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Regulation of Treg cells by cytokine signaling and co-stimulatory molecules

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$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), a vital component of the immune system, are responsible for maintaining immune homeostasis and preventing excessive immune responses. This review explores the signaling pathways of the cytokines that regulate Treg cells, including transforming growth factor beta (TGF-β), interleukin (IL)-2, IL-10, and IL-35, which foster the differentiation and enhance the immunosuppressive capabilities of Tregs. It also examines how, conversely, signals mediated by IL-6 and tumor necrosis factor -alpha (TNF-α) can undermine Treg suppressive functions or even drive their reprogramming into effector T cells. The B7 family comprises indispensable co-stimulators for T cell activation. Among its members, this review focuses on the capacity of CTLA-4 and PD-1 to regulate the differentiation, function, and survival of Tregs. As Tregs play an essential role in maintaining immune homeostasis, their dysfunction contributes to the pathogenesis of autoimmune diseases. This review delves into the potential of employing Treg-based immunotherapy for the treatment of autoimmune diseases, transplant rejection, and cancer. By shedding light on these topics, this article aims to enhance our understanding of the regulation of Tregs by cytokines and their therapeutic potential for various pathological conditions.

KEYWORDS

regulatory T cells, cytokines, signaling pathways, autoimmune diseases, tumors

1 Introduction

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are immunoregulatory cells that express the master transcription factor forkhead box protein 3 (Foxp3) (1). Tregs exhibit persistent and high expression of the interleukin (IL)-2 receptor alpha chain or CD25, as IL-2 is crucial for their survival and proper functioning (2). Tregs refer to those T cell subsets that can regulate or suppress the overreaction of the immune system. Although Tregs account for only 3–10% of the peripheral $CD4^+$ T cell population, they are crucial for maintaining immune tolerance by suppressing the activation, proliferation, and function of effector immune cells. They secrete anti-inflammatory cytokines such as IL-10, IL-35, and transforming growth factor beta (TGF-β) to inhibit immune cells in a contact-

independent manner (3, 4). Additionally, Tregs possess a high level of CD25 surface expression, which leads to the consumption of IL-2 in the surrounding environment. This consumption helps restrict the proliferation and activation of effector T cells (Teffs) (5). Tregs also suppress immune cells through contact-dependent mechanisms involving co-stimulatory molecules, such as cytotoxic T lymphocyte–associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death ligand 1 (PD-L1) (6–10). Through these regulatory mechanisms, Tregs play a crucial role in inhibiting the activity of immune cells, ensuring the balance of the immune system, and preventing the occurrence of excessive immune responses and the development of autoimmune diseases (11, 12).

Tregs can be classified based on their developmental origins (13). Thymus-derived Tregs (tTregs) develop in the thymus, while a small proportion of Tregs is derived from conventional T cells (Tconvs) or peripherally derived Tregs (pTregs) and matures under specific conditions, such as exposure to microbial antigens in the intestinal mucosa (14). In the presence of specific cytokines (i.e., TGF-β and IL-2), antigen stimulation *in vitro* can induce the expression of Foxp3 in Tconvs, which exhibit phenotypic and functional characteristics similar to those of tTregs and pTregs and are called inducible Tregs (iTregs) (15). Subsets of Tregs in peripheral blood exhibit T helper–like characteristics, meaning they share chemokine receptor and transcription factor expression with T helper cells (16). Examples include Th1-Tregs ($CXCR3^+$ $T\text{-}BET^+$ $Foxp3^+$ Tregs) and Th2-Tregs ($CCR8^+$ $GATA3^+$ $Foxp3^+$ Tregs) (17). Another type of T helper–like Treg cell is the follicular regulatory T (Tfr) cell, which suppresses follicular helper T (Tfh) cells. Tfr cells play a critical role in germinal center reactions and antibody production, and defects in Tfr cells lead to antibody accumulation and the occurrence of widespread autoimmune diseases (18–20). These Treg subsets are summarized in Table 1.

The complex interactions among T cell subsets are characterized by diverse functional dynamics. Both iTregs and pTregs typically differentiate from Tconvs, a step critical for peripheral tolerance. It is essential to distinguish tTregs from pTregs and iTregs as they display distinct roles in maintaining central tolerance. The transcription factor Helios has been identified as a biomarker for stable tTregs (32). Additionally, research has uncovered specialized subsets of Tregs, such as Th1-Tregs and Th2-Tregs, each characterized by distinct functions tailored to modulate Th1 and Th2 responses, respectively (33, 34). Indeed, the Treg compartment exhibits a degree of plasticity that enables Tregs to modulate their suppressive functions according to the surrounding microenvironment. For example, interferon gamma (IFN-γ) or IL-27 induce Th1-Tregs with expression of Th1-related molecules, namely chemokine (C-X-C motif) receptor 3 (CXCR3) and T-box gene expressed in T cells (T-bet) (35), which can migrate to sites of Th1 inflammation and suppress Th1 cells effectively (36). Th2-specific Tregs are tailored to suppress Th2 responses, which are associated with allergic inflammation and characterized by Th2 cytokines like IL-4, IL-5, and IL-13, as well as GATA binding protein 3(GATA3) (37, 38).

The development and function of Tregs are heavily reliant on cytokines and co-stimulatory molecules. Exploring their regulatory mechanisms in Tregs is essential for gaining a deeper understanding of immune regulation, the development of related diseases, and the potential for new immunotherapeutic approaches. This review focuses on the role of these signaling pathways in Tregs and their potential implications for future therapeutic strategies.

2 Treg-promoting cytokines

2.1 TGF-β

TGF-β is a multifunctional cytokine produced by macrophages and T cells that plays a critical role in immune regulation. TGF-β exerts its immunoregulatory effects primarily by modulating the development and function of T cell subsets (39). TGF-β induces the expression of Foxp3, a key transcription factor for Tregs, by activating the suppressor of mothers against decapentaplegic (SMAD) signaling pathway (40). This promotes the generation of Tregs, which suppress inflammation and prevent autoimmune reactions. Members of the TGF-β family cooperate with receptors as ligands to form receptor complexes and activate receptor-regulated SMAD (R-SMAD), which cooperates with common mediator SMAD (Co-SMAD) to enter the nucleus. By identifying different SMAD-binding proteins and forming complexes, the specific expression of various target genes is regulated, and multiple biological effects of TGF-β ultimately occur (41). The SMAD family of proteins is the first set of signaling molecules involved in TGF-β signal transduction (40), and this signaling is tightly regulated by inhibitory SMAD (I-SMAD i.e., SMAD6 and SMAD7). The N-terminal domains of SMAD6 and SMAD7 share only 37% homology, while chimeras containing the SMAD7 N-terminal domain and SMAD6 MH2 domain inhibit TGF-β signaling pathways in the same manner as SMAD7 (42). SMAD6 is biased to inhibit signaling pathways induced by the BMP type I receptors ALK-3 and ALK-6. $SMAD6^{-/-}$ mice can develop a variety of cardiovascular diseases (43). SMAD7 inhibits the formation of SMAD complexes and prevents the phosphorylation of SMAD2 and SMAD3, thereby interrupting the signaling cascade and influencing TGF-β signaling and Treg induction (Figure 1) (44).

TGF-β also plays an important regulatory role in the balance between Treg and Th17 cell differentiation (45). Endogenous TGF-

TABLE 1 Treg subtypes and their specific markers and characteristics.

Treg Subtype	Origin	Main Markers
pTregs (21, 22)	Peripheral	GATA3, IRF4, RORC, TBX21, HELIOS
tTregs (21, 23)	Thymus	CCR7, CD45RA, CD31, SELL, NRP1
iTregs (21, 24, 25)	Peripheral	Treg-specific demethylated region (TSDR)
Th1-Tregs (26)	Peripheral	CXCR, Tbet
Th2-Tregs (27, 28)	Peripheral	IL-4, IL-13, IRF4 CCR6-, CXCR3-, CCR4, GATA3
Tr1 (29, 30)	Peripheral	IL-10, CD49b, Lag3, Foxp3-
Tr35 (31)	Peripheral	IL-35, Foxp3-

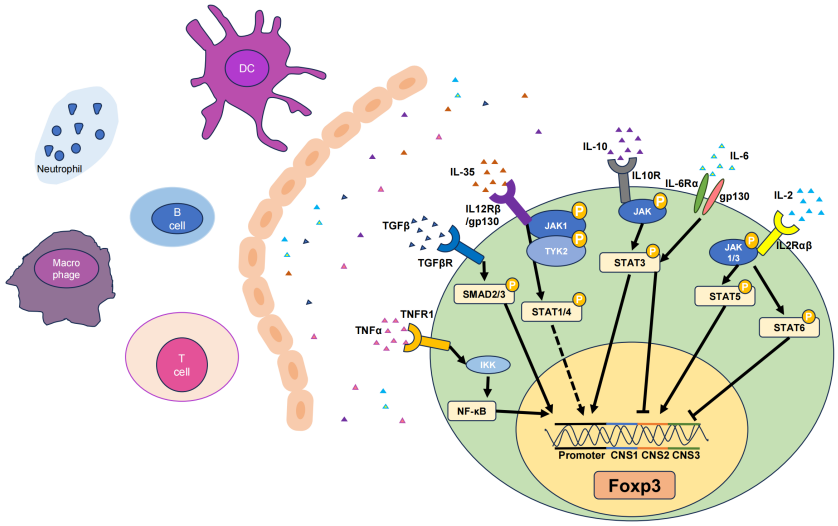

FIGURE 1
Six cytokines that promote Tregs. The TNF-α, TGF-β, IL-6, IL-35, IL-10, and IL-2 pathways can influence their respective receptors to varying degrees, thereby activating different signaling pathways and ultimately upregulating the transcription levels of Foxp3, resulting in increases in the number and stability of Tregs. Among them, there are gene loci that represent the corresponding signaling pathways and functions of different cytokines. Solid lines indicate pathways that have been functionally analysed, while dashed lines represent the effect on the Foxp3 gene without specific key loci identified. JAK, Janus-family tyrosine kinase; CNS, non-coding sequence; TYK2, tyrosine kinase 2. Arrows represent signal path direction, dashed lines represent ambiguity, and horizontal lines represent suppression.

β, along with inflammatory mediators such as IL-6, IL-21, and IL-23, inhibits Foxp3 expression and initiates the differentiation pathway of retinoic acid receptor–related orphan receptor gamma-t (RORγt)–mediated Th17 cells (46). As IL-6 levels decrease during the late phase of inflammation, TGF-β alone promotes the expression of Foxp3 for Treg differentiation and suppresses RORγt to limit Th17 cells, thus maintaining Treg function and controlling the effector cell response for the termination of the immune response (47, 48). Within the cell, the TGF-β/SMAD signaling pathway promotes the expression of Foxp3. SMAD3 can cooperate with nuclear factor of activated T cells (NFAT) to enhance histone acetylation in the Foxp3 enhancer region, thereby inducing Foxp3 transcription (49). Compared to controls, $SMAD3^{-/-}$ mice exhibit a significant reduction in the quantity of Foxp3 induced by TGF-β (50).

The combination of TGF-β and the immunosuppressant rapamycin can significantly promote the proliferation of Tregs, indicating that rapamycin relies largely on TGF-β to exert its immunosuppressive effect (51). Rapamycin is a 32-ring azotriene-containing macrolide that inhibits immunity by inhibiting IL-2 signal transduction by blocking mTOR which is important in IL-2 receptor signaling (52). The IL-2 receptor signaling also leads to the activation of the PI3K/Akt pathway. Akt then activates mTOR by inhibiting the tuberous sclerosis complex (TSC1/TSC2), which normally suppresses mTOR activity. The rapamycin the FK506-binding protein 12 (FKBP12) complex binds to mechanistic target of rapamycin complex1 (mTORC1), inhibits the mammalian target protein of rapamycin (mTOR) pathway, and induces immunosuppression (53). By inhibiting mTORC1, rapamycin effectively blocks the downstream effects of mTOR activation, including protein synthesis and cell cycle progression, which are necessary for T cell proliferation. High-dose rapamycin can promote the proliferation of Tregs by inhibiting the mTOR signaling pathway, thereby significantly inhibiting the progression of experimental autoimmune encephalomyelitis (EAE) in a model and eventually mitigating the incidence and EAE clinical scores of each stage (early onset, peak, and remission) (54).

Many preclinical studies have shown that blocking TGF-β signaling is an effective anti-tumor treatment that can reduce Treg-mediated immunosuppression, increase $CD8^+$ T cell cytotoxicity, promote T cell penetration into the center of the tumor, and thus contribute to strong anti-tumor immunity and tumor regression (55). TGF-β suppresses the immune system by modulating the function of immune cell classes in the tumor microenvironment (TME) (56, 57).

2.2 IL-2

IL-2 is a cytokine mainly produced by activated $CD4^+$ T cells, particularly Th1 subsets (58). In the thymus and peripheral lymphoid organs, IL-2 signaling promotes the differentiation and development of Tregs by binding to the high-affinity IL-2 receptor (IL-2R), which is a heterotrimer consisting of the IL-2R(α/β/γ) subunits. This leads to an increase in Foxp3 gene expression in T cells, promoting the differentiation of $Foxp3^+$ Tregs (59). Upon IL-2 binding to its receptor, the receptor-associated JAKs, specifically JAK1 and JAK3, are activated. JAK1 is associated with the IL-2Rβ chain, and JAK3 is closely linked with the IL-2Rγ chain (60). The activation of JAK kinases is initiated by their phosphorylation, which subsequently enables them to phosphorylate IL-2Rβ and other downstream signaling molecules. This phosphorylation creates docking sites for

signaling molecules containing SH2 domains. Specifically, STAT5 is attracted to the phosphorylated IL-2Rβ chain. Within the STAT5 family, there are two distinct proteins, STAT5A and STAT5B, both of which undergo phosphorylation by the action of JAK kinases (61). Once phosphorylated, STAT5 proteins form homodimers or heterodimers, dissociate from the receptor, and translocate to the nucleus where they bind to specific DNA elements and promote the transcription of target genes (62). Previous studies have revealed that IL-2 signaling activates STAT5 and promotes Foxp3 expression by binding to the intronic enhancer element within conserved non-coding sequence 2 (CNS2) of the Foxp3 gene cluster (Figure 1) (63). This process is essential for maintaining Foxp3 expression in mature Treg cells. Phosphatase 2A (PP2A) is a negative regulator of IL-2 production in Teffs (64) and prevents IL-2Rβ from being clipped from the cell surface by restricting the activity of ADAM metallopeptidase domain 10 (ADAM10) in Tregs, thereby achieving effective IL-2R signaling and ultimately affecting Tregs (65).

IL-2 and IL-2/anti–IL-2 mAb immunocomplexes have been shown to have therapeutic efficacy against autoimmune diseases in preclinical studies via Treg promotions. The complex of IL-2/JES6 (IL-2 and JES6–1 mAb) can selectively expand Tregs for the suppression of autoimmune diseases in EAE (66). With increasing research on the immune regulatory mechanism of low-dose IL-2, IL-2 has gained attention as a potential treatment for various immunological diseases, such as graft-versus-host disease (GvHD) (67), hepatitis C–related vasculitis (68), and type 1 diabetes (69). Low-dose IL-2 promotes a balance between Treg and Th17 cells in patients with Sjögren's syndrome (SS), a condition characterized by dryness (70). In patients with inflammatory myopathy who received 500,000 IU of IL-2 therapy for 5 days, Treg cell numbers significantly increased, while erythrocyte sedimentation rates, muscle enzyme levels, and pain scores significantly decreased (71). Recent clinical studies have shown that low-dose IL-2 is safe and effective against 11 autoimmune diseases, including rheumatoid arthritis (RA) and ankylosing spondylitis (72). Overall, the IL-2 signaling pathway moderates the role of Tregs in immune regulation and self-tolerance by affecting their development, proliferation, survival, and function.

2.3 IL-10

IL-10 is produced by macrophages, monocytes, and T cells and is considered a Th2 cytokine and an anti-inflammatory cytokine. IL-10 signaling requires the presence of cell surface–expressed IL-10 receptors (IL-10R) (73). IL-10 induces STAT3 signaling by phosphorylating the cytoplasmic tails of IL-10R1 and IL-10R2 through Janus-family tyrosine kinase 1 (JAK1) and non-receptor tyrosine-protein kinase (Tyk2), respectively (74). IL-10$^{-/-}$ mice exhibit more severe inflammatory damage, as IL-10 can regulate the function of Tregs through the activation of STAT3, and the lack of IL-10 leads to a significant decrease in STAT3 phosphorylation in microglia. Although the IL-10 promoter lacks binding sites for Foxp3, it does contain binding sites for STAT3, suggesting that Foxp3 may modulate IL-10 expression indirectly through STAT3 signaling. Indeed, Foxp3 is involved in the transcriptional activation of IL-10 by acetylating STAT3 through histone acetyltransferases (HATs), forming the most important connection between IL-10 and Foxp3 (Figure 1) (75). Although IL-10 signaling is not required for the induction of Tregs, it is required for Tregs to mediate their suppressive function. For example, IL-10R is indispensable for Tregs to suppress the autoreactive Th17 response (PMID: 21511185). IL-10 may auto-regulate its expression through a negative feedback loop, which involves the autocrine stimulation of IL-10R and inhibition of the p38 signaling pathway (76). Additionally, IL-10 expression is widely regulated at the post-transcriptional level, possibly involving AU-rich element (77), let-7 (78), or miR-106 (79). Under normal conditions, human iTreg cells produce low levels of IL-10. Inhibiting glycogen synthase kinase-3 (GSK3) can significantly upregulate IL-10 expression in Tregs and promote the generation of IL-10^{+} Foxp3^{+} iTreg cells (80).

IL-10 is predominantly secreted by type 1 regulatory T cells (Tr1 cells). Tr1 cells are typically induced from naïve T cells in the periphery, their differentiation can be driven by several factors, including IL-10, IL-27, and TGF-β (81). The transcription factors (TFs) *c-Maf* interacts with *AhR* to synergistically transactivate the IL-10 and IL-21 promoters, thereby promoting IL-27-induced differentiation of murine Tr1 cells (82). Tr1 cells are characterized by their lack of Foxp3 expression and are identified by the co-expression of CD49b and LAG-3, which serve as distinctive markers in both humans and mice (83). Tr1 cells play a critical immunoregulatory role in promoting tolerance in transplant scenarios, such as renal and pancreatic islet transplantation in humans and mice, and in reducing GvHD following hematopoietic stem cell transplantation, largely through their IL-10 mediated activities and antigen-specific actions (84–86). Tr1 cells have also been shown to ameliorate autoimmune diseases by inhibiting pathogenic Th17 response in experimental autoimmune encephalomyelitis and experimental autoimmune uveitis (87, 88).

IL-10 is a major cytokine involved in Treg-mediated immune regulation and immunosuppression (89). It primarily acts on monocytes and macrophages. IL-10 can inhibit the secretion of the pro-inflammatory cytokines TNF-α and IL-1β by monocytes and macrophages (90). IL-10 also inhibits IL-12 synthesis, hindering the differentiation of Th1 cells (73, 91). Blocking the Treg-mediated suppression of Teffs can be achieved by using anti–IL-10 neutralizing antibodies (92). Treg cells regulate the expression of IL-10 through the transcription factor B-lymphocyte-induced maturation protein-1 (Blimp-1). Mice lacking Blimp-1 in peripheral effector CD4^{+} and CD8^{+} T cells show increased cell numbers, while the overexpression of Blimp-1 in T cells promotes the differentiation of Treg cells and enhances their inhibitory effect on T cell proliferation (93). This highlights the importance of IL-10 in mediating the immunosuppressive function of Treg cells.

In addition, IL-10 has various other regulatory functions. It can induce the differentiation of Th0 cells into helper T cells (Th2), while inhibiting Th1 differentiation, thereby affecting the balance between Th1 and Th2 immune responses (94). IL-10 can also inhibit antigen presentation and prevent monocytes and macrophages from producing pro-inflammatory cytokines. IL-10 can inhibit the secretion of IL-6 and IL-12 by dendritic cells (DCs), thereby suppressing Th17 differentiation (95–97). Compared to

wild-type mice, IL-10$^{-/-}$ mice exhibit more severe arthritis, decreased numbers of Tregs, decreased expression of Foxp3, and increased numbers of Th1 and Th17 cells. This further confirms that IL-10 may work in coordination with Tregs and other immune cells to inhibit the differentiation and development of Th1 and Th17 cells, exerting negative immune regulatory effects (98). The immunostimulatory capacity of IL-10 in the context of immunoregulation has been demonstrated. IL-10 expression in tumor cell lines transfected from IL-10 transgenic mice controls primary tumor growth and reduces the burden of metastasis (99). Recombinant mouse IL-10 has been shown to induce IFN-γ and CD8$^+$ T cell–dependent anti-tumor immunity *in vivo* (100, 101).

2.4 IL-35

IL-35, a member of the IL-12 family, is a heterodimeric protein that consists of p35 and Epstein-barr virus-induced gene 3 (EBI3) (102). It can be secreted by Tregs. IL-35 can be expressed in various tissues and environments, such as the thymus, peripheral lymphoid organs, and inflammatory sites, and influences the generation and maintenance of Tregs by activating the IL-35 receptor (IL-35R) (103). IL-35 not only suppresses effector T cells but also promotes the conversion of CD4$^+$ T cells into IL35-producing induced regulatory T cells (iTr35). IL-35 triggers signal transduction by binding to IL-35R, which is composed of two subunits (i.e. IL-12Rβ2 and IL-27Rα) and subsequently activates the JAK family (102). Specifically, IL-35 induces the phosphorylation and activation of the JAK1 and Tyk2 kinases (104). Activated JAK further phosphorylates and activates STAT proteins, including STAT1 and STAT4 (Figure 1) (105). These phosphorylated STAT proteins undergo conformational changes, form dimers or multimers, and then translocate to the cell nucleus, where they can activate the transcription of the Foxp3.

The function of Tregs proved effective in EBI3$^{-/-}$ and p35$^{-/-}$ mice, that is, IL-35 subunit knockout models, suggesting that IL-35 plays an important role in maintaining the function of Tregs (106). The immunosuppressive capacity of Tregs in EBI3$^{-/-}$ and p35$^{-/-}$ mice was significantly diminished compared to that of Tregs in wild-type mice. In cases of human colon cancer, the expression level of IL-35 in tumor tissues was positively correlated with the degree of malignancy and clinical stage of the tumor. Additionally, a strong positive correlation between the level of IL-35 expression and the number of Tregs in peripheral blood has been noted (107). Therefore, tumor-derived IL-35 may promote tumor growth by recruiting Tregs into the TME (108). Another study conducted by Meghan et al. demonstrated that targeting IL-35 can potentially serve as a therapeutic strategy for tumor suppression. Their research findings revealed that the neutralization of IL-35 led to enhanced tumor control in wild-type C57BL/6 mice when compared to control mice (109).

In summary, IL-35 plays an important role in immune regulation and immune balance by regulating the development of Tregs, enhancing Foxp3 expression and function, and exerting immunosuppressive effects.

3 Treg-inhibitory cytokines

3.1 IL-6

IL-6 is an inflammatory cytokine linked to autoimmune and inflammatory diseases (110). It can be produced by lymphoid and some non-lymphoid cells. It can also be secreted by fibroblasts, endothelial cells, keratinocytes, mesangial cells, and tumor cells. The pro-inflammatory properties of IL-6 include inhibition of the immunosuppressive capacity of Tregs and interference with their differentiation from naïve T cells (111). Studies have shown that high levels of IL-6 and IFN-γ inhibited the expression of Foxp3 during the differentiation of Tregs (112, 113). In early pregnancy, C57BL/6 models, which are susceptible to congenital toxoplasmosis, exhibited elevated IL-6 but reduced expression of Foxp3 in response to congenital toxoplasmosis infection when compared to the infection-resistant Balb/c models (114). *In vitro* activation of purified mouse CD4$^+$ CD25$^+$ Foxp3$^+$ T cells caused their differentiation into Th17 in the presence of IL-6 (47, 115, 116). Therefore, IL-6 is the key factor determining whether naïve CD4$^+$ T cells differentiate into Treg or Th17 cells (117). The deletion of IL-6 and TGF-β in mice contributes to the depletion of Th17 cells, which leads to the failure of EAE modeling (118–120). When IL-6 is present at inflammatory sites, such as sites of mucosal inflammation, it can cause phosphorylation of the downstream STAT3 through tyrosine (121). Hyperactivity of p-STAT3 can increase the expression of the transcription factor RORγt in T cells, followed by a decrease in Foxp3 expression, thus causing CD4$^+$ T cells to differentiate into Th17 cells instead of Tregs (120, 122). Another study reported that when co-cultured with multiple myeloma (MM) cells, bone marrow stromal cells could secrete IL-6 and thereby transform Tregs into Th17 cells, a finding further verified in animal models (123, 124). In preclinical studies, IL-6 monoclonal antibodies demonstrated positive drug synergies (e.g., between bortezomib, melphalan, and dexamethasone), thereby enhancing the effectiveness of MM treatment. This improvement may be attributed to the Treg/Th17 ratio (125, 126). Interestingly, retinoic acid, a metabolite of vitamin A, could regulate TGF-β–dependent immune responses and prevent IL-6 from inducing pro-inflammatory Th17 cells and promoting the differentiation of anti-inflammatory Tregs (127), indicating the complexity of the balance of Th17 cells and Tregs.

3.2 TNF-α

TNF-α is an inflammatory cytokine that mediates inflammation and may cause tissue damage. It is secreted by macrophages, monocytes, neutrophils, CD4$^+$ T cells, and natural killer (NK) cells. While TNF-α is typically known for enhancing immune responses and promoting inflammation, it has also been shown to inhibit Treg function (128). TNF-α suppresses the differentiation and development of Tregs, leading to a reduction in their numbers (129). During Treg differentiation, the presence of TNF-α interferes with the TGF-β signaling pathways. This involves

disruption of the activation and transduction of downstream signaling molecules, such as the phosphorylation and nuclear translocation of the SMAD protein family, subsequently affecting Foxp3 expression and Treg differentiation (129). Previous studies have reported that TNF-α, through tumor necrosis factor receptor 2 (TNFR2) activation, played a role in the expansion and amplification of Tregs (130). Approximately 30–40% of peripheral blood Tregs express TNFR2, which can be upregulated by TNF-α (131). Interestingly, only Tregs that express TNFR2 exhibit strong immunosuppressive activity, while Tregs lacking TNFR2 display minimal to no immunosuppressive activity. Therefore, the TNF–TNFR2 signaling pathway is necessary for maintaining the function and phenotypic stability of Tregs in the body (132). When compared to conventional CD4 single-positive cells, members of the TNF receptor superfamily, including glucocorticoid-induced TNFR-related (GITR), CD134, and TNFR2, are overexpressed on Treg precursor cells. These receptors enhance T cell receptor (TCR) signaling through TGF-β–activated kinase 1 (TAK1) and CD28-dependent signaling pathways. Treg precursor cells lacking TAK1 and CD28 cannot express GITR, CD134, or TNFR2, resulting in the inhibition of $Foxp3^+$ Treg maturation (133).

The systemic injection of an anti–TNF-α neutralizing antibody, such as infliximab, can induce IL-10 in $CD4^+$ T cells and Th17 cells, as well as Aiolos binding of conserved regions of IL-10. However, in the treatment of Crohn's disease with anti–TNF-α therapy, $IL\text{-}17^+$ cells in the intestines of patients decreased significantly after 3 months of treatment, and $Foxp3^+$ cells were unstable. However, the $IL\text{-}17^+/CD4^+$ and $IL\text{-}17^+/Foxp3^+$ ratios were both decreased, suggesting that TNF-α and a balanced relationship between Th17 cells and Tregs are key factors in the treatment of the disease (134–136). TNF-α can damage the function of T cells by enhancing the dephosphorylation of Foxp3; and their function can be restored by TNF-α antagonist therapy, thereby indirectly regulating the interaction between T cells and Th17 and Th1 cells, which affects autoimmune inflammation in RA (137). It is important to note that the inhibitory effects of TNF-α on Tregs may be beneficial in certain contexts, such as enhancing anti-tumor immune responses by reducing the suppressive effects of Tregs (138). The accumulation of Tregs in the TME has been identified as one of the major factors in the initiation and development of immune checkpoint inhibitor resistance (139). The CC motif chemokine receptor 8 (CCR8) is a marker of activated inhibitory Tregs and has a significant impact on the function of Tregs in the TME (140). High levels of TNF-α in the colorectal cancer (CRC) TME upregulate CCR8 expression in Tregs via the TNFR2/NF-κB signaling pathway and Foxp3 transcription factor. Depletion or blockade of TNFR2 inhibits gastrointestinal tumor progression by reducing $CCR8^+$ Treg infiltration, thereby enhancing the efficacy of anti–PD-1 therapy (140, 141).

4 Co-stimulatory molecules

Co-stimulatory molecules, such as CTLA-4 and PD-1 are also crucial for Treg cell activation and function (Figure 2). The B7 family is composed of a group of cell-surface molecules primarily found on antigen-presenting cells (APCs), such as DCs, macrophages, and B cells (142). These molecules provide co-stimulatory or co-inhibitory signals that are crucial for the activation, differentiation, and survival of T cells, thereby playing an essential role in the regulation of T cell–mediated immune responses, especially in the field of immuno-oncology (143). CTLA-4, PD-1, and PD-L1 are the most extensively studied and clinically applied immune checkpoint molecules to date (144). Some clinical trials and studies have shown that the combined use of nivolumab (a PD-1 inhibitor) and ipilimumab (a CTLA-4 blocker) is more effective for patients with advanced melanoma (145). The use of immune checkpoints has ushered in a new era in tumor treatment.

4.1 CTLA-4

CTLA-4 is a co-stimulatory molecule expressed on the surface of effector T cells (146). Interestingly, Treg cells also constitutively

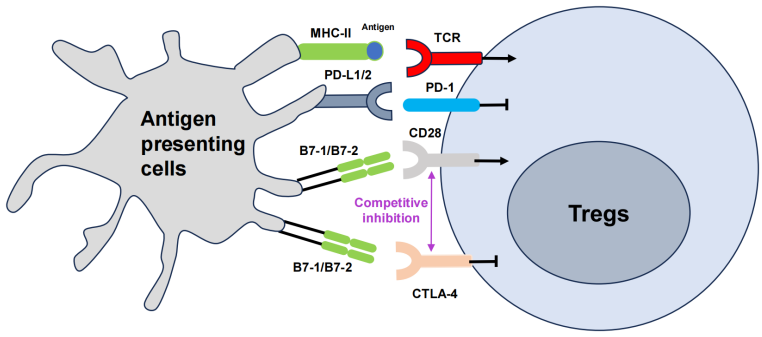

FIGURE 2
The role of co-stimulatory molecules in Treg cells, namely CTLA-4 and PD-1. The T cell receptor (TCR) engages the major histocompatibility complex (MHC)-peptide complex on APCs. The interaction between CD28 on T cells and B7 on APCs triggers costimulatory signaling, which is vital for T cell activation. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) mitigates this activation by outcompeting CD28 for its ligands B7, thus attenuating the costimulatory signal. Concurrently, the interaction of programmed cell death protein 1 (PD-1) on T cells with its ligands PD-L1 or PD-L2 also transmitted by APCs, further modulates immune responses, generally by dampening T cell activity. The arrows represent positive regulation of the Tregs response, and the horizontal lines represent negative regulation.

express CTLA-4 for fine tuning T cell activation through the obstruction of co-stimulatory signals (147). CTLA-4 exhibits higher binding affinity for the co-stimulatory molecules CD80/CD86 than CD28, thereby effectively outcompeting it (148). Additionally, CTLA-4 is involved in the 'trans-endocytosis' of CD80 and CD86 from APCs, further inhibiting their availability for co-stimulatory interactions (147). These actions are crucial for Treg cells to exert control over T cell activation and to prevent autoimmune responses (149). The absence of CTLA-4 disrupts Treg cell function, leading to unchecked proliferation and activation of Tconvs, which can result in autoimmune pathology (150). Researchers have shown that introducing the extracellular domain of CTLA-4 (cdCTLA-4) into mice lacking CTLA-4 fully restores Treg activity, suggesting that cdCTLA-4 is sufficient to provide inhibitory function (151). This implies that CTLA-4 function may not necessarily involve a signal transduction process. In another study, the expression of CTLA-4 in $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells was elevated both in the blood of patients with pulmonary tuberculosis and in the pleural cavity of individuals with tuberculosis pleurisy. Blocking CTLA-4 weakened the ability of $Foxp3^+$ Tregs to suppress the IFN-γ T effector response to the effect of purified protein derivative (PPD) stimulation, and this reversal effect was not consistent with the decrease in IL-10. Blocking CTLA-4 reversed the ability of Tregs to inhibit PPD-driven IFN-γ and IL-2 responses at the mRNA level, while IL-10 and TGF-β did not show significant changes. Blocking CTLA-4 significantly eliminated the inhibitory effect of $Foxp3^+$ Tregs on the PPD-specific T cell proliferation response (152). Therefore, CTLA-4 is a promising new target for immunotherapy for active tuberculosis (153).

Research on CTLA-4 is still in the stage of determining its physical activity. However, although the mechanism is currently obscure, it does not affect the positive clinical effects of anti–CTLA-4 drugs in arousing an immune response and treating tumorigenicity. CTLA-4-targeting agents serve various immunomodulatory roles. Abatacept (Orencia), which includes the CTLA-4 domain, is employed for treating RA and is used to prevent organ transplant rejection, as seen with belatacept (Nulojix). Distinct from ipilimumab (Yervoy), an FDA-approved melanoma treatment that blocks CTLA-4 to stimulate the immune response, abatacept mimics CTLA-4 on T cells. It competes with CD28 for binding to B7 molecules on APCs, thereby blocking the co-stimulatory signal required for T cell activation (154). Belatacept, a derivative of abatacept, with an alteration of merely two amino acids, exhibits a tenfold increase in activity compared to its precursor, more effectively inhibiting the CD28-mediated co-stimulatory signaling of T cells (155). The idea of CTLA-4 target druggability is mainly based on the high-affinity binding of anti-CTLA-4 antibodies to CTLA-4 molecules, mediating Treg depletion or functional blockade, thereby enhancing T cell activation and the immune response to cancer (156). CTLA-4 is involved in maintaining tolerance to autoimmune diseases, such as diabetes, as well as spontaneous abortion tendencies (157–159). Single nucleotide polymorphisms in exon 1 of CTLA-4 have been linked to susceptibility to several autoimmune diseases, including multiple sclerosis (160). The antibody-mediated blocking of CTLA-4 prevents the development of tolerance, enhances the anti-tumor response, and exacerbates autoimmune disease (161). In clinical trials, CTLA-4 development is mainly divided into two treatment modalities: monoclonal antibody or combined with PD-1/PD-L1 monoclonal antibody. Bispecific antibodies have also been constructed by association with other popular targets (162).

4.2 PD-1

PD-1 is a co-inhibitory receptor primarily expressed on activated T cells, B cells, and APCs. PD-1, through binding to its ligands PD-L1 and PD-L2, inhibits T cell activation and function to prevent excessive immune responses (163). Tregs express higher levels of PD-1 on their surfaces compared to $CD4^+$ Th cells (164). This indicates that PD-1 plays an important role in the regulation of Tregs. When PD-1 binds to its ligands, PD-L1 or PD-L2, it exerts inhibitory effects (163). PD-1 activation suppresses signal transduction pathways that activate T cells, leading to reduced cell proliferation and cytokine production, thereby limiting the intensity and duration of immune responses (165). The PD-1 signaling pathway in Tregs can regulate their suppressive function and immune regulatory roles. Evidence from multiple studies indicates that PD-1 inhibits the suppressive abilities of Tregs (166). Isolation of $PD\text{-}1^{hi}$ and $PD\text{-}1^-$ cells from the peripheral blood of healthy individuals has revealed that Tregs with higher levels of PD-1 show diminished suppressive function and elevate production of IFN-γ (167). Mouse model experiments further demonstrate that Tregs lacking PD-1, or those from mice treated with PD-1 blocking antibodies, exhibit enhanced suppressive capabilities (168). In tumor environments, blocking PD-1 not only improves the function of $PD\text{-}1^+$ $CD8^+$ T cells but also intensifies the immunosuppressive effects of $PD\text{-}1^+$ Tregs (169). As a result, the effectiveness of PD-1 inhibitors in treating patients is determined by their complex interplay with both effector T cells and Tregs, which highlights the crucial role of the PD-1 in controlling Tregs.

An *in vivo* mouse MC38 (CRC cell line) subcutaneous transplantation tumor model and an azoxymethane (AOM)/dextran sodium sulfate (DSS)–induced spontaneous CRC model both confirmed that gallic acid can affect Foxp3 protein levels by targeting the expression of ubiquitin specific peptidase 21 (Usp21) in Tregs, inducing the formation of Th1-like Tregs and reducing their immunosuppressive function (170). Simultaneously, gallic acid can enhance the anti-tumor effect of anti–PD-1 immune checkpoint blocking and downregulate the expression of PD-L1 protein in Tregs, indicating that the deubiquitinating enzyme Usp21 can deubiquitinate and stabilize the PD-L1 protein. PD-1 was also expressed on $CD4^+$ $Foxp3^+$ $CXCR5^-$ Tregs and inhibited the activity of lymphocytes by downregulating then maintaining the expression level of Foxp3 protein (171). Neuropilin-1 (NRP-1) plays an essential role in maintaining the stability and function of Tregs within tumors (172). On the one hand, an increase in the NRP-1 phenotype induces the production of IFN-γ in the tumor,

which increases the vulnerability of Tregs, weakens tumor immunosuppression, and enhances antitumor immunity, which is related to the prognosis of melanoma (173) and head and neck squamous cell carcinoma (174). On the other hand, the susceptibility of Treg cells to IFN-γ, which is induced by anti–PD-1 therapy, is a potential mechanism underlying the effectiveness of anti–PD-1 drugs. These drugs stimulate the production of significant quantities of IFN-γ (175). Recently, several studies have reported correlations between tumor PD-L1 expression, objective response rate, and PD-1/PD-L1 inhibitors, suggesting that PD-L1 may become an effective biomarker (176, 177).

5 Potential of Treg-based immunotherapy

The overall outcome of these interactions between Treg subpopulations, cytokines, and co-stimulatory molecules is a finely-tuned immune system that can respond to pathogens aggressively. Based on the characteristics of different subsets of Tregs, different strategies can be utilized to treat autoimmune diseases (178, 179). To date, early clinical trials using Treg cell therapy have shown great promise in the fields of transplantation rejection (180), GvHD (181), and autoimmune diseases (179). However, one of the main challenges in these studies is the isolation of pure Tregs and their expansion to a sufficient clinical dose. The principle of Treg cell therapy is to restore the balance between Teffs and immune regulatory cells by injecting an effective dose of Tregs into the patient's body, thus promoting immunological tolerance (182).

5.1 Autoimmune diseases

Tregs play a pivotal role in upholding immune tolerance to self-antigens, thereby preventing the activation and proliferation of self-reactive T cells that may not be eliminated during thymic selection. They also inhibit APCs, such as DCs, which are instrumental in triggering immune responses (183). Furthermore, Tregs interact with other immune cells, including B cells and NK cells, establishing a balanced immune system (184).

Autoimmune diseases include systemic autoimmune diseases and organ-specific autoimmune diseases. Representative diseases of the former type include systemic lupus erythematosus, SS, and RA (185), and the latter include, for example, type 1 diabetes (186), pemphigus (187), and Hashimoto thyroiditis (188). Treg therapy can alleviate arthritis symptoms by suppressing inflammatory responses and regulating the immune system. In addition, It can regulate intestinal immune balance and reduce intestinal inflammation and tissue damage, thus alleviating inflammatory bowel diseases (such as Crohn's disease and ulcerative colitis) (189, 190). Treg therapy can also alleviate autoimmune hepatitis and improve liver function (191) and systemic lupus erythematosus (192). Finally, Treg therapy can be used for other autoimmune diseases, such as multiple sclerosis (193), myasthenia gravis (194), and autoimmune thyroid diseases (195).

Our recent studies revealed that the intravitreal injection of Tregs resolved ocular inflammation in experimental autoimmune uveitis (116). In patients with RA, synovial Tregs lose their suppressive functions. They fail to inhibit the production of pro-inflammatory cytokines, such as TNF-α and IFN-γ, by other $CD4^+$ T cells and monocytes and to inhibit the proliferation of Teffs (196). In animal models, the adoptive transfer of Tregs significantly reduces disease severity, highlighting the importance of Tregs in controlling abnormal joint inflammatory responses (197). Tregs can also suppress the activity of other immune cells through immunoregulatory molecules, for example, TGF-β and IL–10 produced by Tr1 cells, to inhibit inflammatory reactions and reduce self-attack on joint tissues, thereby alleviating symptoms of RA (198). Other autoimmune diseases, such as inflammatory bowel disease (199), systemic lupus erythematosus (200), and autoimmune thyroid diseases (201), including thyroid nodules, thyroiditis, Graves' disease, and autoimmune hypothyroidism, can all be alleviated by enhancing the immunosuppressive function of Tregs. Today, adoptive Treg therapy has been widely used and tested in autoimmune diseases (Table 2).

5.2 Transplantation

In transplantation, Tregs are critical for promoting graft tolerance and reducing transplant rejection rates. In a study of immune rejection therapy for kidney transplantation, 11 patients were followed for 60 weeks after transplantation to assess immune response, rejection, and renal function. Of these patients, eight were successfully maintained on monotherapy immunosuppression. Additionally, 10 patients who received Tregs treatment could be weaned off immunosuppression to low dose tacrolimus monotherapy within 48 weeks, although eight patients later experienced failure of tacrolimus monotherapy. Despite the need for additional immunosuppressive treatments, all 11 patients in the trial maintained good graft function at the 3-year follow-up time point. The study's authors successfully developed a method for isolating and expanding autologous polyclonal Tregs from a small blood sample and demonstrated the feasibility of this treatment (211).

Graft rejection reactions may occur after bone marrow transplantation, leading to transplant failure. Treg therapy can regulate the immune response after transplantation, reducing the occurrence of graft rejection (212). Amarnath et al. (213) found that Tregs could promote the generation of bone marrow DCs and reduce their ability to stimulate the generation of efficient T cells in GvHD. Tregs have high surface expression levels of PD-L1, which can bind to PD-L1 of DCs, thus inhibiting the activation of T cells and alleviating GvHD (214, 215). In addition, Tregs can express inhibitory molecules (e.g. CTLA-4, LAG-3, and NRP-1) to inhibit T cell activation (216). Tregs can also express CD62L and CCR5, maintain *in vivo* homing characteristics, inhibit the activation of early Teffs, and induce transplantation immune tolerance (217). The development of CAR-T technology has promoted the development of clinical trials using CAR Tregs to treat transplantation immune rejection (218). HLA-A2 is the main

TABLE 2 Animal models of autoimmune disease and inflammation.

Disease	Type	Treg-based immunotherapeutic effects	Influence
Connective tissue autoimmune disease	Systemic lupus erythematosus (202)	Activated Tregs	IFN-γ↓ IL-17↑
	Rheumatoid arthritis (203)	$CD4^+$ $CD25^-$ T & mature tolerant DCs promote $CD4^+$ $CD25^+$ Tregs	TNF-α↓ IL-17↓ IL-6↓ IFN-γ↑ IL-10↑ TGF-β↑
Neuromuscular autoimmune disease	Experimental autoimmune encephalomyelitis (204)	CCL1–Ig promotes $CCR8^+$ Tregs	CD39↑ GranB↑ IL-10↑
	Experimental autoimmune myasthenia gravis (205)	EAMG $CD4^+$ & marrow DCs promote DC EAMG Tregs	Clinical score↓ AChR↓
Endocrine autoimmune disease	Type 1 diabetes (206)	Antigen-specific Tregs	IL-10↑ TGF-β↑ $CD8^+$ T↓ $CD8^+$/$CD4^+$ T↓
	Premature ovarian insufficiency (207)	Activated Tregs	Follicle-stimulating hormone↓ Luteinizing hormone↓ Anti-zona pellucida antibody↓ Estradiol↑ Anti-Müllerian hormone↑
Autoimmune diseases of the digestive system	Autoimmune hepatitis (208)	$CD4^+$ $CD25^+$ Tregs & HSCs promote HSC Tregs	AST↓ ALT↓ Treg/Th17 -
	Ulcerative colitis (209)	Activated Tregs	IL-1↓ TNF-α↓ NO↓ PGE2↓
Other autoimmune diseases	Graft-versus-host disease (210)	Tr1 cells promote Tregs	Th2/Th1↑ Treg/Th17↑

molecular target that causes immune rejection (219). CAR HLA-A2 Tregs can significantly reduce the inflammation and rejection caused by grafts, can promote the immune tolerance of grafts, and are superior to polyclonal Tregs in preventing GvHD caused by donor T cells (220). CAR Treg cell therapy likely has the advantage of avoiding transplant rejection. Early treatment may help prevent the occurrence of rejection reactions, while later treatment may be more suitable for the management of existing rejection reactions.

5.3 Cancer treatment

Tregs, although vital for preserving immune tolerance and preventing autoimmune diseases, can be counterproductive in cancer by inhibiting anti-tumor responses and enabling cancer cells to escape immune surveillance. Consequently, restricting Treg activity is essential for enhancing the immune system's ability to combat cancer. Targeting Tregs also has potential applications in cancer treatment. The goal of targeting Tregs for cancer treatment is to enhance the tumor immune response and suppress tumor growth by modulating the activity of the immune system (221). Immune checkpoint inhibitors have become an important strategy in cancer treatment (222). In this treatment approach, the suppressive effects of Tregs may inhibit the efficacy of immune checkpoint inhibitors. Therefore, reducing or modulating the immunosuppressive effects of Tregs can enhance the efficacy of immune checkpoint inhibitors (182). CpG combined with low-dose anti-OX40/CTLA-4 triple immunotherapy can eliminate Tregs in a tumor and has a curative effect on central nervous system lymphoma in mice (223). In patients with tumors that did not respond to PD-1 monotherapy, the combination of the partial deletion of CARD-containing MAGUK protein 1 (CARMA1) and the sterol regulatory element binding protein (SREBP) inhibitor fatostatin produced a strong antitumor effect (224). This is because, upon the destruction of the CARMA1–BCL10–MALT1 semaphore complex, most tumor-infiltrating Tregs inhibit IFN-γ derived from $CD8^+$ T cells, which inhibits the growth of immunosuppressive M2-like tumor-associated macrophages (TAMs) (225). Fatostatin inhibits SREBP1-mediated fatty acid synthesis, inhibits the occurrence and development of TAMs, and then controls tumor growth.

Chemokine pathway blocking and specific target blocking have also been used to suppress tumors (Table 3) (226). Blocking the migration of Tregs to the TME is a new direction of tumor immunotherapy (227). Tregs in a canine bladder cancer model entered tumor tissue through the CCL17–CCR4 axis, and anti-CCR4 treatment significantly inhibited tumor growth and improved the survival rate. In addition, CCR4 was highly expressed in tumor-infiltrating Tregs (TITRs) in human bladder cancer (228). Another study showed that the number of TITRs in $CD36^{-/-}$ Treg mice was decreased, the anti-tumor activity of tumor-infiltrating lymphocytes was enhanced, and tumor growth was inhibited (229). Neuropilin 1 $(Nrp1)^{-/-}$ Tregs in mice with a partial Nrp1 knockout can prevent wild-type ($Nrp1^{+/+}$) Tregs from performing their immunosuppressive function by secreting IFN-γ, thus promoting the clearance of melanoma (10). New drugs are continuously being developed to inhibit cancers. Some of the difficulties of tumor immunotherapy include eliminating the immunosuppressive effect of the TME and enhancing the specific anti-tumor response. Further effective differentiation between TITRs and tissue-resident Treg

TABLE 3 Methods of Treg-targeted therapy for tumors and their corresponding cytokines.

No.	Therapeutic effect	Drug	Target
1	Treg depletion in the TME	Kinase inhibitor, cyclophosphamide, anti-CD25	IL-2 signaling
2	Halting Treg migration	Anti-CCR4, anti-CCR8	CCR4, CCR8
3	Sensitizing intertumoral Tregs to checkpoint blockade	Anti-TIGIT, anti–LAG-3, LAG-3–Ig fusion protein, nonfucosylated anti–CTLA-4	LAG-3, TIM3, TIGIT, CTLA-4
4	Targeting the co-stimulation of Tregs	GITR agonist, OX40 agonist, ICOS agonist and antagonist, TNFR2 antagonist	GITR, ICOS, OX40, TNFR2, NRP-1
5	Targeting Treg cytokines	Anti–IL-10, anti-GARP, anti–IL-35	IL-10, TGF-β, IL-35
6	Altering Treg fragility	PI3Kδ inhibitor, anti–NRP-1	NRP-1, IFN-γ
7	Targeting Treg metabolism	Meformin, IDO inhibitor, A2AR inhibitor, Orencia, Nulojix	IL-10, CTLA-4, FOXP3

*IL, Interleukin; CD, Cluster of differentiation; CCR, Hemokine (C-C motif) receptor; LAG, Lymphocyte activation gene; TIM, T cell immunoglobulin domain and mucin domain; TIGIT, T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory motif (ITIM) domains; CTLA, Cytotoxic T lymphocyte-associated antigen; GITR, Glucocorticoid-induced tumor necrosis factor receptor; ICOS, Inducible synergistic co-stimulation molecules; OX40, CD134 & TNF receptor superfamily member 4 (TNFRSF4); TNFR2, Tumor necrosis factor receptor; NRP, Neuropilin; TGF, Transforming growth factor; IFN, Interferon; FOX, Forkhead box protein.

phenotypes or transcription levels, as well as a continuous reduction in the dynamic differences in Tregs between preclinical models and patient-derived samples, can provide more therapeutic bases for immunotherapy based on Treg targets.

The exploration and deployment of therapeutics targeting Tregs are extensive, chiefly because these cells express an array of receptors on their surface. The most utilized are agonists of the TNFR superfamily and antagonists of immune checkpoint inhibitors (230, 231). These modulators alleviate autoimmune conditions and bolster anti-tumor responses by influencing Treg function. Next-generation Treg interventions focus on directing Tregs to selectively recognize tissue or organ-specific antigens by incorporating a chimeric antigen receptor (CAR) structure. Engineered CAR-Tregs are also being designed to convert pro-inflammatory cytokine signals into those of IL-2 or IL-10, which intensifies the suppression of inflammation (232).

6 Conclusion

The study of Treg signaling pathways provides a theoretical basis for immune balance and the treatment of autoimmune diseases. By modulating the quantity and function of Tregs, immune system activity can be balanced, inflammation can be alleviated, and the development and progression of autoimmune diseases can be prevented and treated. Preliminary results from clinical trials demonstrate the potential of Treg therapy in the fields of transplant rejection, autoimmune diseases, and cancer. Although the application of Treg therapy poses challenges, personalized treatment strategies and the optimization and monitoring of treatment processes will contribute to improving its safety and efficacy and promoting its further clinical implementation.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

ZY: Writing – original draft, Writing – review & editing. KD: Writing – original draft, Investigation, Software. WC: Writing – original draft, Writing – review & editing.

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