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Combinatorial Antigen Targeting: Ideal Anti-tumor T-Cell Responses and Sensing

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Abstract

T cells expressing chimeric antigen receptors (CARs) are a formidable platform for the study and application of synthetic biology approaches to study customized and flexible control of cellular functions. Recent reports in the journal *Cell* provide a new twist on combinatorial antigen targeting, profiting from the singular cleavage and signaling of the Notch receptor to conditionally express CARs.

The development of customizable strategies for spatiotemporal control of cellular behavior is one of the grand pursuits of synthetic biology and cell engineering across multiple research fields. It is integral to modern cell-based cancer immunotherapy, which well over a decade ago embraced the concept of T cell engineering with artificial antigen receptors [1]. Chimeric antigen receptors (CARs) epitomize this radical departure from classic immunology concepts. CARs are prototypic synthetic receptors wherein the extracellular input is a cell surface antigen and the intracellular output a composite T cell activating signal providing functional and metabolic cues to control T cell fate [2]. When targeting CD19, a cell surface molecule found in most leukemias and lymphomas, CAR T cells have produced remarkable clinical results, validating at once the ‘second generation’ CAR design and the synthetic biology approach to cancer immunotherapy [3].

To attain broader relevance, T cell engineering and CAR therapy in particular, must achieve effective tumor targeting and tumor elimination with minimal or tolerable toxicity. This includes averting ‘on-target/off-tumor’ reactivity (when T cells recognize the targeted antigen on normal tissues) and curbing T cell activation to prevent excessive cytokine secretion, referred to as a ‘cytokine release syndrome’ [3]. In order to achieve optimal T cell reactivity, strategies are needed that will afford both spatial control of T cells (by specifically directing them to tumor sites), as well as temporal control (to further constrain effector T cell function).

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Several approaches have been proposed to regulate CAR T cell function. These include the use of suicide genes (e.g. the inducible caspase-9 (iCasp9) enzyme) to terminate T cell activity [4], bispecific small molecules that transiently bridge antigen and CAR T cells [5], or dimerizing agents that transiently link the antigen-binding and signaling domains of a CAR [6]. The above studies allow remote temporal control of CAR T cell activity, but they do not address spatial control of antigen engagement and tumor selectivity.

To reach the latter goal, strategies engaging two antigens rather than one, provide an interesting paradigm for achieving greater tumor selectivity. One such approach involves the use of inhibitory receptors, known as iCARs, derived from PD-1 or CTLA-4 receptors, which protect normal cells based on iCAR recognition of an antigen present on normal, but not tumor cells [7]. Tumor selectivity can also be enhanced by the use of two receptors with complementary signals: a CAR-mediated minimal T cell activation signal and a chimeric costimulatory receptor (CCR) costimulation signal [8]. This can be achieved provided the two receptors target a pair of antigens co-expressed on tumor, but not normal cells [8] (Figure 1A).

In two recent back-to-back papers published in *Cell*, Morsut *et al.* and Roybal *et al.* take advantage of the synthetic Notch receptor (synNotch) system to achieve spatial control of T cell activation through combinatorial antigen recognition [9, 10]. This approach exploits Notch proteolysis controlled through its transmembrane core domain to induce transcriptional activation, which was previously shown to be modular and amenable to domain swapping [11]. Morsut *et al.* applied the synNotch concept to multiple settings including neuronal differentiation and lymphoid activation. Importantly, they showed that synNotch is cell-to-cell contact-dependent and controls cellular responses in a spatially-defined manner. Using synthetic transcription factors, the output was orthogonal to other signaling pathways, including the intrinsic Notch pathway [9].

Roybal *et al.* further documented the function of a CAR-like synNotch receptor to control the transcription of an authentic CAR, thus utilizing combinatorial antigen recognition to spatially control CAR T cell function [10]. To this end, they constructed synNotch receptors bearing a single-chain variable fragment (scFv), specific for either CD19 or GFP, as well as to Gal4-VP64 or TetR-VP64 which are transcriptional effector domains necessary to induce CAR expression. In this manner, a T cell would only be activated when both the synNotch-ligand and the CAR-ligand were simultaneously expressed on a target cell (Figure 1B). Unlike the iCAR and CAR/CCR strategy, the synNotch/CAR signaling circuit works orthogonally and independently from other intracellular signaling pathways (Figure 1A, B). However, the need for receptor cleavage and transcriptional induction create certain vulnerabilities. For instance, there is a dose/response relationship between ligand concentration and receptor activation (some extracellular domains can display poor inducibility). As such, the induction lag time, level and duration of CAR expression may limit T cell efficacy. Depending on the type of extracellular recognition domain, the synNotch receptor may display variable baseline activity, raising concerns about the system's specificity. Finally, while the use of synthetic transcriptional activators may indeed be orthogonal, the use of natural transcription factors may not be as potent or specific for CAR induction.

When primary CD4⁺ or CD8⁺ T cells engineered with an α -GFP-synNotch switch controlling CD19 CAR/luciferase expression were injected into mice bearing single- or dual- antigen CD19⁺ K562 myelogenous leukemia tumors (on either flank), the CAR/ luciferase signal was strongest in the CD19⁺GFP⁺ tumor. However, a less intense but increasing luciferase signal was also found in CD19⁺GFP⁻ tumors, possibly due to migration of activated CAR T cells from one tumor (dual-antigen) to the other (single- antigen) [10]. The kinetics of the ON-switch were thus critical in determining tumor selectivity and protection of normal tissues. The induction of CAR expression by synNotch-ligand binding would need to be fast enough to engage the tumor while T cells still localized to it. This would then be followed by decayed CAR expression, timely enough to spare healthy tissues expressing CAR target-antigen. In Jurkat T cells, the effective half-time for T cell activation was ~13h and the half-time for CAR expression decay was ~8h. In a subcutaneous two-tumor mouse model using K562 cells as targets, synNotch/CAR-engineered T cells eradicated CD19⁺GFP⁺ tumors and not CD19⁺GFP⁻ tumors [10]. However, the potential toxicity towards a normal tissue remains to be determined.

In principle, the use of synthetic Notch receptor systems has the potential of integrating multiple extracellular inputs, engineer cell signaling cascades and form cellular behavioral patterns in space and time by simply co-expressing multiple synNotch receptors ‘in-line’. This could provide novel opportunities in developmental biology and regenerative medicine and could potentially direct cellular differentiation *in vitro*. Furthermore the ability of synNotch receptor constructs to integrate defined combinatorial environmental cues can be utilized to study specific cell-cell interactions, including the tumor microenvironment. This possibility may however be curtailed by a necessitated multiplicity of chimeric receptors and synthetic transcriptional regulators, previously shown to be immunogenic in non-human primates [12].

Thus, while Wendell Lim’s reports elegantly demonstrate scFv-dependent control of cellular behavior in response to pre-specified extracellular input and orthogonal CAR induction, the translational potential of this strategy remains uncertain. Addressing the concerns regarding strength, duration and specificity of the synNotch system in more stringent and clinically relevant tumor models should be contemplated. Further quantitative studies of CAR expression kinetics and CAR-T cell migration and function will be needed to clarify the system’s clinical applicability. The definition of ‘safe distances’ shielding healthy tissues from off-tumor/on-target toxicity also remain to be explored. Importantly, the use of multiple non-human transcriptional regulators (Gal4, tTA) also raises immunogenicity concerns, and the alternative use of fully human regulatory proteins may undermine orthogonal signaling properties. Evolution itself has not resolved these challenges outside of a handful of receptors. Nonetheless, these powerful studies have at the very least, described a versatile tool for the study of CAR specificity and potency.

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Figure 1. Combinatorial Antigen Targeting Strategies for CAR Therapy

A) The CAR/CCR approach: Full T cell activation requires the combination of CAR-mediated recognition of antigen A and CCR-mediated costimulation conferred by the engagement of antigen B. CAR and CCR signaling must be designed in such a way as to fail initiating T cell activation alone, but at the same time, be able to rescue each other upon simultaneous binding of both target antigens [9]. B) The synNotch/CAR approach: T cell activation requires binding of the synNotch receptor to antigen B, triggering proteolysis of the intracellular transcriptional effector and transcriptional induction of expression of the antigen A-specific CAR [11].