Smart CARS: optimized development of a chimeric antigen receptor (CAR) T cell targeting epidermal growth factor receptor variant III (EGFRvIII) for glioblastoma

Marco Ruella[1,2,3], Bruce L. Levine[1,2,3]

[1]Center for Cellular Immunotherapies, [2]Department of Pathology and Laboratory Medicine, Perelman School of Medicine, [3]Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA 19104-5157, USA

Correspondence to: Marco Ruella, MD. Smilow Center for Transl. Res., 8-196 C, 3400 Civic Center Boulevard, Philadelphia, PA 19104-5157, USA. Email: marco.ruella@uphs.upenn.edu.

Submitted Sep 15, 2015. Accepted for publication Sep 25, 2015.
doi: 10.3978/j.issn.2305-5839.2015.10.11
View this article at: http://dx.doi.org/10.3978/j.issn.2305-5839.2015.10.11

In a recent issue of the journal *Science Translational Medicine*, Dr. Johnson and our colleagues at the University of Pennsylvania reported on the rational development and characterization of humanized anti-epidermal growth factor receptor variant III (EGFRvIII) chimeric antigen receptor (CAR) T cells for glioblastoma (1). In this article, the authors describe the successful preclinical validation of a highly-selective humanized anti-EGFRvIII CAR T cell product that is currently employed in a phase I trial open at the University of Pennsylvania and the University of California San Francisco for patients with glioblastoma (NCT02209376).

CAR T cells are genetically modified patient-derived T cells that express a synthetic CAR comprising an antigen-recognizing domain from a single-chain antibody variable fragment (scFv), fused with the intra-cytoplasmic signaling domains of the T cell receptor complex (CD3 zeta chain) and other co-stimulatory sequences such as CD28 or 4-1BB (2,3). Infusion of anti-CD19 CAR T cells (CART-19 or CTL019) leads to dramatic clinical responses in patients with various types of B-cell neoplasms (4,5), most strikingly in relapsed/refractory acute lymphoblastic leukemia (ALL) (6-9). The successful application of this emerging technology in the treatment of solid tumors requires overcoming significant hurdles including the selection of an appropriate tumor-associated antigen, enhancing tumor infiltration by immune cells, and avoiding immune tolerance (2,10). The choice of the optimal tumor antigen is complicated by the fact that most of the antigens that are expressed in solid tumors are also co-expressed in important normal tissues (off tumor, on target expression), including epithelia. In this scenario EGFRvIII represents a unique opportunity, since it is a neo-antigen, expressed only in cancer cells, with no expression in other normal tissues (11). EGFRvIII is expressed in ~30% of glioblastomas and has been linked to poor long-term survival (12). The mutant EGFR (vIII) results from the deletion of exons 2 to 7 with the subsequent generation of a glycine residue at the junction of exons 1 and 8. This novel protein signals through the RTK/RAS/PI3K pathway and induces increased proliferation and reduced apoptosis in cancer cells (13,14). The idea of targeting EGFR and EGFRvIII for glioblastomas and other solid tumors has been actively pursued by multiple investigators using different approaches such as cancer vaccines (rindopepimut) (15), monoclonal antibodies (cetuximab, mAb806) (16,17) and small molecule inhibitors (gefitinib, erlotinib) (18,19). In the context of the impressive clinical activity of CART-19 against B cell leukemias, the possibility of using the same technology for targeting EGFRvIII in glioblastoma has been hotly pursued (20-23).

In this report, Dr. Laura Johnson and colleagues describe the generation of several anti-EGFRvIII scFv CARs along with extensive screening for specificity against EGFRvIII over wild type (wt) EGFR. Starting from the 3C10 murine clone, eight humanized scFv were generated in order to reduce the possibility an immune response against murine domains in the CAR, as observed in a recent pilot trial of anti-mesothelin murine CAR T cells for pancreatic cancer (24). The optimal co-stimulatory

domain structure of the CAR was also evaluated, comparing a second generation CAR (including 4-1BB and CD3zeta) with a third generation CAR (CD28-4-1BB-CD3z) both *in vivo* and *in vitro*. The second generation 4-1BB-CD3z construct resulted in faster *in vivo* anti-tumor activity and was therefore selected for further studies. Among the 8 humanized scFv, a low affinity scFv (#2173) was selected due to its specificity against EGFRvIII over EGFR wt. This construct was then challenged for its anti-tumor activity *in vitro* and also *in vivo* in three mouse models: two orthotopic models, where human glioma tumor was surgically implanted in the mouse brain, and a subcutaneous tumor model. In all *in vivo* experiments, CART-EGFRvIII cells showed an improved anti-tumor response as compared to control T cells. The highest anti-tumor activity was observed when CART-EGFRvIII cells were administered together with adjuvant chemotherapy (temozolomide).

A fundamental part of this paper is focused on the evaluation of the possible toxicity of this CAR construct, ensuring the selective specificity of #2173 CAR to EGFRvIII over wt EGFR. In contrast to EGFRvIII, wt EGFR is highly expressed in epithelial tissues and, as a consequence, the most common toxicities of the anti-EGFR therapies, like the monoclonal antibody cetuximab, are rash and diarrhea (25). Recent dramatic clinical experiences have shown that the unexpected reactivity of genetically engineered T cells against normal tissues can lead to disastrous consequences (26-29). With this in mind, the lower affinity scFv was chosen for further development to minimize crossreactivity with wt EGFR. This principle in affinity tuning of CARs to mediate potential toxicity to normal tissues has been further elucidated in two other recent studies on CAR T cells targeting wt EGFR (30,31) Johnson *et al*. used extensive *in vitro* and *in vivo* experiments to ensure the specificity of their lead construct (#2173). In particular, since the skin is one the highest EGFR expressing tissues, a novel *in vivo* xenograft model for the evaluation of human skin toxicity was developed. Immunodeficient mice (NSG) were surgically engrafted with human foreskin and after 4-6 weeks, were randomized to receive control T cells (no CAR), cetuximab based CART (recognizing both EGFR and EFGFRvIII) or the lead anti-EGFRvIII CART (#2173). Mice treated with control T cells had no T cell infiltration in the human skin, while cetuximab-CART treated mice had a prominent lymphocytic infiltrate of the epidermis and dermis. Importantly, mice injected with #2173 CAR T cells had mild immune infiltration of the dermis, but the basal cell layer, epidermis and keratinocytes were intact, proving the specificity of this construct for EGFRvIII. The results of the toxicity studies, in addition to high anti-tumor activity observed for the lead CART-EGFRvIII #2173, paved the way for a phase I clinical trial evaluating this cell product in patients with glioblastoma.

The relevance of this preclinical work derives from the highly translational approach undertaken by the authors with the goal of generating the optimal CAR construct in regards to anti-tumor activity and, importantly, safety. The rational development of an ideal CAR T cell product includes the *in silico* selection of the appropriate tumor target, the generation of specific scFv clones, the design and production of the CAR construct ideally testing multiple costimulatory domains, the evaluation of *in vitro* and *in vivo* anti-tumor activity and especially the assessment of potential toxicities. The studies conducted by Johnson *et al*. are a valuable reference in this respect. The authors exhaustively prove that in relevant animal models the lead #2173 anti-EGFRvIII CAR construct does not recognize wt EGFR. Nevertheless, it is certainly true that, as also stated by the authors, the proper assessment of safety and feasibility can only be done in the context of rigorous phase I trials in humans.

The choice of the optimal target is an essential component of the design of a novel CAR T cell product. EGFRvIII has multiple potential advantages: it is expressed in the cell surface, it is a neo antigen only present in cancer cells and not expressed in healthy tissues, it seems to be a driving mutation being expressed also in glioblastoma stem cells (23) and its presence is correlated with poor prognosis (12). However, there are limitations in targeting EGFRvIII: only 30% of glioblastomas are positive and the expression is usually heterogeneous with most tumors having EGFRvIII-positive and negative components. Therefore, targeting only EGFRvIII could potentially lead to escape as already reported in the setting of EGFRvIII vaccines (32), although epitope spreading has been observed in animal models (22).

It is important that the authors also evaluated the combination of anti-EGFRvIII CAR T cells with chemotherapy, an approach that can on the one hand increase response rates and on the other hand can potentially prevent tumor escape. Anti-EGFRvIII CART was tested in combination with a clinically relevant chemotherapeutic agent, temozolomide. Importantly, adjuvant temozolomide led to increased efficacy of anti-EGFRvIII CART. In an era where immunotherapy is increasingly being used for cancer treatment and multiple drugs have been approved by the Food and Drug Administration for clinical use,

the rational simultaneous combination of different agents with diverse mechanism of action is an opportunity to increase response rates. It is likely that the future of cancer therapy will include rationally-designed combinations of immunotherapeutic agents, like CART or checkpoint inhibitors, with other agents with completely different mechanisms of action, including chemotherapy or targeted molecules.

This study also exemplifies a close collaboration between academia and industry, with each group participating based on their expertise and resources. In fact the rational generation of an ideal CAR T cell product is a multi-step process that involves multiple disciplines and requires the collaboration of different research groups as manifested by the now widespread research alliances between Academia and Pharma/Biotech for the development of novel cellular immunotherapies.

There are currently two open clinical trials evaluating anti-EGFRvIII CAR therapy for patients with glioblastoma. A phase I/II trial is open at National Cancer Institute (NCT01454596) and includes the anti-EGFRvIII human 139 scFv, and a retroviral construct with CD28 and 4-1BB costimulatory domains. The other trial that is currently ongoing at the University of Pennsylvania and the University of California San Francisco (NCT02209376) is a phase I trial which includes the humanized clone #2173 (derived from the murine 3C10 clone) and a lentiviral construct with 4-1BB costimulatory domain. The results of these two important trials are eagerly awaited as they will likely prove informative for the future development of CAR therapy for solid tumors.

Acknowledgements

The authors would like to thank Dr. Saar Gill for the productive discussions on this topic and for reviewing the final manuscript. Dr. Ruella is partly supported by the EMD-Serono clinical immunotherapy fellowship from the Society for Immunotherapy of Cancer (SITC) and by a special grant from the Gabrielle's Angel Foundation.

Footnote

Provenance: This is a Guest Editorial commissioned by the Section Editor Tingting Wu, MD (Department of Cardiology, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China).

Conflicts of Interest: The authors receive research funding from Novartis Pharmaceuticals Corporation. The University of Pennsylvania has licensed technology in the field of cell and gene therapy to Novartis. BL Levine declares a financial interest due to intellectual property and patents in the field of cell and gene therapy. Conflict of interest is managed in accordance with University of Pennsylvania policy and oversight.

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Cite this article as: Ruella M, Levine BL. Smart CARS: optimized development of a chimeric antigen receptor (CAR) T cell targeting epidermal growth factor receptor variant III (EGFRvIII) for glioblastoma. Ann Transl Med 2016;4(1):13. doi: 10.3978/j.issn.2305-5839.2015.10.11

