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The promise and potential pitfalls of chimeric antigen receptors

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One important purpose of T cell engineering is to generate tumor-targeted T cells through the genetic transfer of antigen-specific receptors, which consist of either physiological, MHC-restricted T cell receptors (TCRs) or non MHC-restricted chimeric antigen receptors (CARs). CARs combine antigen-specificity and T cell activating properties in a single fusion molecule. First generation CARs, which included as their signaling domain the cytoplasmic region of the CD3ζ or Fc receptor γ chain, effectively redirected T cell cytotoxicity but failed to enable T cell proliferation and survival upon repeated antigen exposure. Receptors encompassing both CD28 and CD3ζ are the prototypes for second generation CARs, which are now rapidly expanding to a diverse array of receptors with different functional properties. First generation CARs have been tested in phase I clinical studies in patients with ovarian cancer, renal cancer, lymphoma, and neuroblastoma, where they have induced modest responses. Second generation CARs, which are just now entering the clinical arena in the B cell malignancies and other cancers, will provide a more significant test for this approach. If the immunogenicity of CARs can be averted, the versatility of their design and HLA-independent antigen recognition will make CARs tools of choice for T cell engineering for the development of targeted cancer immunotherapies.

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Introduction

The advent of effective methods for gene transfer in T cells provides a new means for rapidly generating tumor-specific T cells. T cell engineering also offers a unique means to overcome or circumvent the tolerance mechanisms and immune escape stratagems used by tumors to derail or elude immune responses [1–3]. In principle, genetic reprogramming can be used to enforce tumor antigen recognition, to improve T cell survival, augment T cell expansion, generate memory lymphocytes and offset T cell death, anergy, and immune suppression. Furthermore, the genetic modification of T cells can be employed to enable the tracking of T cell migration *in vivo* and introduce into T cells a safety or recall mechanism to curb T cell responses if needed. The first objective – to afford tumor antigen recognition – is achieved by expressing antigen receptors, which consist of either physiological, MHC-restricted T cell receptors (TCRs) or non-MHC-restricted chimeric antigen receptors (CARs). The latter are the focus of this review.

The first T cell activating receptors can be traced back to the CD3ζ chain fusions that were generated to elucidate the role of the ζ chain [4,5]. These studies showed that cross-linking these fusion receptors was sufficient to provide calcium influx and T cell activation signaling including the initiation of cytotoxicity. Eshhar *et al.* directed such fusion receptors toward haptens by incorporating an immunoglobulin-derived scFv in the extracellular domain of the chimeric receptors, thus enabling the T cells expressing these 'T bodies' to lyse hapten-coated cells [6]. Several groups subsequently confirmed the ability to redirect T cell cytotoxicity using receptors encompassing different scFv's fused to the CD3ζ or Fc receptor γ (FcRγ) cytoplasmic signaling domains. However, as reviewed below, it was not until costimulatory properties were incorporated into the next generation of CARs that a greater strength and quality of antigen-induced signaling could be provided to T cells, which then enabled T cell proliferation and survival upon repeated exposure to antigen. Following this turning point, an impressive array of rapidly developing second generation CARs has been developed and is under intense investigation. Only first generation vectors have been tested in phase I clinical trials, showing so far modest effects. Second generation CARs have just entered the clinical arena.

CARs: the rules of engagement

Most CARs utilize an antibody-derived antigen-binding motif to recognize antigen (Table 1). Others utilize receptor or ligand domains as their targeting moiety, such as heregulin [7] or IL13 [8], that bind to their cognate ligand or receptor counterpart (Table 1). In all of these instances, CARs recognize native cell-surface antigens independently of antigen processing or MHC-restricted presentation. Importantly, CARs therefore do not have to be matched to the patient HLA and can recognize tumors that have downregulated HLA expression [9,10]. The expanding range of CAR specificities is illustrated in Table 1. The cell-surface antigens targeted by CARs

Table 1

Tumor antigens and CAR investigated *in vitro* and *in vivo* in T lymphocytes.

Target antigen	Associated malignancy	Receptor type (other specificity)	*In vivo* studies	Reference
α-Folate receptor	Ovarian cancer	ScFv-FcεRIγ	Phase I	[41]
CAIX	Renal cell carcinoma	ScFv-FcεRIγ	–	[45–47]
		ScFv-CD4-FcεRIγ	Phase I	[42•,48]
CD19	B cell malignancies	ScFv-CD3ζ (EBV)	–	[32]
	B cell malignancies	ScFv-CD3ζ	+	[31•,49]
	B cell malignancies	ScFv-CD28-CD3ζ	+	[25,28,44]
	Refractory Follicular Lymphoma	ScFv-CD3ζ	Phase I	[50]
	CLL	ScFv-CD28-CD3ζ	Phase I	[51]
	B cell malignancies	ScFv-CD28-CD3ζ	+	[27,52]
	ALL	ScFv-41BB-CD3z	–	[53]
	ALL	ScFv-41BB-CD3z	+	[54]
	B cell malignancies	ScFv-CD3ζ (Influenza MP-1)	+	[55]
	B cell malignancies	ScFv-CD3ζ (VZV)	–	[56]
CD20	Lymphomas	ScFv-CD28-CD3ζ	–	[57]
	B cell malignancies	ScFv-CD4-CD3ζ	–	[58]
	B cell lymphomas	ScFv-CD3ζ	–	[59,60]
	Mantle cell lymphoma,	ScFv-CD3ζ	Phase I	[43]
	indolent B cell lymphomas	ScFv-CD28-CD3ζ	–	[23]
		ScFv-CD28-41BB-CD3ζ	–	[23]
CD22	B cell malignancies	ScFV-CD4-CD3ζ	–	[58]
CD30	Lymphomas	ScFv-FcεRIγ	–	[61]
	Hodgkin lymphoma	ScFv-CD3ζ (EBV)	+	[62]
CD33	AML	ScFv-CD28-CD3z	–	[63]
		ScFv-41BB-CD3z		
CD44v7/8	Cervical carcinoma	ScFv-CD8-CD3z	+	[64]
CEA	Colorectal cancer	ScFv-CD3ζ	+	[65–67], [68]
		ScFv-FcεRIγ	+	[68,69]
		ScFv-CD3ε	–	[70]
		ScFv-CD28-CD3ζ	–	[71]
		ScFv-CD28-CD3ζ	+	[72,73]
EGP-2	Multiple malignancies	scFv-CD3ζ	–	[74]
		scFv-FcεRIγ	–	[74,75]
EGP-40	Colorectal cancer	scFv-FcεRIγ	–	[76]
erb-B2	Breast and others	ScFv-CD28-CD3ζ	+	[19,77]
		ScFv-CD28-CD3ζ (Influenza)	+	[78]
		ScFv-CD28mut.-CD3ζ	+	[29]
	Prostate cancer	ScFv-FcεRIγ	+	[79]
erb-B 2,3,4	Breast and others	Heregulin-CD3ζ	–	[80], [7]
		ScFv-CD3ζ	+	[81]
FBP	Ovarian cancer	ScFv-FcεRIγ	+	[35,82,83]
		ScFv-FcεRIγ (alloantigen)	+	[84]
Fetal acethylcholine receptor	Rhabdomyosarcoma	ScFv-CD3ζ	–	[85]
G_{D2}	Neuroblastoma	ScFv-CD28	–	[86]
		ScFv-CD3ζ	–	[32]
		ScFv-CD3ζ	Phase I	[37•]
		ScFv-CD28-OX40-CD3ζ	–	[22]
		ScFv-CD3ζ (VZV)	–	[56]
G_{D3}	Melanoma	ScFv-CD3ζ	–	[87]
		ScFv-CD3ε	–	[87]
Her-2	Medulloblastoma	ScFv-CD3ζ	+	[88]
IL-13R-a2	Glioma	IL-13-CD28-4-1BB-CD3ζ	+	[89]
	Glioblastoma	IL-13-CD3ζ	+	[8,90]
	Medulloblastoma	IL-13-CD3ζ	+	[91]
KDR	Tumor neovasculature	ScFv-FcεRIγ	–	[92]
κ-light chain	B cell malignancies	ScFv-CD3ζ	+	[30]
	(B-NHL, CLL)	ScFv-CD28-CD3ζ	+	[30]
LeY	Carcinomas	ScFv-FcεRIγ	–	[93]
	Epithelial derived tumors	ScFv-CD28-CD3ζ	+	[94]

Table 1 (*Continued*)

Target antigen	Associated malignancy	Receptor type (other specificity)	*In vivo* studies	Reference
L1 cell adhesion molecule	Neuroblastoma	ScFv-CD3ζ	Phase I	[95,96]
MAGE-A1	Melanoma	ScFV-CD4-FcεRIγ ScFV-CD28-FcεRIγ	–	[97]
Murine CMV infected cells	Murine CMV	Ly49H-CD3ζ	+	[98]
MUC1	Breast, Ovary	ScFV-CD28-OX40-CD3ζ	+	[16]
NKG2D ligands	Various tumors	NKG2D-CD3ζ	+	[99–101]
Oncofetal antigen (h5T4)	Various tumors	ScFV-CD3ζ (vaccination)	+	[102]
PSCA	Prostate carcinoma	ScFv-b2c-CD3ζ	–	[103]
PSMA	Prostate/tumor vasculature	ScFv-CD3ζ	+	[18,104,39]
		ScFv-CD28-CD3ζ	–	[21]
		ScFv-CD3ζ	+	[105]
TAA targeted by mAb IgE	Various tumors	FcεRI-CD28-CD3ζ (+ a-TAA IgE mAb)	+	[106]
TAG-72	Adenocarcinomas	scFv-CD3ζ	+	[107,108]
VEGF-R2	Tumor neovasculature	scFv-CD3ζ	–	[109]

(–) *In vitro* studies; (+) *in vivo* animal studies; (Phase I) Phase I clinical trial.

include proteins, carbohydrates, and glycolipids. Most current CARs incorporate an scFv derived from a murine monoclonal antibody. The scFv's are typically cloned from hybridoma RNA, but may also be selected from phage display libraries.

The rules for identifying the best target molecules and corresponding scFv are not yet fully elucidated. Tumor-restricted targets are preferred, but rare. Most well-defined targets are differentiation antigens or cancer/testis antigens, the selection of which depends on the level and frequency of expression on malignant tissues – including cancer stem cells – and their normal counterparts. One may reasonably assume that highly expressed tumor antigens will make better targets, especially if expression is greater on the tumor cells than on normal cells. The threshold antigen density required for optimal CAR-mediated tumor eradication is currently not known.

The optimal range of a CAR's affinity for its target antigen has not been defined either. Whereas the physiological TCRs have affinities in the micromolar range, monoclonal antibodies and scFv's operate in the nanomolar range. Heterodimeric TCRs, however, are coupled to the CD3 complex, a multichain complex optimized to bolster T cell activation following TCR engagement. CARs typically do not associate with the CD3 complex, and it is therefore conceivable that their higher affinity relative to TCRs is important to compensate, partly, for this disadvantage. It is noteworthy that too high an affinity can be detrimental to TCR-mediated antigen recognition [11], which highlights the importance of achieving optimal antigen receptor affinity [12]. One study on CAR affinity reveals the complex relationship been CAR density, antigen density, and CAR affinity [13]. CAR expression levels furthermore impact on T cell effector function [14].

Finally, another emerging factor in choosing an optimal scFv for making a CAR is the position of the epitope on the target molecule. The distance of the epitope to the cell surface appears to matter, as do the length and flexibility of the CAR extracellular hinge region [15,16]. These studies suggest that the CAR-antigen interaction must follow at least some of the structural requirements that apply to TCR-based immune synapses. Optimal interactions may further depend on the transmembrane and cytoplasmic components of the CAR, as well as its monomeric or dimeric structure.

Achieving meaningful signaling with second generation CARs

The first CARs were reported as receptors capable of redirecting the cytotoxic activity of CTL clones and hybridomas [17]. Noticing the lack of data on CAR-induced T cell proliferation, we set out to investigate the proliferative response of zeta chain based CARs in primary T cells. Having expressed a receptor specific for human prostate-specific membrane antigen (PSMA) in mitogen-activated peripheral blood T cells, we found that coculture with PSMA-positive LnCAP cells did allow for initiation of proliferation (typically 2–3 cell divisions), but this was soon followed by T cell death [18]. T cell death could be prevented when a costimulatory signal was coincidentally delivered, which could be achieved by expressing the CD28-ligand B7.1/CD80 on the tumor cells. CAR-transduced T cells activated in this manner were successfully reactivated by a second exposure to antigen, resulting in an absolute increase in T cell number [18]. However, most tumor cells, especially tumor cells *in vivo*, will not express activating costimulatory ligands such as CD80. Furthermore, it is to be expected that CAR-redirected T cells will not engage cross-presented antigen and thus will not have the benefit of dendritic cell-provided costimulation. CARs therefore had to be designed to provide a costimulatory ersatz to address their costimulatory dependence.

The emergence of CARs enabling T cells to survive repeated antigenic stimulation came with the development of CD28-CD3ζ dual-signaling receptors [19–21].

Table 2

CAR cytoplasmic signaling domains investigated in T cells.

	Cytoplasmic signaling domain(s)	Selected references
First generation (single signaling domain)	CD3 ζ	[49,58,87,89,31•,18,32]
	FcεRIγ	[69,75,76,82,92,61,35]
	CD3ε	[70,87]
Second generation (dual signaling domains)	CD28-CD3ζ	[24,73,106,72,19, 110,21,111,25]
	CD134-CD3 ζ	[25,112,22]
	CD137-CD3ζ	[25,112,53]
	ICOS-CD3ζ	[112]
	DAP10-CD3ζ	[25]
Third generation (three signaling domains)	CD28-CD3ζ-Lck	[113,114]
	CD28-CD134-CD3ζ	[16,114]
	CD28-CD137-CD3ζ	[23]

These receptors increased IL-2 secretion in response to antigen and permitted absolute expansion of retargeted T cells in response to antigen in the absence of exogenous costimulation [21]. A number of CD28-CD3ζ fusion receptors have been reported (Table 2), but it is noteworthy that not all of them increase IL-2 secretion [22]. This is possibly due to the different construction designs, which utilize different domains and fusion points. Fusion to different scFv's may also account for different signaling patterns. A side-by-side comparison of different receptors expressed at similar level in the same cells type would be needed to adequately compare different fusion receptors. Significant functional differences between CARs are likely to be found.

In recent years, additional fusion receptors have been reported. Several costimulatory signaling domains, including 4-1BB, OX40, DAP10, and ICOS, have been studied (Table 2). The rationale for each one will not be reviewed here, but the general goal has been to extend the strength of signal afforded by the CAR, augment T cell effector function or extend T cell survival. Many of these newer fusions have not been investigated as extensively as the CD28-CD3ζ receptors and it is fair to say that the jury is not out yet on the relative merits of these different fusions.

More recently, triple-fusion receptors that encompass CD3ζ, CD28, and 4-1BB or OX40 signaling motifs have been reported. These receptors appear to enhance *in vitro* effector functions relative to the dual-fusion receptors [16,22,23], as well as the strength of PI3kinase/Akt activation initiated by contact with antigen (XS Zhong and M Sadelain, unpublished data). These are promising receptors but more studies are needed, including *in vivo* studies, to assess their therapeutic potential. What is clear is that second generation CARs have considerably superior signaling properties compared with their CD3ζ and FCγR forbearers, which opens up real perspectives for the therapeutic use of CARs.

The rising *in vivo* prowess of CARs

A subset of all described CARs has been evaluated in *in vivo* tumor models, investigating either murine or human T cells in xenogeneic tumor models. First and second generation CARs targeting a variety of antigens have been shown to at least delay tumor progression in some animal models (ranging from intraperitoneal cytotoxicity assays to the more convincing systemic models) or in some instances to induce durable remissions in mice bearing established systemic tumors (Table 1). Whereas some first generation CARs have been shown to induce significant responses after intravenous infusion in tumor-bearing mice, it is noteworthy that in every instance where first and second generation CARs were compared, the latter outperformed the former [19,24–30]. Triple fusion receptors are active *in vivo* [16], but comparisons to second generation CARs have not yet been reported.

In xenogeneic models, we showed that human T cells targeted against human CD19 could eradicate systemic Raji tumors (a CD19+ Burkitt lymphoma) in SCID mice [31•]. Importantly, tumor eradication was obtained following a single intravenous infusion of human T cells in the absence of post-infusion cytokine administration to support T cell function. This study also demonstrated that the *in vivo* activity of CAR-transduced T cells depended on the T cell expansion conditions. Thus, T cells activated in the presence of antigen and CD28-mediated costimulation were more effective than T cells expanded by OKT3 antibody and IL-2 (the latter only survived a few days *in vivo*). Furthermore, T cells activated by artificial antigen-presenting cells expressing the CD19 antigen and CD80 were more effective after expansion in the presence of IL-15 and IL-2 than with IL-2 alone [31•]. The crucial impact of *in vitro* T cell activation on the outcome of adoptive T cell therapy should be borne in mind when comparing studies. CARs should thus be compared side-by-side under the same expansion conditions to permit valid receptor comparisons.

Other factors affect CAR function and CAR comparisons. The tumor obviously makes a difference. Thus, eradication of CD19+ Raji tumor cells is easier to achieve than eradication of CD19+ pre-B acute lymphoblastic leukemia cells [25,31•]. This is partly due to the different costimulatory profile of the two tumors [25,31•], but additional factors probably come into play as well, including tissue tropism and intrinsic susceptibility to lysis. Finally, the mouse model also makes a difference. Tumor eradication requires fewer infused T cells in NOD/SCID-γc−/− mice than in SCID-beige mice (J Markley and M Sadelain, unpublished data). The impact of these three parameters – T cell expansion conditions, tumor characteristics, and mouse model – should be taken into account when comparing results obtained with different receptors in murine models.

Table 3

CAR expression in effectors of the innate immune system.

Cell type	Cytoplasmic signaling domain(s)	References
NK cells	CD3ζ	[115–119]
	CD28-CD3ζ	[120,121]
	DAP10	[116]
	CD137-CD3ζ	[116]
	Fc-γ-receptor	[119]
Cytokine-induced killer cells (CIK)	CD3ζ	[122,123]
	DAP10	[123]
	CD137-CD3ζ	[123]
	CD28-CD3ζ	[123]
Monocytes	CD64 (Fc-γ-receptor)	[124]
Neutrophils	Fc-γ-receptor	[119]
	CD3ζ	[119]

Another important question is what cells are better suited for delivering CAR therapy. CARs have been investigated in bulk mouse spleen cells or bulk human peripheral blood T cells, as well as in EBV-specific T cells [32], lymphoid progenitor cells [33,34], unfractionated or Lin–Sca1+ bone marrow cells [14,35]. An important debate in adoptive cell therapies, which applies to CAR therapies like other adoptive cell therapies, is to better define the advantages and disadvantages of naïve, memory, and virus-specific T cells types [36–38]. This is addressed in another article in this issue. It is clear for CAR therapy, as it is for other adoptive T cell therapies, that T cell persistence is an important factor for successful tumor eradication [27,31•,39,40] and that the choice of T cell subset to utilize is one of the important aspects of this therapy.

Finally, it should be noted that other immune cell types than T cells are being investigated using CARs (Table 3).

Clinical studies utilizing first generation CARs

Completed clinical studies are limited to phase I studies evaluating first generation CARs targeting the folate receptor in ovarian cancer [41], carbonic anhydrase in renal cancer [42•], CD20 in lymphoma, [43]) and G_{D2} in neuroblastoma [37•]. The clinical responses have overall been very modest, with the exception of one partial response in the neuroblastoma study. Immunogenicity of CARs was observed in the first two studies, but not the latter two. The renal carcinoma study had to be halted after three patients developed unanticipated cholestasis, an on-target effect due to the high expression of the targeted antigen, carbonic anhydrase, in biliary epithelium. This study has now resumed with the added use of G250 antibody to partially mask the biliary antigen before T cell infusion (C Lamers, unpublished data). It is noteworthy that CAR-directed T cells were active against target antigen-positive T cells that are not typically picked up in imaging studies utilizing the parental monoclonal antibody, lending support to the merits of T cell based therapy.

These studies utilized first generation CARs and suboptimal T cell expansion procedures such as OKT3-mediated T cell expansion [31•,37•]. The field is thus keenly awaiting studies that utilize second generation CARs and improved T cell expansion procedures that provide appropriate costimulation and cytokine stimulation before T cell infusion. Several efforts are under way to address this issue [38,44]. Studies targeting CD19 are especially awaited as these hold the promise of activity in several B cell malignancies. At least seven trials targeting CD19 with second generation CARs are programmed in the US and Europe, with one having already started in chronic lymphocytic leukemia, utilizing a CD19-CD28ζ CAR [51].

Conclusion

In comparison to TCRs, CARs have two major advantages: HLA-independent recognition of antigen, which makes them broadly applicable irrespective of the patient's HLA and enables the recognition of tumor cells that have downregulated HLA expression, and no risk of mispairing with the endogenous TCR. Their signaling capacity was once a concern, but second generation receptors go far beyond what the original zeta chain fusions could achieve, that is, redirect cytotoxicity without permitting T cell expansion and survival upon repeat antigen exposure. CARs are therefore not just an alternative to TCRs but may prove to be superior as therapeutic entities.

Their use is, however, threatened by their potential immunogenicity. Anti-CAR antibody responses have been seen with some CARs, but not universally, which suggests that CAR immunogenicity may be neither universal nor intractable. CARs encompassing humanized scFvs or scFvs derived from human monoclonal antibodies will probably reduce this concern.

The choice of a target antigen is very crucial, as for any immunotherapy. This choice will become even more crucial as potent immunotherapies such as those utilizing second and third generation CARs enter the clinic. The G250 study illustrates the ability of CAR-targeted T cells to seek out normal tissues that express high levels of the targeted antigen [42•]. The targeting of CD19, a cell-surface molecule found on the majority of leukemias and lymphomas, will be an interesting case to follow as CD19 is expressed on normal proB, preB, and B cells but no other hematopoietic progenitor cell types. The several upcoming trials targeting CD19 will provide interesting variations on this one theme, as they utilize slightly different CARs, different scFvs, different vector systems, different T cell expansion methodologies, and different starting T cell subsets to treat different CD19-positive malignancies. The field of CARs is coming of age.

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