P #115

An NFAT Promoter–Based Fluorescent Jurkat Cell Platform for High-Throughput Screening of Chimeric Antigen Receptor (CAR) Constructs

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INTRODUCTION

- CAR T cells have exhibited efficacious treatment of hematological malignancies such as acute myeloid leukemia (AML).
- Upon antigen binding, CARs initiate Ca2+-dependent signaling pathways, leading to an increased intracellular concentration of nuclear factor of activated T cells (NFATs), which stimulates downstream T-cell effector functions.[1,2]
- An objective high-throughput platform to compare CAR-induced T-cell activity has been lacking.

OBJECTIVE

- We report on a CAR screening platform that utilizes an NFAT-sensitive promoter driving fluorescent protein expression in Jurkat T cells.
- The IL2 reporter system (IRS) is employed for the identification of functional CD33 CARs to improve AML CAR T-cell therapies.

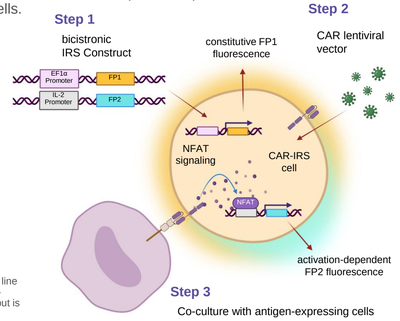
METHODS

- Lentiviral transduction of 2 fluorescent proteins (mOrange2 and mTurquoise2) under either the constitutively active EF1α (FP1) or the NFAT-sensitive minimal IL2 promoter (FP2) into Jurkat cells resulted in 2 IRS cell lines.
- IRS cell function was confirmed by treatment with phorbol myristate acetate (PMA) and ionomycin to induce activation and thus the expression of FP2.
- To screen CARs with previously described potential in AML therapy, IRS cells were transduced with 8 distinct CD33 CAR constructs and cocultured with CD33+ cells (*MOLM13*^{WT}) and CD33 knock-out (*MOLM13* [CD33KO]) cells.
- IRS cell analysis was performed using flow cytometry, fluorescent microscopy, and IncuCyte live cell imaging.

RESULTS

Fig. 1. Illustration of the IL-2 Reporter System

- **Step 1**: Stable integration of the IRS construct into the Jurkat cell genome, which contains FP1 (mOrange) under the constitutively active EF1α promoter, and FP2 (mTurquoise) under the NFAT-sensitive IL2 promoter.
- **Step 2**: After lentiviral transduction, CAR protein expression results in CAR-IRS cells.
- **Step 3**: Coculture of CAR-IRS cells with antigen-expressing cells induces antigen binding and CAR-IRS cell activation. NFAT production activates the IL2 promoter and results in quantifiable FP2 expression, which is used as a proxy for CAR-induced cell activation.



Note: A second Jurkat IRS cell line with FP1-mTurquoise and FP2-mOrange was also generated but is not depicted here.

RESULTS (CONT'D)

Fig. 2. IRS Cell Lines Exhibit FP2 Expression in Response to Activation With PMA/Ionomycin

- Micrographs at 10x magnification show wild-type (WT) Jurkat cells and both IRS cell lines either untreated (−) or treated (+) with PMA/Ionomycin for 24 hours to induce cell activation.
- Cell fluorescence was captured in the 590-nm "red channel" for mOrange expression and 385-nm "blue channel" for mTurquoise expression.
- Untreated cells exhibited FP1 fluorescence only, while treated cells exhibited both FP1 and FP2 fluorescence.

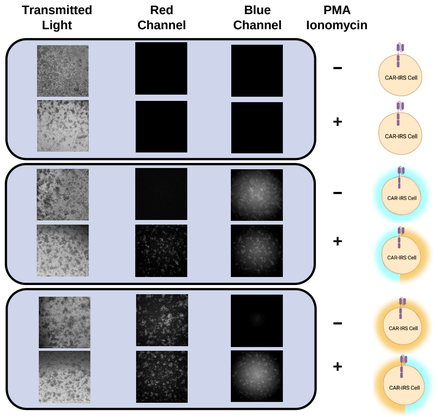


Fig. 3. IncuCyte Live Cell Imaging Detects Robust FP2 Expression Kinetics

- Fluorescence was measured in the IncuCyte (**A**) "blue" channel for mTurquoise, (**B**) "red" channel for mOrange, and (**C**) "blue + red overlap" channels over a 24-hour time course.
- Measurable increase of FP2 fluorescence was observed ~5 hours after treatment for the EF1α_mOrange_IL-2_mTurquoise IRS line and ~10 hours after treatment for the EF1α_mTurquoise_IL-2_mOrange IRS line.
- Peak expression occurred at 13 to 15 hours after treatment, and both cell lines maintained measurable FP2 fluorescence for ≤24 hours after treatment.

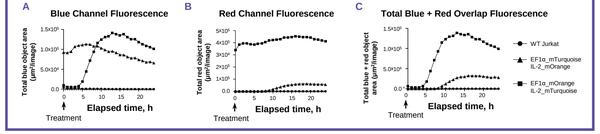


Fig. 4. Gating Strategy of IRS Cell Analysis by Flow Cytometry

- Viable cells were gated for expression of anti-CD33 CAR (CD33 CAR+) and FP1 (FP1+).
- FP1-positive cells were analyzed for FP2 expression (FP2+) upon coculture with *CD33+ MOLM13WT* and CD33KO cells. FP2 expression in response to CD33KO cells was used to determine background.

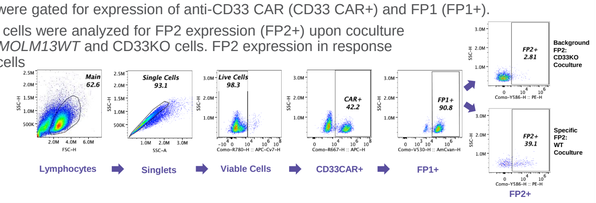
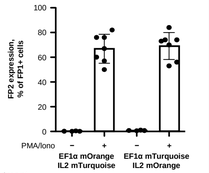


Fig. 5. Flow Cytometry Data Confirm IRS Cell Function After PMA/Ionomycin Treatment

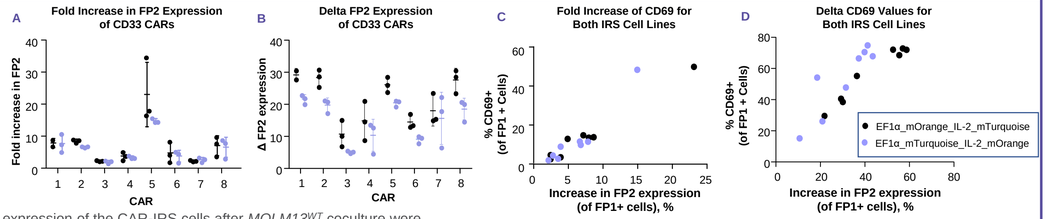
- Both IRS cell lines were either untreated (-) or treated (+) with PMA/Ionomycin for 24 hours and analyzed by flow cytometry.
- FP2 expression increased in about 60% of all FP1+ cells in response to treatment. The range of FP2+ cells was consistent between both IRS cell lines.



Note: CAR expression was not accounted for in this experiment.

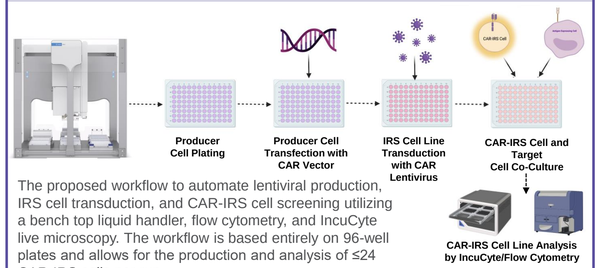
Fig. 6. Flow Cytometry Analysis of 8 Different Anti-CD33 CAR Constructs Indicates CAR-IRS Cell Line Activation Is Reliably Represented by FP2 Expression

- Anti-CD33 CAR-IRS cell lines were cocultured with CD33+ *MOLM13*^{WT} cells and *MOLM13* (CD33KO) cells for 24 hours.



- Differences in FP2 expression of the CAR-IRS cells after *MOLM13*^{WT} coculture were compared to *MOLM13* (CD33KO) coculture as (**A**) fold increase from baseline or (**B**) absolute increase from baseline (delta, Δ).
- Jurkat cells respond to activation by CD69 expression. To correlate CD69 and FP2 expression, cells were also stained for CD69, and the percentage of CD69+ FP1+ cells was plotted over (**C**) fold increase or (**D**) absolute increase in FP2 expression as determined in (**A**) or (**B**), respectively.

Fig. 7. Automating CAR-IRS Cell Line Generation and CAR Activity Screening



The proposed workflow to automate lentiviral production, IRS cell transduction, and CAR-IRS cell screening utilizing a bench top liquid handler, flow cytometry, and IncuCyte live microscopy. The workflow is based entirely on 96-well plates and allows for the production and analysis of ≤24 CAR-IRS cells per run.

CONCLUSION

- IRS cells can be used as an objective, fast, and reliable reporter system for CD33 CAR activity.
- The constitutively expressed FP1 eliminates false negative outcomes and verifies successful transduction of the reporter construct. FP2 expression, driven only in activated cells, represents antigen recognition by and activity of the CAR.
- The use of IRS cells is not restricted to just CD33 CARs but provides a platform for CAR and recombinant T cell receptor screenings against any antigen.
- Efforts are underway to adapt this platform entirely to microplates and a liquid handling system, allowing for high-throughput screens of several AML-targeting CAR constructs.

References

1. Hogan PG. *Cell Calcium.* 2017;63:66-69.
2. Chow C-W, et al. *Mol Cell Biol.* 1999;19(3):2300-2307.

Acknowledgments

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