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Authors

Holohan, Daniel R
Lee, James C
Bluestone, Jeffrey A

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Shifting the Evolving CAR T Cell Platform into Higher Gear

Daniel R. Holohan,¹ James C. Lee,¹ and Jeffrey A. Bluestone^{1,*}

¹UCSF Diabetes Center and Department of Medicine, University of California, San Francisco, 513 Parnassus Avenue, Box 0540, San Francisco, CA 94143, USA

*Correspondence: jeff.bluestone@ucsf.edu
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In this issue of *Cancer Cell*, Zhao and colleagues test various chimeric antigen receptor (CAR) T cells to show that CD28-CD3 ζ CAR T cells that constitutively express 4-1BBL promote T cell expansion and tumor eradication while reducing exhaustion. The results have important implications for the development of effective CAR T cell therapies in cancer patients.

Chimeric antigen receptors (CARs) combine an antibody-derived extracellular domain with intracellular signaling domains that promote immune cell activation and function. T cells transduced with CARs can be effectively redirected to and activated by a target antigen. “Second generation” CARs containing the cytoplasmic signaling domains of CD3 ζ and co-stimulatory (CD28 and 4-1BB) receptors have been used successfully to treat multiple blood cancers (Zhang et al., 2015). The presence of costimulatory domains vastly improved tumor eradication compared to CARs with CD3 ζ alone, largely due to the induction of survival signals and prevention of anergy (Kowolik et al., 2006).

While T cells expressing either CD28- or 4-1BB-containing CARs have achieved many complete remissions, the 4-1BB design appears to favor persistence, with CAR T cells detectable out to at least 6 months in a majority of patients, whereas the CD28 CAR T cells were typically undetectable beyond 3 months (Zhang et al., 2015). Interim analysis suggests that there is less severe cytokine release syndrome and a lower CD19-negative relapse rate using the CD28 CAR T cells (C.H. June et al., 2014, *Am. Soc. Hematol.*, abstract). However, most of the patients in this trial went on to receive curative allogeneic stem cell transplants, so they are lost to long-term follow-up to conclusively determine relapse rate. There also have been no controlled direct comparisons, so differences could reflect other variables including patient population, population of T cells used as therapeutics, CAR transduction approaches, and CD19-specific

antibodies used as the extracellular domains. That said, many groups are forging ahead to develop more effective constructs for human therapy. These efforts will require better and more predictive in vivo models, because several CAR enhancements do not affect in vitro T cell activities such as T cell exhaustion and survival (Long et al., 2015), CAR-T resistance to suppression within the tumor microenvironment, or induction of suppressive regulatory T cells (T_{regs}) (de Aquino et al., 2015).

CAR T cells that constitutively expressed the ligands for CD28 (CD80) and 4-1BB (4-1BBL) also significantly boosted T cell proliferation and tumor eradication in vivo (Stephan et al., 2007). Previous studies revealed that CARs containing both the CD28 and 4-1BB domains enhanced tumor eradication and persistence in mouse models (Zhong et al., 2010). These findings raise the possibility that the CD28 and 4-1BB signals could be delivered either directly into the T cells or indirectly in the tumor milieu to promote aggressive anti-tumor immunity. However, no one had compared whether costimulation provided directly through a CAR confers unique advantages or disadvantages to constitutive ligand expression. In this issue of *Cancer Cell*, Zhao et al. (2015) explore the differences and synergies between CD28 and 4-1BB costimulation in CAR-T cells by systematically exploring third-generation CAR T cells that contain multiple co-stimulatory modules and/or ligands with CD3 ζ . These efforts were coupled with robust in vivo modeling to discriminate between simple enhancement of cytolytic activity and characteristics that reflect CAR supe-

riority in promoting tumor elimination and T cell persistence.

The investigators compared anti-human CD19 CARs containing CD3 ζ alone (19z1), CD28 and CD3 ζ (1928z), or 4-1BB and CD3 ζ (19BBz). They observed no differences in terms of cytolytic activity and a limited enhancement of proliferation and survival of the 19BBz-transduced T cells in vitro. However, by using a novel in vivo “stress test” approach involving the injection of CAR T cells at numbers low enough that CAR T treatment failed in a significant number of animals, a direct comparison of the constructs yielded quite distinct kinetics. CD28-containing CAR T cells favored tumor elimination at earlier time points in the mouse model, whereas 4-1BB CAR-T cells persisted longer and eventually reached the same level of tumor eradication.

To determine if the enhanced cytotoxicity provided by CD28 can be combined with the enhanced T cell proliferation and persistence conferred through 4-1BB, Zhao et al. (2015) designed four additional constructs: a CAR constructed to express both CD28 and 4-1BB costimulatory domains (1928BBz), two CARs that contained either the CD28 or 4-1BB costimulatory domain while expressing the complementary costimulatory ligand (1928z-41BBL and 19BBz-CD80), and a CD3 ζ CAR that expressed both costimulatory ligands (19z1-CD80-41BBL). T cells transduced with the 1928z-41BBL construct resulted in the highest CAR-T accumulation, potent tumor eradication at day 7, and complete remission at the lowest dose of CAR T cells administered. The investigators suggested that the increased efficacy was due to a selective

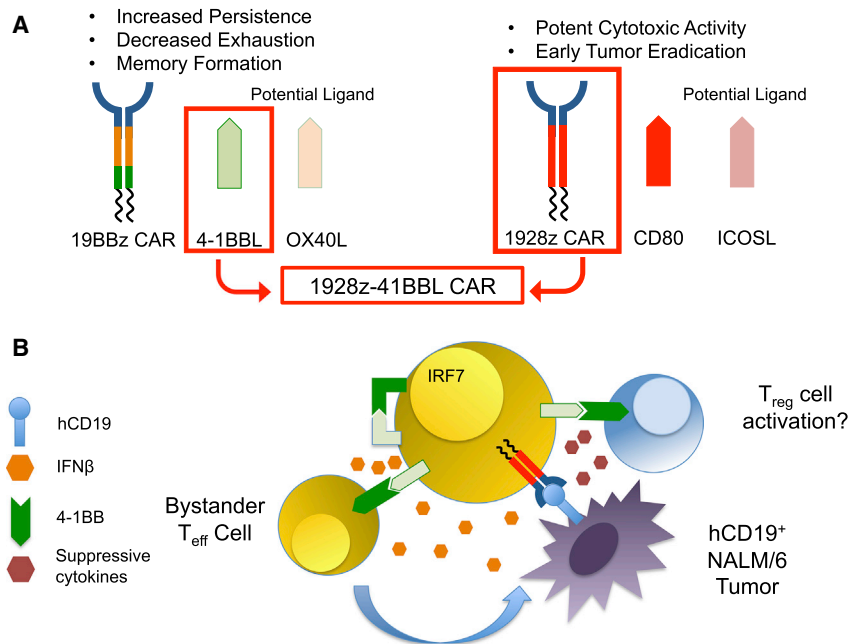


Figure 1. Advancing CAR T Cell Designs

(A) Examples of enhancing features for T cell-based cancer immunotherapy with corresponding second-generation chimeric antigen receptors (CARs), known surface ligands, and potential surface ligands that might enhance these key attributes to maximize CAR T cell efficacy. Highlighted in red is the suggested combination of CAR and surface ligand to maximize CAR T cell efficacy as modeled by [Zhao et al. \(2015\)](#). (B) A model representing a CD4⁺ 1928z-41BBL CAR T cell and its potential interaction partners in the tumor microenvironment based on previous work. This T cell shows the combined benefits of 4-1BB and CD28 costimulation while also having emergent features such as specific cytokine and transcription factor upregulation. A potential interacting cell population, T_{regs}, is also represented that can produce suppressive cytokines and thus alter the *trans* effects of 4-1BBL expression.

accumulation of CD8⁺ 1928z-41BBL T cells and decreased expression of exhaustion markers. These data are consistent with work showing that 4-1BB can alleviate anergy induced by tonic CAR signaling ([Long et al., 2015](#)). Additionally, the authors noted that 1928z-41BBL-expressing T cells express higher amounts of type-I interferon targets, including IRF7 and IFN β , and shRNA knockdown of IRF7 in 1928z-41BBL T cells significantly reduced tumor eradication and mouse survival. This change was associated with decreased IFN γ and granzyme B expression in vitro and could be rescued through exogenous IFN β .

Thus, the enhanced cytotoxicity provided by CD28 signaling can be successfully combined with the increased persistence through 4-1BB signaling to make a more potent CAR T cell ([Figure 1A](#)). The investigators then addressed whether the two signaling pathways needed to go through the same CAR or could be indirectly activated by triggering endogenous CD28 and 4-1BB receptors. The results suggest that 4-1BB signals through

the endogenous receptor may more effectively enhance CAR T cell activity, potentially in a bidirectional manner with direct signaling within the CAR T cells themselves and indirect triggering of other 4-1BB⁺ cells in the tumor microenvironment (either the transduced CAR T cells or other immune cells) ([Stephan et al., 2007](#)). Importantly, incorporation of CD28 in the CAR may be preferred, because it avoids the requirement for CD28 ligand engagement, which can be antagonized by CTLA-4 expression on effector or T_{regs} ([de Aquino et al., 2015](#)).

This study advances the development of next-generation CAR T cells, but there remain questions that will need to be addressed as these new constructs are introduced into clinical applications ([Figure 1B](#)). The immunodeficient mouse model does not address how 4-1BBL expression drives the activation of other immune cells in the tumor microenvironment, such as NK cells and dendritic cells. Immunosuppressive T_{regs} also highly express 4-1BB, although it is unclear how or whether 4-1BB signaling benefits

T_{reg} expansion and activity ([Bartkowiak and Curran, 2015](#)). If this is an issue, it will be important to determine whether the 1928BBz CAR showed less efficacy because of surface expression or a genuine difference in signaling properties, especially considering that the 19BBz and 19BBz-CD80 also show some IRF7 and IFN β production.

Lastly, the in vivo “stress test” model designed by [Zhao et al. \(2015\)](#) could elucidate how to best optimize CAR T cells for treatment-resistant cancers such as solid tumors. The potential bystander effects of IFN β could improve cytotoxicity in the tumor microenvironment and be used to test CARs that would increase efficacy for solid tumors. However, even this model may not reflect the human setting where the complexities of the tumor microenvironment, coupled with distinct expression and signaling of the various co-stimulatory pathways, could give unexpected outcomes. Thus, efforts to explore future generations of CAR constructs will depend on continued innovative approaches that can directly compare individual and combined co-stimulatory domains perhaps based on additional biomarkers that can predict human in vivo efficacy.

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