CAR T-Cells

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Chimeric Antigen Receptor (CAR) T-cells are T lymphocytes that have been specifically engineered to target malignant cells. CARs are synthetic molecules designed to activate T cells in response to a specific antigen, mimicking T cell activation through the T cell receptor (TCR) and associated costimulatory molecules.

Keywords: Chimeric Antigen Receptor T cells ; immunotherapy

1. Introduction

CAR constructs have evolved from the first generation, that included only the signaling endo-domain normally derived from the CD3ζ domain of the TCR or from the γ chain of high-affinity IgE Fc receptor (FcεRI), to second and third CAR generations by adding and combining different co-stimulatory domains with the aim to increase the efficacy and persistence of the CAR T-cells [1]. The therapeutic successes obtained with CAR T-cells, followed by the approval from the American and European medicines regulatory agencies (Food and Drug Administration (FDA) and European Medicines Agency (EMA), respectively) of two CAR T-cell products targeting the CD19 antigen for the treatment of pediatric/young adult B-cell acute lymphoblastic leukemia (Kymriah®) and adult large B-cell lymphoma (Yescarta®) [2][3], are the results of many years of research mainly based on the understanding of T cell biology and of their interaction with the surrounding environment [4][5].

Emerging evidence indicates that the metabolism is a key factor in driving the immune response by regulating the activity and the fate of the T cells. From their naïve to highly differentiated effector function, T cells undergo metabolic reprogramming [6]. This allows the T cells to fulfill the increase in energy demand and to generate the intermediate metabolites necessary for their clonal activation, proliferation and differentiation [7]. Cancer cells undergo also metabolic reprogramming in order to promote and sustain their high proliferation rate and survival [8][9]. Moreover, the metabolic reprogramming of cancer cells contributes to the recruitment of cells with immunosuppressive activity and depletes the microenvironment of metabolites and nutriments, creating conditions particularly hostile for T cells to perform proper effector functions [10][11].

CAR T-cells are specifically designed to target an antigen on the surface of cells and they need to be metabolically fit to reach the tumor, survive in an immunosuppressive microenvironment and display their cytolytic function [12]. Because CAR T-cells are easily “manipulable”, either by genetic modifications or by combination with different therapeutic agents, many efforts are being made to identify and develop new strategies to improve their activity against tumors.

2. Armoring CAR T-cells: Improving the Intrinsic Anti-Tumor Activity through Improved Metabolic Fitness

CAR T-cells, like other effector T cells, require specific metabolic support for optimal performance in terms of proliferation and maintenance of their specific effector and memory functions. Since the therapeutic response of CAR T-cell treatment in patients is strictly linked to their activities and persistence, many efforts have been committed to maximize CAR T-cell efficacy and rendering cells metabolically fit to deal with the tumor.

2.1. Engineering of the CAR Module: Costimulation as Metabolic Support for T Cells

Second and third generation of CARs are composed of a combination of costimulatory domains such as immunoglobulin (Ig) superfamily members, CD28 or inducible T cell costimulatory (ICOS), and the tumor necrosis factor receptor (TNFR) superfamily members 4-1BB, OX40 and CD27. Depending on the costimulatory domains incorporated into the synthetic CAR construct, different signaling pathways are triggered upon antigen activation [13][14][15]. These co-stimulatory domains are particularly implicated in the regulation of T cell metabolic reprogramming, mimicking a physiologic response and improving their persistence, memory and anti-tumor potency.

Antigen activation of second-generation CAR integrating a CD28 cytoplasmic domain (CD28.CD3ζ) enhances the glucose uptake and the aerobic glycolysis, which correlates with an increase in the effector T cell memory population [16]. Glycolysis induction observed after CD28 stimulation appears to be promoted through the activation of PKB/mTOR signaling pathway and activation of HIF1α, the latter being directly involved in the up-regulation of glucose uptake and the expression of glycolytic enzymes [17]. However, other evidence demonstrates that the tonic activation of CAR T-cells with the CD28 endo-domains is responsible for the suboptimal anti-tumor activity observed in vivo as the T cells exhaust rapidly, resulting into a decrease in cell proliferation and cytokine production [17].

In comparison, T cells transduced with second-generation CAR constructs comprising the 4-1BB domain (4-1BB.CD3ζ) have an enhanced mitochondrial biogenesis and oxidative metabolism, which is associated with an increase in cell survival and central memory T cell population. Activation of a 4-1BB.CD3ζ CAR construct targeting CD19 was also reported to counteract the effect of chronic CAR signaling stimulation by decreasing exhaustion and increasing central memory-related markers as well as by inducing a gene expression signature related to hypoxia, metabolism and apoptosis [18]. Activation of 4-1BB increases the metabolic capacity of the T cells through a peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)-dependent mitochondrial fusion and biogenesis mechanisms, via the activation of the p38-microtubule associated protein kinase (MAPK) pathway [17][19].

Therefore, 4-1BB induces a higher mitochondrial oxidative phosphorylation upon activation allowing the generation of memory T cells with a better in vivo persistence phenotype, while CD28 activation increases the aerobic glycolysis path leading to an early dominance of the effector T cells. These differences in T cell phenotypes associated with their metabolic programs are in agreement with clinical observations showing that, T cells transduced with CD19.CAR-4-1BB.CD3ζ construct demonstrate superior efficacy in acute lymphoblastic leukemia than those transduced with the CD19.CAR-CD28.CD3ζ construct [20]. CD28 and 4-1BB endo-domains regulating the effector and the memory T cell phenotypes, respectively, appear both critical for CAR T-cell activity and third-generation CAR built with a combination of these two co-stimulatory domains (CD28.4-1BB.CD3ζ) demonstrated superior anti-tumor efficacy in vitro and in vivo preclinical models compared to their respective second generation CARs (CD28, OX40, 4-1BB) and a third generation CAR encoding for CD28.OX40 costimulatory domains [21]. While CD28 and 4-1BB cytoplasmic domains appear both critical for CAR T-cell activity, other co-stimulatory domains are reported to regulate the metabolism and to potentiate the anti-tumor activity of the CAR T-cells. For instance, dual costimulation of 4-1BB and OX40 in melanoma enhanced glucose uptake, glycolysis, and OXPHOS [22]. OX40, normally induced after T cell activation, regulates Tregs glycolysis and lipid metabolism and promotes T cell expansion and generation of memory cells through a TNF receptor-associated factor 2 (TRAF2)-dependent mechanism [23]. However, Quintarelli et al. demonstrated that OX40 incorporated into third-generation CARs, CD28.OX40.CD3ζ or 4-1BB.OX40.CD3ζ decreases INFγ and IL2 production and the anti-tumor activity when compared to a CAR construct including the combination of CD28.4-1BB.CD3ζ [21].

Another member of the TNFR superfamily costimulatory proteins is CD27, which is normally expressed in resting T cells [24]. Integration of the CD27 cytoplasmic domain into a CAR construct enhances T cell expansion, effector functions as well as survival and augments T cell persistence and anti-tumor activity in vivo. These effects are potentially mediated through the induction of anti-apoptotic proteins of the B-cell lymphoma 2 (Bcl-2) family and the up-regulation of the proto-oncogene serine/threonine-protein kinase Pim-1, particularly involved in the regulation of the oxidative stress and aerobic glycolysis [25][26].

Stimulation of ICOS, a member of the immunoglobulin superfamily costimulatory molecules, switch on the glycolysis and lipogenesis pathways through activation of mTORC1 and mTORC2, as well as the induction of Glut-1 and is a key player for the differentiation and expansion of helper T cells (Th17) [27]. CARs with ICOS cytoplasmic domain are linked to immunotherapies that require Th17 cell function and prevalence [28]. However, second-generation ICOS-based CAR showed to increase the anti-tumor activity and persistence of the transduced T cells when compared to CARs with CD28 and 4-1BB intracellular-domains [29]. Moreover, CAR T-cell persistence and anti-tumor activity were further enhanced when ICOS was combined with the 4-1BB in a third-generation CAR.

All these observations indicate clearly that depending on the co-stimulatory domains integrated in the CAR construct, T cells will activate different metabolic pathways with a particular impact on their functions, fitness and behavior inside the TME.

2.2. Exploiting Transcription Factors and Specific Genes Pathways to Promote Potent Antitumor Activity of CAR T-Cells

Transcription factors regulate the expression of a specific set of genes, some of which are particularly involved in modifying the metabolic states of the T cells. Therefore, different strategies to modulate their activity and modify CAR T-cells transcriptional programs have been used to increase the metabolic fitness and intrinsic anti-tumor activity of engineered T cells.

Kagoya et al. have modified a CAR construct to activate specifically STAT3 [30], a transcription factor involved in the rapid innate immune mitochondria reprogramming and inflammatory response upon antigen stimulation [31]. The modified CAR construct contained a truncated domain of the Il2 receptor and a STAT3 binding tyrosine motif (YXXQ), as well as the co-stimulatory domain CD28. Upon stimulation, STAT3 pathway is activated and the CAR T-cells show potent cytotoxic activity even after repetitive antigen stimulation in vitro resulting in a superior anti-tumor effect in vivo when compared to T cells transduced with a CD28 or 4-1BB second-generation CAR. Over-expression of IL23 is another way to induce STAT3 activation in CAR T-cells. IL23 is constituted by two sub-units, IL23aP19 and IL12bp40, which only assemble upon T cell activation. Xingcong Ma et al. have co-expressed the CAR construct with the P40 subunit, which binds the P19 sub-unit to form IL23 only upon antigen activation [32]. As a result, these IL23-engineered CAR T-cells increase their proliferation rate and lytic activity, as well as decrease the expression of exhaustion markers in vitro. In vivo, the CAR T-cells demonstrate a better tumor control and improve survival. Another approach used to activate the STAT pathway in CAR T cells was to co-expressed together with the CAR a membrane-bound chimeric IL15. The engineered T cells signal through the STAT5 pathway, maintaining a memory-like transcriptional profile and developing a long-term persistence phenotype in vivo [33].

More recently, Kondo et al. have shown that the activation of the Notch homolog 1 facilitates mitochondrial biogenesis, fatty acid synthesis and OXPHOS in CAR T-cells targeting CD19 and leads to the maintenance of a stem cell-memory T cell phenotype [34]. They further demonstrated that the NOTCH effect is mediated through the induction of the transcription factor forkhead box M1 (FOXM1) and the metabolic reprogramming of the T cells. Moreover, overexpression of FOXM1 in CAR T-cells enhances their anti-tumor activity and their stem cell-memory phenotype in an in vivo model of leukemia.

The CAR T-cell metabolic program can also be improved using modified transcription factors as exemplified with the T-box transcription factor TBX21 (T-Bet). T-Bet is normally highly expressed in T cells and is required for the regulation of genes involved in the proinflammatory pathway and the development of T helper (Th) $CD4^+$ cells into a Th1 phenotype [35]. The co-expression of T-bet with a second-generation CAR (CD28.CD3ζ) targeting the B7-H3 antigen has been shown to potentiate CAR T-cell anti-tumor activity. This effect was observed with a T-bet construct deleted in its DNA binding domain (ΔTBOX), indicating that it is unlikely mediated through its direct transcriptional activity. The use of the ΔTBOX construct is known to upregulate the expression of glycolytic pathway genes through the binding of its transactivation domain with other transcription factors, such as BCL6 or NFKB [36][37].

Modifications of the transcription factor activities have been also performed using specific small molecules. The down-regulation of the basic leucine zipper ATF-like transcription factor (BTAF) expression, which cooperates with interferon regulatory factor 4 (IRF4) and nuclear factor of activated T cells (NFAT) to impair $CD8^+$ T cell metabolism and promote exhaustion, has been achieved using JQ1, an inhibitor of the bromodomain containing protein 4 (BRD4) epigenetic regulator [38]. The decrease in BTAF expression following JQ1 treatment led to an increase in glycolysis and OXPHOS, which maintain $CD8^+$ T cells with features of stem cell-like and central memory phenotypes, and co-administration of JQ1 with CAR T-cells enhanced their anti-tumor activity and persistence in vivo. C-Jun over-expression in CAR T-cells has been proposed to be another strategy to negatively regulate the BTAF involvement in T cell exhaustion. C-jun appears to compete with BTAF/IRF at the promoter of the genes switching off BTAF transcriptional activities. Over-expression of C-jun protects CAR T-cells from exhaustion by enhancing IL2 expression and inhibiting the transcription activity of the BTAF/IRF [39].

While the different strategies to manipulate the activity of specific transcription factors have been shown to potentiate CAR T-cells by modifying their metabolism, the efficacy and the safety of these engineered T cells remains to be demonstrated in the clinical arena.

3. Pre-Conditioning CAR T-Cells to Increase Their Metabolic Fitness

The improvement of the quality and fitness of the CAR T-cells before their adoptive transfer is an important step. The composition of the culture medium and the protocol for the expansion of CAR T-cells are crucial to tune specific metabolic programs and enhance CAR T-cell persistence and/or cytotoxic activity and therefore their anti-tumor activity.

The addition of specific cytokines in the culture medium, such as IL7 and IL15, have been carefully chosen to enhance the fitness of the CAR T-cells [40]. IL7 enhances glucose uptake through the STAT5 activation pathway, increasing the survival of the CAR T-cells [41]. On the other hand, IL15 reduces mTOR activity and the expression of glycolytic enzymes, but improves mitochondrial fitness, favoring the stem cell-like properties of CAR T-cells [42]. The inhibition of mTOR activity in IL2 stimulated CAR T-cells with rapamycin or with dichloroacetic acid, both known to block aerobic glycolysis, has shown similar results to IL15 treatment on the CAR T-cell differentiation but impairs their expansion ex-vivo. However, incubation of CAR T-cells with inhibitors of PI3K, upstream regulator of mTOR, increase the naïve and central memory T cell sub-population without affecting ex-vivo expansion. Moreover, PI3K and PKB inhibitors enhance CAR T-cells in vivo persistence and anti-tumor activity [43][44].

The nutritional component of the culture medium also needs to be optimized in order to improve adoptive transfer of T cell therapy. For example, Geiger et al. observed that increasing L-arginine levels lead to phenotypic changes in TCR transgenic $CD8^+$ OT-I T cells [45]. L-arginine enhances T cell metabolic fitness by increasing OXPHOS and decreasing glycolysis and, therefore, induces a central memory-like phenotype that improved persistence and anti-tumor activity in vivo. Thus, CAR T-cells could be pre-incubated with specific metabolites such as L-arginine before their adoptive transfer to the patient. Another strategy, recently highlighted by the work of Fulthan et al. [46], demonstrates that CAR T-cells are susceptible to low arginine level because of the low expression of the resynthesis enzymes, ornithine transcarbamylase and argininosuccinate synthase. Co-expressing these enzymes in a 4-1BB second-generation CAR showed a metabolic rewiring toward arginine and proline, as well as pyrimidine and purine metabolisms. As a result, the proliferation of the modified CAR T-cells is enhanced in vitro and the antitumor efficacy is significantly improved for different in vivo pre-clinical tumor models [46].

Inhibition of the lymphocyte cell-specific protein-tyrosine kinase (LCK) with dasatinib is another way to condition CAR T-cell activity. Dasatinib inhibits LCK-induced CD3ζ phosphorylation of the CAR construct and, thus, blocks CAR T-cell activation, proliferation, cytokine production and anti-tumor activity in vivo without affecting their viability. This blockade is rapidly and completely reversible following removal of dasatinib. Therefore, dasatinib can be used as a pharmacologic on/off switch to control CAR T-cell activity and associated toxicity, such as cytokine release syndrome [47].

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