

Supplemental information

CD99-mediated immunological synapse formation potentiates CAR-T cell function

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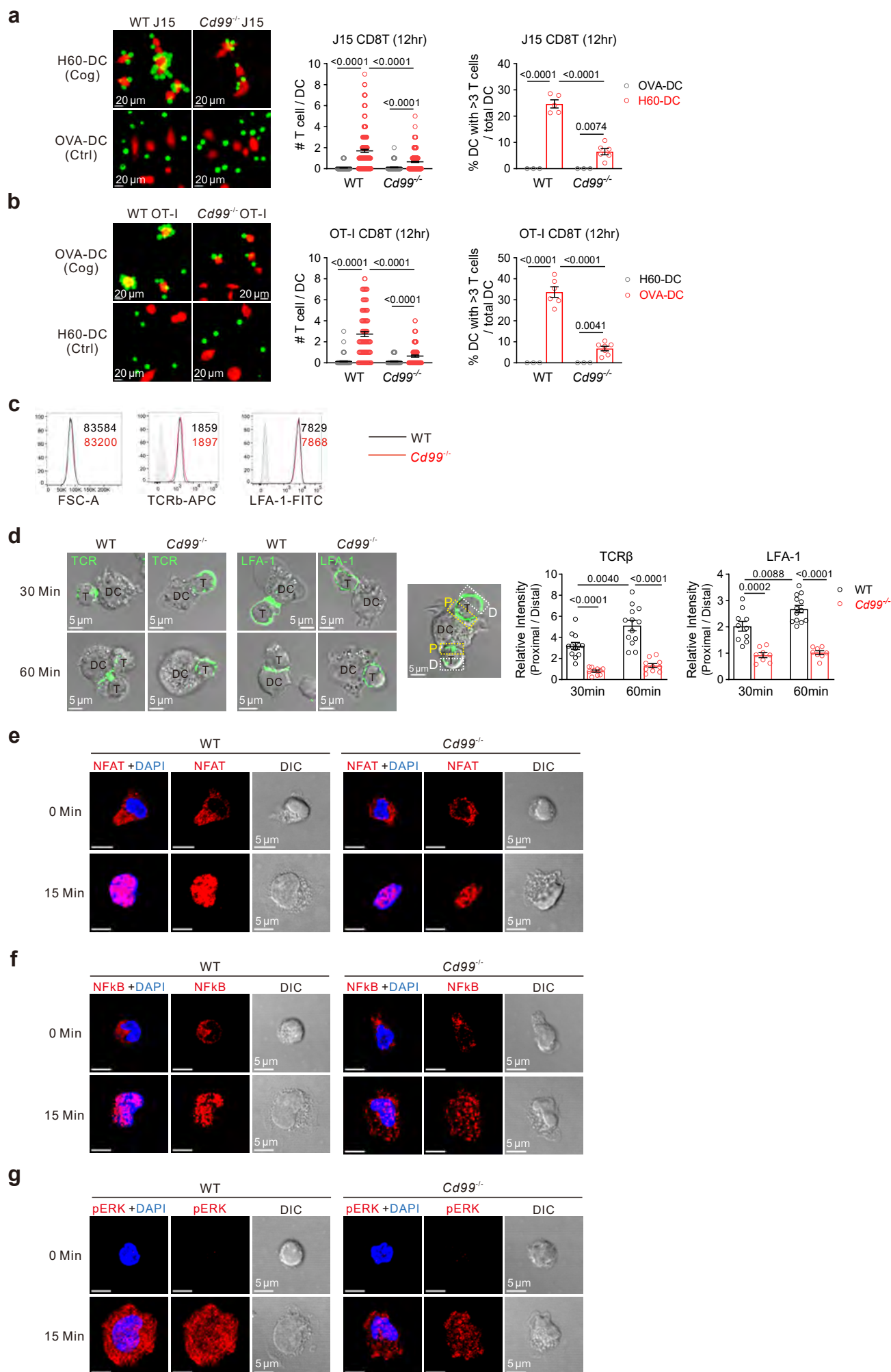
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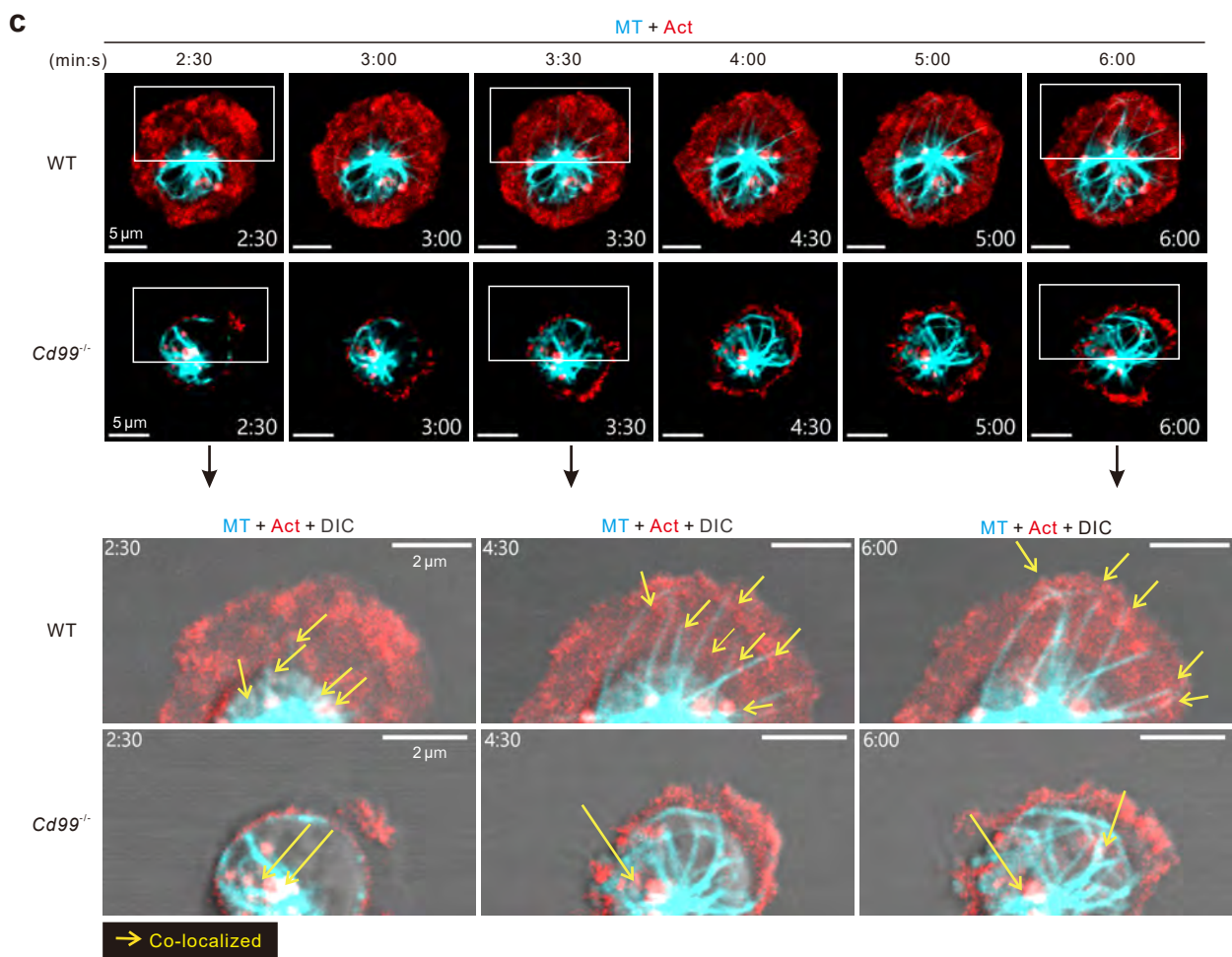
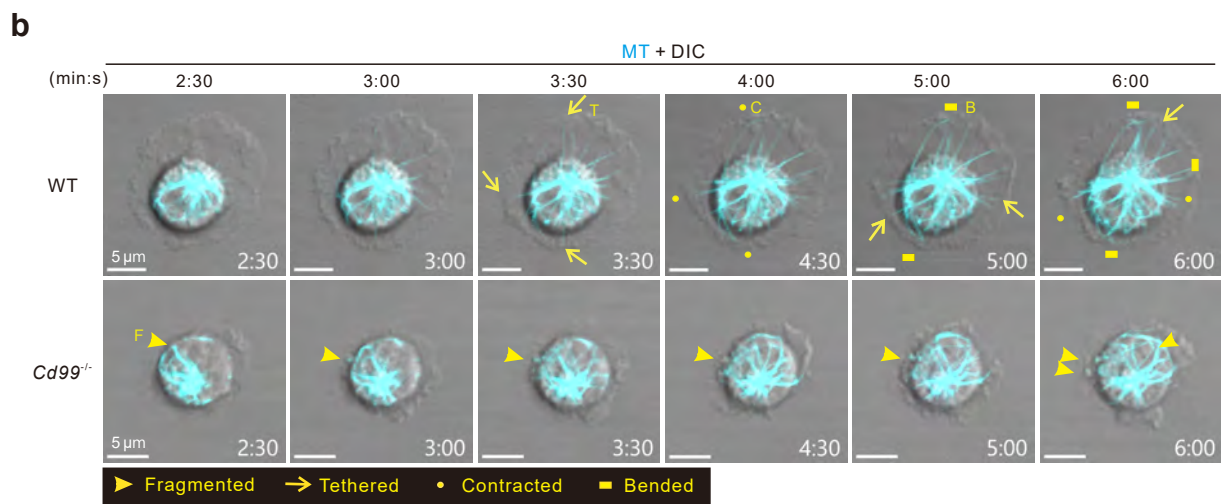
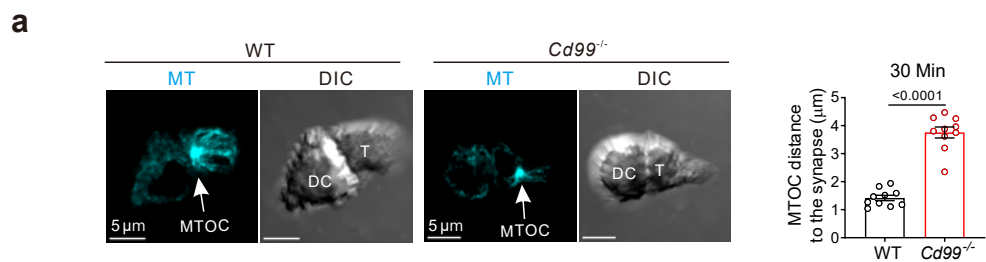
Supplementary Figures 1-8

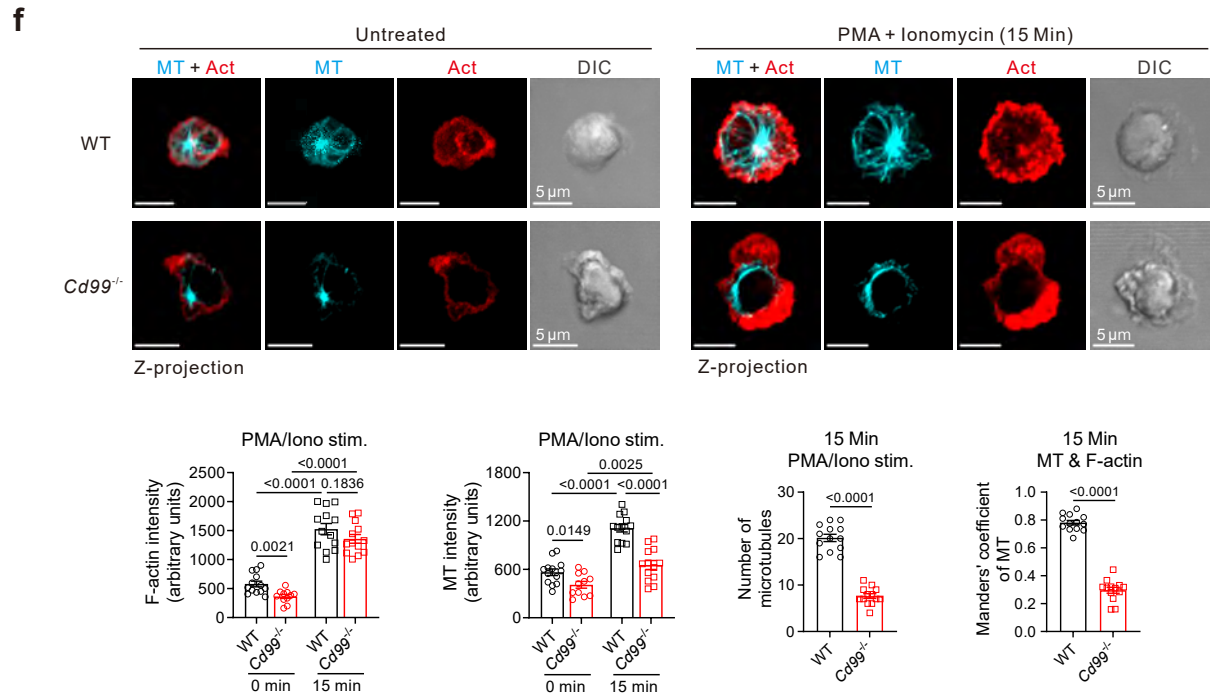
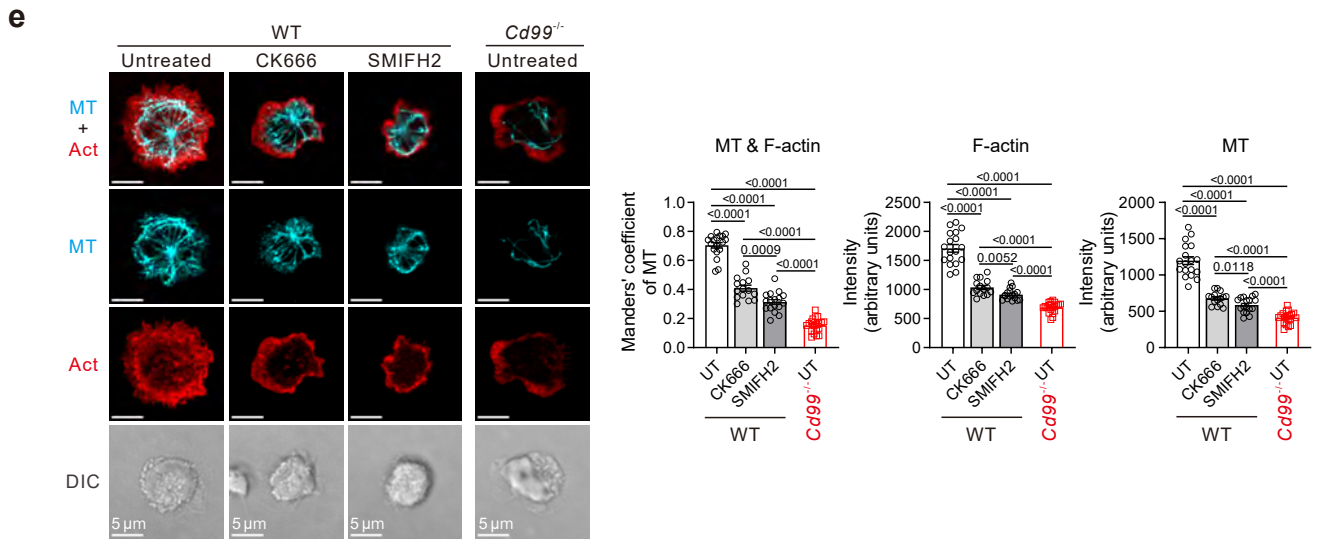
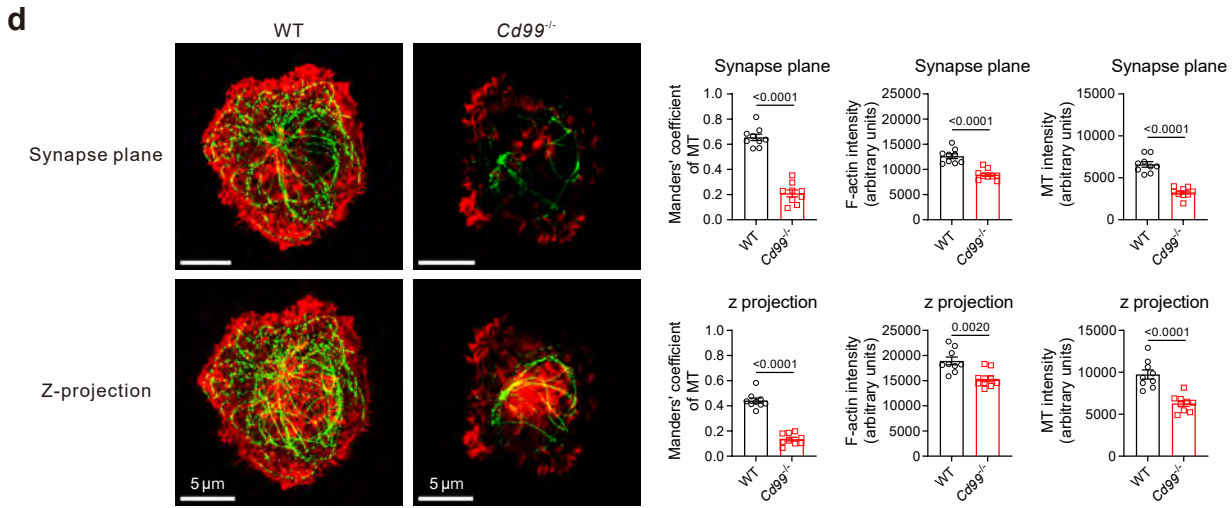
Supplementary Tables 1-2

Supplementary Figures

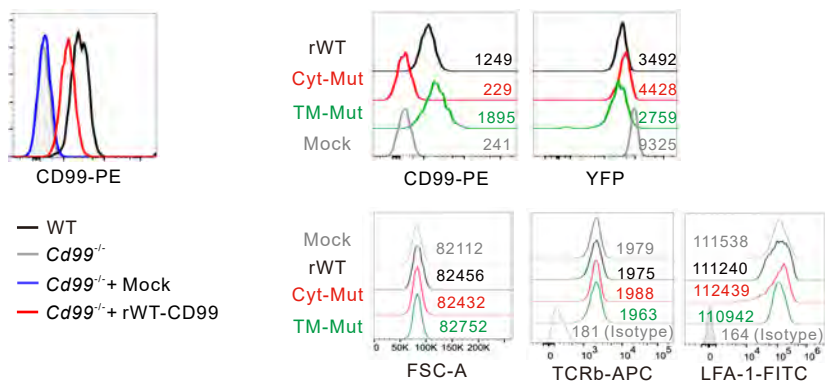
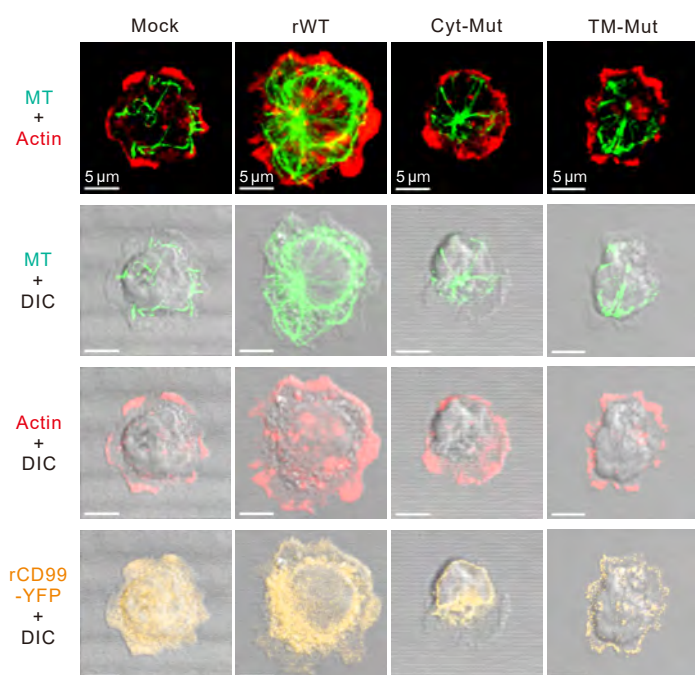
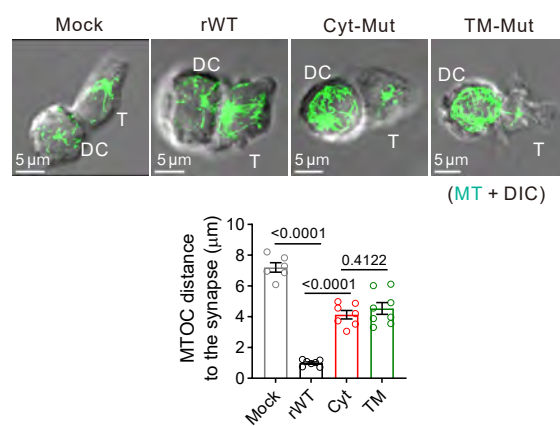
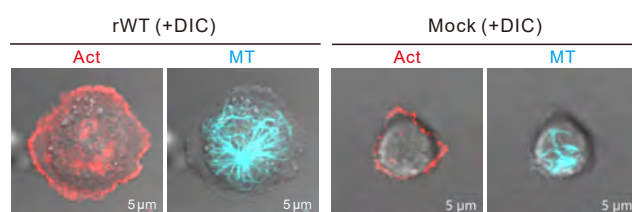
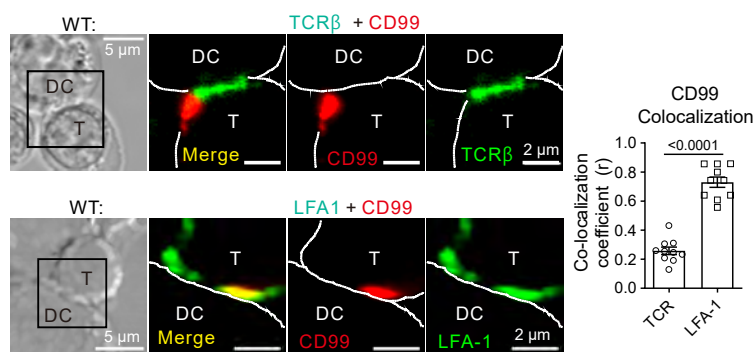
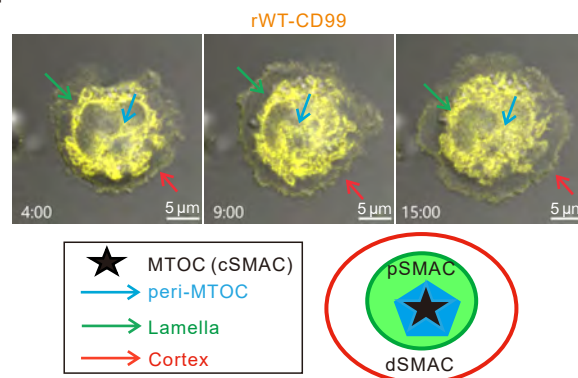
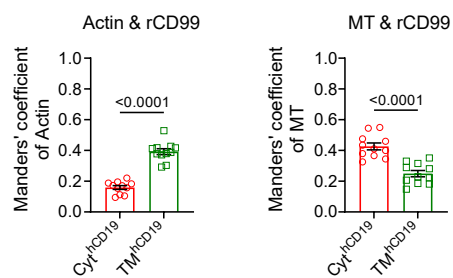
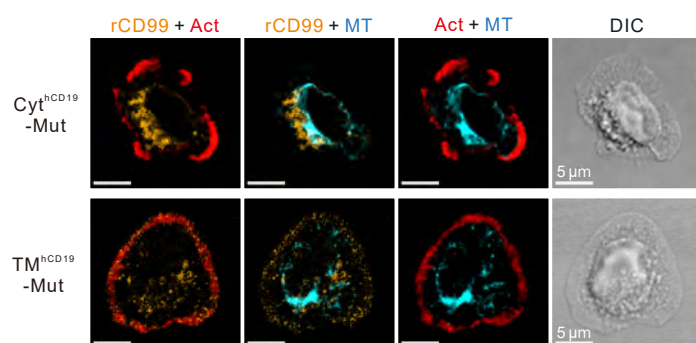


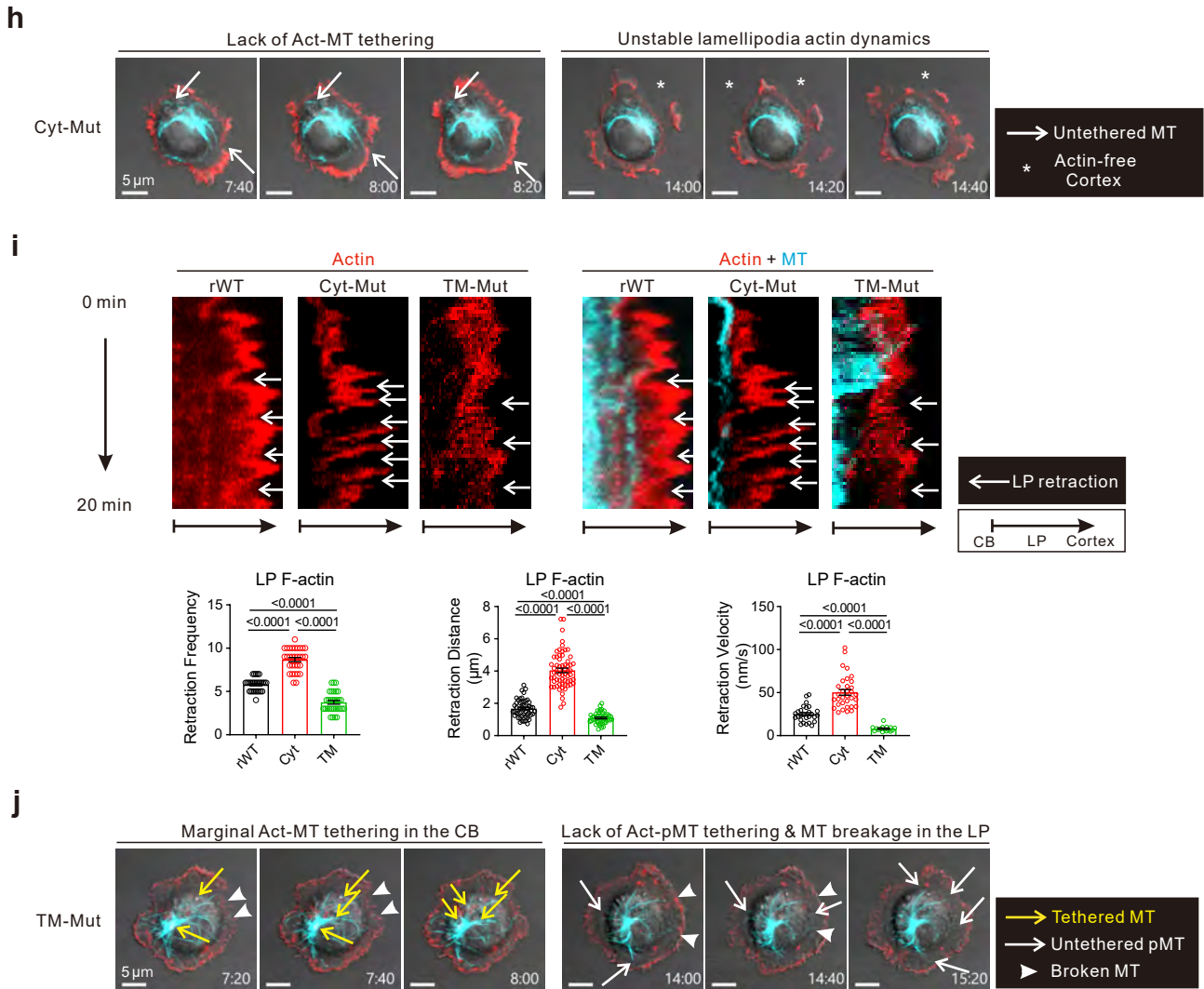
Supplementary Fig. 1. Requirement of CD99 for IS formation. **a, b** Comparison of T-DC conjugate formation between naïve WT and *Cd99*^{-/-} CD8 T cells. Conjugate formation by CMFDA-labeled (green) naïve WT or *Cd99*^{-/-} J15 CD8 T cells (**a**) and OT-I CD8 T cells (**b**) after coincubation with CMTMR-labeled (red) H60-DC or OVA-DC for 12 h. Plots show the number of T cells in contact with one DC ($n = 131, 182, 117,$ and 244 conjugates, from left to right) and the percentage of DCs in contact with more than three T cells in total DC ($n = 3, 5, 3,$ and 6 fields, from left to right). **c** Flow cytometric analysis of WT and *Cd99*^{-/-} CTLs. Single histograms show cell size and surface levels of TCR β and LFA-1. FSC-A and mean fluorescence intensity (MFI) values are noted. **d** Molecular polarization in WT- and *Cd99*^{-/-}-cells after 30 and 60 min of co-incubation with H60-DCs. Confocal images of TCR β or LFA1 staining. The fluorescence intensities of TCR ($n = 12, 10, 13$ and 10 conjugates, from left to right) and LFA-1 ($n = 10, 8, 13$ and 8 conjugates, from left to right) stained in DC-proximal and -distal areas are plotted. **e - g** Representative confocal images illustrating nuclear translocation of NFAT (**e**) and NF κ B (**f**), and ERK phosphorylation (**g**), with DAPI co-staining. Representative single z-section images are shown for clear nuclear localization of NFAT and NF κ B (**e, f**). Images from the synapse plane are presented for cytosolic localization of pERK (**g**). Quantification and statistics are provided in Fig. 1f. Data (**a-g**) are representative of at least three independent experiments. Data (**a, b, d**) are shown as mean $\pm$ SEM. Statistical significance was assessed using unpaired two-tailed *t*-test. Exact *p*-values are presented in the figure. Source data are provided as a Source Data file.





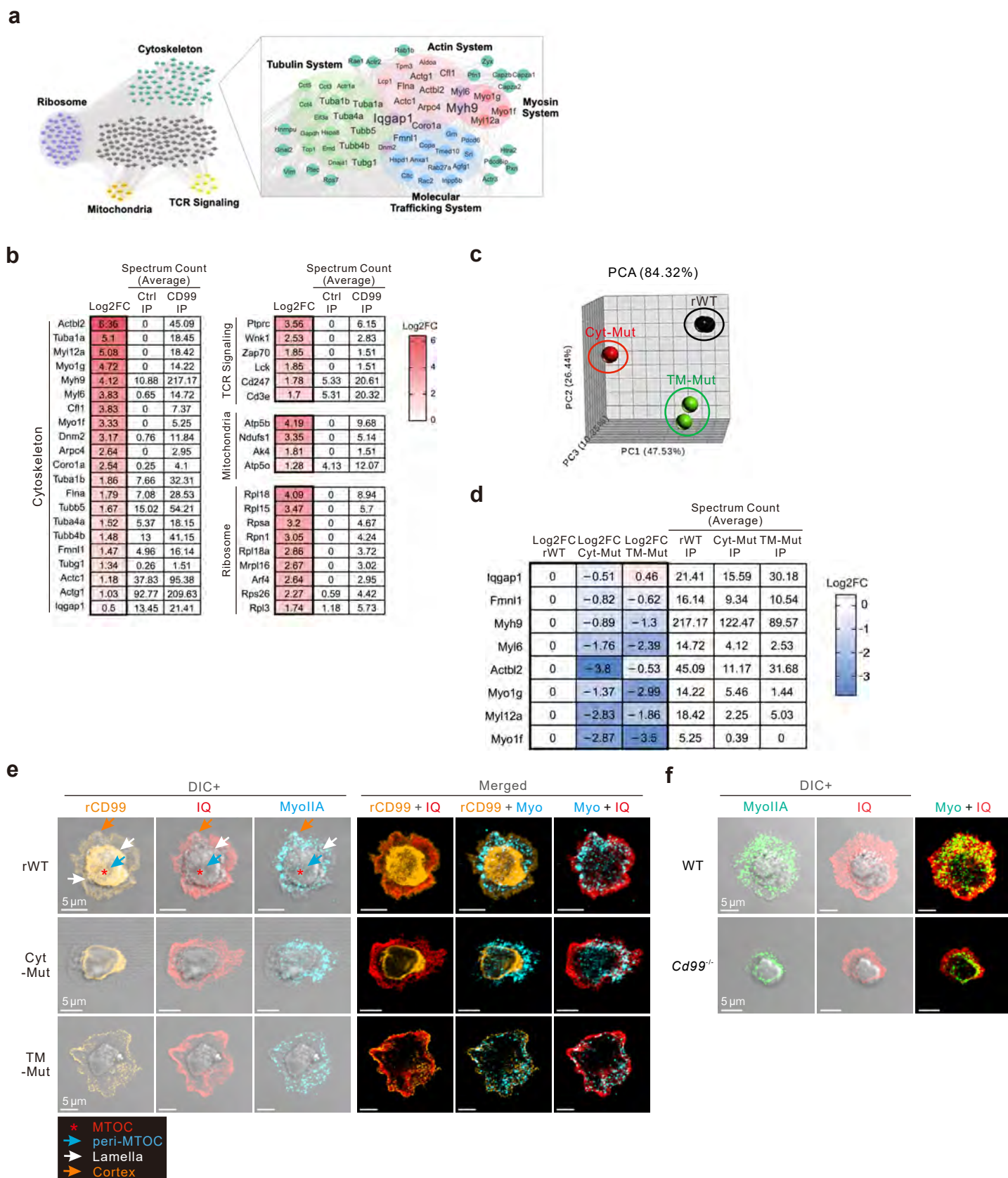
Supplementary Fig. 2. Requirement of CD99 for the co-localization of actin and microtubules, and for microtubule reorganization. **a** MTOC-translocation in WT or *Cd99*^{-/-}-cells coincubated with H60-DC. Representative z-stacked confocal images of microtubules. Plot shows MTOC distance to the synapse site ($n = 10$ conjugates per group). **b, c** Time-lapse confocal images of microtubules in WT and *Cd99*^{-/-}-cells (see Supplementary Movies 2, 3). Representative time-lapse series of microtubule (SiR-tubulin⁺) images overlaid on DIC (**b**) and F-actin (Lifeact⁺) (**c**) images. Zoomed images of actin and microtubules in the boxed areas in the images in the upper panel are overlaid on DIC images in the lower panel (**c**). **d** SIM images of F-actin (red) and microtubules (green), and their colocalization (yellow) in WT and *Cd99*^{-/-}-cells stained with phalloidin and anti- α -tubulin antibody at 15 min after CD3-stimulation. Representative synapse plane images (about $\sim 0.3 \mu\text{m}$ depth) and z-projection images ($\sim 3 \mu\text{m}$ depth; See also Supplementary Movie 4) are shown. F-actin and MT intensities, and their colocalization were quantified ($n = 9$ cells per group). **e** Confocal images of WT-cells pretreated with CK666 (100 μM) or SMIFH2 (10 μM), and untreated WT and *Cd99*^{-/-}-cells following anti-CD3 stimulation for 15 min. F-actin (Act) and microtubule (MT) colocalization and intensities were plotted ($n = 18, 15, 17$, and 20 cells, from left to right). **f** Confocal z-stacked images ($\sim 5 \mu\text{m}$ depth) of WT and *Cd99*^{-/-}-cells with (right panel) or without (left panel) PMA + Ionomycin stimulation for 15 min. F-actin and MT intensities, microtubule number, and actin-microtubule colocalization are plotted ($n = 13$ cells per group). Data (**a-f**) are representative of at least three independent experiments. Data (**a, d-f**) are shown as mean $\pm$ SEM. Statistical significance was assessed using unpaired two-tailed t -test. Exact p -values are presented in the figure. Source data are provided as a Source Data file.

a**b****c****d****e****f****g**



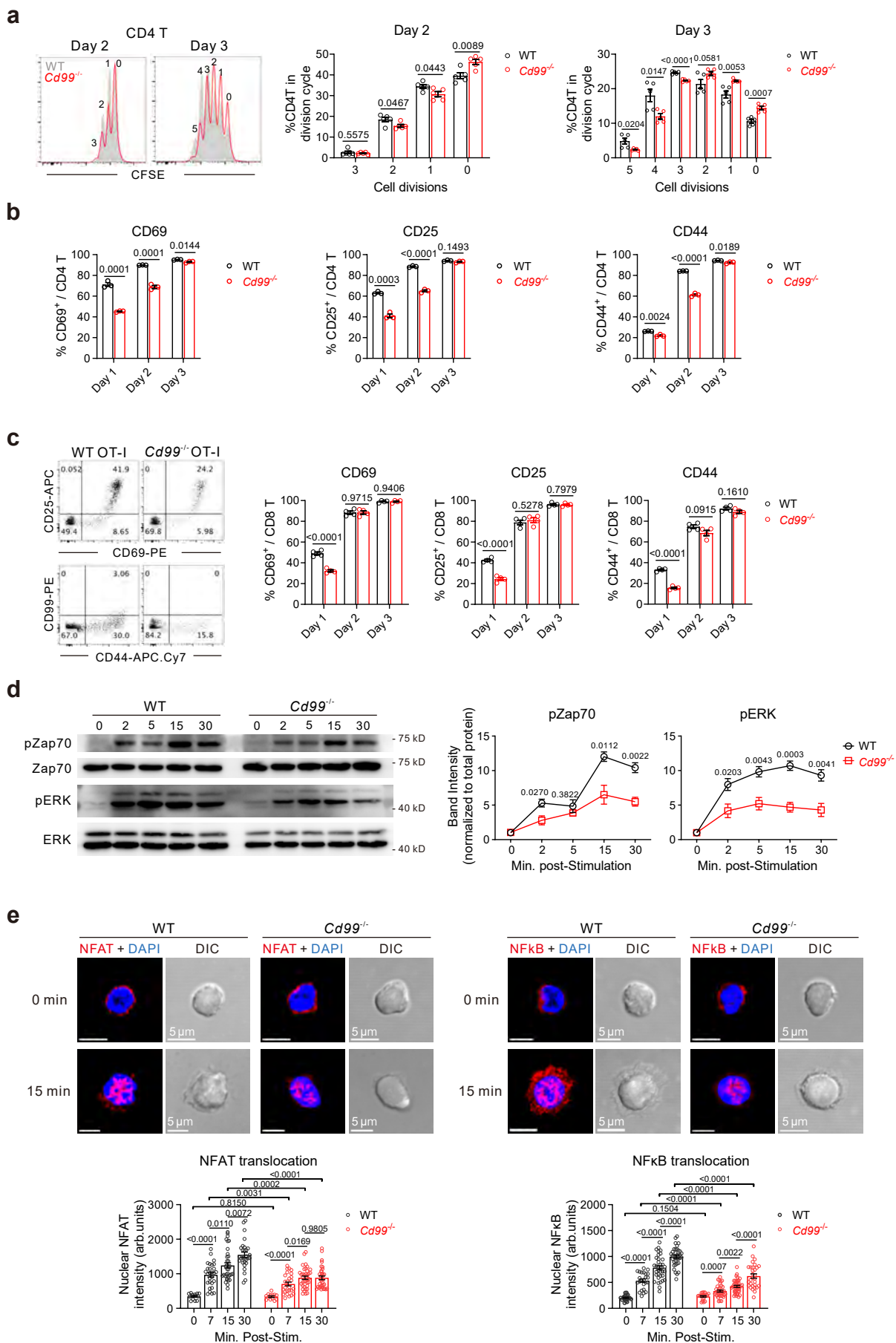
Supplementary Fig. 3. Importance of the cytoplasmic and transmembrane domains for CD99 function. **a** Flow cytometric analysis of cell surface expression of CD99, TCR, and LFA-1, and cell size of rWT-CD99-, Cyt-Mut-CD99-, TM-Mut-CD99-, or mock-transduced (YFP-expressing) cells. MFI values of the molecular expression and FSC-A values are noted. **b** Representative confocal images of F-actin (red), microtubules (green), and YFP (orange yellow) in the transduced cells ($n \geq 5$ cells). Cells were stained with phalloidin and anti- α -tubulin antibody after anti-CD3 stimulation for 15 min. **c** MTOC-translocation in transduced cells. Representative z-stacked confocal images of microtubules after 30 min of incubation of mock-, rWT-CD99-, Cyt-Mut-CD99-, or TM-Mut-cells with H60-DCs. Plot shows MTOC distances (μ m) in T cells to the synaptic site ($n = 7, 7, 8$, and 6 conjugates, from left to right). **d** Time-lapse confocal images of F-actin (Lifeact⁺, red) and microtubules (SiR-tubulin⁺, cyan) on DIC images of rWT-CD99- and mock-transduced cells at 11 min after anti-CD3 stimulation (see Supplementary Movie 5). **e** Confocal images of CD99 with TCR β , or with LFA-1 on the WT-cell surface at the synaptic site. WT-cells pre-stained with anti-CD99 and anti-TCR β antibodies, or with anti-CD99 and anti-LFA-1 antibodies were incubated with H60-DC for 30 min. Colocalization of CD99 with each molecule was quantified by Pearson's coefficient ($n = 10$ cells per group). **f** Time-lapse confocal images of rWT-CD99-YFP in rWT-cells (see Supplementary Movie 6). Time in minutes:seconds. Schematic diagram of the IS substructure. **g** Representative confocal images of cells expressing Cyt^{hCD19}-Mut- or TM^{hCD19}-Mut-CD99. Cyt^{hCD19}-Mut- or TM^{hCD19}-Mut- CD99 (rCD99 in orange yellow), F-actin (in red) and microtubules (in cyan) are shown on black background. Colocalization of F-actin or

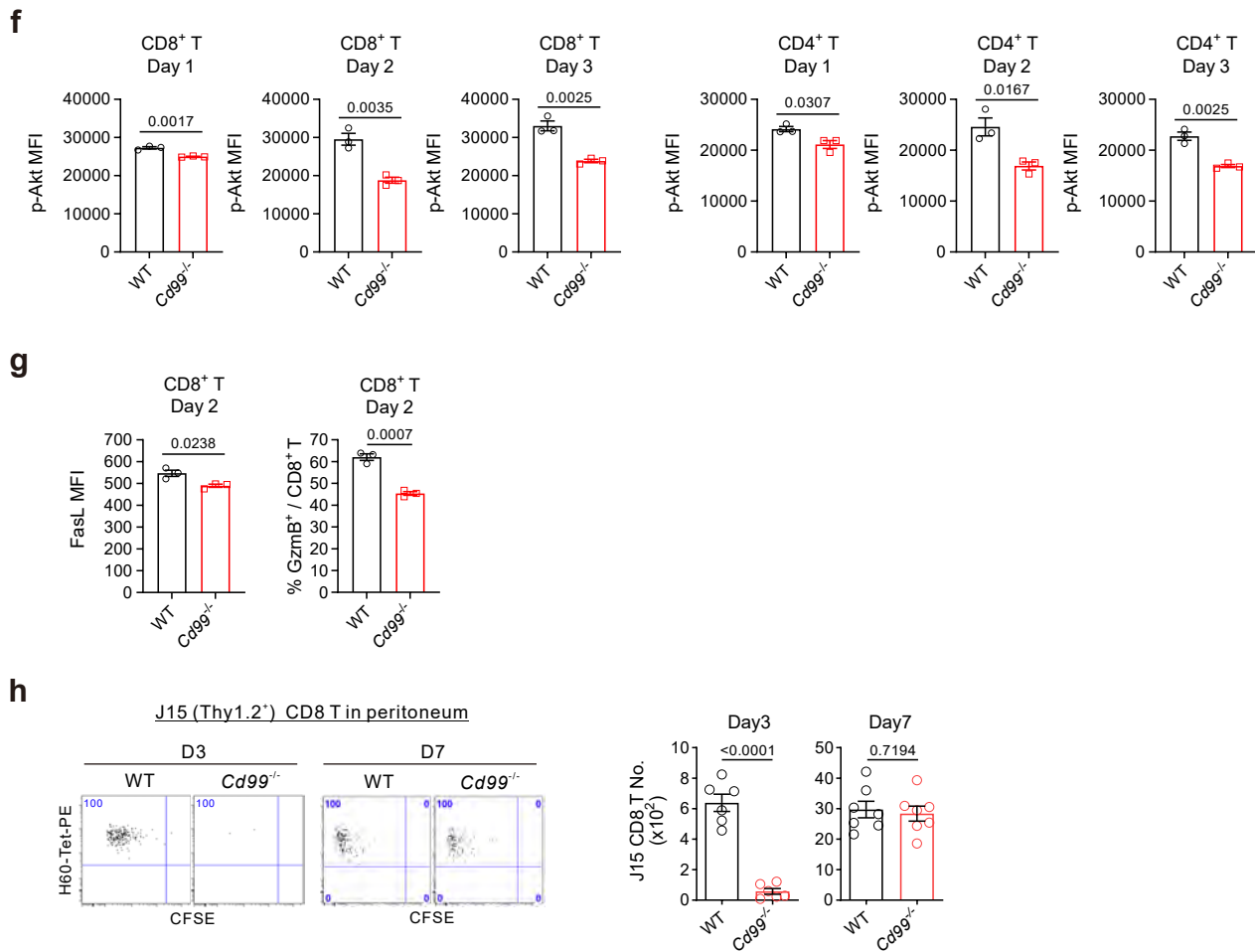
microtubules with Cyt^{hCD19}-Mut or TM^{hCD19}-Mut molecules was quantified ($n = 11$ cells per group). **h - j** Cytoskeleton dynamics in recombinant cells. Time-lapse confocal images of F-actin (Lifeact⁺, red) and microtubules (SiR-tubulin⁺, cyan) in Cyt-Mut-cells (**h**) and TM-Mut-cells (**j**). Time in minutes:seconds. Kymographs of F-actin and microtubule dynamics over 20 min (**i**) along with lamellipodia in rWT-, Cyt-Mut-, and TM-Mut-cells. F-actin retraction frequency over 20 min ($n = 29, 31$, and 31 regions, from left to right), and the retraction distance ($n = 51, 63$, and 47 retractions, from left to right) and velocity per retraction event ($n = 27, 33$, and 16 retractions, from left to right) were quantified. Data (**a-j**) represent at least three independent experiments. Data (**c, e, g, i**) are shown as mean $\pm$ SEM. Statistical significance was assessed using unpaired two-tailed *t*-test. Exact *p*-values are presented in the figure. Source data are provided as a Source Data file.



Supplementary Fig. 4. Identification of IQGAP1 and Myosin IIA as domain-mediated interaction partners of CD99. **a, b** Network analysis of the CD99 interactome. Diagram of CD99 interactome components (**a**). Average normalized spectrum counts denoting the abundance of protein interactions (**b**), as detected by CD99 LC-MS. The log2 fold changes (FCs) in protein expression between samples immunoprecipitated with anti-YFP and isotype IgG antibodies

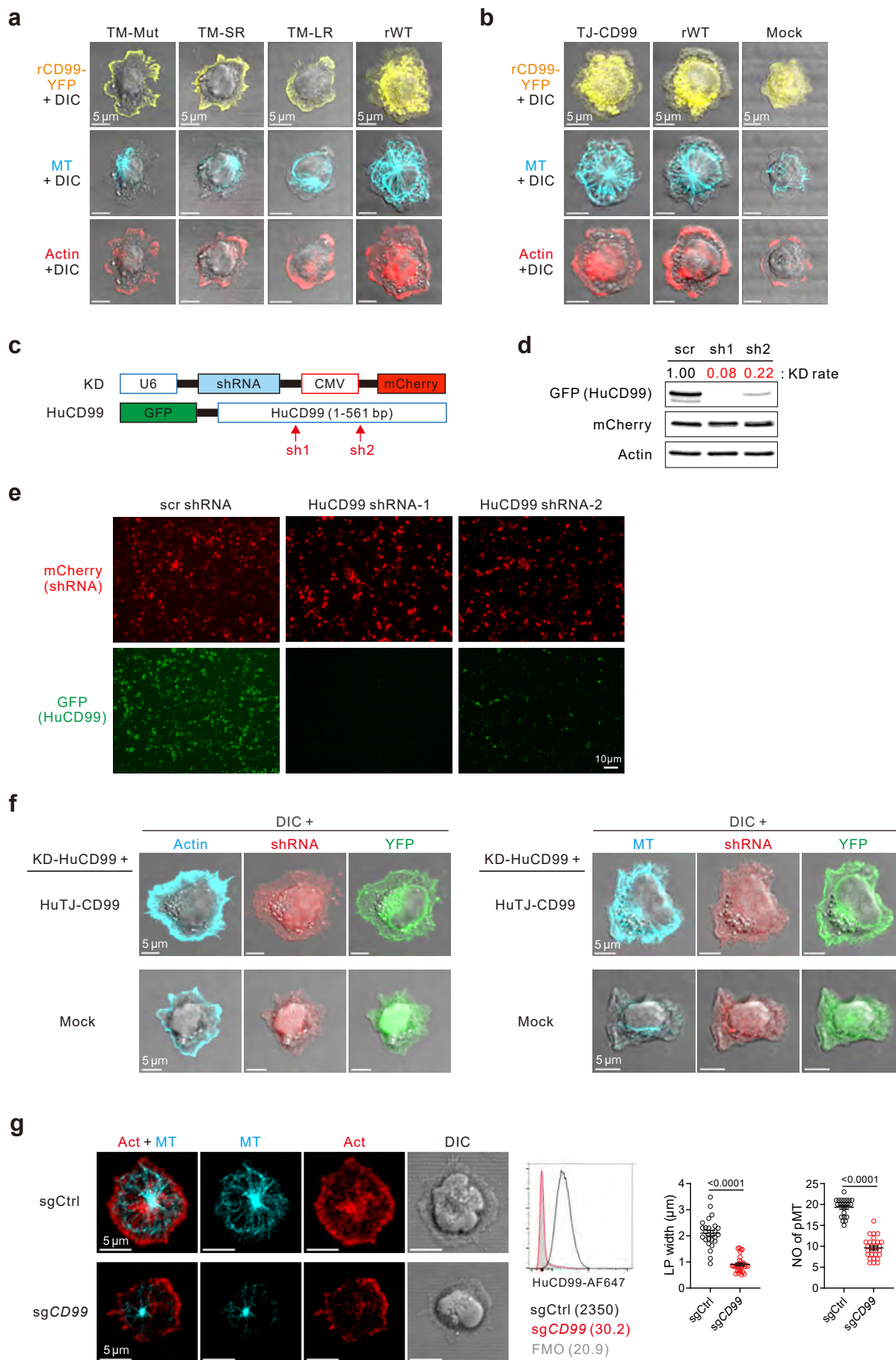
are listed. **c** Principal component analysis (PCA) of the interactomes of rWT-, Cyt-Mut-, and TM-Mut-cells. Analysis was performed using proteomics datasets and visualized in a three-dimensional plot. **d** Average normalized spectrum counts of rWT-CD99, Cyt-Mut-CD99- or TM-Mut-CD99-interacting cytoskeletal proteins. The $\log_2$ FCs in protein expression in Cyt-Mut or TM-Mut versus rWT interactomes are listed. Proteomics data (**a-d**) are representative of two biological replicates. Two independent LC-MS experiments were conducted for each sample. **e** Confocal images (related to Fig. 4f) of recombinant CD99-YFP (orange yellow), IQGAP1 (red), and MyoIIA (cyan) in rWT-, Cyt-Mut-, or TM-Mut-cells. **f** Confocal images (related to Fig. 4i) of MyoIIA (green) or IQGAP1 (red) in WT or *Cd99*^{-/-} cells. Data (**e, f**) are representative of three independent experiments.



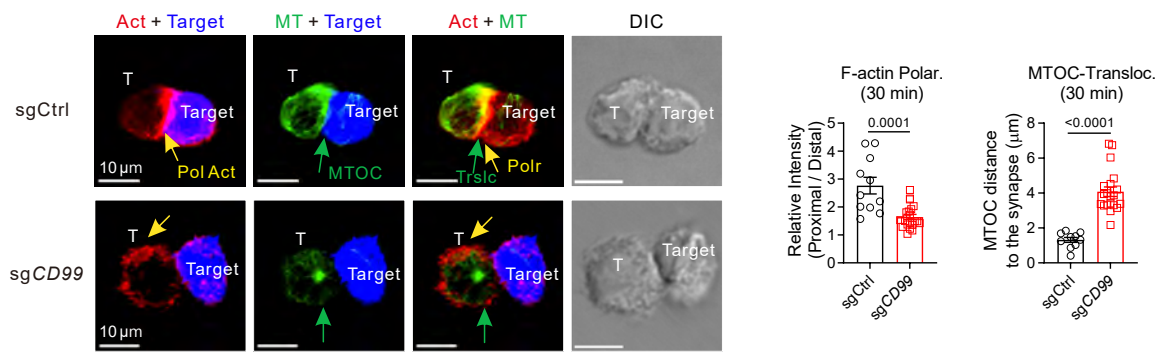


Supplementary Fig. 5. Ineffective T-cell activation and in vivo immunity in the absence of CD99. **a, b** Comparison of in vitro activation of CD4⁺ T cells from WT and *Cd99*^{-/-} mice. Flow cytometry profiles of CFSE dilution ($n = 5$ mice per group) (**a**) and the expression of CD69, CD25, and CD44 ($n = 5$ mice per group) (**b**) by anti-CD3/CD28 stimulated WT and *Cd99*^{-/-} T cells. Plots of the proportions (%) of T cells in each diluted CFSE peak (**a**) and marker-positive cells (**b**). Data (**a, b**) are pooled from two independent experiments ($n = 2 - 3$ mice/ group/experiment). **c** Activation of WT and *Cd99*^{-/-} OT-I CD8 T cells cocultured for 24 h with OVA-DCs. Flow cytometric profiles of CD69, CD25, and CD44 expressed on T cells. The percentages of CD69⁺, CD25⁺, and CD44⁺ cells in OT-I CD8 T cells are plotted using data pooled from two independent experiments ($n = 2$ mice/group/experiment). **d** Immunoblots of total and phosphorylated Zap70 and ERK in WT and *Cd99*^{-/-} CD8⁺ T cells stimulated with anti-CD3 and CD28 antibodies for the indicated times (min). Plots of phosphorylated Zap70 or ERK band intensities relative to the pre-stimulation (0 min) intensities, using data pooled from four to five independent experiments. **e** Representative confocal images showing nuclear translocation of NFAT (left) and NFκB (right) in primary T cells from WT or *Cd99*^{-/-} mice after anti-CD3 stimulation. Single z-section images were used for nuclear localization, and nuclear NFAT ($n = 15, 13, 28, 27, 35, 37, 29$, and 35 cells, from left to right) and NFκB ($n = 18, 20, 24, 36, 35, 40, 36$, and 30 cells, from left to right) intensities were quantified. Data represent two independent experiments. **f, g** Flow cytometric analysis of Akt phosphorylation by CD4⁺ and CD8⁺ T cells (**f**), and FasL and Granzyme B expression by CD8⁺ T cells (**g**) from WT or *Cd99*^{-/-} mice after anti-CD3/CD28 stimulation ($n = 3$ mice per group). Data represent two independent experiments. **h** Flow cytometric profiles of H60-tetramer binding and CFSE-dilution by WT or *Cd99*^{-/-} J15 CD8⁺ T cells in the peritoneum of adoptive hosts immunized against H60 on days 3

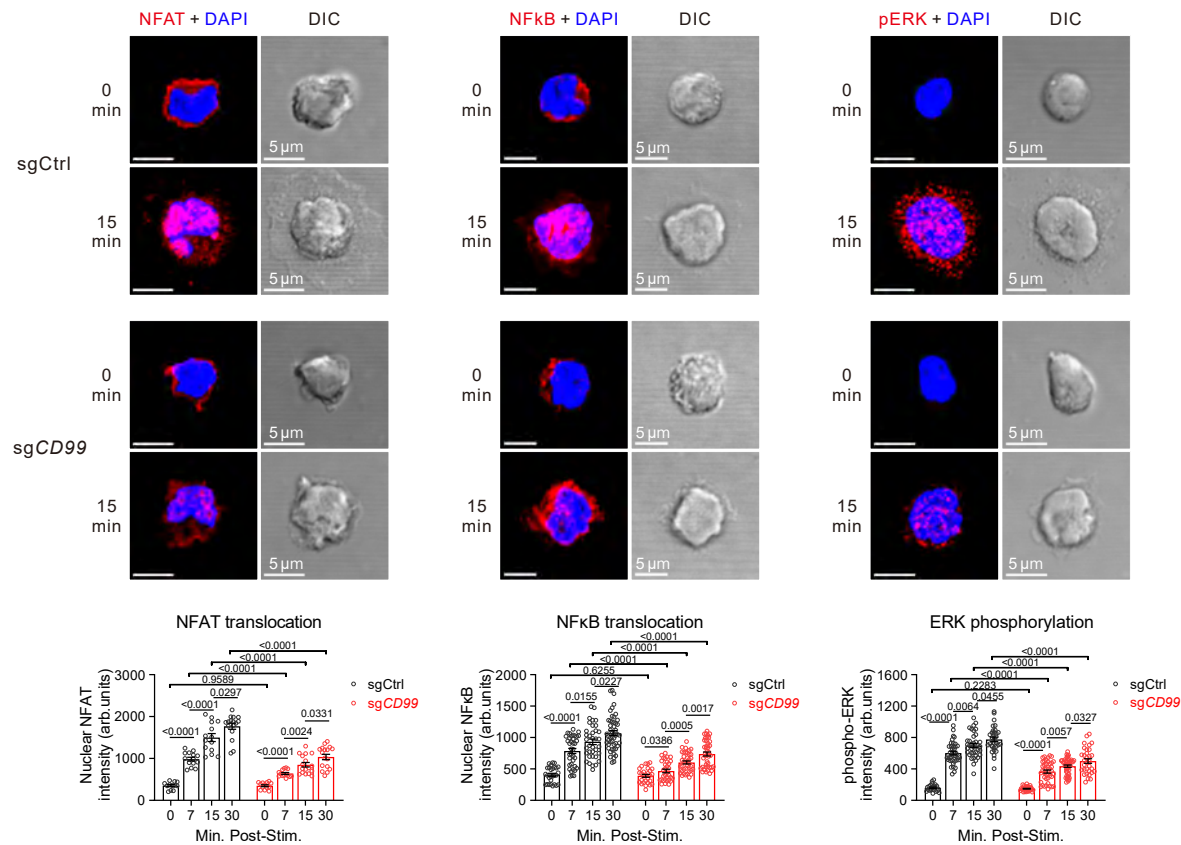
and 7 post-immunization. Plots of WT and *Cd99*^{-/-} J15 CD8⁺ T cell numbers in the peritoneum from three independent experiments ($n = 2 - 3$ mice/group/ experiment). Data (**a-h**) are shown as mean $\pm$ SEM. Statistical significance was assessed using unpaired two-tailed *t*-test. Exact *p*-values are presented in the figure. Source data are provided as a Source Data file.



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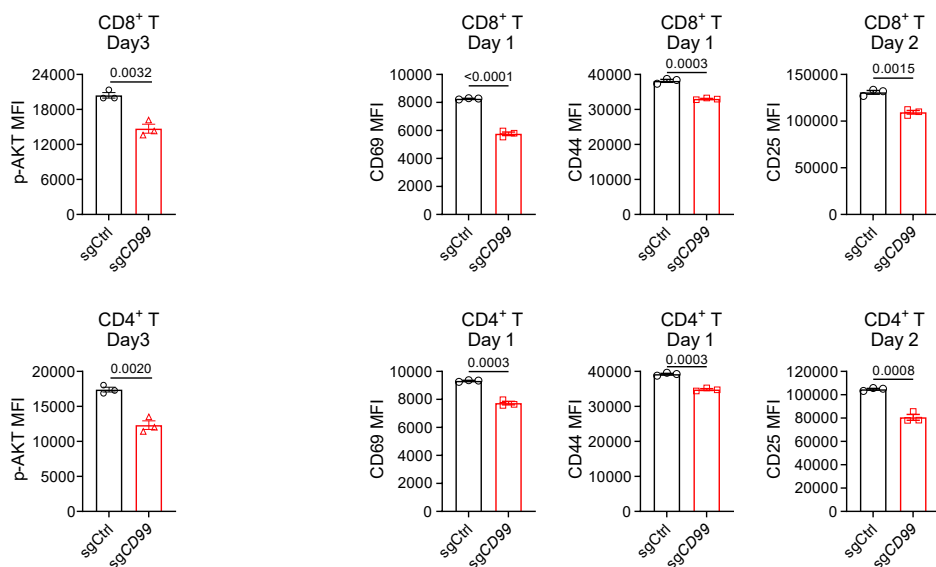


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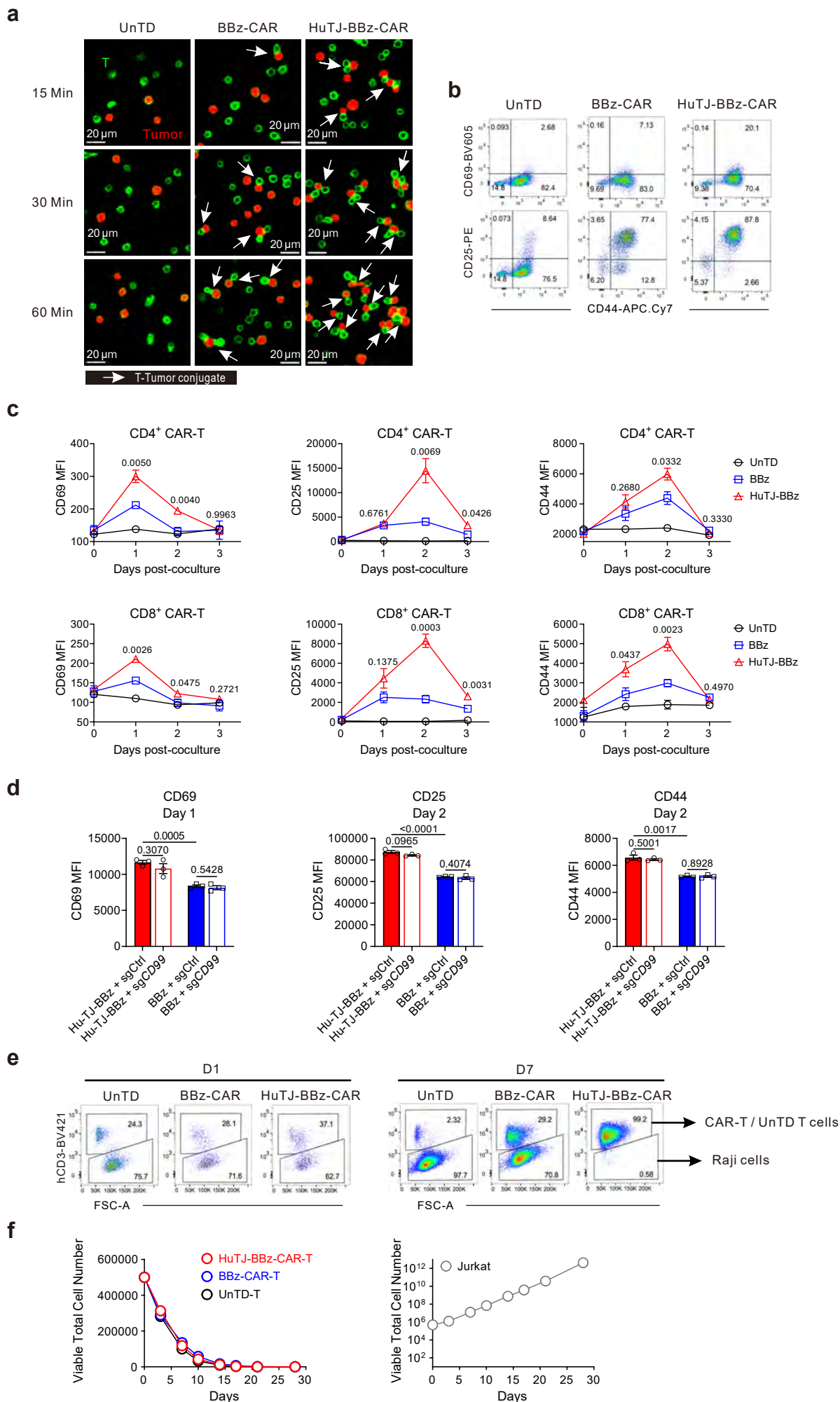


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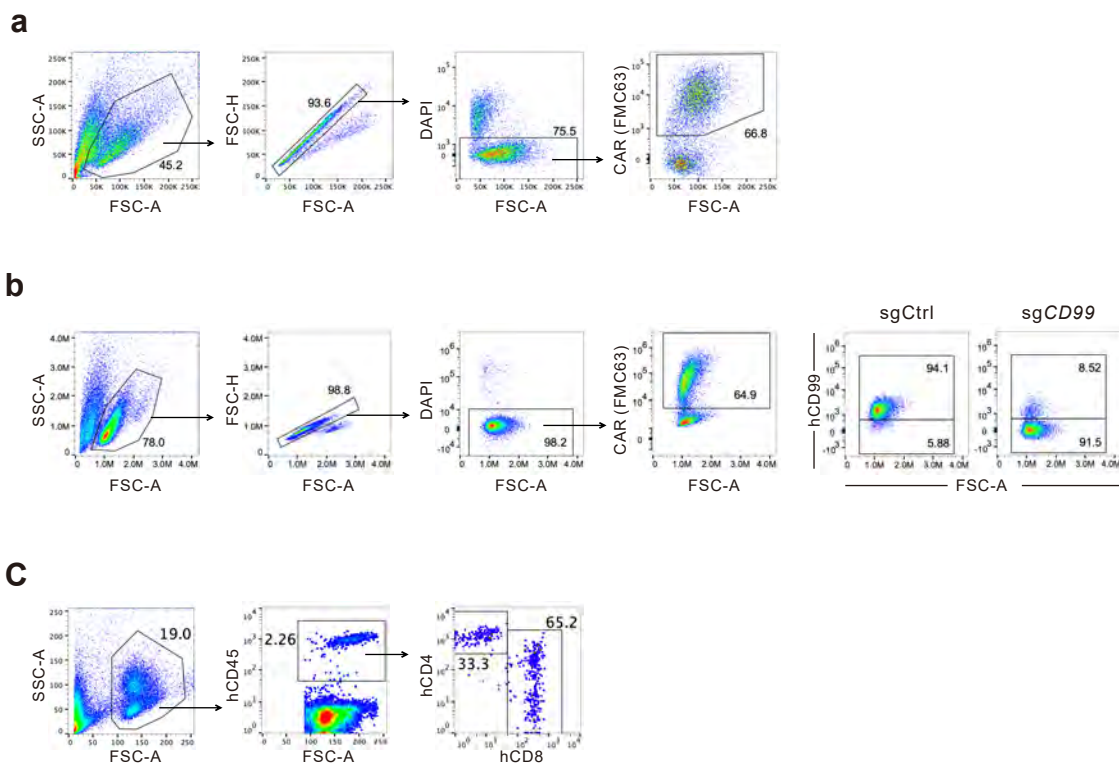


Supplementary Fig. 6. Identification of transmembrane and juxtamembrane domains as minimal essential domains of mouse and human CD99. **a** Confocal images (related to Fig. 6b) of recombinant CD99-YFP (yellow), F-actin (red), and microtubules (cyan) overlaid on DIC images of *Cd99*^{-/-}-cells transduced to express TM-Mut-CD99, TM-SR-CD99, TM-LR-CD99, or rWT-CD99. **b** Confocal images (related to Fig. 6d) of YFP (yellow), F-actin (red), and microtubules (cyan) overlaid on DIC images of TJ-CD99, rWT-CD99, or mock-transduced *Cd99*^{-/-}-cells. **c - e** Validation of human CD99 knockdown (KD) efficiency. Schematic diagram of the DNA constructs for mCherry and shRNA expression in human CD99-KD, and for the expression of GFP-tagged human CD99 (HuCD99) (**c**). Western blotting (**d**) and fluorescence microscopic analyses (**e**) of HuCD99 (GFP) and shRNA (mCherry) expression in HEK293T cells transiently transfected with the KD and HuCD99 plasmids. Relative CD99-band intensities of knockdown cells to those of scramble-transfected cells are denoted as KD rate. **f** Confocal images (related to Fig.6f) of F-actin or microtubules (cyan), shRNA (mCherry, red), and HuCD99 (YFP, green) in HuTJ-CD99- and mock-transfected KD-HuCD99-Jurkat cells. **g - i** Confocal images of CD99-deleted (sg*CD99*) or control- (sgCtrl) human alloreactive T cells after stimulation with anti-CD3 for 15 min (**g, i**) or after coincubation with target allogeneic cells for 30 min (**h**). Flow cytometric single histogram shows CD99 surface expression on sgCtrl or sg*CD99* cells, with MFI and the fluorescence minus one (FMO) values indicated in parentheses (**g**). F-actin ring width ($n = 26, 30$ cells), pMT number ($n = 22, 28$ cells), and F-actin and MTOC polarization ($n = 11, 20$ conjugates) were quantified (**g, h**). Representative single-section images of NFAT ($n = 12, 13, 14, 14, 14, 17, 16,$ and 17 cells, from left to right) and NF κ B ($n = 28, 28, 37, 32, 36, 38, 50,$ and 43 cells, from left to right) translocation, and ERK phosphorylation ($n = 23, 31, 38, 42, 37, 46, 32,$ and 32 cells, from left to right) are shown, and the corresponding values are plotted (**i**). **j, k** Levels of AKT phosphorylation (**j**) and surface expression of CD69, CD25, and CD44 (**k**) on peak days after stimulation with anti-CD3 of sgCtrl or sg*CD99* cells. Cells were analyzed by flow cytometry, and the MFI values are plotted ($n = 3$ replicates per group). Data are representative of three (**a, b, g-k**) or two (**d-f**) independent experiments. Data (**g-k**) are shown as mean $\pm$ SEM. Statistical significance was assessed using unpaired two-tailed *t*-test. Exact *p*-values are presented in the figure. Source data are provided as a Source Data file.



Supplementary Fig. 7. Potentiated IS formation and antitumor effects via CD99 domain-incorporated CAR design.

a Confocal images of untransduced (UnTD) T, BBz-CAR-T, and HuTJ-BBz-CAR-T cells (green; F-actin) after coincubation for 15, 30, or 60 min with CMTMR-labeled CD19⁺ Raji tumor cells (red). The coincubated cells were stained with phalloidin (green). CAR-T cells forming conjugates with the tumor cells are indicated by white arrows. Data are representative of two independent experiments. **b** Flow cytometry profiles (related to Fig. 7d) of the expression of CD69, CD25, and CD44 on UnTD T, BBz-CAR-T, and HuTJ-BBz-CAR-T cells after 2 days of coincubation with Raji cells. Data are representative of three independent experiments. **c** Plots of MFI values of CD69, CD25, and CD44 expressed on CD4⁺ or CD8⁺ fractions of UnTD T, BBz-CAR-T, or HuTJ-BBz-CAR-T cells after coculture with Raji cells ($n = 4$ replicates per group). Data are representative of three independent experiments. **d** Expression of activation markers on BBz-CAR-T and HuTJ-BBz-CAR-T cells with control (sgCtrl) or endogenous CD99 gene deletion (sgCD99) after co-culture with Raji cells. The expression levels of CD69, CD25, and CD44 were assessed by flow cytometry and the MFI values were plotted ($n = 3$ replicates per group). Data are representative of four independent experiments. **e** Flow cytometry profiles (related to Fig. 7e) of residual tumor cells and CAR-T cells on days 1 and 7 post-coculture. **f** Kinetic plots of viable cell counts for UnTD-T, BBz-CAR-T, or HuTJ-BBz-CAR-T cells cultured in 10% RPMI-1640 medium without CAR stimulation and cytokines. No significant differences were observed between groups. Data are from two independent experiments (triplicate/group/experiment). Data (**c**, **d**, **f**) are shown as mean $\pm$ SEM. Statistical significance was assessed using unpaired two-tailed t -test (**c**, **d**), and one-way ANOVA with Dunnett's post-hoc test (**f**). Exact p -values are presented in the figure. Source data are provided as a Source Data file.



Supplementary Fig. 8. Gating strategies used for flow cytometric analysis. **a** Representative gating strategy for analyzing the activation status of CAR-T cells, as shown in Fig. 7d and Supplementary Fig. 7b. **b** Representative gating strategy for analyzing the activation status of endogenous CD99-deleted CAR-T cells, as shown in Supplementary Fig. 7d. **c** Representative gating strategy for quantifying CAR-T cell numbers in host peripheral blood, as shown in Fig. 8e.

Supplementary Table 1. Oligonucleotide list

Primers for CD99 knockout mice genotyping	
mCD99 Exon_F	TCCCCCCAGACCCCAACATGAA
mCD99 Exon_R	ACCTGAGTCTCCGTGTGGCCTT
mCD99 Zeocin_F	GCGTTACCCAACTTAATCG
mCD99 Zeocin_R	TGTGAGCGAGTAACAACCCG
Oligonucleotides for human CD99 knockdown	
hCD99 shRNA-1	GATAAATCGAAACCACCATCC
hCD99 shRNA-2	TCCTTCTCCACCTGAAACGC
scramble shRNA	TCGCATAGCGTATGCCGTT
hCD99 sgRNA	CGGCGACCAGAACACCCAGC

Supplementary Table 2. Antibodies information

Antibody	Clone	Company	Catalog#	Dilution
PE/Cy7 anti-mouse CD8a	53-6.7	BD Biosciences	552877	1:2000
FITC anti-mouse TCR beta	H57-594	Thermo Fisher Scientific	11-5961-81	1:1000
FITC anti-mouse CD11a (LFA-1)	2D7	BD Biosciences	553120	1:1000
PE anti-mouse ICAM-1	YN1/1.7.4	Thermo Fisher Scientific	12-0541-81	1:500
APC anti-mouse CD25	PC61.5	Thermo Fisher Scientific	17-0251-81	1:2000
APC/Cy7 anti-mouse/human CD44	IM7	BioLegend	103028	1:2000
eFluor450 anti-mouse CD45.1	A20	Thermo Fisher Scientific	48-0453-82	1:500
PE anti-mouse CD69	H1.2F3	BioLegend	104508	1:2000
BV605 anti-mouse CD90.2	30-H12	BioLegend	105343	1:4000
PE anti-mouse CD99		R and D systems	FAB3905P	1:500
APC anti-mouse CD178 (FasL)	MFL3	BioLegend	106609	1:500
BV421 anti-human CD3	UCHT1	BioLegend	300434	1:500
Alexa Fluor 647 anti-human CD99	DN16	Novus Biologicals	NB100-64930AF647	1:1000
BV510 anti-human CD8	SK1	BioLegend	344732	1:500
APC/Cy7 anti-human CD4	RPA-T4	BD Biosciences	557871	1:500
BV605 anti-human CD69	FN50	BD Biosciences	562989	1:250
PE anti-human CD25	M-A251	BioLegend	985802	1:1000
Biotinylated anti-FMC63	Y45	Acro Biosystems	FM3-BY54	1:50
PE/TexasRed Streptavidin		BD Pharmigen	551487	1:2000
PE Streptavidin		BD Pharmigen	554061	1:8000
7-AAD Viability Staining Solution		BioLegend	420404	5ul/sample
123count eBead Counting Beads		Thermo Fisher Scientific	01-1234-42	10ul/sample
anti-Tubulin, alpha		Abcam	ab18251	1:1000
anti-Tubulin, alpha	DM1A	Millipore	05-829	1:500
anti-NFAT1	D43B1	Cell Signaling Technology	5861	1:200
anti-NFkBp65	D14E12	Cell Signaling Technology	8242	1:200
anti-phosphor-Erk1/2	D13.14.4E	Cell Signaling Technology	4370	1:200
anti-GFP	3E6	Thermo Fisher Scientific	A11120	1:500
anti-IQGAP1	C-9	Santa Cruz Biotechnology	sc-376021	1:200
anti-Myosin IIA		Cell Signaling Technology	3403	1:200
Alexa Fluor 594-conjugated phalloidin		Thermo Fisher Scientific	A12381	1:1000
Alexa Fluor 647-conjugated phalloidin		Thermo Fisher Scientific	A22287	1:1000
Alexa Fluor 488 goat anti-mouse IgG		Thermo Fisher Scientific	A11001	1:2000
Alexa Fluor 555 goat anti-rabbit IgG		Thermo Fisher Scientific	A21430	1:2000
Alexa Fluor 594 goat anti-mouse IgG		Thermo Fisher Scientific	A11005	1:2000
Alexa Fluor 647 goat anti-rabbit IgG		Thermo Fisher Scientific	A21245	1:2000