Overview and Recent Advances of Regulatory T cell Therapy in Solid Organ Transplantation

Haideh Namdari,[1] Ahmadreza Sadeghi,[1] Alireza Heidary Rouchi[2]

[1]Iranian Tissue Bank and Research Center, Tehran University of Medical Sciences, Tehran, Iran
[2]Liver Transplantation Research Center, Tehran University of Medical Sciences, Tehran, Iran

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Solid organ transplant recipients are in high demand for developed immune-modulating agents to control allo-immune responses following transplantation. The immunosuppressive agents offer the recipients improved short-term graft survival; nonetheless, this benefit is tempered by unavoidable long-term adverse events of these medications. Active control of allo-response using therapeutic cell transfer has gained much attraction during the last few years. It is widely established that regulatory T cells (Tregs) control immune responsiveness to allo-antigens and contribute to the induction of tolerance. Here, it is aimed to review recent results regarding Tregs and chimeric antigen receptor (CAR) Tregs therapy in solid organ transplantation and discuss strategies to overcome technical challenges of developing successful Tregs/CAR Tregs therapy.

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Review

INTRODUCTION

Solid organ transplantation is considered as the therapeutic modality of choice for patients suffering end-stage organ failure.[1,2] Key challenges facing organ transplantation, are the cumbrous and costly procedure accompanying with organ shortage.[3] Moreover, to manage rejection and prevent graft loss, life-long immune-suppression is mandatory, which can cause several adverse effects.[3-5] Thus, the ultimate goal of transplantation is operational tolerance, whereby patients maintain normal graft function, preferably without immunosuppression.[3] Regulatory T cells (Tregs) account for 5 to 10% of peripheral $CD4^+$ T cells subpopulation in human and mice.[6] There are two major subpopulations of Tregs, natural Treg cells (nTregs) that develop in the thymus and induced Treg cells (iTregs) which arise in the periphery from naïve conventional $CD4^+$ T cells during the immune response.[7] It is a well-established fact that nTregs differentiation is dependent on the strength and duration of the T cell receptor (TCR) signaling.[8,9] As a matter of fact, the strength of TCR signals that CD4 single positive (SP) cells receive drives their fate in the thymus. Strong signals end to negative selection of CD4 SP cells, while intermediate TCR signals result in differentiation of these cells into Tregs.[10] nTregs which particularly express the transcription factor Forkhead box protein P3 (Foxp3) are considered crucial for the maintenance of self-tolerance. Foxp3 gene is essential for regulating Tregs gene expression and is in charge of suppressive functions of these cells.[11] It has been shown that thymic Tregs possess a completely demethylated Treg-specific demethylated region (TSDR), which is essential for generating stable Tregs.[12,13] All things considered, both Foxp3 expression and TSDR demethylation are crucial for nTreg lineage commitment.[14]

Differently from nTregs, $Foxp3^+$ iTregs can differentiate from naïve $Foxp3^-CD4^+$ T cells in the periphery and their generation is likely promoted through TCR interaction of high affinity with non-self-antigens.[15,16] Far away TCR signaling, suboptimal co-stimulation and the combination of TGF-β and IL-2 promote the differentiation of iTreg cells as well.[17,18] Treg cells have been explained to perform their suppressive function via diverse mechanisms. This subset exerts their

suppressive functions either through contact dependent mechanisms (such as inhibitory receptors and effector cell cytolysis by releasing granzyme and perforin) or contact independent mechanisms (deprivation of IL-2 and production of immunoregulatory cytokines such as TGFβ, IL-10, and IL-35) .[19] It is confirmed by several studies that Tregs contribute to the generation of tolerance and play a critical role in preventing allograft rejection.[14,20] In the setting of transplantation, "linked suppression" and "infectious tolerance" are two mechanisms of Treg-induced tolerance, which actively provide a rationale to harness their suppressive activities for the induction of transplantation tolerance. "*Linked or bystander suppression*" denotes that Tregs can function in an antigen non-specific manner, by that enabling Tregs of one antigen specificity to suppress T effectors (Teffs) of many other antigen-specificities. In consequence, to prevent allograft rejection, Tregs do not require recognition of all major and minor histocompatibility antigens. Superiorly, Treg-induced tolerance can be sustained long-term via infectious tolerance, by virtue that Tregs by producing immunosuppressive molecules within the microenvironment can confer a state of tolerance onto naïve T cells and promote the production of iTregs with different antigen specificity. In this framework, Tregs specific to alloantigen Y are able to suppress rejection of graft that expresses both alloantigens Y and X. Gradationally, alloantigen X–specific Tregs generate and maintain tolerance in the absence of the alloantigen Y–specific Tregs.[4] Intriguingly, infused Tregs may not necessarily need to survive for a long time but for long enough to confer a suppressive ability to other immune cells in the inflamed tissue. [20,21] Given these, utilizing non-modified or modified Tregs, aiming to establish or improve transplantation tolerance following solid organ transplantation has gained increasing attention in recent years.[22] Here, we review the studies in which Tregs and CAR Tregs therapy used to induce alloantigen-specific tolerance in transplantation and discuss challenges and overcoming strategies for developing successful Tregs/CAR Tregs therapy.

HOW TO IMPROVE TOLERANCE BY TREG CELLS?

In organ transplantation, the primary target of adaptive immunity is the major histocompatibility complex (MHC) antigen on the surface of donor cells. In general, allo-recognition can be divided into three pathways: direct, semi-direct, and indirect. In direct pathway, intact donor MHC antigens are presented by donor antigen presenting cells (APCs). In semi-direct pathway, donor MHC antigens are presented by recipient APCs as intact cell membrane fragments, whereas in indirect allo-response, donor MHC antigens are processed into peptides and presented by recipient APCs on self-MHC molecules to T cells.[23] In point of fact, direct and semi-direct antigen presentation is mainly involved in acute rejection.[23] Early acute rejection might be mediated predominantly by direct and semi-direct pathways because at that time high density of allo-MHC molecules are displayed on donor-derived passenger APCs.[24-26] In contrast, activation of $CD4^+$ T cell population through the indirect pathway is more common in chronic or late acute rejection.[25,27,28] It is well-established that before the arrival of Tregs, host Teffs migrate to the graft site at first.[4] Following the identification of donor MHC II antigens through direct and semi direct pathways, $CD4^+$ Teffs become activated. Activated $CD4^+$ Teffs not only contribute to acute allograft rejection but also vastly raise graft rejection by helping direct alloantigen-reactive $CD8^+$ T cells. Upon activation, direct alloantigen-reactive $CD8^+$ T cells target MHC I alloantigen in the graft and further decrease graft survival.[25] Conversely, indirect alloantigen-reactive $CD4^+$Teffs by providing help for $CD8^+$ T cell allo-immune responses contribute to chronic rejection.[25] Worthy of note, indirect pathway $CD4^+$ T cells are also crucial helper cells for B cell alloantibody production which in turn leads to acute alloantibody-mediated rejection.[25,29]

These findings propose that to suppress both acute and chronic graft rejections, Tregs require direct, semi-direct, and indirect allo-specificity.[25,30] Principally, direct alloantigen-reactive Teffs and Tregs are more abundant than indirect alloantigen-reactive Teffs and Tregs in human blood.[28,30-32]Thus, direct alloantigen-reactive Teffs are able to respond to the transplant without undergoing clonal expansion in lymph nodes.[4] There is preclinical evidence that direct donor-alloantigen-reactive Tregs (dar-Tregs) regulate graft rejection by first infiltrating to the graft to limit organ injury and

then migrating to regional lymph nodes and contribute to the induction and maintenance of tolerance.[4] Remarkably, alloantigen specificity of Tregs for the induction and maintenance of tolerance might be different. This indicates that induction of tolerance requires Tregs with direct alloantigen specificity, while maintenance of tolerance needs Tregs with indirect alloantigen specificity. Induction of tolerance by direct Tregs not only serves to regulate graft damage and rejection but also allows indirect-alloreactive Tregs to expand and establish long-term graft tolerance.[4] It is noteworthy, during the allo-immune response both direct and indirect Tregs proliferate and migrate into the organs but they are not able to completely regulate allo-immune responses, and as a result, organ injury occurs.[33] Hence, in order to generate immune tolerance, Teffs responses need to be attenuated by Tregs.[4] One useful approach to the establishment of tolerance and prevention of rejection is to dominate Tregs in the graft site by preventing Teffs graft infiltration.[4,34-36] Another strategy is to increase Tregs number[4] which can be simply augmented through the promotion of endogenous Tregs or by infusion of *ex vivo*-expanded Tregs; nonetheless, each strategy has its own cons and pros.[4] Compared with promoting endogenous Tregs, infusion of *ex vivo*-expanded Tregs provides the advantages of manageable Treg specificity, dosage, and therapy timing.[22]

Various protocols compliant with Good Manufacturing Practices (GMP) have been designed for *ex vivo* expansion of Tregs either polyclonally or antigen-specifically.[22,37,38] For *ex vivo* expansion of polyclonal Tregs (poly-Tregs), Tregs isolated from peripheral blood mononuclear cells (PBMCs) are activated and expanded using anti-CD3/CD28 beads and interleukin 2 (IL-2).[39] Alternatively, exposing Tregs to allogenic APCs during *ex vivo* expansion leads to the proliferation of dar-Tregs.[22] It is important to emphasize that Tregs therapy with poly-Tregs demands a large number of cells. Besides, undesirable effects such as systemic immune-suppression are most likely to occur after the adoptive cell transfer of poly-specific Tregs.[19] Whereas, the therapeutic application of dar-Tregs has lower non-specific immune-suppression than poly-Tregs and also minimizes the costs, as well as reduces the number of cells required for infusion.[36,40-42] Moreover, dar-Tregs efficiently infiltrate to sites of inflammation, where they can induce bystander suppression, and result in less non-specific immune-suppression.[43] Eventually, it is more convenient to apply these cells, especially in the settings that target antigen, and the time of antigen exposure is known, taking transplant condition into consideration.

TREG CELL THERAPY IN SOLID ORGAN TRANSPLANTATION

Promising results in treating inflammatory and autoimmune diseases have encouraged the clinical use of Tregs for developing transplantation tolerance.[39] In various studies, the safety profile and potential efficacy of adoptive Tregs transfer to prevent Graft versus Host Disease (GVHD) after hematopoietic stem cell transplantation have been shown.[44-50] Likewise, it has been demonstrated that poly and dar-Tregs can prevent graft rejection after solid organ transplantation,[51-53] proposing that reinforcing Treg-mediated suppression might be an interesting approach for developing transplantation tolerance (Table 1A). In order to evaluate the safety and efficacy of *ex vivo*-expanded poly- Tregs and direct dar-Tregs, clinical trials applying these cells in kidney and liver transplant recipients have been approved[4] (Table 1B). Concisely, autologous $CD4^+$ $CD25^+$ $CD127^-$ Tregs are separated by

Table 1. Various Studies and Clinical Trials on Treg Therapy in Solid Organ Transplantation

A. Infusion of Tregs in Solid Organ Transplantation			
Study Design	**Phase**	**Outcome**	**Ref**
A single infusion of 0.43 × 10^6 to 6.37 × 10^6 dar-Tregs /kg of on day 13 post-liver transplantation	I/IIA	Utilizing *ex vivo*-expanded dar-Tregs is safe and effective for minimization of immunosuppressive drugs and development of operational tolerance in liver recipients with non-immunological liver diseases	51
A single infusion of 320 × 10^6 $CD4^+$ $CD25^+$ $CD127^{low}$ poly-Tregs to kidney transplant recipients on immune-suppression with sub-clinical graft inflammation	I	No infusion-related reactions and no infections or malignancies observed during the one-year follow-up period	52

Table 1. Continued

Study Design	Phase	Outcome	Ref
A single infusion of 0.5, 1, and 5 × 10^9 poly-Tregs into three groups of kidney recipients 60 days post-transplantation	I	* All doses of Treg therapy examined were safe with no adverse effects over two years post-transplant * Expanded Tregs potently suppressed allo-responses and promoted de novo generation of Tregs in the recipient's allo-responders *in vitro* **In vivo*, expanded Tregs augmented circulating Treg levels in a constant manner	53
B. Ongoing and Completed Clinical Trials Using Tregs to Prevent Rejection in Solid Organ Transplantation			
Study Design	**Phase**	**Aim**	**Trial ID**
A single infusion of 1 to 10 × 10^6 $CD4^+$ $CD25^+$poly-Tregs /kg 5 days post renal transplantation	I/II	To examine the safety and feasibility of Treg populations	NCT02129881
Injection of escalating doses of 0.5, 1 and 3 × 10^6 $CD4^+$ $CD25^+$poly-Tregs /kg to kidney recipients	I/II	To investigate the safety and the ability of poly-Tregs to polarize the immunological response towards graft acceptance	NCT02371434
A Single infusion of 300 × 10^6 and 900 × 10^6 dar-Tregs ten days post renal transplantation	I/II	To investigate the safety and feasibility of dar-Tregs	NCT02244801
A single infusion of 300 × 10^6 and 900 × 10^6 belatacept (CTLA4 blocking Ig) anergized dar-Tregs ten days after kidney transplant	I/II	To investigate the safety and feasibility of dar-Tregs	NCT02091232
Two injections of 200 × 10^6 $CD4^+$ $CD25^+$ $CD127^{low}$ $foxp3^+$ poly-Tregs 30 days and six month post renal transplantation	I /II	To develop a new strategy that will be more effective in preventing organ rejection	NCT01446484
Infusion of $CD4^+$ $CD25^+$ poly-Tregs 2 month post renal transplantation	I	To assess the safety profile of Treg adoptive cellular transfer	NCT02145325
A single infusion of poly-Tregs in renal transplant recipients	N. A	To evaluate the safety and effectiveness of collecting, expanding and infusing Tregs to renal transplant recipients who are using Zortress (Everolimus) as immunosuppressive therapy	NCT03284242
Infusion of 5 to 10 x 10^6 cells /kg autologous expanded Tregs 6 months post-renal transplantation	IIb	To demonstrate the efficacy of Treg-based immunotherapy	ISRCTN11038572
A single infusion of 320 × 10^6 $CD4^+$ $CD25^+$ CD127-poly-Tregs to subjects with inflammation on their six month surveillance biopsy post renal transplantation	I	To test the safety of the experimental therapy of a single infusion of Tregs	NCT02088931
A single infusion of 400 × 10^6 polyclonal and dar-Tregs to subclinical inflammation in kidney transplantation on their six month surveillance biopsy post renal transplantation	I/II	To determine the safety and efficacy of autologous polyclonal and dar-Tregs in renal transplant recipients with subclinical inflammation	NCT02711826
A single infusion of 1 × 10^6 and 4.5 × 10^6 $CD4^+$ $CD25^+$ poly-Tregs /kg 12 weeks post-liver transplantation	I	To test the safety of poly-Tregs	NCT02166177
A single infusion of 400 × 10^6 dar-Tregs to liver transplant recipients	I/II	To test the safety and feasibility of dar-Tregs infusion	NCT02474199
A single infusion of 50, 200, and 800 × 10^6 dar-Tregs to three cohorts of liver transplant recipients	I	To test the effects of receiving one of three different doses of dar-Tregs while taking a specific combination of drugs after liver transplantation	NCT02188719
A single infusion of 2.5 to 500 × 10^6 donor alloantigen-specific Tregs (arTreg-CSB) to liver transplanted patients	I/II	To test immune-suppression withdrawal in liver transplant recipients by using Tregs	NCT03577431
Multiple infusions of 1 × 10^6 $CD4^+$ $CD25^+$ CD127-darTregs/kg at several intervals to liver transplant recipients	I	To test multiple infusions of dar-Tregs on tolerance induction	NCT01624077

Abbreviations: dar-Tregs, donor alloantigen reactive Tregs; Poly-Tregs, *ex vivo*-expanded polyclonal Tregs; N.A., not applicable; arTreg-CSB, alloantigen-reactive T regulatory cells costimulatory blockade.

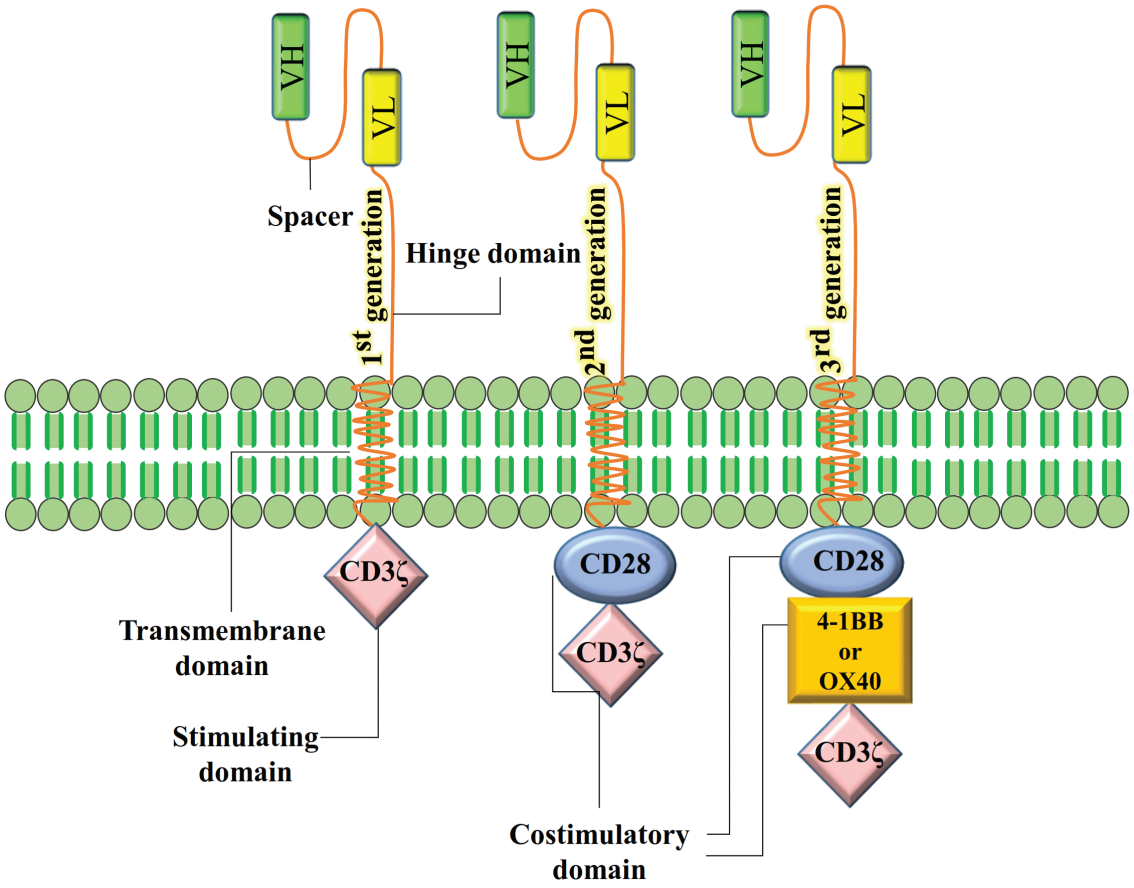
apheresis. Of note, though Tregs can be isolated from various sources including peripheral blood, umbilical cord blood and discarded thymuses, they commonly isolated from peripheral blood

for clinical application. Following isolation, Treg enrichment is performed using immunomagnetic selection (CliniMACS, Miltenyi BioTec). After they get enriched, Tregs must be expanded *ex vivo* by either polyclonal or antigen-specific stimulation for 2 to 3 weeks. Addition of mTOR inhibitors and IL-2 is crucial during this step. Expanded cells require meeting the release criteria such as: high viability and low level of contamination with $CD8^+$ and $CD19^+$cells. Quality assessed products then administrated into the lymphodepleted patients.[22,54,55]

Notably, in these trials, Tregs are administered into patients at different times. Theoretically, Tregs could be transferred to transplant recipients at any time, including at the time of organ transplantation or even as rejection rescue therapy. For some reasons; however, the focus of Tregs therapy has been Treg infusion near the time of implantation. First, since the ischemia-reperfusion injury triggers immune responses,[56-59] the immediate post-transplant period may be an excellent therapeutic window of Tregs therapy. Second, the period after induction therapy may also be a suitable time to change the balance of Tregs and Teffs towards an increased regulatory response. Additionally, it may preferentially change the balance between naïve/effector T cells during immune reconstitution.[60-62] Fourth, lympho-depleting agents such as rabbit anti-thymocyte globulin (ATG) might exert beneficial effects on Tregs.[63] However, despite such theoretical benefits of Tregs therapy following lympho-depletion, data from non-human primate models are contentious.[63,64] This controversy implies that several factors may affect the usefulness of administered Tregs. For instance, inflammation induced following ischemia may attract infused Tregs into the organs, but several factors may impede the functions of these cells. Whereas, some depleting agents promote Treg expansion, others such as calcineurin inhibitors will deplete the infused Tregs. Remarkably, severe inflammation after transplantation inhibits *de novo* Treg generation and promotes Tregs conversion into Teffs. Therefore, it appears that the ideal time for infusing Tregs is when there is low level of inflammation in the graft.[4] Another important consideration is that various doses of Tregs had been administered in the patients in these trials. Although it has been shown that increasing Tregs to at least 30% of T conventional seems effective to improve graft survival, the optimal dose of Tregs is not clear yet.[22] Furthermore, though in most studies a single infusion of Tregs have been applied,[21] it is still a matter of debate whether a single or multiple Tregs infusions are adequate to provide long-lasting protection.[65]

More Importantly, all these studies depend on expanding Tregs with APCs to proliferate direct dar-Tregs, possibly because these cells can be produced more readily than indirect dar-Tregs.[25] It is worthy of note that compared with direct dar-Tregs, Tregs with indirect allo-specificity have lower precursor frequency in the original polyclonal cells; thus, they cannot be efficiently expanded *in vitro* using allogeneic APCs.[20] Besides, antigen-specific Treg expansion by APC requires massive *in vitro* expansion, which in turn results in loss of Foxp3 [66], telomere length shortening, and decreased *in vivo* survival.[67] Therefore, alternative strategies such as genetic engineering required generating antigen-specific Tregs specific for a particular antigen. There are currently three approaches to generate antigen-specific Tregs *in vitro*. One strategy is via genetic modification of TCR on these cells. This strategy has been investigated in GVHD and solid organ transplant rejection.[68,69] Although Tregs engineered with TCRs seem to be promising,[19,20] issues such as TCR mispairing and MHC-restriction limit their therapeutic application.[70] An alternative strategy is to convert antigen-specific Teffs into Tregs via enforced over-expression of Foxp3. This approach has been applied in various studies; nonetheless, it poses the potential risk of generating unstable or intermediate Teffs in converted cell populations.[20] The third strategy is to use genetically modified CAR Tregs, which are MHC-independent and can be used in a larger number of patients.[19,71] Main studies and clinical trials on Treg therapy in the context of solid organ transplantation is outlined in Table 1.

Antigen specificity is a crucial factor for successful Tregs therapy, therefore re-educating Tregs via a CAR against a transplant-relevant antigen confers to the patient a long-term graft function. Generally, CARs contain an extracellular single-chain variable fragment (scFv) antigen–binding domain against the desired surface antigen bound to the intracellular signaling domains of T cells (Figure 1).[71] In brief, PBMCs separate from leukapheresis or peripheral



Schematic Representation of Chimeric Antigen Receptor (CAR). CARs consist of a binding domain, a hinge domain, a TM, and the signaling domain. Commonly the binding moiety involves the variable regions of heavy (VH), and light chain (VL) of a monoclonal antibody joined together by a flexible spacer to form an scFv molecule. The first-generation CAR is comprising a signaling domain that is composed of CD3ζ-chain or similar signaling domains. In second- and third-generation CARs, one or more costimulatory signaling domains are included (e.g., CD28, 4-1BB (CD137), OX-40 (CD137), within their signaling domain (Abbreviations: TM, transmembrane domain; scFv, single-chain fragment variable).

blood. Tregs isolated from PBMCs and then expand by anti-CD3/28 beads and IL-2 (with or without rapamycin). The resultant cells are then transduced using a GMP-grade viral vector expressing the CAR construct. The developed CAR-Treg product is further expanded by continuous medium supply and re-stimulation with GMP-compliant anti-CD3/CD28 magnetic beads and (high) exogenous IL-2. QC assays for sterility, identity, and functionality are implemented at the end of the manufacturing workflow. The final CAR-Tregs product can be further expanded until the desired number of cells is reached then cryopreserved or immediately infused back into the patient.[39]

Generally, three generations of CARs have been developed to enhance the survival and function of CAR-engineered cells. In first generation CARs the signaling domain comprises that of the ζ chain of the CD3 complex. Second-generation CAR constructs contain the CD3ζ signaling domain and one co-stimulatory domain such as CD28, 4-1BB, or OX40. Third-generation CAR utilizes multiple co-stimulatory domains, such as 4-1BB or Ox40 (Figure 1).[72]

Although Tregs can have a suppressive function in an antigen non-specific manner, CAR-mediated activation of cells is antigen-specific, such that the level of local antigen expression determines whether the cell becomes activated.[20] Direct recognition of an antigen on target cells by CARs could also lead to Treg cell-mediated targeted killing via the perforin/granzyme B pathway. CARs can also provide a unique opportunity to target Tregs to the site of tissue destruction or transplanted tissues.[20] There have been studies conducted using CARs Tregs in the context of autoimmunity,[73-77] leading

researchers to test the hypothesis that this method could be useful in the setting of transplantation.[71] Compared with poly-Tregs, CAR Treg-mediated immune-suppression is more specific with fewer adverse effects.[19] Moreover, CAR Tregs are more effective in suppression of allo-responses than poly-Tregs as antigen-specific CAR Tregs tend to infiltrate to a target organ containing a specific antigen and activate via their CARs.[19,78] It is well accepted that by engineering Tregs to express CAR, these cells can confer specificity for donor MHC-I antigen.[79] Of note, MHC-specific CAR Tregs identify donor MHC antigens by the direct and semi-direct routes, but not by indirect pathway; therefore, contribute more efficiently in the prevention of acute *vs*. chronic rejection. It would seem likely; however, that anti-donor MHC CAR Tregs activated in the graft overcome chronic rejection.[40] Given that human leukocyte antigen (HLA)-A mismatching is often related to poor outcomes after transplantation, HLA-A2 is considered a potential target antigen for generating antigen-specific Tregs to induce transplantation tolerance.[19,79,80] A2-CAR Tregs are able to provoke a vigorous immune response against a single HLA mismatch.[71] Results of a research performed by MacDonald *et al.* demonstrated that HLA-A2–specific CAR Tregs (A2-CAR Tregs) were suppressive and more potent at inhibiting GVHD than unmodified Tregs in the immunodeficient NSG (NOD scid gamma) mice,[71] showing the rationale for engineering Tregs to express a CAR as a means of developing therapeutic antigen-specific cells. In another study, data revealed that Tregs modifications to express an HLA-A2–specific CAR alter nTregs specificity without alteration of their phenotypes and stability.[80] In addition, activation of the A2-CAR Tregs resulted in more potent cell proliferation, up-regulation of CD39 molecule, and inhibited allo-specific Teffs expansion *in vitro* more potently than that of unmodified nTregs. Moreover, A2-CAR-Tregs infiltrated to skin grafts and contributed to the survival of HLA-$A2^+$ human skin grafts.[80] These findings were proved by Boardman and colleagues who found that human A2-CAR Tregs were able to preferentially migrate to HLA-$A2^+$ tissues and inhibit rejection of skin transplants in a human skin xenograft transplant model.[79] Antonio *et al.* developed monoclonal antibody (mAb) CAR Tregs expressing a fluorescein isothiocyanate (FITC) –targeted CAR that could be activated flexibly by numerous mAbs conjugated to FITC. They confirmed that mAbCAR Tregs maintained their phenotypes and regulatory functions. Furthermore, mAbCAR Tregs inhibited GVHD and prolonged survival of islet and secondary skin allografts in mice.[81] Recently, it has been shown that A2-CAR $CD8^+$ Tregs maintain their original phenotype and are potent suppressors of allo-immune responses triggered by HLA-A*02 mismatch *in vitro* and *in vivo*.[40] Dawson *et al.*, by using a panel of 20 humanized A2-CARs (hA2-CAR), showed that humanization would reduce HLA-A cross-reactivity. Moreover, they found that hA2-CAR Tregs were different in terms of A2-CARs expression, their ability to bind to HLA-A*02:01, as well as stimulation of human Treg suppression *in vitro*. Also, hA2-CAR Tregs infiltrated into $A2^+$ allografts, suppressed HLA-$A2^+$ cell-mediated xenogeneic GVHD, and reduced rejection of human HLA-$A2^+$ skin allografts.[82] Despite the above-mentioned encouraging results, care should be taken while interpreting the results of studies employed a xenogeneic GVHD model to investigate the efficacy of CAR Tregs *in vivo.* This is due to the fact that xenogeneic disease and human GVHD or GVHD in allogeneic transplant models are different.[78] Notably, human Tregs in such hosts survive for a short-term; thus, they are active only during the initiation phase of the disease, but not for the long term.[78] Safety aspects of CAR Tregs in HLA-A2–transgenic NSG mice, should also be interpreted with caution. Despite lack of tissue injury following A2-CAR Tregs, it should be noted that immune-deficient NSG mice lack essential cells and effector mechanisms/molecules involved in tissue damage following recognition of HLA-A2 on target tissues by CAR Tregs.[78] Another important consideration is environmental differences, meaning that immunological experiences are highly complex in human patients when compared with laboratory pathogen-free rodents. Both time and immunological challenges may affect tolerance.[83] Also, there are still several un-answered questions that remained to be answered. First, will A2-CAR Tregs maintain their stability and function during *ex vivo* expansion and *in vivo* graft recognition? Second, will A2-CAR Tregs create HLA-A2–specific tolerance in immune-competent hosts? Third, would A2-CAR Tregs therapy be as effective in stopping allograft rejection after initiation?

CHALEENGES WITH TREGS CAR TREGS CELL THERAPY

While preclinical data provide a clear rationale for Tregs adoptive transfer to develop transplantation tolerance, there are challenges in the clinical translation of this technology.[4] The current Tregs/CAR Tregs therapy encounters various challenges such as cell isolation, purity, yield, expansion, vector production and transduction, stability, *in vivo* persistence, trafficking, product release testing and variation in cell product.[3,39] Table 2 files several essential obstacles of Tregs/CAR Tregs manufacturing procedure.

CONCLUSION

It is well recognized that Tregs promote immune

Table 2. Strategies to Overcome Challenges Associated with Tregs/CAR Tregs Therapy

Challenges	Overcoming Strategy(s)	Considerations	Ref
Tregs isolation using GMP-compliant semi-closed MACS devices has problems such as: Lack of identification of $CD25^{hi}$Tregs Lack of selection of Tregs based on multiple parameters Low purity of Tregs	- To solve the problem applying FACS (MACSquantTyto cell sorter) has various benefits including: - High post-sort purity by using more selective markers, such as $CD45RA^{+}$ and $CD127^{low}$ - Treg isolation based on the highly researched markers of suppression, stability, and specificity - High yields for *ex-vivo* expansion	Effective preclinical sorting strategies based on FACS that isolate highly pure Tregs (> 98%) are not yet GMP-compliant	3, 39, 68, 84-86
Contamination of the isolated Tregs with Teffs	-Use of thymus-derived natural Tregs as starting population -Addition of the rapamycin during Treg expansion	As memory Tregs are most prominent in adults, use of thymus-derived natural Tregs for Tregs/CAR Tregs therapy is associated with lower yields	3, 39, 80
Lack of *in vivo* persistence of Tregs/CAR Tregs	Below strategies are found to improve *in vivo* persistence of CAR-T cells: Administration of low-dose IL-2 Inclusion of both CD28 and CD137 costimulatory domains in CAR construct Incorporation of IL-2 receptor β-chain in CAR, telomerase reverse transcriptase co-transduction or treatment with PI3Kinhibitor		19, 20, 39, 87
Lentiviral-based manufacturing strategies for CAR T-cell production are time consuming, labor-intensive and expensive and also carry the risk of causing oncogenic changes due to random integration of the transgene	- Use of safe and cost-effective non-viral approaches - Use of plasmid-based sleeping beauty system - Use of genome editing technologies such as CRISPR-Cas9	Regarding gene editing strategies it remains to investigate how these strategies affect Tregs homeostasis and function	20, 39
Applying medium containing either fetal calf serum or human serum during Tregs/CAR Tregs expansion step, have limitations regarding biosafety and sufficient supply for scaling up	Optimization of serum-free, xeno-free medium for Tregs/CAR Tregs expansion		39
Instability of Tregs/CAR Tregs *in vivo*	- Develop more stringent guidelines to release high-standard products - Generating a suitable *in vivo* condition by selecting therapy timing and concomitant immunosuppression - Monitoring Tregs/CAR Tregs products after infusion - Transcriptional, post-translational and epigenetic editing - Addition of rapamycin during Tregs/CAR Tregs expansion	PTregs are less stable than nTregs under lymphopenic conditions, suggesting that nTregs may represent a better population for ACT	4, 20, 39, 88
Low number of Tregs in adult peripheral blood	- Polyclonal expansion of Tregs *ex vivo* - Utilizing UBC to isolate Tregs has numerous advantages including: a) A large number of Tregs can be isolated from one unit of UBC b) $CD25^{high}$Tregs are purified more readily from UCB c) Tregs isolated from UCB are devoid of $CD25^{+}$ memory T cells	No GMP expansion protocol has been developed yet	3, 14, 68, 89

Table 2. Continued

Challenges	Overcoming Strategy(s)	Considerations	Ref
Tregs/CAR Tregs homing capacity	Use of below approaches to dictate the homing potential of therapeutic cells: Gene-editing technologies Culturing Treg together with certain cytokine combinations during *ex-vivo* expansion Combining ectopic chemokine receptor, cytokine receptor or transcription factor expression with antigen specificity via a CAR or TCR		14, 20, 39, 54, 70
QC assays for release of Tregs/CAR Tregs products	- Standardization of QC assays - Determining the optimal dose of Tregs/CAR Tregs products for clinical application - Designing robust and predictive functional assays - Rapid formulation procedure to maintain the viability of cell product - Standardization of cryopreservation		20, 22, 39, 80
- High batch-to-batch variation for each Treg product - The limited source of autologous Tregs	- Use of third-party allogeneic CAR Tregs products represent many benefits such as: - Opening the possibility of using cells isolated from sources other than peripheral blood - One donor could provide standardized and low-cost therapeutic cells for multiple patients - To maximize the quality of the CAR T cell product, donors with a T cell phenotype associated with superior T cell function can be chosen - Re-dosing for the same patient can be done more readily	- Patient's T-cells will reject infused third-party non-HLA matched CAR T cells - TCRαβ on third-party CAR Tregs recognizes HLA mismatch and results in pan-immunosuppression Note: Generating HLA I /TCR negative Tregs products will offer unique opportunities in the development of off-the-shelf products	20, 70, 90-92

Abbreviations: GMP, good manufacturing practice; FACS, fluorescence-activated cell sorting; UBC, umbilical cord blood; ACT, adoptive cell therapy; QC, quality control.

tolerance by controlling immune responsiveness to alloantigens. In the light of this evidence, utilizing non-modified or modified Tregs, in order to establish or improve transplantation tolerance following solid organ transplantation has gained increasing attention during the past years. It is important to emphasize that Tregs therapy with poly-Tregs needs a large number of cells and result in non-specific immune-suppression. Contrarily, therapeutic application of dar-Tregs has lower side effects and also minimizes the costs, as well as reduces the number of cells required for infusion. Due to difficulties with the expansion of dar-Tregs by allogeneic APCs, alternative approaches such as genetic engineering of Tregs with genes encoding CARs to generate antigen-specific Tregs *in vitro* have gained increasing attention over recent years. Despite the growing knowledge of Tregs specificity and functionality, as well as success in developing of Tregs/CAR Tregs product, creates a unique opportunity to induce tolerance in the transplant recipient, there are still challenges in the clinical translation of this technology. The current Tregs/CAR Tregs therapy faces various essential challenges such as cell isolation, purity, yield, expansion, vector production/transduction, stability, *in vivo* persistence, trafficking, product release testing and variation in cell product. In view-point of improving the outcome of Tregs/CAR Tregs therapy issues such as the longevity of Treg-induced tolerance, the toxicity of Tregs/CAR Tregs products, the long-term impact of the therapy against infections and malignancies, the function of Tregs/CAR Tregs, the effective concomitant immune-suppression, Tregs/CAR Tregs tracking *in vivo*, and the proper target antigen and co-stimulatory signaling domain for CAR Tregs also required to be addressed in upcoming years.[3,4,21,22] Upon removing these obstacles, Treg therapies by Tregs/CAR Tregs products to alleviate or even achieve the aim of complete weaning in transplantation settings would become unlimited.

GLOSSARY

Fluorescein Isothiocyanate (FITC)

A derivative of fluorescein with an isothiocyanate reactive group, rendering it reactive towards amine and sulfhydryl groups found in biomolecules. FITC is used in wide-ranging applications including flow cytometry.[93]

NOD Scid Gamma (NSG) Mouse

A brand of immunodeficient laboratory mice lacking mature T, B, and natural killer cells. These mice are also deficient in multiple cytokine signaling pathways with defected innate immunity. [94]

AUTHOR CONTRIBUTIONS

HN and AHR reviewed the literature, wrote, revised, and edited the article. AS contributed to the final revision of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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Correspondence to:
Alireza Heidary Rouchi, MD, PhD
Liver Transplantation Research Center, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran 1419733141, Iran
Tel: 0098 21 61192659
Fax: 0098 21 66581657
E-mail: rouchiah@tums.ac.ir

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