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REVIEW

Type 1 regulatory T cells: a new mechanism of peripheral immune tolerance

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The lack of immune response to an antigen, a process known as immune tolerance, is essential for the preservation of immune homeostasis. To date, two mechanisms that drive immune tolerance have been described extensively: central tolerance and peripheral tolerance. Under the new nomenclature, thymus-derived regulatory T (tT_{reg}) cells are the major mediators of central immune tolerance, whereas peripherally derived regulatory T (pT_{reg}) cells function to regulate peripheral immune tolerance. A third type of T_{reg} cells, termed iT_{reg}, represents only the *in vitro*-induced T_{reg} cells[1]. Depending on whether the cells stably express Foxp3, pT_{reg}, and iT_{reg} cells may be divided into two subsets: the classical $CD4^+Foxp3^+$ T_{reg} cells and the $CD4^+Foxp3^-$ type 1 regulatory T (Tr1) cells[2]. This review focuses on the discovery, associated biomarkers, regulatory functions, methods of induction, association with disease, and clinical trials of Tr1 cells.
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INTRODUCTION

The induction and formation of peripheral immune tolerance is essential to maintaining stability of the immune system. In addition to the immune system's major roles in regulating clonal deletion, antigen sequestration, and expression at privileged sites, a variety of regulatory immune cells have critical roles in maintaining peripheral immune tolerance. Over the past two decades, the peripherally derived regulatory T (T_{reg}) cell subset $CD4^+Foxp3^-$ type 1 regulatory T (Tr1) cells have received increasing attention in medical research, particularly their role in peripheral immune tolerance. Tr1 cells are induced by chronic activation of $CD4^+$ T cells by antigen in the presence of interleukin-10 (IL-10) and are thought to represent a new subset of $CD4^+$ T cells in humans and mice.[3] The immunomodulatory functions of Tr1 cells make them a promising target for the treatment of autoimmune diseases, cancer, the prevention of organ transplant rejection, and other immune-associated disorders. In 2013, the characteristic cell-surface markers for Tr1 cells in humans and mice were identified ($CD4^+$ $CD49b^+$ $LAG\text{-}3^+$ $CD226^+$).[4] This development provides an encouraging basis for further study of Tr1 cells.[4] Currently, scientists have induced Tr1 cells using several *in vivo* and *in vitro* methods to explore the unknown biological functions of these cells and ultimately to apply this knowledge to the treatment of associated diseases.

THE DISCOVERY OF TR1 CELLS

Tr1 cells were first described by Roncarolo *et al.* in 1997, who demonstrated that a subset of $CD4^+$ T cells suppressed antigen-specific T-cell responses and prevented colitis. This $CD4^+$ T-cell population differed significantly from the $CD4^+Foxp3^+$ T_{reg} cells, largely due to the $Foxp3^-$ phenotype.[3] These $CD4^+Foxp3^-$ T_{reg} cells appeared to exert their immunosuppressive functions through high expression of IL-10.[3] Before 2013, immunologists distinguished Tr1 cells from other $CD4^+$ T-cell populations by the unique cytokine expression profile $IL\text{-}10^{++}$ $TGF\text{-}\beta^+$ $IFN\text{-}\gamma^+$ $IL\text{-}5^+$ $IL\text{-}4^-$ $IL\text{-}2^{low/neg}$.[3,5] In 2013, a unique panel of Tr1 cell-surface markers was shown by Roncarolo *et al.*, which were regarded as results of unique transcription factors. However, no uniquely expressed transcription factor has been identified in Tr1 cells so far. The recent discovery of Tr1 cell-surface markers, however, will facilitate *in vitro* purification and *in vivo* tracking of Tr1 cells. For example, this ability will be useful in patients with autoimmune diseases or graft versus host disease (GVHD) with induced immune reconstitution.

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THE ASSOCIATED BIOMARKERS OF TR1 CELLS

There are many biomarkers associated with Tr1 cells, including cell-surface molecules, cytoplasmic molecules, and transcription factors.

1. Cell-surface and cytoplasmic molecules associated with Tr1 cells

To identify human and mouse Tr1 cells, Roncarolo *et al.* emphasized that both CD49b and the lymphocyte activation gene-3 (LAG-3) are indispensable.[4] LAG-3 is a membrane protein in Tr1 cells with a negative regulatory effect on TCR-mediated signal transduction in human and mouse cells; when it becomes a soluble molecule, LAG-3 activates dendritic cells (DCs) and enhances the antigen-specific T-cell response in mice.[4,6,7] CD49b belongs to the integrin family and is a receptor for many (extracellular) matrix and non-matrix molecules.[8–10] It has been widely reported that Th17 cells can produce IL-17A, IL-17F, and IFN-γ by co-stimulation CD3 monoclonal antibody (mAb) and CD49b.[10] Half of memory T cells express CD49b and produce high levels of TNF-α while the remainder are CD49b-negative and secrete IL-10.[11] CD49b provides little contribution to the differentiation and function of Tr1 cells.[4]

In addition to CD49b and LAG-3, Tr1 cells express co-stimulatory molecules. When activated via stimulation of the T-cell receptor (TCR), Tr1 cells may produce normal levels of molecular markers, such as CD40L, CD69, CD28, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4/CD152), programmed cell death protein 1 (PD-1), and human leukocyte antigen-DR (HLA-DR).[5] Moreover, Tr1 cells express high levels of regulatory factors, such as glucocorticoid-induced tumor necrosis factor receptor (GITR), OX40 (CD134), and tumor-necrosis factor receptor (TNFRSF9).[12,13] In addition, Kohyama *et al.* demonstrated that Tr1 cells produce substantial levels of inducible co-stimulator (ICOS).[14] In 2006, a transcriptome analysis of human Tr1 cells revealed an overexpressed integrin CD18.[15] In 2014, Schuler *et al.* reported that in a tumor microenvironment, Tr1 cells derived from $CD4^+CD25^-$ T cells *in vitro* co-expressed the immunosuppressive surface molecules CD39 and CD73 and produced adenosine (ADO) and prostaglandin E2 (PGE2).[16–18] Fousteri *et al.* demonstrated that Tr1 cells proliferated and gained tolerance to transplantation of pancreatic islets in protein tyrosine phosphatase non-receptor 22 (PTPN22) knockout mice.[19]

Compared with traditional $CD4^+CD25^+T_{reg}$ cells, Tr1 cells normally do not express CD25 or Foxp3. Tr1 cells are distinct from the traditional $CD4^+CD25^+T_{reg}$ cells because of their unique cytokine expression profile, denoted as $IL\text{-}10^+$ $TGF\text{-}\beta^+$ $IFN\text{-}\gamma^+$ $IL\text{-}5^+$ $IL\text{-}4^-$ $IL\text{-}2^{low/neg}$.[3,5]

2. Transcription factors associated with Tr1 cells

A number of transcription factors, such as the cellular homolog of the avian virus oncogene musculoaponeurotic fibrosarcoma (c-Maf), the aryl hydrocarbon receptor (AhR), interferon regulatory factor 4 (IRF4), the repressor of GATA-3 (ROG), and early growth response protein 2 (Egr-2) have been proposed as transcription biomarkers for Tr1 cells.[13,20–22] In mouse $CD4^+$T cells, IL-6-associated signaling resulted in an evident increase of IL-10 mRNA levels in an IL-2- and IL-21-dependent pattern.[20] At the molecular level, IL-6 signaling drives expression of c-Maf, AhR, and IRF4, all of which are crucial transcription factors for IL-10 secretion and Tr1 cellular differentiation.[20] Furthermore, it has been demonstrated that the transcriptional effects of IL-6 and IL-2 are mediated by the signal transducer and activator of transcription 3 (STAT3) and STAT5, respectively.[20] Activated STAT3 and STAT5 can both directly bind to *Il10* and *c-Maf* promoters; thus, combined STAT5 and STAT3 activities might optimally activate these promoters and those of *Ahr* and *IRF4*.[20,23–26]

Moreover, ROG is a transcriptional factor associated with Tr1 cells in mice.[13] However, ROG is not specific for Tr1 cells, as it is rapidly induced in other T helper (Th) cells upon activation.[22] Stimulation of naive $CD4^+$ T cells in mice with IL-27 leads to phosphorylation of STAT3, which drives expression of B lymphocyte-induced maturation protein 1 (*Blimp1*) and *Il10* genes via an Egr2-dependent pathway.[21] Iwasaki *et al.* have reported that the transcription factor Egr-2 is required for Blimp-1-mediated IL-10 production in IL-27-activated Tr1 cells and in $CD4^+CD25^-LAG\text{-}3^+Foxp3^-$ T cells.[27,28] Other researchers showed that AhR is highly expressed and three molecules (c-Maf, IL-21, and ICOS) are required for IL-27 activation of Tr1 cells.[29,30]

The underlying mechanism of Tr1-cell induction by IL-6 and IL-27 initially involves the expression of IL-10. As IL-10 is critical to the differentiation of Tr1 cells, we believe that enabling the secretion of IL-10 will promote the differentiation of Tr1 cells. However, at this point the transcription factor that controls Tr1 cells differentiation is unknown.

IN VITRO INDUCTION OF TR1 CELLS

It is important to note that the induction of Tr1 cells by self or non-self-antigens in the presence of IL-10 *in vivo* may not apply to Tr1-cell induction *in vitro*.[5] As previously mentioned, the differentiation of Tr1 cells is affected by many factors, including the intensity of TCR stimulation, cytokines, co-stimulatory molecules, chemokines, complement, *in vivo* or *in vitro* conditions, and the use of human or mouse cells. Unfortunately, the main contributing factor is unclear in the complex microenvironment in the body.

Given the important role of Tr1 cells in regulation of autoimmune diseases and other disorders, the successful induction of Tr1 cells *in vitro* is essential for studying and elucidating the pathogenic mechanisms of autoimmune diseases and for developing appropriate therapeutics. Many factors participate in the differentiation of Tr1 cells. First, induction of Tr1 cells is dependent on specific subsets of antigen-presenting cells (APCs), including immature dendritic cells (iDCs), plasmacytoid DCs (pDC), and IL-10-treated tolerogenic DCs.[31] Second, many studies have reported that cytokines are also essential to Tr1-cell induction, such as IL-21, IL-6, IL-27, and especially IL-10.[20,26,30,31] Third, co-stimulatory molecules such as ICOS ligand (ICOS-L), CD46, CD2, and CD55 are involved in the differentiation of Tr1 cells.[30,32–34] It has also been

demonstrated that chemokines and B cells are involved in the induction and proliferation of Tr1 cells. Carrier *et al.* showed that the long-term interactions of GITR/GITRL signaling boosted Tr1-cell differentiation and expansion in mice *in vivo*.[35] Zohar *et al.* believed that chemokine (C-X-C motif) ligand 11/chemokine receptor 3 (CXCL11/CXCR3) interaction may induce transformation of $CD4^+$ T cells to Tr1 cells.[36] Hsu LH *et al.* found that B-1a cells stimulated naïve $CD4^+CD25^-$ T cells to become $Foxp3^-$ T cells with high production of IFN-γ and IL-10 but tiny amounts of IL-4.[37]

In the following passages, we cite three methods for the induction of Tr1 cells to illustrate critical factors involved in the process of Tr1-cell differentiation and to provide useful information for the study of Tr1 cells.

Stimulation of naive T cells by iDCs

This method was originally described in a study of the induction of Tr1-cell differentiation by iDCs.[38] Peripheral blood mononuclear cells (PBMCs) were incubated for 2 h at 37°C. iDCs were induced from $CD14^+$ monocytes by the addition of 25 ng/ml of recombinant human IL-4 (rhIL-4) and 50 ng/ml of recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) for 5 days. Media was replenished on day 2 and day 4 after isolation of cells by replacing half of the media with fresh complete media containing rhIL-4 and rhGM-CSF. The iDCs (1×10^5) were collected on day 6 and co-cultured with purified naive $CD4^+$ T cells (1×10^6 cells). On day 7, the co-cultures were supplemented with 40 IU/ml rhIL-2 and the cells were cultured for an additional 7 days. T cells were then collected, washed, and re-stimulated with iDCs for 3 days. Cultures were then supplemented with rhIL-2 (40 IU/ml), and the resultant Tr1 cells were collected after an additional 4 days.

IL-10-expressing lentiviral vector transduction of human $CD4^+$ T cells

Human $CD4^+$ T cells were transduced with IL-10-expressing lentiviral constructs to induce Tr1 cells in a report in *Molecular therapy* in 2012 by Roncarolo and colleagues.[39] Purified $CD4^+$ T cells were pre-activated for 2 days with soluble anti-CD3 and anti-CD28 mAb and rhIL-2. The activated cells were infected with a LV-IL-10-GFP ($CD4^{LV\text{-}IL\text{-}10}$) construct overnight (multiplicity of infection (MOI): 20). Purified $CD4^{LV\text{-}IL\text{-}10}$ T cells were cultured in an allogeneic feeder cell system containing irradiated PBMCs (10^6/ml), irradiated JY cells (10^5/ml), and soluble anti-CD3 monoclonal antibodies (1 μg/ml). After 12 days of culture, $CD4^+$ T cells were isolated by flow cytometry and 2×10^5 cells in each well were activated with anti-CD3 mAb (10 μg/ml) and soluble anti-CD28 mAb (1 μg/ml) in the presence of rhIL-2 (100 U/ml) in complete X-VIVO medium. After 3 days, 3H-thymidine (1 μCi/well) was added to the culture and incubated for 16 h before the proliferative and suppressive functions of Tr1 cells were evaluated.

Tr1-cell induction with immunosuppressive drugs

Immunosuppressive drugs have been shown to stimulate the induction of Tr1 cells from naïve $CD4^+$ T cells isolated from both human and mice.[40]

Inducing mouse Tr1 cells. Purified ovalbumin (OVA)-specific naive $CD4^+$ T cells (2×10^5) were cultured with splenic APCs (5×10^6, γ-irradiated, 3000 rad) and the $OVA_{323–339}$ peptide (0.6 M), and cells were stimulated with plate-bound anti-CD3 mAb (10 μg/ml), anti-CD28 mAb (1 μg/ml) in the presence of vitamin D_3 (4×10^{-8} M) and dexamethasone (10^{-8} M). Ontertiary Tr1-cell cultures were collected, washed, and re-stimulated (1×10^6/ml) by plate-bound anti-CD3 mAb (2 μg/ml), anti-CD28 mAb (2 μg/ml). PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of vitamin D_3 (4×10^{-8} M), dexamethasone (10^{-8} M) and brefeldin A were used as a treatment before performing flow cytometry.

Inducing human Tr1 cells. $CD4^+$ T cells were removed from the mixture with Dynabeads to leave only $CD4^+CD45RA^+$ T cells. These cells were stimulated with plate-bound anti-CD3 mAb, soluble anti-CD28 mAb (2 μg/ml), rhIL-2 (50 U/ml) and vitamin D_3 (10^{-7} M) or dexamethasone (5×10^{-8} M) in the presence of neutralizing anti–IL-4 (5 μg/ml), anti–IFN-γ (5 μg/ml), and anti–IL-12 (5 μg/ml) mAb. After four rounds of treatments with vitamin D_3 and dexamethasone, cells were re-stimulated with anti-CD3 mAb, anti-CD28 mAb, and rhIL-2 for 18 h prior to phenotypic testing and other experiments.

The first method is the mixed lymphocyte reaction (MLR), which uses iDC and IL-2/IL-10 to stimulate Tr1-cell differentiation and proliferation. The MLR method is the most common in Tr1-cell differentiation. The additional two described methods use anti-CD3 mAb joined with anti-CD28 mAb to stimulate naive T cells, thereby significantly increasing Tr1-cell quantity. The second described method involves promoting IL-10 expression using gene transfection, wherein Tr1-cell differentiation pathways are enhanced in an IL-10-dependent manner. The third method involved the induction of immunosuppression and included an explanation that Tr1-cell differentiation may rely on specialized conditions.

FUNCTIONS AND RELATED MECHANISMS OF TR1 CELLS

The function of regulatory T cells (T_{reg}) is the suppression of T effector cells and the inhibition of an undesirable immune response. However, thymus-derived regulatory T (tT_{reg}), peripherally derived regulatory T (pT_{reg}) and Tr1 cells have different effects on their targets. First, Tr1 cells are Ag-specific T_{reg} that can be generated both *in vivo* and *in vitro*. Tr1 and pT_{reg} may be anergic to the different self or non-self-antigens in different disease environments, although most of the tT_{reg} cells are activated by autoantigens in the thymus.[5] Second, tT_{reg} cells exert regulatory immune functions throughout the whole body and contribute to homeostasis, whereas Tr1 cells are thought to regulate local immune microenvironments where specific

antigens exist.[41–43] Third, depending on the individual potentials, Tr1 or pT_{reg} cells may inhibit autoimmune diseases within a limited range, so it is beneficial to study the mechanisms of associated diseases to facilitate the development of clinical therapy.[44] Moreover, studies of the epigenetic status of DNA, most tT_{reg} and pT_{reg} cells contain T_{reg}-specific de-methylated regions (TSDR), which are critical to stable expression of Foxp3 and the development and functions of most tT_{reg}/pT_{reg} cells.[45,46–48] However, the transcription factor of Tr1 cell has not been determined. Finally, patients with systemic lupus erythematosus (SLE) seem to show more effective inflammation control by tT_{reg} than Tr1 cells.[49] Thus, different types of T_{reg} may have specific roles in distinct disease models.

Regarding the molecular and cellular mechanisms involved in the Tr1 cell function, at least four important mechanisms have been addressed. First, Tr1 cells suppress T-cell and APC responses primarily via the secretion of IL-10.[50–53] The activation of the IL-10/IL-10R pathway between Tr1 cells and APCs cause upregulation of the immunomodulatory expression molecules immunoglobulin-like transcript-3 (ILT3), ILT4, and HLA-G on DCs. These molecules inhibit DCs maturation and induce T-cell tolerance.[50–53] Tr1-cell associated bystander suppression and infectious tolerance via IL-10 have been reported in studies of the treatment of refractory Crohn's disease.[44] Meanwhile, a longitudinal prospective study showed that in the initial stage of HIV infection, Tr1 cells controlled the general immune activation through IL-10 production.[41] Second, researchers demonstrated that Tr1 cells released granzyme B (GZB) and perforin and that cell–cell contacts via CD2/CD58 and CD226/CD155 led to the death of myeloid APCs.[3,54,55] Third, Tr1 cells inhibit T-cell responses through the Tr1-cell-DC-effector T-cell pathway. The expression levels of different co-stimulatory or inhibitory molecules such as CTLA-4, PD-1, and ICOS in Tr1 cells exerted immunomodulatory effects similar to traditional $Foxp3^+$ T_{reg} in this pathway.[56,57] The PD-1/PD-L pathway mediated by cell–cell contact between Tr1 cells and APCs indirectly inhibits the activation and proliferation of self-reactive T cells.[58,59] In this process, the CTLA-4/CD80 pathway exhibited a synergistic effect.[58,59] Furthermore, interactions between Tr1 cells and DCs indirectly inhibited priming of the effector T cells via downregulation of the expression levels of MHC class II and co-stimulatory molecules or anti-inflammatory molecules such as ICOS-L, IL-1 receptor antagonist, and PD-1L on DCs.[50–53] Lastly, in terms of metabolic regulation, Tr1 cells express the ectoenzymes CD39 and CD73, which produce ADO through the hydrolysis of extracellular ATP and exert immunosuppressive functions in tumor microenvironments through the disruption of the metabolic state of effector T cells.[16]

TR1 CELL-RELATED DISEASES

Several different research groups have demonstrated that Tr1 cells play significant roles in the prevention and treatment of autoimmune diseases, organ transplantation, and chronic inflammatory diseases by suppressing the immune response of effector T cells and memory T cells and regulating immune tolerance in the periphery.

Tr1 cells and tumor immunity

Tr1 cells are activated in the peripheral blood of patients with head and neck squamous cell carcinoma, where they may function as mediators of immune suppression, thereby promoting tumor progression.[18] Immature DCs induce the differentiation of Tr1 cells from naive $CD4^+$ T cells through antigen-specific presentation of inhibitory cytokines, particularly, IL-10. This process might result in the expansion of tumor cells in the microenvironment.[18] Tumor-cell antigens induce the differentiation and proliferation of T_{reg} cells, which mediates immune tolerance and permits immune evasion of tumor cells.[18]

Tr1 cells and graft versus host diseases

Tr1 cells contribute to the mediation of immune tolerance in the microenvironment of organ transplants. In patients with type 1 diabetes (T1D), allogeneic islet transplantation can restore insulin production. Transplantation of polyclonal Tr1 cells might contribute to transplantation tolerance, which was indicated in a non-lineal mouse model of diabetes.[60] Roncarolo *et al.* demonstrated that antigen-specific Tr1 cells were superior to the polyclonal Tr1 cells in this model.[60] In 2015, Fousteri G *et al.* demonstrated that PTPN22 deficiency strengthened transplant tolerance to pancreatic islets by increasing the number of $Foxp3^+$ T_{reg} and Tr1 cells.[19] Additionally, following the achievement of immune tolerance in patients with severe combined immunodeficiency (SCID), who underwent stem-cell transplants expressing mismatched HLA, the cytokine profiles of T cells isolated *in vivo* are similar to those of antigen-specific Tr1 cells.[3] Moreover, clonal Tr1 cells were found to prevent skin graft rejection in a mouse model of transplantation.[13]

Tr1 cells and autoimmune diseases

In a murine model of SCID with inflammatory bowel disease (IBD), the co-transfer of Tr1 cells (pre-induced *in vitro* with IFN-α and IL-10) and pathogenic $CD4^+CD45RB^{high}$ T cells prevented disease progression.[3] This result was only observed when Tr1 cells were activated by antigen-specific TCR stimulation.[3] In another study, Tr1 cells that were induced by *Bifidobacterium breve* (an intestinal bacteria) alleviated the development of intestinal inflammation.[61] In recent years, Tr1 cell-related immunotherapy has reached the point of clinical trials for treatment of IBD.[44,62]

TR1 CELLS IN CLINICAL TRIALS

The immunosuppressive properties of Tr1 cells contribute to the downregulation of the immune response by maintaining peripheral immune tolerance and suppressing autoimmune diseases. Consequently, Tr1 cells have been suggested as a potentially useful treatment of various immune-related disorders in the clinic. The Tr1 cell-associated clinical trials in humans are ongoing and published results indicate that the

use of Tr1 cells in this field is safe and efficient for use in organ transplants.

In 2014, Bacchetta *et al.* reported a small proof-of-concept clinical trial, in which IL-10-anergized T cells (Tr1 cells) and hematopoietic stem cells were transferred into patients with hematologic malignancies.[63] Of the 12 patients involved, four showed recurrence and survived beyond day 100, three failed to achieve the desired immune reconstruction and were prone to infection, four reached the target immune reconstitution with long-term optimal performance status, and the remaining patient obtained immune reconstitution although ultimately died of infection.[63] Compared with the previous clinical data on the risk of such procedures at an advanced stage of the disease, the therapy appears effective, safe, and promising.[64,65]

A phase I/II clinical trial was launched to determine whether OVA-specific Tr1 cells are effective in the treatment of refractory Crohn's disease. Crohn's disease is a multifaceted disease and each individual is likely to have a genetic susceptibility.[66] The theoretical basis for this treatment is that Tr1 cells induce bystander suppression and infectious tolerance by producing IL-10 and induce tolerance of T cells.[44] However, this is still an ongoing trial currently lacking conclusive results.

CONCLUSIONS

This review article introduces the historical discovery, associated biomarkers and regulatory functions of Tr1 cells. In addition to the related diseases and clinical implications of Tr1 cells, methods for *in vitro* induction of Tr1 cells were addressed. The continuing research of Tr1 cells is foundational for studying the mechanisms of Tr1 cell-associated immune disorders and will be crucial for developing effective treatments for these disorders.

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