Fine tuning CARs for best performance

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Abstract

Chimeric antigen receptors (CARs) allow redirection of T cells against any surface antigen. However, CARs require optimization to achieve activity against low density antigens. Heitzeneder *et al* perform an iterative adjustment of CAR components to reach a design for targeting GPC2 that shows potent pre-clinical activity in neuroblastoma models.

Main Text

CARs typically consist of an antibody-derived single-chain variable fragment (scFv), an extracellular spacer region (referred to as hinge), a transmembrane (TM) domain, a T cell receptor derived-CD3ξ signaling domain, and usually one or two costimulatory domain(s) (for example CD28 or 41BB) (Figure 1). CAR T cells have proven to be highly effective as a treatment for B cell malignancies. This success has fueled efforts to translate this same approach to other cancers including solid tumors.

At first sight, it appears seductively simple to construct a CAR against any target antigen of interest. Indeed, rudimentary CARs will demonstrate killing when tested in *in vitro* studies at high effector to target ratios. However, as often, the devil is in the detail and not 'one-size fits all'. As shown by Heitzeneder *et al* in this issue of Cancer Cell - careful selection of the optimal CAR design for a given tumor target is required to achieve potent and persistent activity in a setting that recapitulates the human disease.

Heitzeneder *et al* select oncofetal target cerebroglycan (GPC2) to develop CAR T cells as treatment for childhood solid tumor neuroblastoma based on its consistent expression on neuroblastoma but highly restricted postnatal expression on healthy tissues. They set out to generate a GPC2-CAR following the standard CAR design as first described by Campana(Imai et al., 2004). While this GCP2-CAR is effective against targets that have been engineered to overexpress GPC2, activity is lacking against targets expressing native levels of GPC2. Using three cell lines with low, medium and high levels of GPC2 expression as targets, the authors

demonstrate the dependency of GPC2-CAR T cell function upon antigen density: while $GPC2^{hi}$ tumor cells can be well controlled *in vitro* and *in vivo*, this GPC2-CAR design fails to control expansion of $GPC2^{mod}$ or GPC^{lo} tumor cells.

To gain a better understanding of the clinical relevance of this inability to target $GPC2^{mod/lo}$ tumors, the authors measure the density of GPC2 expression on several neuroblastoma cell lines, patient-derived xenografts (PDXs) and bone marrow samples from patients with metastatic neuroblastoma. This analysis shows that neuroblastoma metastases express a consistent, but low-density level of GPC2, like the $GPC2^{lo}$ cell line used in the GPC2-CAR functional studies. Importantly, while expression of GPC2 on metastatic tumor tissue shows little interpatient variability, expression is significantly lower as compared to PDX samples - a finding confirmed for several other neuroblastoma target antigens. These results stress the importance of careful assessment of target antigen density on representative tumor samples and selection of tumor models that recapitulate clinically relevant target antigen expression to inform the selection of the optimal CAR format.

Tuning of the GPC2-CAR to a threshold of activity matched to the clinically relevant expression range of GPC2 on neuroblastoma was hence required. Whereas T cell receptors can effectively respond to low antigen levels, some authors have found that CARs need around a 100-fold higher level of target expression to function(Salter et al., 2021). However, threshold of CAR activity has multiple determinants including scFv affinity, epitope location, hinge and transmembrane domains, and type and conformation of endodomains. Each of these CAR components influences the formation of an immunological synapse and resulting triggering of receptor signaling.

Through a process of iterative adjustments Heitzeneder *et al* develop a CAR design which grant the required potency against the low-density target GPC2. In the initial GPC2-CAR, CD8α-derived hinge and TM domains are used to link the scFv to 41BB and CD3ζ endodomains. The authors had shown previously that replacement of CD8α TM with that of CD28 increased the potency of a CD19-CAR(Majzner et al., 2020) and this is mimicked here with a CD28 TM-based GPC2-CAR. To further improve this CAR design, the 41BB endodomain is also replaced with a CD28 endodomain. These combined adjustments to the CAR structure improve the ability of the GPC2-CAR to lyse $GPC2^{lo}$ targets. In 'stressed' *in vitro* and *in vivo* models based

on low effector to target ratios, the CAR containing the CD28-derived TM and endodomains are better able to control tumor growth as compared to the CD28TM-41BBζ GPC2-CAR.

While use of a CD28 endodomain can enhance CAR potency, this can be at the cost of impaired engraftment and long-term persistence of CAR T cells as previously described(Long et al., 2015). Indeed, the authors show that in a $GPC2^{lo}$ *in vivo* model, the use of a CD28 endodomain markedly reduces CAR T cell engraftment as compared to when 41BB is used as an endodomain. Engineering of T cells with components in addition to the CAR is used to counteract this impaired engraftment. The authors here apply their recently developed approach to overexpress the transcription factor c-Jun in CAR T cells which supports extensive T cell proliferation without acquiring an exhausted phenotype(Lynn et al., 2019). Combining this approach with the CD28TM/endodomain, GPC2-CAR induces rapid and sustained clearance of $GCP2^{lo}$ targets in multiple *in vivo* models and greatly enhances long-term CAR T cell persistence. In a subgroup of mice treated with CD28TM/endodomain CAR T cells, recurrence of neuroblastoma tumors with ultralow expression of GCP2 is observed. This acquired resistance is absent when using the GPC2-CAR incorporating CD28TM/endodomains combined with c-Jun overexpression, demonstrating the potent and persistent activity of this CAR design in the context of low-density target antigen.

In summary, this paper sets a standard for pre-clinical CAR development. It provides an approach to selection of the optimal CAR for a given target density using iterative design adjustments, tested in biologically relevant models. The ability to finetune CARs broadens the repertoire of suitable CAR targets. On one side of the spectrum are now well-established targets like CD19, which are highly expressed on tumor cells and favor targeting with a low potency 41BBξ CARs,(Ghorashian et al., 2019) and on the other side targets like GPC2, expressed at low density and requiring increased CAR potency with CD28 TM and signaling domains. Inclusion of other described variations of hinge and transmembrane domains(Alabanza et al., 2017; Majzner et al., 2020; Ying et al., 2019) as well as intracellular signaling domains(Feucht et al., 2019; Salter et al., 2021) in addition to the ones utilized by Heitzeneder *et al* to construct the described GPC2-CAR, provides a CAR component repertoire likely capable of tuning each CAR to the required potency for a given target be it expressed at low, medium or high density. In addition to these CAR-intrinsic approaches, 'next-generation' CAR designs which combine CARs with expression of additional components such as transcription factors(Lynn et al., 2019)

to adjust further aspects of T cell biology, enhance the ability to achieve potent and persistent CAR T cell function. Establishing a ‘toolkit’ of different scFv binders, hinges, and transmembrane and signaling domains alongside additional encoded components, will enable construction of ‘next generation’ CARs and selection of the optimal component combination through testing in biologically relevant models (Figure 1). This way CAR designs for each given tumor type can be finetuned taking multiple variables including tumor antigen density and differential expression compared to healthy tissues into consideration and informing selection of the optimal format to be taken forward for clinical testing.

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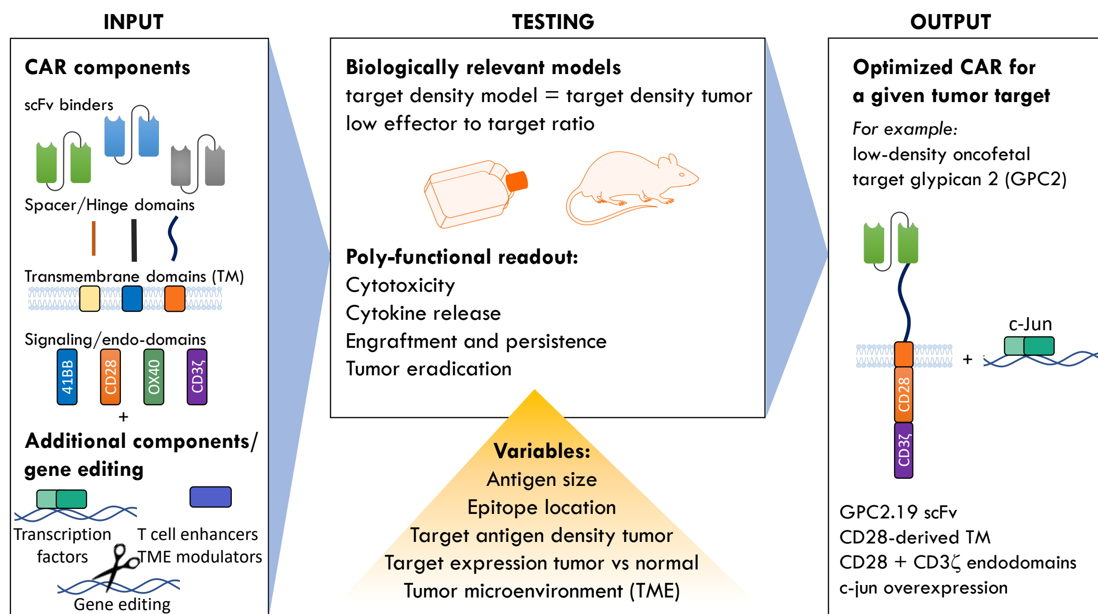

Legend Figure 1: Approach to finetuning CAR design for a given tumor target.

Left panel. Chimeric antigen receptors are constructed through linking single chain variable fragments (scFv) derived from target antigen-specific antibodies with hinge and transmembrane regions to signaling domains. A growing number of variants for each of the CAR components is available. CAR constructs can further incorporate gene editing or co-expression of additional components such as transcription factors or enhancing modules (i.e. cytokine signals, decoy receptors to confer resistance to inhibitory factors present in the tumor microenvironment) to optimize T cell function. **Central panel.** Variables as listed need to be considered to determine which CAR design is optimal for a given tumor type. Iterative testing of CAR prototypes in biologically relevant models using a polyfunctional readout of CAR T cell efficacy can inform the selection of the optimal CAR design finetuned for a given tumor target. **Right panel**. The optimized CAR result as demonstrated by Heitzeneder *et al* for oncofetal target glypican 2.