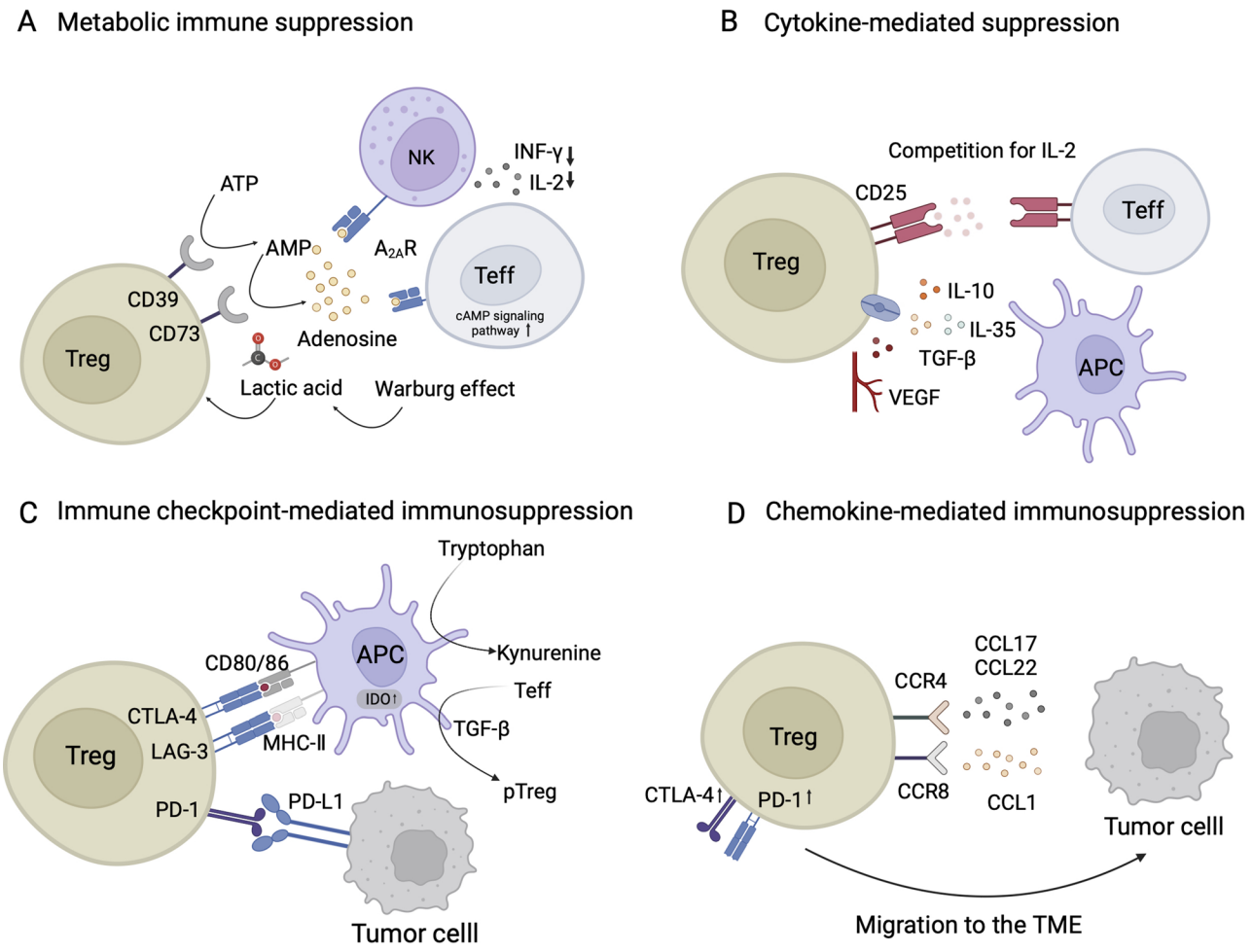

Fig. 3 Tregs in antitumor immunity regulation. **A**. Tregs degrade ATP to produce adenosine via CD39 and CD73, and adenosine inhibits the function of NK and effector T cells through A2aR.At the same time, tumor cells cause lactate accumulation due to Warburg effect, which promotes the generation of Tregs. **B**. Tregs compete with effector T cells for IL-2 through CD25 and secrete inhibitory cytokines such as IL-10, IL-35, TGF-β, and VEGF, Suppressing the immune response and promoting tumorigenesis. **C**. Tregs exhibit CTLA-4, LAG-3, and PD-1 on their surface, which interact with molecules on tumor cells and antigen-presenting cells (APC), suppressing effector T cells. **D**. Tregs respond to chemokines such as CCL17, CCL22, and CCL1 by expressing receptors like CCR4, CCR8, and PD-1, leading to their migration to the tumor microenvironment (TME)

In TME, under lactate-rich and hypoxic conditions, FoxP3 expression alters the metabolic modalities of Tregs, enabling them to function normally in a low-glycemic and high-lactic acid environment, thus adapting better to the TME [57]. In contrast, glycolysis-dependent Teffs are suppressed. Additionally, studies have shown that tumor cells and associated cells can secrete factors like TGF-β and IL-10, inducing Tregs' expression of FoxP3 and increase in number [58]. Activation of inflammatory factors and immune cells may also regulate FoxP3 expression [59]. Therefore, targeted therapy against various sources of FoxP3 in the TME holds significant potential value. AstraZeneca has developed antisense oligonucleotides targeting FoxP3 (AZD8701), a therapy specifically targeting Tregs and has been evaluated in patients with advanced solid tumors (NCT04504669), revealing potential to enhance $CD8^+$ T cell activation. Simultaneous studies in the A20 tumor model that combined FoxP3 ASO with anti-PD-1 treatment demonstrated that FoxP3 ASO significantly inhibited tumor growth and increased the number of complete responses (CR) or near-complete responses (near-CR) in mice [60]. In addition, a novel chemically modified self-delivered antisense oligonucleotide (FANA ASO) reduced the mouse tumor volume by targeting FoxP3 and reduced the mRNA level of FoxP3 and the number of Tregs. Several immune checkpoints, including CTLA-4, Tim-3, PD-1, LAG-3, and TIGIT, were down-regulated and are currently being investigated in combination with ICIs [61].

CD25+ tregs

CD25, the α-chain of the IL-2 receptor, is predominantly expressed on Tregs and forms the IL-2 receptor along with the β-chain (CD122) and the γ-chain (CD132) [62].

However, not all CD25-expressing cells are Tregs, necessitating their identification in combination with other markers [63]. IL-2 serves multiple roles as a cell growth factor, including promoting antibody-secretion of B cells, activating Teffs and NK cells and promoting Treg cell growth and differentiation [64]. CD25 binds to receptors on Tregs, activating Janus kinase [65], which leads to the phosphorylation of signal transducer and activator of transcription 5 (STAT5). Activated STAT5 then binds to the promoter of FoxP3 and CNS2, enhancing transcriptional activation and expression in Tregs [66]. In the TME, Tregs' high CD25 expression allows IL-2 a strong affinity for its receptor IL-2Rαβγ, competing for IL-2 and inhibiting Teffs that require IL-2 signaling for survival and function [67]. This mechanism enables Tregs to protect the tumor from the immune system by suppressing the immune response.

Despite the crucial role of the molecule, Treg-targeted therapies that focus on CD25 exhibit limited clinical efficacy. The primary issue is that systemic Treg depletion induces severe responses of inflammation and autoimmune, while also disrupting IL-2 signaling in Teffs, instead of selectively acting on Tregs. This unintended action undermines therapeutic efficacy [68]. This challenge is prevalent across all Treg-targeted therapies. Hence, designing optimized anti-CD25 antibodies that specifically target Tregs presents a promising strategy. 7D4, an IgM antibody, binds to CD25 without inhibiting IL-2/IL-2R signaling. It represents a novel type of optimized antibody. RG6292, derived from 7D4, selectively depletes Tregs via antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) while sparing $CD4^+$ and $CD8^+$ Teffs [69]. Moreover, studies in mouse models demonstrated that combining optimized anti-CD25 antibodies with PD-1 inhibitors significantly amplifies anti-tumor effects and improves ICB efficacy [70, 71]. h7B7-15 S, a humanized anti-CD25 monoclonal antibody that does not block IL-2, further enhances Treg depletion when paired with anti-CTLA-4, leading to substantial improvements in the remodeling of the tumor immune microenvironment [72].

GITR+ tregs

GITR is a constituent of the tumor necrosis factor superfamily which exhibits elevated expression levels under the influence of FoxP3 in mature Tregs [73]. Within the TME, the interaction of GITR on Tregs with its natural ligand initiates the activation of several pivotal cellular signaling cascades, including the NF-κB signaling pathway, the MAPK pathway, and the PI3k/pkB pathway [74]. These signaling cascades are crucial for the activation, survival and functionality of Tregs, in addition to facilitating the proliferation and effector capacities of Teffs [75].

Studies have indicated variability in GITR expression on tumor-infiltrating Tregs and lymphocytes across different tumor types, highlighting that patients with NSCLC, renal cell carcinoma and melanoma might benefit from anti-GITR therapy [76]. Helios plays a key role in ensuring that Tregs maintain their stable suppressive phenotype by enhancing FoxP3 expression, while the downregulation of Helios expression following GITR activation induces a Th1 effector-like phenotype in Tregs, thereby destabilizing FoxP3 expression and compromising their suppressive function within the tumor microenvironment [3, 77].

GITR's dual functions influencing both Tregs and Teffs make it a compelling and important target in the field of tumor immunotherapy [78]. Research concerning GITR agonist monoclonal antibodies showed that although efficacy has been seen with combination therapy with ICIs in phase I/II trials, they do not appear to be as effective as monotherapy, but rather respond to combination therapy, particularly with the addition of PD-1 blockade. The addition of PD-1 blockade may produce synergistic and complementary antitumor effects by reversing $CD8^+$ T cell exhaustion [74, 79]. DTA-1, an antibody to rat immunoglobulin g2a of GITR, significantly prolonged survival and induced durable tumor-specific immunity in a mouse ID8 ovarian cancer model with combined anti-PD-1 and GITR therapy. Combination therapy reduced tumor burden [80]. In phase I trials, TRX518 (anti-GITR) was administered alone or in combination with PD-1 inhibitors such as pembrolizumab or nivolumab. Considerable clinical responses were demonstrated when TI-Tregs were significantly depleted while $CD8^+$ T cell infiltration was increased, suggesting that combination therapy may be particularly effective when targeting tumors with high Treg content [81].

CCR4+ tregs

CCR4 is found on Tregs and other Th cells and interacts with the ligand CCL17 and CCL22 [82]. This receptor manifests elevated expression in T-cell malignancies such as adult T-cell leukemia/lymphoma and cutaneous T-cell lymphomas [83]. Within the TME, tumor cells and tumor-associated immune cells secrete CCL17 and CCL22. Tregs exhibiting high expression of CCR4 are recruited into the TME through recognizing and binding to these chemokines, thereby facilitating immune evasion from cancer [84]. The team led by Christine Ménétrier-Caux has demonstrated the CCL22-mediated recruitment of Tregs in breast cancer across two different studies [85]. At the same time, the study found that when cancer patients receive ICB treatment, it will lead to the activation of inflammatory responses in the TME,

the expression of pro-inflammatory and immunomodulatory signaling pathways, including CCL17 and CCL12. This promotes the migration of $CCR4^+$ Tregs into the tumor will promote tumor progression and resistance to ICB treatment [86, 87].

Therefore, anti-CCR4 monoclonal antibodies play a crucial role in evoking and enhancing anti-tumor immunity in cancer patients by selectively depleting Tregs. For instance, mogamulizumab is a fully humanized and deglycosylated monoclonal anti-CCR4 antibody. The researchers conducted a phase I clinical study to evaluate the safety and efficacy of nivolumab and mogamulizumab monoclonal antibodies in patients with advanced solid tumors. Analysis of paired biopsy samples from 12 patients showed that in most cases, TI-Tregs decreased and $CD8^+$ T cells increased. Solid objective responses were observed in tumors such as hepatocellular carcinoma and NSCLC [88]. However, David S. et al.'s study on patients with locally advanced or metastatic solid tumors found that the synergistic effect of mogamulizumab combined with nivolumab was not observed. This may be since CD8 and NK cells also express CCR4, resulting in additional consumption [89, 90], which requires further research and verification. However, it is essential to recognize that, due to the high expression of CCR4 in skin tissue, mogamulizumab treatment may result in skin-related adverse reactions and necessitates careful monitoring [91].

CCR8+ tregs

CCR8 serves as a chemokine receptor that binds to its ligand CCL1, thus mediating cell chemotaxis [92]. CCR8 is recognized as a potential specific marker for TI-Tregs and is selectively enhanced by Tregs within tumors across a broad spectrum of human cancer types, including breast, hepatocellular, colorectal, NSCLC and metastatic melanoma [93]. CCR8 is predominantly expressed in Tregs expanded in tumors, with negligible expression in tumor-infiltrating effector T cells (TI-Teffs) or peripheral Tregs in both human and mice [94]. Meanwhile, $CCR8^+$ Tregs are deemed as a stable subtype possessing enhanced immunosuppressive capacity, and their frequency increases with disease progression [95]. Although evidence suggests that the CCR8 signaling pathway is not essential for Tregs to exert tumor suppressive immunity and may not be involved in the recruitment of tumor Tregs, targeting CCR8 remains a critical component in tumor therapy [94], thereby serving as an effective strategy for TI-Treg cell targeting. Anti-cancer therapeutic antibodies targeting CCR8 can eliminate tumor-associated Tregs via Fc region-mediated ADCC and ADCP [96]. Therefore, anti-CCR8 antibodies may mitigate the adverse effects of extensive Treg depletion induced by anti-CTLA-4 or anti-PD-1 therapy and prevent the onset of fatal autoimmune reactions. S-531,011 is identified as a monoclonal antibody, which uniquely interacts with CCR8 among all known chemokines and possesses potent ADCC activity that neutralizes the CCL1-CCR8 signaling pathway, resulting in the depletion of Tregs within the tumor and producing a significant anti-tumor effect [97]. Van Damme et al. demonstrated in a study using NSCLC mouse models that the combination of anti-CCR8 antibodies and PD-1 monoclonal antibodies yielded enhanced therapeutic benefits [93]. Similarly, a phase 1/2 clinical trial report indicated that LM-108, an Fc-optimized anti-CCR8 monoclonal antibody exhibited encouraging anti-tumor efficacy in gastric cancer patients resistant to PD-1 therapy when administered in combination with anti-PD-1 antibodies [98].

CD39 / CD73+ tregs

CD39 and CD73 are nucleic acid ectonucleotidases that play pivotal roles in immune regulation and the TME. These enzymes are predominantly expressed in Tregs and regulate the immune response by synergizing within the TME [99]. Initially, CD39, a nucleotide triphosphatase, progressively degrades extracellular ATP into ADP and AMP, marking a crucial initiating step. Subsequently, CD73 efficiently transforms AMP into adenosine [100] which is a potent immunosuppressive molecule that can diminish the anti-tumor immune response by binding to adenosine receptors 2a (A2aR) or 2b (A2bR) on the surface of $CD8^+$ T cells, NK cells, and APCs, thereby inhibiting their activation and cytotoxic functions [101]. This mechanism further compromises the immune system's capacity to attack the tumor, offering a pathway for the tumor to circumvent immune surveillance [102].

At the same time, it affects the body's immunotherapy. The activation of adenosine/A2AR signaling can increase the expression of immune checkpoints on the surface of immune cells, including PD-1, CTLA-4 and LAG3, while promoting Treg proliferation and secretion of immunosuppressive factors (including TGFβ and IL-10) [103]. The high expression of CD39 and CD73 is usually induced by hypoxic conditions within tumors and plays an important role in creating an immunosuppressive microenvironment, thereby reducing the efficacy of ICB [104]. When the adenosine level in the TME is high (such as breast cancer and lung cancer), or when tumors grow rapidly and promote TME hypoxia, the use of anti-CD39 and CD73 antibodies can help enhance the efficacy of ICB [105]. For example, high levels of CD73 are expressed in EGFR-mutated NSCLC, thereby inhibiting T cell activity. The combination of anti-PD-L1 and anti-CD73 therapy significantly improved T cell responses and reduced tumor growth in EGFR-mutated NSCLC compared with either therapy alone [106].

CTLA-4+ treg

CTLA-4 is an inhibitory receptor expressed on the surface of T cells and plays an important role in activating Tregs and maintaining the stability of the immune system [107]. In the TME, Tregs express significantly more CTLA-4 than Teffs [108].CTLA-4 on the surface of Tregs competes with the co-stimulatory molecule CD28 for binding to the B7 molecule (CD80/CD86) on the surface of APCs. When CTLA-4 binds to the B7 molecule, it deprives CD28 of the required co-stimulatory signals, effectively inhibiting T cell activation [26, 109]. Meanwhile, CTLA-4 also carries CD80 and CD86 on the surface of antigen-presenting cells into Tregs through endocytosis [110], which reduces the number of co-stimulatory molecules on the surface of APCs and indirectly decreases T cell activation and expansion. CTLA-4 is essential for Tregs function because T cells require essential amino acids, such as tryptophan, for protein synthesis and metabolic activities. CTLA-4 induces the production of indoleamine-2,3-dioxygenase (IDO) through interaction with APCs, which promotes catabolism of tryptophan and generates pro-apoptotic metabolites that inhibit the activation of effector T cells [17, 111]. In addition, CTLA-4 signaling can interfere with the proximal signaling of T cell receptor and CD28 [112]. All these findings suggest that CTLA-4 serves as an important target for clinical therapy and provides new avenues for the treatment of various types of cancer.

TIGIT+ tregs

TIGIT, a member of the immunoglobulin superfamily featuring immunoglobulin and immunoreceptor tyrosine kinase structural domains, is extensively expressed across the immune system, encompassing Teffs, NK cells and Tregs [113]. Elevated TIGIT expression levels on Tregs in peripheral blood mononuclear cells of both healthy individuals and cancer patients correlate with hypomethylation of the TIGIT locus and the binding of FoxP3 to Tregs. Furthermore, TIGIT expression is notably upregulated within the TME [114]. TIGIT manifests immunosuppressive effects by binding to two ligands, CD155 (PVR) and CD112 (PVRL2), thereby inhibiting T and NK cell activation through competition for ligands with other molecules, such as CD226 (DNAM-1) or CD96 [115]. Within the TME, TIGIT binding to its ligands activates signaling pathways resulting in Treg attachment and stimulates the expression of the effector molecule fibrinogen-like protein 2, thus promoting Treg cell-mediated suppression of Teff proliferation [116]. Additionally, researchers have observed that TIGIT$^+$ Tregs in peripheral and tumor sites demonstrate increased expression of various characteristic marker genes, such as FoxP3, Helios, CTLA-4, PD-1, and lymphocyte activation gene-3 (LAG-3) [117]. This shows that blockade of TIGIT increasesthe immune-suppressing ability of Tregs, thereby affecting the efficacy of ICB therapy.

Therefore, blockade of TIGIT has become a key area of research in recent years following anti-PD-1 therapy. Dual blockers of PD-1 and TIGIT are a promising approach for tumor immunotherapy. Dual PD-1/TIGIT blockers boost the growth and activity of tumor-specific CD8$^+$ T cells and tumor-infiltrating lymphocytes (TILs) compared with a single blocker [118]. Tiragolumab, a popular inhibitor of the TIGIT target, has shown encouraging clinical results in combination with PD-1 or PD-L1, particularly in NSCLC [119]. The latest Phase II study found that Tiragolumab, which activates tumor and circulating myeloid cells via the Fc receptor, greatly improved the objective remission rate and progression-free survival [120].

Other types of treg

Indeed, there are other types of Tregs that play a crucial role in regulating tumor immunity. LAG-3 is a cell surface protein which is prominently expressed on Tregs. Typically, LAG-3 attaches to MHC II molecules on the surface of antigen-presenting cells and delivers inhibitory signals that interfere with CD4-MHC II interactions, thus inhibiting T cell activation and facilitating tumor cell evasion of immune attacks [121, 122]. Within the TME, TGF-β+Tregs suppress the function of effector T cells through the release of TGF-β, which additionally induces the differentiation of undifferentiated T cells into Tregs, thus promoting tumor invasiveness and metastatic ability [123]. The dysfunction of TIM-3$^+$ Tregs and CD8$^+$ tumor-infiltrating T lymphocytes, as well as the expansion of Tregs, are positively correlated [124]. Cluster of differentiation 103 is recognized as a hallmark of TI-Tregs and facilitates their specific migration and localization within tissues, notably in sites such as the intestinal mucosa, thereby enhancing the inhibitory effect upon Teff proliferation [125, 126]. CXCR3$^+$ Treg specifically binds to IFN-γ-related ligands such as CXCL9, CXCL10 and CXCL11, and continuously migrates to the TME, which can not only guide immune activation in an inflammatory environment, but also enhance the immunosuppressive function of Tregs in tumors. It has a dual role and is closely related to tumor immune escape. Focusing on the expression of different genes and molecules may provide a deeper understanding of the immunoregulatory mechanisms within the TME, potentially leading to the development of new therapeutic strategies designed to modulate the function of these cells to enhance the anti-tumor immune response.

Tregs in ICB

Molecules such as CTLA-4, PD-1, and PD-L1 operate through co-inhibitory signaling pathways and is also a key target for ICB therapy at the same time. ICB therapy blocks the interaction of these molecules to enhance Teffs' activity and improve their ability to attack tumors (Fig. 4). After treatment, changes in the number and function of Tregs within the TME are influenced by various factors, including Treg molecular expression, tumor type, metabolic regulation, and individual immune responses [3]. Current study aims to investigate how these factors contribute to variations in therapeutic efficacy and design new therapies with improved anti-tumor efficacy without overexposing patients to irAEs.

Surface biomarkers and antibody molecular mechanisms

Tregs and CTLA-4 blockade immunotherapy

During ICB treatment, the expression level of specific targets in Tregs and their molecular mechanisms are crucial to the therapeutic effect. Blocking different targets leads to varying effects. Initially, CTLA-4 inhibitors were believed to primarily function by reactivating dysfunctional Teffs. However, later studies revealed that CTLA-4 is predominantly expressed by Tregs within the TME [127]. Anti-CTLA-4 therapy promotes Treg depletion by inhibiting CD80/86-CTLA-4 interactions or through ADCC and ADCP, thus enhancing T cell activation [128, 129]. In anti-tumor immunity, the differential expression of CTLA-4 in Tregs and Teffs allows anti-CTLA-4 monoclonal antibodies to selectively deplete Tregs while preserving activity of Teffs, thereby enhancing anti-tumor immune responses [130] .

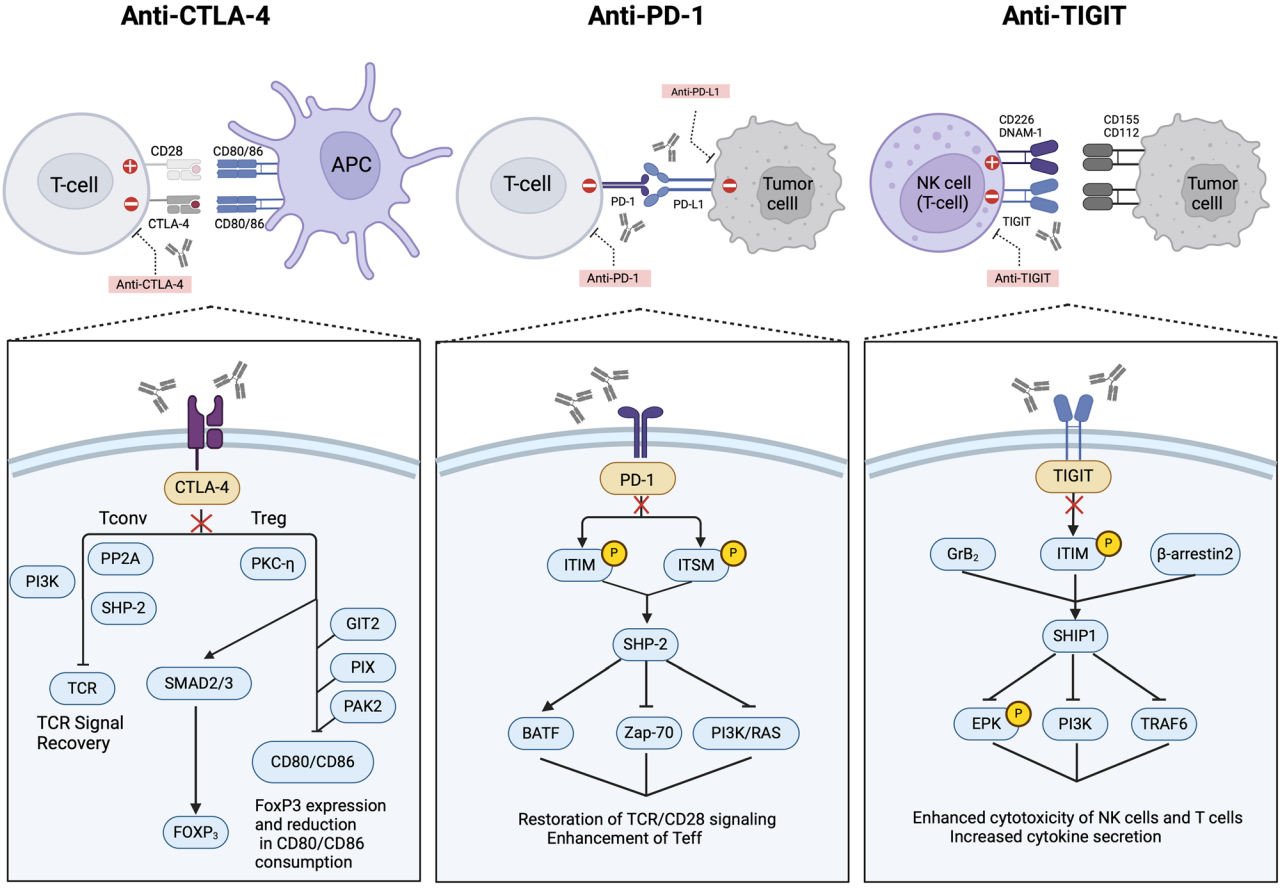

Fig. 4 The mechanisms of Tregs in immune checkpoint blockade therapy. Anti-CTLA-4 enhances the recovery of T-cell receptor (TCR) signaling by blocking CTLA-4 on T cells, thereby boosting the activity of Teffs. It also reduces the competition for CD80/CD86 by Tregs, indirectly decreasing the expression of FoxP3 and the immunosuppressive function of Tregs; Anti-PD-1 disrupts the interaction between PD-1 on T cells and its ligand PD-L1 on tumor cells, restoring TCR/CD28 signaling and enhancing the function of effector T cells (Teff). This enhancement also involves affecting downstream pathways such as PI3K/RAS, thereby improving cell survival; Anti-TIGIT blocks TIGIT, thereby enhancing the cytotoxicity of natural killer (NK) cells and T cells, and increasing cytokine secretion. It also restores the competitiveness of CD226, DNAM-1. This is achieved by inhibiting molecules that interact with TIGIT, such as SH2 domain-containing proteins (SHIP1), and intracellular T-cell signaling pathways like EPK and PI3K

However, the precise contribution of anti-CTLA-4 to overall antitumor efficacy remains a subject of debate. Studies have indicated that with employment of anti-CTLA-4 treatment, the number of Tregs in the TME may not decrease significantly [26]. For instance, quantitative immunohistochemical analysis of patients with melanoma, prostate cancer, and bladder cancer after ipilimumab treatment revealed increased infiltration of $CD4^+$ and $CD8^+$ T cells in the tumor, whereas the number of $FoxP3^+$ Tregs remained mostly unchanged [131]. Consequently, some studies suggest that anti-CTLA-4 antibodies may alter the migration patterns and activation status of Tregs. This effect may be attributed to the CTLA-4 blockade removing constraints on Treg expansion, leading to an increase in peripheral blood Tregs that subsequently replenish Tregs within the tumor [132, 133]. This also explains the failure to detect substantial TI-Treg depletion following treatment with depleting anti-CTLA-4 antibodies. Furthermore, Francesco et al. demonstrated in a mouse subcutaneous tumor transplantation model that Tregs regulate their population size by relying on CD28 costimulatory signals to deplete CD80 and CD86 expression. CTLA-4 inhibitors disrupt this balance, potentially resulting in excessive Treg proliferation within tumors [134]. Moreover, non-selective CTLA-4 targeting weakens the anti-tumor response because other immune cells in the TME, such as activated Teffs and DCs also express CTLA-4 [135].

Anti-CTLA-4 antibodies exert anti-tumor effects by binding to Fc receptors (FcγRs) via their Fc regions and this mechanism has become a significant hotspot in recent years. Research has shown that anti-CTLA-4 antibodies initiate ADCC or ADCP by binding to FcγRs, leading to the clearance and depletion of Tregs [136]. The rate of Treg cell depletion is influenced by the IgG isotype of anti-CTLA-4 antibodies and the polymorphisms of FcγRs [137]. For instance, tuvirumab, an IgG2 antibody targeting CTLA-4, has low affinity for activating FcγRs and primarily functions by blocking CTLA-4 signaling, with weak ADCC-mediated Treg clearance capabilities. In contrast, ipilimumab (IgG1) exhibits higher affinity for FcγRs, effectively inducing ADCC and promoting Treg cell depletion [136, 138]. Thus, selecting the appropriate antibody structure for specific tumor types is essential. However, if anti-CTLA-4 antibodies preferentially bind to inhibitory FcγRIIB on the surface of Tregs, it may reduce the effectiveness of antibody-mediated Treg clearance, ultimately diminishing the efficacy of ICB therapy [139]. Optimizing the Fc region structure of antibodies to balance Treg clearance efficiency and minimize side effects remains a critical focus in antibody drug development. For example, XTX101 is designed with an Fc enhancement region and is covalently linked to a masking peptide that blocks the complementarity determining region. This design enables it to be specifically activated in the TME, thereby minimizing systemic side effects [140].

Tregs and PD-1 blockade immunotherapy

PD-1 is an inhibitory costimulatory molecule that is broadly expressed on the surface of Teffs [141]. PD-L1 is a transmembrane protein typically expressed on tumor cells and specific immune cells, including DCs and macrophages [142]. PD-L1 upregulates FoxP3 expression in Tregs and influences anti-tumor therapies. Numerous studies suggest that elevated PD-L1 expression correlates with poor patient prognosis [143]. When PD-1 interacts with PD-L1 on tumor cell surfaces, it results in the inactivation of Teff function, thereby promoting tumor immune evasion. Blocking the PD-1/PD-L1 interaction reactivates Teffs, thereby strengthening the immune system's response to tumor cells [144]. PD-1/PD-L1 blockers represent the most extensively researched ICB therapies to date. Studies indicate a significant improvement in median overall survival in metastatic NSCLC to 21.9 months [145]. Toor et al. demonstrated that pembrolizumab, an anti-PD-1 antibody, inhibited FoxP3 expression on Tregs when applied to peripheral blood mononuclear cells from melanoma patients. This inhibition reduced the immunosuppressive function of Tregs [146]. Additionally, blocking PD-1 with pembrolizumab monoclonal antibody in melanoma patients showed similar results [147]. Unlike CTLA-4, PD-1 expression on Teffs within the TME is typically higher than on Tregs. Consequently, the primary aim of anti-PD-1 therapy is to relieve the inhibition of Teffs and restore their anti-tumor activity [148]. However, unchecked Tregs may impair treatment efficacy. This may be because that anti-PD-1 antibodies not only enhance $CD8^+$ T cell activity but also activate $PD\text{-}1^+$ Tregs through TCR and CD28 signaling pathways, thus maintaining their immunosuppressive effects [149, 150]. However, in patients unresponsive to PD-1/PD-L1 ICIs, elevated numbers of $PD\text{-}1^+$ Tregs are linked to reduced therapeutic efficacy [148, 151]. Geels' team therefore showed that Treg cell accumulation after PD-1 blockade may be indirectly related to activated $CD8^+$ T cells. IL-2 production by $CD8^+$ T cells lead to upregulation of ICOS by TI-Treg, thereby promoting their accumulation. Administration of ICOSL inhibitors prior to anti-PD-1 therapy reduces Treg cell accumulation while significantly enhancing the efficacy of anti-PD-1 therapy in immunogenic melanoma [152]. Gulijk et al. found that in mice models, anti-PD-L1 treatment preferentially activated Tregs in resistant tumors. In contrast, Teffs were not activated in the TME, which is a key factor contributing to treatment resistance [153].

Hyperprogressive disease (HPD) refers to the phenomenon where tumors in cancer patients experience rapid

acceleration following ICB treatment [154]. Kamada's team found that in patients with advanced gastric cancer, approximately 10% of anti-PD-1 treatments lead to HPD [150]. PD-1 blockade may promote the proliferation of highly suppressive PD-1$^+$ eTregs in HPD, which in turn diminishes the efficacy of ICB therapy. Therefore, the presence of actively proliferating PD-1$^+$ eTregs in tumors serves as a reliable indicator of HPD [155]. Research indicated that the ratio of the frequency of PD-1$^+$CD8$^+$ T cells to the frequency of PD-1$^+$ Tregs in the TME served as a more reliable predictor of the clinical effectiveness of PD-1 blockade therapies compared to the predictors PD-L1 and tumor mutational load [148]. In a mouse model, single-cell analysis revealed that PD-1 signaling promotes lipid metabolism, proliferation, and inhibitory pathways in TI-Tregs. Conditional deletion or blockade of PD-1 diminishes TI-Treg function while enhancing anti-tumor immunity [156]. Thus, combined approaches targeting PD-1 and other Treg markers that promote Teff activity while suppressing Treg functionality could be crucial for enhancing the efficacy of anti-PD-1 therapy.

TGF-β promotes PD-L1 expression through the MRTF-A/NF-κB pathway, leading to immune escape in NSCLC [157]. In the TME, TGF-β induces naive T cells to differentiate into iTregs, while Tregs enhance immunosuppression by expressing TGF-β [158]. Blocking TGF-β enhances the effect of anti-PD-1/PD-L1 therapy. The fusion protein M7824, which blocks both PD-L1 and TGF-β, prolonged survival and induced long-term effective anti-tumor immunity in a mouse model [159]. Y332D inhibited both TGF-β and VEGF signaling, exhibiting enhanced anti-cancer activity when combined with PD-1 inhibitors [160]. Anti-PD-1 treatment up-regulates other suppressor molecules on T cells (e.g., TIM-3, LAG-3, TIGIT), which increases the suppressive capacity of Tregs [115, 161]. Clinical trials targeting these suppressor molecules in combination with anti-PD-1 have shown promising efficacy [162]. The effects of anti-PD-1/PD-L1 antibody therapy on Tregs are multifaceted and complex, requiring further research to clarify their relationship.

Interactions between CTLA-4 and PD-1 blockade immunotherapy

Anti-CTLA-4 primarily acts during the initial activation phase of T cells by blocking the interaction between CTLA-4 and B7 molecules. This process occurs primarily in the lymph nodes, where it promotes anti-tumor effects by enhancing naïve T cell activation and reducing the suppressive function of Tregs [163]. In contrast to anti-CTLA-4, PD-1 expression on effector T cells in the TME is typically higher than on Tregs, and anti-PD-1 therapy mainly restores Teff function [164] Clinically, anti-PD-1 therapy has demonstrated superior efficacy compared to anti-CTLA-4 therapy [165]. Anti-CTLA-4 therapy affects not only Tregs in the TME but also systemic Tregs, leading to their depletion and frequently causing irAEs such as colitis, rash, and hepatitis [166]. Consequently, anti-PD-1 therapy has gained wider clinical application. Subsequent clinical trials have confirmed that combining anti-CTLA-4 and anti-PD-1 therapies significantly enhances efficacy. This combined strategy enhances CD4$^+$/CD8$^+$ T cell infiltration into tumor tissues, amplifies co-stimulatory signals and promotes the infiltration and activity of T cells and other immune cells within the TME [163, 167].

However, when the traditional anti-CTLA-4 antibody, ipilimumab, is administered alongside anti-PD- (L)1 monoclonal antibody therapy, the likelihood of irAEs rises. Generally, more than 50% of patients encounter irAEs, which signifies a markedly higher incidence than that observed with either treatment alone [168]. Therefore, this adverse effect can be mitigated by altering the structure of the previously mentioned anti-CTLA-4 antibody. AGEN1181 is a novel anti-CTLA-4 antibody that exhibits increased affinity for FcγRIIIa due to the S239D/I332E mutations and is capable of selectively depleting TI-Tregs and diminishing systemic immune activation. It has confirmed clinical efficacy in patients who have undergone multiple treatments, either as a monotherapy or in combination with the anti-PD-1 antibody Balstilimab [169]. Botensilimab, another engineered anti-CTLA-4 antibody, has shown sustained clinical responses across nine distinct immune-resistant or poorly immunogenic tumor types when combined with Balstilimab in patients with advanced solid tumors, establishing a foundation for future trials [170].

Metabolic regulation and TME environmental factors

Amino acid metabolism

In the TME, besides cell surface biomarkers, the unique metabolic mechanisms of Tregs and environmental factors also play a crucial role. In amino acid metabolism, the tryptophan-kynurenine (Trp-Kyn) pathway is closely associated with local immunosuppression within the TME. Tryptophan degradation inhibits the mTORC1 signaling pathway, activating GCN2 and resulting in T cell cycle arrest. Meanwhile, kynurenine and its metabolites act as potent agonists of the aryl hydrocarbon receptor (AhR), further activating Tregs and myeloid-derived suppressor cells (MDSCs), thereby inducing immune regulation [171, 172]. Targeting this metabolic pathway may enhance the efficacy of ICB. A phase II/III study assessed the efficacy of Indoximod (an IDO inhibitor) in combination with pembrolizumab for advanced melanoma treatment. The clinical trial (NCT02073123) reported an objective response rate of 56% and a complete response rate of 19%, demonstrating promising therapeutic efficacy [173].

Glycolysis and lactate metabolism

Glycolysis plays a crucial role in the efficacy of ICB therapy. Cancer cells rely on aerobic glycolysis to consume glucose for survival, leading to a substantial reduction in glucose levels within the TME [174]. Reduced glucose in the TME impairs mTOR activity, glycolysis, and IFN-γ production in TILs, which subsequently results in diminished TIL efficacy [175]. Tregs predominantly rely on oxidative phosphorylation to maintain their suppressive function, whereas Teffs depend on glycolysis and are more susceptible to damage [176]. The lactic acid produced during metabolism influences Treg activity in ICB therapy through several mechanisms. Roberta et al. found that blocking CTLA-4 promotes metabolic adaptability in T cells within tumors exhibiting low glycolytic activity, destabilizing Tregs and shifting them towards an inflammatory phenotype that produces cytokines like IFN-γ and TNF. This transformation enhances immune cell infiltration and improves therapeutic outcomes, particularly in tumors with glycolytic defects [177]. Additionally, Ding et al. demonstrated that lactate promotes RNA splicing and CTLA-4 expression in TI-Tregs via the lactate-FoxP3-USP39-CTLA-4 signaling axis, maintaining the immunosuppressive function of Tregs and impacting ICB efficacy. Thus, in tumors with low glycolytic activity, the efficacy of anti-CTLA-4 therapy is enhanced, and combining CTLA-4 blockade with glycolysis inhibitors may offer additional therapeutic benefits [178]. During anti-PD-1 treatment, in a low-glucose and high-lactate environment, eTregs take up lactate via MCT1 and upregulate PD-1 expression, whereas $CD8^+$ T cells exhibit the opposite PD-1 expression pattern. This divergence contributes to the failure of high-glycolytic tumors to respond to PD-1 inhibitors, ultimately driving disease progression [179]. In an NSCLC model, combining the glycolysis inhibitor IACS-010759 with a PD-1 inhibitor demonstrated superior efficacy compared to monotherapy [180]. For different tumor types, a detailed analysis of their TME is essential to formulate personalized treatment strategies.

Hypoxic environment

Hypoxia is one of the main markers that distinguish solid tumors from normal tissues [181]. It promotes the polarization of tumor-associated macrophages (TAMs) via hypoxia-inducible factor, facilitating their shift to the immunosuppressive M2 phenotype. M2-like macrophages secrete immunosuppressive cytokines, including IL-10 and TGF-β, which promote Treg cell activity and diminish the efficacy of ICB [182, 183]. Additionally, hypoxia can activate the transcription factor HIF-1α, inducing FoxP3 expression and promoting the differentiation of $CD4^+$ T lymphocytes into Tregs [184]. HIF-1α also upregulates PD-L1 expression, enabling Tregs to more effectively suppress T cell function, thereby limiting the efficacy of ICB therapy in this immunosuppressive environment. Studies have shown that directly eliminating TME hypoxia can improve cancer immunotherapy in mice [185, 186]. Li et al. demonstrated in a mouse model that the combination of hyperbaric oxygen therapy and chemotherapy agents, such as teniposide, against hepatocellular carcinoma can activate the cGAS-STING signaling pathway, thereby enhancing anti-tumor immune responses and increasing sensitivity to PD-1 antibody immunotherapy [187]. Bailey's team found that targeting HIF-1α reduced PD-L1 expression on tumor cells and tumor-infiltrating myeloid cells. However, through an IFNγ-dependent mechanism, it elevated PD-L1 expression in normal tissues, abolished the PD-1/PD-L1 checkpoint in the TME, diminished Treg-mediated immunosuppression and enhanced immune tolerance checkpoint activity in normal tissues [188]. Therefore, HIF-1α inhibitors, such as PX-478, may be ideal partners for CTLA-4 targeted immunotherapy, offering potential therapeutic synergy [189].

Improve ICB efficacy by treg changes

Presently, researchers are exploring therapies capable of selectively depleting Tregs within tumors while augmenting ICB efficacy without triggering autoimmune responses. An optimal target would be one highly expressed on intra-tumoral Tregs, making the combination of Treg cell depletion with ICIs a potentially superior therapeutic approach [190]. Simultaneous targeting of two inhibitory receptors to enhance anti-tumor immune responses. Clinical agents under development, targeting molecules like CD25, CCR4, OX40, ICOS, or GITR, are being considered for use in conjunction with ICIs (Table 1). In the previous sections, we described a variety of existing popular targets and analyzed their relationship with regulatory Tregs in ICB therapy, highlighting the positive effects of combination therapy. Nevertheless, researchers are concurrently investigating novel Treg cell targets to attain highly specific targeting of intra-tumoral Tregs, thereby enhancing the efficacy of ICB.

Itahashi et al. found that the Basic Leucine Zipper ATF-like Transcription Factor (BATF) plays a vital role in activating Tregs in mice and the TME by analyzing TAC-seq and ChIP-seq data. Additionally, $BATF^+$ Tregs were linked to poor clinical responses to PD-1 blockade [191]. Tregs activated by BATF showed higher expression of genes related to TCR and NF-κB signaling. Moreover, BATF might affect how Tregs move into the tumor area by downregulating specific chemokine receptor signaling genes [191]. Therefore, focusing on Tregs with high BATF expression alongside PD-1 blockade therapy is an important direction for future research. Additionally, endoglin acts as a co-receptor for TGFβ through immune

Table 1 Recent experiments on ICB tumor therapy were associated with the corresponding Treg cell changes

Treatment Plan	Trial phase Trial registration	Tumor types	Effects	Tregs
Ipilimumab + Cetuximab + Radiotherapy	phase 1 NCT01935921	Locally advanced head and neck cancer	3-year PFS: 61%; 3-year DFS: 72%; 3-year OS: 72%	Elimination of the less inhibitory Treg population may alter the balance of immune surveillance and immune escape
Camrelizumab + Apatinib + Oxaliplatin	Phase II NCT03878472	Locally advanced gastric cancer	ORR: 28% CPR: 15.8% MPR: 26.3%	Treg became more enriched in the tumor microenvironment post-treatment in some non-major pathological response (non-MPR) cases
Nivolumab + Ipilimumab	Phase II/III NCT03048474.	Malignant pleural mesothelioma	PFS: 6.25% OS: 23%	Treatment caused changes in the Treg cell ratio and proliferation rate, reflecting the adjustment of the immune response
Ipilimumab + Rituximab	Phase I NCT01729806	Relapsed / Refractory B-cell lymphoma	mPFS: 2.6 months ORR: 24%	The CD45RA-Treg cell ratio increases after treatment and may help to predict treatment response
Mogamulizumab + Durvalumab	Phase I NCT02301130	Advanced solid tumors	ORR: 5.3% mPFS: 1.9months mOS: 8.9months	Most patients are almost completely depleted of peripheral eTreg with reduced intratumoral Treg
Ipilimumab + Nivolumab + Anthracycline-based	Phase II NCT03409198	Metastatic hormone receptor-positive breast cancer	mPSF: 5.1months CBR: 55%	Addition of the drug to chemotherapy did not improve efficacy but caused a decrease in Treg levels, indicating a degree of immunomodulatory activity
Nivolumab + Abemaciclib + Endocrine therapy	Phase II JapicCTI-194,782 jRCT2080224706 UMIN000036970	HR-positive HER2-negative metastatic breast cancer	ORR(FUL): 54.5% ORR(LET): 40.0% DCR(FUL): 90.9% DCR(LET): 80.0%	Lower number of treg cells. Combination therapies have potential immunomodulatory effects, and monitoring Treg levels may help to understand therapeutic efficacy
Mogamulizumab + Nivolumab	Phase I/II NCT02705105	Locally advanced or metastatic solid tumors	ORR: 10.5% mPFS: 2.6months mOS: 9.5months	Treatment causes depletion of CCR 4 + effector Treg in the peripheral blood of most patients, benefiting Nivolumab play a role
Mogamulizumab + Nivolumab	Phase I NCT02476123	Advanced or metastatic solid tumors	Differ from one another	The observed depletion of effector Tregs in the peripheral blood and in the tumor microenvironment, may enhance the patient immune response
Pembrolizumab + Low-dose Cyclophosphamide + G100 (TLR4 agonist)	Phase II NCT02406781	Advanced pretreated soft tissue sarcoma	mPFS: 1.8months mOS: 10.6months	The reduced CD8 / FoxP 3 + CD4 ratio indicates an increase in Treg cells and may affect the limited efficacy of the treatment
Pembrolizumab + Pelareorep	Phase II NCT03723915	Advanced pancreatic adenocarcinoma	mPFS: 1.9months mOS: 6.3months,	The abundance of Treg cells decreased in the peripheral blood of the CBR group and not in Treg cells in the peripheral blood of the NR
Anti-GITR-TRX518 + Nivolumab	Phase I NCT02628574	Advanced solid tumors	DCR: 50% ORR: 12.5%	The Treg cells were initially reduced, but increased after the TRX518 dose
Durvalumab + Tremelimumab	phase II NCT02592551	Malignant Pleural Mesothelioma	mOS<14.0months DFS<8.4months	Both ICB regimens induced CD8 T-cell infiltration into MPM tumors but did not alter CD8/Treg ratios

Table 1 (continued)

Treatment Plan	Trial phase Trial registration	Tumor types	Effects	Tregs
Tiragolumab + Atezolizumab	Phase IB NCT02802098	Advanced HER2-negative breast cancer	mPFS: 3.5 months mOS: 11 months	Decreased circulating Treg cells in non-progressors, suggesting enhanced immune response against the tumor.

mechanisms. Research has shown that Tregs expressing endoglin are present in both mouse and human rectal cancer tissues. A study by Schoonderwoerd and colleagues found that combining anti-endoglin and anti-PD-1 antibodies significantly improved treatment outcomes in various colorectal cancer models [192]. Li and others demonstrated that targeting Bcl6 in Tregs effectively slowed tumor growth and enhanced the effectiveness of ICB treatment when used with anti-CTLA-4 or anti-PD-1 therapies. These new targets show strong antitumor effects, expanding options for ICB therapy and offering new possibilities for future clinical use [193].

Tregs not only promote tumor escape but also help control immune-related toxicity in ICB therapy [194]. The number of Tregs is often not reduced under ICB therapy, and the modulation of either the number or function of these cells to enhance the effects of ICB therapy is a hot topic. In different tumor microenvironments, significant variations exist in the phenotype and function of Tregs. Recently, single-cell RNA sequencing technology has unveiled the diversity of Tregs in different tumor types [195]. For example, Tregs in breast cancer demonstrate high PD-1 and PD-L1 expression [196], while those in ovarian cancer display high PD-1 and 4-1BB expression [197]. This diversity can result in varying behaviors across different tumor microenvironments, potentially impacting ICB efficacy. In common tumors such as those of the lung and liver, Tregs play an immunosuppressive role. However, in colorectal cancer and head and neck tumors, Tregs are associated with a better prognosis, partly by suppressing microbial-induced inflammation and reducing susceptibility to tumors [198, 199]. Tregs situated in various parts of the tumor exhibit distinct functions. In colorectal cancer, Tregs in the tumor mesenchyme contribute to a better prognosis compared to those in the tumor nests and swelling margins [200]. For example, CCR8, targeting tumor-expanding Tregs specifically while preserving peripheral Tregs, represents an important candidate therapeutic target for future therapies. Surface Oncology has developed SRF114, a highly selective humanized anti-CCR8 desialylated antibody, currently undergoing a phase I clinical trial (NCT05635643). Preliminary results indicate that in patients with head and neck squamous cell carcinoma, SRF114 effectively reduces TI-Tregs and modifies the TME to facilitate immune attack [201].

Additionally, the number or function of Tregs can be modulated using low-dose adjuvant therapy or immunomodulators, enhancing ICB efficacy. Using low-dose chemotherapeutic agents or radiotherapy can selectively reduce the number of Tregs and, when combined with ICB therapy, decrease the immunosuppressive effect of Tregs [202, 203]. For example, the combination of Ipilimumab and Nivolumab with anthracycline chemotherapeutic agents (e.g., adriamycin) and Cyclophosphamide for metastatic hormone receptor-positive breast cancer has demonstrated clinical benefits following the discontinuation of chemotherapy in some patients, despite a higher risk of high-grade adverse events [204].

Other immunomodulators, including variants of IL-2, are primarily intended to enhance Teffs over Tregs, or to indirectly influence Treg cell function by modulating other immunosuppressive factors, such as PD-1/PD-L1 [205]. A low-affinity IL-2, developed by Ren et al. and combined with an anti-PD-1 antibody, targeted intratumorally infiltrating $CD8^+$ T cells, without significantly affecting Tregs. This combination therapy not only significantly boosted the antitumor effect but also synergized with anti-PD-L1 therapy to overcome tumor resistance to immune checkpoint inhibition without significant toxicity [144]. Another study investigated the use of a PD-1-linked IL-2 agonist to enhance effector T-cell antitumor capacity and effectively overcome immunosuppression in chronic infection and cancer [206]. Other recent studies such as the use of MALT 1 inhibitors to reprogram Tregs to lose their immunosuppressive function and secrete INF-γ, thereby enhancing the immune response to tumors. This approach has shown potential benefits in combination with anti-PD-1 in animal models and is being evaluated in clinical trials [207].

Challenges and future directions

While ICB stands as a groundbreaking advancement in antitumor therapy, enhancing therapeutic outcomes, it also presents several limitations, particularly in terms of the role of Tregs. Initially, certain patients might develop resistance to ICB over time, a process underpinned by numerous intricate mechanisms [208]. Firstly, tumor-intrinsic factors, including insufficient tumor antigenicity, defective INF-γ signaling, and the absence of endogenous MHC, can contribute to ICB resistance [209]. Additionally, external factors in the TME, such as

immunosuppressive Tregs, myeloid-derived suppressor cells (MDSCs), and TGF-β, also contribute to ICB resistance through their inhibitory effects [210]. Metabolic alterations within the TME further impair immune cell function, exacerbating drug resistance [211]. This article had explored the impact of Treg cell-mediated immunosuppression on treatment response and discusses potential strategies to overcome this challenge. Furthermore, ICB therapy can lead to chronic immune-related adverse events impacting various organs, such as the endocrine and rheumatologic systems, potentially affecting as many as 40% of patients [212, 213]. Moreover, the absence of reliable biomarkers for predicting patient responses to ICB therapy hinders the prediction of outcomes with ICB combination therapies and the discovery of novel targets [214]. Ultimately, the efficacy of ICB therapy exhibits considerable variation among patients and across different types of cancer, with some experiencing significant benefits while others see no response [209]. These challenges underscore the necessity for additional research and clinical trials to address these issues, enhance both the efficacy and safety of ICB therapy, and broaden its applicability across a more diverse patient demographic.

To achieve this goal, it is imperative to explore the intricate mechanisms of action of Tregs and the signaling pathways that connect their isoforms with distinct effector features. Understanding how Tregs influence the efficacy of ICB is essential to fully leveraging Tregs as an immunotherapeutic target [3, 215]. Future research endeavors might concentrate on the development of drugs designed specifically to target Treg in the TME. Such drugs, including antibody-drug conjugates, immunotoxins, and small interfering RNA conjugates, aim to either eliminate Tregs or inhibit their functions without compromising other immune cells [216].

Furthermore, targeted drug delivery systems represent a promising approach to specifically eliminate Tregs from the tumor environment, thereby bolstering the body's anticancer response and augmenting the efficacy of ICB [217]. Particularly noteworthy is the advancement of nanodrug delivery systems, which encapsulate drug carriers within nanoparticles to accurately target tumor sites, enhancing drug stability and biocompatibility, extending the drug action duration, and significantly lowering toxicities. Nanoparticles in NDDS are specifically designed to target Tregs [218]. For example, in tumor models such as breast cancer 4T1 and colon cancer CT-26, the tumor-activated biomimetic lipoprotein carrier system is used to break through the intratumoral delivery barrier, efficiently deliver immunogenic cell death (ICD) inducers to intratumoral tumor cells and activate antitumor resistance. The tumor immune response weakens the dominance of Tregs and significantly enhances the therapeutic effect of immune checkpoint blockade (ICB) such as anti-PD-1 [219]. By combining targeted delivery systems with ICB therapy, it is possible to overcome the immunosuppression caused by Tregs, thereby improving the patient's response rate to immunotherapy. This strategy is expected to become an important tool in future cancer immunotherapy [220].

Considering the variability among individuals, developing personalized medical strategies that adapt treatment regimens to the unique immune microenvironment and Treg characteristics of each patient is crucial. This development requires evaluating the efficacy and safety of diverse therapies via large-scale, multicenter clinical trials [221]. Research into Treg utilization in ICB therapy represents a rapidly advancing field, confronted with myriad challenges. By integrating the latest advancements in biotechnology, clinical trials, and data analysis, there is an expectation of significantly improving personalized oncology treatment strategies, enhancing therapeutic efficacy, and reducing side effects in the future.

Conclusion

In summary, Tregs embody a paradoxical nature, serving as a double-edged sword by both mitigating undue activation of the immune system to avert autoimmune diseases and facilitating tumor progression. As research into Tregs advances, a growing repertoire of Treg markers has been delineated within tumor tissues, offering insights into their roles and mechanisms of action in tumorigenesis. These markers hold potential not only as diagnostic biomarkers for cancer but also as therapeutic targets. Nevertheless, the translation of therapeutic interventions from disease samples, cellular, and animal models to clinical application remains limited. This gap underscores the need for extensive research to unearth the clinical utility of Tregs in cancer diagnostics and treatment. The challenge of indiscriminately depleting Tregs, which may trigger immune dysregulation, accentuates the importance of devising strategies that precisely target tumor-associated Tregs without compromising systemic immune homeostasis. ICB therapy has marked a significant leap in reinvigorating the immune system's capacity to detect and eradicate cancer cells, addressing a pivotal tumor immune evasion tactic. Despite this progress, ICB therapy encounters obstacles, including therapy resistance and adverse effects. The trajectory of ICB therapy hinges on the adoption of personalized medical strategies, bolstered by genomic analysis, biomarker identification, and a nuanced understanding of the tumor microenvironment. In particular, the role of Tregs in modulating immune responses and their potential to diminish ICB therapy's effectiveness is a pivotal research avenue, heralding new discoveries and enhancements in oncology. Furthermore, advancements in gene editing and cell engineering technologies portend the

development of highly specialized Tregs, paving the way for more targeted and efficacious cancer therapies in the foreseeable future.

Abbreviations

Treg	Regulatory T
ICB	immune checkpoint blockade
CCL22	C-C motif chemokine ligand 22
CCR4	C-C motif chemokine receptor 4
TGF-β	transforming growth factor-β
IL-35	interleukin-35
IL-10	interleukin-10
IL-2	interleukin-2
CD25	cluster of differentiation 25
CTLA-4	cytotoxic T-Lymphocyte-Associated protein 4
CD80	cluster of differentiation 80
APCs	antigen presenting cells
TCR	T cell receptor
CD28	cluster of differentiation 28
tTreg	thymus-derived Tregs
pTreg	peripherally induced Tregs
nTregs	natural Tregs
FoxP3$^+$ Treg	forkhead box P3$^+$ Treg
iTreg	induced regulatory T cells
CD39	cluster of differentiation 39
CD73	cluster of differentiation 73
PD-1	programmed death-1
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TIGIT	T cell immunoreceptor with IG and ITIM domains
GITR	glucocorticoid-induced TNFR-related protein
OX40	tumor necrosis factor receptor superfamily member 4
4-1BB	tumor necrosis factor receptor superfamily member 9
CCR8	C-C motif chemokine receptor 8
TME	tumor microenvironment
STAT5	signal transducer and activator of transcription 5
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCP	antibody-dependent cell-mediated phagocytosis
CTL	cytotoxic T lymphocyte
NSCLC	non-small cell lung cancer
IDO	indoleamine-2,3-dioxygenase
LAG-3	lymphocyte activation gene-3
ICIs	immune checkpoint inhibitors
PD-L1	programmed cell death-ligand 1
ICIs	immune checkpoint inhibitors
irAEs	related adverse events
TI-Tregs	tumor-infiltrating Tregs

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AZ, TF, YL and GY conceptualized and designed the study and reviewed and revised the manuscript. AZ and TF were involved in data acquisition and analysis.GY, CL and ZJ supervised the study. All of the authors have read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

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