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Review

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Review

Regulatory T-cell dysfunction and its implication for cell therapy

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Summary

Regulatory T cells (Tregs) are a subtype of CD4+ T cells that can mediate immune tolerance by a multitude of immunomodulatory mechanisms. Treg-based adoptive immunotherapy is currently being tested in multiple phases I and II clinical trials in transplantation and autoimmune diseases. We have learned from the work done on conventional T cells that distinct mechanistic states can define their dysfunctions, such as exhaustion, senescence, and anergy. All three can negatively impact the therapeutic effectiveness of T-cell-based therapies. However, whether Tregs are susceptible to such dysfunctional states is not well studied, and results are sometimes found to be controversial. In addition, Treg instability and loss of FOXP3 expression is another Treg-specific dysfunction that can decreasein their suppressive potential. A better understanding of Treg biology and pathological states will be needed to compare and interpret the results of the different clinical and preclinical trials. We will review herein Tregs' mechanisms of action, describe different T-cell dysfunction subtypes and how and if they apply to Tregs (exhaustion, senescence, anergy, and instability), and finally how this knowledge should be taken into consideration when designing and interpreting Treg adoptive immunotherapy trials.

Keywords: regulatory T cells (Tregs), exhaustion, senescence, anergy, cell therapy

Abbreviations: 4-1BB: tumor necrosis factor ligand superfamily member 9; $A_{2A}R$: adenosine 2a receptor; APC: antigen-presenting cell; CAR: chimeric antigen receptor; CD: cluster of differentiation; CKD: chronic kidney disease; CNS: non-coding sequence elements; CTLA4: cytotoxic T-lymphocyte-associated protein 4; DC: dendritic cell; DCAF1: DDB1 and CUL4 associated factor 1; DNMT: DNA methyltransferase; EOMES: eomesodermin gene; FOXP3: forkhead box p3; GARP: glycoprotein A repetitions predominant; GSH: glutathione; HDAC: histone deacetylase; IDO: indoleamine 2,3-dioxygenase; IL-R: interleukin receptor; IPEX: immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome; iTregs: induced Tregs; LAG-3: lymphocyte-activation gene 3; LAP: latency associated peptide; LFA-1: leukocyte function-associated antigen-1 factor; LKB1: liver kinase B1; MHC: major histocompatibility complex; NFAT: nuclear factor of activated T cells; NK: natural killer; nTregs: natural Tregs; $p16^{Ink4a}$: cyclin-dependent kinase inhibitor 2A; $p19^{Arf}$: p19 alternative reading frame protein; $p21^{Cip1}$: cyclin-dependent kinase inhibitor 1; PD: programmed cell death; PD-L: programmed cell death ligand; PI3K/Akt/mTOR: phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR); pTregs: peripherally derived Tregs; RA: retinoic acid; RNA-seqRNA sequencing; ROS reactive oxygen species; SA-β-gal: senescence-associated beta-galactosidase; STAT5: signal transducer and activator of transcription 5; T1D: type 1 diabetes; T-BET: T-box transcription factor TBX21; Tconv: conventional T cells; TCR: T-cell receptor; TE: transendocytosis; TET: ten-eleven translocation; TGF: transforming growth factor; Th: helper T cell; TIGIT: T-cell immunoreceptor with Ig and ITIM domains; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; TOX: thymocyte selection-associated high-mobility group box gene; Treg: regulatory T cell; TSDR: Treg-Specific demethylated region; tTregs: thymic-derived Tregs.

Introduction

Around 50 years ago, a new type of T cell was discovered and named suppressor T cells [1]. These cells were further described and labeled regulatory T lymphocytes (Tregs) in the 1990s by the work of Dr. Shimon Sakaguchi and his team [2, 3]. They can be identified by a high level of the IL-2 Rα (CD25) [2, 3], low expression of IL-7Rα (CD127) [4], and high expression of forkhead box p3 (FOXP3) transcription factor [5, 6]. *FOXP3* is located on the X-chromosome and plays a crucial role in their development and identity [7]. However, its role in mature cells was recently challenged [8]. Mutations in *FOXP3* lead to the development of the Immunodyregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, which is characterized by an abundance of autoimmune diseases such as autoimmune enteropathy, endocrinopathies, and eczematous dermatitis [9]. The equivalent mouse model of IPEX syndrome is the "scurfy" mouse, which likewise develops multi-organ lymphocytic infiltration and severe autoimmune-related dysfunctions [10].

Tregs are classified into two main Treg subtypes; thymic-derived ((tTregs), also known as natural Tregs, (nTregs)) and induced (pTregs or iTregs). iTregs are derived from naïve conventional CD4+ T cells in the periphery and, under certain circumstances and factors such as TGF-β and IL-2, start to express FOXP3 and obtain the regulatory phenotype [11]. nTregs are more stable over time, unlike -iTregs that could reverse into a pro-inflammatory phenotype in an inflammatory environment [12]. While no markers have been established

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to distinguish nTregs from iTregs, some studies suggest that Helios and Neuropilin-1 expression in nTregs indicates their thymic origin, with some controversy between mice and human results [13–16].

The promoter and three non-coding sequence elements (CNS1, CNS2, and CNS3) of the *FOXP3* gene have an interesting dynamic toward Treg differentiation and proliferation. CNS2, also known as TSDR (Treg-Specific Demethylated Region), interacts directly with FOXP3 to stabilize Tregs promotion [17]. nTregs have a demethylated state of TSDR, but iTregs lack complete demethylation of this region, which reduces their phenotypic stability. TSDR deletion does not lead to excessive autoimmunity, and thus does not seem necessary for Tregs. However, its role is linked to many other regulatory factors that are essential for Treg differentiation and function [18]. Histone modifications of CNS1 by downstream signaling of TGF-β, retinoic acids, rapamycin, and others also lead to the successful production of iTregs [17]. Treg epigenome is crucial for their identity, and therapies aiming at increasing Treg production and function through histone/protein deacetylases (HDACs) inhibition, which alters FOXP3 post-translational modifications, are currently being tested (reviewed in [19]).

Treg adoptive immunotherapy was proposed decades ago to promote tolerance in auto-immune diseases and transplantation. In the last 20 years, it has been tested in multiple phase I/II clinical trials, assessing the feasibility and safety of this therapy in many diseases including transplantation [20], graft-versus-host disease, type I diabetes, inflammatory bowel disease, amyotrophic lateral sclerosis, autoimmune hepatitis, Alzheimer's disease, acute respiratory distress syndrome, pemphigus, and B-cell acute lymphocytic leukemia [21]. To translate to clinical use, phase III clinical trials assessing their efficacy will need to be done. The focus placed on the balance between apoptosis and proliferation [22] or metabolic regulations [23, 24] of Tregs brought us closer to understanding Treg fitness. However, most biological dysfunctions associated with chronic diseases in Tregs have yet to be clearly established. This review will thus tackle current knowledge of the different mechanisms for Treg dysfunction, their definition, and their implications for cell therapy.

Tregs mechanism of action

Tregs can promote the suppression of conventional T cells either directly, or through their interaction with other cells such as dendritic cells (Fig. 1). First, they can secrete soluble mediators such as TGF-β, IL-10, and IL-35, which serve as immunosuppressive signals for pro-inflammatory T cells (reviewed in [25]). TGF-β can also play a role in the generation of iTregs by regulating the expression of FOXP3 (11). Other mediators released by Tregs include granzymes and perforin leading to cytolysis of the targeted cell [26].

Interleukin-2 (IL-2) consumption represents one of the first described non-antigen-specific Treg's mechanism of action. IL-2 is a cytokine first discovered and characterized by Dr Robert Gallo's team in the early 1980s [27]. Tregs, as opposed to conventional T cells (Tconvs), are defined by constitutively high expression of IL-2Rα (CD25), a high-affinity IL-2 receptor. IL-2 is crucial for their survival and proliferation and induces the expression of FOXP3 [28]. Tregs, however, do not secrete IL-2 and need to scavenge it from their environment to survive. By doing so, the higher consumption of IL-2 by Tregs empties this local "reservoir" for conventional T cells [29] and starves them, leading to their death. Indeed, $CD4^+$ and $CD8^+$ Tconvs need autocrine and paracrine IL-2 for cell-fate decisions following their antigen-receptor activation [30–32]. Tregs can also deplete the microenvironment of extracellular ATP through CD39, CD73, and adenosine 2a receptor ($A_{2A}R$). Adenosine release can be regulated by soluble or membrane-bound expression of CD39 and CD73 on T cells by transforming ATP/ADP into AMP via their ectonucleotidase cascade [33, 34]. Adenosines and $A_{2A}R$ have been identified for downregulating proinflammatory responses in Tconv [35]. Constitutive high expression of $A_{2A}R$ in Tregs, along with its expression of CD39/CD73, effectively reduces the use of ATP by Tconv, limits their activation, and induces iTregs differentiation [36]. Another mediator-related mechanism of Tconv suppression by Tregs belongs to the consumption/reduced availability of cysteine either by directly oxidizing it into sulfate or inhibiting glutathione (GSH) synthesis in DCs via CTLA-4-CD80/86 interaction [37]. Consumption of cysteine is needed for the one-carbon metabolic network (1CMet), essential for effector T cells' redox balance, DNA methylation, and other synthetic processes [38].

As mentioned above, Tregs also have an impact on DCs, leading to the inhibition of their maturation and function. This, in turn, leads to lower conventional T-cell activation and proliferation. Expression of CD80/86 co-receptors by DCs is important for Tconv antigen-receptor signaling activation. Tregs, however, express high levels of CTLA-4 that bind to CD80/86 on DCs, competing with CD28 (activation co-receptor) expressed by Tconvs [39]. Tregs can also remove CD80/86 costimulatory molecules at the surface of DCs via trogocytosis, where part of DCs' surface membranes are taken by Tregs, reducing the available receptors for Tconv activation [40]. Transendocytosis (TE) was also previously reported as a CTLA-4-related mechanism to reduce CD80/86 availability. Unlike trogocytosis, TE leads to the capture of CTLA-4's ligand and its destruction via endocytosis and lysis [41]. These mechanisms of Tconv suppression have been associated with many autoimmune diseases such as T1D [42].

Other costimulatory molecules expressed by Tregs such as LAG-3, TIGIT and PD-1 can interact with antigen presenting cells (APC)s and contribute to their immunosuppressive effect [43, 44]. LAG-3 is a homolog of the CD4 receptor with a higher affinity for MHC-II [45] and can inhibit DCs' activation upon engagement with their MHC-II [46]. Upregulated in activated Tregs, LAG-3 does not seem critical for suppression but highly reduces Tconv activation by blocking MHC-II-TCR interaction [47]. T-cell immunoreceptor with Ig and ITIM domains (TIGIT), expressed on T and NK cells, interacts with DCs to induce a suppressive phenotype in these APCs. While not affecting their maturation state, DCs interacting with TIGIT produce higher levels of IL-10 and significantly reduce their proinflammatory cytokines production [44]. Programmed death 1 (PD-1)'s role in Tregs is more controversial. It can mediate tolerance through its interaction with PD-L1/PD-L2 on DCs by inducing immunotolerant DCs. The PD-1/PD-L1 axis is also implicated in iTreg development [48]. However, PD-1 expression in Tregs can inhibit their activation and suppressive capacity [49]. Tregs can also cause a downregulation of CD80/86 on DCs via the leukocyte function-associated antigen-1 factor (LFA-1),

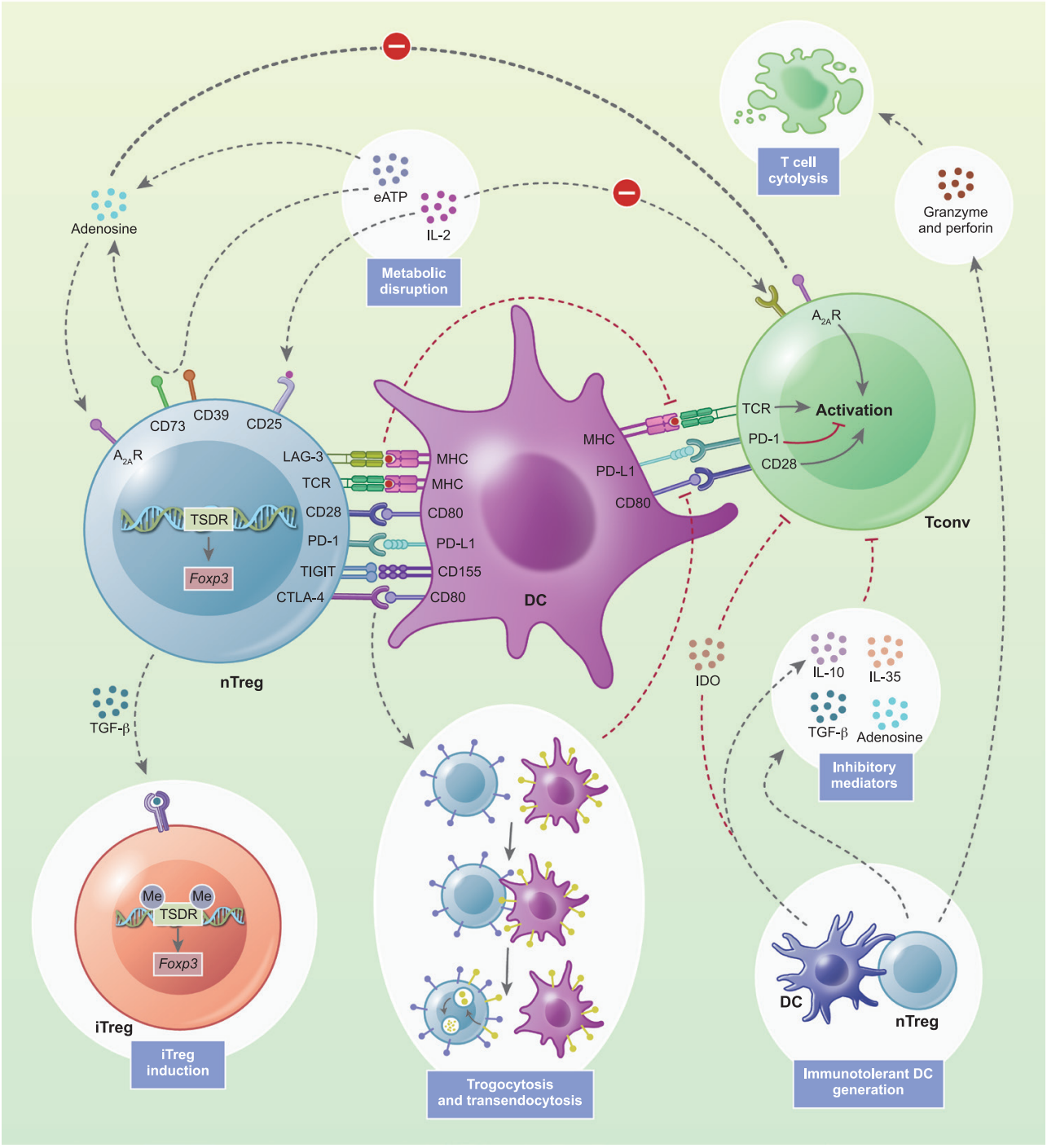

Figure 1. Regulatory T cells mechanisms of action. Regulatory T cells (Tregs) can mediate immune tolerance through different mechanisms of action. First, they can lead to the production of inhibitory mediators such as IL-10, IL-35, TGB-β, and adenosine. They can also mediate cytolysis through granzyme and perforin secretion and lead to metabolic disruption by depleting the environment in IL-2 and extracellular ATP (eATP). Then, they can lead to the generation of induced Tregs through TGF-β production. Finally, they can mediate their tolerance through their impact on other cells, such as dendritic cells (DC)s. Indeed, they generate more tolerogenic DCs through binding, trogocytosis and transendocytosis of CD80/86 with CTLA4. Other inhibitory co-receptors such as LAG-3, PD-1, and TIGIT also have an immunomodulatory impact on DCs. nTregs: natural regulatory T cells; iTregs: induced regulatory T cells; DC: dendritic cells; MHC: Major Histocompatibility Complex.

which is important for their aggregation on DCs and work synergistically with the CTLA-4 signaling pathway [50]. Furthermore, Tregs can lead to the secretion of indoleamine 2,3-dioxygenase (IDO) by DCs via CTLA-4 signaling. This enzyme converts tryptophan into kynurenine and leading to Tconv starvation and cell death [51].

All known antigen- and non-antigen-specific immunosuppressive mechanisms guide the global tolertogenic function of Tregs against allo- and autoimmunity. Highly diverse in their suppressive abilities , Tregs can also maintain homeostasis through “infectious tolerance.” This process involves mechanisms described above to transfer specialized suppressive

abilities of Tregs to other lymphoid populations either indirectly, via soluble mediators, or directly through interaction with DCs [52]. The efficient creation of a regulatory milieu is one of Tregs' strengths and is also a reason for its high potential for use in transplantation [53].

Tregs in chronic diseases

As Tregs are important to maintain homeostasis and tolerance, deficits or dysfunctions consequently lead to autoimmune diseases, or alloimmune reactions in the context of transplantation. A decrease in Treg number or imbalance between Tregs and proinflammatory T cells such as Th1 and Th17, correlates with the development and progression of rheumatoid arthritis, type 1 diabetes, chronic kidney disease (CKD), and many other autoimmune and inflammatory chronic conditions [54–57]. Chronic inflammation and changes in the microenvironment associated with those diseases represent a bidirectional relationship for their impact on Tregs' phenotype and functions [58], and the consequences on Treg biology are only scarcely studied.

Treg dysfunction

Through the evolution of adoptive cell therapies with conventional T lymphocytes, potential dysfunction-associated phenotypes were studied to optimize cell manufacturing and engineering. The degree of overlap between the different subtypes of cell dysfunction remains a matter of debate and the transferability of those concepts to Treg biology is still mostly unknown. We will summarize herein the current body of literature and identify knowledge gaps in Treg biology (Fig. 2).

Exhaustion

Persistent antigen exposure and chronic activation can lead to a stage of exhaustion. T cells in this state are described as being functionally hyporesponsive and secrete fewer cytokines (reviewed in [59]). They are characterized by the expression of inhibitory co-receptors such as CTLA-4, PD-1, LAG-3, and TIM-3 that compete with their activation signaling pathways. Specific transcription factors, namely T-BET, EOMES, TOX and others, have been described as feed-forward mechanisms of persistent T-cell exhaustion phenotype, at different stages of exhaustion (reviewed in references [60, 61]). Stress responses induced by excessive stimulation also impact mitochondrial and epigenetic cross-talks leading to higher histone acetylation, methylation, and DNA methylation amongst others (reviewed in reference [62]).

There is now a growing body of evidence that exhaustion also exists in Tregs. Although shown to proliferate better than Tconvs in homeostatic conditions [63] *in vitro* Tregs are known to become hypoproliferative upon antigenic and IL-2 stimulation [64]. Since they also mediate their immunosuppressive effects through the expression of inhibitory co-receptors such as PD-1, LAG-3, and CTLA-4, illustrating the difference between a suppressive and an exhausted Treg phenotype requires a more complex approach than with Tconv. Lowther *et al.* showed that PD-1^{hi} Tregs had a reduced suppression of CD4+ Tconv, possessed a molecular exhaustion signature, and secreted IFN-γ [65]. However, their suppressive abilities were tested without the presence of DCs, that is important for PD-1-mediated mechanisms of suppression. Still, their results align with those of Hiroyoshi Nishikawa's team who showed that mouse Tregs deficient in PD-1 signaling were more proliferative and immunosuppressive. They suggested that patients with a high number of PD-1^{+} Tregs could have a paradoxical response to PD-1 blockade manifested by a rapid cancer progression, as the therapy might not only reinvigorate Tconvs but Tregs as well [66].

Recently, we used a model known to induce exhaustion in conventional T cells, to study the existence and characteristics of Treg exhaustion, i.e., Tregs expressing a tonic signaling Chimeric Antigen Receptor (CAR). We showed that tonic-signaling-CAR Tregs acquired a phenotype similar to what is seen in exhausted Tconvs and had important changes in their transcriptome, metabolism, and epigenome [67]. Indeed, they expressed PD-1, TIM-3, and TOX, but also showed Treg-specific changes such as high expression of 4-1BB, LAP, and GARP. In addition, they remained suppressive *in vitro* but were not functional *in vivo* [67].

Senescence

In conventional T cells, senescence can be defined as irreversible, permanent cell-cycle arrest, usually in correspondence to telomere shortening. Upon activation of DNA damage response, senescent T cells can be identified through an increase in β-galactosidase activity and dysfunctional mitochondria [68]. Immune aging has a direct correlation with low-grade chronic inflammation found commonly in the elderly [69]. Exposure to stress factors in addition to repeated stimulation can also induce "premature" senescence (reviewed in reference [70]). Although Tconvs are known to stay viable and metabolically active (reviewed in reference [68]), the senescent phenotype in Tregs has not been well characterized.

Previous studies showed no significant difference in the Tregs reservoir with age (reviewed in reference [69]), but later studies in mice indicate reduced generations of these cells and of their immunosuppressive potential with time [71]. RNA-seq of less proliferative aged Tregs showed upregulation of gene signatures related to senescence such as $p16^{Ink4a}$, $p19^{Arf}$, and $p21^{Cip1}$. In fact, Tregs have been shown to possess shorter telomeres and manifest a more severe aging phenotype than Tconvs [72]. With Tregs senescing faster than their Tconvs counterparts, an imbalance between Th17/Tregs could explain the low-grade inflammation "inflammaging" often found in the elderly [73]. A few studies in mice and humans have found that DCAF1 downregulation associated with tissue aging can also be found in Tconvs as well as Tregs. Deficiency of DCAF1 has been associated with elevated reactive oxidative species (ROS) levels, increased senescence-associated-β-gal activity, and upregulation of $p16^{Ink4a}$ in T cells [72]. Pathways needed for the regulation of ROS are found to be altered in aging Tregs such as PI3K/Akt/mTOR and DNA damage/p53 response pathways. AMP-activated protein kinase (AMPK) is also downregulated in "inflammaging" associated with DNA damage. Impaired AMPK signaling pathway leads to reduced STAT5 phosphorylation and altered IL-2R function thus affecting Treg survival and immunosuppressive functions [74].

In the elderly, larger differences between nTregs and iTregs are thought to derive in part from the lack of proper demethylation of the *Foxp3* region [73]. Such epigenetic alterations associated with aging and inflammation could be caused by altered fatty acid and protein metabolisms [73, 75, 76].

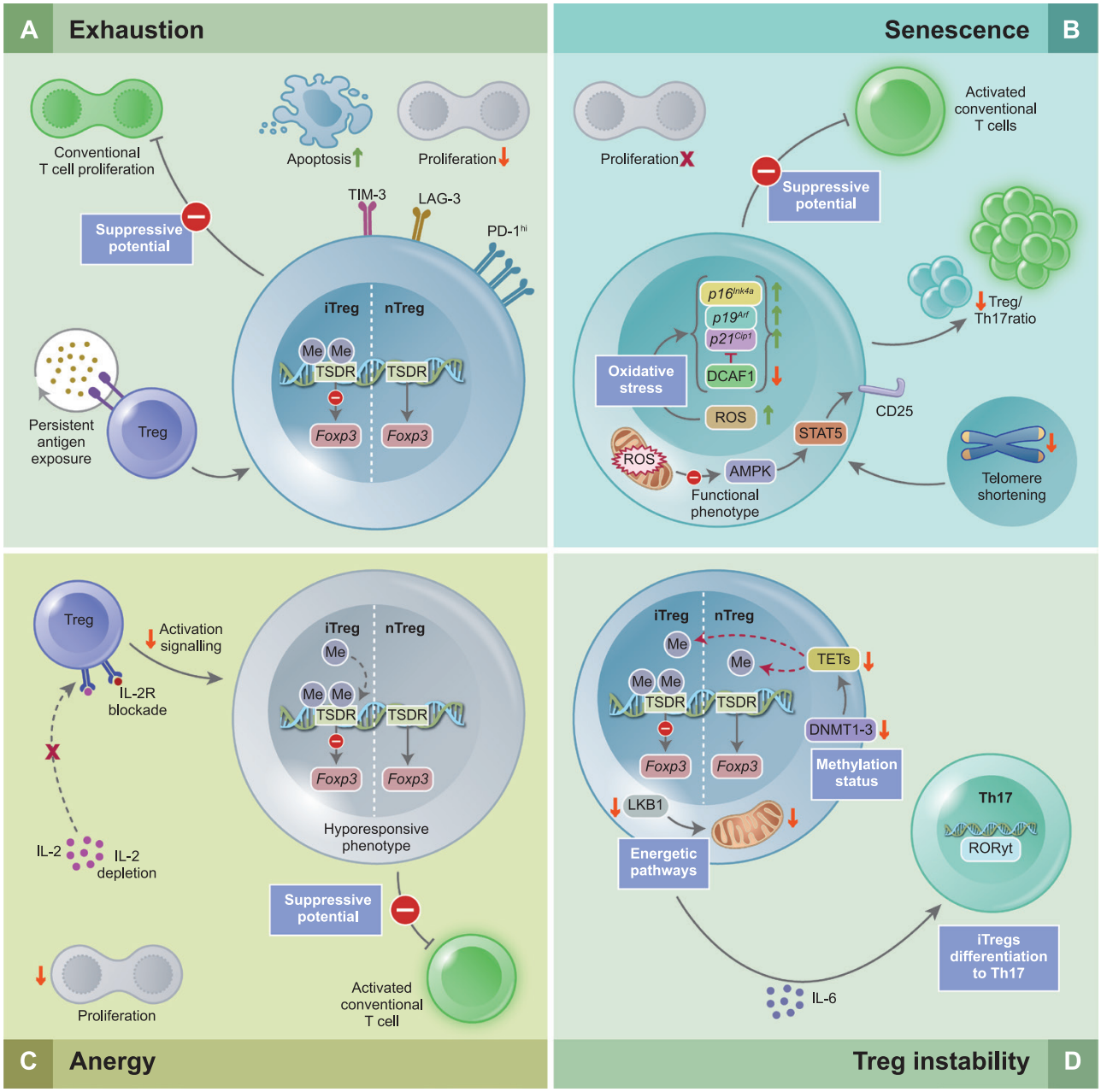

Figure 2. Mechanisms of regulatory T-cell dysfunction. Cell mechanisms responsible for Tregs' loss of tolerance potential are led by four main dysfunctional states. (A) Exhaustion is driven by a persistent antigen exposure and leads to an increased susceptibility to apoptosis, a decreased proliferation rate, and the expression of inhibitory receptors such as PD-1, LAG-3, and TIM-3. Exhausted Tregs are believed to be less suppressive *in vivo*. (B) Senescence, resulting in part from telomere shortening due to age and inflammation, is characterized by an ability to maintain oxidative stress protection. DNA stability for cell survival and suppressive potential is negatively affected. Senescent cells are also believed to be less suppressive. As Tregs are more susceptible to senescence than conventional T cells, it could explain the imbalance between Th17 and Tregs ratio in the elderly. (C) Anergy is induced by a lack of proper costimulation. Reduced downstream signaling from CD25 (IL-2R) leads to hyporesponsiveness and downregulation of their suppressive potential. (D) Treg instability. Other important regulators of Treg lineage stability and functions, such as the metabolic mediator LKB1 and epigenetic modifiers such as DNMTs and TET enzymes, have important roles in the maintenance of FOXP3 expression and Tregs' identity. Also, IL-6 and an inflammatory environment could skew iTreg differentiation towards Th17. Treg: regulatory T cells; nTregs: natural Tregs; iTregs: induced Tregs; DNMT: DNA methyltransferase.

Indeed, lipid metabolism is proposed to be a critical regulator and target of senescent cells (reviewed in [77]).

Anergy

Dysfunction in T cells classified as anergy is when an antigen encounter will be followed by functional hyporesponsiveness [78]. These anergic T cells remain alive but cannot play their intrinsic role. There are, precisely, two states of anergy. One is known as clonal anergy, or growth arrest, which arises when previously activated T cells incompletely activate due to an issue in their downstream pathways. Adaptive tolerance, or *in vivo* anergy, happens when T cells are exposed to a costimulation-deficient or inhibition-rich environment (reviewed in [79]). Like senescence, the definition of anergy in

Tregs is another controversial concept, since in nature they can be considered anergic as they cannot produce their own IL-2 but depend on it to survive and proliferate [78].

Tregs cannot expand via TCR signaling alone which is why they capture IL-2 in their environment with high affinity IL-2R [3]. Absence of IL-2 for other Tconvs can lead to anergy in these T cells and induce tolerance [80]. Calcineurin inhibitors, used for the prevention of solid organ graft rejection, collaterally impair Treg responsiveness via anergy-inducing mechanisms. By inhibiting the activation of the nuclear factor of activated T cells (NFAT) this drug suppresses the production of IL-2 and other cytokines decreasing abruptly the number of Tconv and Tregs in the periphery [81]. Basiliximab, a monoclonal antibody that targets IL-2Rα used in kidney transplant recipients to prevent graft rejection, reduces T-cell proliferation but alters Tregs as well [82]. Inversely, the use of exogenous low-dose human recombinant IL-2 promotes Treg activation, proliferation, and reversion of their anergic-like phenotype [83].

Treg instability

Other mediators are important for Treg stability and function but are not related yet to one of the typical dysfunction subtypes cited above. Involved in metabolic homeostasis of T cells, the liver kinase B1 (LKB1) seems particularly important for Treg lineage stability. Indeed, LKB1-deficient Tregs produce Th1 and TH17 cytokines, are less suppressive [84], and express less FOXP3 [85]. LKB1 acts mainly as a metabolic sensor and is critical to maintaining cellular metabolism [24]. As LKB1 serves serine homeostasis, so does glutathione (GSH) which also leads to the downregulation of FOXP3 and decreased suppressive functions when deficient in Tregs [86].

Numerous epigenetic modulators impact Treg stability and function. DNA methyltransferases 1-3 (DNMT1-3), while not essential for nTregs TSDR's methylation status, are important regulators of immunosuppressive gene expression in Tregs [87]. TET enzymes that are known to oxidize DNA-methylation reactions also contribute to Tregs lineage stability and impact the demethylation of the TSDR region. Recent studies indicate that DNMTs and TETs are often downregulated in diseases associated with chronic inflammation such as cardiovascular diseases [88].

Proinflammatory mediators imbalance with regulatory mediators in the environment can also turn Tregs pathogenic with the loss of FOXP3 expression, transforming them into "exTregs" [89, 90]. Although, it is thought that this phenomenon only affects iTregs because nTregs' FOXP3 expression is deemed too stable [91]. In some instances, inflammatory cytokines such as IL-6 have been shown to reduce Treg suppressive functions and to stimulate their differentiation toward Th17 effector cells. However, as Tregs are naturally more self-reactive compared with conventional T cells, the Tregs-turned-Th17 can cause autoimmunity [92].

Cell therapy

In the last decade, T-cell adoptive immunotherapy revolutionized cancer care. That being said, the potency of this approach was shown to be limited by different states of T-cell dysfunction, namely terminal differentiation, exhaustion, senescence, and activation-induced cell death [59]. Targeting those dysfunctional states is an efficient way to increase immunotherapy therapeutic efficacy. However, as most of those concepts have been studied in conventional T cells, the transferability of such an approach to Tregs is still unknown. In addition to a clear definition of Treg fitness and dysfunction, we also need to acknowledge the different factors influencing cell therapy products; cell source, expansion protocols, and cell engineering.

Cell source

Tregs can be either isolated from peripheral blood, thymus, or cord blood. New methods of genetic engineering also open the possibility of genetic reprogramming of conventional T cells or pluripotent stem cells (reviewed in reference [93]). Peripheral blood Tregs are the most used. However, it is still a heterogeneous population and cell surface markers to isolate them varies. Most commonly, people use $CD4^{+}CD25^{hi}$. The use of $CD127^{low}$ and $CD45RA^{hi}$ (naïve) can prevent contamination with activated conventional $CD4^{+}$ cells that can upregulate CD25 [94, 95]. A more stringent definition thus increases Treg purity to the detriment of cell number, which can be an issue for cell therapy.

The thymus is an excellent source of Tregs in terms of yield, stability, and suppressive abilities [96]. Thymic Tregs can be collected at the time of pediatric cardiac surgeries as this organ is routinely removed and discarded otherwise. Thymic Tregs are currently being tested in pediatric heart-transplanted children, one of the only autologous options (NCT04924491) [97]. Their broader utilization will reside in third-party "off-the-shelf" cell therapy, which comes with a risk of decreased cell survival or off-target effects.

Another similar strategy is the use of Tregs from umbilical cord blood, which shares the advantages with thymic Tregs of being mostly naïve [98]. However, the main limitation resides in the need of a high count of cells and third-party therapies. They were tested in phase I clinical trials in graft-versus-host disease [99, 100]. It is now possible to genetically modify cells to be antigen-specific with the use of transgenic T-cell receptors (TCRs) or chimeric antigen receptors (CARs). One of the main advantages of antigen-specific Tregs is its lower cells need, at an estimated 10th of the common dose. With smaller doses needed, there is a possibility for umbilical cord blood use to resurface.

When using autologous Tregs, the patient's disease and immunosuppressive therapy also need to be considered. Recently, Tang *et al.* reported the results of the ARTEMIS trial, which tested the use of autologous donor alloantigen-reactive Treg therapy in liver transplant recipients [101]. Cells were collected between 2 and 6 years after transplant, in patients on immunosuppressive drugs. They experienced major manufacturing problems as only two cell products out of nine reached the attended cell number and an additional three expanded enough for a partial dose. Their results were explained by a selective reduction of donor-reactive Tregs after transplant and generalized Treg activation and senescence [101].

Expansion protocol

Many different expansion protocols are currently being tested and used [102]. The activation, cytokines, media, supplements, and feeder cells can have an impact on Tregs. However, their implication is poorly studied. Recently, MacDonald *et al.* showed that cell density and feeding frequency have not only an impact on growth rate but also viability and FOXP3 expression [103]. Julia Polanski's group also studied the impact

of repetitive cycles of stimulation on Treg DNA methylation profiles. They showed hypomethylation in the promoter of genes implicated in Tconv exhaustion and increased TSDR methylation indicating possible destabilization [104]. This important study was the first, to our knowledge, to study the epigenetic consequences of an expansion protocol. Bruce Blazar's group also studied the impact of adding rapamycin to their expansion protocol and showed that the transcriptome of Tregs stimulated once or twice without rapamycin had a transcriptome enriched for exhaustion genes and were not as stable as opposed to Tregs expanded through up to five rounds of stimulation with rapamycin [105]. Dr Soldevila's group also used rapamycin for long-term expansion of allo-specific Tregs (4 weeks). Their Tregs were suppressive, but long-term expansion led to an increase in methylation of the TSDR [106]. In conclusion, the consequences of the different expansion protocols on Treg phenotype, function, transcriptome, and epigenome thus need to be further studied.

Cell engineering

Cell engineering can be used in Tregs to either alter their target/specificity, homing, cytokine production, identity (FOXP3 expression), etc [93]. With almost endless possibilities, the associated impact of cell engineering on Treg biology is only sparsely studied. Recently, we studied the impact of a CAR with tonic-signaling on Treg biology and showed that it could drive Treg exhaustion similarly to what was observed in Tconvs. Their phenotype, transcriptome, metabolism, and epigenome were changed with persistent signaling. When those cells were adoptively transferred to humanized mice, they could no longer prevent graft-versus-host disease [67]. Similarly, Lamarthée and colleagues saw a dramatic impact of a CAR with tonic-signaling (4-1BB) on Tregs with an associated reduction in their suppressive capacities [107]. A comprehensive study of the impact of cell engineering on Treg biology should thus be done before testing them in clinical trials.

Conclusion

Treg adoptive immunotherapy is a promising approach to induce tolerance in auto- and alloimmune diseases. To critically analyze the results of the many phase I/II clinical trials being conducted, we need a better definition of Treg fitness. As Tregs differ from conventional T cells in terms of their biology, mechanisms of action, metabolism, and epigenome, we currently do not know if and how the current definitions of cell dysfunctions apply to Tregs. With the era of cell engineering and the corresponding impact on cell biology, this cannot be overlooked.

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Ethical Approval

As we did not present any original data, we did not need ethical approval, patient consent, clinical trial registration, or to adhere to animal research guidelines.

Conflict of Interests

C.L. holds a patent on an HLA-A02 CAR technology.

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Data Availability

Not applicable.

Author Contributions

N.V. and C.R.C. wrote the manuscript. C.L. wrote and critically reviewed the manuscript.

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