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Review

Functional Design of Chimeric T-Cell Antigen Receptors for Adoptive Immunotherapy of Cancer: Architecture and Outcomes

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Abstract. *Adoptive immunotherapy using genetically modified T-cells with a chimeric antigen receptor (CAR) is a promising modality for cancer treatment, because the CAR-grafted T-cells can directly recognize and kill tumor cells, expressing a specific tumor-associated antigen (TAA), in a human leukocyte antigen (HLA)-independent manner. Optimal molecular designs of the CAR and a careful choice of the target TAA are requisite to attain a significant response in CAR-mediated therapy. This review provides a brief overview of the past studies and the present state of CAR research, especially focusing on the development of the CAR protein architecture.*

For decades, adoptive immunotherapy has received much attention as a means of cancer treatment (1-4). The adoptive transfer of *ex vivo*-cultured tumor-infiltrating lymphocytes (TILs) isolated from patients with malignant melanoma has been shown to mediate tumor regression (5-7). The capability of $CD8^+$ cytotoxic T-cells among the TILs to recognize and destroy tumor cells is dependent upon the expression of a human leukocyte antigen (HLA)-antigen complex on the surface of target cells. However, it is very difficult to achieve a robust clinical response following adoptive immunotherapy for most malignancies, not only because the isolation and expansion of tumor-reactive TILs are technically difficult and are not possible in all patients, but also because many tumor cells have acquired the ability to escape from immune surveillance by the down-regulation or mutation of HLA molecules (8, 9).

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In order to overcome the obstacles associated with TIL therapy, gene-therapeutic approaches for the redirection of T-cells to defined tumor-associated antigen (TAA) have been developed. One sophisticated strategy involves engineering of autologous T-cells with a chimeric antigen receptor (CAR), which generally consists of a TAA-binding moiety, an extracellular hinge region, a transmembrane (TM) domain, and intracellular signaling elements (Figure 1). This article provides an overview of the functional designs of the CAR and the results of clinical trials employing these CARs.

The TAA-Binding Moiety of the CAR

Species of TAA-binding moieties. A single chain variable fragment (scFv) derived from the heavy and light chain variable regions of a TAA-specific monoclonal antibody is commonly utilized as the extracellular TAA-binding moiety (Figure 1). Since the scFv can directly recognize the TAA on tumor cells, independently of antigen processing and HLA-restricted antigen presentation, the potential target-molecules are not limited to protein and peptide antigens, but rather comprise of various surface marker molecules that are uniquely or dominantly expressed on tumor cells, including the carbohydrate portion of glycoconjugates, such as glycoproteins, glycolipids, and gangliosides (10-12). While scFvs have been widely used in many CARs, some groups have utilized receptor or ligand proteins for the CAR-targeting moiety because the respective corresponding ligands or receptors are often overexpressed on tumor cells. For example, binding peptide against vascular endothelial growth factor receptor (VEGF) (13), anti-integrin $\alpha5\beta6$ peptide (14), heregulin (15), interleukin (IL)-13 mutein (16), and the NKG2D receptor (17, 18) have been introduced into CARs and successfully used for retargeting of genetically modified T-cells, resulting in tumor regression.

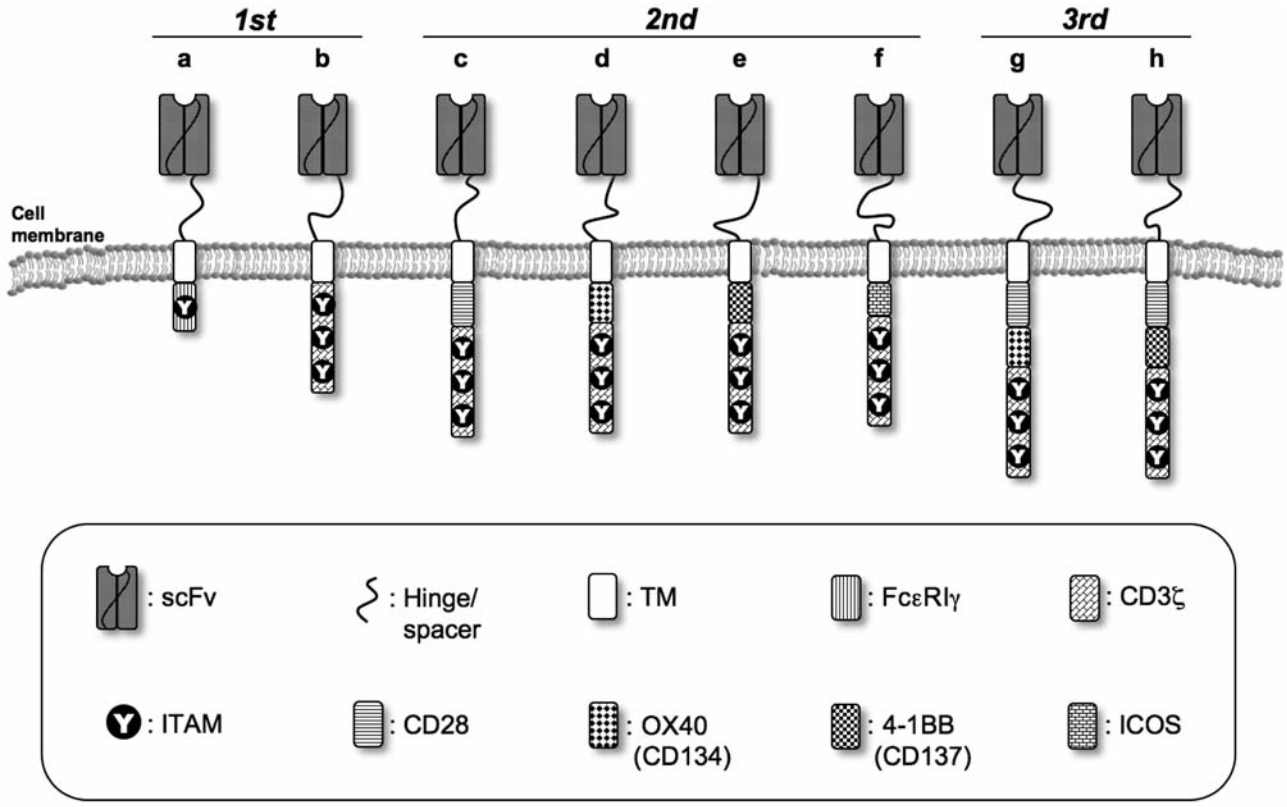

Figure 1. *A schematic representation of the structures of the first-, second-, and third-generations of CAR proteins. scFv, Single chain variable fragment; TM, transmembrane domain; ITAM, immunoreceptor tyrosine-based activating motif; ICOS, inducible co-stimulator.*

Urbanska and colleagues recently reported a pioneering strategy for expanding the flexibility of T-cell-targeted antigen specificity (19). They developed a novel CAR, specific for biotinylated molecules. This biotin-binding immune receptor (BBIR) is composed of an extracellular-modified avidin linked to an intracellular T-cell signaling domain. BBIR-grafted T-cells could target cancer cells pre-labeled with any specific biotinylated molecule, such as monoclonal antibodies, aptamers, or other tumor-specific ligands. The versatility of this method enables the sequential or simultaneous targeting of multiple TAAs using single transfected T-cells, thereby reducing not only the cost and time associated with CAR-engineered T-cell therapy, but also decreases the ability of tumors to evade eradication.

Targeting and initiating factors of the TAA-binding moiety. It is obvious that the binding affinity of a TAA-targeting domain is a critical factor for optimal T-cell activation through the CAR (20, 21). The magnitude of the CAR-mediated T-cell response is also dependent on the effectiveness of the surface expression of the CAR on T-cells, the antigen density on tumor cells, and the accessibility to the epitope (22-24). Using a series of anti-ErbB2 CARs containing an scFv with dissociation constants ranging from 10^{-7} to 10^{-11}, Chmielewski and co-workers demonstrated that the activation threshold of the CARs was inversely correlated with their binding affinity, but the maximum level of T-cell activation was essentially the same and independent of the affinity (21). Moreover, they also showed that T-cells with a high-affinity CAR were activated in a similar fashion against target cells with different amounts of the surface antigen, while T-cells with a low-affinity CAR were activated exclusively by tumor cells with a high amount of ErbB2. On the other hand, Turatti *et al.* reported that when the lytic potential of low- and high-affinity CARs was evaluated, their efficiency was comparable under conditions where there was high antigen and CAR expression, whereas a low-affinity CAR was more efficient than a high-affinity CAR, under conditions of limited antigen and CAR expression (20). Regardless of this, by careful optimization of the surface expression levels and the antigen binding affinity of the CAR, it will be possible to generate genetically modified T-cells to selectively target the cells with antigens that are overexpressed in tumor tissues, but which are also found at low levels on normal cells.

Antigenicity problems associated with the TAA-binding moiety in humans. Another issue associated with the targeting domain of CARs is their antigenicity in humans. The majority of CARs reported so far contain an scFv moiety that originated from murine-derived or humanized antibodies for the specific recognition of TAAs, which might trigger a host immune response and have inherent risks for the production of human anti-mouse antibodies (HAMA). The HAMA-related immune responses may limit the clinical use of CARs (25, 26). Utilizing fully human antibodies to construct CARs is likely to be a straightforward strategy for lowering the immunogenicity and minimizing the risk of a host-versus-graft response, but this needs to be further assessed in preclinical and clinical investigations (27, 28).

Extracellular Hinge Region of the CAR

The hinge/spacer region between the targeting moiety and the TM domain is important for ensuring the suitable positioning of the binding domain during the antigen–CAR interaction. Several studies have demonstrated that efficient antigen recognition by CAR-grafted T-cells depends on the position of the epitope on the target molecule (29-31). Guest *et al*. demonstrated that CARs containing scFvs specific for membrane-distal epitopes were well-activated with or without a spacer region, whereas CARs with scFvs against membrane-proximal epitopes were activated only when they had a flexible hinge region (30). Similar results were also reported by James *et al*. They clearly showed that the T-cell activation through an anti-CD22 CAR, with a spacer derived from the IgG1 Fc region, was dependent on the distance of the cognate epitope from the target cell membrane (31). Therefore, the signal strength of CARs might be modulated by differential choices of target epitopes, with respect to the distance from the cell membrane, allowing discrimination between targets with disparate antigen density.

TM Domain of the CAR

Most CARs include a TM domain derived from type I membrane proteins, such as CD3ζ, CD4, CD8, or CD28. The TM domain of CARs is not well studied compared to the other domains. However, the TM domain seems to be a pivotal region for CAR function, especially with regard to the oligomeric status of CARs (32, 33). Bridgeman and colleagues have demonstrated that CARs containing a CD3ζ TM domain homodimerized and were incorporated into the endogenous T-cell receptor (TCR) complex (33). Mutations of the CAR TM domain that abrogated these interactions resulted in a reduced functional capacity of the CAR to respond to the antigen, indicating that the complex formation with endogenous TCRs is beneficial for optimal T-cell activation. We also observed potential cross-activation of endogenous TCRs by the antigen–CAR interaction (34).

Intracellular Signaling Elements of the CAR

The first-generation CAR. A great deal of attention has been devoted to the intracellular signaling module of CAR because of its central role in effective T-cell activation, proliferation, and survival. The concept of the CAR was initially introduced by Eshhar *et al*., and the first-generation CARs transmit their signal through only a single signaling domain, derived from the high-affinity receptor for IgE (FcεRIγ; Figure 1, a) or the CD3ζ chain (Figure 1, b) (35, 36). The domain contains one or three immunoreceptor tyrosine-based activating motif(s) [ITAM(s)] for antigen-dependent T-cell activation. The ITAM-based activating signal is able to endow T-cells with the ability to lyse the target tumor cells and secret cytokines in response to antigen binding (37). Initial *in vivo* studies demonstrated that T-cells with the CD3ζ-containing CAR exhibited better tumor eradication than T-cells with the FcεRIγ-CAR (38), and thus, to date, the signaling domain derived from CD3ζ has been utilized in almost all CARs. However, the antitumor effects of the first-generation CARs were found to be limited (39-41). The ITAM-based signal alone induces T-cell anergy, thus leading to inefficient cytokine secretion, poor proliferation, and the activation-induced cell death of CAR-grafted T-cells (40, 42).

Second-generation CARs. It has been clearly demonstrated that complete T-cell activation and the prevention of apoptosis require a co-stimulatory signal *via* the CD28-B7 system, in addition to the CD3ζ signal (43). According to this two-signal model for T-cell activation, a co-incidentally delivered CD28 co-stimulatory signal by B7-expressing target cells, greatly augmented the cytokine secretion and the antitumor activity induced by CAR-transduced T-cells (42). However, the expression of such co-stimulatory ligands is not expected on most tumor cells. Therefore, in order to improve the function and overcome the limitations of the first-generation CARs, the new CARs had to be designed to be able to provide a co-stimulatory signal in a cis-fashion. Therefore, the second-generation CARs were developed to contain a second signaling domain for efficient co-stimulation, especially of CD28-mediated signals (Figure 1, c). For optimal surface expression, the CD28 signaling domain needs to be placed at a membrane-proximal site (44). In fact, most second generation CARs reported so far have such a molecular format (10).

In response to the target antigen, genetically modified T-cells with the second-generation CD28-containing CARs exhibited enhanced production of cytokines [*e.g*. IL-2, interferon (IFN)-γ, and granulocyte macrophage colony-stimulating factor, reduced activation-induced cell death, sustained proliferation, and tumor lytic activity in both *in vitro* and *in vivo* investigations (45-47). Recently, further second-signaling domains derived from co-stimulatory

proteins, such as OX40 (CD134; Figure 1, d), 4-1BB (CD137; Figure 1, e), and inducible co-stimulator (ICOS; Figure 1, f) have been investigated (48). OX40 and 4-1BB are members of the tumor necrosis factor (TNF) receptor family. They recruit TNF receptor-associated factor (TRAF) 1/2 adaptor proteins, which in turn activate crucial downstream players such as extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (49). Another B7 family member, ICOS, like CD28, has an important role in regulating the T-cell proliferation, survival, and effector functions (43, 50). All of the newer second-generation CARs are able to transmit the co-stimulation signal to induce significant cytokine secretion and up-regulation of anti-apoptotic proteins [*e.g.* B-cell lymphoma-extra large (Bcl-xl)], and lead to enhanced proliferation. However, it has been found that co-stimulation with CD28 is superior, in many cases, compared with the other molecules, especially in terms of IL-2 production (48, 51).

Third-generation CARs. In order to further improve and optimize the CAR design, third-generation CARs with a tripartite signaling domain, which consists of a CD28, CD3ζ, OX40 or 4-1BB signaling region, have recently been reported (Figure 1, g and h). The results from investigations of the third generation CARs remain somewhat controversial, but several studies showed that these CARs have improved signaling capacities relative to the second-generation molecules. Pulè *et al*. reported that a third-generation CAR with OX40 enabled T-cells to enhance the production of IL-2 and TNF-α, sustain clonal expansion, and significantly retain cytolytic activity compared with second generation CARs without OX40 (52). Zhao *et al*. compared a series of second and third generation CARs specific for ErbB2, and demonstrated that T-cells engrafted with a third-generation 4-1BB-containing CAR, showed the greatest lytic activity *in vitro*, enhanced cytokine production, and dramatic tumor growth inhibition in an *in vivo* mouse model (53). Notably, a recent report by Hombach *et al*. demonstrated that T-cell activation by a second-generation CD28-CD3ζ CAR induced the production of the repressive cytokine IL-10, which compromises T-cell immunity, whereas additional OX40 co-signaling by a third-generation CD28-CD3ζ-OX40 CAR repressed IL-10 production without affecting the secretion of pro-inflammatory cytokines (54).

Clinical Studies Using CAR-grafted T-Cells

To date, several clinical studies have been conducted to evaluate the efficacy of CAR-grafted T-cells in cancer therapy (55-58). Early trials utilizing first-generation CARs did not demonstrate any remarkable response. However, several groups have recently reported encouraging clinical outcomes in CAR-mediated adoptive T-cell immunotherapy for a variety of cancers, including melanoma (59), colorectal cancer (60), indolent B-cell non-Hodgkin's lymphoma (61), synovial sarcoma (62), neuroblastoma (63), chronic lymphocytic leukemia (64, 65), and follicular lymphoma (66). Based on the pre-clinical results, the second- and third-generation CARs seem highly promising. However, it is possible that the basal activity of CARs with multivalent signaling endodomains is up-regulated, resulting in a reduction of the activation threshold in CAR-grafted T-cells. This might cause nonspecific T-cell activation, leading to damage to normal tissues with no or low TAA expression, namely, off-target or on-target/off-tumor toxicity, so greater attention needs to be paid to such possible problems.

It was reported that a serious adverse event occurred in one patient treated with T-cells, engineered with a third-generation anti-ErbB2 CAR gene (67). The authors speculated that this was due to a severe cytokine storm caused by CAR-mediated on-target/off-tumor toxicity. Although not mentioned in this review, many other issues concerning the pre-clinical and clinical outcomes also need to be taken into account (*e.g.* the method of CAR gene transduction, conditions of *ex vivo* cultivation/expansion of CAR-grafted T-cells, and the type of T-cell subset to be engrafted) (3, 57, 68).

Conclusion

Since the field of CAR research has quickly progressed within a comparatively short period of time, there might have been a tendency to impatiently pursue a curative effect in cancer patients. Therefore, in addition to detailed and careful clinical investigations, there is an urgent need to develop a more competent and safer architecture of CAR, *i.e.* fourth-generation CARs, the design of which will require further basic studies, in order to explore the spatiotemporal dynamics of CAR-mediated molecular events and to more fully define the molecular basis of T-cell activation by CARs.

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