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Review

Chimeric antigen receptor-redirected T cells return to the bench

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ABSTRACT

While the clinical progress of chimeric antigen receptor T cell (CAR-T) immunotherapy has garnered attention to the field, our understanding of the biology of these chimeric molecules is still emerging. Our aim within this review is to bring to light the mechanistic understanding of these multi-modular receptors and how these individual components confer particular properties to CAR-Ts. In addition, we will discuss extrinsic factors that can be manipulated to influence CAR-T performance such as choice of cellular population, culturing conditions and additional modifications that enhance their activity particularly in solid tumors. Finally, we will also consider the emerging toxicity associated with CAR-Ts. By breaking apart the CAR and examining the role of each piece, we can build a better functioning cellular vehicle for optimized treatment of cancer patients.

1. Introduction

Chimeric antigen receptors (CARs) are fusion proteins where the binding site of a monoclonal antibody (Ab) is fused to intracellular signaling molecules. Upon engraftment in T cells, CARs direct their antigen-specificity toward antigens expressed on the cell surface of tumor cells. CARs thereby provide T cells with major histocompatibility complex (MHC)-independent cytotoxic activity and co-stimulation impartial from ligands expressed by tumor cells.

Among cancer cell-based therapies, CAR T lymphocytes (CAR-Ts) are currently perceived as the most promising therapeutic approach as emphasized by the significant investment in CAR-Ts by pharma companies. The clinical impact of CAR-Ts, especially for lymphoid malignancies, has been extensively summarized in other recent review articles [1–4]. We will instead outline molecular aspects of the design of CAR molecules that affects their function as well as additional features of CAR-Ts. These emerging data should likely encourage investigators to revisit the bench to further assess the basic biology of these molecules and their effects on T cells upon engraftment to better understand clinical results.

The application of CAR-Ts in solid tumors is still in its infancy, but it is clear that the results in the clinical arena of solid tumors are not comparable to the experience in the lymphoid malignancy setting [5,6]. This likely stems from solid tumors posing extra barriers of complexity as compared to liquid tumors. While the combination of CAR-Ts with other immunomodulatory or biological agents can overcome some of the extra barriers, in this review we will discuss how genetic engineering of CAR-Ts has been implemented to enhance their functions. The clinical success of CAR-Ts in solid tumors may require more efforts and multiple exploratory phase I studies to optimize a larger therapy scope, but there is no reason to lose enthusiasm for this technology in the more challenging solid tumor setting.

The great majority of effective anti-tumor agents cause side effects, and CAR-Ts do not make an exception as objective tumor regressions are frequently achieved with accompanying toxicities. Some of these toxicities were in some way anticipated, but others were unexpected. We will also discuss efforts that investigators are making to limit or contain the toxicities of CAR-Ts.

1.1. CAR design

The antigen specificity of CARs is derived from mouse monoclonal Abs, humanized Abs or fully human Abs. Specifically, the variable regions of the heavy and light chains of Abs are cloned in the form of single-chain variable fragments (scFv) and joined through an hinge and a transmembrane domain to intracellular signaling molecules of the T-cell receptor (TCR) complex and co-stimulatory molecules. A significant amount of *in vitro* experiments indicate that CARs when sufficiently expressed on the cell surface of either $CD4^+$ or $CD8^+$ T cells can promote cytotoxic function as long as either the ζ-chain or the FcεRI of the TCR complex is included, and regardless of which molecular design is used to assemble the

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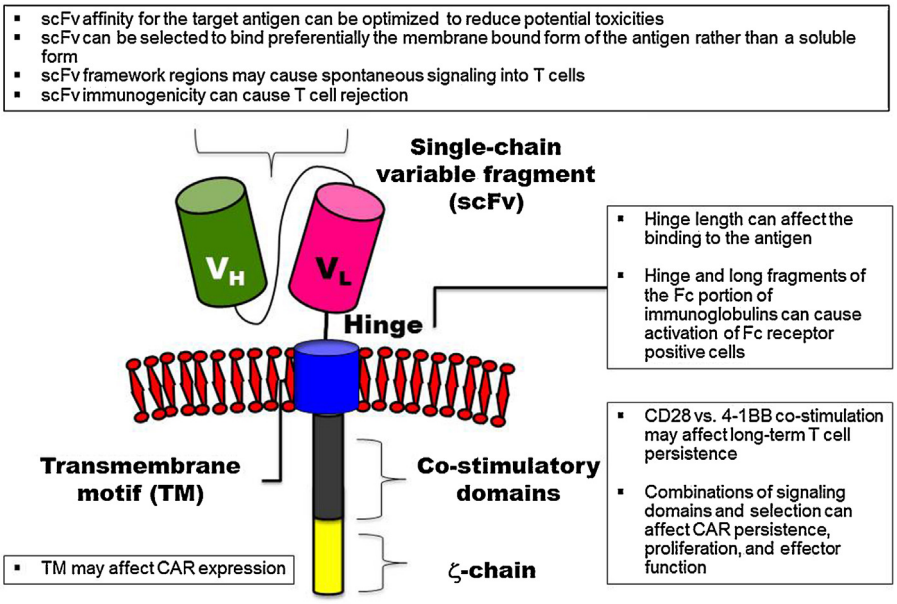

Fig. 1. Construction of CAR molecules. Schematic representation and functional characterization of CAR molecules.

CAR. However, from their original introduction as T-bodies in the late eighties [7], the design of CAR molecules has evolved significantly in the past 10 years [8,9]. Emerging data shows that the individual fragments included in these chimeric proteins can affect the functionality and survival of T lymphocytes. We outline below some of the developing preclinical and clinical data in which the specific design of CARs can be linked to T cell function or survival. For convenience, we have grouped these components into two sections, the "Extracellular Region" that includes the signal peptide, the scFv and the hinge, and the "Intracellular Region" that includes the transmembrane domain and signaling domains (Fig. 1).

1.1.1. Extracellular region

The native signal peptide of a protein is an N-terminal short sequence necessary for the translocation of the nascent precursor protein to the endoplasmic reticulum membrane and to the secretory pathway. Although signal peptides of different proteins accomplish the same function in eukaryotic cells, their sequences are not highly conserved [10]. In addition, the signal peptide is added "ectopically" to the scFv for the CAR assembling, and different sequences have been used. At the moment it is unknown if signal peptide sequences are more suitable for CAR assembly versus others.

The scFv is the portion of the CAR that determines its antigen specificity. It is fair to say that currently all the scFv used in preclinical and clinical studies to assemble CARs derive from Abs for which the sequences of the variable regions were known, or from Ab sequences obtained from available mouse hybridomas [8,9,11–14]. Although conflicting results have been reported, it is becoming evident that we may need to revisit the use of available scFv for the generation of CARs and consider that new scFv must be generated and tailored to CAR application [15]. First and foremost, the affinity of the scFv for the target antigen needs to be optimized for tumor antigens that can be expressed at low levels on normal tissues, in an attempt to minimize potential toxicities [16]. In addition, since some target antigens can also be detected in soluble form at different levels in cancer patients, it may be relevant to consider the cloning of a scFv that has a greater affinity for the membrane bound form of the antigen rather than the soluble form, to enhance the specificity of the binding to tumor cells [13,17,18]. In some instances it has been reported that the framework regions of specific scFv may cause a spontaneous antigen-independent signaling of CARs, leading to T cell exhaustion [19], or constitutive proliferation of CAR-Ts especially when CD28 is used as a co-stimulatory moiety [20]. The clinical implications of these results remain conflicting, especially taking into consideration that a certain level of antigen-independent growth of CAR-Ts carrying the 4-1BB co-stimulatory moiety is considered a positive factor [21], and led to sustained clinical responses [3]. The immunogenicity of scFv of mouse origin or junctional regions may also emerge as an obstacle for the long term persistence of CAR-Ts [22]. While preconditioning regimens used in patients before the infusion of CAR-Ts may allow their survival for weeks, we cannot exclude the possibility of CAR-T rejection in immune reconstituted hosts by either B or T cells.

The hinge region of CARs has received significant attention in the past few years. The hinge has generally been considered a portion of the molecule empirically used to provide flexibility to the scFv. The addition of long hinges derived from human immunoglobulins (Igs) was also used as an opportunity to insert into CARs a fragment that could allow for their detection on the cell surface of T cells, particularly when Abs to detect the scFv were not functional or available [12,13,23]. In the majority of cases, CAR-Ts show robust cytotoxic activity in *in vitro* experiments, regardless of the type of hinge used for the CAR assembly, and thus this component was not really considered to have any critical or specific function. However, it turned out that the hinge region can indeed have a significant impact on CAR-T properties. For instance the length of the hinge derived from Igs may require optimization in relation to the location of the epitope within the molecule that the scFv is targeting [24,25]. More surprisingly, hinge/spacer containing Fc portions of Igs included into CARs seem to retain some of their native properties in full antibodies. For instance the IgG1 Fc portion included into CARs can still bind with the Fc receptor expressed by monocytes/macrophages and cause their activation [26]. In mouse experiments the reactivity of the IgG1 portion appears to reduce the anti-tumor activity of CAR-Ts likely due to the activation and exhaustion mediated by macrophages [27]. An extra level of complexity as far as the hinge is concerned, is that in some CAR designs the hinge used does not belong to the Igs but to other molecules such the native hinge of the CD8α molecule [28]. The CD8α hinge contains cysteine and proline residues known to play a role in the interaction of the CD8

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