Abstract Number 292

Functional screen to optimize logic gate potency and selectivity

Martire S[1], Suryawanshi V[1], Wang X[1], Mcelvain M[1], Gill T[1] , Riley T.P [1], Xu H[2], Netirojjanakul C[1], Kamb A[1]

[1]A2 Biotherapeutics, Inc., Agoura Hills, CA USA , [2]Current address: Port Therapeutics 1927 Paseo Rancho Castillo Los Angeles, CA USA

Summary

Logic-gated cell therapy systems are considerably more complicated than conventional therapeutics and, therefore, more challenging to optimize. Here we describe the design and testing of a flow-cytometry-based screening system to rapidly select functional inhibitory receptors from a pooled library of candidate constructs. In proof-of-concept experiments, this approach identifies inhibitory receptors that can operate as NOT gates when paired with activating receptors.

Introduction

Logic-gated cell therapy systems are challenging to optimize, due to the complexity of the mechanism. One type of logic gate , the NOT gate, responds to two inputs: the presence of antigen A and absence of antigen B. The best studied NOT gate, the Tmod^{TM}, consists of an activator (e.g., a chimeric antigen receptor [CAR]) that is co-expressed with an inhibitory receptor, or blocker (1) (**Figure 1**). Progress has been made toward understanding the properties of Tmod (2,3). However, the complexity of the system limits the amount of structure-activity information that can readily be obtained.

Here, we developed a high-throughput screening to optimize the Tmod system. This approach utilizes FACS (fluorescence-activated cell sorter) combined with fluorescent reporters to select for cells that contain the gene of interest. As a proof of concept, we designed libraries of blockers and identified ones that function in the context of Tmod. Jurkat coculture assays were used to confirm top hits from the screen. In addition, future applications may include acquisition of large-scale data sufficient to train neural network models, bypassing the empirical process of Tmod optimization (4).

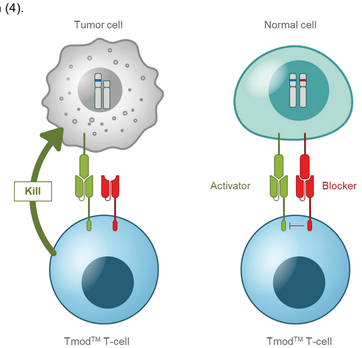


Figure 1. A schematic representation of the Tmod system
Tmod, which consists of an activator and a blocker, selectively kills tumor cells that express only the activator antigens, while protecting normal cells expressing both activator and blocker antigens.

Engineering of the Jurkat Reporter Cell Line

Figure 3. The Jurkat cell line used in this study was engineered to fit for the purpose of enriching blockers by FACS. Jurkat cells containing the fluorescent reporter (GFP), which is located downstream of NFAT-regulated promoter.

Validation of the Jurkat Reporter Cell Line

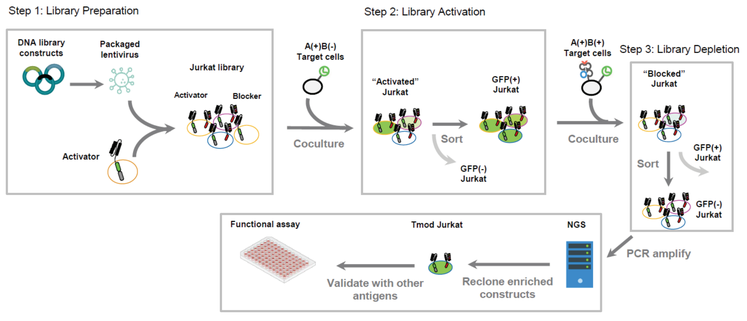
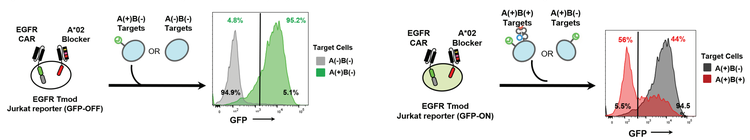


Figure 2. Step 1-Library Prep: A library of blockers is cloned into a lentiviral vector and transduced into an optimized GFP-reporter line of Jurkat cells, which express a CAR activator. **Step 2-Activation:** Jurkat cell library is exposed to target cells expressing only the activator antigen (A(+)B(-)) and sorted using FACS to isolate the activated GFP(+) population. **Step 3-Depletion:** The GFP(+) Jurkat cells are exposed to target cells expressing both activator and blocker antigens (A(+)B(+)) and sorted to isolate GFP(-) fraction. **Step 4-Analysis and Verification:** Cells are collected and analyzed by NGS to identify candidate blocker sequences enriched during the process.

Validation of the Jurkat Reporter Cell Line

Figure 4. Quantitatively measuring activation and blocking windows by flow cytometry using EGFR-CAR. GFP fluorescence is measured after 18hrs of coculture with HeLa target cells.



$$\text{Activation} = \frac{\%\ \text{GFP (+) cocultured with A(+)B(-)}}{\%\ \text{GFP (+) cocultured with A(-)B(-)}}$$

$$\text{Blocking} = \frac{\%\ \text{GFP (-) cocultured with A(+)B(+)}}{\%\ \text{GFP (-) cocultured with A(+)B(-)}}$$

Activation: HeLa cells that express A antigen (EGFR) were presented to the Jurkat reporter cell lines (transduced with EGFR CAR), to activate the NFAT promoter and GFP signal.

Blocking: Jurkat cells that express both the EGFR CAR and an HLA-A*02 blocker were cocultured with HeLa cells that express both target antigens (A(+)B(+)).

Kinetics of Activation and Blocking with Jurkat Line

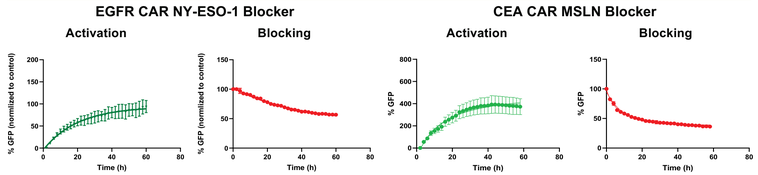


Figure 5. Kinetic Imaging assay to confirm induction of the Jurkat reporter cell line over time. Measured by imaging the GFP signal every 2 hours on an IXM imager using 2 different Tmod activator/blocker pairs.

NY-ESO-1 Blocker Library Construction

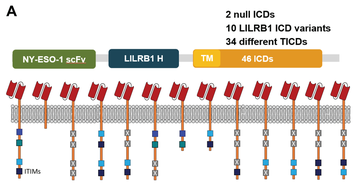


Figure 6. (A) 46 Variants of the NY-ESO-1 Blocker
The blocker library comprised of 46 constructs with NY-ESO-1 scFv, LILRB1 hinge (H) and transmembrane domains (TM), and 46 different intracellular domains (ICDs) (2 null ICDs, 10 LILRB1 ICD variants, and 34 different ICDs)

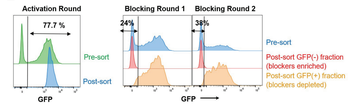


Figure 6. (B) Jurkat reporter cells that express an EGFR CAR and blocker library were cocultured with A(+) HeLa cells that express EGFR antigen, sorted for the GFP(+) population to enrich cells able to activate. After recovery in media, activated cells were cocultured with A(+) B(+) HeLa cells that express EGFR antigen and NY-ESO-1 trimer antigen and sorted for GFP(+) and GFP(-) fractions. Flow plots show the gates for the activation step and the blocking rounds.

Enrichment analysis by NGS

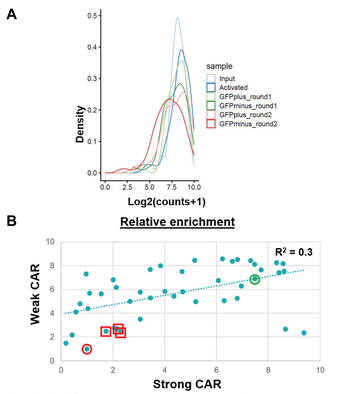


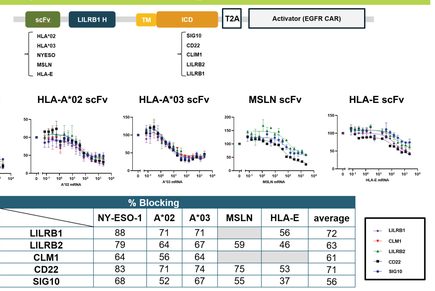
Figure 7. (A) NGS generates reads that are mapped to the library sequences comparing counts and frequencies of each variant. >90% of reads were successfully aligned to the reference library. **(B)** Scatter plot of enrichment values for 2 EGFR CARs (one strong and one weak activator). The LILRB1 wild-type positive control is circled in green. The negative control is in red. The 3 variants with <2 WT ITIMs are boxed in red.

Ranked List by Enrichment Relative to the Negative Control

STRONG EGFR CAR		WEAK EGFR CAR	
Gene	Enrichment vs. Negative Control	Gene	Enrichment vs. Negative Control
EPOR	20.6	G6B	8.6
SCIMP	10.2	SIGL9	8.5
CD244	9.4	CEAM1	8.5
SLAF5	8.7	PILRA	8.4
CD22	8.6	SHPS1	8.4
SIG10	8.6	CLIM1	8.3
LIRB2	8.4	SIG10	8.2
CLIM1	8.3	LAIR1	8.1
LIRB1 positive control	7.7	CD33	8.0
KIR3DL2	7.5	MPZL1	7.7
LAIR1	7.5	LIRB1 positive control	7.7

Figure 8. Ranked list of library constructs from strong EGFR CAR and weak EGFR CAR experiments (top 10 out of 46 are shown). Enrichment is calculated as the ratio of variant frequencies in the round 2 sample divided by the frequency in the activated sample prior to round 1. This enrichment is divided by the negative control (LILRB1 with truncated ICD) to give enrichment relative to the negative control.

Functional Assay to Confirm Blocking

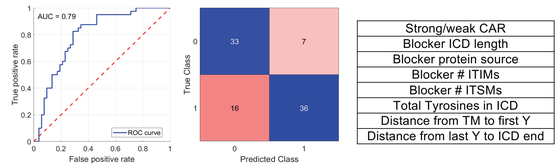


C

% Blocking						
	NY-ESO-1	A*02	A*03	MSLN	HLA-E	average
LILRB1	88	71	71		56	72
LILRB2	79	64	67	59	46	63
CLM1	64	56	64			61
CD22	83	71	74	75	53	71
SIG10	68	52	67	55	37	56

Figure 9. Functional assay to confirm blocking of enriched ICDs using 5 blockers. **(A)** Diagram of the 5 blocker constructs that were chosen and cloned into a single vector containing 5 different scFvs fused to the LILRB1 backbone plus the EGFR CAR. **(B)** Functional assays in Jurkat NFAT-luciferase reporter cells using HeLa target cells that express the different activator antigens, titrated with blocker antigen mRNA. The plots show comparable dose-dependent blocking of all the constructs tested. **(C)** Percent blocking of 5 activators with selected enriched blocker constructs.

Machine Learning Models Identify Predictors of Enrichment



- Strong/weak CAR
- Blocker ICD length
- Blocker protein source
- Blocker # ITIMs
- Blocker # ITSMs
- Total Tyrosines in ICD
- Distance from TM to first Y
- Distance from last Y to ICD end

Figure 10. To further analyze the data from enrichment, a linear regression machine learning models were developed to predict enrichment. 8 features were selected to construct a model predicting successful or unsuccessful enrichment for each blocker and CAR pair. Most models predicted the blocker behavior with 70-80% accuracy.

References
1. **DiAndreth, B.,** et al., *The Tmod cellular logic gate as a solution for tumor-selective immunotherapy.* Clin Immunol, 2022. 241: p. 109030.
2. **Marry, D.,** et al., *Robust In Vitro Pharmacology of Tmod, a Synthetic Dual-Signal Integrator for Cancer Cell Therapy.* Front Immunol, 2022. 13: p. 826747.
3. **Partin, A.C.,** et al., *Geometric parameters that affect the behavior of logic-gated CAR T cells.* Front Immunol, 2024. 15: p. 1304765.
4. **Martire et al.** *High-throughput screen to identify and optimize NOT gate receptors for cell therapy* 2024 (submitted)

Presented at the Society for Immunotherapy of Cancer 39th Annual Meeting, November 8–10, 2024