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☐ 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.

A statistical method was not used to predetermine sample size. A minimum of triplicates was chosen to allow for calculation of statistics. In the majority of instances, data were collected using flow cytometry where > 770 events were collected to characterize a distribution of the data. Biologically independent experimental triplicates then allowed for statistical analysis of selected features of the data distributions.

2. Data exclusions

Describe any data exclusions.

No data were excluded from the analyses.

3. Replication

Describe whether the experimental findings were reliably reproduced.

All attempts at replication were successful.

4. Randomization

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

For in vitro experiments, samples were allocated to identical cell-culture flasks/wells in a spatial pattern that facilitated organization. There is no reason to believe the spatial location of the sample influenced experimental results. When evaporation was a concern (such as for multi-day experiments), volumes were regularly assessed and uniformly corrected.

5. Blinding

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

The same experimentalist labeled and processed in vitro samples. While strict blinding and de-identification was not performed, all authors agree that care was taken to uniformly process all samples in an experiment when acquiring data. Uniform data processing was applied to experimental samples regardless of whether they were control or test samples. Since the results reported are either entirely quantitative (i.e., not subjective in nature) or unambiguous in nature (e.g., either fluorescent or not under confocal microscopy), blinding was unnecessary for the experiments performed.

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.	FlowJo (TreeStar) was used to analyze flow cytometry data. Software for the ChemiDoc XRS+ System was used to image blots and FIJI/ImageJ was used to analyze blots. NIS Elements 4.2 was used to help acquire microscope images and FIJI was used to analyze microscopy data. FCAP Array v3.0.1 was used to analyze data collected with the BD Cytometric Bead Array kit. R 3.3.2 was used to plot equations. R 3.3.2 and Microsoft Office Excel were used to calculate statistics.

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.	No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	All antibodies in this study were validated first by the manufacturer. For flow cytometry and microscopy experiments, antibodies were also internally titrated in our lab. Titration experiments involved staining positive and negative samples with solutions containing an antibody at various dilutions. Antibodies were subsequently used at dilutions that provided the widest dynamic range in the titration study. The following antibodies were used in this study: Flow cytometry: Antibodies that bind CD69 (clone FN50, BioLegend), DYKDDDDK (clone L5, BioLegend; or #130-101-571, Miltenyi Biotec), EGFR (Erbitux, Bristol-Myers Squibb), TNF-α (clone Mab11, BioLegend), IFN-γ (clone 4S.B3, BioLegend), and IL-2 (clone MQ1-17H12, BioLegend). Western Blots: Primary antibodies for CD3ζ (#551034, BD Biosciences), phosphorylated SMAD2/3 (clone D27F4, Cell Signaling Technology), CD19 (#AP1494a, Abgent), and GAPDH (clone GAPDH 71.1, Sigma-Aldrich), as well as anti-mouse or anti-rabbit IgG (H+L) secondary antibodies (Jackson ImmunoResearch). Microscopy: ZAP70 antibody (clone IE7.2, Thermo Fisher Scientific).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Primary T-cell lines were freshly isolated with the RosetteSep CD4+ or CD8+ Human T-cell Enrichment Cocktail (Stemcell Technologies) from healthy donor whole blood obtained from the UCLA Blood and Platelet Center. Jurkat Clone E6-1 cells, HEK293T cells, and HepG2 cells were obtained from ATCC (TIB-152, CRL-11268, and HB-8065 respectively). The EGFP NFAT reporter Jurkat cell line was a gift from Dr. Arthur Weiss (University of California, San Francisco). The EGFP NFκB reporter Jurkat cell line was a gift from Dr. Xin Lin (MD Anderson). The TM-LCL cell line, an Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line, was a gift from Dr. Michael Jensen (Seattle Children's Research Institute). The OKT3+ TM-LCL cell line was a gift from Dr. Stephen Forman (City of Hope National Medical Center).

b. Describe the method of cell line authentication used.

When isolating primary T cells, isolated fractions were confirmed to have CD3 and CD4 or CD8 expression. Generation of cell lines expressing transgenes were verified by flow cytometry to assess transgene expression. NFAT and NFκB reporter Jurkat cell lines were verified through their ability to produce EGFP upon antigen stimulation. The TM-LCL and OKT3+ TM-LCL lines had been confirmed by flow cytometry to express CD19 and CD20, and the OKT3+ TM-LCL line was further confirmed to nonspecifically stimulate T-cell activation (presumably via OKT3 binding to T-cell receptors). Cell lines received from ATCC were certified by ATCC and no further authentication was performed on these cell lines.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cell lines were tested periodically for mycoplasma contamination. Any cell line found to be mycoplasma positive was discarded.

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 the specific cell lines used are listed as commonly misidentified. HEK cells, the parental line of HEK293T that we used, are listed. We used HEK293T as a means for protein and lentivirus production. This cell type is commonly used for such purposes due to their susceptibility to transient transfection and the ease in routine culture. Notably, the exact biology of the cell line is less important than the eventual purity of the generated protein and virus products.

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.

No animals were used.

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.

Study did not involve human participants.

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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.	Samples contained either pure populations of immortalized cell lines or primary T cells. Primary T cells were initially isolated from human whole blood with the RosetteSep CD4+ or CD8+ Human T-cell Enrichment Cocktail (Stemcell Technologies) and explanded using CD3/CD28 Dynabeads (ThermoFisher Scientific) at a 1:1 cell:bead ratio. Prior to flow cytometry, samples were washed in PBS + 2% FBS and stained with antibodies if needed. For intracellular staining, cells were first fixed with 1.5% formaldehyde and permeabilized with ice-cold methanol prior to any antibody staining.
6. Identify the instrument used for data collection.	A Miltenyi MACSQuant VYB was used to collect flow cytometry data.
7. Describe the software used to collect and analyze the flow cytometry data.	Data was collected with the Miltenyi MACSQuant VYB and its native operative system and software. Data files were analyzed using FlowJo for Windows.
8. Describe the abundance of the relevant cell populations within post-sort fractions.	Cell lines that were sorted were sorted to near purity (96-100%). Purity was assessed using flow cytometry to detect the presence of a fluorescent protein or surface-stained marker present in the population of interest.
9. Describe the gating strategy used.	Initial FSC-Area/SSC-Area gates are drawn to remove debris and dead cells. A subsequent FSC-Area/FSC-Height gate indicates the singlet events. Multicolor samples are compensated with the help of single-color controls. The boundaries between "positive" and "negative" cell populations are defined by either an identically treated true-negative sample or the same sample stained with an isotype control antibody if no true-negative is available.

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