Signal 1 boosters for Tmod: addressing the next obstacle in cell therapy for solid tumors

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ABSTRACT

Background: Access to tumor antigens in solid tumors is one of the major impediments to progress in oncology, exemplified by the stark difference between the success of cell therapies for blood cancers vs. solid tumors. To overcome this obstacle, cell therapy research and development has explored a wide range of methods to enhance potency without worsening toxicity. Special attention has been paid to onboard potency boosters expressed by engineered immune cells, because they more readily avoid systemic toxicity. Investigation has focused primarily on signals 2 (co-stimulatory factors) and 3 (cytokines), but other mechanisms have also been considered, including expression of transcription factors, anti-apoptotic proteins, and TGFβ pathway inhibitors. Despite the magnitude and range of these efforts to boost potency, little attention has been directed at enhancers of signal 1, beyond designs intended to improve acute sensitivity of chimeric antigen receptors (CARs) and t-cell receptors (TCRs).

Methods: Three different small-molecule-regulated systems were explored as inducible modules to regulate tonic signaling by CARs and TCRs. Acute and long-term behavior was examined, with an emphasis on inducer-dependent activation of immune function. Fusions of known members of the TCR signaling pathway were compared to identify modules with different functional properties; for example, background signaling level and ligand-induced induction ratio.

Results: A variety of constructs, including fusions to LAT, SLP76, CD3ε, and CD3ζ were shown to affect tonic signaling in a fashion that could be controlled by small-molecule inducers. Acute effects measured in Jurkat cells were also observed in primary T cells. Three clinically-tested classes of small molecule were able to act with their cognate ligand-binding fusion proteins as signal 1 boosters, demonstrating flexible small molecule control of the booster. This tunable-booster approach was extended to Tmod™, a dual-receptor NOT gate based on the LIR-1 inhibitory receptor (Hamburger AE, et al. Mol Immunol. 2020).

Conclusions: We have developed an approach to mitigate a major challenge for solid tumor cell therapy—access to tumor antigens in the blood. The system utilizes signal 1 agonists and is tunable, flexible, and modular. These signal 1 constructs can be added to CARs, TCRs, and other receptor systems to mimic an antigen stimulus with a small molecule, thereby triggering tonic (antigen-independent) signaling that reversibly activates the cells in the absence of antigen.
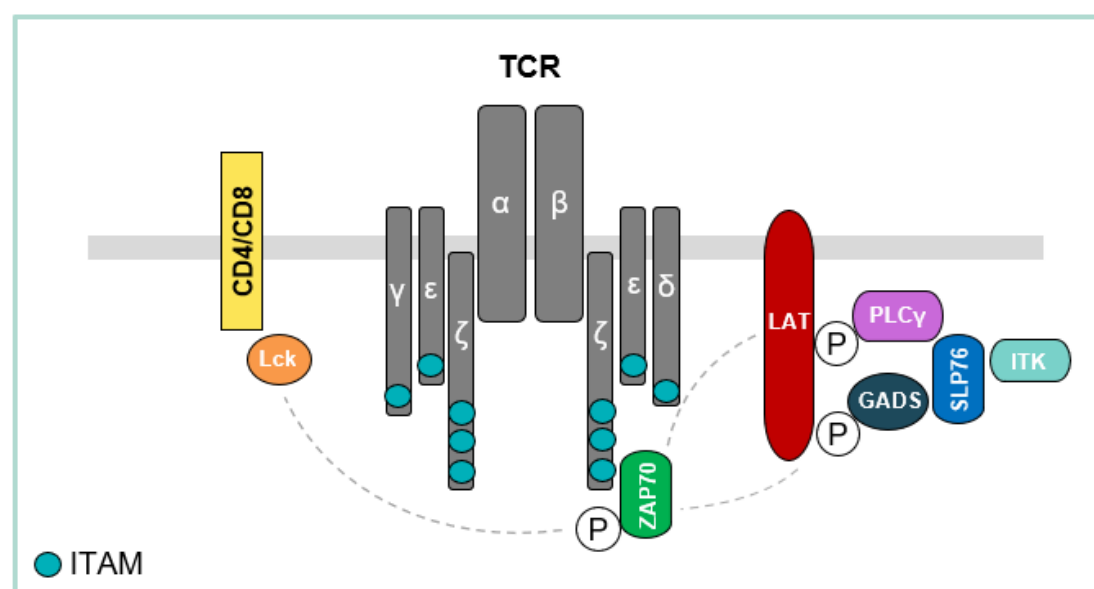
Figure 1: T-cell receptor signaling

Figure 1: A schematic depicting T-cell activation and a portion of proximal signaling molecules involved in TCR signaling. Upon antigen recognition, CD4 or CD8 engage with Lck resulting in recruitment of ZAP70 to the TCR. ZAP70 phosphorylates LAT, that then recruits several signaling proteins regulating TCR responses (e.g. GADS, PLCγ, SLP76, ITK, etc.).

CAR EXPRESSION CORRELATES WITH TONIC SIGNALING

Tmod is a dual-receptor NOT gate system with an activator (accelerator) and a blocker (brake) module [1]. In the context of Tmod, higher levels of CAR (activator) expression increases tonic signaling as measured by CD25 expression (**Figure 2**).

Figure 2: Increasing CAR expression increases tonic signaling in cells engineered with Tmod
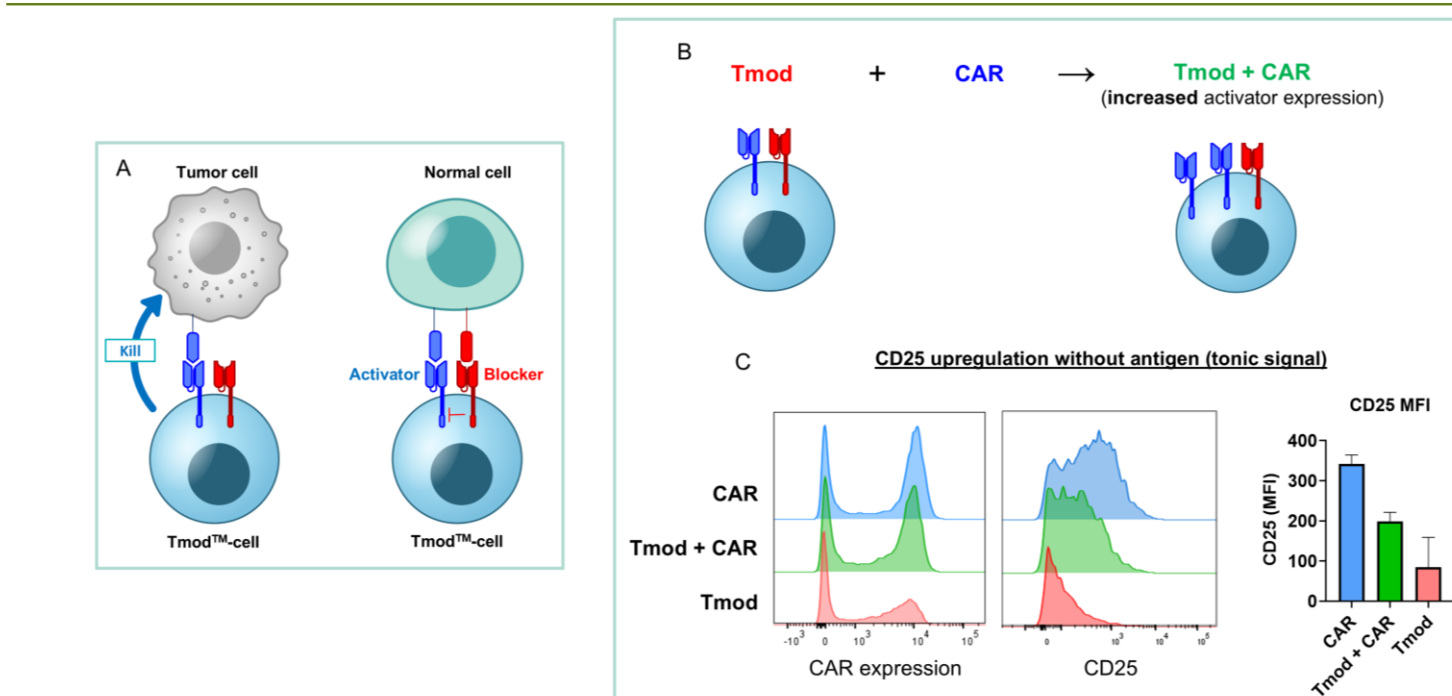

Figure 2: (A) Tmod cells target a surface antigen present on normal and tumor cells and a second surface antigen present in normal tissues that triggers the blocker, preventing cytotoxicity [1]. (B) Additional CAR constructs were transduced with Tmod constructs to increase the amount of activator expression. (C) Increased activator expression (left) correlated with antigen-independent signaling measured via CD25 MFI (center, right).

SIGNAL 1 BOOSTERS ARE INDUCIBLE WITH FKBP FUSIONS

Some efforts to boost performance of CAR-Ts have centered on downstream elements of TCR signaling (e.g., LAT, SLP76, Lck, etc. [2,3]. These have in some cases produced new designs for CARs that extend beyond the classical ITAMs of TCRs. LAT is an adaptor molecule phosphorylated after TCR activation that serves as a scaffolding complex for the recruitment of enzymes and signaling molecules involved in downstream events of T-cell activation (**Figure 1**). A LAT-FKBP fusion protein can be brought to CAR-FKBP via dimerization of FKBP domains with the small molecule rimiducid [4], triggering signaling in the absence of antigen (**Figure 3A**). The addition of rimiducid induced a dose-dependent increase in signaling up to 3-fold (**Figure 3B**).

Figure 3: Addition of rimiducid to Jurkat cells harboring FKBP fusions triggers tonic signaling
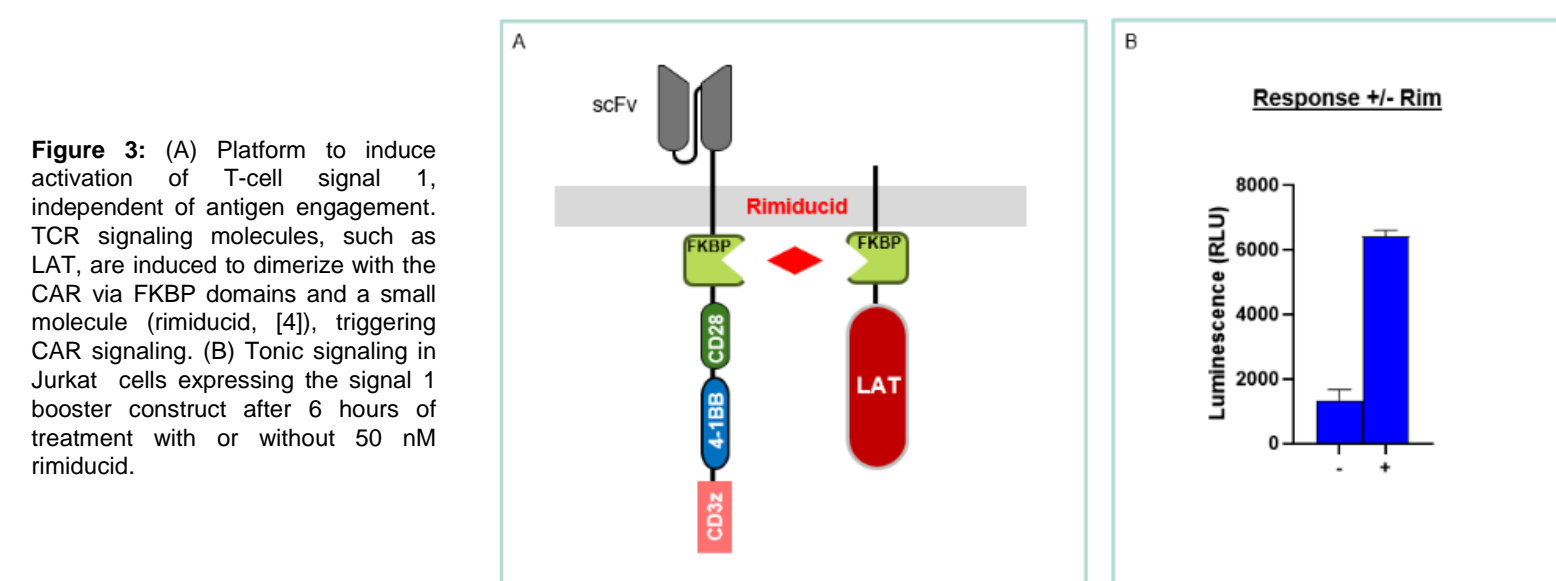

Figure 3: (A) Platform to induce activation of T-cell signal 1, independent of antigen engagement. TCR signaling molecules, such as LAT, are induced to dimerize with the CAR via FKBP domains and a small molecule (rimiducid, [4]), triggering CAR signaling. (B) Tonic signaling in Jurkat cells expressing the signal 1 booster construct after 6 hours of treatment with or without 50 nM rimiducid.

SIGNAL 1 BOOSTERS WORK WITH TMOD

The LAT-FKBP fusion protein also enhanced signaling in the context of Tmod (**Figure 4**). LAT, without an FKBP domain in combination with Tmod, increases baseline signaling, but a dose-dependent signal of rimiducid-induced oligomerization is achieved with Tmod and LAT containing FKBP (**Figure 4**).

Figure 4: Signal 1 booster in the context of Tmod

Figure 4: Signaling in Jurkat cells expressing Tmod and the LAT-FKBP construct after 6 hours of treatment with titrated rimiducid.

A RANGE OF SIGNAL 1 BOOSTERS HAVE BEEN IDENTIFIED

FKBP fusions can function as rimiducid-dependent signal 1 boosters in many contexts. Similar to activation of CARs, an FKBP domain can be added to CD3ε to oligomerize the endogenous TCR and other proximal signaling proteins involved in T-cell activation (**Figure 5**).

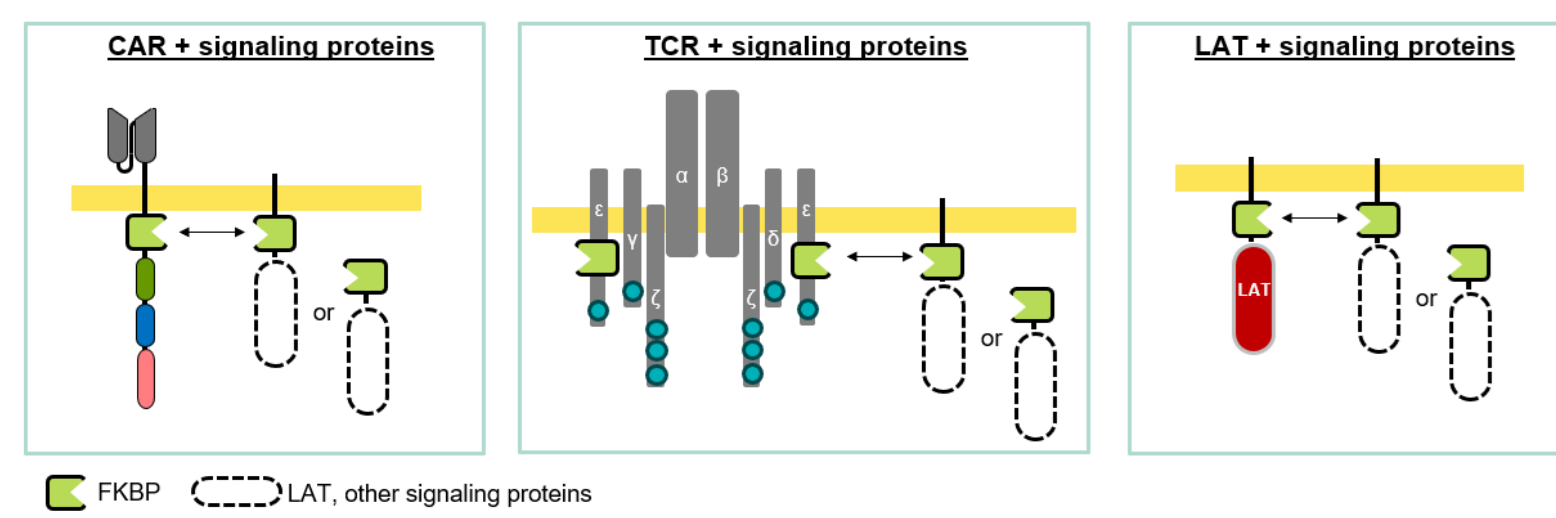**Figure 5: Signal 1 molecules can be recruited to the CAR/TCR/LAT**

Figure 5: A schematic demonstrating how signal 1 fusions to FKBP can be applied across several activation strategies. LAT or other signaling proteins can be oligomerized with the CAR (left), the TCR (middle), or LAT (right).

Figure 6: A range of signal 1 boosters have been identified

SLP76 is a component of the ITAM signaling pathway and is recruited to the LAT complex after T-cell activation (**Figure 1**). Inclusion of SLP76 with an FKBP domain can enhance activation and is one of many examples of a signaling protein that can be oligomerized with the CAR, TCR or LAT (**Figure 6**).
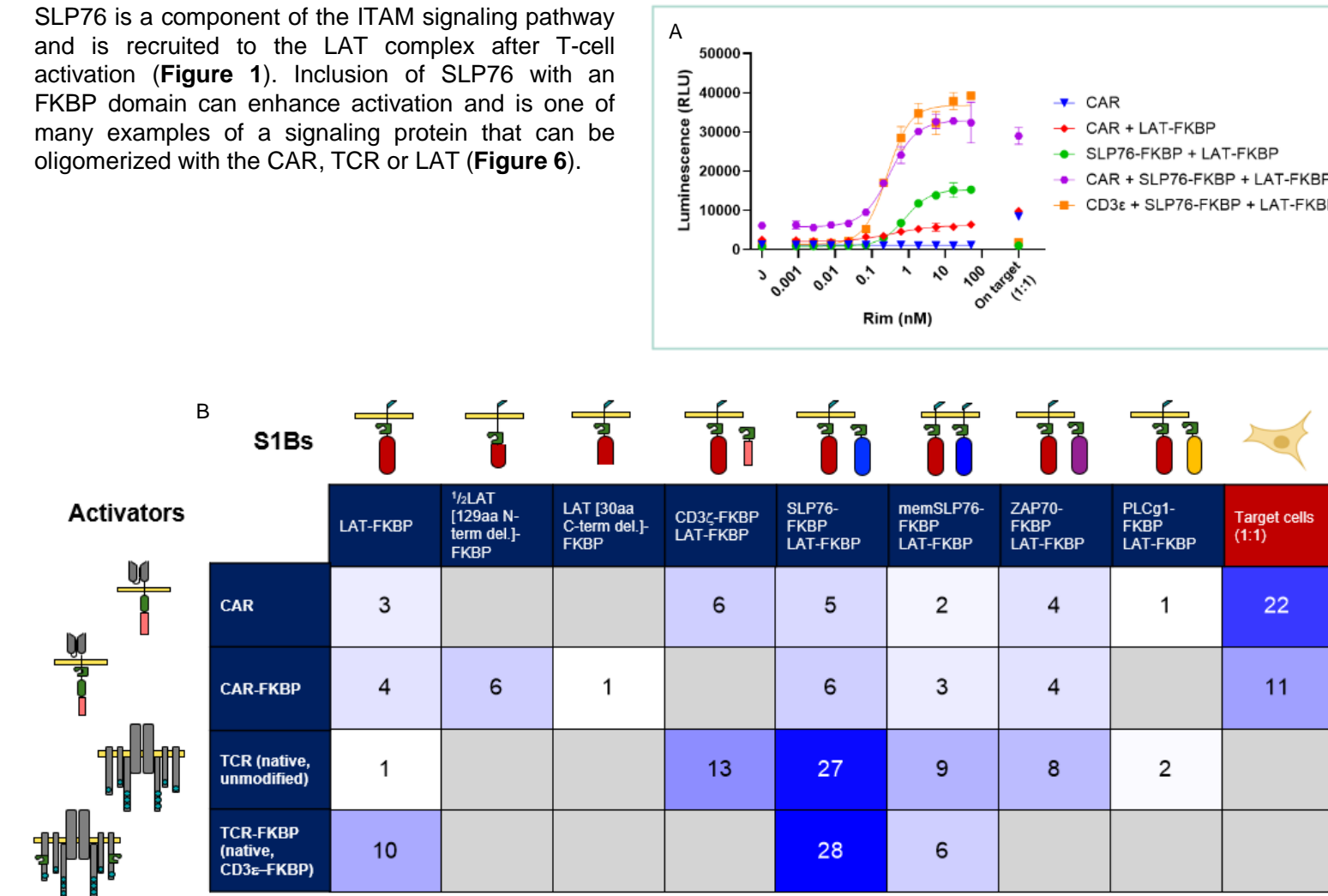

Figure 6: (A) Signaling in Jurkat cells expressing CAR or CD3ε and the LAT-FKBP construct or that of another proximal signaling molecule with an FKBP fusion, after 6 hours of treatment with titrated rimiducid. (B) A table summarizing different induction ratios of signal 1 boosters in Jurkat cells (+/- rimiducid; mem: membrane bound).

Activators	LAT-FKBP	¹/₂LAT [129aa N-term del.]-FKBP	LAT [30aa C-term del.]-FKBP	CD3ζ-FKBP	SLP76-FKBP LAT-FKBP	memSLP76-FKBP LAT-FKBP	ZAP70-FKBP LAT-FKBP	PLCg1-FKBP LAT-FKBP	Target cells (1:1)
CAR	3			6	5	2	4	1	22
CAR-FKBP	4	6	1		6	3	4		11
TCR (native, unmodified)	1		13	27	9	8	2		
TCR-FKBP (native, CD3ε-FKBP)	10			28	6				

Visit our other poster (Abstract #4072) to hear about a high-throughput screen to identify and optimize signal 1 boosters.

THREE INDUCIBLE SYSTEMS WORK WITH SIGNAL 1 BOOSTERS

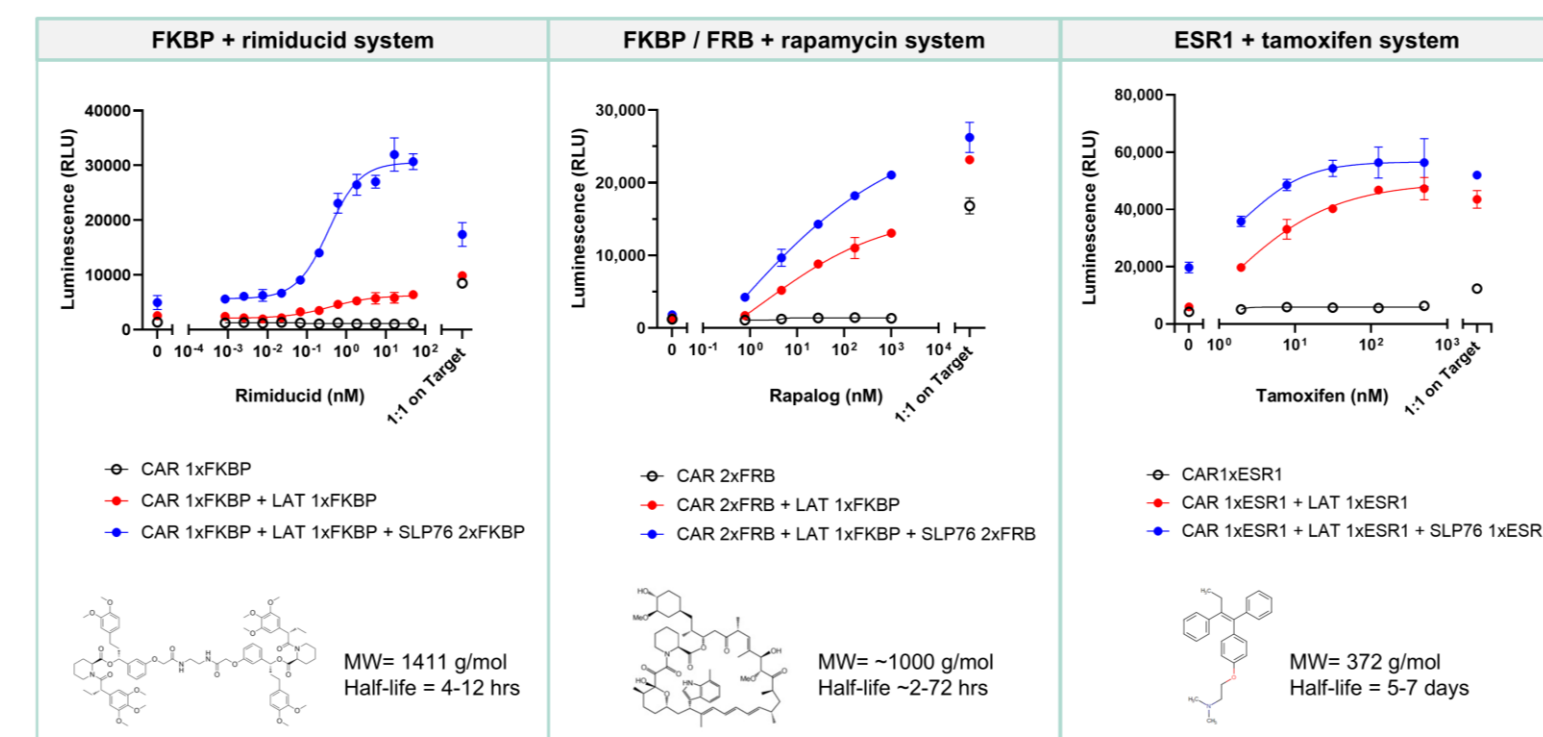### Figure 7: At least three different clinically-approved molecules work as S1B inducers

Figure 7: Signaling in Jurkat cells expressing a CAR and the signal 1 boosters LAT ± SLP76 with titrated small molecule rimiducid (left), rapamycin analog (rapalog, center) (FRB: FKBP-rapamycin-binding), or tamoxifen (right) (ESR1: estrogen receptor).

SIGNAL 1 BOOSTERS INDUCE IFN-γ SECRETION IN PRIMARY T CELLS

LAT-FKBP and SLP76-FKBP also induced interferon-gamma (IFN-γ) secretion in primary T cells. IFN-γ is a cytokine that plays a role in many functions of T cells including activation, proliferation and cytolytic activity [2]. Donor T cells co-transduced with CAR and LAT- and SLP76-FKBP fusions secrete IFN-γ in a dose dependent manner when incubated with titrated rimiducid (**Figure 8**).

Figure 8: Addition of rimiducid to primary T cells harboring FKBP fusions triggers signaling
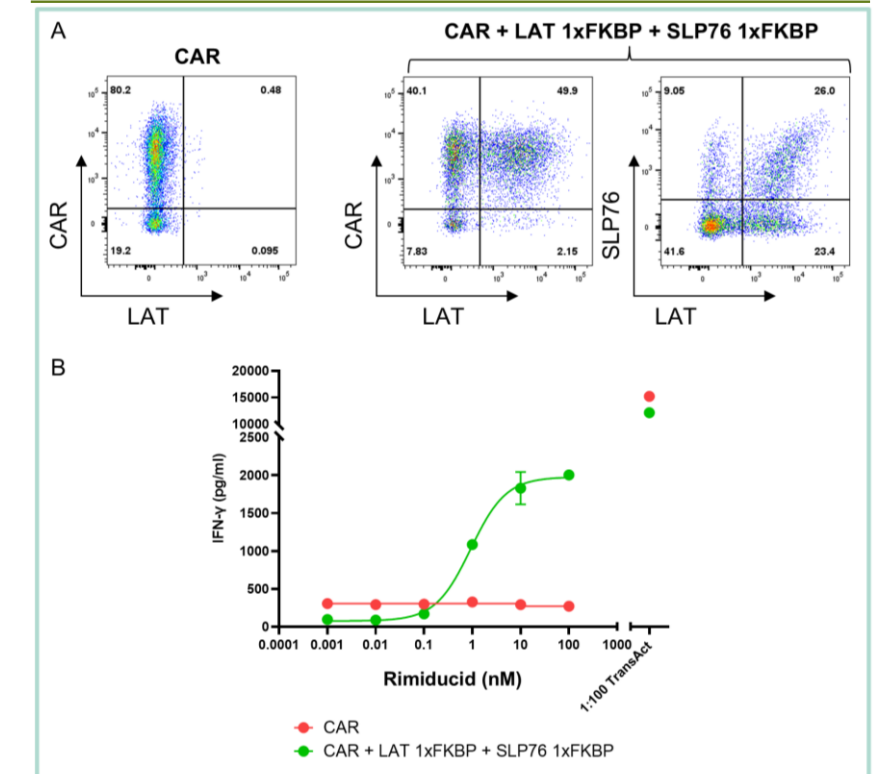

Figure 8: (A) Co-transduction and expression of CAR, LAT-FKBP, and SLP76-FKBP lentivirus in primary T donor cells measured by flow cytometry. (B) Signaling in donor T cells after 24 hours of treatment with titrated rimiducid as measured by secreted IFN-γ. 1:100 TransAct is included as a control.

TUNED SIGNAL 1 AND SIGNAL 3 IN IMMUNE CELLS

With the selective power of Tmod, an inducible system can be utilized to combine several boosters to enhance potency. An NFAT-regulated signal 3 cytokine booster can be combined to amplify activation by the signal 1 booster (**Figure 9**).

Figure 9: Development of a cell therapy that has tumor selectivity with tunable activity
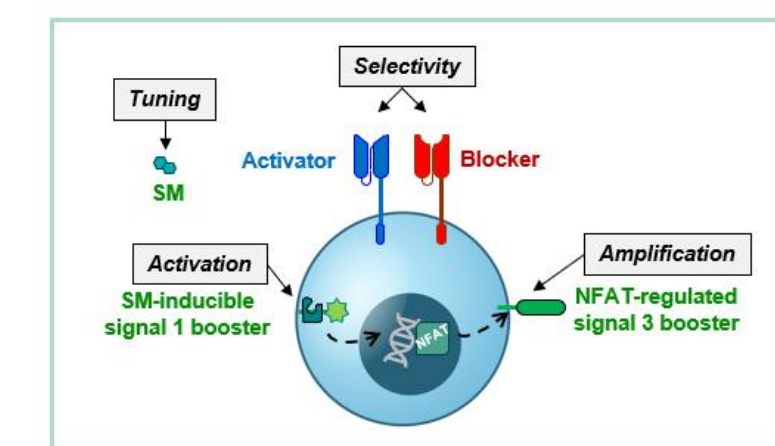

Figure 9: Tmod constructs engineered with selectivity via activator and blocker receptor, a signal 1 booster under the control of a small molecule (SM) which in turn controls a downstream NFAT-regulated signal 3 booster.

CONCLUSIONS:

- Access to tumor tissues that express target antigen is severely restricted by the blood vessel walls.
- With limiting antigen, it is unclear how antigen-dependent boosters can be brought into action.
- We address this problem by mimicking antigen with a small molecule that triggers signaling by the CAR or TCR.
- The signal 1 booster allows tuned stimulation of T cells.

References

1. Hamburger AE, et al. Mol Immunol. 2020;128:298 310.
2. Mock JY et al. ImmunoHorizons. 2021; 5: 349-359.
3. Tousley AM et al. Nature. 2023; 615: 507-516.
4. National Center for Biotechnology Information. PubChem Compound Summary for CID 16135625, Rimiducid. https://pubchem.ncbi.nlm.nih.gov/compound/Rimiducid. Accessed June 24, 2024.