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Adoptive Treg Cell-Based Immunotherapy: Frontier Therapeutic Aspects in Rheumatoid Arthritis

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Abstract

The major current focus on treating rheumatoid arthritis is to put an end to long-term treatments and instead, specifically block widespread immunosuppression by developing antigen-specific tolerance, while also permitting an intact immune response toward other antigens to occur. There have been promising preclinical findings regarding adoptive Treg cells immunotherapy with a critically responsible function in the prevention of autoimmunity, tissue repair and regeneration, which make them an attractive candidate to develop effective therapeutic approaches to achieve this interesting concept in many human immune-mediated diseases, such as rheumatoid arthritis. *Ex vivo* or *in vivo* manipulation protocols are not only utilized to correct Treg cells defect, but also to benefit from their specific immunosuppressive properties by identifying specific antigens that are expressed in the inflamed joint. The methods able to address these deficiencies can be considered as a target for immunity interventions to restore appropriate immune function.

Keywords

adoptive cell therapy, expansion of Treg cell, Foxp3, immunotherapy, induced antigen-specific Treg cell, regulatory T cell, rheumatoid arthritis, Treg, Treg cell immunotherapy, Treg cell modulation

Rheumatoid arthritis (RA) is considered a chronic disease that has been faced with many challenges in the time to treatment initiation and also in the treatment process such as the physical condition, the specific genetic profile, the environmental factors, the presence of auto-antibodies and even the disease manifestations in the RA patient that have been comprehensively studied (1). The treatment protocols of RA have changed during the last years with more emphasis on preventing joint damage and functional impairment. Pharmacotherapy is initiated at the time of diagnosis, with the goal of hampering disease progression or reducing its pace. European League Against Rheumatism and the American College of Rheumatology has provided algorithms for the use of conventional and biologics disease-modifying antirheumatic drugs for treating RA patients in different stages of

disease activity (2,3) which primarily are based on applying a general suppression of the immune system (4). However, despite therapeutic advances, many patients still suffer from disability which could be a reason for the importance of time to initiate treatment, urgent triage and identification of related factors in facilitating early treatment of rheumatic disease (5). Therefore, the ultimate aim of treating RA is to maintain safe homeostasis, especially tolerance against self-antigen (Ags) and discontinuation of treatment. In this regard, Treg-based therapy is of high interest since Treg cells have undeniable potential roles to control the immune system hyperactivation seen in autoimmune diseases without concomitant immunosuppression. Function and frequency defects of Treg cells have been described, along with some commonly used biologic treatments that can target these cells (4,6,7). Here, we aim to summarize and discuss the emerging *in vitro* and *in vivo* approaches to expand the highly enriched Treg cells population, as well as their associated challenges in the field-including barriers and strategies to overcome these barriers for restoring defective self-tolerance-and focusing on their application in RA therapy.

Description of Treg cells

Treg cells are a specialized subpopulation of T cells that downregulate immune response via suppression of activation, proliferation and cytokine production of CD4⁺, CD8⁺ T cells, B cells and dendritic cells (DCs) as an important 'self-check' built into the immune system. Treg can be identified by flow cytometry based on the specific surface and intracellular biomarkers expression. It should be noted that both Treg and effector T (T_{Eff}) cell populations are derived from the same progenitor cells in the bone marrow (BM), which become committed to their specific lineage based on CD4 and CD25 biomarkers expression in the thymus (8). Therefore, it is very difficult to discern Treg from T_{Eff} cells, making them difficult to study. Though some studies have referred to Forkhead box P3 (FoxP3) as a specific marker in this lineage, it has been reported that Foxp3 expression level is deferent between resting and activating Treg cells (9). On the other hand, Foxp3 express in activated conventional Tcells (T_{Cons}) transiently results in unspecific staining of human T_{Eff} cells (10,11) and is highly along with hypo-responsiveness to these cells (12). Foxp3 - a member of the Fox family of transcription factors - is defined by a conserved 110 amino acid residue encompassing the DNA binding domain of the winged helix structure and generally involved in gene expression, function and survival of Treg cells (13). However, this intracellular marker can be used as a good marker for MOUSE CD4⁺ CD25⁺ Treg cells but is not a specific marker to stain HUMAN Treg cells (14). Hence, selected surface markers including CD4⁺CD25^{+/high}, CD127^{low/-}, CD62 ligand, Integrin Eα (CD103), CTLA-4 (CD152), GITR (TNFRSF18), Neuropilin and CD45RO could serve as surrogate markers for identifying Treg cells in routine clinical practice, in addition to intracellular staining of Foxp3, (15,16). It should be noted that the CD45RA and CD45RO markers are used for distinguishing naive Treg (CD45RA⁺FoxP3^{low}) from activated memory Treg (CD45RA⁻FoxP3^{high}) cells (17). These cells have been broadly classified as natural Treg (nTreg) and induced Treg (iTreg) when generated *in vitro*, or peripheral Treg when generated *in vivo*. Although the contribution of nTreg and iTreg cells in maintaining tolerance is unknown, both share a similar inhibitory function, albeit with some epigenetic differences between them, for example, the former having more stable Foxp3 expression and wider demethylation. iTreg cells have recently been described as a regulatory subset that complements the regulatory responses of nTreg cells (18).

Role of Treg cells in RA

The role of Treg cells has been studied in many autoimmune diseases, such as RA, using laboratory-developed methods. Recently, several articles have indicated that Treg cells are responsible for suppressing inflammatory events directly and/or indirectly at the site of inflammation (19). Some reported effects of Treg cells in improving autoimmune diseases are shown in Figure1. In general, human and mouse Treg cells are very similar in molecular characteristics and can be identified in both peripheral blood and synovium of RA patients (20). Fluctuations in the number of Treg cells simultaneously in peripheral blood and inflammation sites-affected by

disease activity and progression have been reported in many studies (20-22). Therefore, it seems that compensation of immune deficiency using adoptive T cell therapy is the best way to optimize the immune balance induced by Treg cells failure.

Figure 1. The immunodominant strategies for Treg cells in the suppression of immune responses. A. Induction of tolerogenic DC, which induces anergy in activated T cells. B. Depletion of IL-2 from the environment, which increases T cells' susceptibility to death by PD-L1-expressing tolerogenic DCs. C. B-cell suppression via TGF- β secretion, which inhibits the production of autoantibody. DC: Dendritic cell.

Peripheral blood

Overall, controversial results are presented for Treg cells frequencies in the peripheral blood circulation of RA patients (20,23). The usage of different *in vitro* methods to identify Treg cells as well as immunosuppressive medication application-especially biological agents such as anti-TNF- α (infliximab, etanercept) (24)-can be mentioned as the main reasons for this inconsistency. According to the reported findings, the question still remains whether the enhanced Treg cells are specific or nonspecific. In the animal models, the absence of a significant rise in Ag-specific Treg cells in the peripheral blood can be noted for the following reasons: the use of a certain antigen such as collagen type II (CII) to induce RA (25) and, likely, the infiltration of Ag-specific Treg cells into the involved site. In human RA disease, observations are different due to the multifactorial nature of the disease (26), the probability of creating several Ag-specific Treg cells clones, as well as unknown normal or abnormal physiological mechanisms. However, these findings suggest that likely to be a correlation between the improving of RA disease and increasing the Treg cells number or enhancing Treg cells suppressive function.

Synovium

It should be noted that the increase of Treg cells in synovial fluid is usually associated with unchanging or even decreasing in the frequency of these cells present in peripheral blood of patients with RA (27,28). This phenomenon occurs because of the high-level expression of chemokine receptors on Treg cells, which recruit them to the site of inflammation (29). Thereby, some researchers have reported that Treg cells infiltrate at higher frequencies in synovial fluid and synovial membrane than in peripheral blood (PB) and relative Treg frequency is significantly higher in synovial membrane (19). It has been shown that synovial infiltrated Treg cells exhibit active phenotype with significant expression of CD152, GITR, OX40 (CD134) and potentially were able to exert a considerable suppressive effect on T_{Eff} cells and cytokines production *in vitro* (20,30). Therefore, it can be concluded that these cells have infiltrated to inhibit inflammation in the affected joint, but are unable to inhibit host T_{Eff} cells for various reasons. These reasons include heterogeneity of Treg cells population (27) and the presence of abundant inflammatory cytokines that can convert Tregs to T_{H17} cells, while also making T_{Eff} cells resistant to Treg cell-mediated suppression (28).

Deficiency of Treg cells in RA

The crucial role of CD4⁺ T cells in the pathogenesis of several autoimmune diseases has been well accepted, including in that of RA. Therefore, therapeutic strategies focus on modulating the CD4⁺ T cells' activity through a variety of methods; one of these methods is the use of regulatory cells (31). Although there are reports of apparent deficiencies in the Treg cells numbers (32) and functions (33) in the peripheral blood of RA patients compared with the healthy donor, several other studies have reported different results indicating the similar (34) or increased Treg cells percentages in peripheral blood of RA patients (23). It seems that the origin of this discrepancy is in the used methods for separation and identification of Treg cells. In most studies, Treg cells are identified using Foxp3 expression, whereas it has been found that recently activated T cells also express Foxp3 (35,36). Therefore, for the separation of these populations and excluding activated conventional T cells, the cytokine production profile within the Foxp3 population should be examined, which were seen to be negligible in Treg cells (37). Besides, there is conclusive evidence that Treg cells in RA patients may be ineffective due to the CTLA-4 deficiency or excessive expression of IL-6 in the inflammatory site (38,39). In this case, the results are contradictory and some researchers have shown isolated Treg cells from RA patients are able to effectively inhibit the proliferation of conventional T cells (20). One characteristic of Treg cells biology is functional instability and conversion phenotypically into T_{Eff} cells against pro-inflammatory cytokines, such as TNF- α and IFN- γ (4).

Therapeutic potential of Treg cells in RA

The regeneration of Treg cells function has been considerably reported following the use of biological treatments in patients with RA. Hence, multiple approaches have been designed to increase the number and to recover the function of Treg cells (4). Existing evidence of the potential of Treg cells in treating RA patients can be explained in two sections, covering both *in vivo* and *ex vivo* evidence.

In vivo evidence

It has been reported that some pharmacological and biological agents that are used to treat RA probably exhibit their therapeutic effect through the modulation of Treg cell activity and frequency *in vivo*, although it is worth mentioning that these medications are mostly not designed with the intention to modulate Treg cells. The spectrum of these medications is wide and does not include only current approved medications for treatment (Table1). In conclusion, targeting Treg/T_{H17} cell balance is one of the most common therapeutic approaches that has been used in recent years to treat RA. Therefore, selective targeting of Treg cells may provide potentially powerful tools for the treatment of inflammatory arthritis diseases.

Table 1. Therapeutic agents that involve some level of Treg cells modulation, either numerously or functionally.

Class	Mechanism of effect	Effects on regulatory T cell	Ref.
TNF blockade	Induction of Foxp3 expression by phosphorylation of the <i>Foxp3</i> gene	Increases the proportion of circulating Treg cells Induces novel differentiated iTreg cells	(40,41)
Anti-IL-6	Rebalance Foxp3/Ror- γ t expression ratio	Increases Treg/T _{H17} ratio by suppresses T _{H17} generation Induces Treg cells differentiation	(42)
CTLA-4-Ig	Blocks T cell activation by binding to CD80/CD86 ligands Promoting DC to express IDO Preventing CD95-mediated apoptosis	Induces new iTreg cells population Increases Treg cells proportion Activates existing Treg cells	(43,44)

Anti-CD3	Remove CD3 through internalization Induces Foxp3 expression on CD8 ⁺ T cells	Increase the number of circulating CD8 ⁺ Treg cells Inhibit CD4 ⁺ T cell function and proliferation	(45)
Anti-CD4	Increases Treg/T _{H17} ratio	Induces immunological tolerance Prevents T _{H17} polarization Decreases circulating CD4 ⁺ T cell Depressed <i>in vitro</i> T cell responses	(46)
Anti-IL-17	Increases Treg/T _{H17} ratio	Inhibition of pro-inflammatory T _{H17} pathway	(47)
IL-2	Activate the STAT5 transcription factor	Promotes Treg cells activation and expansion	(48)
Rapamycin	Blocking AKT-mTOR-SMAD3 signaling axis Inducing Foxp3 expression	Suppresses T _{Eff} cell proliferation Induces Treg cells differentiation	(49)
TGF	Induction of Foxp3 expression	Induces resistant Treg cells differentiation	(50)
ATRA	Prevent ROR γ t expression Activate the ERK1/2 signaling pathway	Prevents T _{H17} cells differentiation Induces Ag-specific Treg cells	(51)
VIP	Increasing Foxp3 and TGF- β 1 expression Induction of suppressive soluble factors secretion	Increases Treg cells numbers and suppressive activity Deviated the immune response toward the T _{H2} subsets	(52)
AM	As an intraperitoneal neuropeptide occasion T _{H1} switch to T _{H2}	tDC Induce Treg cells	(53)
Ucn2	Aa an endogenous immune-medodulatory factor with anti-inflammatory effect	Ucn2 induce Treg cell with increase the secretion of IL-10/TGF- β 1	(54)
HDAC	Open chromatin architecture by hyper-acetylation on H4 Acetylation of Foxp3 protein	Increases numbers of Foxp3 T cells Enhanced Treg cells suppressive capacity	(55)
IVIG	Induction of COX2-dependent PGE2 in human DC	Stimulates Treg cells proliferation	(56)

Ag: Antigen; AM: Adrenomedullin; ATRA: All *trans*-retinoic acid; CD: Cluster of differentiation; DC: Dendritic cell; iTreg: Induced Treg cell; IVIG: Intravenous IgG; PGE2: Prostaglandin E2; tDC: Tolerogenic dendritic cell; T_H: T helper cell.

Ex vivo evidence

Adoptive transfer of either nTreg or iTreg cells can rescue Scurfy mice and prevent many autoimmune diseases (57), suggesting that manipulation of these cells may have the potential to treat or cure autoimmune diseases. In this regard, removal of Treg cells before immunization or prior to the onset of the disease increases the incidence and severity of the disease. This process could be slowed down by transferring of Treg cells (58,59). Although most studies revealed that the therapeutic effects of adoptive nTreg cells transferring are unsatisfactory on established collagen-induced arthritis (CIA) to suppress progression, it can markedly prevent the development of CIA. The inability of Treg cells to exactly reverse disease does not necessarily indicate that Treg cells cannot control arthritis. Importantly, when repressing arthritis in these models, Treg cells not only control T cells and B cells, but also prevents joint damage by directly suppressing osteoclast-mediated bone destruction (60). We demonstrate an alternative model of inflammatory arthritis, CIA, in which specific Treg cells for CII is accumulated in joints significantly higher in compared with nonspecific Treg cells (61). In addition, these cells apply Ag-dependent repression, which was not detected by polyclonal Treg cells (4).

Adoptive therapy of Treg cells in RA

Recent studies have highlighted the fact that Treg cells play an important role in modulating immune responses. Hence, adoptive Treg cells therapy-with the ultimate goal of reaching satisfactory clinical outcomes-tends to become more attractive in treating RA. It has been suggested in several studies that early adoptive transfer of Treg cells could be beneficial in preventing the early auto-inflammatory events, which initiate the disease (6,62,63). The results provide a proof-of-concept that adoptive cell therapy (ACT) of Treg cells could be an effective therapeutic option for autoimmune diseases. The important issues that should be considered in ACT of Treg cells are schematically shown in Figure2. Theoretically, Treg cells can be obtained from a donor (allogeneic) or as a patient (autologous) and each of these approaches can be divided into two groups, including polyclonal and Ag-specific Treg cells therapy. In most initial animal studies, polyclonal cells derived from healthy animals have been used. This method is used in inbred or after developing an intensive form of inbred animals that allow the transfer from one animal to another (61). Given today's immunological knowledge, it seems very unlikely that this strategy to be successful in humans. However, due to the substantial dependence of ACT treatment effect on the Ag-specific and the infusion time of Treg cells, there is already a significant change in the use of Ag-specific cells derived from the infected animals (63) or collagen-specific T-cell receptor (TCR) transgenic mice (62). Before the implementation of ACT in the clinic, some technical issues should be resolved. If the goal is obtaining Ag-specific Treg cells, at the initial step, the most specific pathogenesis-responsible Ags to activate the most specific Treg cells must be identified. Next, DCs that could express these rare organ-specific auto-antigens should be detected and extracted in order to activate Treg cells in coculture (64). The next issue is the effective expansion of Ag-specific Treg cells without losing their specificity or general functional capacities. It is worth mentioning that several factors including vitamin D3, glucocorticoids, IL-10, TGF- β , vasoactive intestinal peptide and so on, induce Treg cells indirectly by the generation of tolerogenic DCs, being a mechanism to drive antigen-specific Treg-mediated responses (65,66). The sufficient number of infused cells is the other question that should be answered in order to reach a successful ACT for RA patients (67).

Figure 2. The steps that must be considered in the adoptive Treg cell-based immunotherapy.

Polyclonal Treg cells ACT

Treg cells can be isolated from peripheral blood and cultured under special conditions in order to produce a large number of them (68,69). However, the methods used to purify Treg cells (based on magnetic or flow cytometric methods) are limited (70) and the presence of non-Foxp3 cells may be harmful, especially if the interest is the Ag-specific Treg cells production. Although there are several reports of successfully treated using adoptive polyclonal Treg cells therapy in CIA models following Treg cells aggregation in inflamed joint (71,72)

and suppression of osteoclast formation (73), transfer of polyclonal Treg cells turned to be futile for two main reasons. First, the efficacy of polyclonal Treg cells transferring depends on the time and stage of arthritis. Thus, the improvement of the disease is confined to using this method in the early stages of the disease (74). Second, a large number of polyclonal Treg cells should be used to reduce the severity of disease (75). However, such a large number of polyclonal Treg cells transferring could cause temporary suppression of the immune system, as well as increased inflammation due to the conversion of transmitted Treg cells into the T_{H17} cells (76).

Ag-specific Treg cells ACT

Increasing the number of Treg cells in the inflamed joint indicates that both specific and non-specific Treg cells can migrate to the inflamed site. Nevertheless, the need to detect Ag with a specific TCR results in prolonged homing of Ag-specific Treg cells in inflammatory lesions, unlike the polyclonal Treg cells. Evaluation of in vivo Ag-specific Treg cells dynamics showed that they maintained their phenotype in the absence of target Ag and despite the ability to proliferate, revealed a late phase suppressive activity following target Ag immunization (77). Monoclonal Treg cells lines against a single Ag specificity derived from several ways, including:

- Mucosal tolerance by oral administration of self-Ag (78).
- Converting the isolated patient's autoreactive T cells into Treg cells using genetic methods (61).
- Chemically cross-linking auto-Ags to the cell surface of antigen-presenting cells(79).
- Augment immunosuppressive capacity of Treg cells through direct contact with mesenchymal stromal cells (MSCs) (80).

Although the first procedure is a potent way to induce Ag-specific Treg cells (78), additional considerations are two major difficulties in applying Ag-specific Treg cells. The first is the extremely low number of each Ag-specific subpopulation and second, the wide variety of target auto-Ags are involved in the development of RA and the main Ag is unknown. Although the enhancement of Treg cells suppressive capability with MSCs via PD-1/B7-H1 interaction, provided a promising approach, the more research is needed to prove the immunosuppressive capacity of MSCs (80). Taken together, our findings, as well as others, strongly suggests that Ag-specific Treg cells-by inhibiting Ag-specific T_{Eff} cells without suppressing of all T_{Eff} cells-can be suitable cellular vehicles for locally targeted immunotherapy consistent with a good safety profile of therapy in terms of low systemic side effects (61,81).

Useful subpopulations of Treg cells for ACT

nTregs or iTregs

Treg cells can be generally grouped into two categories, natural or induced. The therapeutic potential of both nTreg and iTreg subgroups has been well demonstrated by improving or preventing many autoimmune diseases in animal models. Although there are no specific phenotypic biomarkers that differentiate iTreg from nTreg cells, there are still significant differences in development and function between these cells, despite them sharing some similar phenotypic and functional characteristics (82). Briefly, the differences and similarities between these two subpopulations of Treg cells are shown in Table2. Generally, given the properties of iTreg cells-especially stability in an inflammatory environment-it seems that iTreg cells may have a favorable therapeutic effect both on inhibiting the onset and preventing established autoimmune disease (61).

Table 2. Differences and similarities of both natural Treg and induced Treg cells.

	Characteristics	nTreg	iTreg
Differences	Low frequency and poor growth	Yes	No
	Suppression of Ag-specific T _{Eff} cells	No	Yes

	Resistance to inflammatory cytokines	No	Yes
	Dynamic alteration to T _{H17} cells	Yes	No
	Converted into T _{H2} cells	Yes	No
	Converted into T _{H1} cells	Yes	No
	Suppressed the disease progression	No	Yes
	Complete Demethylation of Foxp3 locus	Yes	No
Similarities	Suppressed the disease onset	Yes	Yes
	Expression of CTLA-4, GITR, CCR4,	Yes	Yes
	Produce immunosuppressive cytokines	Yes	Yes
	Cell-cell contact as a suppressive mechanism	Yes	Yes
	Cytokine production as a suppressive mechanism	Yes	Yes

Ag: Antigen; iTreg: Induced Treg cell; nTreg: Natural Treg; T_{Eff}: Effector T cell; T_H: Helper T cell.

T follicular regulatory cells

The formation of high levels of various autoantibodies is one of the key elements in the process of RA pathology, which could affect future therapeutic options. Forming autoantibodies depends on lymphoid follicular germinal centers (GCs) and requires interaction between several cell types, including GC B cells, T follicular helper (T_{FH}) and T follicular regulatory (T_{FR}) cells. Notably, T_{FR} is an important component of the GC which interacting with T_{FH} and/or B cells, leading to suppress antibody production (83). An imbalance between helper and regulatory function could lead to dysregulation of GC response and excessive autoantibodies production. Therefore, enhancing function or number of T_{FR} cells could be an effective strategy for developing T_{FR} and T_{FH} balance and controlling the antibody production (84), restoring immune homeostasis and thereby improving the outcome of RA (85).

Protective Treg₁₇ cells

T_{H17} cells can be divided into two distinct lineages either protective or pathogenic cells depending on environmental conditions. In this sense, it has been shown that protective cells termed Treg₁₇ cells induced by exposure to a combination of TGF- β plus IL-6 cytokines (82,86). The profile of cytokines produced from two distinct lineages are clearly different from each other. So that the Treg₁₇ cells express immune system-suppressing cytokines such as IL-10 and IL-21, along with high ROR γ T expression and Aryl hydrocarbon receptors (87,88). Therefore, Treg₁₇ cells with regulatory and inhibitory activity against pathogenic T cells, can be considered as a candidate for ACT in established autoimmune diseases.

Useful subsets of Treg cells for ACT

Based on our current understanding, the Treg cells family consists of several heterogeneous subsets, such as CD4⁺, CD8⁺, CD4⁺ CD8⁻, $\gamma\delta$, Type 1 regulatory T (T_{R1}) and natural killer T cells, each of which plays a fundamental role in maintaining the normal immune homeostasis against auto-Ags (89). In addition to the CD4⁺ Treg cells, regulatory roles of other subsets, as well as CD8⁺ Treg cells, have been elucidated in the regulation of autoimmune disease (62,90,91). CD8⁺ Treg cells have a unique inhibitory mechanism- suppression of follicular helper T cells (T_{HF})-despite their mechanism in part shared with CD4⁺ Treg cells (92). Therefore, CD8⁺ Treg cells that can target only the self-reactive CD4⁺ T cells and, thereby, complement CD4⁺ Treg cells suppressor activity and serve a vital role in self-tolerance. Given the growing general research interest on CD8⁺ Treg cells leading to the identification, proliferation and maintenance of other Treg cells subsets is likely to result in the innovation of novel therapeutic strategies for human inflammatory diseases (93).

Approaches to modulate Treg cells

There are several different methods to directly or indirectly expand Treg cells for the treatment of arthritis. The straight approach includes expansion and induction of Treg cells *in vivo* or *in vitro* by immunomodulatory

compounds followed by reinfusion into the patient, which is also known as *ex vitro*, because the cells are treated outside the body. In addition to the direct strategies, indirect approaches can also be taken to enhance Treg cells function through *in vivo* approaches, which include reducing the pro-inflammatory environment, enhancing responsiveness of effector cells to suppression and using of specific Treg cells proliferation stimulants via targeted gene therapy, all of which help to increase Treg cells population and function in patients with autoimmune disease (94).

In vivo

In recent years, *in vivo* expansion of Treg cells has been one of the conventional approaches to treat autoimmune disease. Although expanded Treg cells can improve established CIA, they are polyclonal and have the ability to suppress the overall immune system. The most promising approach to *in vivo* expansion and induction of Treg cells is utilizing several immuno-reactive or inhibiting the pro-inflammatory agents. The most extensively studied Treg cells enhancing agents will be discussed in section 4 and are summarized in Table1.

In vitro

Increasing the understanding of immune system regulation by the Treg cells has made it feasible to induce controlled hemostasis via manipulating Treg cells *in vitro*. *In vitro* methods for inducing Treg cells can be divided into two groups. In the first group, Treg cells were isolated from an individual and subsequently expanded in the presence of anti-CD3/anti-CD28 and IL-2 (61). Remarkably, it has been reported that this protocol has the ability to increase cells number up to several 1000-times without losing the inhibitory potential of Treg cells.

In the second group, the discussion is about the creation of Treg cells from non-Treg cells. This protocol eliminates the difficulty of requiring a large number of Treg cells for therapeutic purposes. In general, expansion via *in vitro* methods has the advantage that the product can be continuously analyzed in terms of functional and phenotypic activity and the dose can be carefully controlled. Yet, despite the acceptable positive therapeutic effects of adoptively transferred Treg cells to animal models of diabetes or lupus, some of the problems and potential risks of using Treg cells in the treatment of autoimmune diseases remain. These issues include contamination with T_{Eff} cells due to marker similarities in the first category and conversion of expanded Treg_s into T_{Eff} cells under special conditions in both categories has made it difficult to generalize this therapy to the clinic. Therefore, due to the complexity of Treg cells induction *in vitro*, the development of established standard protocols to optimize isolation, expansion and stability of expanded Treg cells is still a fundamental requirement. A number of direct and indirect laboratory methods used to increase the number and function of Treg cells are briefly explained.

Enhancing the responsiveness to Treg cells-mediated suppression

It is noted that T_{Eff} cells are resistant to inhibition by Treg cells in some autoimmune diseases, including RA, which can be achieved by the production of pro-inflammatory cytokines-like TNF- α and INF- γ -and the recruitment of protein kinase C- θ (PKC θ) to the immunological synapse (95). Eventually, this leads to inhibition of Treg cells-mediated suppression and uncontrolled activation of T_{Eff} cells. Therefore, increasing the responsiveness to Treg cells-mediated suppression is one of the indirect methods for improving Treg cells function. Hence, blocking these pro-inflammatory cytokines reduces the resistance of T_{Eff} cells to suppress and, thereby, increases the control of inflammation by Treg cells.

Expansion of Treg cells

The major problem with the clinical use of Treg cells is their low frequency in the blood. Hence, expansion of purified Treg cells in the *in vitro* method using anti-CD3/anti-CD28 antibodies along with IL-2 or rapamycin and cell-based artificial antigen-presenting cells that expressed the high-affinity fragment crystallizable receptor and CD86 is a potential solution to overcome this problem (96,97). The advantages of this method include the potential for multiplying Treg cells up to several 1000-times, as well as the evaluation of the phenotype and

function of these cells before injection. The capability to efficiently produce functional Treg cells could transform the treatment of autoimmunity by providing a proven cellular therapy (79).

Genetic engineering of Treg cells

To ensure that Treg cells suppress T_{Eff} cells at the site of inflammation, a variety of strategies have been proposed to regulate the amount and functionality of Treg cells - for example, ectopic expression or the acetylation modulation of Foxp3 (75). It is possible with this type of gene therapy approach that additional genes could be added to the therapeutic construct to address additional genetic defects identified in RA patients (4).

Promoting Treg cell stability

The maintenance and stability of iTreg cells phenotype and function have recently become a controversial topic in clinical applications of Treg cell-based immunotherapy. The constant expression of Foxp3 plays a critical and proven role in the differentiation and function of iTreg cells, so that impaired Foxp3 expression especially under inflammatory milieu leads to reprogramming and converting to inflammatory T_{Eff} cells, as well as enhancing autoimmunity. However, several directly or indirectly genetic strategies have been introduced to produce stable iTreg cells. For instance, CD28 superagonist stimulation *in vitro*, in the absence of CD3 ligation, is more efficient in promoting polyclonal Treg cells proliferation and prevention of pro-inflammatory cytokine expression, such as IL-17, as compared with anti-CD3/antiCD28-stimulated Treg cells (98,99). Combined stimulation with rapamycin and TNF receptor type II ($TNFR_{\text{II}}$) agonist antibody enhanced hypo-methylation of the *Foxp3* gene, thus promoting Treg cell stability (100).

Conclusion

The causal link between defects of Treg cells and autoimmunity established the stage for using adoptive Treg cells therapy to treat animal models of autoimmunity, such as RA. Although the number of Treg cells may be different in RA patients, the suppressive activity of Treg cells in autoimmune disorders appears to be limited. However, the induction of peripheral immunological tolerance using adaptive Treg cells transfer has notably increased immunotherapy expectations for the reversion of ongoing RA (Figure3). Polyclonal Treg cells are particularly effective for autoimmune diseases involving larger tissues, compared with smaller tissues, despite their potential ability to suppress natural immune function. Given that Treg cells have the ability to detect Ags specifically through TCR, the development of a new class of ACT that directs to a specific site or detect certain Ag may be an ideal opportunity to directly modify the pathological immune response by enhancing Ag-specific Treg cells activity. The encouraging findings emphasize the need to explore the therapeutic potential of Ag-specific iTreg cells in established immune-mediated diseases because fewer cells are needed to exert inhibitory effects and produce a more localized and targeted suppression. However, the potential of other Treg cell subunits, especially $CD8^{+}$ Treg cells, should not be overlooked, because it is likely that either one of the well-known or new subunits may be able to complement or induce the inhibitory function of $CD4^{+}$ Treg cells, even in low cell numbers.

Figure 3. A 'plan' for the Treg cells generation and the adoptive Treg cell-based immunotherapy of rheumatoid arthritis. In immunotherapy, the first step is the mass production of Treg cells that can be termed as (1) *in vivo* : injection of biological medication that exerts part of their effect by increasing the number or function of Treg cells and (2) *ex vitro* : T cells can be harvested either from tissue-infiltrated lymphocytes (TILs) or peripheral blood lymphocytes (PBLs) and re-injected into the patient subsequently of some gene-modification and expansion processes that have been developed in an attempt to improve the adoptive cell therapy of Treg cells therapeutic effects. The processes include: A. selection of cell populations. CD4 + CD25 + CD127 low nTreg cells can be sorted and expanded for 7-14days. B. Production of iTreg cells. It can be produced by genetic engineering techniques such as transferring of viral vectors carrying the Foxp3 gene construct. C. Production of antigen-specific iTreg cells. This step can be used for both TIL cells (because they are naturally specific for inflamed joint antigens) and polyclonal iTreg cells approaches. In contrast, specificity can be established in PBLs, either through co-culturing with a single auto-antigen or genetic engineering. Selection of cell subsets. Subsets of Treg cells can be sorted by flow cytometry. iTreg: Induced Treg cell; nTreg: Natural Treg cell.

Future perspective

Numerous studies based on Treg cell immunotherapy indicate a remarkable increase of interest in Treg cells as a smart medication because Treg cells apply multiple molecular mechanisms to suppress unwanted immune responses in RA. Currently, the application of these cells requires overcoming some major problems including, time and cost reduction of producing enough Ag-specific Treg cells property. According to advances in the molecular basis of RA immunopathogenesis, it will probably lead to identify the involved Ags. in the near future. Therefore, developing strategies to overcome existing pitfalls will facilitate the process of Treg cell immunotherapy moving to the clinic. Meanwhile, advances in the development of chimeric antigen receptors and genetic management to increase the viability, functionality and specificity of Treg cells is likely to increase efficiency as well as facilitate the production of these cells.

Executive summary

- Despite advances in the developments of biologic agents, the ideal outcome remains suboptimal. Therefore, notable challenges arise in the usage of these agents in rheumatoid arthritis (RA) treatment.
- The most important of which are extensive immunosuppression and increased opportunistic infections associated with long-term use of biologic agents.
- There is, therefore, more effort to develop protocols that address harmful responses in RA specifically and maintain homeostasis.

- The presence of regulatory cells in the structure of the immune system with unique functions has highlighted the effort to use these cells in the treatment of arthritis.
- In this review, we summarize the practical opinion of how Treg cells came to be recognized as an attractive smart cell to develop a specific RA treatment and express the available approaches in the enrichment of Treg cells population *in vitro* and *in vivo* conditions.
- Adoptive Ag-specific Treg cells immunotherapy attracts the major interest to modify the immune response in RA.
- To target autoimmune response, further efforts have been made to expand Treg cells *in vivo* and *in vitro*.
- Treg sublinages are probably involved in improving inhibitory function hence, their therapeutic potential should not ignore.

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