

SUPPLEMENTARY MATERIAL

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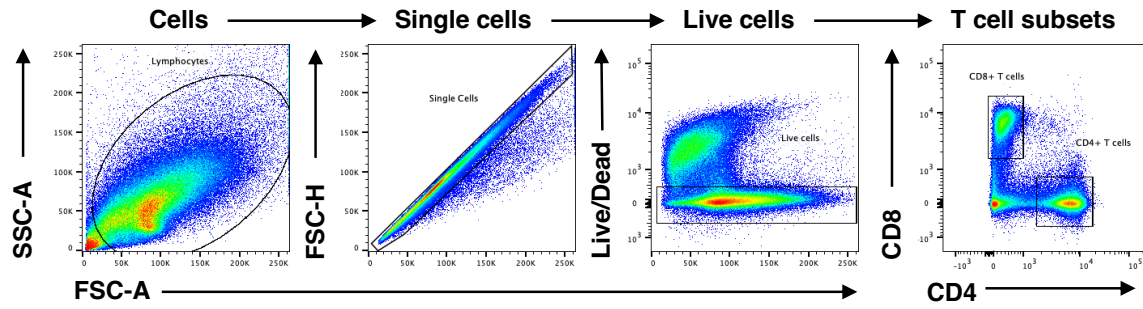


Figure S1 Gating strategy for CD4⁺ and CD8⁺ T cells using conventional flow cytometry after in vitro T-cell-specific activation of PBMCs

Total cells are gated based on size (forward scatter, FSC-A) and granularity (side scatter, SSC-A) to remove debris. Single cells are determined by their direct proportionality between FSC-area (FSC-A) and FSC-height (FSC-H). Live cells are identified by their absence of fluorescence of a membrane-permeable dye. T cell subsets are then identified by their high expression of either CD4 or CD8.

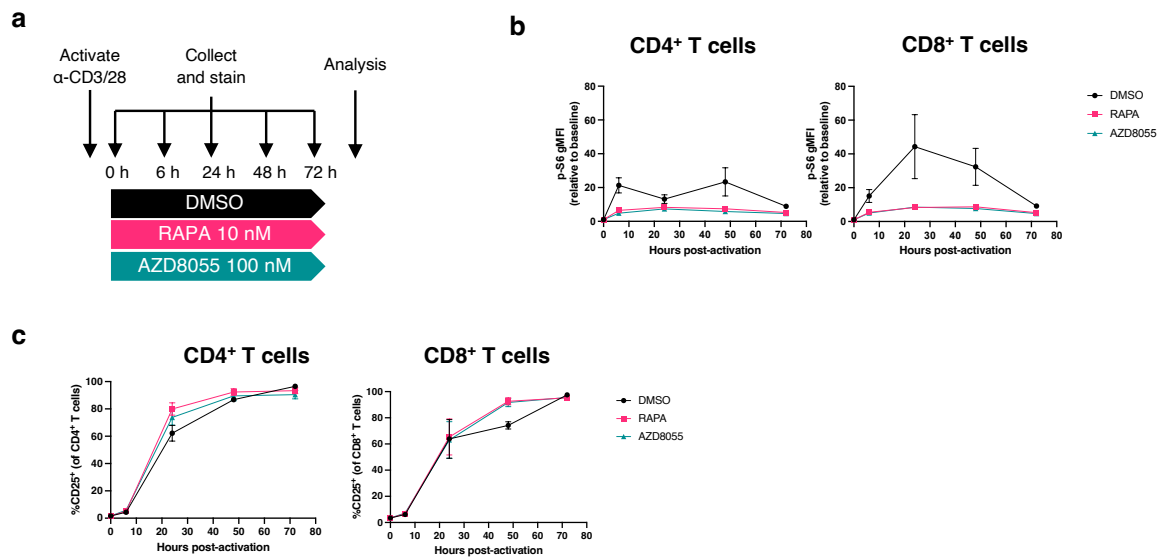


Figure S2 Effects of mTOR inhibitors on human T cell activation over 3 days

(a) Experimental design for 3-day T-cell-specific activation of PBMCs from healthy donors with 1 μ g/ml α -CD3/28 each, in the presence of 10 nM rapamycin (RAPA), 100 nM AZD8055, or DMSO control, with analysis by flow cytometry. (b) p-S6 geometric mean fluorescence intensity (gMFI) in total CD4⁺ (left) and CD8⁺ T cells relative to baseline. (c) Proportion of cells positive for (C) CD25 across the 3-day activation in flow cytometry-gated CD4⁺ or CD8⁺ T cells.

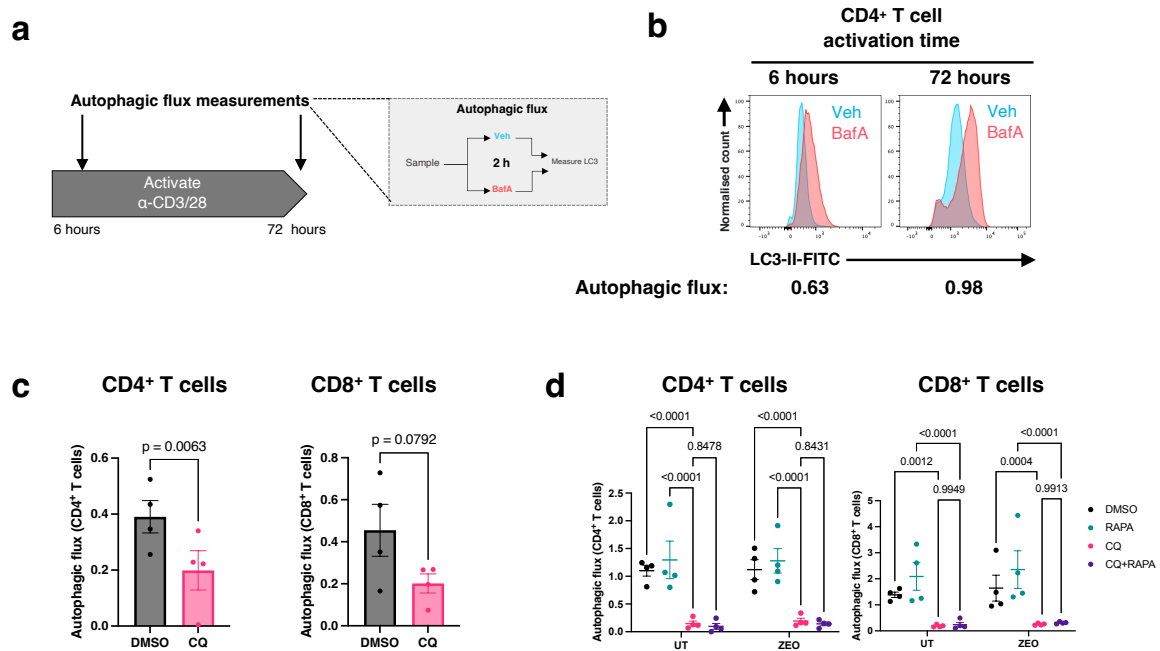


Figure S3 Flow cytometry-based measurement of autophagic flux

(a) Experimental design in which PBMCs from healthy donors underwent T-cell-specific activation with 1 μ g/ml α -CD3/28. At 6 hours and 72 hours of activation, cells were retrieved and treated with either 10 nM bafilomycin A₁ or DMSO vehicle control (Veh) as indicated, and autophagic flux measured using a flow cytometry-based LC3 assay. (b) Representative fluorescence histograms of LC3 levels in gated CD4⁺ T cells after 6 and 72 hours of activation as in (a), with autophagic flux indicated below. Representative of 3 independent experiments. (c-d) Autophagic flux in CD4⁺ and CD8⁺ T cells undergoing 3-day activation in the presence of (c) chloroquine (CQ, 10 μ M) or DMSO vehicle control or (d) following zeocin treatment (or untreated) after 3-day activation in chloroquine (CQ, 10 μ M), rapamycin (RAPA, 10 nM), or both (CQ+RAPA), n=4 healthy donors. P-values are derived from a paired t-test (c) or two-way ANOVA with Tukey's multiple comparisons test (d).

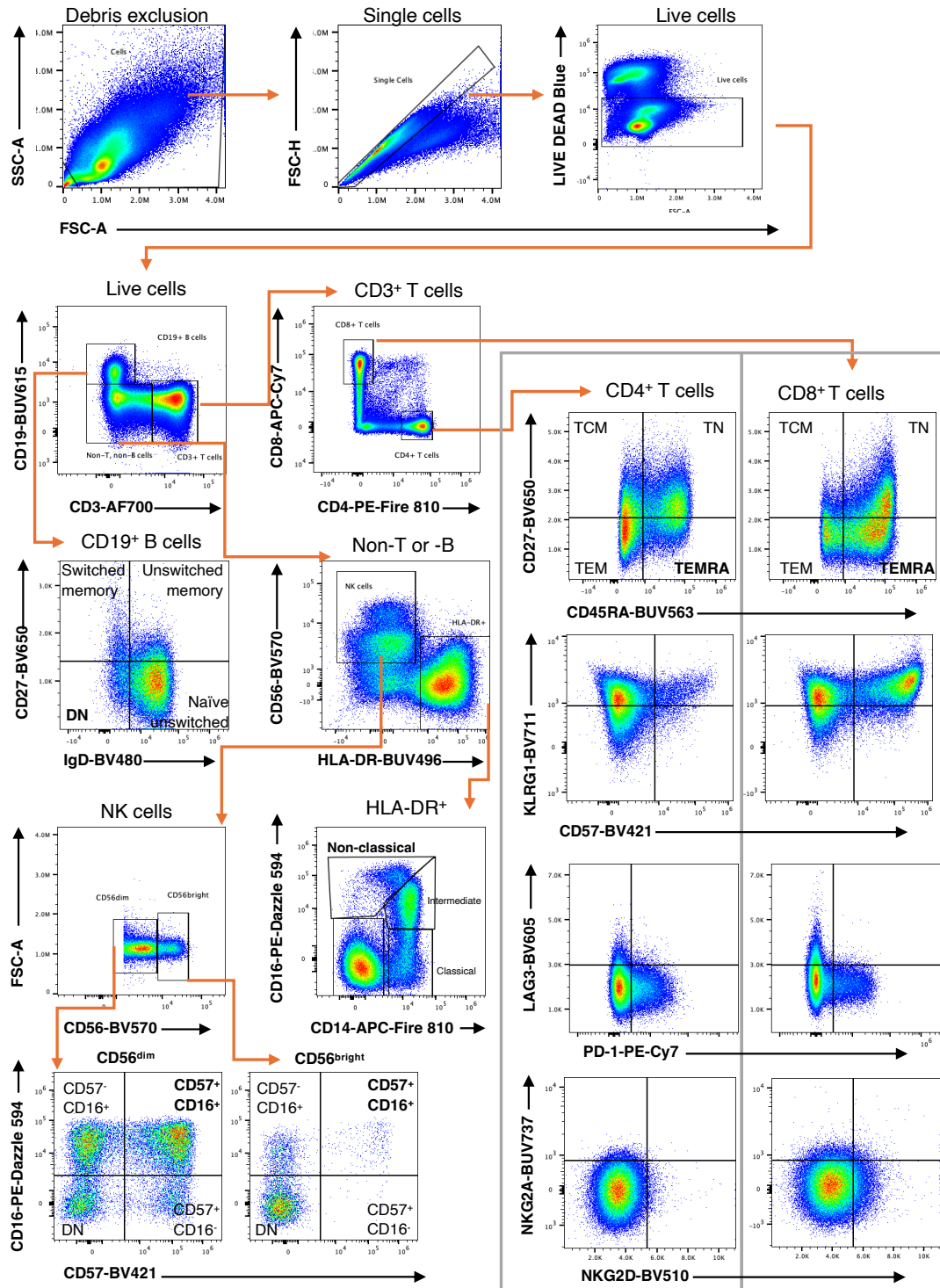


Figure S4 Gating strategy for PBMCs using 27-colour spectral flow cytometry

Gates are indicated. Orange arrows indicate where a population has been further gated upon.

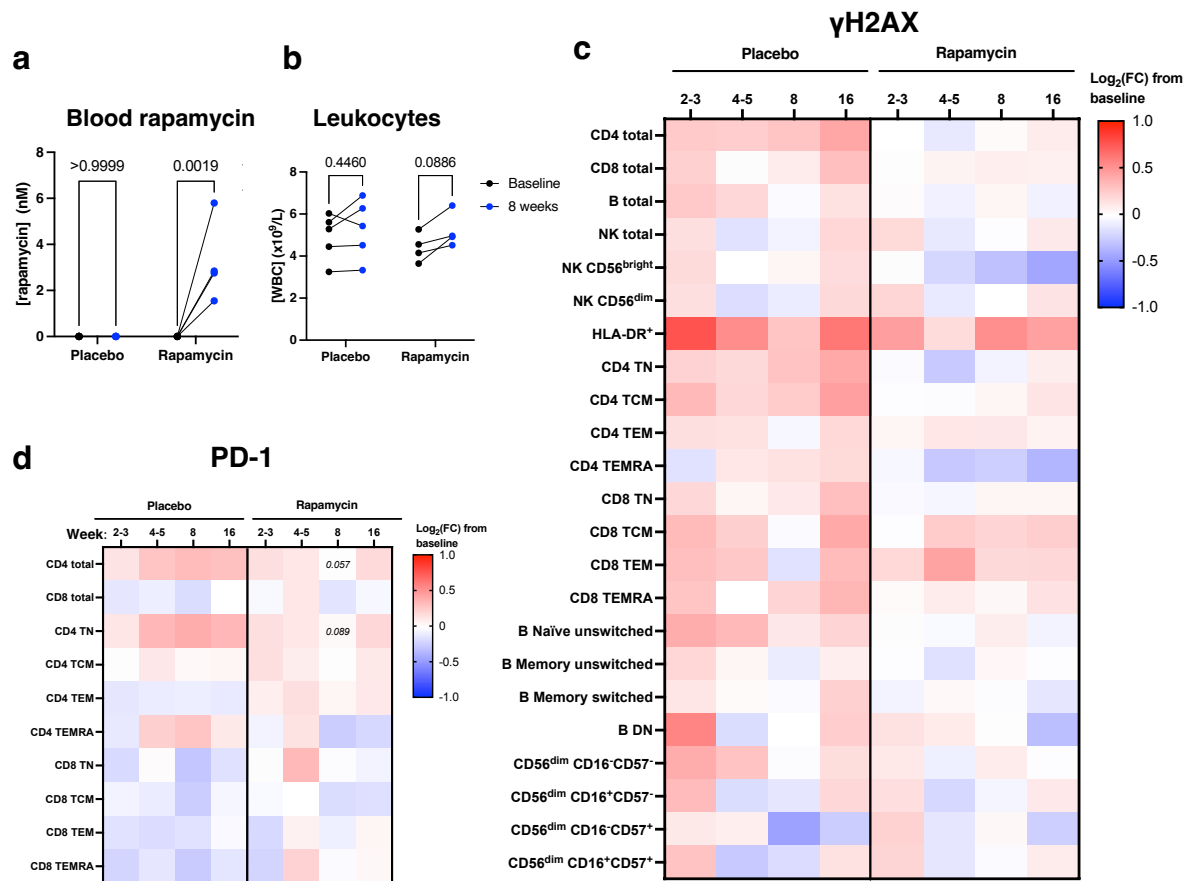


Figure S5 *In vivo* rapamycin treatment in older humans

(a-b) Blood concentration of rapamycin (a) or white blood cells (b) in participants of placebo and rapamycin groups at week 8 of the study (n=5 placebo, n=4 rapamycin). (c) γ H2AX geometric mean fluorescence intensity across immune subsets in the rapamycin trial. (d) Proportion of T cells positive for PD-1 across defined T cell subsets in participants in rapamycin (n=4) and placebo (n=5) groups. In (c-d), each value is expressed as $\log_2(\text{fold change})$ from baseline for each participant. Statistical tests in (c-d) are derived from an unpaired t-test between placebo and rapamycin at each time point. *P*-values in (a-b) are determined by two-way ANOVA with Šidák's multiple comparisons tests.

Table S1 Details of drugs used in cell culture experiments

Name	Target	Final concentration	Solvent	Manufacturer	Cat. code
Zeocin	DSB inducer	200 μ g/ml	H ₂ O	Invitrogen	R25001
Rapamycin	mTORC1 inhibitor	10 nM	DMSO	Alfa Aesar	J67452
AZD8055	Pan-mTOR inhibitor	100 nM	DMSO	Strattech Scientific	A8214-APE-10mM
Bafilomycin A ₁	Autophagy inhibitor	10 nM	DMSO	Sigma	B1793-10UG
Hydrogen peroxide	Oxidative stress	25 μ M	PBS	Sigma	H1009-100ML
Chloroquine	Autophagy inhibitor	10 μ M	DMSO	Sigma	C6628-25G
Cycloheximide	Protein translation inhibitor	50 μ g/ml	DMSO	Sigma	239763-M

N.B. DMSO vehicle controls always contained 0.1% DMSO (v/v) in the cell culture media.

Table S2 Details of antibodies used in flow cytometry – see separate excel file.