

Regulatory T Cells in Cancer Immunotherapy: From Tumor Microenvironment Dynamics to Bispecific Antibody Approaches

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Abstract

Regulatory T (Treg) cells are crucial in maintaining immune homeostasis and preventing autoimmunity¹. However, within the tumor microenvironment (TME), Tregs shift their role towards immune suppression. In cancer, the immune system develops resistance, contributing to a suppressive TME. The TME plays a vital role in tumorigenesis², and tumor-infiltrating lymphocytes (TILs) are key components of this environment. These lymphocytes consist of various cell types, including natural killer (NK) cells, CD8+ cytotoxic T cells, CD4+ helper T cells and regulatory T cells. Various interactions and immune responses occur between tumor cells and neighboring cells through the circulatory and lymphatic systems. These interactions include the upregulation of immune checkpoint molecules, the secretion of immunosuppressive cytokines, and alterations in the metabolic environment of effector T cells, among other mechanisms. This facilitates the tumor cells to evade immune detection, escape the immune system, and promote tumor growth and survival. In recent years, immunotherapy has emerged as one of the most promising treatments for cancer, significantly transforming the therapeutic landscape of cancer care. By harnessing the power of the immune system, immunotherapies target and combat tumor cells. Regulatory T cells have become key targets in the development of new immunotherapeutic strategies due to their pivotal role in immune evasion of cancer cells³. In this review, we delve into the molecular characteristics and immunosuppressive mechanisms of Tregs in cancer, with a focus on key molecules CD25 (IL-2 receptor α chain) and TIGIT (T cell immunoreceptor with Ig and ITIM domains), which play significant roles in tumor immune evasion. We also discuss the potential of bispecific antibodies (BsAbs) as a therapeutic treatment for tumor by selectively targeting Treg markers (CD25 and TIGIT) within the tumor microenvironment.

Molecular Characteristics of Regulatory T (Treg) cells

Regulatory T (Treg) cells are a distinct lineage of T cells within the adaptive immune system, critical for preserving immune balance, ensuring immune tolerance and preventing autoimmune responses. According to the site of development, Tregs cells are classified into: thymic Tregs (tTregs), peripheral Tregs (pTregs) and induced Tregs (iTregs). tTregs are generated in the thymus following exposure to self-antigens, accompanied by an enrichment of T-cell receptors (TCRs), while pTregs rise in the periphery in response to specific antigens. In contrast, induced Tregs (iTregs) develop upon antigen stimulation along in the presence of a unique cytokine environment found in TME⁴. Natural Tregs (nTregs) are primarily responsible for preserving self-tolerance and preventing autoimmunity, whereas iTregs maintain peripheral tolerance at mucosal interfaces and in response to external antigens.

The Treg subset is characterized by high expression of the

Forkhead box protein 3 (FoxP3) transcription factor and the CD25 receptor. FoxP3 is crucial for the development and stabilization of the suppressive activity of Tregs⁵, and CD25 is a key component of the interleukin-2 (IL-2) receptor which enables Treg cells to respond to IL-2⁶, a cytokine essential for sustaining their suppressive function. Additionally, CD4+CD25+ FoxP3+ Tregs express other characteristic receptors, including glucocorticoid-induced TNFR-related gene (GITR), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and CD39. These markers distinguish Tregs from other immune cells and contribute to immune evasion and promoting tumor progression.

Immunosuppressive Functions of Tregs in the Tumor Microenvironment (TME)

Regulatory T (Treg) cells play a complex role in cancer, with their function often shifting from immune protection to tumor promotion. Within the tumor microenvironment (TME), Tregs contribute to tumor growth and survival through several mechanisms (Fig 1) as outlined below:

A) Recruitment and Infiltration of Treg Cells into the TME⁷: Various chemotactic molecules and their receptors are involved in the recruitment and infiltration of regulatory T (Treg) cells into the TME. For instance, CCR8 which is selectively upregulated in intratumoral Treg cells, mediates chemotaxis toward CCL1, a chemokine secreted by tumor cells. Similarly, CCR4, another key chemokine receptor binds to CCL22 and CCL17, facilitates the trafficking and accumulation of Treg cells into the TME. Additionally, CCL5 activated by cancer-associated FOXP3, is responsible for the infiltration of FOXP3+ Treg cells into tumor, as observed in Pancreatic Ductal Adenocarcinoma (PDAC). This chemokine-receptor interaction facilitates an immunosuppressive environment.

B) Upregulated expression of checkpoint molecules such as PD-1 (Programmed Cell Death Protein 1), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), TIGIT (T cell immunoreceptor with Ig and ITIM domains), LAG-3 (Lymphocyte Activation Gene-3), TIM-3 (T cell immunoglobulin and mucin domain-containing protein 3), and VISTA (V-domain Ig suppressor of T cell activation)⁸:

- i. PD-1 engages with its ligands PD-L1/PD-L2, which are expressed on activated antigen-presenting cells (APCs), leading to suppression of T-cell responses⁹.
- ii. CTLA-4 competes with CD28, a co-stimulatory molecule that normally promotes T-cell activation for binding to ligands on APCs, thereby blocking T-cell activation.
- iii. TIGIT interacts with CD112 and CD155 ligands found on APCs, inhibiting T cell activation and function.
- iv. LAG-3 inhibits the expression of MHC class II molecules in dendritic cells (DC), suppressing T-cell activation.

- v. TIM-3 is activated via binding to its ligand galectin, which induces death and apoptosis of effector T cells.
- vi. VISTA binds to V-set and immunoglobulin domain containing 3 (VSIG-3), causing inhibition of effector T-cell proliferation and reducing their cytokine release. In naïve T cells, VISTA promotes differentiation into regulatory T cells (Tregs), contributing to immune suppression.

C) Secretion of immunosuppressive cytokines including TGF- β (Transforming Growth Factor), IL-10 (Interleukin-10), IL-35 (Interleukin-35): These cytokines directly suppress the activity of T and B lymphocytes, dendritic cells (DCs), and macrophages.

- i. TGF- β promotes the expansion and stability of Tregs while inhibiting effector T-cell (Teff) activation¹⁰. The soluble form of TGF- β activates the PI3K (Phosphoinositide 3-Kinase) signaling pathway, upregulating FoxP3 expression and inducing the loss of PTEN, leading to the expansion of Treg cells. In contrast, the membrane-bound form (GARP/TGF- β complex) of TGF- β binds to TGF- β receptors (T β RI and T β RII) on CD4+ and CD8+ T cells, blocking their activation and proliferation⁸.
- i. IL-10 suppress the IFN γ dependent activation of APCs, downregulates the expression of MHC class II molecules and CD86 on the surface dendritic cells (DCs) and macrophages¹¹, thereby impairing their antigen-presenting capability and leading to a compromised immune response.
- i. IL-35 triggers the conversion of T cells into a suppressive Treg population and downregulates interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α) secretion, which hampers antigen-specific antitumor T cell responses¹². Additionally, IL-35 promotes the expression of multiple checkpoint inhibitory receptors (PD1, TIM3, LAG3) on T cells, leading to exhaustion of intratumoral T cells¹³.

D) Direct cell-to-cell contact inhibition: Treg cells secrete perforin which forms pores in the membranes of target cells, allowing granzymes A and B to enter and induce apoptosis in CD4+ and CD8+ T cells, monocytes, and dendritic cells¹⁴. Additionally, Tregs express galectin-1 and utilize the TRAIL-DR5 pathway and Fas/FasL interactions to trigger apoptosis in target cells¹⁵. Together, these mechanisms help Tregs to eliminate immune cells that might otherwise target tumor cells.

E) Metabolic Disruption of Teff cells: TME is often hypoxic and enriched with Treg cells that highly express FoxP3. These Tregs can induce adenosine-mediated

immunosuppression via the ectoenzymes CD39 and CD73, which concert extracellular adenosine triphosphate (ATP) to adenosine, an immunosuppressive mediator. Adenosine binds to the A2A receptor on Teffs, triggering the accumulation of cyclic adenosine monophosphate (cAMP) and the activation of protein kinase A (PKA) signaling. This results in the suppression of Teff proliferation, cytokine production, and TCR-mediated signaling¹⁶. Additionally, Tregs disrupt the metabolic environment by consuming IL-2 (interleukin-2), a cytokine critical for Teffs survival,

proliferation, and function. Due to high expression of CD25, Tregs enhances IL-2 uptake leading to a cytokine sink within the TME. This deprivation of IL-2 hinders Teff activation and the subsequent immune responses needed to target and eliminate tumor cells¹⁷.

F) Promotes M2-Like Tumor-Associated Macrophages (TAMs) Polarization: Regulatory T (Treg) cells secrete IL-13 and suppress the secretion of IFN γ (interferon- γ) by CD8+ T cells, which promotes the activation of

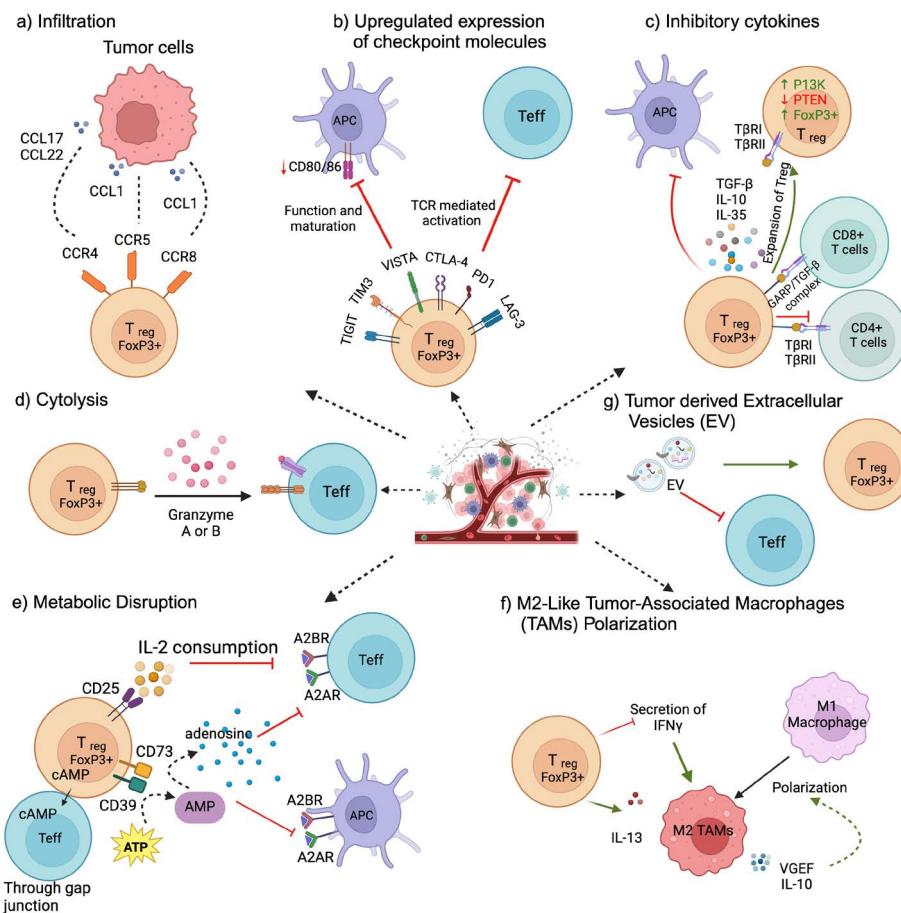


Figure 1: Immunosuppressive mechanisms of Treg cells in the Tumor Microenvironment: A representation of the different mechanisms of regulatory T cells Treg. a) Chemokine-mediated recruitment and infiltration of Treg cells, which express chemokine receptors (e.g., CCR4, CCR8, CCR5, GPR15), into the tumor microenvironment (TME). b) Upregulated expression of checkpoint molecules like PD-1, CTLA-4, TIGIT, LAG-3, TIM-3, and VISTA suppress T-cells and dendritic cells activation and function. c) Treg cells secreted immunosuppressive cytokines include TGF- β , IL-10, and IL-35 which promote the conversion of Tconv cells to Treg cells and suppress the activation of Teff cells (CD8+ T cells/ CD4+ T cells) and APC function. d) Treg cells secrete perforin and granzymes, which induce apoptosis in Teff cells. e) Metabolic disruption includes apoptosis triggered by IL-2 cytokine deprivation through binding to high-affinity CD25 (IL-2 receptor α), Teff cell inhibition via cyclic AMP (cAMP), and immunosuppression caused by the conversion of ATP to adenosine through CD39 and/or CD73, which binds to adenosine receptor 2A (A2AR) and impairs Teff cell function. f) Treg cells suppress IFN γ from CD8+ T cells and secrete IL-13, promoting M2-like TAM within TME. These M2-TAMs secrete pro-angiogenic factors and immunosuppressive cytokines, enhancing tumor angiogenesis, invasion, and metastasis, polarization of M1-macrophage to M2 -TAM in the TME. g) Treg cell-derived EVs (Treg-EVs) from tumor cells suppress Teff cell activation and promote immunosuppression via the accumulation of Tregs. Treg, Regulatory T cell; Teff, Effector T cell; APC, Antigen-presenting cell; PD-1 (Programmed Cell Death Protein 1), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), TIGIT (T cell immunoreceptor with Ig and ITIM domains), LAG-3 (Lymphocyte Activation Gene-3), TIM-3 (T cell immunoglobulin and mucin domain-containing protein 3); VISTA (V-domain Ig suppressor of T cell activation); TGF, transforming growth factor; IL, interleukin; GARP, Glycoprotein A repetitions predominant; TGF- β Transforming growth factor beta; T β RI/II, TGF- β receptor type I/II; A2AR/A2BR, adenosine A2A/A2B receptor; TAM, Tumor associated macrophages; IFN γ , Interferon-gamma; VEGF, Vascular endothelial growth factor. Created by using BioRender.com.

SREBP1 (sterol regulatory element-binding protein-1)-mediated fatty acid synthesis in M2-like tumor-associated macrophages (TAMs)¹⁸. These M2-type TAMs exhibit an immunosuppressive phenotype, secreting pro-angiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and platelet derived growth factor (PDGF), which promote tumor angiogenesis and vascular formation. They also secrete immunosuppressive cytokines like transforming growth factor-beta (TGF- β) and IL-10 that promotes the polarization of M1 macrophage to M2-TAM. Additionally, M2 like TAMs release proteases that degrade the extracellular matrix, aiding in tumor cell invasion and metastasis. Indirectly, Tregs foster the accumulation of M2 like TAM within TME.

G) Extracellular Vesicles (EVs): EVs are the key components of TME in cancer. Treg cell derived EVs (Treg-EVs) have the ability to regulate the immune responses by inducing gene silencing through microRNAs (miRNAs), modulating the activity of surface proteins, and transmitting enzymes¹⁹.

CD25 and TIGIT: Key Regulators of Tregs Function and Tumor Immune Evasion

Understanding the molecular markers and pathways that influence regulatory Tregs function is essential for developing new therapeutic strategies. Two key molecules involved in Tregs functionality and tumor immunity are CD25 and TIGIT. CD25, the IL-2 receptor α chain, plays a vital role in the function of Tregs and in regulating immune responses within the TME. It is highly expressed on activated T cells and is essential for maintaining immune tolerance and preventing autoimmune reactions. In cancer, the expression of CD25 on Tregs, along with CD25-mediated signaling, contributes to immune suppression within the TME through multiple mechanisms.

A) Induction of Tregs proliferation and activity: CD25 on Tregs binds to IL-2, a cytokine essential for their survival, proliferation, and function. The binding of IL-2 activates signal transducer and activator of transcription (STAT5) through phosphorylation, leading to its dimerization and translocation into the nucleus. This process is essential for maintaining FoxP3 expression, which is a key transcription factor required for Tregs development and their suppressive activity²⁰. Additionally, phosphatase and tension homology (PTEN) plays a key role in maintaining Tregs stability by inhibiting the activation of the PI3K/Akt signaling pathway²¹. The PI3K/Akt pathway is involved in regulating various cellular processes, including cell growth, survival, and metabolism. PTEN stabilizes the expression of crucial Tregs markers, particularly CD25 and Foxp3 thereby supporting immune suppression and Tregs stability.

B) IL-2 Sequestration and Effector T Cells

Suppression: The high expression of CD25 on Tregs results in the excessive consumption of IL-2, reducing its availability for effector T cells (Teffs). IL-2 is essential for the activation, proliferation, and survival of Teffs, including CD8+ cytotoxic T cells and CD4+ helper T cells, which are crucial for anti-tumor immunity. Lack of cytokine induces apoptosis of Teff cells²², thereby inhibiting their function and suppressing the immune response against tumor cells.

TIGIT plays a key role in regulating both adaptive and innate immune responses. It is an immunoglobulin (Ig) superfamily receptor expressed on CD8+ T cells, CD4+ T cells, natural killer (NK) cells, follicular T helper cells and regulatory T cells (Tregs). Within the TME, TIGIT is crucial for immune suppression through several mechanisms.

A) Suppression of T cells activation: TIGIT binds to its ligand CD155, which is expressed on tumor cells, dendritic cells (DCs), and other cells within the TME, triggering a signaling cascade that shifts DCs to a tolerogenic phenotype. This reduces the secretion of pro-inflammatory cytokine like IL-12, while increasing the immunosuppressive cytokines such as IL-10. Consequently, the ability of DCs' to activate T cells is impaired, limiting immune responses against tumor cells²⁴.

B) Disruption of CD226 (DNAM-1)-Mediated Activation: CD226 is an activating receptor that enhances immune cell adhesion and cytokine production by binding to CD155, which helps activate T cells and NK cells. TIGIT competes with CD226 for binding to CD155, with higher binding affinity, thereby preventing CD226 from initiating its pro-inflammatory signaling. This competition reduces the anti-tumor immune response.

C) Suppression of NK cell activity: TIGIT also inhibits NK cell-mediated cytotoxicity against tumor cells by binding to CD155, thereby preventing NK cell degranulation and cytokine production. This contributes to a reduced ability of the immune system to eliminate tumor cells.

D) Promotion of Tregs function: Expression of TIGIT is upregulated on Tregs which promotes the secretion of immunosuppressive cytokines such as IL-10 and the expression of checkpoint inhibitors like PD-1, CTLA-4, and LAG-3. Together, these factors inhibit the activation and function of effector T cells (Teffs) while suppressing proinflammatory responses.

Bispecific Antibodies: A Novel Strategy to Target Tregs in Solid Tumors

Numerous studies have explored the role of regulatory T cells (Tregs) in cancer, leading to the development of immunotherapies targeting Tregs and other immune components, including checkpoint inhibitors, immune modulators, and tumor-associated antigens. Targeting Tregs with monoclonal antibodies (mAbs) through

inhibition of CTLA4, PD-1, LAG3, and TIGIT has initially transformed treatment²⁵. However, these approaches have faced limitations due to poor targeting of intratumoral Tregs and the widespread expression of surface markers on both Tregs and activated effector T cells. This led to unintended elimination of Teffs and an imbalance in the immune system, potentially causing excessive immune activation or autoimmune reactions. To address these challenges, bispecific antibodies (BsAbs) have developed. BsAbs enhance specificity by simultaneously recognizing two different antigens and can target cells in both cis and trans orientations, improving precision in tumor targeting and immune response while reducing off-target effects. As a result, the development of these molecules has accelerated. By 2023, several bispecific antibodies were approved for the treatment of cancer and non-oncology indications²⁶.

CD25 and TIGIT are markers for Tregs in the tumor microenvironment (TME). Xin Wei et al. leveraged the co-expression properties of CD25 and TIGIT on intratumoral Tregs, rather than on peripheral Tregs, to develop a bispecific antibody (bsAb) targeting both markers²⁷. The therapeutic potential of targeting both CD25 and TIGIT was confirmed through co-expression profiling of these markers in Teffs and murine Tregs using tumor models such as MC38, EG.7, and CT26, as well as pan-cancer T cell atlas and single-cell RNA sequencing (scRNA-seq) of human data. These analyses revealed an enrichment of CD25 and TIGIT in Tregs within the TME and across various cancer types.

The authors developed a mouse bispecific antibody (bsAb), NSWm7210, which demonstrated a six-fold stronger affinity for mCD25+ mTIGIT+ double-positive CHO cells. *In vivo*, NSWm7210 exhibited enhanced antitumor activity, significantly reducing tumor growth in four syngeneic tumor models (MC38, EG.7, CT26, and MCA205) through interactions of the Fc region with Fcγ receptors on immune cells, as well as through antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP), thereby conferring therapeutic efficacy. NSWm7210 promotes the activation of effector cells, with an increase in secretion of interferon (IFN)-γ in CD8+ T cells and NK cells while also depleting intratumoral Treg cells within the TME. For translational purposes, the authors developed seven anti-hCD25xTIGIT bispecific antibodies (bsAbs), designated as NSWh7211–NSWh7217. *In vivo*, NSWh7216 selectively binds to intratumoral Tregs and suppresses established tumors without impacting the number of peripheral Tregs. This highlights the potential of NSWh7216 to overcome the issue of Teff depletion, thereby enhancing tumor-specific immune responses while minimizing systemic immune system disturbances.

Key Takeaways and Future Outlook

The exploration of Tregs in cancer has paved the way for the development of targeted immunotherapies aimed at modulating the TME to enhance immune responses. CD25 and TIGIT, as key markers, are involved in regulating Treg function and facilitating tumor immune evasion. Targeting these markers on Tregs within the TME, bispecific antibodies (BsAbs) have emerged as a promising strategy to overcome the limitations related to off target and autoimmune response in patients. The bispecific antibody NSWm7210, developed by Xin Wei et al. has demonstrated enhanced antitumor activity by selectively targeting CD25 and TIGIT on Tregs within the TME. Additionally, the development of NSWh7216 further reinforces the potential of bispecific antibodies in therapeutic efficacy through improved tumor targeting and immune cell activation. Notably, NSWh7216 selectively targets intratumoral Tregs while preserving peripheral Tregs and effector T cells (Teff), thereby mitigating the risk of Teff depletion.

In combination with immune checkpoint inhibitors (ICIs), NSWh7216 holds the potential to significantly enhance the therapeutic efficacy of immune checkpoint blockade therapies. Moreover, other markers such as CTLA-4 and CCR8, which are highly expressed on Tregs along with CD25, which is low on Teffs, could be explored as potential targets for developing bispecific antibodies (bsAbs). These advancements underscore the promise of bispecific antibody-based treatments in cancer immunotherapy, offering new avenues for precise and effective interventions aimed at reprogramming the immune system to combat cancer while minimizing adverse side effects.

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