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Heterogeneity and subtypes of $CD4^+$ regulatory T cells: implications for tumor therapy

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In the conventional view, $CD4^+$ regulatory T cell (T_{reg}) represents a subset of lymphocytes that involve the perception and negative regulation of the immune response. $CD4^+T_{reg}$ plays an important role in the maintenance of immune homeostasis and immune tolerance. However, recent studies have revealed that $CD4^+T_{reg}$ do not suppress the immune response in some diseases, but promote inflammatory injury or inhibit tissue remodeling, suggesting the functional heterogeneity of $CD4^+T_{reg}$. Their involvement in tumor pathogenesis is more complex than previously understood. This article reviews the relevant research on the heterogeneity of $CD4^+T_{reg}$, subtype classification, and their relationship with tumor therapy.

KEYWORDS

functional subtypes, tumor microenvironment, clinical trials, regulatory T cells, forkhead box protein 3

1 Introduction

$CD4^+$ regulatory T cell (T_{reg}) was first identified by Sakaguchi et al., who discovered a population of $CD4^+CD25^+$T cells in the thymus of mice that could suppress the progression of autoimmune diseases (1). Traditionally, $CD4^+T_{reg}$ has been believed can suppress the activities of T cells, B cells, natural killer (NK) cells, and other immune cells. Functional impairment of $CD4^+T_{reg}$ is a significant contributing factor to the development of autoimmune diseases (2). Studies in the field of tumor microenvironment (TME) have shown that tumor cells exploit the immunosuppressive capacity of $CD4^+T_{reg}$ in TME to evade immune surveillance and promote tumor progression (3). Therefore, targeting $CD4^+T_{reg}$ in tumor therapy holds significant potential for development.

Given the crucial role of $CD4^+T_{reg}$ in tumor progression, it is essential to gain a deeper understanding of the distinct functional subtypes of $CD4^+T_{reg}$. Recent advances in functional studies and single-cell sequencing have provided novel insights into the functional heterogeneity of $CD4^+T_{reg}$ (4, 5) and identified new molecular targets for $CD4^+T_{reg}$ in TME (6). This review aims to summarize the relevant research, including the

functional heterogeneity and subtyping of $CD4^+T_{reg}$, and provide an overview of clinical trials targeting $CD4^+T_{reg}$.

2 Classification of $CD4^+T_{reg}$

$CD4^+T_{reg}$ can be classified into two main categories based on their cellular origins: natural-occurring T_{reg} (nT_{reg}) and peripheral T_{reg} (pT_{reg}). nT_{reg} are generated in the thymus, the precursor cells of T_{reg} mature into $CD4^+CD25^+Foxp3^+T_{reg}$ following stimulation by IL-2/STAT5 and IL-4 signals (7). On the other hand, pT_{reg} are derived from nT_{reg} or initial $CD4^+T$ cells in the periphery under specific cytokine or molecular stimuli (8). Additionally, there is a subset of $CD4^+T_{reg}$ known as induced T_{reg} (iT_{reg}), which can be generated *in vitro* experiments. These three types of cells constitute the classical classification of $CD4^+T_{reg}$. Subsequent research has further refined this classical classification based on Foxp3 expression. For example, iT_{reg} can be further divided into Foxp3 $+iT_{reg}$ and Foxp3-iT_{reg}, with the latter including Type I Treg (Tr1) cells (9), T helper 3 (Th3) cells (10), and IL-35-induced $CD4^+T_{reg}$ (iTr35) (11). Table 1 summarizes the common types of $CD4^+T_{reg}$ and their corresponding phenotypes.

3 Heterogeneity and subtypes of $CD4^+$ T_{reg}

3.1 Subtypes of $CD4^+T_{reg}$

Previous studies on $CD4^+T_{reg}$ have primarily focused on $CD4^+CD25^+Foxp3^+T_{reg}$. However, emerging evidence suggests functional heterogeneity within this population, and relying solely on the $CD25^+Foxp3^+$phenotype may lead to contradictory conclusions in studies investigating the same disease. Therefore, the establishment of a reliable subtyping strategy is crucial. Currently, two mainstream classification systems are widely used: functional subtypes proposed by the Sakaguchi et al. (16) and the Th-like T_{reg} subtypes based on cytokine expression (17).

Sakaguchi et al. proposed a subdivision of $CD4^+T_{reg}$ based on CD45RA expression in 2009, further categorizing them into three functional subsets: subset I consists of $CD45RA^+Foxp3^{lo}/CD25^{lo}$ resting T_{reg} (rT_{reg}); subset II comprises $CD45RA^-Foxp3^{hi}/CD25^{hi}$ effector T_{reg} (eT_{reg}), also known as activated T_{reg} (aT_{reg}); and subset III includes $CD45RA^-Foxp3^{lo}/CD25^{lo}$ non-T_{reg} (16). Functionally, rT_{reg} possess a certain degree of immunosuppressive capacity and express markers of naive cells such as CCR7 and CD62L. They can differentiate into eT_{reg} upon antigen stimulation. Subset of eT_{reg} exhibit stronger proliferation and immunosuppressive abilities but are more prone to apoptosis. Non-T_{reg}, which were previously considered as cells secreting pro-inflammatory cytokines such as IFN-γ and IL-17 without immunosuppressive capabilities, were found to exhibit significant functional heterogeneity by Cuadrado et al. using mass spectrometry. $CD127^+$ non-T_{reg} and $CD127^-$ non-T_{reg} displayed characteristics of conventional T cells and eT_{reg}, respectively. Furthermore, the $CD127^-$ subset can be further subdivided based on CD49d and CCR4 expression, with $CD127^-CCR4^+CD49d^-$ cells showing high levels of IL-2 expression, while $CD127^-CCR4^-CD49d^+$ cells exhibit elevated levels of IFN-γ and IL-17 (18).

In addition to the classification proposed by Sakaguchi et al., Halim et al. categorized $CD4^+T_{reg}$ into four distinct subtypes, termed Th-like T_{reg}, based on their cytokine secretion profiles and transcription factor expression in 2017. These subtypes include Th1-like T_{reg} ($CCR4^+CCR6^-CXCR3^+$, primarily secreting IFN-γ and TNF-α), Th2-like T_{reg} ($CCR4^+CCR6^-CXCR3^-$, primarily secreting IL-2, IL-4, IL-5, and IL-13), Th17-like T_{reg} ($CCR4^+CCR6^+CXCR3^-$, primarily secreting IL-17A/IL-17F), and Th1/17-like T_{reg} ($CCR4^+CCR6^+CXCR3^+$, secreting both IFN-γ and IL-17A without significant statistical differences compared to other subsets) (17). Each Th-like T_{reg} subset exhibited transcription factor expression and cytokine secretion patterns similar to their corresponding Th cell counterparts. Furthermore, all Th-like T_{reg} subtypes demonstrated immunosuppressive capabilities. However, this classification did not investigate the stability of Foxp3 expression in different subtypes, leading to inconsistent conclusions in various studies utilizing this classification system.

Besides to the aforementioned common classifications, the research conducted by Bluestone et al. revealed a stronger correlation between CD127 and Foxp3 expression levels compared to CD25. Furthermore, the suppressive function of the $CD4^+CD127^{lo/-}$ subset was found to be superior to that of the $CD4^+CD25^+$ subset (19). Currently, the phenotype of $CD4^+CD25^+CD127^{lo/-}$ is widely used for isolation of $CD4^+T_{reg}$.

TABLE 1 Characteristic markers of different $CD4^+$Treg subtypes.

Subtype of T_{reg}	Phenotype	Effector molecules	Transcription factors	Reference
nT_{reg}	$CD4^+CD25^+Foxp3^+Helios^+CTLA4^+$ $Nrp1^+$	TGF-β, IL-10, CTLA4, IL-35, LAG3, LAP, etc.	Foxp3	(12, 13)
$Foxp3^+iT_{reg}$	$CD4^+CD25^+Foxp3^+Helios^+CTLA4^+$ $Nrp1^-$	Similar to nT_{reg}	Foxp3	(13)
Tr1	$CD4^+Foxp3^-CD49b^+LAG3^+$ $CD226^+$	TGF-β, IL-10, PD-1, CTLA4, etc.	c-Maf, AhR, IRF4	(9)
Th3	$CD4^+CD25^-CD69^+Foxp3^-LAP^+TGF$-β+	TGF-β	Not specified	(14)
iTr35	$CD4^+Foxp3^-Ebi3^+p35^+IL$-$10^-TGF$-$β^-$	IL-35	Not specified	(11)
T_{reg} induced by B cells	$CD4^+CD25^+Foxp3^-$ $LAG3^+ICOS^{+②}PD1^+GITR^{+⑧}CD134^+$	IL-10, PD-1, CTLA4, LAG3, etc.	Not specified	(15)

3.2 Foxp3 stability and the function of $CD4^+T_{reg}$

Current evidence suggests that stable expression of Foxp3 is closely associated with the immunoregulatory function of $CD4^+T_{reg}$. Therefore, the main factors regulating Foxp3 stability may be crucial for the heterogeneity of $CD4^+T_{reg}$ function, such as local inflammatory cytokine stimulation or epigenetic regulation.

Under stimulation from the inflammatory microenvironment, the expression of Foxp3 in some cells may be affected, leading to the loss of negative immune regulatory function. Early *in vitro* studies have shown that CD4+CD25- T cells can transiently express CD25 and Foxp3 upon IL-2 or TGF-β stimulation, but these T cells do not possess immunosuppressive abilities (20). Recent research by Yi et al. has found that, under the stimulation of the inflammatory cytokine IL-6, TIGIT-positive $CD4^+T_{reg}$ can stably express Foxp3 and maintain immunosuppressive function, while TIGIT-negative unstable cells lose Foxp3 expression and transition into an effector T cell phenotype (21). These results suggest that $CD4^+T_{reg}$ with stable Foxp3 expression may possess immune regulatory capabilities, while cells with unstable Foxp3 expression may transition into other types of T cells under microenvironmental influences.

In recent years, the role of epigenetic regulation in Foxp3 expression has become a new research focus, with DNA methylation being the most common modification. The gene *Foxp3* consists of 11 coding exons, 3 non-coding exons, and 10 introns. Within this gene region, there are 3 conserved non-coding sequences (CNS), with CNS2 containing numerous cytidine-phosphate-guanine (CpG) sites. These sites are methylated in effector T cells but exhibit demethylation in $CD4^+$Treg cells (22), thus being referred to as the T_{reg}-specific demethylated region (TSDR). Sakaguchi et al. previously discovered that there are differences in the methylation levels of the TSDR at the Foxp3 locus. TSDR in rT_{reg} and eT_{reg} exhibited demethylation, while TSDR in non-T_{reg} had a higher degree of methylation, suggesting that Foxp3 expression is more stable in rT_{reg} and eT_{reg} (16). Additionally, a recent study found that the CD28-PKC-NF-κB signaling pathway inhibits demethylation in the CNS2 region of peripheral $CD4^+T_{reg}$, causing unstable Foxp3 expression in pT_{reg} cells and subsequently affecting their immunosuppressive function (23). Furthermore, Ohkura et al. discovered a close relationship between single nucleotide polymorphisms in the epigenetic regulatory region of $CD4^+T_{reg}$ and susceptibility to autoimmune diseases (24). Apart from CNS2, two other CNS regions have been shown to play important roles in stable Foxp3 expression and immune tolerance: CNS1, located in the promoter region of the Foxp3 gene, was found to be associated with pT_{reg} differentiation and maintenance of fetal immune tolerance (25); and CNS3 mediates the clonal expansion of the $CD4^+T_{reg}$ receptor repertoire, thereby controlling excessive immune responses of autoreactive T cells and maintaining immune homeostasis (26). Additionally, research by Kitagawa et al. found that the transcription factor Satb1 can bind to a $CD4^+T_{reg}$-specific super-enhancer and a newly discovered conserved non-coding region upstream of the Foxp3 promoter prior to $CD4^+T_{reg}$ activation, promoting the development of $CD4^+T_{reg}$. This newly discovered region has been named CNS0 (27).

In addition to methylation, acetylation and histone modifications have also been shown to be associated with the stable expression of Foxp3. During the acetylation process, histone acetyltransferases (HATs) and histone deacetylases (HDACs) influence Foxp3 acetylation levels from different directions. Two studies by Li and Tao demonstrated that HAT TIP60 and class II HDACs (HDAC7 and HDAC9) can form complexes with Foxp3, regulating the immunosuppressive capacity of $CD4^+T_{reg}$ (28, 29). Other studies further investigated the impact of acetylation on $CD4^+T_{reg}$ function in specific diseases. Su et al. found that rheumatoid arthritis patients have a TIP60 functional deficiency, leading to instability of Foxp3 expression and impaired suppressive function of $CD4^+T_{reg}$ (30). Jiang et al. discovered that the acetyltransferase p300/CBP-associated factor (PCAF) can acetylate *Foxp3* and enhance its transcriptional activity, thereby promoting the suppressive function of $CD4^+T_{reg}$ (31). These studies suggest that acetylation and deacetylation processes play a critical role in regulating the stability and function of Foxp3 in $CD4^+T_{reg}$. In the field of histone modifications, substantial progress has been made in understanding the role of Enhancer of zeste homolog 2 (EZH2). EZH2 is a catalytic enzyme of the polycomb repressive complex 2 (PRC2) responsible for the methylation of histone H3 lysine 27 (H3K27) at the *Foxp3* locus, leading to the formation of H3K27me3 histone modification. Studies by DuPage et al. have demonstrated that Ezh2 is involved in stabilizing the expression of Foxp3 in a mice model, selective deletion of *Ezh2* in $CD4^+T_{reg}$ resulted in the development of autoimmune diseases and accompanied by reduced stability of $CD4^+T_{reg}$ (32). This study suggests that Ezh2 may play a crucial role in maintaining T_{reg} cell function. Goswami et al. found that selective deletion of *Ezh2* in $CD4^+T_{reg}$ delays tumor progression in mice, while the use of Ezh2 inhibitors in wild-type mice enhances the function of cytotoxic T lymphocytes (CTLs) and promotes the efficacy of anti-CTLA-4 antibodies, thus inhibiting tumor progression (33). These findings indicate that the histone modifications mediated by EZH2 may represent another important mechanism regulating the function of $CD4^+T_{reg}$.

4 Heterogeneity of $CD4^+T_{reg}$ in the TME

Infiltration of $CD4^+$Tregs in TME suppresses local anti-tumor immune responses and is associated with poor prognosis in various types of cancer. The heterogeneity of $CD4^+T_{reg}$ offer diverse mechanisms that contribute to the modulation of anti-tumor immune responses and facilitate immune evasion. Therefore, accurate characterization of different $CD4^+T_{reg}$ subsets in TME is crucial for gaining insights into the formation of the immunosuppressive TME as well as for improving patient prognosis.

The classification of Tregs subsets proposed by Sakaguchi et al. have been widely used to study the heterogeneity of $CD4^+T_{reg}$ in TME. Saito et al., based on the classification, divided colorectal cancer into two types: Type A, characterized by predominant infiltration of eT_{reg} and correlated with poor prognosis, and Type B, characterized by predominant infiltration of non-T_{reg} and correlated with better prognosis (34). Similarly, studies on

different types of tumors have consistently shown that eT_{reg} are the major population in TME and correlated with poor prognosis (35, 36). Wang et al. found a high overlap between the phenotypes and TCR repertoire of tumor-infiltrating $CD4^+T_{reg}$ and peripheral eT_{reg} in patients with breast cancer. They proposed a cytokine signal index (CSI) based on the responsiveness of various peripheral $CD4^+T_{reg}$ subsets to four cytokines, the CSI of eT_{reg} could effectively predict patient prognosis and relapse (37).

Limited research focused on Th-like T_{reg} in TME. Mizukami et al. have shown that tumor-infiltrating $CD4^+T_{reg}$ express CCR4 in lymphoma and gastric cancer (38). Halim et al. demonstrated that $CCR4^+T_{reg}$ in the TME predominantly exhibit Th2-like characteristics, they also discovered a significant elevation of CCR8 expression on Th2-like T_{reg} (17), consistent with the single-cell sequencing results reported by De Simone (39). Van Damme et al. also confirmed that tumor-infiltrating $CCR8^+T_{reg}$ was highly activated and immunosuppressive in both human and mouse tumors. Selective depletion of this subset resulted in increased responsiveness to immunotherapy (40). Downs-Canner et al. discovered that Th17 cells can differentiate into Th17-like T_{reg} in ovarian cancer, thereby suppressing immune responses (41).

The emergence of single-cell RNA sequencing technology has advanced our understanding of the functional subgroups of $CD4^+T_{reg}$ in tissue microenvironments, particularly in tumor-infiltrating sites. Several studies analyzed the immune cell infiltration in head and neck squamous cell carcinoma (HNSCC), non-small cell lung carcinoma (NSCLC), and breast cancer, respectively. Cillo et al. categorized $CD4^+T_{reg}$ infiltrating HNSCC into six subsets, with subsets 2 and 4 representing early-stage $CD4^+T_{reg}$ with upregulated IFN-related signaling pathways, while subsets 3 and 6 represented late-stage $CD4^+T_{reg}$ with upregulated TNF-related signaling pathways (42). Guo et al. observed high expression of *Foxp3*, *IL-2RA*, and *IKZF2* in tumor-infiltrating $CD4^+T_{reg}$, as well as increased expression of *CTLA4*, *TIGIT*, and *TNFRSF9* in tumor-infiltrating $CD4^+T_{reg}$ in NSCLC specimens. Further investigation revealed that the $TNFRSF9^+$subset within tumor-infiltrating $CD4^+T_{reg}$ exhibited potent immunosuppressive abilities (43). Azizi et al. identified five subsets of tumor-infiltrating $CD4^+T_{reg}$ in samples of breast cancer and analyzed the expression of immune checkpoint-related genes within each subset. The results showed that all subsets exhibited high expression of *CTLA4* and *GITR*, with three subsets showing elevated expression of *TIGIT* (6). The widespread adoption of single-cell sequencing technology enables a more comprehensive analysis to the expression of $CD4^+T_{reg}$-related genes in tissue microenvironment, facilitating the identification of biomarkers that effectively represent $CD4^+T_{reg}$ functionality. However, these findings still require further validation through functional experiments.

5 Clinical trials targeting $CD4^+T_{reg}$ in cancer

Targeting $CD4^+T_{reg}$ has the potential to become an important approach in immunotherapy as tumor cells in TME can evade immune surveillance by recruiting immune-suppressive cells such as $CD4^+T_{reg}$. Considering the functional subgroups of $CD4^+T_{reg}$, the primary challenge lies in selectively targeting immunosuppressive subsets while minimizing the impact on other subpopulations, thus reducing the potential for undesirable autoimmune reactions. Early clinical trials primarily targeted CD25 as a marker for T_{reg}, drugs such as denileukin and daclizumab have been reported to reduce the proportion of $CD4^+CD25^+T_{reg}$ and enhance the response rate to tumor vaccine (44). However, CD25 is not a specific marker to $CD4^+T_{reg}$, and the combination of $CD25^-$targeted therapy with tumor vaccines can suppress the generation of tumor-specific T cells (45), limiting the application of CD25 antibodies.

CCR4 is predominantly expressed on the surface of eT_{reg} (46), and previous studies indicated that blocking CCR4 can inhibit the accumulation of eT_{reg} in TME (35). Therefore, targeting CCR4 has shown certain efficacy. Mogamulizumab, a monoclonal antibody targeting CCR4, has been approved by the FDA for the treatment of cutaneous T-cell lymphoma (47). A clinical trial combined mogamulizumab with PD-1 inhibitor (NCT02476123) found an objective response rate of 12% in various types of locally advanced/metastatic solid tumors. The combination significantly reduced the proportion of eT_{reg} in both peripheral blood and tumor-infiltrating lymphocytes (TIL). Moreover, the combination therapy increased the proportion of $CD8^+$T cells in TME regardless of tumor response, indicating the potential of CCR4 targeting therapy (48).

Inhibition of immune checkpoint receptors including CTLA-4, TIM-3, and LAG-3 had shown promising effects in clinical trials or pre-clinical studies. CTLA-4 is a co-inhibitory molecule expressed on the surface of $CD4^+T_{reg}$ and negatively regulates T cell activation (49). Studies in patients with metastatic melanoma have shown that the CTLA-4 antibody ipilimumab can prolong overall survival, but it is also associated with severe adverse events in 10% to 15% of patients (50). Recent clinical trials have found that PD-1 antibody has a lower incidence of adverse events compared to ipilimumab(KEYNOTE-006, NCT01866319) (51), and combination of nivolumab and ipilimumab (CheckMate 067, NCT01844505) can further extend overall survival in patients with melanoma (52). The data from this trial suggest that CTLA-4 antibodies may potentially serve as adjunctive therapy to enhance the efficacy of PD-1 antibodies. TIM-3 is expressed on innate immune cells. Although TIM-3 is not a specific marker on the surface of $CD4^+T_{reg}$, recent studies have identified $TIM\text{-}3^+CD4^+T_{reg}$ within TME played a crucial inhibitory role by inducing exhaustion in effector T cells and $TIM\text{-}3^+CD4^+T_{reg}$ might be a potential therapeutic target (53). Combination therapy targeting TIM-3 and PD-1 mAb has already been tested in several solid tumor, and an ongoing Phase 1 clinical trial is currently evaluating the dosage and anti-tumor efficacy of a humanized anti-TIM-3 antibody in advanced solid tumors (NCT02817633) (54). LAG-3 is expressed on activated T cells including $CD4^+T_{reg}$ (55) and LAG-3 can hinder the proliferation of effector T cells and dendritic cells, while promoting the differentiation of eT_{reg} (56). Currently, there are two clinical trials investigating the efficacy of anti-LAG-3 alone or in combination with PD-L1 antibody in patients with advanced solid tumors (NCT01968109, NCT03156114) (57). OX40 is a costimulatory molecule expressed mostly on activated effector T cells and nT_{reg} (58). Pre-clinical studies have demonstrated the immunosuppressive function of $OX40^+T_{reg}$ and anti-OX40 could improve tumor control in mouse models (59, 60). A recent phase I

clinical in HNSCC patients (NCT02274155) demonstrated that anti-OX40 administration was well tolerated and increased the infiltration of activated $CD4^+$ and $CD8^+$T cells (61). This data indicates the potential clinical utility of anti-OX-40, but further clinical trials are needed to confirm its efficacy.

Above clinical trials aim to reverse the immunosuppressive state of TME by inhibiting immunosuppressive cells such as $CD4^+T_{reg}$. Optimization of combination therapies that effectively target $CD4^+T_{reg}$ while enhancing anti-tumor immune responses represents a promising avenue for the improved treatment of cancer. However, it is crucial to take into account the pathological subtype of cancer and adopt a targeted approach towards suppressive $CD4^+T_{reg}$, rather than indiscriminately depleting all T_{reg} or other effector T cells. These considerations highlight the importance of further evaluation when developing novel therapeutic strategies in this context.

6 Conclusion

Although $CD4^+T_{reg}$ represent a small proportion of lymphocytes, their immunoregulatory role in the TME is crucial. Effectively distinguishing the distinct functional heterogeneity of $CD4^+$Treg subsets is a pressing challenge. Currently, the subtypes proposed by Sakaguchi et al. is the most widely used and exhibits good consistency in research conclusions. However, the subtypes of Th-like T_{reg} reflects the plasticity of $CD4^+T_{reg}$ in TME and still faces inconsistent findings across different studies. Furthermore, numerous clinical trials targeting $CD4^+T_{reg}$ have been conducted, highlighting the pressing need to accurately inhibit specific functional Treg subsets involved in immunoregulation. In future research, rational subgroup analysis based on different surface markers is essential for a better understanding of the role of $CD4^+T_{reg}$ in the TME.

Author contributions

HL: Conceptualization, Project administration, Writing – original draft. YX: Conceptualization, Formal analysis, Writing – review & editing. CL: Conceptualization, Writing – review & editing.

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Conflict 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.

Publisher's note

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