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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.

Keywords

Chimeric antigen receptor; Adoptive Immunotherapy

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

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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 CARTs 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 CARTs.

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 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 (Figure 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 co-receptor and MHC molecules, which may also not surprisingly affect CAR signaling. More basic signaling experiments are needed to fully discover the function of this fragment in CARs.

1.1.2 Intracellular Region—The transmembrane motif (TM) anchors the CAR to the plasma membrane. The native TM portion of CD28 is generally used in CARs in which CD28 also provides the co-stimulation, while the TM of CD8α is generally used when 4-1BB is the co-stimulatory endodomain (28–30). We have previously observed that in CAR-Ts expressing only the ζ-chain without co-stimulatory domains the type of TM used (ζ vs. CD28) affects the stability of the surface CAR expression (9), but it remains unexplored if using CD28 versus CD8α TM may affect CAR assembling and signaling.

T cell activation requires at least two signaling events: the engagement of the TCR and a separate co-stimulatory signal. While T cells receive both signals by antigen-presenting cells, they usually do not receive co-stimulation by tumors lacking the ligands that engage the cognate co-stimulatory molecules. The design of CARs has quickly evolved from the simple incorporation of the ζ-chain (1^{st} generation CARs) to the incorporation either of a single co-stimulatory domain (for example CD28 or 4-1BB)(2^{nd} generation) (28;29;31), or the incorporation of two co-stimulatory endodomains (CD28/OX40 or CD28/4-1BB) (32;33) (3^{rd} generation). Together with co-receptors such as CD8, these signaling moieties produce downstream activation of kinase pathways, which support gene transcription and functional cellular responses. The rational for adding co-stimulatory endodomains to CARs was supported by mechanistic studies showing activation of proximal signaling proteins related to either CD28 (Phosphatidylinositol-4,5-bisphosphate 3-kinase) or 4-1BB/OX40 (TNF-receptor-associated-factor adapter proteins) pathways, and MAPK and Akt activation (29;32). In some models the use of a combination of early (CD28) and late (4-1BB/OX40) costimulatory molecules (3^{rd} generation CARs) has evidenced a greater strength of signaling and persistence (33). However, it is also evident that this assumption does not apply to all circumstances, further underlining that the co-stimulation of CARs needs to be assessed in the context of all the other molecular components of the CAR, including its affinity to the target antigen and the structural characteristics of the targeted epitope. The clinical impact of the most suitable CAR co-stimulation still remains difficult to assess. In lymphoid malignancies targeted with CD19-specific CARs, while it is obvious that co-stimulation is required to promote T cell expansion (23), clinical responses in patients with acute

lymphoblastic leukemia are attainable with CARs containing either CD28 or 4-1BB (3;34;35). The long-term follow up of patients receiving CD19-specific CAR-Ts containing the 4-1BB without any additional treatment post T cell infusion shows long-term persistence and biological function of these cells, as demonstrated by the prolonged B cell aplasia (3). More recently, it has been suggested that 4-1BB co-stimulation, as compared to CD28, may promote longer survival of CAR-Ts by ameliorating the T cell exhaustion caused by persistent CAR signaling (19). Although a highly competitive scenario for the marketing of CD19-specific CAR-Ts is currently in place, proper comparative studies would help in defining the role of the specific co-stimulation. As far as solid tumors are concerned, clinical trials are still ongoing in different tumor settings using different CARs. These studies will be instrumental in assessing the role of CAR co-stimulation in solid tumors.

1.2 CAR-T products

1.2.1 Generation of CAR-T products for clinical use—In the most simplistic and frequently used approach, CAR-Ts are generated starting from unselected peripheral blood mononuclear cells that are activated using Abs specific for CD3 and CD28 either plate-bound or on coated iron-beads (23;34;36;37). The delivery of the CAR transgene into activated T cells is generally achieved using either gamma-retroviral or lentiviral vectors (23;34;36;37). Currently there is no apparent clinical benefit from either type of vector, despite gamma-retroviruses and lentiviruses having significant intrinsic differences. In addition, gamma-retroviruses use the native virus promoter for CAR expression, while lentiviruses use an internal promoter for gene expression.

The methodology used for the *in vitro* expansion phase of CAR-Ts is also extremely variable between academic center in terms of media components and continuous CD3/CD28 stimulations versus only cytokine supply. In general recombinant IL-2 is the most widely used growth factor to support T cell expansion (23;34;36;37). Most protocols aim at keeping CAR-Ts in culture less than 3 weeks to limit aging and loss of proliferative potential of the CAR-Ts once infused into patients. However, time of culture may vary due to the starting number of cells and the dose of T cells planned to be infused.

Non-viral gene transfer approaches, such as the Sleeping Beauty Transposon/Transposase System, are also used to transfer CARs. In general, given the relatively low efficiency of DNA integration, this method requires a longer period of culture for CAR-Ts to expand to numbers sufficient for infusion (38). Ongoing clinical trials will provide insights on whether this approach has comparable activity to viral-based gene transfer. Other non-viral gene delivery systems such as the prokaryotic type II CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats CRISPR-associated 9) will significantly extend the ease in which the human genome can be edited in both stem cells and T lymphocytes (39).

1.2.1 CAR-T subsets and anti-tumor activity—The composition of T cell subsets of CAR-T products used in clinical trials is extremely heterogeneous. However, it seems appropriate to conclude that CAR-Ts expanded *ex vivo* and infused into patients are largely composed of a heterogeneous proportion of $CD4^+$ and $CD8^+$ T cells which have the

phenotypic characteristics of circulating effector (T_{EFF}) and effector-memory (T_{EM}) cells, while a minority resembles central-memory (T_{CM}) cells (23;34;36;37).

The clinical experience with adoptive transfer of tumor infiltrating T lymphocytes (TILs) in melanoma patients and mouse models of adoptive T cell transfer are instrumental in clarifying the critical role of T cell subsets required for tumor responses. In TILs both telomere length and frequency of $CD8^+CD27^+$ T cells correlate with superior persistence and anti-tumor activity of these cells in patients (40). In an effort to maintain these biologic characteristics in the infused product, short-term culture approaches were implemented and moved to clinical practice (41). In mouse models, the engraftment and antitumor activity of tumor-specific T cells inversely correlate with the differentiation status of the infused cells, being naïve T cells (T_N) superior to central memory $T_{CM,}$ and T_{CM} superior to $T_{EM(42)}$. More recently another subset of mouse and human T cells, identified within the CD8 compartment, defined T memory stem (T_{SCM}) cells, was found to exhibit even more enhanced self-renewal and survival capacities and the multi-potent ability to derive all memory and effector T cell subsets (43). These properties make T_{SCM} cells an ideal T cell population to employ in CAR-Ts in clinical trials, where therapeutic outcomes are highly dependent on engraftment and persistence of the transferred T cells. In the cell subset arena, we were the first to report that the presence of $CD4^+$ T cells within the CAR-T products correlated with the *in vivo* persistence of GD2-specific CAR-Ts in patients with neuroblastoma (6). In lymphoma patients, we found that the number of $CD8^+CD45RA^+CCR7^+$ CD19-specifc CAR-Ts, which resemble T_{SCM}, highly correlated with superior *in vivo* expansion (44).

One of the challenges for the near future is to integrate these observations to generate better CAR-T products, whilst avoiding excessive complexity that can drastically reduce scalability of the process, and containing the manufacturing costs. Numerous cytokines profoundly affect T-cell development and differentiation. IL-2, IL-7, IL-15, and IL-21 are members of a cytokine family whose heteromeric receptors share the common γ chain (γc). Each cytokine has been described as a T-cell growth factor and each has been used to augment T-cell anti-tumor immune responses. IL-2 is the predominant cytokine used for CAR-T manufacturing as for a long time was the only available clinical grade cytokine (3;23;34;36). We and others have found that *in vitro* IL-7 and IL-15 preserve a higher frequency of T_{SCM} in CAR-Ts allowing for enhanced T cell persistence and anti-tumor activity in preclinical models (44;45). IL-7 and IL-15 instead of IL-2 have been recently incorporated in the manufacture CAR-Ts for clinical use. Similarly, exploring other cytokines may provide simple methods to generate more effective CAR-Ts. Investigators have also implemented a scalable approach to generate CAR-Ts starting from $CD62L^+$ cells selected from the peripheral blood, followed by an adjustment of the ratio of $CD4^+$ and $CD8^+$ T cells in the final product (46). The development of automated and closed cell separation system may further facilitate the implementation of the genetic modification of desirable T cell subsets. Finally, taking in consideration that specific biologic characteristics of these subsets may have a favorable impact in specific tumors compared to total $CD3^+\alpha\delta TCR^+$ polyclonal T cells, many investigators are pursuing other cell type for CARs engraftment, including virus-specific T cells (5;47), NK cells (48), γδT cells (49) and NKT cells (50).

1.3 CAR-Ts and beyond

The successful use of CAR-Ts in lymphoblastic leukemia is likely partially contingent on choosing CD19 antigen as a target. The CD19 expression is indeed restricted to B lymphocytes and CD19-specific CAR-Ts encounter leukemic blasts or normal B lymphocytes immediately in the blood stream, thus receiving immediate activation, or in the bone marrow, where adoptively transferred T cells can reliably accumulate. By contrast, the selections of appropriate antigens and bio-distribution for CAR-Ts remain challenging in solid tumors. Several articles already underlined the critical role of antigen selection in solid tumor to minimize the damage or destruction of normal tissues (8;9;51), and thus we will only discuss studies in which CAR molecules have been coupled with other genes to enhance their functions.

1.3.1 CAR-T trafficking to tumors—In solid tumors, CAR-Ts circulating in the blood stream must extravasate to tumor sites and then counter a complex and frequently dynamic environment that can make CAR-Ts non-responsive. Taking into consideration the physiologic process of peripheral tissue infiltration of antigen-specific T cells in pathological conditions, such as inflammation, impaired migration and infiltration of CAR-Ts in solid tumors must account for some of the limited clinical benefits so far reported (5;6;52). Several preclinical examples have reported the feasibility of coupling CAR-Ts with chemokine receptors matched to the chemokine that tumor or stromal cells release, to allow specific and enhanced migration of CAR-Ts at the tumor site (53;54). CAR-Ts may also be modified to restore the expression of enzymes that digest components of the extracellular matrix and facilitate their infiltration within the stroma (55). Alternatively, CAR-Ts may be combined with other treatment modalities that help these cells to perfect their trafficking as recently demonstrated by using armed oncolytic viruses (56).

1.3.2 CAR-T survival and inhibition—When CAR-Ts effectively reach the tumor upon intravenous inoculation, several tumor escape mechanisms may then hamper and reduce their longevity or function within the tumor. Several additional genetic modifications of CAR-Ts have been reported to counter the plethora of inhibitory mechanisms that tumor cells employ to block immune responses. These genetic modifications can be summarized in strategies aimed at (1) directly promoting CAR-T survival, or (2) reverting or blocking tumor-associated inhibitory molecules.

Addition of cytokines or cytokine receptors have been proposed to create T cells with either an autonomous cytokine fuel or the capability to use surrounding cytokines. IL-2, IL-7, IL-15 and IL-21 have been coupled into CARs to promote the proliferation and survival of CAR-Ts (57;58). IL-15 is perhaps the most promising cytokine for partnering with CAR-Ts, to promote their local survival and growth while avoiding systemic toxicity. When expressed by CAR-Ts, IL-15 promotes T-cell proliferation, prevents apoptosis and exhaustion and overcomes T-cell inhibition mediated by regulatory T cells (57;59). Alternatively, CAR-Ts can be manipulated to express cytokine receptors such as IL-7Rα that sustain their proliferation in response to the homeostatic cytokine IL-7 (60).

To revert or block tumor-associated inhibition, CAR-Ts have been coupled with the constitutive or inducible release of IL-12, although this last modification was only achieved by the use of two separated vectors (61;62). Ectopic expression of IL-12 by CAR-Ts promotes Th1 differentiation and helps in triggering the elimination of tumor-infiltrating macrophages, dendritic cells, and myeloid derived dendritic cells(63). Taking in consideration the blocking effects of Programmed Death PD ligand 1 (PD-L1) receptor, CAR-Ts have been engineered to transform the negative signal of PD-1 in T cells in a positive signal by substituting the intracytoplasmic domain of PD-1 with co-stimulatory signals (64).

1.4 CAR-Ts and toxicities

1.4.1 On-target toxicity and cytokine release syndrome—As evidenced in many effective treatments in cancer patients, objective tumor regressions in response to CAR-Ts are accompanied by toxicities. At the moment CAR-Ts pose two main safety concerns. The first relates to the inability of CAR-Ts to distinguish between tumor and normal tissues when the targeted antigen is shared between them ("on-target/off-tumor" toxicity). The second concern relates to the systemic perturbation of the immune system, known as cytokine release syndrome (CRS), systemic inflammatory response syndrome or cytokine storm, consequence of the rapid and robust *in vivo* expansion of CAR-Ts.

On-target toxicity such as B-cell aplasia induced by CD19-specific CAR-Ts was expected since the CD19 antigen is homogeneously expressed by both normal and malignant B lymphocytes (37). By contrast, the lethal on-target toxicity reported using a 3rd generation HER2/*neu*-specific CAR was unanticipated based on the safety data accumulated in clinical trials in which the same antigen was targeted using the monoclonal Ab trastuzumab (65). More recently, another clinical study showed that patients with osteosarcoma can be safely infused with T cells expressing a different 2nd generation HER-2/*new*-specific CAR (52). However, this second study did not show significant clinical benefits, and thus it remains to be confirmed whether a therapeutic window can be obtained with HER-2/*new*-specific CAR-Ts.

CRS is a clinical condition characterized by fever and hypotension that, in severe cases, leads to multiple organ failure. This toxicity correlates with the *in vivo* expansion of infused CAR-Ts, which causes a general perturbation of the immune system, and release of high levels of pro-inflammatory cytokines, such as TNF-α and IL-6 (37). This toxicity has been observed after the administration of CD19-specific CAR-Ts regardless of the CAR design or co-stimulation (either CD28 or 4-1BB) used.

1.4.2 Control on-target toxicity and cytokine release syndrome—Limiting or controlling on-target toxicities and CRS remains a critical aspect for the development of CAR-T therapies. On target toxicities are in general predictable in the case of antigens that are lineage-restricted in the hematopoietic system and hematologic malignancies since the expression profile of these antigens have been extensively characterized. By contrast, the on-target toxicity remains challenging when pursuing antigens in solid tumors since, with few

exceptions, molecules overexpressed by tumor cells are also found, although at different levels, in some normal tissues.

To prevent on-target toxicities, the accurate screening of the antigen expression in different tissues is critical and can be facilitated by taking into account the publically available mRNA and protein Atlas (51). It is however also important to consider that different Abs with the same antigen-specificity may have different reactivity and thus the screening for the antigen expression must be performed using the same antibody from which the scFv of the CAR is derived. As mentioned in the CAR design paragraph, the screening of scFv with different affinities may help in discriminating between normal and tumor cells expressing different levels of the antigen, although this may also increase the chance of tumor escape.

The assessment of the potential toxicity of CAR-Ts targeting antigens shared with normal tissues can be facilitated in the clinical setting by the infusion of T cells that transiently express the CAR, for example after electroporation of mRNA encoding the receptor (66). In addition, there are significant efforts to further engineer CAR-Ts by including safety switches that allow the drastic elimination of CAR-Ts in case of severe on-target toxicity. Vectors in which a CAR is combined with safety switches, such as the inducible caspase9 gene (67) (activated by a chemical inducer of dimerization) or a truncated form of the EGF receptor R (activated by the monoclonal antibody cetuximab) (68), are currently in clinical trials.

In further preclinical assessments, investigators have proposed to control the on-target toxicity by allowing CAR-Ts to exploit their full cytotoxic function only when an appropriate combination of antigens is recognized in tumor cells as compared to normal cells (69), or combining inhibitory chimeric antigen receptors (iCARs) to self-regulate CAR-T effector function taking advantage of the physiologic inhibitory effects of PD-1 and CTLA-4 (70). The elegant modeling used in these preclinical studies can be recapitulated within the heterogeneous human malignancies however need further validation in clinical studies.

The CRS toxicity caused by CAR-Ts poses a different level of attention as compared to on-target toxicities and may require different approaches to be controlled. While severe on-target toxicities for non-hematopoietic tissues are life threatening side effect that must be avoided, clinical data indicates that CRS is frequently associated with ongoing effective clinical responses (34;37). However, severe CRS can be lethal if not effectively controlled. Blocking the IL-6 receptor using the clinically available anti-IL-6 Ab tocilizumab has been used to successfully control severe CRS (3;71), but the development of alternative strategies that can more precisely control the dynamic of CAR-T expansion *in vivo* is desirable. Safety switches that rapidly eliminate CAR-Ts can also be effective in controlling CRS. For instance, it was recently reported that a clinical scenario resembling CRS occurring after allogeneic stem cell transplant was rapidly controlled by the selective *in vivo* activation of the inducible caspase9 (72). However, while the complete elimination of CAR-Ts is desirable to control potentially lethal on-target toxicities, the total elimination of CAR-Ts in patients developing severe CRS is not desirable since this will also compromise the ongoing anti-tumor effects. The activity of the inducible caspase9 as compared to all other clinically

available safety switches can be pharmacologically modulated by dosing the CID (73) and may thus provide means to control CRS without completely abrogating CAR-Ts. Finally, recent effort to generate CARs in which intracellular signaling are controlled by small molecules that allow heterodimerization of the signaling components may further create CAR-Ts in which proliferation and effector function can be tightly controlled(74).

Conclusions

CAR-Ts and checkpoint inhibitors are conquering a significant space in the treatment of patients with a variety of malignancies. While we find that some tumors are more susceptible than other to immunotherapy approaches, a better understanding of the mechanisms underlining the clinical success or failure of checkpoint regulations are critical to continue implementing more advanced strategies. This concept is particularly true for CAR-Ts a scenario where a more rational and systematic approach in designing and assessing the functionality of these molecules can only be guided by a more precise definition of how these molecules interact with the complex network of T cell signaling, activation and differentiation. In addition, CAR-Ts likely need to be associated with other biological components or molecules to achieve robust clinical benefits in patients with solid tumors.

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Highlights

- Examination of key molecular attributes of CARs that influence functionality
- Factors to consider in building a CAR
- Coupling CARs with additional molecules to enhance their function
- CAR-T toxicities

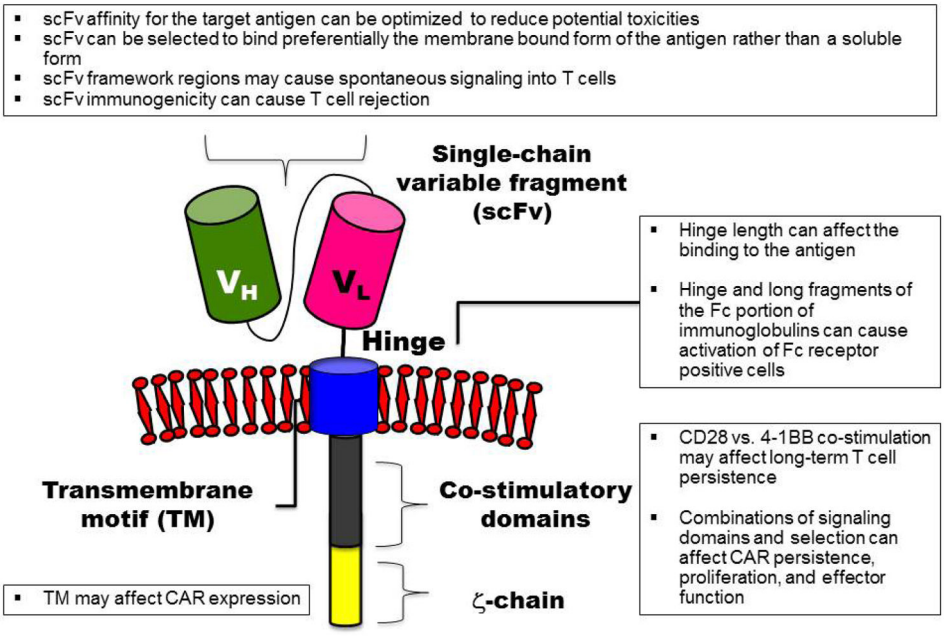

Figure 1. Construction of CAR molecules

Schematic representation and functional characterization of CAR molecules