Chapter

Type 1 Regulatory T Cells and Their Application in Cell Therapy

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Abstract

Critical roles of regulatory T cells (Tregs) in the maintenance of immune homeostasis by controlling unwanted types of immune responses have been well documented. Therefore, Treg-based therapeutic strategies for inflammatory diseases have long been investigated. Type 1 regulatory T (Tr1) cells and Foxp3$^+$ Tregs are two major subsets of regulatory CD4$^+$ T cells. In contrast to Foxp3$^+$ Tregs, the master transcription regulator for Tr1 cells still remains elusive. Nevertheless, Tr1 cells are generally defined as a specialized subset of CD4$^+$ T cells, which are induced in the periphery during antigen exposure in tolerogenic condition. As one of their key features, Tr1 cells express immunosuppressive cytokine IL-10, which can repress the function of effector immune cells independently of Foxp3 expression. In this book chapter, we discuss the recent developments in the field of Tr1 cells, including major characteristics of Tr1 cells, methods for Tr1 induction as well as their therapeutic potentials in immune-mediated diseases.

Keywords: Tr1 cells, Tregs, IL-10, Foxp3, CD49b, LAG-3, cell therapy, immune regulation

1. Introduction

The immune system is a delicate network consisting of a variety of cellular and molecular components that are designed to protect the host by clearing invading foreign pathogens as well as altered self antigens [1]. In addition, immune system is also equipped with a fine-tuned regulatory machinery that can maintain the balance between activation and suppression of immune responses to achieve immune homeostasis and tolerance.

Studies on the mechanisms of immune regulation have revealed multiple different cell types, including subsets of T cells [2, 3], B cells [4, 5], NK cells [6, 7], and myeloid-derived suppressor cells (MDSCs) [8, 9], with immune regulatory function. Among them, two types of regulatory CD4$^+$ T cells, namely type 1 regulatory T (Tr1) cells and Forkhead box protein P3$^+$ regulatory T cells (Foxp3$^+$ Tregs) are best studied so far. These two types of Tregs have, to some extent, overlapping functions in immune regulation. For example, they both can downregulate unwanted types of immune responses and play important roles in the maintenance of immune tolerance in general. However, mounting evidence suggests that Tr1 cells and Foxp3$^+$ Tregs are distinct populations of regulatory CD4$^+$ T cells [10] and more importantly, they can also display different immune regulatory properties [11–13]. For example, human Tr1 cells, but not Foxp3$^+$ Tregs, have been reported to secrete IL-22 and protect gut epithelial cells from TNFα-induced damage [14, 15].

Foxp3 is known to be the master transcription regulator for Foxp3$^+$ Tregs [16–18]. Both naïve and memory CD4$^+$ T cells are known to differentiate into Tregs with induced Foxp3 expression [19]. Mutation in the *FOXP3*/*Foxp3* gene gives rise to hyperactive T cell responses [16, 20]. In contrast, though many transcription factors have been reported to transactivate *IL10*/*Il10* gene, which plays an important role in the differentiation of Tr1 cells, the master transcription regulator for Tr1 cells is still under investigation. Therefore, several key features have been proposed to identify Tr1 cells. First, Tr1 cells produce predominantly IL-10 and TGFβ. However, Tr1 cells can also express different amounts of other cytokines, including IFNγ [21, 22], depending on the microenvironments where Tr1 cells localize. Second, Tr1 cells exhibit suppressive functions without constitutive Foxp3 expression. IL-10 expressed by Tr1 cells plays a major role in Tr1 cell-mediated immune suppression. Third, CD49b and Lymphocyte-activation gene 3 (LAG-3) have been proposed as surface markers for both human and mouse Tr1 cells [10]. However, whether they contribute to the immunosuppressive functions of Tr1 cells remains elusive so far. In addition, CD49b and LAG-3 are also expressed by other cell types, including CD8$^+$ T cells and B cells, which can also express IL-10. In addition to CD49b and LAG-3, Tr1 cells can express other surface proteins, including CTLA-4, PD-1, T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), CD226, inducible T cell costimulator (ICOS), and CCR5. Of note, these surface molecules can be expressed not only by Tr1 cells, but also by Foxp3$^+$ Tregs and non-Treg populations, depending on the immune context [23].

Herein, we review the major characteristics of Tr1 cells and different experimental methods to induce Tr1 cells both *in vitro* and *in vivo*. We also summarize animal models and human diseases in which Tr1 cells are indispensable in controlling inflammatory immune responses. In addition, we recapitulate clinical trials using Tr1 cells as immunotherapeutics. Lastly, we discuss the future perspectives and major questions to be addressed in the field of Tr1 cells.

2. Tr1 cells and Foxp3$^+$ Tregs are distinct populations of regulatory T cells

Chronic stimulation of naïve CD4$^+$ T cells from both human and mouse in the presence of IL-10 has been reported to induce IL-10-producing antigen-specific immunosuppressive T cells *in vitro* [24]. More importantly, this induced CD4$^+$ T cell population can prevent the development of colitis *in vivo* induced by pathogenic CD4$^+$CD45RB^{hi} splenic T cells in mice [24]. Based on these findings, such antigen-specific IL-10-producing immunoregulatory CD4$^+$ T cells are therefore designated Tr1 cells [24]. Of note, the term "Tr1 cells" was coined several years before the initial studies reporting Foxp3$^+$ Tregs [16, 17], therefore, the question whether Tr1 cells and Foxp3$^+$ Tregs could be distinct populations of regulatory T cells remained unsolved at that time.

By utilizing the IL-10 reporter mice, in which the cellular source of IL-10 can be detected, the presence of Tr1 cells is further investigated *in vivo* [25, 26]. In a steady state, Tr1 cells have been found in multiple tissues of mice, including small intestine and spleen [10, 26]. More importantly, in the IL-10 and Foxp3 dual-reporter mice, the presence of Foxp3$^-$IL-10$^+$ Tr1 cells and Foxp3$^+$IL-10$^{+/-}$ Tregs, as well as their different distributions in tissues and developmental origins under steady state condition have been observed *in vivo*, suggesting that Tr1 cells and Foxp3$^+$ Tregs are different subsets [10, 26]. Of note, there is only low and transient Foxp3 expression upon activation of Tr1 cells, and functional Tr1 cells have been generated *in vitro* from Foxp3-mutated

$CD4^+$ T cells of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients [13], indicating that in contrast to $Foxp3^+$ Tregs [16, 17], Tr1 cells do not require Foxp3 for their development or suppressive function [13].

Taken together, all these findings have identified that immunosuppressive Tr1 cells are different T cell population from $Foxp3^+$ Tregs. More importantly, Tr1 cells also play important roles in the induction and maintenance of immune homeostasis.

3. Experimental induction of Tr1 cells in animal models *in vivo* and in humans *in vitro*

So far, multiple methods have been reported to induce Tr1 cells (**Table 1**). Different microbial components have been shown to induce and further promote Tr1

Reagent and Method	Mouse	References	Human	References
Heat-killed *Mycobacterium vaccae* suspension	+	[27]		
Bordetella pertussis filamentous hemagglutinin	+	[28]		
Cholera toxin	+	[29, 30]		
V. filiformis lysate	+	[31]		
Lactobacillus pentosus KF340	+	[32]		
B. breve	+	[33]		
Microbiota-derived short-chain fatty acids	+	[34]		
PEGylated G-CSF	+	[35]		
Bowman-Birk inhibitor	+	[36, 37]		
Rapamycin + IL-10	+	[38]		
Rapamycin + G-CSF	+	[39, 40]		
Vitamin D3 + dexamethasone	+	[41]	+	[41]
IL-10	+	[24]	+	[24]
TGFβ	+	[26, 42]		
IL-27	+	[43–49]	?	[50, 51]
IFNα			+	[52, 53]
IL-6			+	[54]
DC-ASGPR agonist			+	[22, 55]
ICOS–ICOSL ligation	+	[56]	+	[57]
Indoleamine-2,3-dioxygenase (IDO)			+	[58]
Activin-A	+	[59]	+	[60]
Retinoic acid (RA)	+	[61]	+	[62]
CD2–CD58 ligation			+	[63]
Co-stimulation of CD46			+	[64]
Co-stimulation of CD55			+	[65]
Artificial APCs			+	[66, 67]

Table 1.
Factors that promote the generation of Tr1 cells in mice and humans.

cell differentiation in mice *in vivo*. For example, subcutaneous injection of heat-killed *Mycobacterium vaccae* suspension induces allergen-specific IL-10-producing Tr1-like cells that can protect mice against airway inflammation [27]; treatment with filamentous hemagglutinin from *Bordatella pertussis* inhibits IL-12, but induces IL-10 expression by dendritic cells (DCs), which in turn direct naïve $CD4^+$ T cell differentiation into Tr1 cells in the respiratory tract [28]; immunization of mice with antigen in the presence of cholera toxin gives rise to antigen-specific Tr1 cells [29, 30]; *Vitreoscilla filiformis* lysate can induce tolerogenic DCs and further drive the differentiation of murine Tr1 cells to suppress effector T cells and inflammation *in vivo* [31]; *Lactobacillus pentosus* KF340 can modulate DCs to promote Tr1 cell response, which can prevent systemic inflammation in a mouse model of atopic dermatitis [32]; oral administration of *Bifidobacterium breve* has been reported to mitigate intestinal inflammation in mice via the induction of Tr1 cells [33]; gut microbiota-derived short-chain fatty acids can enhance microbiota antigen-specific Tr1-like cell induction and inhibit murine colitis induced by dextran sulfate sodium [34].

In addition, pharmacological approaches, including PEGylated G-CSF [35], Bowman-Birk inhibitor, which is a soybean-derived serine protease inhibitor [36, 37], rapamycin combined with IL-10 [38] or G-CSF [39, 40], and a combination of immunosuppressive drugs vitamin D3 and dexamethasone [41], have been reported to induce Tr1 cells, therefore promote transplantation tolerance or suppress autoimmunity in mice *in vivo*.

IL-10 is known to be the primary cytokine that can drive the generation of both mouse and human Tr1 cells [24]. In addition, TGFβ secreted by $CD4^-CD8^-CD11c^+$ splenic DCs has been reported to induce the development of mouse Tr1 cells, which can mediate immune suppression *in vivo* [42]. In contrast, IFNα, but not TGFβ, can act synergistically with IL-10 to induce the generation of human Tr1 cells from naive $CD4^+$ T cells *in vitro* [52].

Of note, IL-10 administration alone failed to induce T cell tolerance in animal models of transplantation [68, 69] and autoimmune diseases [70, 71]. Results from these studies suggest that the induction of immune tolerance *in vivo* via Tr1 cell differentiation might require chronic antigen-specific stimulation in the presence of IL-10. Therefore, systemic administration of IL-10 alone may not be sufficient to control inflammatory immune response, and therefore fail to establish immune tolerance *in vivo* [24].

IL-10-producing macrophages with M2 phenotype have recently been reported to play an important role in immune tolerance via induction of Tr1 cells in the mouse model of allogeneic pancreatic islet transplantation [72]. In addition, multiple studies have suggested that IL-10 expressed by DCs plays a critical role in Tr1 cell induction. For example, IL-10-producing $CD11c^{lo}CD45RB^{hi}$ plasmacytoid-like DCs in mouse lymph nodes and spleens have been reported to induce immune tolerance through the enhancement of Tr1 cell differentiation *in vivo* [73]. Repetitive stimulation of human naïve T cells with immature or mature DCs differentiated in the presence of exogenous IL-10 has also been reported to promote the differentiation of Tr1 cells *in vitro* [74, 75]. In addition, targeting self- and foreign antigens to myeloid DCs via C-type lectin receptor DC-ASGPR using antigen-antibody fusion proteins, which can stimulate IL-10 expression by DCs, has been shown to elicit antigen-specific immunosuppressive Tr1 cells from naïve and memory human $CD4^+$ T cells *in vitro* as well as in non-human primates *in vivo* [22, 55]. Furthermore, a subset of IL-10-producing human DCs, namely DC-10, has been discovered in the peripheral blood *in vivo* [75]. In addition, DC-10 can also be generated from monocytes *in vitro* in the presence of

IL-10 [75]. More importantly, DC-10 isolated from either peripheral blood or generated *in vitro* can induce antigen-specific Tr1 cells via an IL-10-dependent manner, which further supports the importance of DC-derived IL-10 in the induction of Tr1 cells [75–77].

In addition to DC-derived IL-10, inducible T cell costimulator ligand (ICOSL) expression by mouse pulmonary DCs plays an important role in the induction of IL-10-producing Tr1-like cells, as interruption of the ICOS–ICOSL signaling suppresses Tr1-like cell induction and blocks the development of tolerance to allergen in mouse *in vivo* [56]. Human plasmacytoid DCs (pDCs) express enhanced level of ICOSL upon maturation, which is known to promote the differentiation of IL-10-producing Tr1-like cells [57]. Furthermore, mature pDCs isolated from the peripheral blood of rheumatoid arthritis patients have high levels of indoleamine-2,3-dioxygenase (IDO), which can promote Tr1 cell differentiation [58]. However, whether ICOSL and IDO expression by pDCs is associated with IL-10 requires further investigation.

Of note, Tr1 cell development in the gut-associated lymphoid tissues in mice does not require IL-10 but mainly depends on TGFβ for their induction and/or maintenance, suggesting that other cytokine(s) could compensate for the absence of IL-10 to induce Tr1 cells [26]. Subsequent studies have further reported that IL-27 plays a critical role in inducing mouse Tr1 cells. Indeed, short-term activation of murine T cells in the presence of IL-27 results in the induction of Tr1 cells *in vitro* and *in vivo* [43–49]. In comparison, the role of IL-27 in inducing human Tr1 cells has been less studied. In humans, plasma IL-27 has been found to be correlated with peripheral $CD4^+IL\text{-}10^+$ T cells in Sjögren Syndrome patients [78]. Positive correlation between serum IL-27 and frequency of $CD4^+CD45RA^-CD49b^+LAG\text{-}3^+$ Tr1 cells in the peripheral blood has been reported in the severe forms of paracoccidioidomycosis [79]. In addition, IL-27 has been reported to induce IL-10-producing Tr1-like cells from human naïve $CD4^+$ T cells *in vitro* [50, 51], however, the suppressive function of these human Tr1-like cells requires further investigation.

In addition to cytokines mentioned above, retinoic acid (RA) has been reported to induce antigen-specific Tr1 cells in mouse *in vivo*, which is further enhanced by co-administration of IL-2 [61]. Immunization of mice with autoantigens in the presence of RA and IL-2 can suppress the development of autoimmunity in the mouse models of experimental autoimmune encephalomyelitis (EAE) and autoimmune uveitis [61]. In addition, RA by itself is sufficient to induce retinaldehyde dehydrogenase (RALDH) expression and endow human DCs with tolerogenic properties to elicit Tr1-like cells *in vitro* [62]. Aerobic glycolysis has also been reported to support Tr1 cell differentiation through a metabolic program controlled by hypoxia-inducible factor 1 alpha (HIF-1α) and aryl hydrocarbon receptor (AHR) [80], suggesting that both immunological and metabolic signals in a specific microenvironment can play pivotal roles in regulating Tr1 cell induction.

Furthermore, activation of human T cells via CD2 results in human Tr1 cell induction [63]. IL-10 is reported to downregulate the expression of costimulatory molecules CD80 and CD86 without affecting CD58/LFA-3 expression on antigen-presenting cells (APCs) [81]. Costimulation of human $CD4^+$ T cells via CD2 by its ligand CD58 induces the differentiation of Tr1 cells independently of IL-10 [63], suggesting that CD2 costimulation triggers an intrinsic signaling pathway resulting in Tr1 cell differentiation. In addition, costimulation of human naive $CD4^+$ cells through CD46 [64] or CD55 [65] can induce $CD4^+$ T cells to display a Tr1 cell phenotype. However, the precise mechanisms in which signals via CD46 and CD55 contribute to the induction of Tr1 cells remain to be determined.

4. Enigmatic lineage-defining transcription factor for Tr1 cells

So far, there is no master transcription regulator confirmed for either human or mouse Tr1 cells. Current understanding of mechanisms underlying the induction of Tr1 cells is mainly limited to IL-10 gene transactivation, and a number of transcription factors have been reported in this process (**Table 2**).

In mouse, IL-27 can promote IL-10 production by $CD4^+$ T cells through activation of STAT1 and STAT3 and drive the differentiation of Tr1 cells [44, 48, 82, 97]. Similarly, other cytokines that can activate STAT3, including IFNα and IL-6, have also been reported to promote Tr1 cells [52–54, 83].

In addition, IL-27-mediated signaling cascade through early growth response 2 (Egr-2) and B lymphocyte induced maturation protein-1 (Blimp-1) has been reported to play an important role in inducing mouse Tr1 cells. Retroviral gene transfer of Egr-2 can convert mouse naïve $CD4^+$ T cells into IL-10-producing and LAG-3-expressing antigen-specific immunosuppressive T cells *in vivo* [84]. Subsequent study has further revealed that IL-27 is sufficient to induce Egr-2, IL-10, and LAG-3 expression in mouse naïve $CD4^+$ T cells, whereas induction of IL-10 and Blimp-1 by

Markers	Mouse	References	Human	References
CD49b	+	[10]	+	[10]
LAG-3	+	[10]	+	[10]
CD226	+	[10]	+	[10]
Kinases and Transcription Factors				
STAT1	+	[48, 82]	+	[51]
STAT3	+	[44, 54]	+	[51, 83]
Egr-2	+	[47, 84]		
Blimp-1	+	[47, 85, 86]		
c-Maf	+	[45, 46, 86]	+	[60]
AHR	+	[45, 46]	+	[60]
IRF4	+	[87]	+	[60]
ITK	+	[87]	+	[87, 88]
Eomes	+	[89]	+	[89, 90]
ROR-α	+	[91]	+	[91]
IRF1	+	[92]		
BATF	+	[92]		
HIF-1α	+	[80]		
Mechanisms of Suppression				
Cytokines (IL-10 and TGFβ)	+	[24, 41]	+	[24, 74]
Killing of APCs (GzmB and perforin)			+	[93]
Cell-cell contact (CTLA-4 and PD-1)			+	[94, 95]
Metabolic disruption (CD39 and CD73)	+	[80]	+	[96]

Table 2.
Cellular and molecular features of mouse and human Tr1 Cells and their mechanisms of action.

IL-27 is dependent on Egr-2 [47]. Deficiency of Blimp-1 in mouse $CD4^+$ T cells results in impaired IL-10 production, whereas Blimp-1 overexpression has been reported to promote the Tr1 cell phenotype in effector T cells [98]. Blimp-1 is also found to promote IL-10 production by Tr1 cells in mouse models of malaria and visceral leishmaniasis [85]. A recent study has examined the transcriptional network driven by IL-27 across different mouse T cell subsets and identified multiple regulators of IL-10 expression [86]. Two central regulators, *Prdm1* (Blimp-1) and *Maf* (musculoaponeurotic fibrosarcoma, c-Maf) are found to cooperatively drive the expression of signature genes induced by IL-27 in Tr1 cells and mediate IL-10 expression in all T helper cells [86]. More importantly, genetic depletion of *Prdm1* and *Maf* in T cells, but not either alone, results in the development of spontaneous colitis in mice, which underscores the importance of the crosstalk between *Prdm1* and *Maf* in the maintenance of immune homeostasis *in vivo* [86].

In addition, IL-27 is also reported to induce the expression of c-Maf, which acts in synergy with AHR, to promote IL-10 expression and differentiation of mouse Tr1 cells [45, 46]. Mice with impaired AHR signaling in $CD4^+$ T cells show lower IL-10 production and resistance to IL-27-mediated mitigation of EAE [46]. Furthermore, IL-27-driven c-Maf expression has been reported to transactivate IL-21 production [45, 46]. IL-21 by itself fails to induce Tr1 cells from native $CD4^+$ T cell, but it serves as an autocrine growth factor for the expansion as well as maintenance of Tr1 cells induced by IL-27, which is evidenced by the observation that loss of IL-21 signaling in $CD4^+$ T cells results in the inhibition of IL-27-driven generation of IL-10-producing T cells *in vitro* and in IL-21R-deficient mice *in vivo* [45]. Nonetheless, the detailed roles of IL-27 in the induction and activation of Tr1 cells in humans remain to be investigated.

Treatment of human naive T cells with activin-A, a member of the TGFβ superfamily, has been reported to induce the activation of interferon regulatory factor 4 (IRF4) [60]. IRF4, along with AHR and its binding partner, AHR nuclear translocator (ARNT), forms a tripartite transcription factor complex that is necessary for the differentiation and effector functions of human Tr1 cells [60]. In addition, IRF4 is also involved in the functional development of mouse Tr1 cells [87]. IL-2-inducible T-cell kinase (ITK) downstream of T cell receptor is found to serve a critical role for the activation of Ras/MAPK/IRF4 signal cascade, which further enables the functional development of Tr1 cells [87, 88]. Furthermore, adoptive transfer of human Tr1 cells induced by activin-A to a humanized mouse model of allergic asthma has been shown to provide the protection against major disease manifestations [60]. Activin-A is also reported to induce a population of antigen-specific IL-10-producing regulatory $CD4^+$ T cells, possibly representing Tr1 cells, which can protect against Th2-associated airway hyperresponsiveness and allergic airway disease in mice [59].

Other transcription factors, including Eomesodermin (Eomes) [89, 90], retinoic acid-related orphan receptor α (ROR-α) [91], have also been proposed to transactivate IL-10 expression in $CD4^+$ T cells and promote Tr1 cell differentiation. Furthermore, it has been reported that interferon regulatory factor 1 (IRF1) and basic leucine zipper ATF-like transcription factor (BATF) are induced early on during IL-27-induced Tr1 differentiation and act as pioneering factors for the differentiation of Tr1 cells in mouse [92]. BATF prepares the genomic landscape for the binding of additional transcription factors necessary for the development of Tr1 cells, and IRF1 specifically transactivates of the *Il10* gene for Tr1 cell differentiation in mice [92].

With the findings of these transcription factors in IL-10 gene transactivation, however, the lineage-defining transcription factor for mouse and human Tr1 cells is still elusive, which remains a key question to be answered in the study of Tr1 cells.

5. Phenotype of Tr1 cells

Though the immunosuppressive functions of Tr1 cells have been reported both *in vitro* and *in vivo*, their phenotype, in contrast, remains poorly defined. Coexpression of CD49b and LAG-3 has been proposed as the surface markers for both human and mouse Tr1 cells [10]. It has been known that CD49b is expressed on memory T cells [99], while LAG-3 is expressed on activated $CD4^+$ and $CD8^+$ T cells. In addition, high level of LAG-3 is also expressed by other immune cells, such as $Foxp3^+$ Tregs and IL-10-producing B cells [100, 101]. Therefore, the single use of CD49b or LAG-3 is not sufficient to define a pure population of functional Tr1 cells or separate these cells from other T helper cells or Treg cells. Of note, coexpression of CD49b and LAG-3 is found not limited to the $Foxp3^-$ Tr1 cells but is also observed in $Foxp3^+$ Tregs and $CD8^+$ T cells that produce IL-10 [102]. Indeed, IL-10-producing Tr1 cells, $Foxp3^+$ Tregs and $CD8^+$ T cells are all capable of co-expressing CD49b and LAG-3 *in vitro* when differentiated under IL-10-inducing conditions, and *in vivo* upon pathogenic encounter or infection in the pulmonary mucosa [102]. Therefore, it is highly recommended that a deliberate and precise gating strategy will need to be made to isolate $CD49b^+LAG\text{-}3^+$ memory Tr1 cells with the exclusion of B cells, $CD8^+$ and $Foxp3^+$ cells.

In addition to CD49b and LAG-3, Tr1 cells can express many other surface molecules, including PD-1, CTLA-4, TIGIT, TIM3, ICOS and CD226, as well as ectoenzymes CD39 and CD73, depending on the immune context [103, 104]. Although expression of such additional inhibitory receptors by Tr1 cells is generally in line with their immunosuppressive function, it is also necessary to realize that their expression is not specific to Tr1 cells.

6. Mechanism of Tr1 cells in immune suppression

The regulatory function of Tr1 cells requires their activation via TCR by cognate antigen recognition. In addition, Tr1 cells can also display bystander immunosuppressive activity to proximal T cells regardless of their antigen specificity. This indicates that activated Tr1 cells can regulate immune responses via both antigen-specific and non-specific manners (**Figure 1** and **Table 2**). Upon activation, human and mouse Tr1 cells secrete IL-10 and TGFβ [24, 41, 74], which suppress T cell responses directly and indirectly. IL-10 can limit the magnitude of immune responses by reducing the surface expression of MHC class II molecules [105–107], co-stimulatory molecules [108, 109], as well as the secretion of pro-inflammatory cytokines by APCs, followed by the suppression of effector T cell responses [104]. TGFβ expressed by Tr1 cells can also repress APC functions and inhibit T cell proliferation and cytokine production [52]. Granzyme B (GzmB) and perforin expressed by Tr1 cells can selectively kill APCs via both cognate and non-cognate mechanisms [93]. Cytolysis of the APCs can consequently suppress antigen-specific T cells and bystander T cells [110].

In addition to secretion of soluble factors including cytokines and enzymes, expression of inhibitory molecules PD-1 and CTLA-4 by Tr1 cells can repress effector T cells via cell contact-dependent manner, which is evidenced by that finding that blockade of CTLA-4 or PD-1 can decrease the suppressor activity of human Tr1 cells [94, 95]. Expression of ectoenzymes CD39 and CD73 [23, 103], though not exclusive to Tr1 cells as mentioned earlier, can facilitate Tr1-mediated suppression of effector T cells via metabolic disruption [80, 96]. In addition, IL-10-producing $Foxp3^-$ Tr1-like cells have also been reported to downregulate B cell antibody production due to low or no expression

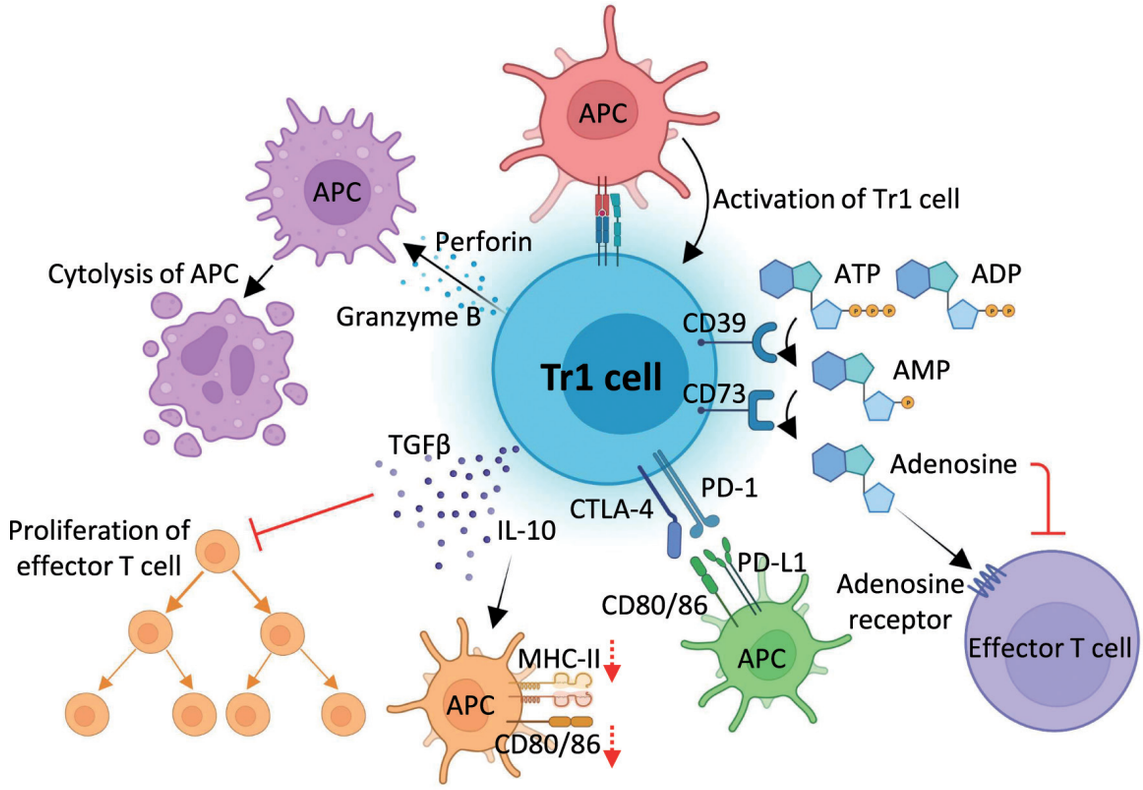

Figure 1.
Suppressive mechanisms of Tr1 cells. Upon activation, Tr1 cells suppress immune responses both directly and indirectly. The secretion of granzyme B and perforin can induce cytolysis of APCs, resulting in inhibition of both antigenspecific T cells and bystander T cells. TGFβ secreted by activated Tr1 cells can inhibit T cell proliferation and cytokine production while IL-10 can downregulate expression of MHC-II molecules, co-stimulatory molecules and production of pro-inflammatory cytokines by APCs. Tr1 cells can also inhibit APC-induced effector T cell activation via cell-cell contact involving CTLA-4 and PD-1. Ectoenzymes CD39 and CD73 expressed by Tr1 cells can mediate the suppression of effector T cells via metabolic disruption.

of CD40L [111]. Taken together, Tr1 cells can exhibit their immunosuppressive function through multiple mechanisms. In future studies, it would be of significance to investigate whether the suppressive mechanisms of Tr1 cells in different clinical settings are associated with the stage of disease progression and pathological microenvironment.

7. Therapeutic potentials of Tr1 cells

The immunoregulatory capacity of Tr1 cells has been tested in multiple different murine models of inflammatory diseases. Foxp3$^-$ Tr1 cells isolated from the intestine of *Il10*^{eGFP}*Foxp3*^{RFP} double reporter mice have been shown to suppress colitis caused by the transfer of pathogenic Th17 cells in an IL-10-dependent manner *in vivo* [112]. In a mouse model of multiple sclerosis, transfer of OVA-specific Tr1 cells can prevent EAE development when antigen-specific Tr1 cells are activated by intracranial injection of OVA [41]. In a mouse GVHD model, Tr1 cells have been reported to constitute the most abundant regulatory population after allogeneic bone marrow transplantation [89]. More importantly, transfer of purified populations of Tr1 cells can significantly suppress GVHD and contribute significantly to transplant survival [89]. In acute and chronic collagen-induced arthritis mouse models, transfer of collagen type II-specific Tr1 cells can reduce the incidence and clinical symptoms of arthritis in both preventive and therapeutic settings, with a significant impact on collagen type

II-specific antibodies. Importantly, injection of collagen-specific Tr1 cells can significantly decrease the proliferation of antigen-specific effector T cells *in vivo* [113].

Human gliadin-specific Tr1 cell clones generated *in vitro* from the intestinal mucosa of celiac patients in remission have been shown to inhibit pathogenic T cell response to dietary gliadin [114]. With good manufacturing practice (GMP)-compatible protocols to differentiate and expand human Tr1 cells *in vitro*, Tr1 cells are also being used as a therapeutic product in clinical applications. IL-10-anergized donor T cells which contain Tr1 cells specific for recipient alloantigens generated *in vitro* have been tested in controlling GVHD in a clinical trial in which patients with high-risk/advanced stage hematologic malignancies received haplo-identical HSCT [115]. Patients had mild to moderate GVHD and showed rapid immune reconstitution [115]. Donor-derived T cells remained hyporesponsive to recipient alloantigens *in vitro* and an increase in cells with Tr1 cell signatures has been observed over time in recipients. Results from this study give the first indication of the feasibility of Tr1 cell-based immune therapy and show promise for the future use of Tr1 cells as treatment for hematologic malignancies and immune-related diseases [115]. T-allo10, as an improved cell product generated by stimulation of donor T cells with host-derived DC-10 in the presence of IL-10, contains a higher percentage of Tr1 cells (up to 15% of $CD49b^+$ $LAG\text{-}3^+$ Tr1 cells) [23], when compared with the previous generation of IL-10-anergized T cells (containing <5% Tr1 cells) used in the clinical trial mentioned above. The overall immunological outcome of T-allo10 in controlling GVHD in patients who received mismatched HSCT for the treatment of hematologic malignancies is still under investigation in an ongoing Phase I trial (ClinicalTrials.gov Identifier: NCT03198234).

In addition to GVHD in HSCT, the therapeutic effect of Tr1 cells in controlling graft rejection in solid organ transplantation is also being investigated. The protocol for generation of recipient-derived donor-specific Tr1 cells for kidney transplantation has been developed [116]. In addition, *Drosophila*-derived artificial APCs have been developed to induce antigen-specific Tr1 cells [66]. Schneider *Drosophila* cells transfected with a transmembrane form of a murine anti-human CD3 monoclonal antibody, together with human CD80 and CD58, as well as human IL-2 and IL-4, have been shown to expand a large number of antigen-specific Tr1 cells [66]. Using this method, Tr1 cells have also been tested to treat inflammatory disease in a phase I/IIa clinical study, in which OVA-specific Tr1 cell clones generated *in vitro* using artificial APCs have been adoptively transferred to patients with refractory Crohn's disease [67]. Patients were fed with OVA-enriched diet to ensure activation of OVA-specific Tr1 cells migrating to the gut. Administration of these OVA-specific Tr1 cells to patients with refractory Crohn's disease was well tolerated and had dose-related efficacy. Though the clinical effect was limited, reaching the maximum at 5 weeks after treatment and declining thereafter, the OVA-specific immune response correlated with clinical outcomes, supporting immunosuppressive function of OVA-specific Tr1 cells [67].

Induction of stable and sustained expression of IL-10 by conventional $CD4^+$ T cells has been developed as an alternative strategy to generate a large number of Tr1 cells. The lentiviral vectors encoding both human IL-10 gene and a marker GFP gene of selection have been tested to induce Tr1 cells [117]. It has been reported that lentiviral vector-mediated human IL-10 gene transfer converts conventional human $CD4^+$ T cells into Tr1-like cells, namely $CD4^{IL\text{-}10}$ cells. These cells resemble Tr1 cells phenotypically and functionally as they express large amount of IL-10, repress T cell responses *in vitro*, and more importantly, prevent xenogeneic GVHD development and progression *in vivo* [117]. Subsequent study has further reported that in addition to suppress T cell responses both *in vitro* and *in vivo*, $CD4^{IL\text{-}10}$ cells were also capable

of killing myeloid leukemia cells in an HLA class I-dependent but Ag-independent manner [118]. This new generation of Tr1 cell product paves the way for adoptive cell therapy with Tr1 cells in patients undergoing allogeneic organ transplantation and HSCT [15, 119, 120].

Furthermore, utilization of artificial chimeric antigen receptors (CARs) to redirect regulatory T cell specificity towards pathogenic cell populations and antigens has also provided new insights in designing and implementing the next generation of Tr1 cells, CAR-Tr1 cells, for the treatment of transplantation rejection, autoimmunity, and leukemia [121–123]. In addition, genome editing techniques (including the application of CRISPR–Cas9) are under investigation to further enhance the specificity and immune regulatory functions of Tregs [122]. Together, all these progresses will certainly further increase the therapeutic value of Tr1 cells.

8. Conclusion

In the last two decades, the immune suppressive functions of Tr1 cells have been demonstrated both *in vitro* and *in vivo*. Studies from different groups have shown that Tr1 cells are able to prevent and constrain undesirable immune responses in different disease contexts, and therefore promote immune tolerance. These important discoveries have led to the idea that Tr1 cells could serve as a therapeutic product to promote and restore immune tolerance in transplantation, as well as in inflammatory and autoimmune diseases. The completed clinical trials have shown, to some extent, the safety of Tr1 cell-based therapy and further indicated the therapeutic potentials. Different methods are being developed to generate better Tr1 cell products. However, with all these advances, questions on Tr1 cells including whether they represent an established T cell lineage and whether Tr1 cells induced *in vitro* can maintain long-term immunoregulatory functions due to possible plasticity and repolarization *in vivo* remain unanswered. In addition, there is controversy over how well CD49b/LAG-3 surface co-expression signature defines circulating Tr1 cells in healthy individuals as many labs, including ours, struggle to use CD49b and LAG-3 to isolate Tr1 cells from bulk culture and it may not be the best approach when compared to the clinically applicable IL-10 cytokine capture method [14]. Future studies will need to elucidate the key molecules, including better and more stringent cell surface marker(s) as well as lineage-defining transcription factor(s) of Tr1 cells. In addition, a deeper and more comprehensive understanding on the biology of Tr1 cells is also necessary to deliver safer and more effective Tr1 cells that can be used to treat different diseases which require long-term regulation of inflammatory immune responses.

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Conflict of interest

The authors declare no conflict of interest.

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