Adoptive Chimeric Antigen Receptor T Cell (CAR-T) and Treg Cell-Based Immunotherapies: Frontier Therapeutic Aspects in Cancers

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Received: 14 Feb. 2021; Accepted: 27 Sep. 2021

Abstract- Based on this point that some cancers do not appropriately respond to conventional therapy, and there is the possibility of relapse, immunotherapy is currently under investigation. Cancer immunotherapies are widely recognized as transformational for several cancers and enable to move to the front-line therapy with few side effects. One of its new branches is treatment with T-cells that have been changed their receptor. The research on these cells is generally according to the design of a receptor against a specific tumor antigen. Also, manipulation of regulatory T-cell (Tregs), as the barriers to proper immune responses in the tumor microenvironment, will promote Tregs-targeted therapeutic opportunities and improve the efficacy of the current cancer treatment, such as radiation and chemotherapy. This review attempts to show novel insights into the roles of Tregs in cancer which can be considered a promising anticancer therapeutic strategy for targeting them and approaches for the generation of tumor antigen-specific T lymphocytes (AST) using chimeric antigen receptors.

Acta Med Iran 2021;59(10):570-577.

Keywords: Receptors; Chimeric antigen; T-lymphocytes; Regulatory; Tumor microenvironment; Immunotherapy; Adoptive

Introduction

Immunotherapy is based on T-cell receptors, such as T-cells with the chimeric antigen receptor (CAR) and tumor-associated-antigen (TAA) T-cells that lead to increased graft-versus-leukemia (GVL) effect (1). T-cells having CAR are used to treat relapsed diseases and as an auxiliary treatment for high-risk patients after bone marrow transplantation (1).

Like all treatments, toxicity is a serious concern for T-cell receptor-based immunotherapy that occurs in two ways. At first, when a non-malignant host tissue expresses the same antigens associated with the tumor, second, the cytokine release syndrome (CRS) happens (2). Actually, CRS occurs in T-cell immunotherapy with the proliferation of T-cells in vivo. The released cytokine in these conditions is IL-6. Tocilizumab is a monoclonal antibody against IL-6 commonly used as first-line treatment for CRS and corticosteroid therapy in the second line of treatment (3). CRS can be due to massive T-cell stimulation and the secretion of inflammatory cytokines such as IL-2, IL-7, IL-15, IL-21, and IFN-β, which increase the expression of programmed cell death protein 1 (PD-1) (2).

According to the results reported by Vinay Prasad in 2017, Tisagelnelcleucel is the first anti-CD19 CAR T-cell approved by the FDA (4). In a study, a complete remission (CR) in a large population of B-ALL recurrence patients treated with CD19 CAR T has been seen (5). However, CD19 CAR T may be associated with toxicity such as high fever, hypertension, hypoxia, and neurological disorders. The CRS syndrome has been observed in clinical and laboratory studies in a specific and small group of patients (6). This current study highlights approaches for targeting Tregs present within the tumor microenvironment (TME) regarding their

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primary roles in cancers and the generation of antigen-specific T lymphocytes (AST) using CAR.

The major effects of Tregs in cancers

Tregs are able to inhibit undesirable immune responses to insider antigens, allergens, and normal flora. They are commonly seen in TMEs of solid tumors and help the cancerous cells escape from host immunity. Repression mechanisms of Treg are as follows: cell-to-cell contact with inhibitory surface molecules; depriving IL-2 of effector T-cells by highly expressed IL-2Rs on Tregs; releasing inhibitory cytokines and other mediators such as IL-10 and TGF-β, IL-35, and gerenzyme (7,8). Extensive studies have been performed evaluating the clinical potential of Tregs-targeted treatment, including Anti-CTLA-4, Anti-LAG-3, Anti-GITR, Anti-CD25, Anti-TGF-β and, Anti-TIM-3. The effect of each transcription factor, secretory soluble mediators, and inhibitory receptors of Treg cells has been explained in Table 1.

PD-1 and Cytotoxic T lymphocyte antigen (CTLA)-4 are inhibitor receptors that regulate T-cell activation negatively (9). PD-1 increases the levels of Indoleamine 2,3-dioxygenase (IDO), which plays an essential role in increasing the exhausted T-cell, regulatory T-cells (Tregs), and is associated with incremented metastasis (10,11). CTLA-4 is a target gene of the transcription factor Foxp3 (12) and is thus expressed predominantly on Tregs. CTLA-4 was the first inhibitory immune checkpoint to be identified (13). Anti-CTLA4 antibodies (Ipilimumab (IgG1) and Tremelimumab (IgG2)) inhibit the function of Treg and lead to anti-tumor function in patients with metastatic melanoma (14). Studies in mice have shown that anti-CTLA-4 antibodies can lead to Fc-mediated depletion of intra-tumoral $FOXP3^+$ Tregs selectively (15). Tregs are effective barriers to anti-tumor immunity; therefore, anti-CTLA-4 antibodies have a therapeutic effect in cancer by blocking the function of CTLA-4 in these cells or by inducing selective depletion of Tregs in the tumor microenvironment (16).

In the presence of TGF-β, the interaction of PD1 with PDL-1contributes to the reduction of the PI3K / AKT signaling pathway and consequently limits T-cell responses to the tumor antigen. Also, this binding inhibits expansion, survival, and effector T-cells functions, inducing the differentiation into the $FOXP3^+$ Tregs and promoting the apoptosis of tumor infiltrative lymphocytes (TIL) in TME (17). Moreover, the high level of PD-1 expression reflects T cell exhaustion status (17). Increasing TIL is associated with a better prognosis in various solid tumors, which indicates an active immune system that controls tumor growth (18). The anti-PD-1 antibody, Nivolumab, is another critical safety checkpoint to block the PD-1/PD-L1 immunity (19). It induces anti-tumor immune responses, diminishes the count and activity of Tregs in TME (20).

Chemokines are also indispensable factors that can influence immune cell infiltration into the tumor and regulate angiogenesis, proliferation, and survival of tumor cells. The targeting of chemokines, if accompanied by existing treatments for cancer, can have synergistic effects on immune responses (21). CCL22 chemokines derived from the tumor cells associated with CCR4 at the surface of Tregs induce the migration of Tregs to the TME. Therefore, several studies have shed new light on CCR4/CCL22 as therapeutic targets in breast and ovarian cancers (22,23). Presenting many Tregs in gastric and ovarian cancers was related to poor prognosis (18).

Although many $FOXP3^+$ Tregs in human ovarian cancers have a higher degree of cell death than $FOXP3^-$ Tregs, these cells still sustain and amplify their suppressor capacity (8).

The death susceptibility of apoptotic Tregs is influenced by an impaired nuclear factor (erythroid-derived 2)-like 2 (NRF-2) mRNA, protein, and transcriptional activity, resulting in attenuated protection against reactive oxygen species (ROS) enriched in the TME. Consequently, cell membrane damage and eventually apoptosis occur (8,24) CD73 and CD39 receptors on the apoptotic Tregs surface cause ATP-to-adenosine conversion and mediate immunosuppression via the adenosine and A_{2A} pathways. Thus, the data support a model wherein Tregs in TME have the ability of suppressive function through oxidative stress (25) (Figure 1).

Neuropilin (NRP) is expressed by most Tregs and is vital for their survival and stability in the TME, but they are not essential for peripheral immune tolerance. Treg-restricted deletion of NRP1, as an interferon-gamma (IFN-γ) producer method, induces the formation of fragile Tregs, so identifying these cells can improve immunotherapy for human cancer. Tregs deficient NRP1 produces IFN-γ required to respond to anti-PD-1. It increases anti-tumor immunity and can remove the tumor quickly (26). IFNγ can limit Treg production and drive Treg fragility to promote anti-tumor immunity. Treg fragility is needed to respond appropriately to immunotherapy based on targeting PD-1 (27).

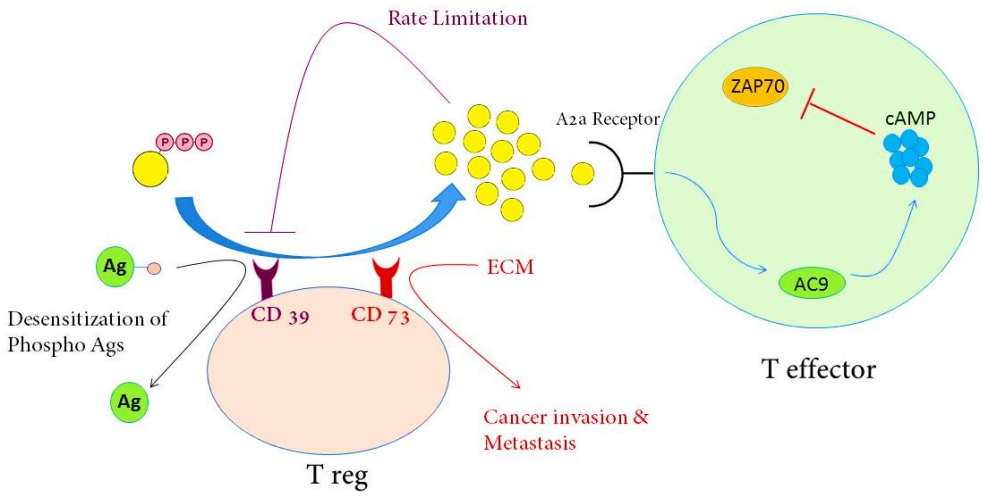

Figure 1. Treg-mediated inhibitory effects on other immune cells and its key ectoenzymes. CD73 and CD39 receptors on the Treg surface cause ATP-to-adenosine conversion. The adenosine influences the A2a receptor via extracellular space and activates it. The concentration of cAMP is increased by AC9 receptor, which results in inhibition of TCR signaling by inhibiting zap70 phosphorylation. CD73 alone can play a role in the development of metastatic and invasive cancer. CD39 is the rate-limiting enzyme regulated by high-dose adenosine and can dephosphorylate and cause desensitization of phospho-antigens. ATP: Adenosine-triphosphate, cAMP: Cyclic adenosine monophosphate, AC9: Adenylyl cyclase 9, TCR: T-cell receptor, ZAP70: Zeta-chain-associated protein kinase 70, ECM: Extracellular matrix

Table 1. The important transcription factors, secretory soluble mediators, and inhibitory receptors of regulatory T-cell

Transcription Factor			References
Foxp3		Foxp3 is the main regulator in the development and function of Treg, which is controlled by RUNX-CBFβ, CREL, STAT5, TCR, IL2, and cAMP.	28
Secretory soluble mediator	**cAMP**	Tregs have large amounts of cAMP that transmit them via gap junction to the effector T-cells and cause inhibitory activity on these cells.	29,30
	TGF-β	TGF- β secreted by Tregs is an inhibitory cytokine important for Treg-induced inhibition of the exocytosis of granules and the cytolytic function of $CD8^+$ T cells	31
	NRP-1	NRP-1 **is** an imperative factor for the Treg cell to prevent anti-tumor responses.	32
	Amphiregulin	Amphiregulin is an activating ligand of the epidermal growth factor receptor. On the other hand, it promotes cancer and, by maintaining the health of the tissue, prevents tumor progression.	33-35
	IL-34	IL-34 is a Treg-specific cytokine and mediates immune tolerance	7
	IL-35	Treg-derived IL-35 is an anti-inflammatory cytokine such as TGF-β and IL-10. It is very high in the tumor environment and leads to increase exhausted T cells lost robust effector functions.	36
Inhibitory Receptor	**CD39, CD73**	CD39 and CD73 receptors convert ATP/ADP to Adenosine, which results in inhibition of TCR signaling.	37
	CTL-4	Loss of CTLA4 in Tregs leads to increasing Treg number and impaired suppressive activity of them.	38
	PD-1	PD-1 is a cell surface receptor that weakens adaptive immune responses.	39
	TIGIT	TIGIT is expressed on natural Tregs and causes Treg differentiation and has a crucial role in immunosuppression.	40
	LAG-3	LAG-3 is essential for Tregs-mediated suppression, but its role remains controversial.	41,42
	Tim-3	Tim-3 is an immune checkpoint receptor alongside LAG-3 and TIGIT, which induces T cell tolerance.	43

Treg: Regulatory T cell; TCR: T cell receptor

Chimeric antigen receptors as a new era in tumor immunotherapy to redirect antigen-specificity

The CAR is constructed with fusion proteins, including one domain to identify antigen and the co-stimulatory receptors. CAR T-cell therapy uses gene transfer technology to T-cells of patients as scheduled for CAR expression to target specific tumor antigens. Therefore, it combines the specificity of antibodies, the property of cytotoxicity, and the memory T-cells. CAR generally consists of a sequence of a variable single-chain derived from mouse antibodies (human antibody is less immunogenic) which also expresses extracellular,

intramembrane, and intracellular signaling domains of T receptor, and it can have CD3 and the ζ chain (44) (Figure 2). The method used to expand the T-cell before the injection is substantial to determine its effectiveness in the body. A study showed that T-cells from a patient with lymphoma, leukemia, myeloma, HIV, or specific T-cells against an external antigen were expanded with an anti-CD3 and anti-CD28 monoclonal antibody, which were linked covalently to the magnetite bead. Thus, these T-cells had anti-tumor activity in vivo and in vitro (45).

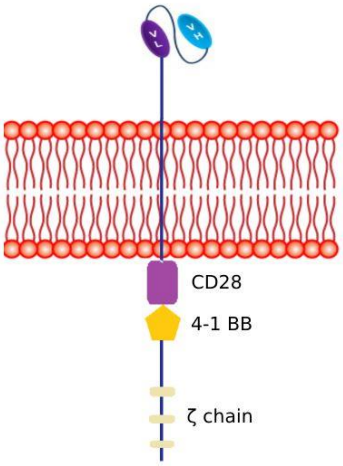

Figure 2. Structure of third-generation CAR T-cell. CAR T-cells have a single chain against a specific antigen. They also express an extracellular, intermembrane, and intracellular signaling domain, and the CD28 and 4-1BB co-stimulatory receptor

In another study, two populations of T-cells were selected. The first group consisted of first-generation T-cells, and the second included second-generation T-cells with CD28 co-stimulatory receptors in patients with chronic lymphocytic leukemia (CLL) or non-Hodgkin lymphomas (NHLs). According to this study, the stability of second-generation T-cells is more than the first generation. In another study, a lentiviral vector was used instead of a 4-1BB stimulator for signaling, and better results in two-thirds of CLL patients were seen (46).

Studies have demonstrated that CAR T-cells containing CD28 co-stimulatory domain increased the genes associated with the exhaust T-cell, while the 4-1BB co-stimulatory domain (TNFSF9) reduced these T-cells. CD8 membrane domain was replaced with cytoplasmic and intramembrane signaling domains of the CD28 co-stimulatory molecule to send a second message in CAR T. Consequently, it increased the anti-tumor effect of 19-28zCAR cells in B leukemia (6,47).

CAR T-cell is a living drug. The physiological mechanisms, such as T-cell homeostasis, memory formation, and antigen-driven expansion, occur when CAR T-cell is injected. This event is one of the attractions of using it (48). New CARs have improved clinical responses due to the combination of CAR T-cells with treatments such as checkpoint inhibitors or oncolytic viruses, vaccines, and cytokines that enhance the therapeutic efficacy of tumor cancers (49). Therefore, research on this issue demonstrates that the combination of drugs can be fruitful in the treatment of disease.

Generally, the efficacy of CAR T-cell therapy in solid tumors is lower compared to hematological malignancies due to immunity of tumor microenvironment, endothelial and fibroblast T-cells, extracellular matrix molecules, and cytokines. EGFRvIII (expressed in glioblastoma), ERBB2, mesothelin (which is increased in mesothelioma, pancreatic, ovarian, and lung cancers) (50-52), prostate stem cell antigen (PSCA), and prostate-specific membrane antigen (PSMA) are examples of antigens that have been investigated in CAR T-cell therapy (53,54,16). The specificity of this therapeutic approach can be enhanced by a further study on the detection of specific antigens in various tumors. Actually, the suitable target epitope for CAR T-cell therapy is an antigen that not only has a critical role in the maintenance and propagation of the malignant phenotype and is expressed on every tumor cell, but also is absent on normal tissues (47,55). Generally, as heterogeneous antigen is expressed on solid tumors and low-level TAAs are expressed on healthy tissues, detecting proper TAA for CAR T-cell therapy has been harder for solid tumors than for hematological malignancies (56). It is advisable to target several tumor antigens or use epigenetic modifiers to enhance TAA expression to eliminate TAA and prevent the escape of

high tumor antigen from the immune system. For example, Azacitidine and Sodium Valproate are used to increase the expression of melanoma-associated antigen (MAGE) and melanoma-associated antigen A4 (MAGE A4) antigens in acute myeloid leukemia or myeloma (57), and Hodgkin lymphomas, respectively (58). Thus, the expression of specific antigens in the tumor can be increased to improve the efficacy of this treatment through utilizing auxiliary agents.

In order to target several tumor antigens, in designing CAR T, several different CARs or a single bi-specific construct can be considered in a T-cell (59). To expand the capability of CAR T-cells, a Universal CAR system is introduced that can simultaneously respond to multiple antigens and have the ability to switch targets without re-engineering the T-cells by simply switching the soluble adaptor in case of antigen escape or insufficient tumor response (60). This CAR is a two-component receptor system composed of a universal receptor (zipCAR) expressed on T-cells and a tumor-targeting scFv adaptor (zipFv). The general idea is that an adaptor CAR, with specificity against a particular tumor antigen, links to the soluble adapter (60). The use of combined special soluble adaptors targets multiple tumor antigens concomitant, so this approach is a fantastic strategy to dispel solid tumor heterogeneity. However, the clinical possibility of such universal CAR platforms remains to be determined (60).

After the effects of the T-cells with CAR, it is necessary to remove these cells to create a balance in the body and prevent their disproportionate effect. One strategy for removing CAR T after the completion of the work is the induction of the Clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR- associated 9 (Cas9) system, which results in apoptotic paths being activated (49,61) (Figure 3).

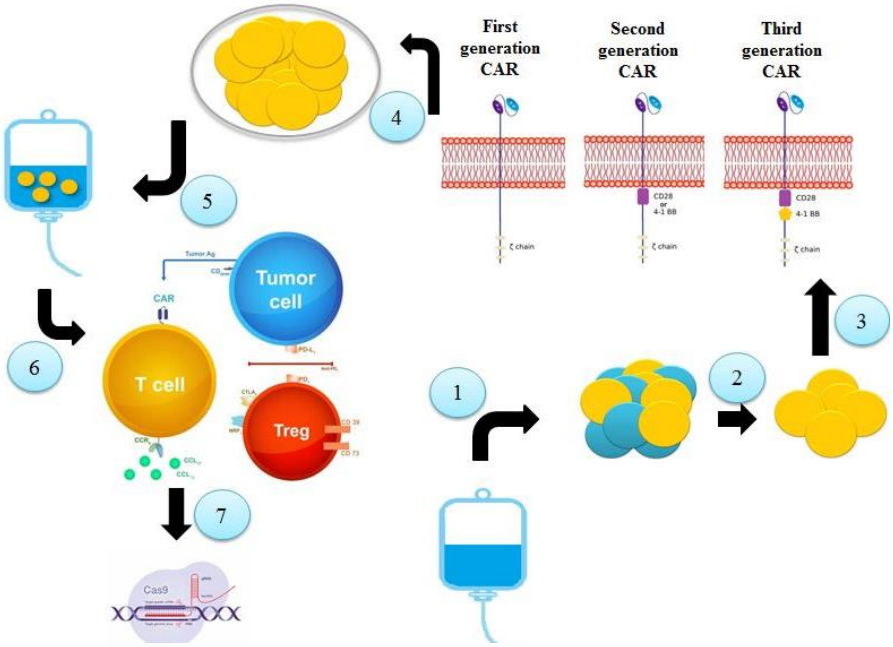

Figure 3. The stages of production, operation, and destruction of CAR T-cell. First (stage 1), during the process of leukopheresis, the white blood cells are separated, then (stage 2) the lymphocytes are isolated from these cells, and the CAR genes are introduced into them (stage 3). The CAR is classified into three categories of the first, second, third generation, the difference being whether there is a co-stimulating receptor or not. Then they are grown in vitro (stage 4) and injected into the patient (stage 5). These CARs in the patient's body go to the tumor microenvironment and react to a specific antigen or cell based on the receptor (stage 6). Ultimately, they are destroyed by the CRISPER system to prevent their high response (stage 7). CAR: Chimeric antigen receptor, Treg: Regulatory T-cell

Cancer immunotherapy with either direct targeting or modulating the immune system holds significant promise for patients with cancer, improving survival rates and decreasing late effects. A more generalized use will likely need to enhance efficacy by not only targeting specific antigens that are present on a patient's tumor but also the use of therapeutic agents such as checkpoint inhibitors, bispecific antibodies, and attenuating their impact by depletion of Tregs resulting in elicit a more robust response (Figure 4). We are optimistic that identifying

ways to incorporate immunotherapies into current conventional treatment regimens will significantly impact the quality of life for patients with cancer.

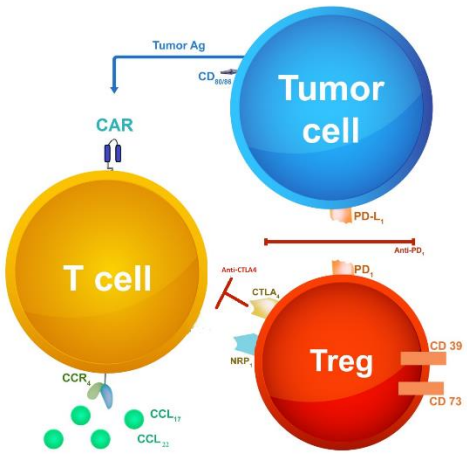

Figure 4. The interaction between CAR T, tumor cells, and Treg cells. Tumor Ag: Tumor Antigen, Treg: Regulatory T-cell, CAR: Chimeric Antigen Receptor

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