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Review

Regulatory T-cells: The Face-off of the Immune Balance

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Abstract

Regulatory T-cells (Tregs) play a crucial role in maintaining immune homeostasis, ensuring a balanced immune response. Tregs primarily operate in an antigen-specific fashion, facilitated by their distinct distribution within discrete niches. Tregs have been studied extensively, from their point of origin in the thymus origin to their fate in the periphery or organs. Signals received from antigen-presenting cells (APCs) stimulate Tregs to dampen inflammation. Almost all tumors are characterized by a pathological abundance of immune suppression in their microenvironment. Conversely, the lack thereof proves detrimental to immunological disorders. Achieving a balanced expression of Tregs in relation to other immune compartments is important in establishing an effective and adaptable immune tolerance towards cancer cells and autoantigens. In the context of cancer, it is essential to decrease the frequency of Tregs to overcome tumor suppression. A lower survival rate is associated with the presence of excessive exhausted effector immune cells and an increased frequency of regulatory cells. However, when it comes to treating graft rejection and autoimmune diseases, the focus lies on immune tolerance and the transfer of Tregs. Here, we explore the complex mechanisms that Tregs use in human disease to balance effector immune cells.

Keywords: regulatory T-cells (Tregs); immune imbalance; Foxp3; natural Treg (nTreg); induced Treg (iTreg); thymic Treg (tTreg); peripheral Treg (pTreg)

1. Introduction

Immune regulation balances pro-inflammatory and anti-inflammatory immune responses. Classifications characterize the immune compartments in an effort to improve understanding of the mechanisms involved [1]. Regulatory T-cells (Tregs) are known for their powerful function in regulating the immune system and improving self-tolerance, which is pivotal in immune homeostasis [2,3]. Tregs account for 3–10% of the peripheral $CD4^+$ T-cell population in humans [4]. Tregs are initially observed in the thymus during embryogenesis, specifically at the 12th week of gestation, and their levels remain constant throughout pregnancy and infancy [4]. The immunosuppressive characterization of Tregs from the thymus during fetus development showed early expression of forkhead box P3 (Foxp3) and other markers associated with their function, such as glucocorticoid-induced TNFR-related protein (GITR) and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [5].

Tregs are derived from either the thymus, referred to as (tTregs), or conventional T-cells in tissues outside the thymus, known as peripheral Tregs cells (pTregs). The tTregs typically possess T-cell receptors (TCRs) with higher affinity for autoantigens compared to conventional T-cells and pTregs [6]. In both homeostatic and inflammatory conditions, the conversion of conventional $CD4^+$ T-cells into pTregs occurs on interaction with self-antigens or exogenous antigens and in the presence of transforming growth factor beta (TGF-β) [7]. The presence of pTregs is important for regulating peripheral tolerance, particularly in the gut, lungs, and skin [8]. It is unknown how conventional T-cells turn into pTregs, especially in humans. *In vitro* stimulation of $CD4^+$ T-cells with TGF-β and interleukin-2 (IL-2) leads to the generation of induced $Foxp3^+$ Treg cells (iTregs) [9]. On transfer to mice, iTregs exhibit suppressive abilities, confirmed by their transcriptional profile, which differs from both pTreg and tTregs [10]. However, these cells do not possess the ability to suppress immune responses [11]. Despite this, there are no definitive markers to distinguish Tregs of different origins in either humans or mice [12]. While the gene expression profiles of pTreg and tTreg cell lineages in mice are similar, there are some discernible differences. Their TCR repertoires are different, showing that TCR affinity is one of the things that sets tTreg and pTregs apart [13]. It was discovered that the best results for pTreg induction were obtained through TCR stimulation with low doses of high-affinity peptide. The efficient Foxp3 expression is largely dependent on determining the optimal ratio of antigen concentration to TCR avidity, which is determined by TCR-peripheral major histocompatibility complex (pMHC) affinity. The regulation of this process is influenced by surface ligands like CD28 and soluble mediators TGF-β and retinoic acid [14]. Since the cascades

of interactions are vast, we are going to focus in our article on the origin, biological role, and clinical benefits of Tregs cells.

2. Origin and Biology of Regulatory T-cells

2.1 Thymic Development of Treg

The development of Tregs in the thymus during the neonatal period leads to dominant tolerance [15]. Only 2–3% of developing $CD4^+$ single positive (SP) thymocytes are represented by thymic Tregs. Their development is propelled by the combination of a strong TCR stimulation and a CD28 co-stimulatory signal [15]. The progression of $CD25^+$ $Foxp3^+$ Tregs into a mature stage is enhanced by a third signal mediated by γ-chain cytokines, which also promotes their survival [16]. The tTreg has been found to be a more varied and ever-changing population. The ontogeny of Tregsin the thymus plays a crucial role in shaping their phenotypic and functional diversity [17]. Thus, Tregs show a highly self-reactive TCR repertoire, distinct from conventional T-cells, which contributes to their functional diversity [18,19].

Treg development is dependent on TCR/CD28 stimulation and cytokine signaling, both of which follow a two-step model. First, induction of CD25 occurs in $CD25^-$ $Foxp3^-$ $CD4^+$ SP thymocytes through moderately to slightly strong binding on TCR signals. The outcome of this process is the generation of $CD25^+$ $Foxp3^-$ Treg precursors, commonly referred to as $CD25^+$ Tregs. The binding to the TCR signal is required to be lower than the clonal deletion binding of SP $CD4^+$ thymocytes [18,19]. The CD28- CD80/86 co-stimulatory axis plays a crucial role in Treg development, starting from their precursor stage and facilitating the induction of Foxp3 expression [20]. The binding of TCR signaling when associated with CD28 drives to the upregulation of tumor-necrosis factor receptor superfamily (TNFRSF) members, which are TNFR2, GITR and OX40 [21]. Second, the maturation of $CD25^+$ Treg through stimulatory γ-chain cytokines, like IL-2, -4, -7, -9, -15, and -21, to express the intracellular $Foxp3^+$ [22]. Among all γ-chain cytokines, only IL-2 and IL-15 are required for Tregs maturation and to protect Tregs against the pro-apoptotic effects of Foxp3 [23]. By activating GITR, OX40, or TNFR2, the conversion of $CD25^+$ Treg into mature Tregs is enhanced during this second stage, allowing for heightened responsiveness to IL-2 [21]. The source of IL-2 is puzzling, as it is initially thought to be secreted by thymic dendritic cells [24], which are precisely assigned to self-reactive $CD4^+$ thymocytes [15]. Later it was found that stromal cells such as cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC) are the main source for both IL-2 and IL-15 as well as different levels of major histocompatibility complex (MHC) class II molecules [25].

Identification of another Treg precursor subset, which expresses a low level of Foxp3 and lack CD25 ($CD25^-$ $Foxp3^{low}$) suggests another developmental pathway [17]. When migrating from thymus, it is found that $CD25^+$ Treg differentiates into mature Treg. These findings confirm that both $CD25^+$ and $Foxp3^{low}$ Treg can be the sources of the generation of the mature Tregs. $Foxp3^{low}$ Tregs appear to be dependent on IL-15 for survival, and IL-2 is necessary for maturation into Tregs. The development of periphery $CD25^+$ Treg is likely dependent on IL-2. Therefore $CD25^+$ Tregs show a higher affinity for self-antigens than $Foxp3^{low}$ Tregs [17].

2.1.1 Subsets of $CD4^+$ Tregs

$CD4^+$ Tregs were phenotypically known as $CD4^+$ $CD25^+$ $Foxp3^+$ cells. Based on surface marker expression, different classifications emerged. The first functional classification was based on the cytokine expression of Th-like Treg subtypes [26]. In this classification, CD45RA is crucial for the division of $CD4^+$ Tregs. This subset is further subdivided into three subsets. First is the resting (rTreg) or naïve Tregs with phenotype $CD45RA^+$ $Foxp3^{low}$ $CD25^{low}$. The second is the effector Tregs (eTregs) with phenotype $CD45RA^-$ $Foxp3^{high}$ $CD25^{high}$ that has strong inhibitory and stabilizing functions. The third is non-Tregs (noTregs) with phenotype $CD45RA^-$ $Foxp3^{low}$ $CD25^{low}$ that mainly secrete inflammatory cytokines and promote the immune response [27]. The rTregs can suppress the immune system as it expresses CD62L and the IL-7 receptor (CCR7), which are indicators of naïve cells. Upon antigen stimulation, they differentiate into eTregs, which exhibit stronger immunosuppressive and higher proliferation capabilities. These eTregs are short-lived and more prone to apoptosis. Unlike the earlier identified Tregs subsets, non-Tregs are categorized as Tregs and have the ability to secrete pro-inflammatory cytokines like interferon-γ (IFN-γ) and IL-17, despite not having immunosuppressive capabilities [15]. A subset of non-Tregs possess $CD127^+$ which is like conventional T-cells, while another subset does not possess $CD127^-$ which is similar to eTregs. The $CD127^-$ subset is subdivided into $CD127^-$ $CCR4^+$ $CD49d^-$ cells that have high levels of IL-2 and $CD127^-$ $CCR4^-$ $CD49d^+$ cells that have high levels of IFN-γ and IL-17 [28].

Another classification is based on the cytokine secretion profiles and transcription factor expression (Table 1, Ref. [27,30–42]). $CD4^+$ Treg has been classified into four distinct subtypes. The first, Th1-like Tregs secrete IFN-γ and tumor necrosis factor-α (TNF-α) ($CCR4^+$ $CCR6^-$ $CXCR3^+$). The second, Th2-like Tregs secrete IL-2, IL-4, IL-5, and IL-13 ($CCR4^+$ $CCR6^-$ $CXCR3^-$). The third, Th17-like Tregs secrete IL-17A/IL-17F ($CCR4^+$ $CCR6^+$ $CXCR3^-$). The fourth, Th1/17-like Treg which is secreting IFN-γ and IL-17A ($CCR4^+$ $CCR6^+$ $CXCR3^+$) [29]. The similarity between Th-like Treg subset and their corresponding Th cell counterparts was evident in their shared transcription factor expression and cytokine secretion patterns.

Table 1. Overview of the immunophenotypes of Tregs subsets based on maturation origin, function, and markers for differentiation.

Treg subset	Origin	Phenotype	Function	Markers for Differentiation	References
Natural or Naïve Tregs (nTregs) Cells	Thymus	$CD4^+CD25^+Foxp3^+$	Maintain self-tolerance and immune homeostasis by suppressing immune responses via direct cell-cell contact and secretion of inhibitory cytokines like IL-10 and TGF-β.	Express $CD4^+$, $CD25^+$, $Foxp3^+$, $Helios^+$, $CTLA\text{-}4^+$, $GITR^+$.	[27,30–32]
Induced Tregs (iTregs)	Peripheral tissues from nTregs	$CD4^+CD25^+Foxp3^+$	Induced in response to specific antigens and cytokines. Suppress immune responses and maintain tolerance.	Express $CD4^+$, $Foxp3^+$, $CD25^+$, $CTLA\text{-}4^+$, $ICOS^+$.	[27,31,33–35]
Type 1 Regulatory T (Tr1) Cells	Peripheral tissues from CD4 T-cells	$CD4^+CD25^-CD49b^+LAG\text{-}3^+$	Suppress immune responses through the secretion of IL-10 and TGF-β particularly in the gut.	Do not express $Foxp3^-$ but produce high levels of IL-10, IFN-γ, GITR, and TNFSRF9.	[33,34]
T Helper 3 (Th3) Cells	Peripheral tissues from CD4 T-cells	$CD4^+CD25^-Foxp3^-LAP^+$	Regulate mucosal immunity and oral tolerance primarily through TGF-β production.	$CD4^+$, TGF-β^+, $Foxp3^{+/-}$.	[36–38]
$CD8^+$ Regulatory T-Cells	Thymus and peripheral tissues from $CD8^+$ T-cells	$CD8^+CD25^+Foxp3^-$	Suppress immune responses through direct cytotoxic activity or cytokine production. They can inhibit the function of other T-cells and APCs.	Express CD25 and Foxp3 in some cases.	[39,40]
Double Negative Regulatory T- (DN Tregs) Cells	Develop from $CD4^-$ $CD8^-$ T-cells in Peripheral tissues	$CD3^+CD4^-CD8^-TCR\alpha\beta^+/\gamma\delta^+$	Suppress immune responses through cytokine production and direct cell-cell contact. They are involved in controlling autoimmune responses and graft-versus-host disease.	T-cells are expressing the $\alpha\beta$ or $\gamma\delta$ T-cell receptor (TCR), but not the CD4 nor the CD8 co-receptors.	[41,42]

CD25, IL-2 Receptor Alpha; Foxp3, Forkhead box P3; CTLA-4, Cytotoxic T-Lymphocyte Associated Protein 4; GITR, Glucocorticoid-induced tumor necrosis factor receptor; LAG-3, Lymphocyte Activation Gene-3; TGF-β, Transforming Growth Factor Beta; TNFRSF9, tumor-necrosis factor receptor; LAP, Latency-associated peptide; ICOS, Inducible co-stimulator; IL-10, interleukin 10; IFN-γ, interferon-γ; APCs, antigen-presenting cells.

In both classifications, Foxp3 expression was not associated with any immunosuppressive potentiality, which leads to conclusions not in line with majority of studies that have employed Foxp3 to demonstrate immunosuppressive capacity. Still, the most acceptable phenotype for $CD4^+$ Treg isolation and detection is $CD4^+$ $CD25^+$ $CD127^{low/-}$, because $CD127^{low/-}$ cells have higher suppressor potentiality than $CD25^+$ subsets [29]. Different classes of Tregs were characterized in Table 1.

2.1.2 Foxp3 Functional Role in Tregs

Foxp3 is essential for deciding which lineages to commit to throughout the formation of tTregs in the thymus. In addition, this mechanism is responsible for the maintenance of the extrathymic tTregs population and ensuring the continuous expression of genes that are fundamental in defining the specific characteristics of the Tregs signature [43]. Under homeostatic conditions, the majority of Tregs maintain their commitment to the tTregs lineage even after leaving the thymus. Despite this, a notable fraction (10-20%) of $Foxp3^+$ Tregs experience a loss of Foxp3 expression, leading them to become exhausted Tregs [44]. This population mainly originates from activated conventional T-cells that exhibit temporary expression of Foxp3, as well as from pTregs [8]. Foxp3 plays a crucial role in determining the lineage commitment of Tregs. Foxp3 is not the only factor that influences the Treg gene program and suppressor activity (Fig. 1) [45].

Foxp3 is responsible for maintaining the stability of the core suppressive mechanism of Tregs, ensuring that peripheral inflammatory and non-inflammatory signals do not interfere with it [46]. Tregs driven by Foxp3 are characterized by a distinct gene signature. They express a gene set associated with an activation program that is shared with conventional T-cells [47]. Foxp3 is essential for the establishment of suppressive activity of Tregs since a frameshift mutation of Foxp3 leads to immune dysregulation and polyendocrinopathy, enteropathy X-linked (IPEX) syndrome [48]. It is possible to repair the severe autoimmunity of Treg-deficient mice and efficiently restore their suppressive function by restoring Foxp3 transcription [48]. This suggests that there is a mechanistic role of Foxp3 in the establishment of Tregs, that grants periphery Tregs suppressive activity. It also implies that there are multiple molecules and mechanisms that likely play a role in regulating the function of both tTregs and pTregs.

When non-self-antigens are recognized, thymus derived Tregs develop based on their interactions with self-peptide-MHC complexes in the presence of TGF-β for induction [49]. Both tTregs and iTregs express Foxp3 as the most important and lineage-specific transcription factor [50]. *Foxp3* gene contains a highly conserved CpG-rich region in the intron (+4201 to +4500) that is termed the Treg-specific demethylated region (TSDR). TSDR is differentially methylated and fully de-methylated in tTregs ($CD4^+$ $CD25^+$) [49–52]. The TSDR demethylation process is closely linked to the high and stable expression of Foxp3, which is why it is commonly used to identify Tregs. A quantitative methylation-specific droplet digital PCR (ddMSP) assay was established for assessment of TSDR Foxp3 methylation status in *ex vivo* expanded nTregs as a marker for nTreg stability [30]. The presence of IL-2 is vital for both the homeostatic maintenance and thymic development of Tregs [53]. IL-2 is produced by $CD4^+$ T-cells, not the regulatory T-cells [35,54]. Binding of IL-2 to surface ligand IL-2Ra or CD25, phosphorylates signal transducer and activator of transcription 5 (STAT5). STAT5 binds to both the promoter and intronic elements of the *Foxp3* gene, and activates the transcription of tFoxp3 [35]. The absence of either IL-2 or IL-2 receptor results in the enlargement of peripheral lymphoid organs, impaired activation-induced cell death, and the occurrence of autoimmune disorders. These effects are linked to a decrease in the generation of Tregs [55]. Tregs can lose their Foxp3 expression and adopt the role of autoimmune effector cells in certain situations [56,57]. *Foxp3* gene expression handles the maturation functions and phenotype of Tregs. When TGF-β is secreted, retinoic acid-related orphan receptor gamma (RORγT) is expressed stimulating the differentiation of Tregs and Th17 T-cells. Foxp3 has the ability to suppress the function of RORγT and promote the differentiation of Tregs. However, if inflammatory cytokine IL-6 inhibits the function of Foxp3 and activates the Th17 differentiation pathway. Consequently, achieving the right equilibrium between Foxp3 and RORγT expression is of utmost importance in determining the destiny of CD4 T-cells and the subsequent immune response [57].

The signaling pathway mediated by TGF-β is common to both Th17 and Tregs. In the presence of pro-inflammatory cytokines released in infection, naïve $CD4^+$ T-cells differentiate into Th17 cells which promote inflammation, produce IL-17, IL-22, and IL-23, and recruit neutrophils. In contrast, due to the absence of inflammation, TGF-β drives differentiation into Tregs, which produce anti-inflammatory cytokines IL-10 and TGF-β [58].

Metabolic pathways can determine the Th17/Treg balance. Naïve T-cells rely on oxidative phosphorylation and fatty acid oxidation or become anabolic to match cell proliferation and growth. Activated naïve T-cells rely on mammalian target of rapamycin (mTOR) as a critical regulator of differentiation and function. The proper complex function of the two mTOR complexes is necessary for glycolysis upregulation and specific effector subset differentiation. Without this, CD4 T-cells cannot activate glycolytic machinery for effector function, leading to a regulatory phenotype [59]. On the other hand, Tregs metabolize fatty acids, amino acids, and glucose, besides carrying out oxidative phosphorylation. Rapamycin enhances Foxp3 expression and expands tTregs. Therefore, faulty mTOR activity af-

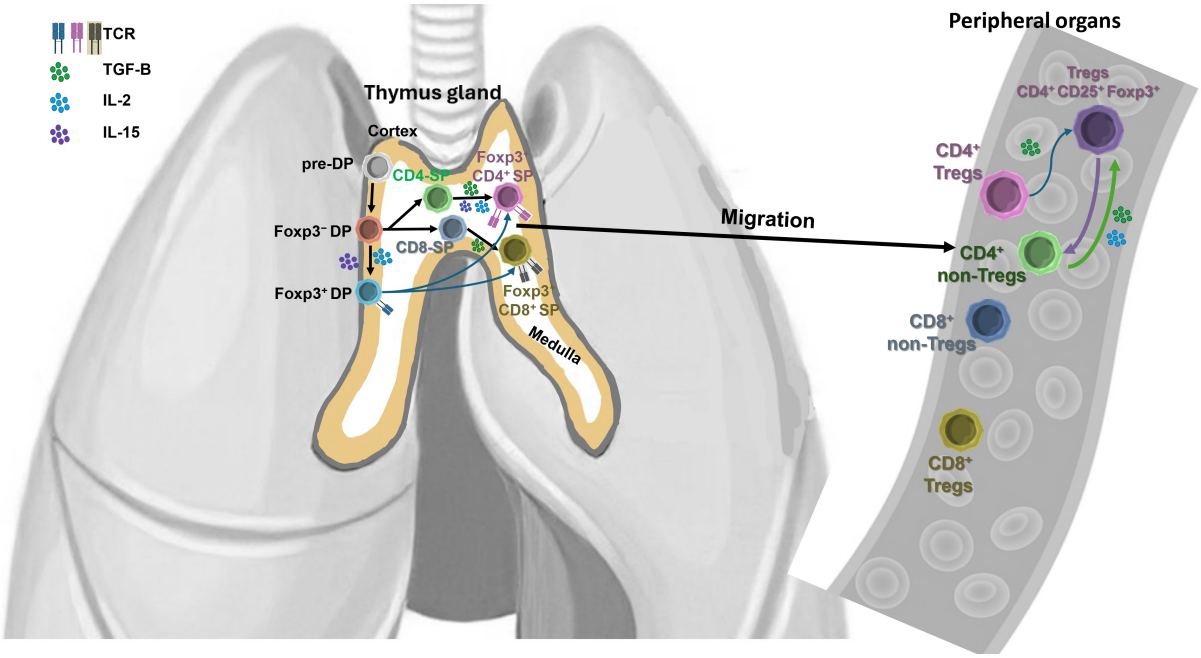

Fig. 1. Tregs thymic development, excursion to periphery, cytokine profiling, and Foxp3. In the cortex of thymus, pre-double positive T (pre-DP)-cells are matured and Foxp3 is not yet expressed. Foxp3$^-$ DP cells are committed to be either traditional T-cells or tTregs, depending on the presence of IL-2 and IL-15. Foxp3$^-$ DP or Foxp3$^+$ DP cells are ready to migrate to the medulla of the thymus for maturation. In the medulla, Foxp3$^-$ DP mature to become naïve CD4 single positive T-cells (CD4-SP) or CD8 single positive T-cells (CD8-SP), which then migrate to the peripheral organs. A subset of CD4-SP and CD8-SP cells are converted to serve as Tregs expressing Foxp3. When Foxp3$^+$ DP migrate from the cortex to medulla, they become Foxp3$^+$ CD4 T-cells or Foxp3$^+$ CD8 T-cells. TCR, T-cell receptor.

fects the Th17/Treg balance by enhancing T-cell sensitivity to TGF-β, resulting in insensitivity to proinflammatory cytokines and STAT3 signaling [60].

2.1.3 Molecular Signature of Tregs

Tregs modify their phenotypes without compromising their suppressive function. They express transcription factors and chemokine receptors associated with various T-cell types, but they do not produce inflammatory cytokines due to Foxp3-dependent repression [61]. These events enable Tregs to express a Th-determining transcription factor and migrate to the site of inflammation. Tregs in healthy tissues are involved in immune suppression, tissue repair, and other non-immune activities [62]. Skeletal muscle Tregs produce amphiregulin to aid muscle repair [63]. The molecular signature of Tregs differs with the pathogenic condition. The transcriptomic profile of Foxp3$^+$ Tregs from individuals with colorectal cancers compared to cells from people without cancer. Tregs showed upregulation of chemokine receptors -4, -1, -2, and -7, and cytokines IFN-γ, IL-10, IL-22, as well as chemokine ligands 1 and 10 (Fig. 1) [64]. In terms of functionality, the molecular signatures may serve as a roadmap for the successful functioning of Tregs in maintaining homeostasis and suppressing the immune system.

Foxp3 is being now studied in the development and function of Tregs [65]. Foxp3 is the primary transcription factor accounting for Treg function [15]. Mutations in the *Foxp3* gene have a detrimental effect on the function of Tregs, with a particular impact on their ability to suppress immune responses [63,65]. Foxp3$^+$ nTregs are highly stable and can effectively prevent autoimmune diseases in animal models [33,34,63]. Upon constant exposure to self-antigens and microbial antigens, their functionality becomes stable through flexibility of their highly proliferative state [66]. However, *Foxp3* gene expression alone was reported insufficient to maintain Treg function. In line with this, 70% of genes were noted to vary between conventional T-cells and Foxp3$^+$ naïve Tregs. *Ikzf2* (Helios), *Ikzf4* (Eos), *CTLA-4*, *NFAT*, *Nr4a2*, and *AP-1* and TNF-receptor superfamily are examples of gene differences that are correlated with and/or were able to enhance Foxp3 transcription in Tregs [67]. At a Treg precursor stage, histone modifications occur before Foxp3 expression, and the genes reach their peaks of expression prior to Foxp3 induction [63].

The signature of specific genes affects the behavior and pathological role of Tregs. For example, in mice with psoriasis, mutations in inhibitor of Kappa B Kinase beta (IKBKB) disrupted the balance between pro-inflammatory (TNF and IFNγ) and anti-inflammatory cytokines (IFNα, IFNβ, IL-12p70, IL-1b, and IL-4), resulting in systemic inflammation and psoriatic arthritis. The activity of IKBKB

not only control the number of Tregs but also determines the growth of a specific group of Tregs found in tissues [68].

2.2 Peripheral Tregs Source and Induction

The development of Tregs, both in the thymus (tTregs) and in the periphery (pTregs), is triggered by the induction of Foxp3 in response to antigen exposure. TGF-β induces Foxp3 expression and suppressive activity in conventional T-cells *in vitro*. This suggested the possibility of extrathymic generation of Tregs from naïve T-cells (Fig. 1) [69]. However, the mechanisms by which signals induce the development of pTregs *in vivo* have yet to be precisely elucidated. The induction of pTregs is associated with subimmunogenic antigen exposure and foreign antigen presence in a lymphogenic environment. pTregs can be a significant part of Tregs, particularly in tissues like the lamina propria, and are found in various conditions. pTregs have a vital role in regulating autoimmune responses. Nrp-1^{low} Foxp3^{+} cells undergo upregulation of neuropilin receptor (Nrp-1) while developing in the thymus, which helps distinguish pTregs from tTregs in circulating cells in non-inflamed tissues. Notably, pTregs display the ability to upregulate Nrp-1 specifically during inflammation [70].

pTregs are identified as true Tregs with the expression of canonical Treg markers like CTLA-4, GITR, and CD103 and their correlation with IL-2 [15]. Non-immunogenic antigen delivery methods are the most effective triggers for the induction of pTregs [71]. When tTregs are employed for *in vitro* assays, the pTregs are obtained and accompanied with highly effective suppressive function. Although TGF-β induced iTregs show some suppressive activity, they do not completely adopt the transcriptional signature that is typical of Tregs, highlighting the differences between iTregs and pTregs [72].

By examining different cell populations, a fascinating variation was found that sheds light on the inherent adaptability of pTregs. Helios, which is an ikaros family transcription factor, is a specific marker for tTregs. Helios is highly expressed on Foxp3^{+} Tregs in the thymus [73]. Approximately 70% of pTregs express Helios, which can be used to distinguish genotypically between tTregs and pTregs [74].

2.3 Functional Hallmarks of Treg Subsets

2.3.1 The Role of Tregs in Immune Tolerance

When stimulated with CD28, TCR signaling, or IL-2, Tregs undergo differentiation into mature or eTregs, which exhibit strong suppressive activity. These eTregs express more immunosuppressive molecules like Human Leukocyte Antigen – DR isotype (HLA-DR), CTLA-4, Helios, and T-cell immunoreceptor with Ig and ITIM domains (TIGIT), chemokine receptor 4 (CCR4), C–X–C chemokine receptor Type-4 (CXCR4), and CXCR5, to enhance infiltration of Tregs into tumor microenvironment (TME) and maturation of nTregs to become eTregs (Fig. 2) [75]. This type of infiltration of eTregs promotes immune tolerance.

Tregs exhibit diverse inhibitory activities for efficient immune cell control [76] including expression of immune suppressive cytokines, such as IL-10 and TGF-β, and depletion of effector T-cell essential cytokine, IL-2 [77,78]. Tregs suppressive effects can also be achieved by degrading pro-inflammatory mediators and inactivating APCs by eliminating CD80 and CD86. Tregs have multiple roles in immune suppression, including increasing negative signals like indoleamine 2, 3-dioxygenase [79], and conveying inhibitory signals through the process of trogocytosis, which includes the transmission of MHC class II [80]. By performing these various functions, the inflammatory microenvironment can be altered, resulting in the recruitment of other immunosuppressive cell types to amplify and extend the tolerogenic effects [77].

Long-term tolerance can be achieved through adoptive transfer of Tregs and depletion of Tregs did not affect tolerance in this situation. T-cells from immuno-tolerant mice can establish long-term tolerance in recipient animals, demonstrating the long-term tolerogenic qualities of Tregs to surrounding immune cells both directly and indirectly, and ensuring the durability of their effect [33,34].

2.3.2 The Role of Tregs in Modulating the Innate Immune Response

The innate immune response is finely tuned by regulatory Tregs, which work to maintain immune homeostasis and modulate the activities of other immune cells, through suppressing effector cell functions, controlling antigen presentation, controlling cytokine production, maintaining tissue integrity, and via their interaction with innate-like T-cells [80,81].

Tregs help prevent excessive inflammation and tissue damage that can occur during immune responses by dampening the activity of the macrophages, dendritic cells (DCs), and natural killer (NK) cells [81]. Tregs inhibit the maturation and antigen-presenting function of DCs, which modulate the activation of T-cells and other effector cells of the innate immune system. Tregs controls other immune subsets by the production of anti-inflammatory cytokines, such as IL-10 and TGF-β [82]. Tregs prevent immune-mediated damage to tissue integrity, which is especially important in tissues damage infections or autoimmune reactions. The interaction between Tregs and innate-like T-cells, such as $\gamma\delta$ T-cells and NK T-cells, plays a role in fine-tuning the innate immune responses within diverse tissues and under varying physiological circumstances [80].

Treg can exert their anti-inflammatory and protolerogenic effects by modulating the behavior of neutrophils. During immune/inflammatory responses, neutrophils and different Treg subtypes establish a complex crosstalk. Lipopolysaccharide (LPS) or CD3/CD28 lig-

ation triggers the activation of Tregs, resulting in the expression of a range of immune suppressive pathways in neutrophils through various mediators, alongside the promotion of their apoptosis [83]. Culturing human neutrophils with activated Treg led to elevated levels of anti-inflammatory molecules such as IL-10, TGF-β1, indoleamine-2,3-dioxygenase (IDO), and haemoxygenase 1. The secretion of CXCL8 by Tregs has helps recruit neutrophils. The close spatial distribution of neutrophiles and Foxp3$^+$ Tregs hint that both cells modulate CD4$^+$ T-cell differentiation via programmed death-ligand 1 (PD-L1)/PD-1 interactions [84]. In mice with autoimmune hepatitis, autoantigen-specific Tr1 cells and B regulatory cells (Breg) worked together to attract neutrophils to the liver and transform them into myeloid-derived suppressor cell (MDSC) subtypes using granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, and TGF-β [85].

2.3.3 The Role of Tregs in Tissue Repair and Homeostasis

Tregs possess the capability to restore injured tissues through the production of healing molecules like amphiregulin, and tissue-regulatory protein peroxisome proliferator–activated receptor γ (PPARγ) [86]. Tissue-resident Tregs can effectively regulate immune responses involving different innate and adaptive immune subsets by adjusting local conditions [87]. Thus, the current challenge to developing Treg-based therapeutics is how to harness the properties of these cells to durably re-establish immune tolerance in acute and chronic inflammatory diseases [11]. Tregs polypharmaceutical attributes are hard to imitate with drugs. For instance, medications that target inflammatory signaling pathways are not as safe as nTregs in preventing infections and cancers, and they only address a portion of the effects induced by Tregs [11,27].

Tregs play a crucial role in tissue repair and regeneration by regulating inflammation and orchestrating the activity of both innate and adaptive immune systems [83]. Following tissue injury, a symphony of immune responses is set in motion until a new tissue is regenerated. Tregs play a role in each of the various stages. Tregs can counteract the onset of inflammation by suppressing the secretion of inflammatory cytokines such as IL-6, IFN-γ, TNF-α, and IL-1β, and by preventing neutrophil extravasation through the release of IL-10 [88]. Furthermore, Tregs have the capacity to trigger neutrophil apoptosis and promote the uptake of dead neutrophils by macrophages. At the same time, Tregs inhibit monocyte function, enhance their longevity, and secretion of anti-inflammatory cytokines like IL-4, IL-10, and IL-13 [89]. Tregs have the natural ability to quell inflammation mediated by CD4 and CD8 T-cells, employing cytokines such as IL-10, TGF-β, and IL-35. The overall effect of these Treg-mediated mechanisms is the inhibition of neutrophil, inflammatory macrophages, CD4, and CD8 T-cell activity, which supports the process of tissue repair and regeneration [83].

In skeletal muscle, Tregs are linked to mesenchymal stromal cells, nerves, and IL-33 secretion. All are connected when, through calcitonin-gene-related peptide, Tregs accumulated [90]. IL-33 acts on Tregs containing the ST2 receptor encoded by the *IL1rL1* gene compared to that of Tregs in lymphoid tissue. IL1rL1 is upregulated in Tregs isolated from damaged muscle [91]. Tregs in muscles have been found to express significant amounts of amphiregulin [92]. By directly interacting with satellite cells, these specialized Tregs have an impact on supporting muscle regeneration. The administration of amphiregulin normalized the muscle transcriptome during muscle repair. Amphiregulin also enhances myogenic differentiation [92]. Treg depletion in injured muscle was associated with less tissue regeneration, prolonged inflammation, and impaired production of myogenic transcription factors, macrophages polarization from M1 to M2 phenotype [93]. Tregs protect against tissue deterioration in neurodegenerative [89], cardiac [94], lung [95], autoimmune [27], atherosclerotic [86], and skin diseases [96].

2.3.4 The Role of Tregs in Immune Regulation During Infections

Tregs have a crucial role in preventing immunopathogenic reactions to various viral, bacterial, fungal, protozoal, and helminth infections [97]. During acute infection, Tregs inhibit the accumulation of cytotoxic CD8$^+$ T-cells. Second, Tregs produce IL-10 that promotes the maturation of memory T-cells [15]. Different Treg populations that emerge during acute infection with *Listeria monocytogenes* [98]. These populations have distinct effects on regulating CD8$^+$ T-cell responses at various stages, including the priming and contraction phases [99]. Different Treg subpopulations separated from several time points of the same animal model were clonally unique, suggesting that they most likely came from different cell progenitors [100].

During chronic infection, limiting Treg number boosts immune responses mediated by cytotoxic CD8$^+$ T-cells, leading to improved control of the infection [101]. The generation of clones of Tr1 cells that produce IL-10 was only observed in situations involving persistent infection [102,103]. Meta-analyses compared the role of Tregs in acute versus chronic infections. Increased CD4$^+$ Treg frequencies were noted in chronic hepatitis B virus (HBV) infection, pointing to the role of Tregs in disease progression, viral load, absence of therapy, and risk of hepatocellular carcinoma [104]. Tregs attracted CD4$^+$ and CD8$^+$ T-cells to the liver through chemokines CCL17 and CCL22 and reduced their inflammatory response in cases of chronic hepatitis C virus (HCV) infection, leading to the prolonged existence of pathogens [15]. On the other hand, Tregs may be functional in reducing the amount of liver damage caused by HCV [105–107].

$CD4^+$ Tregs mediated inconclusive anti-HIV immune responses and were comparatively more abundant in the mucosa and bloodstream [108]. $CD4^+$ Tregs decreased HIV replication in T-cells *in vitro* by altering ectonucleotide levels through CD39 and by transferring cAMP through gap junctions formed with conventional T-cells [109]. Blocking CD39 restored the ability of HIV-gag-stimulated $CD8^+$ T-cells to produce cytokines [110]. By interfering with the immunological synapse, they inhibited the spreading of virus from DCs to T-cells [109]. There is a positive correlation between the frequency of $CD4^+$ $CD39^+$ Tregs and HIV viral load and disease progression [108]. These somewhat contradictory data may be resolved by distinguishing between acute and chronic infection [108].

2.3.5 The Role of Tregs in Transplantation

An understanding of Tregs stimulated efforts to treat autoimmune disorders, organ transplant rejection, and inflammation-related neurodegenerative diseases. This is a result of extensive comprehension of the molecular and mechanistic aspects of Treg biology (Fig. 2) [27]. High expression of IL-2Rα (CD25) is a characteristic of Tregs which is involved in immune regulation. Low-dose IL-2 provides selective advantages to Tregs in proliferation and survival. IL-2 and IL-2 muteins both raise the proportion of Tregs in individuals with autoimmune diseases and graft-versus-host disease (GvHD). These therapies have shown clinical efficacy [111].

The advantage of Treg therapies lies in their capacity to educate and propagate endogenous cells to exhibit suppressive activities, thereby facilitating long-term tissue protection even in the absence of survival of the infused Tregs [112]. Preclinical research showed that Tregs have the ability to prevent and reverse disease. Adoptive Treg therapy prevented GvHD in individuals after allogeneic hematopoietic stem cell transplant. Tregs were applied to r transplant- and autoimmune-related diseases [113]. Tregs were infused efficacious without adverse side effects such as systemic immunosuppression [114]. As well, Tregs were used in the treatment of solid organ transplantation [113,115–119], spontaneous abortion [114], and autoimmune disease [14,120–123].

2.3.6 Tregs and Metabolic Crosstalk

Tregs can modify their metabolic functions, including glycolysis, oxidative phosphorylation (OXPHOS), fatty acid oxidation (FAO), and amino acid metabolism, in order to meet their energy needs. Nevertheless, there is disagreement and a lack of clarity about the connections between these processes and the underlying mechanisms [124]. The metabolic program of the cell is influenced by its activity state, and varies between naïve, activated, and memory cells. To illustrate, when cells are in a resting state which needs energy to maintain survival and circulation, they rely on energy sources from OXPHOS such as ATP [125,126]. Through the TCR and co-stimulatory CD28, effector T-cells switch from OXPHOS to glycolysis [125,127]. Upon their activation, cells use glutaminolysis in addition to glycolysis to generate energy [125,126]. Tregs exhibit a distinct metabolic profile compared to other T-cell subsets. Initially, they employ glycolytic metabolism for activation, migration, and proliferation. However, they subsequently undergo a metabolic shift, becoming independent of glucose and relying on the oxidation of lipids and pyruvate [128,129].

Tregs that proliferate exhibit heightened glucose transporter 1 (GLUT1) expression and mammalian target of rapamycin (mTOR) activity, resulting in reduced suppressive capacity and simultaneous downregulation of Foxp3 expression [114,116,127–129]. Tregs are regulated glycolytically by different mechanisms, such as the phosphoinositide 3-kinase (PI3K)-Akt-mTOR signaling network. This pathway enhances the glycolytic rate of Tregs and significantly influences their differentiation and functional stability [130]. The PI3K-Akt-mTOR pathway is regulated by many factors including AMP-activated protein kinase (AMPK), phosphatase and tensin homolog (PTEN), and hypoxia-inducible factor 1 α (HIF-1α) [131]. AMPK is a metabolic energy regulator of both glycolysis and FAO in Tregs. When it is stimulated to increase the ratio of AMP/ATP, catabolism is activated [132,133].

Tregs were generated from human $CD4^+$ cells by inhibition of fatty acids binding proteins. This dysregulated mitochondria, decreased OXPHOS, and increased glycolytic pathways [134]. The persistence of eTregs is linked to mitochondria, as they acquire energy through FAO. The transfer of mesenchymal stem cell mitochondria to $CD4^+$ T-cells aids in the differentiation of Tregs, providing relief from GvHD [135,136]. Mitochondrial complex III and mitochondrial transcription factor A prevented DNA hypermethylation to suppress Foxp3 expression [137].

The pro-migratory molecule lymphocyte function-associated antigen 1 when stimulated by its ligand, increases iglucose uptake [138]. Multiple metabolic processes rely on the participation of amino acids. Immune homeostasis and responses are regulated by the availability and metabolism of amino acids. Treg generation and function is linked to amino acid transporters, such as those responsible for branched-chain amino acids (glutamate, glutamine, and glutathione). Furthermore, the catabolism of tryptophan and arginine was noted [127]. Maintaining cholesterol balance is essential for Tregs as it impacts their lipid metabolism, biofilm and lipoprotein composition, mTOR-class 1 activation, and immune synapse formation [139]. The rise in cholesterol levels in cells interferes with mTOR signaling, leading to the promotion of Tregs. Insufficient lipids disrupt the mevalonate pathway, resulting in protein modification [140] and increases PD-1 and eTreg numbers [141].

2.4 Face-off Roles of Tregs in Immune Balance

2.4.1 Tregs in Cancer versus Autoimmune Diseases and Graft Rejection

Tregs are essential for the development of immunotherapies against cancer and autoimmune diseases. In cancer models, Treg depletion induced an anti-tumor immune response [142,143]. Tregs are important players that can either contribute or protect against diseases. This raises the question of the dual role of these cells. Within the hepatocellular tumor microenvironment, the frequency of $\gamma\delta$ T-cells decreased and was inversely correlated with the number Tregs. This phenomenon may be attributed to the suppressive action of Tregs, mediated by TGF-β and IL-10, on the cytotoxic anticancer $\gamma\delta$ T-cells [144]. Alternatively, $\gamma\delta$ T-cells may become tumor-derived $\gamma\delta$ Tregs and promote the tolerance of DCs and T-cells [145]. Tregs abundance in the tumor microenvironment is correlated with poor prognosis and is found to suppress $CD8^+$ T-cells numbers [142]. Depletion of Tregs by anti-chemokine (CCR4) antibody results in a favorable immune response [146]. On the other hand, in the tumor microenvironment, metabolites like IDO and adenosine stabilized Treg function. Due to accelerated cancer cell metabolism, glycolysis is decreased and replaced by increased fatty acid metabolism. Within this microenvironment, Tregs actively absorb the lactic acid that is generated, eliminating cMyc-mediated expression by Foxp3. This, in turn, leads to a rise in oxidative phosphorylation and the oxidation of nicotinamide adenine dinucleotide [115,118]. The rise in central memory Tregs following T-cell engagers immunotherapy, points to a more balanced bone marrow in individuals with acute leukemia. Thus, Treg expression can have positive therapeutic effects [147]. Currently, anti-Treg-CCR4 mAb (mogamulizumab) for advanced or recurrent solid tumor substantially decrease eTregs in periphery [148]. The $CCR8^+$ receptor was increased in Tregs. This encouraged, binding to chemokine CCL1 (secreted by $CD11b^+$ $CD14^+$ myeloid cells) to increase Tregs infiltration in breast cancer. The communication between CCL1 and CCR8 boosts the levels of Foxp3 through the STAT3. Activated $CCR8^+$ Tregs effectively suppress the immune response against tumors by stimulating ATP-adenosine metabolism through CD39, as well as by secreting IL-10 and granzyme B [149]. Tregs inhibit excessive activation of effector T-cells through the suppression of TCR and CD28 signals and inducing dysfunctional exhaustion to T-cells [150]. Through the expression of PD-1 on Tregs, immune suppression in the tumor microenvironment can overcome therapeutic interventions (Fig. 2) [151]. Tregs in mesenteric lymph nodes and colon cancer expressed IL-17 receptor-A (IL-17RA) and ablating IL-17RA increased IL-17 cells and exacerbated tumor development. When IL-17RA is lost in tumor Tregs, it reduced RNA splicing downregulation of several RNA binding proteins to deregulate immune actions in colorectal cancer [152].

Tregs are one of the main gatekeepers of the immune system and serve as a protector in preventing and treating autoimmune diseases. In glomerulonephritis, Tregs provided protection against renal tissue injury that is linked with pathogen driving Th1 and Th17 effector cell activation [153,154]. The incidence of most autoimmune diseases is somehow correlated with dysfunction of suppressor immune cells, mainly Tregs [155]. The mutation of the autoimmune regulator gene in Tregs leads to loss of normal immune tolerance and increased the incidence of autoimmune polyendocrine syndrome type 1 (APS-1) [155,156]. Furthermore, a close link between Tregs malfunction and the type 1 diabetes was noted. Specifically, adoptive transfer of genetically engineered Tregs in non-obese diabetic mice limited disease [157]. Inflammatory and autoimmune diseases are now treated by adoptively transferred and genetically altered Tregs (Fig. 2) [158].

Germane to this, most autoimmune diseases and transplantation rejection emerges from abnormal immune tolerance as well as deficiency or malfunctions of normal existing Tregs in tissue and periphery [159–161]. Tregs induce immune suppression to other immune subsets by crosstalk though cytokines, chemokines, and cell-to-cell contacts, such as Tregs crosstalk towards T-cells [162], myeloid cells [163], B-cells [164], NK cells [165], and $\gamma\delta$ T-cells [166]. In autoimmune diseases, Tregs are lower frequencies with higher inflammatory pathways, for example: autoreactive T-cells [167], uncontrolled myeloid cells [168–170], uncontrollable B-cells [171], pro-inflammatory NK cells [172], self-reactive DCs [173] and $\gamma\delta$ T-cells [174] (Fig. 2).

2.4.2 Tregs in the Newborn, Youth, and Elderly

Ageing enhances Treg senescence and limits proliferation [175]. Tregs migrated less and did not regenerate muscle in aged animals [176]. In order individuals, Tregs had less ability heal lung damage caused by influenza [177]. Differentiation of Tregs diminished with age, which is significant when comparing the differentiation of naïve T-cells from aged mice to those of young animals [178]. Similarly, a reduction in the *de novo* induction of antigen specific Tregs in the aged mice was less compared to young animals [179].

Retinaldehyde dehydrogenase 2 (RALDH2) was decreased in DCs from mesenteric lymph nodes (MLN) from older mice [15]. Additionally, $CD11b^-$ $CD103^+$ PD-$L1^{high}$ DCs, characterized by elevated RALDH2, were fewer [179] in conjunction with TGF-β, RALDH2-mediated retinoic acid production allows MLN DCs to promote Treg development [180]. Despite a decrease in the generation of tTregs and pTregs, elevated numbers of Tregs in the spleen and lymph nodes of aged mice were noted [181].

The accumulation of Tregs appears to be age dependent, with middle-aged mice exhibiting Treg levels that are in between those of young and old mice [182]. For obvious reasons, Tregs are assessed in human blood sam-

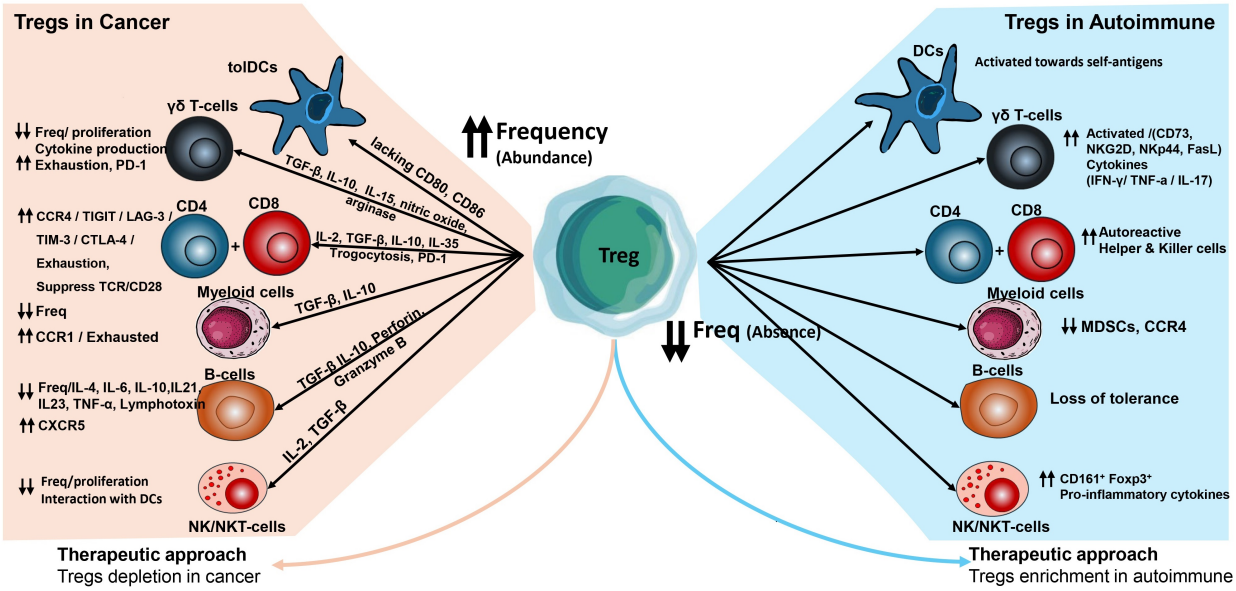

Fig. 2. Role of Tregs in protection from autoimmune diseases and cancer development. In cancers, Tregs induce immune suppression of other immune subsets through cytokines, chemokines, and cell-to-cell contacts, tolDCs, Tolerogenic dendritic cells; CCR, chemokine receptor; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CXCR1, C–X–C chemokine receptor 1; MDSCs, myeloid-derived suppressor cells; NK/NKT-cells, natural killer; PD-1, programmed cell death protein 1; TNF-α, tumor necrosis factor-α.

ples. More aTregs (Foxp3^{high} CD45RA^{-}) and less rTregs (Foxp3^{low} CD45RA^{+}) were noted in blood from older individuals [181,183]. Related to this, Tregs were more numerous in the skin of older subjects and could account, in part, for fewer in the circulation [184].

3. Future Research Directions in Current Treg-Targeting Therapies

Technology advancements and a better understanding of Treg biology will likely drive the development of Treg-targeting therapeutics in several fascinating ways.

A Precision Medicine: Treg-targeting therapies may be more effective and less likely to cause side effects if they are customized to each individual based on their unique immune profile and genetic background. This is becoming more possible with advances in proteomics and genomic technology [185].

B Combination Therapies: Combining Treg-targeting strategies with other immunotherapies to increase therapeutic efficacy, such as checkpoint inhibitors or chimeric antigen receptor (CAR)-T cell therapy [186]. The goal is to produce synergistic effects that enhance anti-tumor or autoimmune responses. Combination checkpoint inhibitor therapy slowed tumor growth by blocking several pathways, such as PD-1 (Nivolumab), LAG-3 (Relatlimab), and CTLA-4 (Ipilimumab) [187]. The FDA approved six CAR-T therapies for cancer including Kymriah (tisagenlecleucel), Tecartus (brexucabtagene autoleucel), Yescarta (axicabtagene ciloleucel), Abecma (idecabtagene vicleucel), Breyanzi (lisocabtagene maraleucel), and Carvykti (ciltacabtagene autoleucel) [186].

C Nanotechnology: Using nanoparticles (NPs) and nanocarriers to precisely deliver drugs or therapeutic agents to Tregs to improve efficacy and lower systemic toxicity [188–190]. NPs can administer monoclonal antibodies (anti-PD1). Other NP formulations deliver small interfering RNAs to disrupt immunological checkpoints [191,192]. Using NPs, antigen, such as CAR-encoding DNA *in vivo* and CAR-encoding mRNA, can be delivered to T-cells [193]. NPs that release TGF-β and IL-2 can increase the number of Tregs *in vivo*, reducing lupus symptoms [194]. Poly(lactic-co-glycolic) acid (PLGA) NPs have been used to administer immunomodulators and prevent allograft rejection [195].

D Gene Editing Technologies: Tregs can be precisely modified using tools like CRISPR/Cas9. This could involve engineering Tregs to either enhance their suppressive functions for autoimmune diseases or reduce their inhibitory effects for cancer treatment. CRISPR/Cas9 can edit both primary T-cells and engineered T-cells, including CAR-T and TCR-T, *in vivo* and *in vitro* to regulate T-cell differentiation and activation [196]. Tregs can more effectively detect islet-associated antigens and improve the immune-suppressive environment using CRISPR-Cas9 to replace endogenous TCR with islet-specific TCR and stable Foxp3 expression [197,198]. CRISPR/Cas9-edited dual-targeted (CD19/CD22) CAR-T, was safe and

efficient in individuals with B-cell acute lymphoblastic leukemia (B-ALL) [196].

E Novel Drug Development: Novel pharmaceuticals or biological treatments that precisely modulate Treg survival or function are being developed. These may provide more efficient and selective modulation of Tregs.

F Selective Targeting: Developing therapies that target pathogenic Tregs precisely while protecting Tregs that maintain normal immune tolerance. This may lessen adverse effects and improve safety profiles [11].

4. Conclusions

Integrating regulatory T-cells into medicine requires careful consideration and is not straightforward. Clinical correlative studies should be considered when examining the delicate immunological balance of Tregs in their macro- and microenvironments. Varying roles of regulatory T-cells are found in many situations and diseases, in aging, between sexes, and potentially underestimated factors. Tregs crosstalk to other immune cells through complicated network mechanisms. This is necessary for a balanced immune reaction. Sometimes Tregs have a beneficial role and sometimes a harmful role. In autoimmune diseases, Tregs are not of adequate number or function mainly secondary to hyperactive immune cells recognizing self-antigens. Consequently, proinflammatory cytokines and chemokines are secreted to augment the immune reaction. Enrichment of the affected organ with autologous Tregs might restore the immune balance. In cancers, Tregs are abundant and under the control of cancer cells to maintain a balanced less severe tumor immune response. In this case, depletion of Tregs from the immune compartments of the tumor may increase cancer killing.

Author Contributions

MS, AME, and MIH, Conceptualization, writing, and editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

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Conflict of Interest

The authors declare no conflict of interest.

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