Supplementary Materials for

Calreticulin-Targeting L-Asparaginase-Flagellin Conjugate Enhances *Salmonella*-Mediated Antitumor Efficacy

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Materials and Methods

Measurement of binding affinity using surface plasmon resonance analysis

Binding kinetics of CRT3LFP to CRT were determined by surface plasmon resonance using a Biacore X100 instrument (Cytiva, USA). rCRT was immobilized on a Series S Sensor Chip CM5 via amine coupling to a density of approximately 1,000–2,000 response units (RU). CRT3LFP was prepared in running buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 0.05% Tween-20] and injected as analyte in a multi-cycle kinetic series at concentrations ranging from 0.3 to 130 nM (two-fold serial dilutions). Association was monitored for 180 - 300 seconds, and dissociation for 600 - 1,200 seconds at a flow rate of 30 - 50 μL/min. The surface was regenerated with 10 mM glycine-HCl pH 2.0 - 2.5 (or the appropriate regeneration condition). Sensorgrams were fitted to a 1:1 Langmuir binding model using Biacore X100 Evaluation Software to derive the association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant ($K_D = k_d/k_a$).

L-ASNase activity assay

L-ASNase activity was assessed following the method of Wriston and Yellin[51], which measures ammonia liberation via nesslerization. The reaction mixture comprised 1.5 mL of 0.04 M L-asparagine in 0.05 M Tris-HCl buffer (pH 8.6) and 0.5 mL of enzyme solution, resulting in a total volume of 2 mL. This mixture was incubated at 37°C for 30 min, and the reaction was terminated by adding 0.5 mL of 1.5 M trichloroacetic acid (TCA). A control was prepared by adding TCA to the enzyme solution prior to mixing with the substrate. Post-reaction, the mixture was centrifuged at 10,000×g for 15 min to remove precipitated proteins, and the supernatant was collected. For ammonia quantification, 0.5 mL of supernatant was diluted to 7 mL with distilled water, followed by the addition of 1 mL of Nessler's reagent. After 20 min, OD480 was measured. Ammonia concentration was determined using a standard curve established with ammonium sulfate solutions. One international unit (IU) of L-ASNase activity is defined as the enzyme quantity catalyzing the release of 1 μmol of ammonia per minute at 37°C and pH 8.6.

Protein stability assay in serum

100 μL of CRT3LFP [60 μg or 8 IU in PBS (pH 7.4)] was mixed with the same volume of mouse blood serum in a low-binding 1.5-mL microcentrifuge tube and incubated at 37°C. The

aliquots (10 μL) were withdrawn at predefined time points and immediately cooled on ice. L-ASNase activity of each aliquot was determined as described above. The enzyme activity percentage = [(enzyme activity in CRT3LFP + serum) - (enzyme activity in serum alone) at the indicated time]/[(enzyme activity in CRT3LFP + serum) - (enzyme activity in serum alone) at t = 0] x 100. Recombinant L-ASNase was used as positive control.

FlaB activity assay

HEK293/mTLR5 cells (3×10^4) were plated in 96-well plates and incubated overnight at 37°C. The cells were transiently transfected with 100 ng of pNiFty(2)-SEAP (Cat. code: 293-htlr5, InvivoGen, Hong Kong), a plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) after activation of the mTLR5-NF-kB signaling pathway, in 10 μL Turbofectin reagent (Cat. code: TF81001, Origene, USA). After incubation for a day, the cells were replaced with 180 μL of fresh DMEM supplemented with 10% heat-inactivated FBS and then treated with 20 μL of 4 nM CRT3LFP. The purified FlaB was used as a positive control. After 6 h of incubation, 20 μL of culture supernatants were transferred into each well of a 96-well plate, and 180 μL of alkaline phosphatase detection reagent QUANTI-Blue™ (InvivoGen, USA) was added to each well. After 3 h, the OD620 value of each well was measured using a microplate reader to quantify SEAP activity.

***In vitro* binding assay of CRT3LFP against the tumor cells treated with CNC018**

CT26 or MC38 cells (2×10^5 cells) were cultured overnight in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere on 6-well plates. The medium was removed by aspiration, and fresh DMEM without antibiotics containing CNC018 (2×10^6 CFU/mL) was added to each well. The cells were incubated for 22 h, followed by a 2 h treatment with penicillin-streptomycin (LS203-01, Welgene, Korea) to be a final 1% concentration. Then, the cells were detached using 0.05% trypsin-EDTA (LS-015-01, Welgene, Korea) and washed three times with FACS buffer (1% FBS in PBS; OPBDPWO50, Welgene, Korea). To assess CRT exposed on the cell surface, cells were treated with 50-fold diluted PE-conjugated anti-CRT antibody (19780S, Cell Signaling, USA) for 30 min and incubated on ice. The same cells were treated with 100 nM CRT3LFP as well as DGRLFP and CRT3LP for 1 h on ice. All the treated cells were washed five times with FACS buffer and treated with an anti-His tag monoclonal

antibody (1:1,000 dilution, ab245114, Abcam, USA) for 1 h. After simple washing, the cells were treated with 5 μg/mL Alexa Fluor 488-conjugated secondary antibody (A11008, Thermo Scientific, USA) for 1 h. Fluorescence signals of the cells were analyzed using a FACS Canto II flow cytometer (BD Biosciences, USA) and FlowJo software (Becton, Dickinson & Company, USA).

Confocal immunofluorescence imaging analysis

CRT3LFP, as well as DGRLFP and CRT3LP bound to CRT exposed on cell membranes, were visualized as previously described using confocal immunofluorescence microscopy[6]. CT26 and MC38 cells ($1x10^4$) were cultured overnight on cover glass and treated with CNC018 ($1x10^5$) at 37°C for 22 h. Thereafter, the cells were fixed with 1% paraformaldehyde (PFA; Biosesang, South Korea) for 15 min and then sequentially stained with anti-CRT antibody (1:1000 dilution) on ice for 1 h and Alexa Fluor 488-conjugated secondary antibody (5 μg/mL) for 1 h. The same processed cells were incubated with 100 nM recombinant proteins on ice for 1 h and then sequentially stained with anti-His tag monoclonal antibody (1:1,000 dilution) for 1 h and Alexa Fluor 488-conjugated secondary antibody (5 μg/mL) for 1 h.

Cell membranes were labeled with Alexa Fluor 555-conjugated wheat germ agglutinin (WGA) (1:5,000 dilution; Thermo Fisher Scientific, USA). Samples were washed with cold 1% Tween-PBS between each step. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Cat# P36931, Thermo Fisher Scientific, USA). The staining with anti-CRT antibody (1:1000 dilution) was also done using sequential staining with anti-CRT and Alexa Fluor 488-conjugated secondary antibodies.

Imaging was performed using an LSM510 confocal microscope (ZEISS, Jena, Germany), and data were analyzed with ZEN-LSM imaging software (ZEISS, Jena, Germany).

Cell viability assay

CT26 and MC38 cells (10^4) were plated in three wells of a 96-well plate and cultured at 37°C. The next day, the culture medium was replaced by 100 μL of fresh media containing CNC018 bacteria (10^5 CFU). After 18 hours of culture, penicillin-streptomycin was added to each well to achieve a final concentration of 1%, and the culture continued for an additional 2 hours. The

PBS-washed cells were treated with 5 IU/mL of CRT3LFP as well as CRTLP and DGR-LFP and cultured for 4 h at 37°C. Then, CCK-8 reagent (10 μL) was added to each well and incubated at 37°C for 1-2 h. The OD450 value at each well was measured as a viable cell level by a SpectraMax M2 microtiter plate reader (Molecular Devices, USA).

In vivo toxicity assay of CRT3LFP in mice

Female 7-week-old BALB/c mice (Samtako, Korea) were implanted subcutaneously (*s.c.*) with CT26 cells ($5x10^5$) in the right flank. When the tumor volumes reached approximately 100-120 mm^3 in volume, CRT3LFP (4, 8, or 12 IU) was *i.p.* injected into mice. PBS was used as an untreated control. Body weight, tumor size, and survival of mice were observed at the indicated days.

Supplementary figures

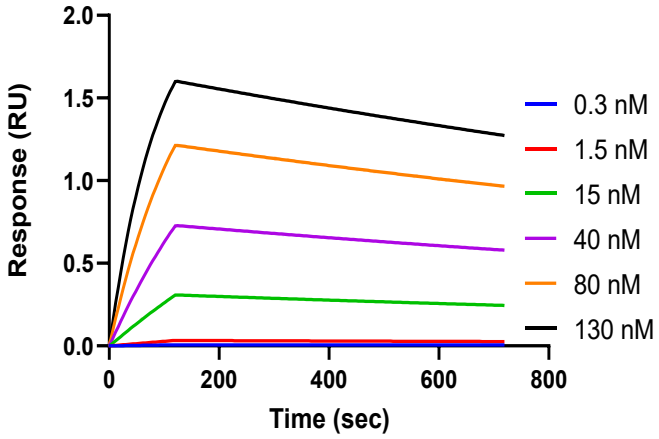


Figure S1. The binding affinity of CRT3LFP to calreticulin (CRT) is determined by surface plasmon resonance (SPR). Sensorgrams showing the real-time binding of CRT3LFP (analyte) at various concentrations (0.3 nM, blue; 1.5 nM, red; 15 nM, green; 40 nM, purple; 80 nM, orange; 130 nM, black) to immobilized CRT (ligand). The curves display association and dissociation phases, with global fitting to a 1:1 binding model yielding an equilibrium dissociation constant ($\mathbf{K}_D$) of 4.52×10^{-9} M, an association rate constant (k_a) of 8.51×10^4 $M^{-1}s^{-1}$, and a dissociation rate constant (k_d) of 3.85×10^{-4} s^{-1}.

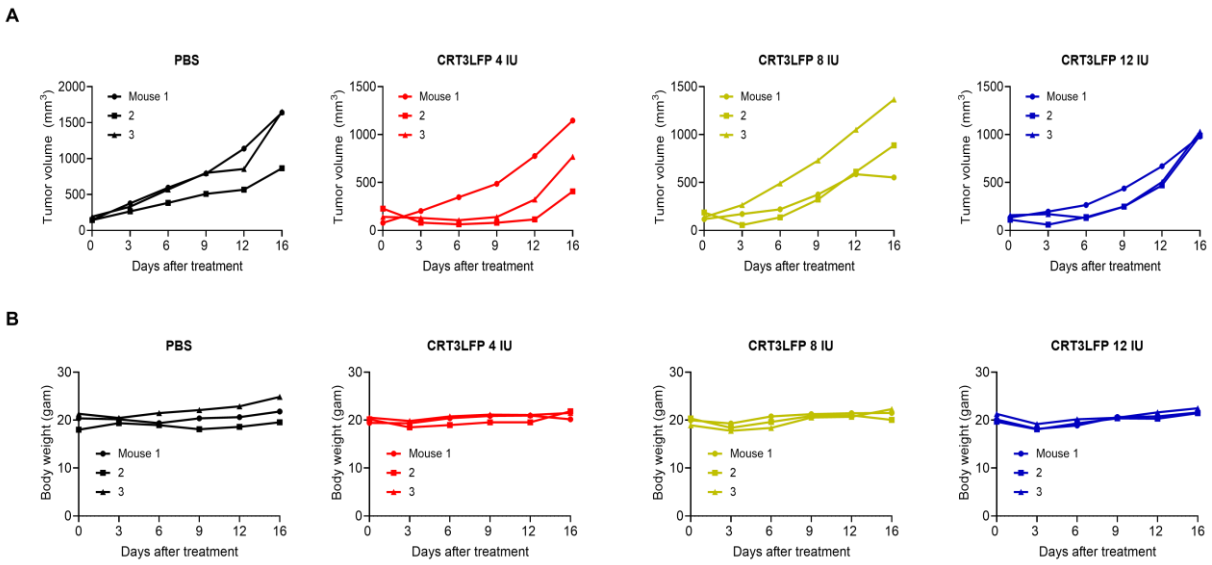
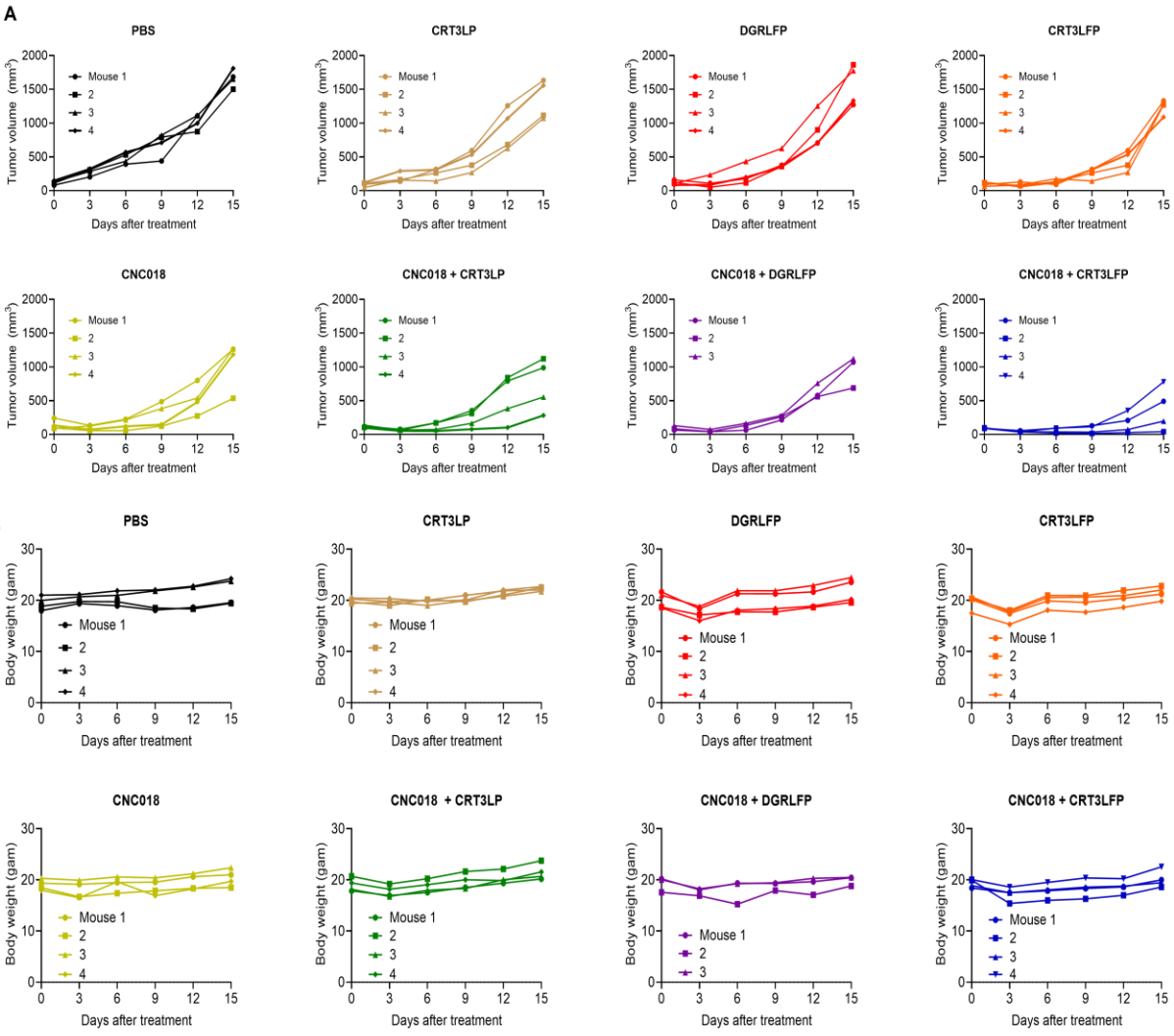


Figure S2. Evaluation of tumor growths and body weight changes in individual CT26 tumor-bearing mice treated with CRT3LFP (related to Figure 4).

(**A**) Tumor growth curves in individual mice treated with PBS or CRT3LFP (4 IU, 8 IU, or 12 IU).

(**B**) Body weight changes in individual mice treated with PBS or CRT3LFP (4 IU, 8 IU, or 12 IU).


A
PBS
CRT3LP
DGRLFP
CRT3LFP
CNC018
CNC018 + CRT3LP
CNC018 + DGRLFP
CNC018 + CRT3LFP
Tumor volume (mm3)
Days after treatment
Mouse 1
2
3
4
B
Body weight (gam)

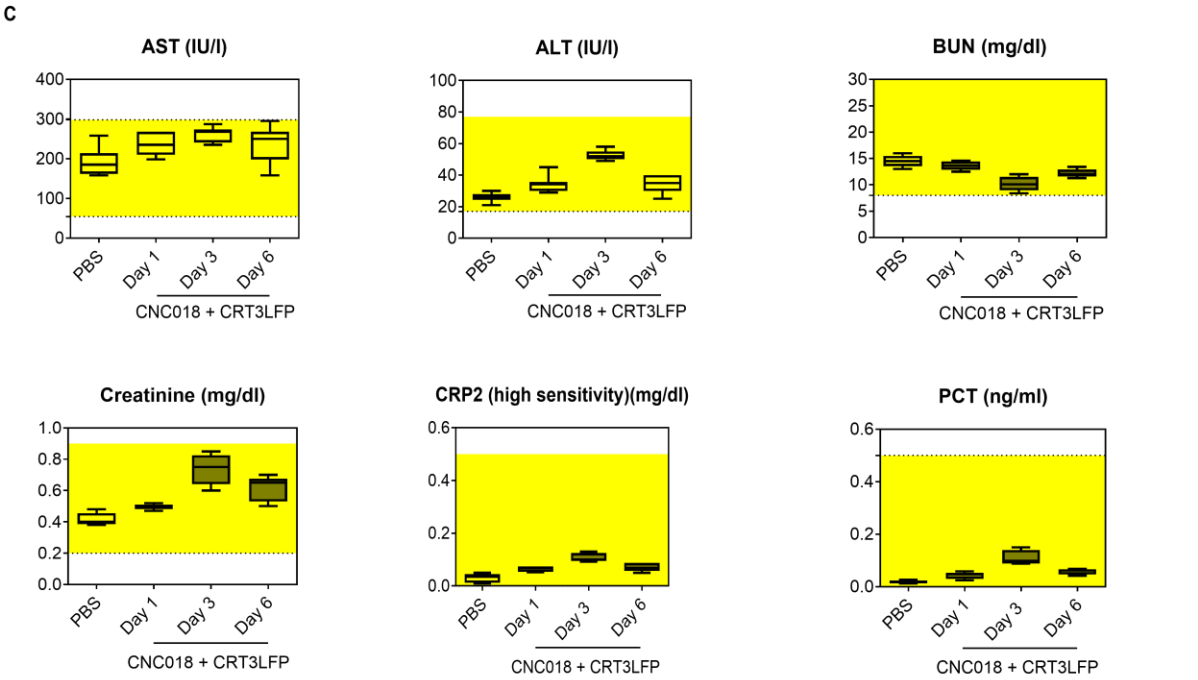

C
AST (IU/l)
ALT (IU/l)
BUN (mg/dl)
Creatinine (mg/dl)
CRP2 (high sensitivity)(mg/dl)
PCT (ng/ml)
PBS
Day 1
Day 3
Day 6
CNC018 + CRT3LFP

Figure S3. Therapeutic effects of CNC018 and CRT3LFP co-treated in individual CT26 tumor-bearing mice (related to Figure 5). (**A**) Individual tumor growth curves in CT26 tumor-bearing mice co-treated with the indicated treatments. CNC018 ($2x10^7$ CFU) was *i.v.* administered on day 0. The proteins (CRT3LP, DGR-LFP, and CRT3LFP; 8 IU/injection) were *i.p.* given five times with a 1-day interval from day 1. PBS was treated as a control. (**B**) Body weight changes in individual mice across treatment groups listed in (A). (**C**) Systemic toxicity profile in CT26 tumor-bearing mice co-treated with CNC018 ($2x10^7$ CFU) and CRT3LFP (4 IU/injection) combined with CNC018. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, plasma C-reactive protein (CRP), and procalcitonin were measured on days 1, 3, and 6 post-treatments. Sera of PBS-treated mice were controlled. Boxes indicate the interquartile range; whiskers denote the 10th and 90th percentiles. Reference ranges: ALT 17–77 IU/L, AST 54–298 IU/L, blood urea nitrogen 8–33 mg/dl, creatinine 0.2–0.9 mg/dl, CRP < 0.5 mg/dl, procalcitonin < 0.5 ng/ml. Yellow-shaded areas indicate the normal range for each parameter.

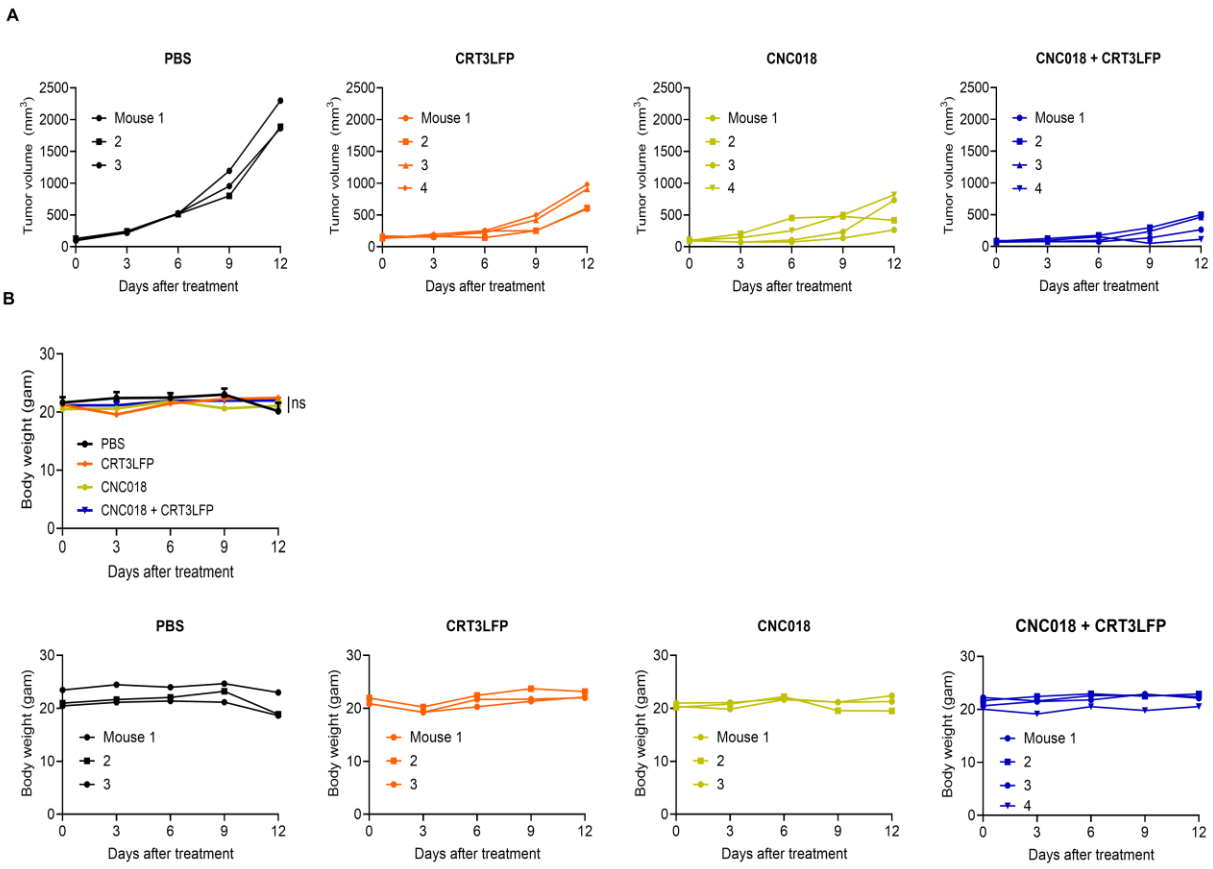


Figure S4. Therapeutic effects of CNC018 and CRT3LFP co-treated in individual MC38 tumor-bearing mice (related to Figure 5E, G). CNC018 ($2x10^7$ CFU) was *i.v.* administered on day 0. CRT3LFP (8 IU/injection) was *i.p.* given five times with a 1-day interval from day 1. (**A**) Individual tumor growth curves in MC38 tumor-bearing mice. (**B**) Individual body weight changes in mice of (A).

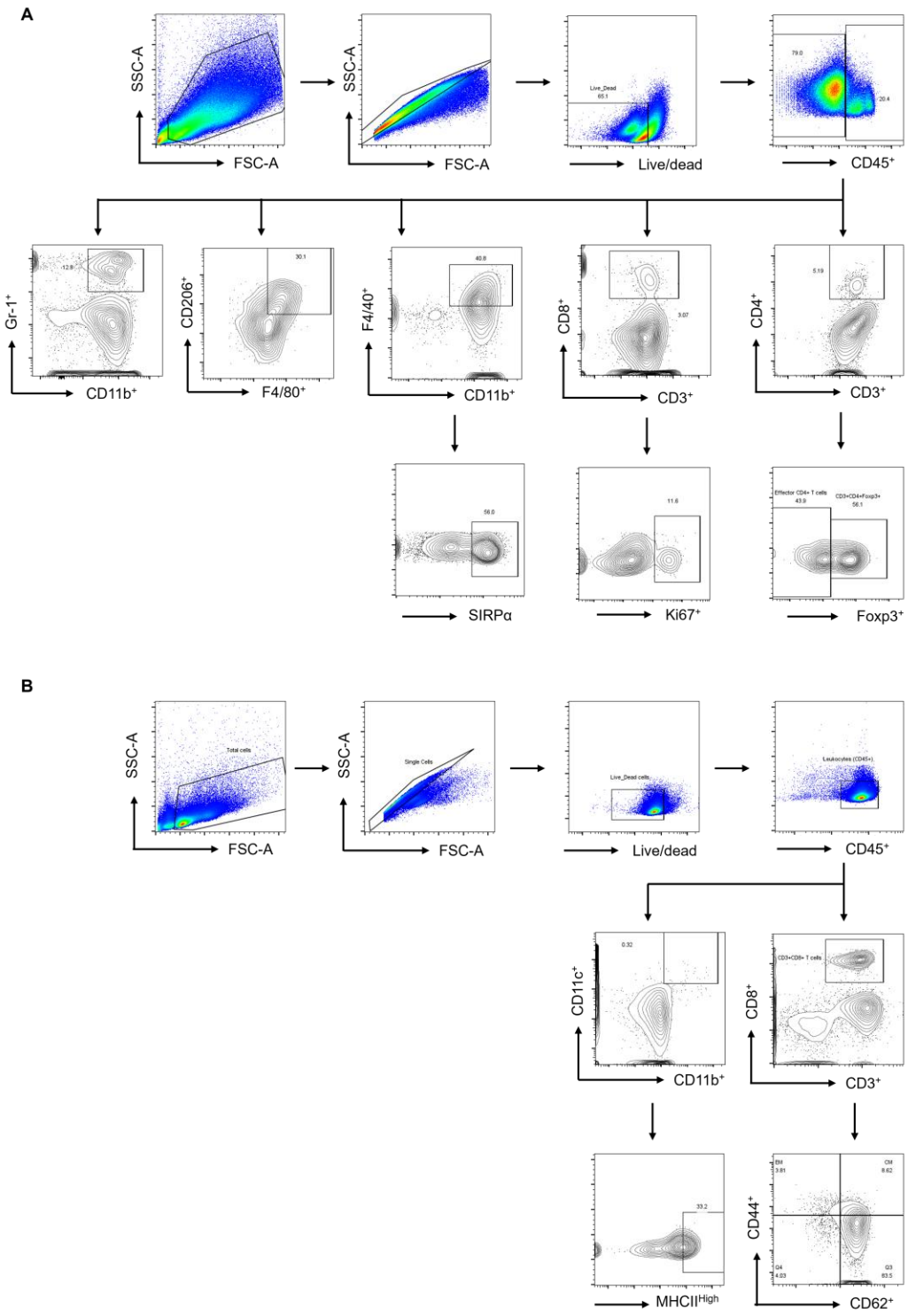


Figure S5. Gating strategies of flow cytometry analysis in CT26 tumor tissues and tumor-draining lymph nodes (TdLNs) (related to Figure 6A-H). After harvesting cells in tumor tissues and TdLNs, they were stained with a Live/Dead cell kit, anti-CD45 (leukocyte marker), anti-CD16/CD32 (to block non-specific Fc receptor binding), and additional immune cell-specific antibodies. (**A**) Gating strategy for tumor tissue analysis. (**B**) Gating strategy for TdLN analysis

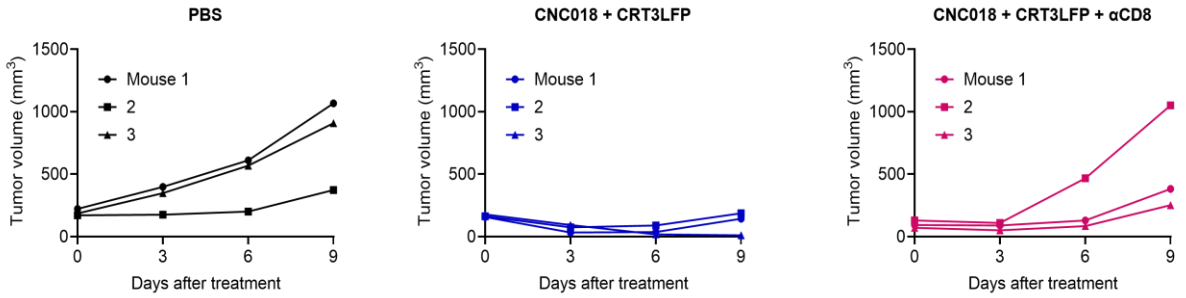


Figure S6. CD8⁺ T cell depletion effect in individual CT26 tumor-bearing BALB/c mice co-treated with CNC018 and CRT3LFP (related to Figure 6J). CT26 *s.c.* tumor-bearing BALB/c mice were *i.v.* treated with CNC018 (2×10^7 CFU) on day 0, and subsequently *i.p.* treated with CRT3LFP (4 IU/injection) five times from day 1 with a 1-day interval. Anti-CD8 (αCD8) antibody (200 μg/injection) was *i.p.* administrated three times with a 3 - 4 days interval. Individual tumor growth curves are shown.

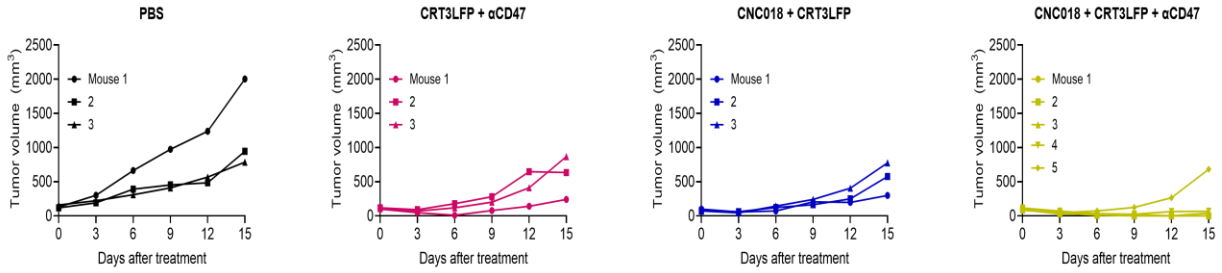


Figure S7. CD47 blockade enhances antitumor therapeutic effect in mice co-treated with CNC018 and CRT3LFP (related to Figure 7E). Mice bearing CT26 tumors (~100-120 mm³) received CNC018 (2×10^7 CFU, day 0, *i.v.* injection) and/or CRT3LFP (4 IU, five-time *i.p.* injections with a 1-day interval from day 1). αCD47 antibody (200 μg/injection) was *i.p.* administered five times with a 2-day interval from day 0. Individual tumor growth curves of CT26 tumor-bearing mice are shown.

Table S1. Antibodies used in this study.

Ab ID	Ab description	Manufacturer/Catalogue number	Application
Calreticulin (D2E6) XP Rabbit mAb	Monoclonal Ab	Cell Signaling/12238	Elisa
Anti-Calreticulin	Monoclonal Ab	Thermal Fisher/MA5-15382	
L-asparaginase	Polyclonal antibody	Abcam/ab55824	
Calreticulin (D3E6) XP® Rabbit mAb (PE Conjugate)	Clone D3E6	Cell Signalling/19780S	Flow cytometry
Rabbit (DA1E) mAb IgG XP® Isotype Control (PE Conjugate)	Clone DA1E	Cell Signalling/5742S	
Anti-His tag	Monoclonal Ab	Abcam/ab245114	
Goat anti-rabbit secondary antibody, Alexa Flour 488		Thermo Scientific/A11008	
Anti-mouse CD16/32	TruStain FcX™ Fc blocking solution	BioLegend/101320	
Live/dead Fixable aqua dead cell stain kit		Invitrogen/L34957	
Pacific Blue anti-mouse CD45.2 antibody	Specific monoclonal Ab	BioLegend/109820	

Anti-mouse CD3-FITC	Clone 17A2	eBioscience/11-0032-82	
Anti-mouse 11b-FITC	Clone M1/70	BioLegend/101205	
Anti-mouse CD8a-PE	Clone 53-6.7	eBioscience/12-0081-82	
PE anti-mouse CD172a (SIRPα) Antibody (PE Conjugate)	Clone P84	Biolegend/144012	
Rat IgG1, κ Isotype Ctrl Antibody (PE Conjugate)	Clone RTK2071	Biolegend/400408	
Anti-mouse CD47 Antibody (PE Conjugate)	Clone miap301	Biolegend/127508	
Rat IgG2a, κ Isotype Ctrl Antibody (PE Conjugate)	Clone RTK2758	Biolegend/ 400508	
Anti-mouse CD4-APC	Clone GK1.5	BioLegend/100412	
Anti-mouse CD49-APC	Clone DX5	BioLegend/108909	
Anti-mouse CD206-APC	Clone B7-H1	BioLegend/C068C2	
Anti-mouse CD11c-APC	Clone N418	Biolegend/117310	
Anti-mouse granzyme B-PerCP-Cy5.5	Clone QA16A02	BioLegend/372212	

Anti-mouse CD62L-PerCP-Cy5.5	Clone MEL-14	BioLegend/104432	
Anti-mouse MHCII-PerCP-Cy5.5	Clone M5/114.15.2	BioLegend/107625	
Anti-mouse Ly-6G-PerCP-Cy5.5	Clone RB6-8C5	BioLegend/127615	
Anti-mouse F4/80-PerCP-Cy5.5	Clone BM8	BioLegend/123128	
Anti-mouse Ki67-PE-Cy7	Clone SolA15	Thermo Fisher Scientific/25469882	
PE/Cy7 anti-mouse CD152	Clone UC10-4B9	BioLegend/106313	
Anti-mouse CD8 PE-Cy7	Clone 53-6.7	eBioscience/25-0081-82	
Anti-mouse CD11c PE-Cy7	Clone N418	Thermo Fisher Scientific/25-0114-82	
Anti-mouse PD-1-APC-Cy7	Clone 29F.1A12	BioLegend/135224	
Anti-mouse CD44-APC-Cy7	Clone IM7	BioLegend/103028	
Anti-mouse CD86-APC-Cy7	Clone GL-1	BioLegend/105029	
Anti-mouse CD8α	Clone 2.43	Bio X Cell/BP0061	$CD8^+$ T depletion
Anti-mouse CD47	Clone MIAP301	Bio X Cell/BE0270	In vivo CD47

			blockade
Recombinant anti-6X His tag antibody		Abcam/ab245114	Western blotting
L-asparaginase	Polyclonal antibody	Abcam/ab55824	
Rabbit anti-FlaB	Polyclonal Ab.	A gift from Dr. Joon Haeng Rhee, Chonnam National University Medical school[52]	
Anti-rabbit secondary antibody, HRP		Invitrogen/31460	
Asparaginase antibody (HRP)		GenTex/GTX40848	
Goat anti-rabbit secondary antibody Alexa Flour 488		Thermo scientific/A110088	Confocal immunofluorescence imaging
Wheat germ agglutinin-Alexa Flour 555 Conjugate		Thermo scientific/W32464	

Table S2. CDI value of CRT3LFP combined with CNC018 bacteria in CT26 and MC38 tumor models

Average tumor volume (mm^3)	Control (PBS)	A (CRT3LFP)	B (CNC018)	AB (CRT3LFP plus CNC18)	CDI	Interpretation
CT26 tumor volume (day 15)	1661.725	1241.525	1063.05	377.45	0.475	Synergism
MC38 tumor volume (day 9)	983.67	357.25	337.75	108.5	0.885	Synergism

CDI, coefficient of drug interaction; CDI = (AB/control)/[(A/control) x (B/control)]