

Supporting Information

A Ca-Mg Synergistic Agonist for Trade-off CD8 T Cell Immunomodulation to Enhance Solid Tumor Immunotherapy

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Supplementary Figures

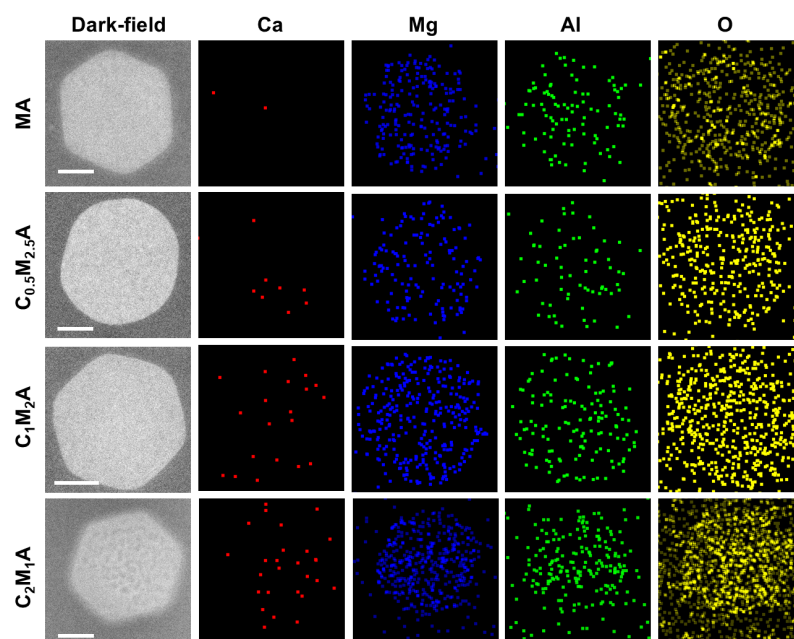


Figure S1. Energy-dispersive X-ray spectroscopy elemental mapping of MA and C_xM_{3-x}A.

Scale bar = 50 nm.

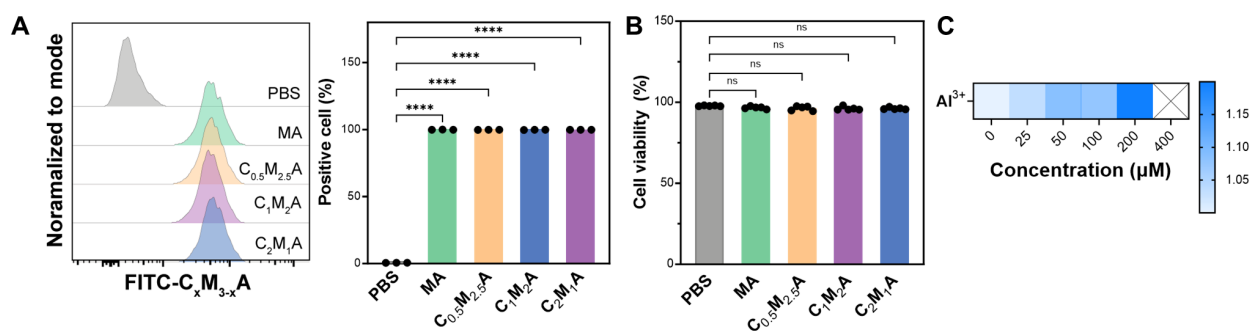


Figure S2. The cytotoxicity and cellular uptake of MA and C_xM_{3-x}A. Primary CD8⁺ T cells were incubated with the indicated formulation (100 μg mL⁻¹) for 24 h. The cellular uptake efficiency (A), and the cytotoxicity (B) of CD8⁺ T cells. (C) The relative CD8⁺ T cells viability after treating with concentration-varied Al³⁺. Statistical significances were computed via one-way ANOVA test. Data are shown as the mean ± s.d. (n = 3). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

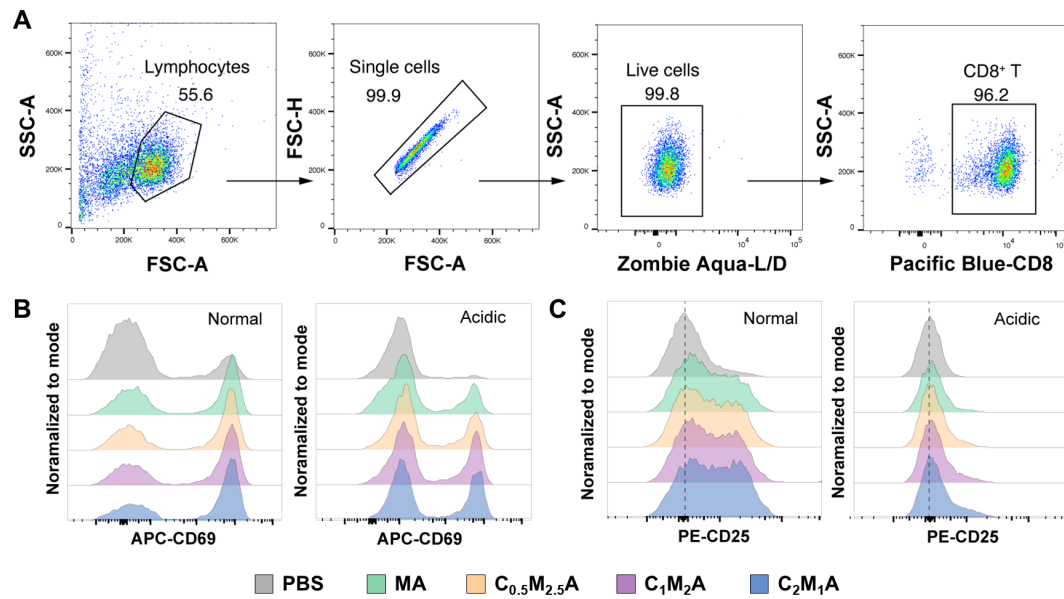


Figure S3. Expression levels of activation markers on CD8⁺ T cells treated with C_xM_{3-x}A (100 $\mu\text{g mL}^{-1}$). (A) Gating strategy used for flow cytometry analysis. (B-C) Representative histogram plots of CD69 (B) and CD25 (C) expression under normal and acidic condition.

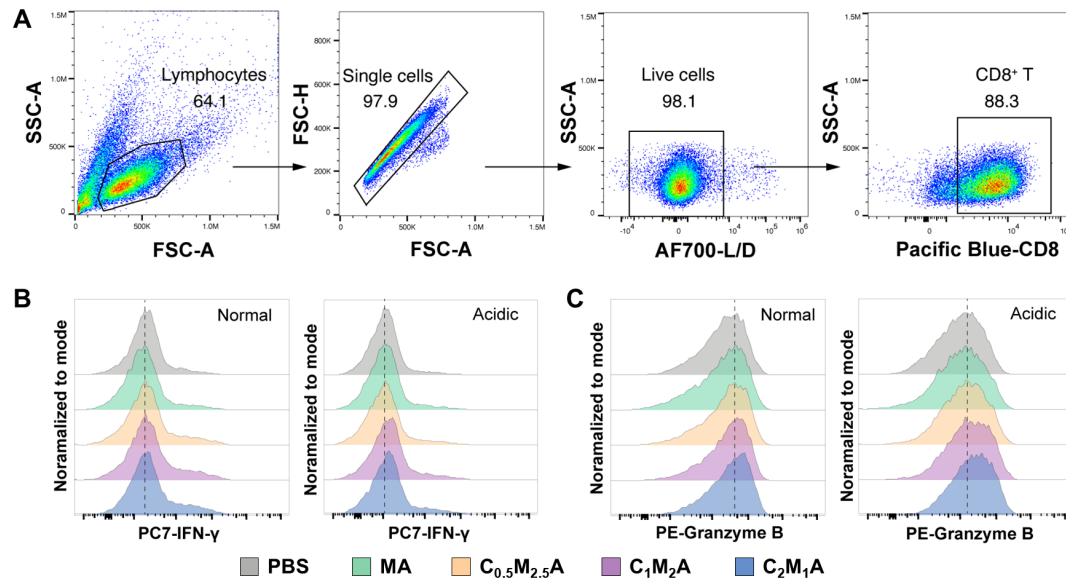


Figure S4. Expression levels of effector molecules on CD8⁺ T cells treated with MA and C_xM_{3-x}A (100 $\mu\text{g mL}^{-1}$). (A) Gating strategy used for flow cytometry analysis. (B-C) Representative histogram plots showing IFN- γ (B) and Granzyme B (C) expression under normal and acidic condition.

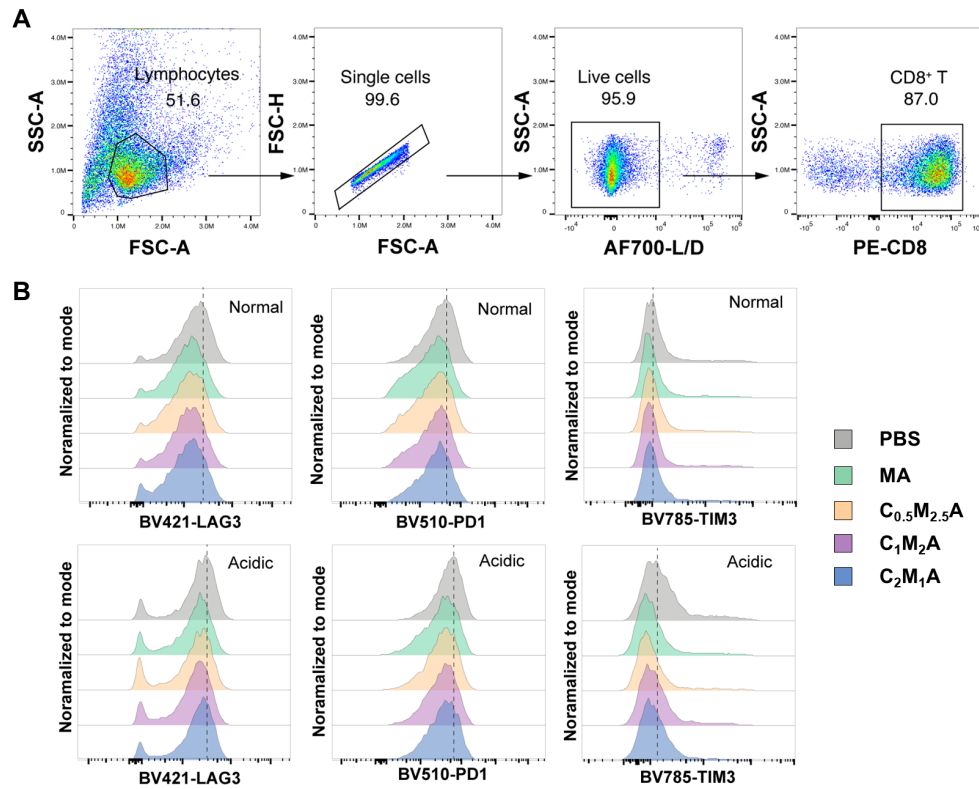


Figure S5. Expression levels of exhaustion markers on CD8⁺ T cells treated with MA and C_xM_{3-x}A (100 μ g mL⁻¹). (A) Gating strategy used for flow cytometry analysis. (B) Representative histogram plots showing PD1, LAG3 and TIM3 expression under normal and acidic condition.

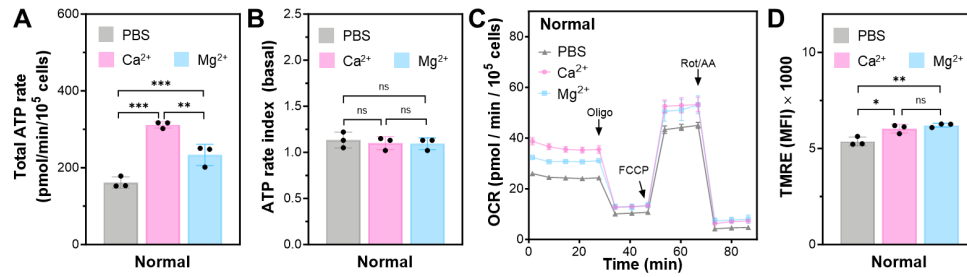


Figure S6. Free ions promote CD8⁺ T cell metabolism. The total basal ATP production rate (A) and ATP rate index (B) of CD8⁺ T cell with indicated treatment. (C) Representative oxygen consumption rate (OCR) in CD8⁺ T cells treated with CMA under normal condition. Sequential injections of oligomycin (Oligo), FCCP, and rotenone /antimycin A (Rot/AA) are indicated by black arrows. (D) Mitochondrial membrane potential (TMRE staining) in CD8⁺ T cells treated with free ions. Data are shown as the mean $\pm$ s.d. (n = 3). Statistical significance was tested by one-way ANOVA with Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

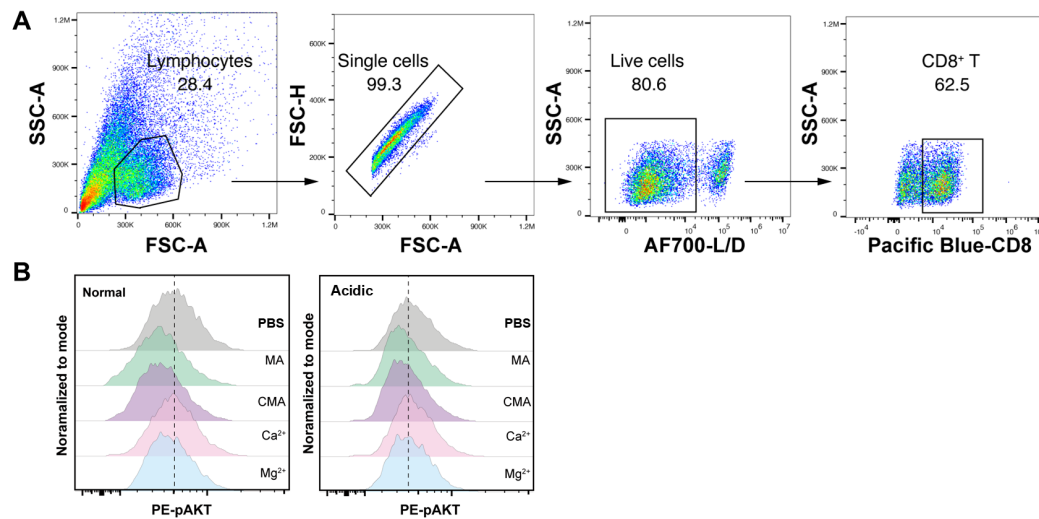


Figure S7. pAKT levels in CD8⁺ T cells treated with CMA (100 $\mu\text{g mL}^{-1}$). (A) Gating strategy used for flow cytometry analysis. (B) Representative histogram plots showing pAKT expression under normal and acidic condition.

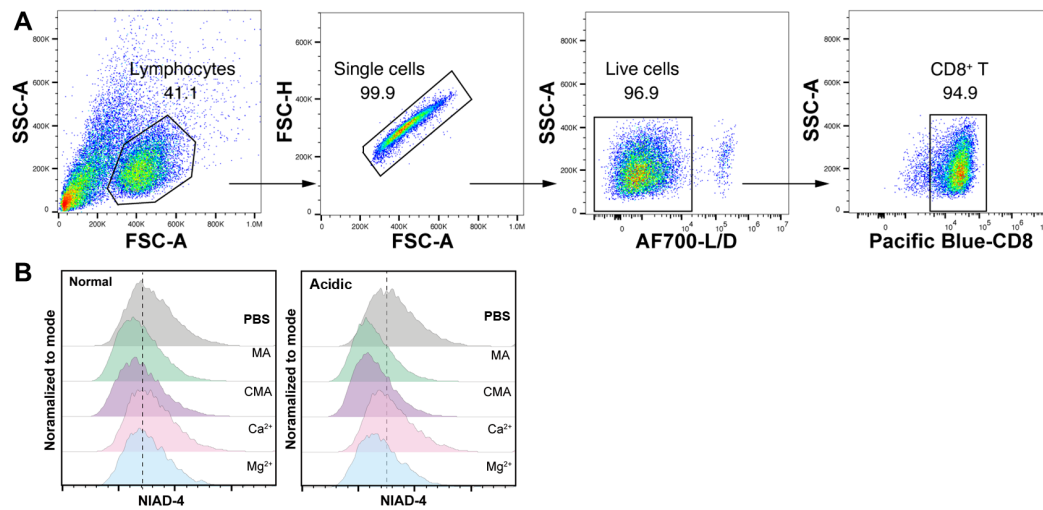


Figure S8. Protein aggregation levels in CD8⁺ T cells treated with CMA (100 $\mu\text{g mL}^{-1}$). (A) Gating strategy used for flow cytometry analysis. (B) Representative histogram plots showing NIAD-4 staining under normal and acidic condition.

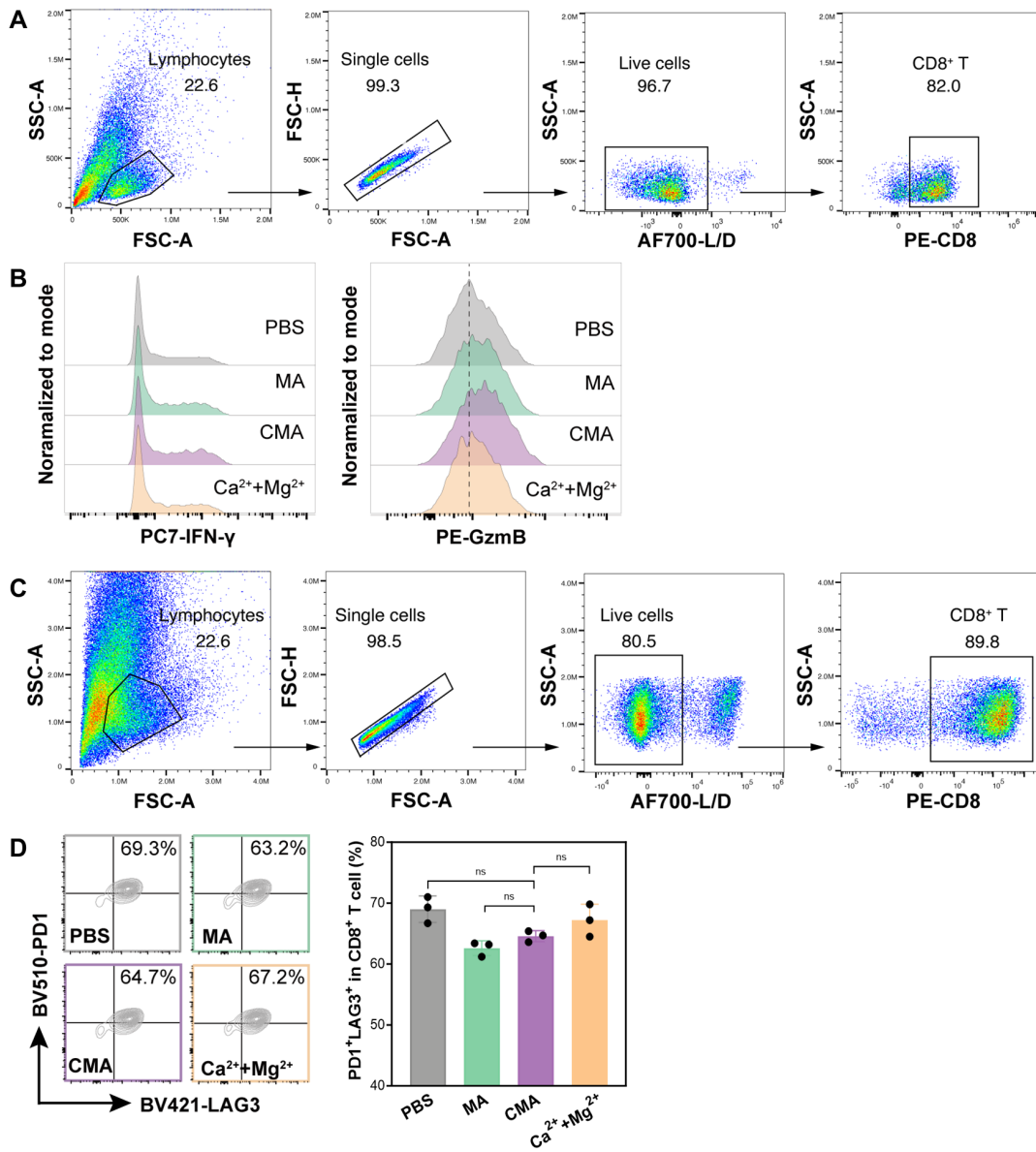


Figure S9. CMA pretreatment prevents CD8⁺ T cell functional impairment induced by lactic acid. (A) Gating strategy for flow cytometric analysis of OT-1 CD8⁺ T cells. (B) Representative histogram plots showing IFN-γ and granzyme B expression after challenged with lactic acid. (C) Gating strategy for flow cytometric analysis of OT-1 CD8⁺ T cells. (D) The proportion of exhausted T cells in pretreated CD8⁺ T cells after challenged with lactic acid (10 mM, pH = 6.5). Data are presented as the mean ± s.d. (n = 3). Statistical significance was tested by one-way ANOVA with Tukey's post hoc test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

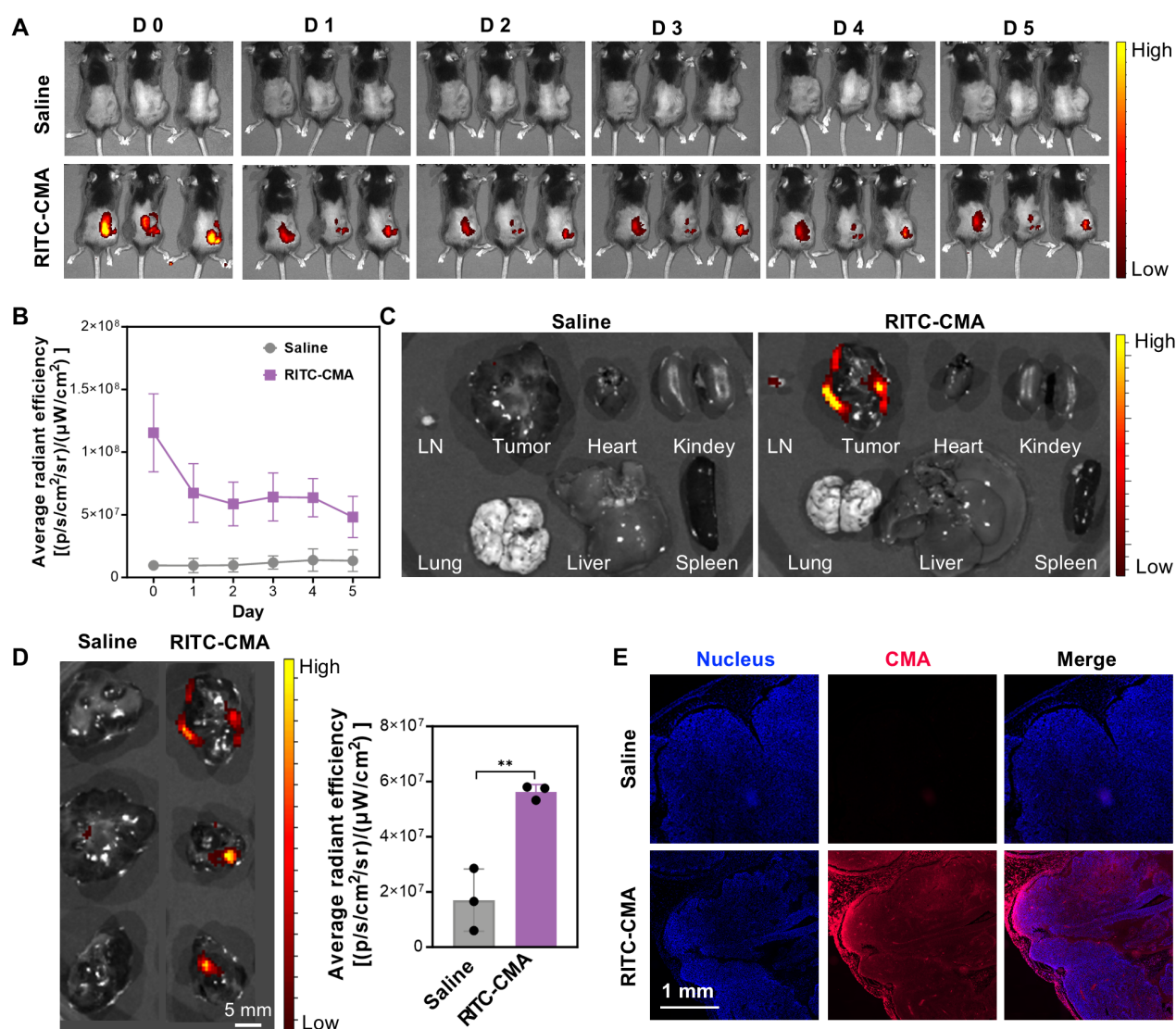


Figure S10. Biodistribution of CMA in vivo. (A) Representative fluorescence images showing RITC-labeled CMA accumulation in tumors over 5 days, acquired using an in vivo imaging system (IVIS). (B) Quantitative analysis of fluorescence signals from (A). (C) Ex vivo fluorescence images of major organs and tumors harvested 5 days post-administration. (D) Fluorescence imaging and quantitative analysis of CMA accumulation in tumors. Scale bar = 5 mm. (E) Representative fluorescence images of tumor sections showing RITC-CMA localization (red) and nuclei stained with DAPI (blue). Scale bar = 1 mm. Data are presented as the mean $\pm$ s.d. (n = 3).

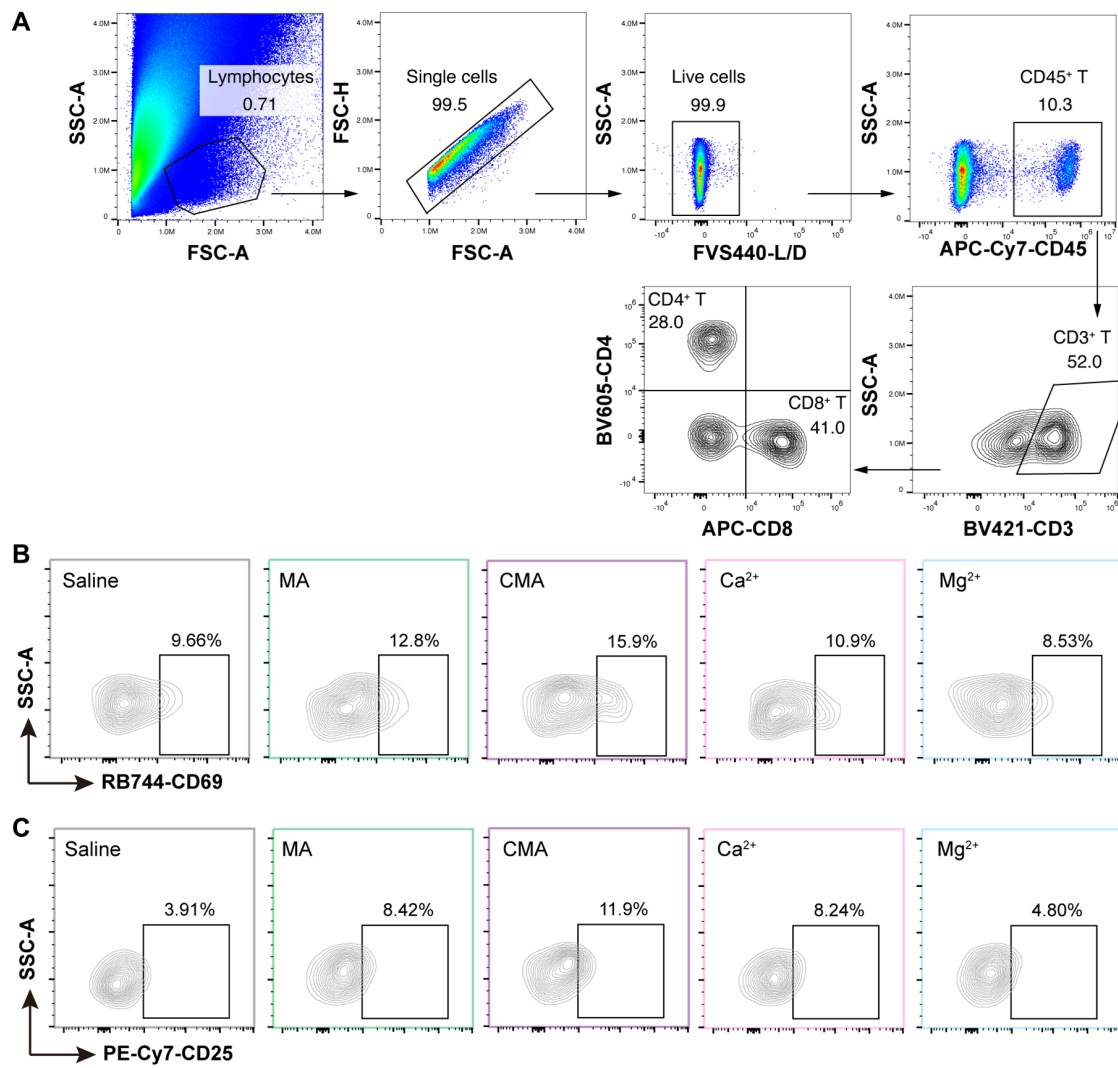


Figure S11. Proportions of activated CD8⁺ T cells within the TME. (A) Gating strategy for flow cytometric analysis of tumor-infiltrating CD8⁺ T cells. (B, C) Representative flow cytometry plots of CD8⁺ CD69⁺ (B) and CD8⁺ CD25⁺ (C) T cells in tumors.

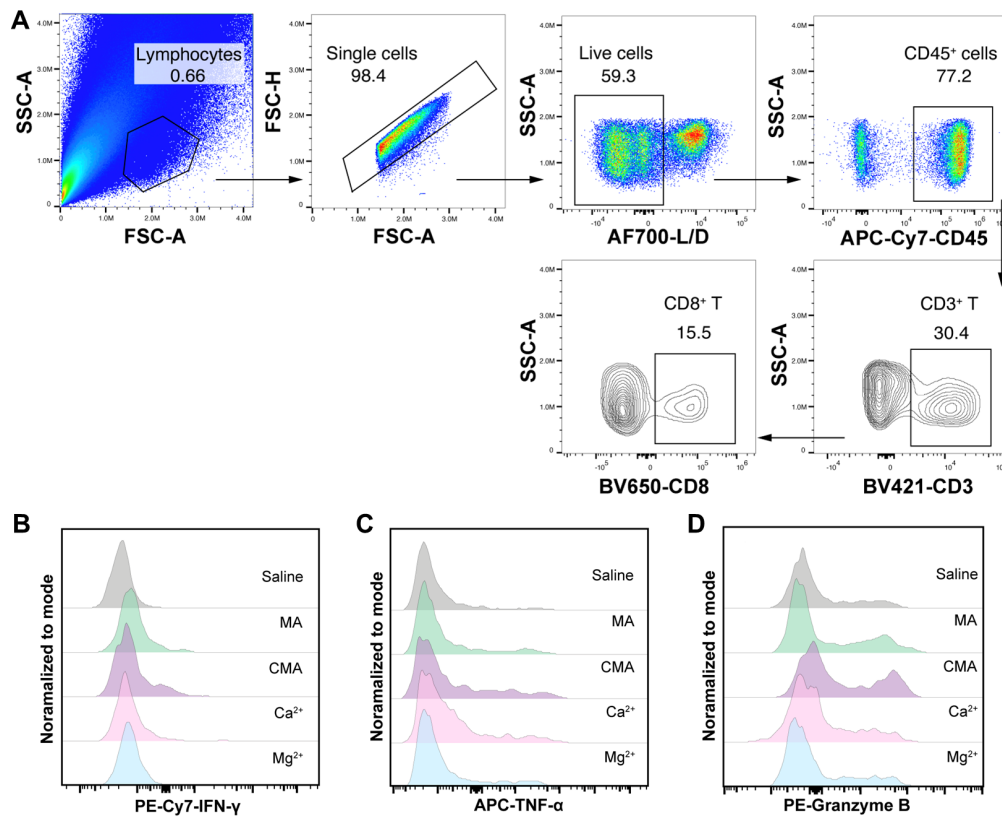


Figure S12. Expression of effector molecules in CD8⁺ T cells within the TME. (A) Gating strategy for flow cytometric analysis of tumor-infiltrating CD8⁺ T cells. (B-D) Representative flow cytometry plots showing the expression of IFN-γ (B), TNF-α (C) and Granzyme B (D) in CD8⁺ T cells.

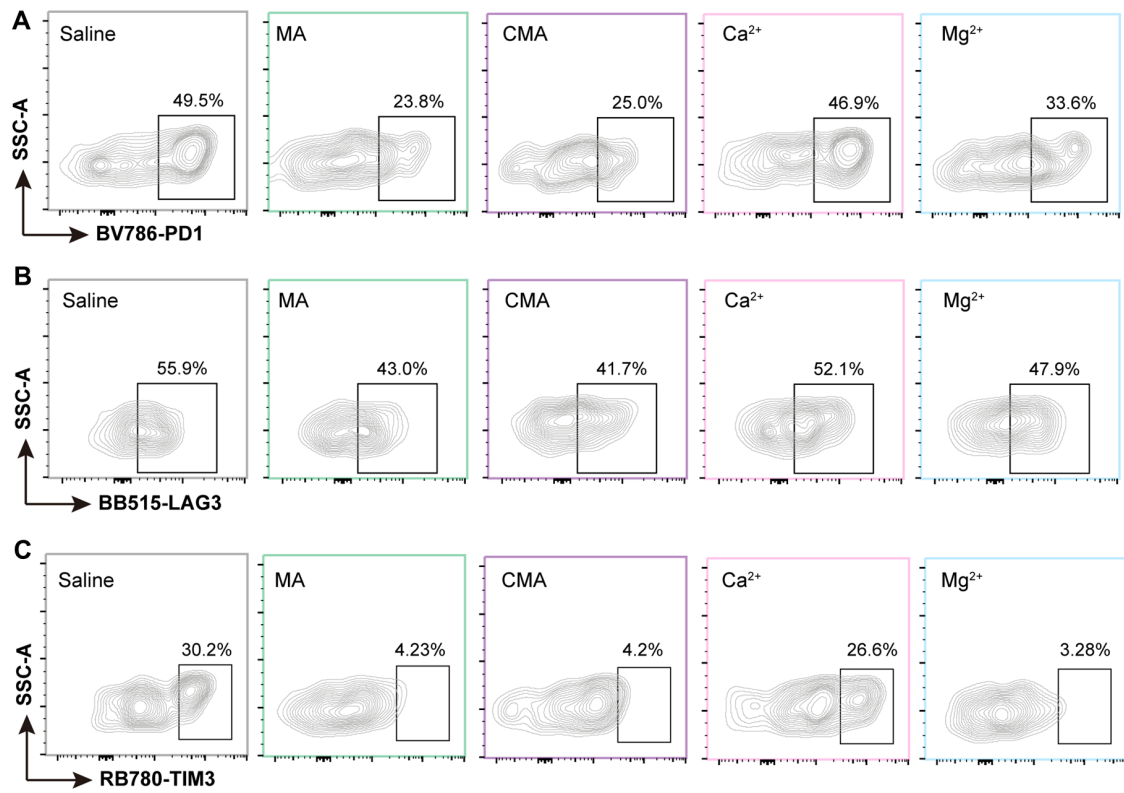


Figure S13. Proportions of exhausted CD8⁺ T cells within the TME. Representative flow cytometry plots of CD8⁺ PD1⁺ (A), CD8⁺ LAG3⁺ (B) and CD8⁺ TIM3⁺ (C) T cells in tumors.

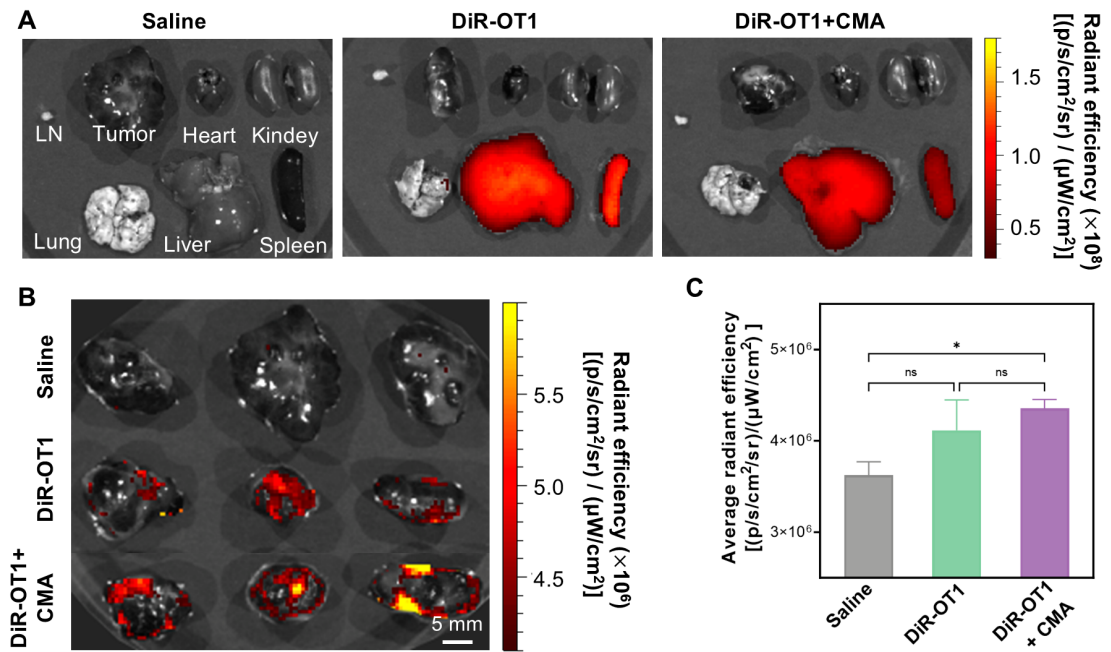


Figure S14. Biodistribution of retro-orbitally injected OT-1 CD8⁺ T cells. (A) Representative image of DiR-labeled cell accumulation in major organs. Scale bar = $0.3 \sim 1.8 \times 10^8$ (p/s/cm²/sr)/(μ W/cm²). (B, C) Representative image (B) and quantitative analysis (C) of DiR-labeled cell accumulation in tumors. Scale bar = $4.1 \sim 6.0 \times 10^6$ (p/s/cm²/sr)/(μ W/cm²). Data are presented as the mean $\pm$ s.d. (n = 3). Statistical significance was tested by one-way ANOVA with Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

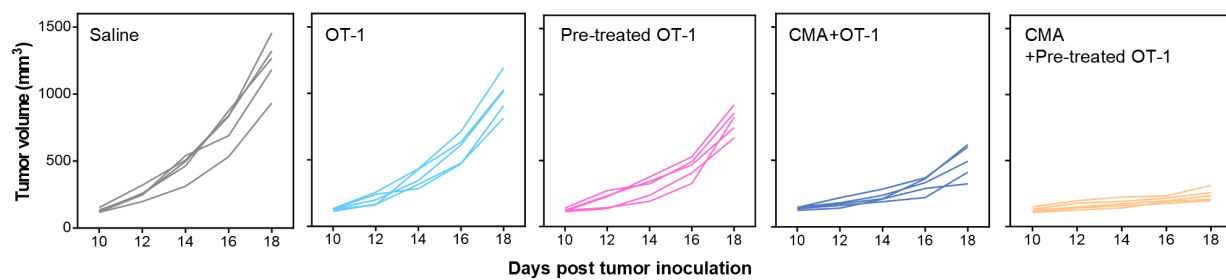


Figure S15. Individual tumor growth curves following indicated treatment (n = 5).

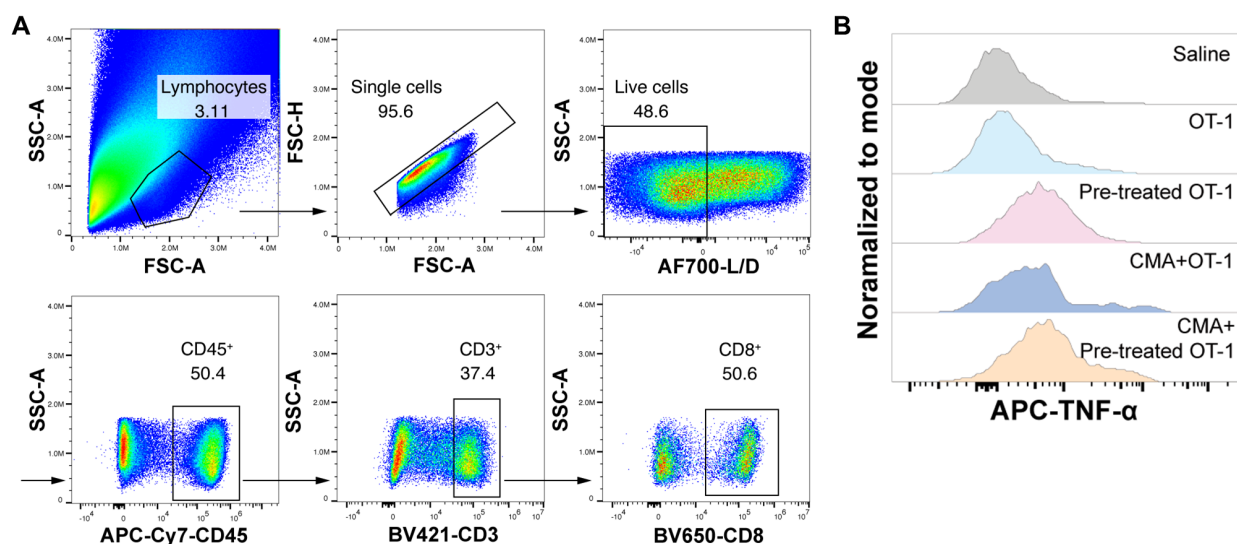


Figure S16. Effector molecular expression in CD8⁺ T cells within TME. (A) Gating strategy for flow cytometric analysis of CD8⁺ T cells in TME. (B) Representative flow cytometry histograms of TNF-α expression in CD8⁺ T cells within TME following indicated treatments.

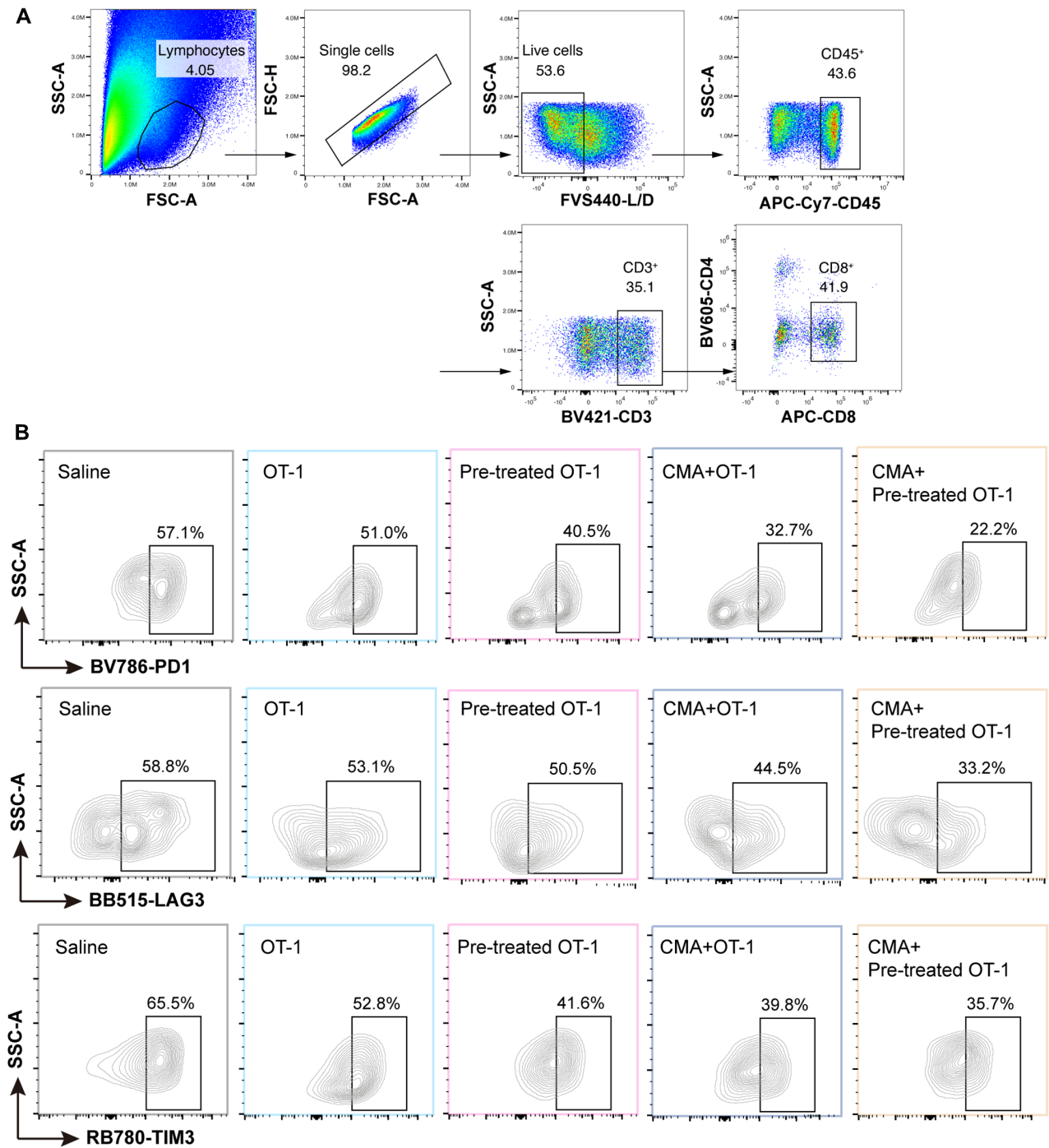


Figure S17. Expression of exhaustion markers in CD8⁺ T cells within TME. (A) Gating strategy for flow cytometric analysis of CD8⁺ T cells in TME. (B) Representative flow cytometry plots of PD1, LAG3 and TIM3 expression in CD8⁺ T cells within TME following indicated treatments.

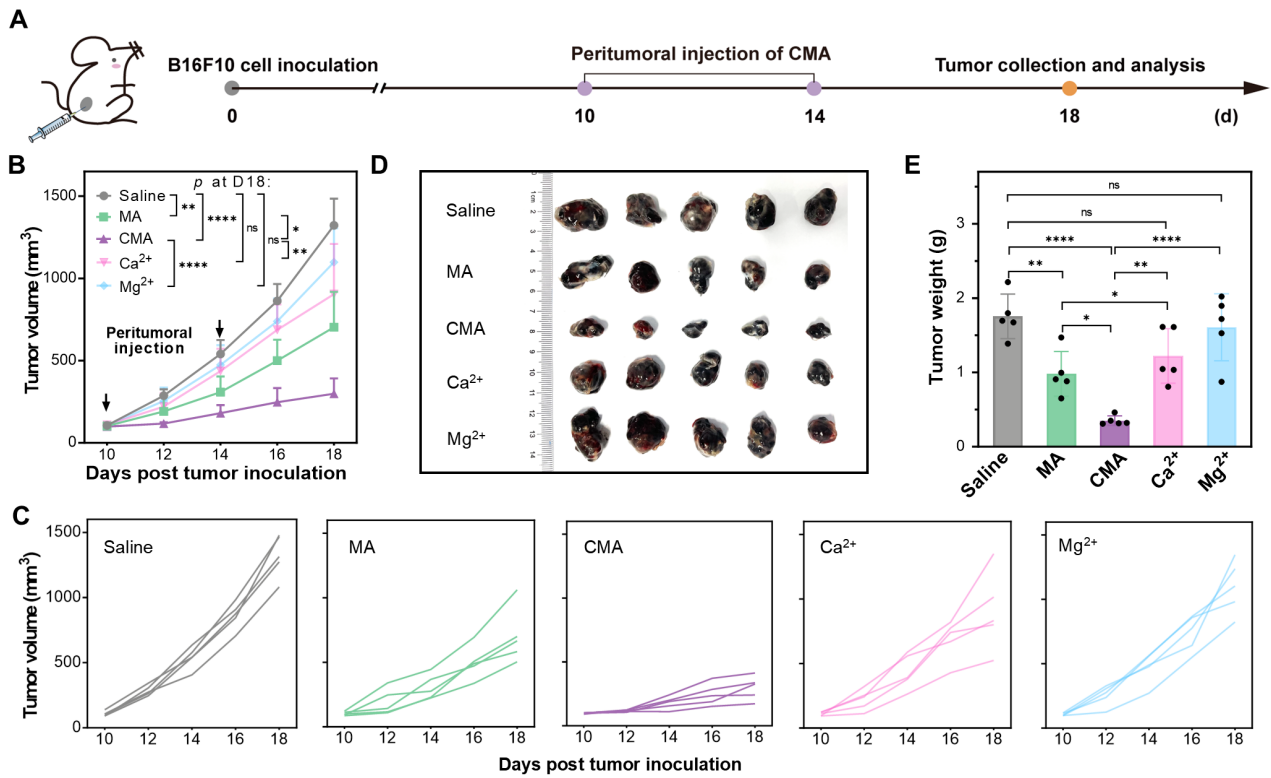


Figure S18. Therapeutic efficacy of CMA in B16 tumor-bearing mice. (A) Schematic timeline of treatment. (B, C) Mean (B) and individual (C) tumor growth curves following CMA treatment. (D) Representative tumor images at day 18 post-treatment. (E) Tumor weights at day 18 post-treatment. Data are presented as the mean $\pm$ s.d. ($n = 5$). Statistical significance was tested by one-way ANOVA with Tukey's post hoc test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

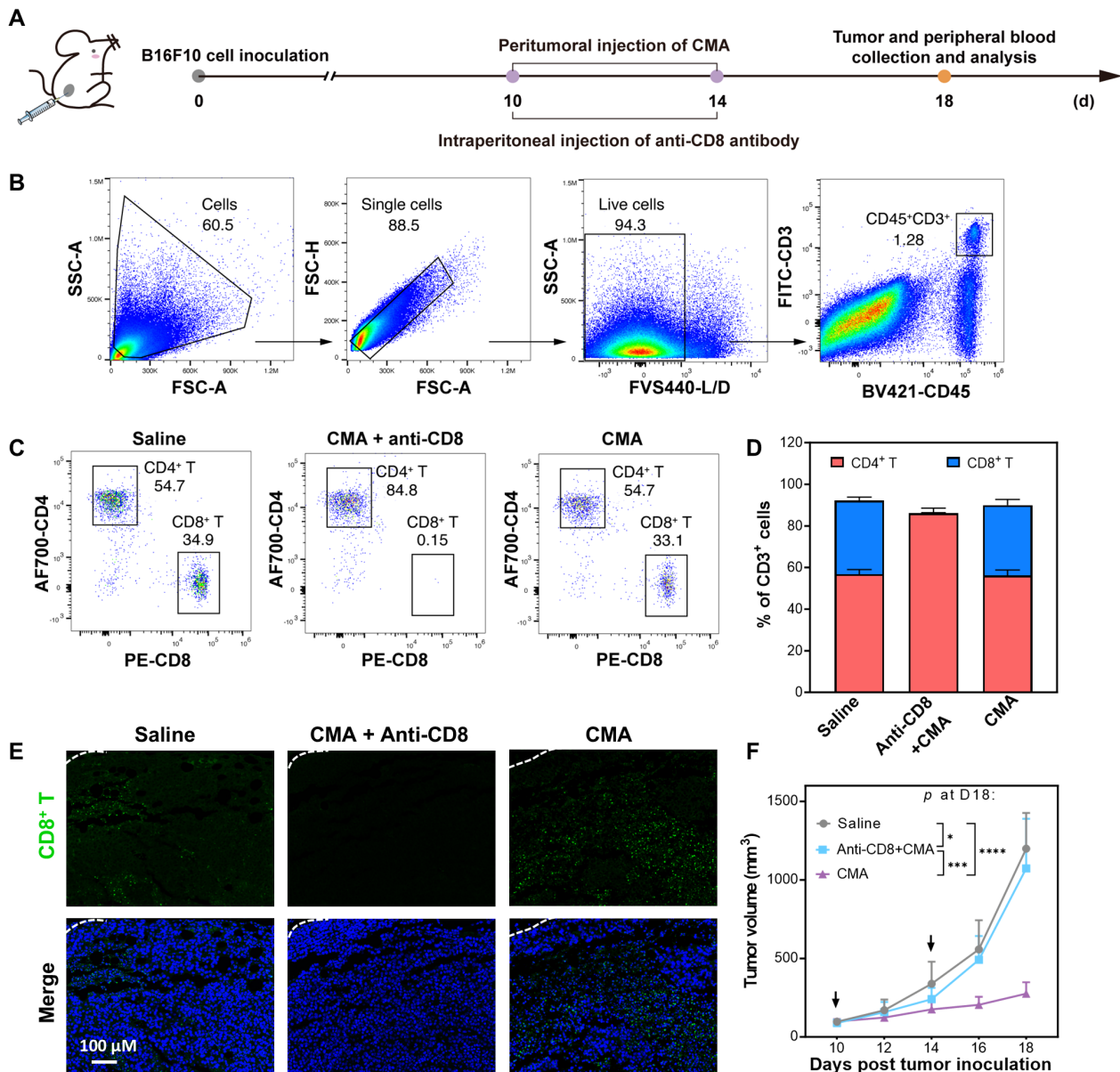


Figure S19. In vivo CD8⁺ T cell depletion. (A) Schematic timeline of treatment. (B) Gating strategy for flow cytometric analysis of peripheral blood. (C) Representative flow cytometry plots showing the proportion of CD8⁺ T cell in peripheral blood at day 18. (D) Quantitative analysis of CD8⁺ T cell proportion from (C). (E) Representative immunofluorescence images of tumor sections showing CD8⁺ T cell infiltration (green) with nuclei stained by DAPI (blue). Scale bar = 100 μ m. (F) Mean tumor growth curves over time in CD8⁺ T cell-depleted mice treated with CMA. Black arrows indicate peritumoral injection of CMA and intraperitoneal administration of anti-CD8 antibody. Data are presented as the mean $\pm$ s.d. ($n = 5$). Statistical significance was tested by one-way ANOVA with Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

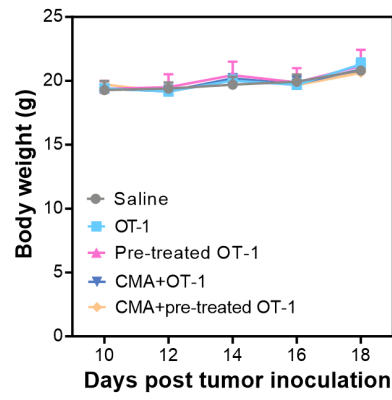


Figure S20. Body weight changes of B16-OVA tumor-bearing mice over time following indicated treatments. Data are presented as the mean $\pm$ s.d. (n = 5).

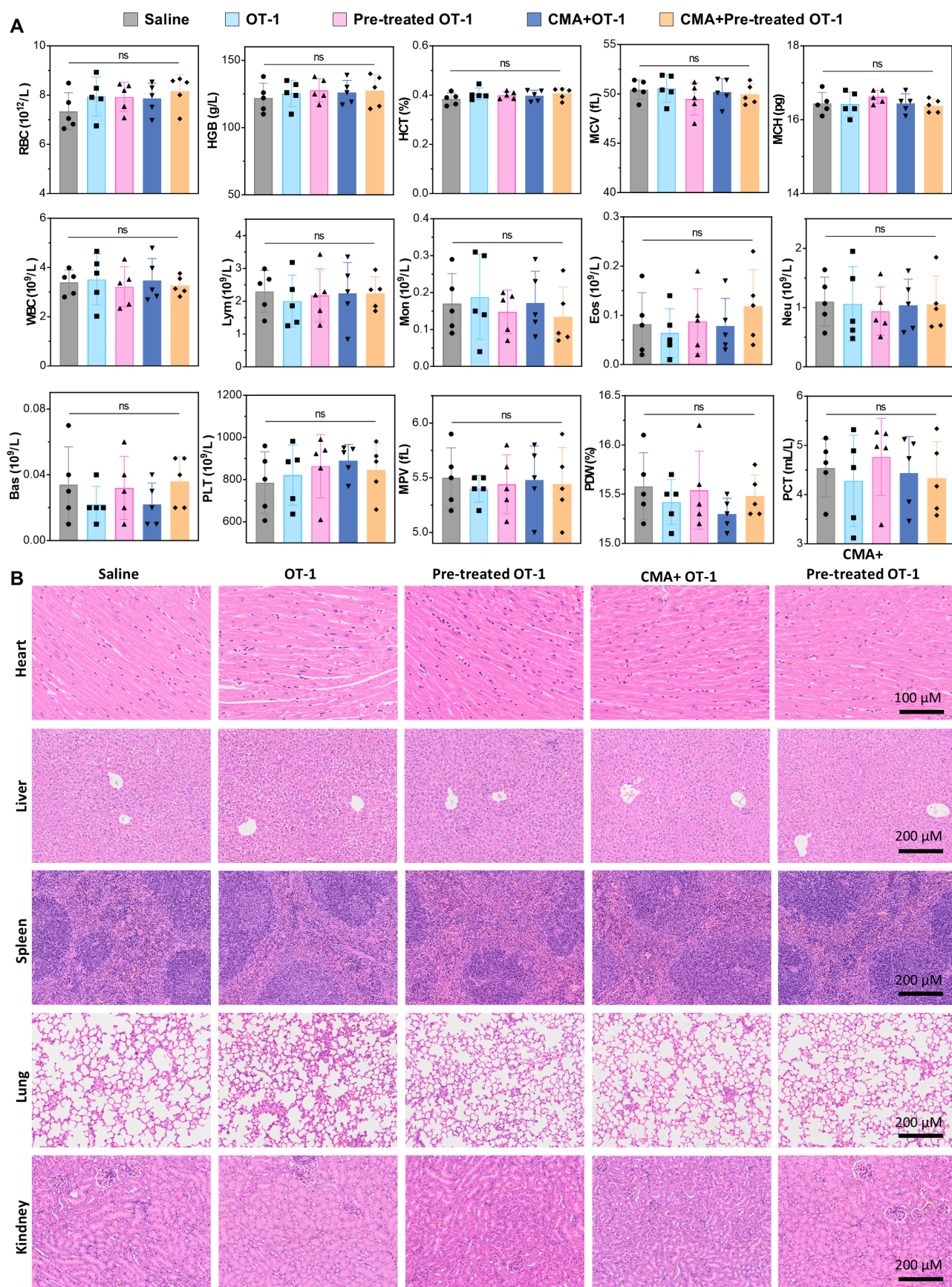


Figure S21. In vivo biosafety evaluation of CMA pretreated OT-1 T cells. (A) Complete blood count analysis of mice treated with CMA pretreated OT-1 T cells at day 18. Statistical significance

was tested by one-way ANOVA with Tukey's post hoc test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. (B) Representative H&E staining of major organs (heart, liver, spleen, lung, and kidneys) harvested from mice at day 18 post-treatment.

Supplementary Tables

Table S1. The size, zeta potential, and molar ratio of MA and C_xM_{3-x}A.

Sample	Size	Zeta potential	Molar ratio (Ca/Mg/Al)
MA	140.0 ± 1.8	41.6 ± 0.7	0:2.7:1
C _{0.5} M _{2.5} A	162.1 ± 1.3	47.0 ± 0.4	0.15:2.86:1
C ₁ M ₂ A	144.3 ± 1.5	42.5 ± 0.5	0.26:2.30:1
C ₂ M ₁ A	146.4 ± 2.1	33.5 ± 0.7	0.58:1.99:1

Data are presented as the mean ± s.d. (n = 3).

Table S2. Ion release from CMA in buffers of varying pH after 24 h.

pH buffer	Released ion amount after 24 h (µg/mg CMA)	
	Ca ²⁺	Mg ²⁺
pH=7.4	3.03 ± 0.13	0.16 ± 0.07
pH=6.5	17.07 ± 0.94	8.99 ± 0.26
pH=5.5	18.15 ± 0.45	23.6 ± 0.62

CMA was used at 100 µg mL⁻¹. Data are presented as the mean ± s.d. (n = 3).

Table S3. Antibodies and dyes used for flow cytometry analysis.

Antibody / Dye	Clone	Cat	Supplier
CD3	145-2C11	100359	BioLegend
CD28	37.51	102121	BioLegend
CD16/32	93	101320	BioLegend
PB-CD8	53-6.7	100725	BioLegend
PE-CD8	53-6.7	553032	BD Bioscience
FITC-CD8	53-6.7	100706	BioLegend
APC-CD8	53-6.7	553035	BD Bioscience
BV650-CD8	53-6.7	100742	BioLegend
APC-Cy7-CD45	30-F11	557659	BD Bioscience
BV421-CD45	30-F11	557659	BioLegend
BV421-CD3	145-2C11	562600	BD Bioscience
FITC-CD3	17A2	100204	BioLegend
BV650-CD11c	HL3	564079	BD Bioscience
AF700-CD11b	M1/70	101222	BioLegend
APC-Fire810-F4/80	BM8	123166	BioLegend
AF700-CD4	GK1.5	100430	BioLegend
BV605-CD4	RM4-5	563151	BD Bioscience
AF647-anti-firefly luciferase	EPR17789	AB237252	Abcam
APC-CD69	H1.2F3	104514	BioLegend
RB744-CD69	2F3	756897	BD Bioscience
PE-CD25	3C7	101904	BioLegend
PC7-CD25	PC81	552880	BD Bioscience
PC7-IFN- γ	XMG1.2	505826	BioLegend
PE-granzyme B	QA16A02	372208	BioLegend
APC-TNF α	MP6-XT22	506308	BioLegend
BV786-PD1 (CD279)	J43	744548	BD Bioscience
BV510-PD1 (CD279)	29F.1A12	135241	BioLegend
BV421-LAG3	C9B7W	125221	BioLegend

(CD223)			
BB515-LAG3	C9B7W	564672	BD Bioscience
(CD223)			
BV785-TIM3	RMT3-23	119725	BioLegend
(CD366)			
RB780-TIM3	25F1D6	569810	BD Bioscience
(CD366)			
PE-pAKT	A21001C	606553	BioLegend
Zombie Aqua Fixable	/	423102	BioLegend
Viability Kit			
Fixable Viability Stain	/	564997	BD Bioscience
700			
Fixable Viability Stain	/	566332	BD Bioscience
440UV			
Fluo-4-AM	/	F14201	Invitrogen
TMRE	/	HY-D0985A	MedChemExpress
NIAD4	/	HY-111263	MedChemExpress
