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Corresponding author(s): James A. Wells

☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

Describe how sample size was determined.

Each well of the CAR T-cell activation studies contained 50,000 CAR T-cells and 100,000 K562 cells. These ratios and values were chosen based on previously reported protocols.

2. Data exclusions

Describe any data exclusions.

No data was excluded.

3. Replication

Describe whether the experimental findings were reliably reproduced.

The CRISPRa cellular studies were performed as four independent replicates for the single concentration experiments (Figure 3b), and three independent replicates for the drug titration experiment (Figure 3c). CAR T-cell activation studies were performed as three independent replicates (Figure 4c). Cell viability assays were performed as three independent replicates (Supp Figure 11). The data was very reproducible in all cases.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

N/A

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

N/A

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	RStudio, FlowJo, and GraphPad Prism

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	We will make the plasmids necessary for AbCID generation available to the community under MTA.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	As part of this study we generated and validated 10 unique Fabs for use in our AbCIDs. In addition, we utilized the following commercially available Antibodies: (HRP)-conjugated anti-Fab monoclonal antibody (Jackson ImmunoResearch 109-036-097) anti-AviTag antibody (GenScript mouse mAb, A01738) Myc-Tag Mouse mAb Alexa Fluor647 conjugate (Cell Signaling 2233S) APC anti-human CD69 Antibody (Biolegend 310910) BD Human IL-2 ELISA Kit

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	The NFAT reporter Jurkat cells utilized were a generous gift from Arthur Weiss. The K562 and HEK293T cells utilized were from frozen stocks maintained by the Wells lab.
b. Describe the method of cell line authentication used.	No further authentication of the cell lines was performed before use.
c. Report whether the cell lines were tested for mycoplasma contamination.	No test for mycoplasma contamination was performed.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	None of these cell lines were utilized.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	N/A

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	N/A

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Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.	Jurkat cells expressing CARs were mixed with antigen positive (CD19+) or antigen negative (CD19-) K562 target cells at a ratio of 1:2. Bispecifc antibody (AZ1-αCD19) or Fab (AZ1) and ABT-737 or DMSO was diluted in media and added to cell mixtures. After overnight incubation at 37 ˚C, cells were pelleted by centrifugation. NFAT-dependent GFP reporter expression was quantified by flow cytometry using a FACSCanto II (BD Biosciences). CD69 expression was quantified by immunofluorescence flow cytometry using a FACSCanto II (BD Biosciences) using an APC anti-human CD69 Antibody (Biolegend 310910).
6. Identify the instrument used for data collection.	FACSCanto II (BD Biosciences)
7. Describe the software used to collect and analyze the flow cytometry data.	BD FACSDIVA was used to collect all flow cytometry data. All flow cytometry data analysis was performed using FlowJo software and all plots were generated using Prism software (GraphPad).
8. Describe the abundance of the relevant cell populations within post-sort fractions.	Data is provided in Figure 4, Supplementary Fig. 10, and Supplementary Fig. 15.
9. Describe the gating strategy used.	CAR T-cell activation assay gating scheme. Jurkat - NFAT Reporter cells and K562s were assessed in isolation (Top) to determine a Live Cell (Jurkat Only) gate. For co-culture experiments, live Jurkat cells were gated (Gate 1), and GFP (Gate 2) and CD69 staining (Gate 3) positive populations were quantified.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☒