Figure S1

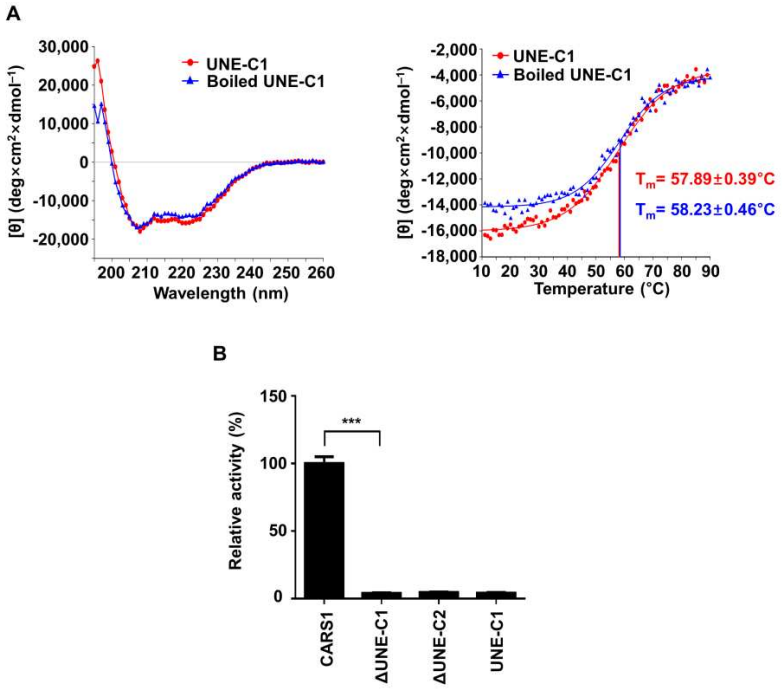
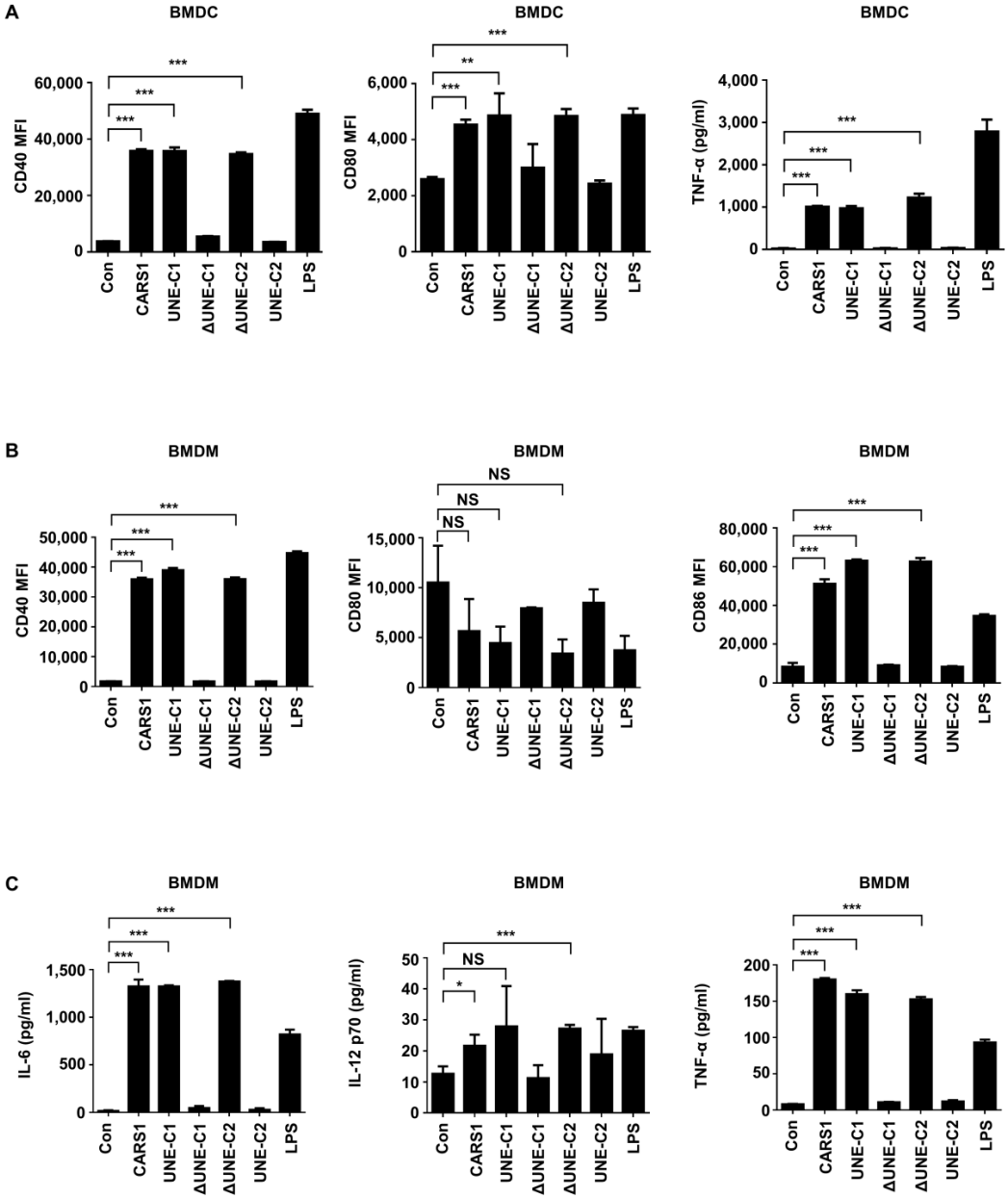


Figure S1 UNE-C1 has an unusual thermostability and involves in protein translation (A) Far-UV Circular dichroism (CD) spectroscopy analysis of UNE-C1 and boiled UNE-C1 at 195-260 nm wavelength. Thermal unfolding CD spectra and melting temperature (T_m) of UNE-C1 and boiled UNE-C1 at 222 nm wavelength. The CD spectra of UNE-C1 and boiled UNE-C1 are shown in red and blue colors, respectively. The CD spectra were measured in triplicate. (B) The enzymatic activity of CARS1 fragments was determined. 100 nM of each protein was incubated with reaction mixtures for 10 minutes and aminoacylated cysteine was quantified. The enzymatic activities are expressed as the relative percentage compared with full-length CARS1. Results are presented as mean ± SD and statistical significance was analyzed with student's t-test (*** $p < 0.001$).

Figure S2


A
BMDC
CD40 MFI
60,000
40,000
20,000
0
***
***
***
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
BMDC
CD80 MFI
6,000
4,000
2,000
0
***
**
***
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
BMDC
TNF-α (pg/ml)
4,000
3,000
2,000
1,000
0
***
***
***
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
B
BMDM
CD40 MFI
50,000
40,000
30,000
20,000
10,000
0
***
***
***
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
BMDM
CD80 MFI
15,000
10,000
5,000
0
NS
NS
NS
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
BMDM
CD86 MFI
80,000
60,000
40,000
20,000
0
***
***
***
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
C
BMDM
IL-6 (pg/ml)
1,500
1,000
500
0
***
***
***
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
BMDM
IL-12 p70 (pg/ml)
50
40
30
20
10
0
***
NS
*
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS
BMDM
TNF-α (pg/ml)
200
150
100
50
0
***
***
***
Con
CARS1
UNE-C1
ΔUNE-C1
ΔUNE-C2
UNE-C2
LPS

13

Figure S2 UNE-C1 induces functional activation of APCs (A) *in vitro* generated BMDCs were treated with UNE-C1 for 24 h. Co-stimulatory molecules were analyzed from the gated $CD11c^+$ population. The expression levels of CD40 and CD80 were evaluated by flow cytometry and the secretion level of TNF-α in the supernatants were quantified by ELISA. (B, C) *In vitro* generated BMDMs were treated with different fragments of CARS1 for 24 h. Co-stimulatory molecules were analyzed from the gated $CD11b^+$ $F4/80^+$ population. The expression levels of CD40, CD80, and CD86 (B) were evaluated by flow cytometry and the secretion levels of IL-6, IL-12p70, and TNF-α (C) were measured by ELISA. Results are presented as mean ± SD and statistical significance was analyzed with student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure S3

A

Gene Symbol	Fold Change
nos2	954.8
cxcl9	72.3
cxcl11	48.7
Il-6	38.1
irf7	36.4
cxcl10	35.2
ccl5	21.4
cd40	7.7
Il-12a	7.1
Il-12b	6.7
cd86	6.3
cd80	2.1

B

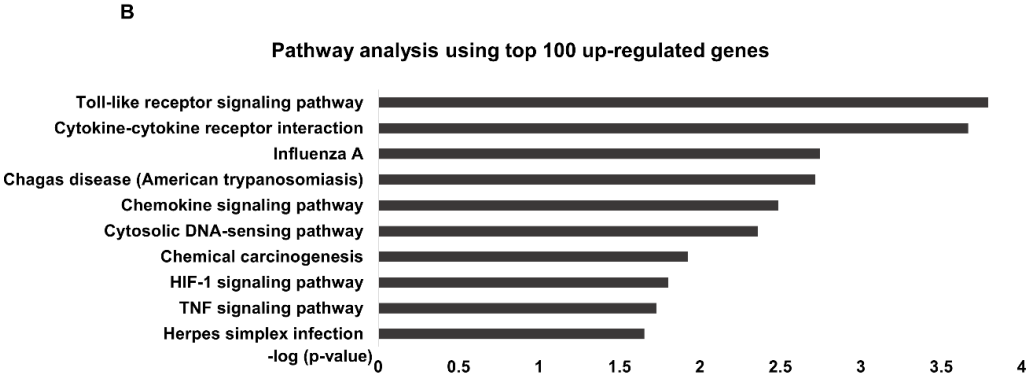


Figure S3 UNE-C1 stimulates the functional activation of APCs via TLR2/TLR6

(A, B) RNA-sequencing was conducted to assess UNE-C1 mediated immune responses. Demonstration of genes related to pro-inflammation or involved in TLR signaling pathways (A). Analysis of KEGG pathways generated from top 100 genes up-regulated by UNE-C1 treatment (B).

Figure S4

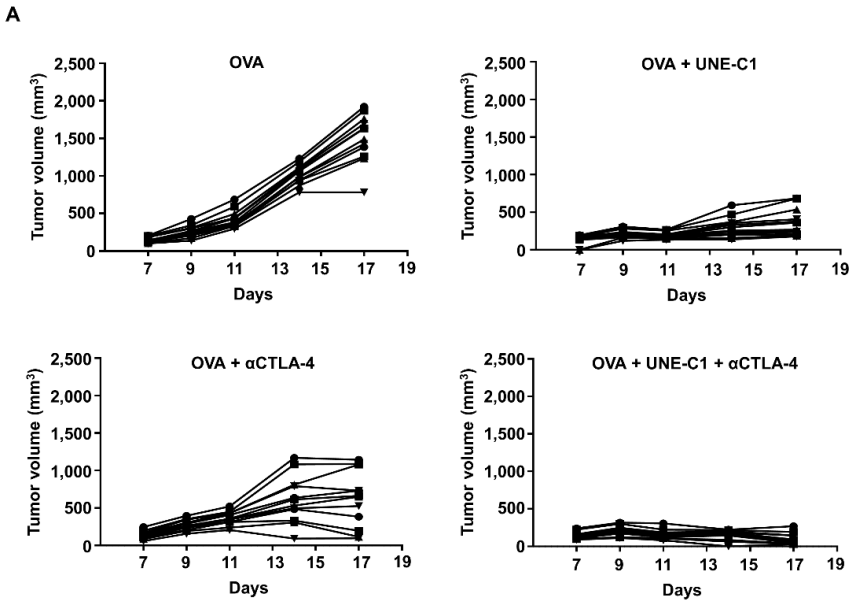


Figure S4 Combination therapy of anti-CTLA-4 antibody and UNE-C1 immunization induces further tumor regression (A) C57BL/6 mice were implanted with 1 × 10^6 of E.G7-OVA cells. OVA + UNE-C1 were injected s.c. on days 3 and 10. Anti-CTLA-4 antibody was injected i.p. on day 3, 6, 9, 12, and 15 to check the synergy effect. Individual tumor growth curves of OVA, OVA + UNE-C1, OVA + αCTLA-4, and OVA + UNE-C1 + αCTLA-4 groups are shown.

Supplementary Methods

Cell culture

HCT116, THP-1, Daudi, and Jurkat cells were cultivated in RPMI media containing 10% fetal bovine serum (FBS) and 50 µg/ml of streptomycin and penicillin. RAW264.7 cells were grown in DMEM media with the same supplements as described above. THP-1 cells were differentiated using 50 ng/ml of PMA (Sigma-Aldrich). Primary cells (BMDCs and BMDMs) were obtained from female C57BL/6 mice and cultured in RPMI complete media. GM-CSF (R&D Systems) or M-CSF (R&D Systems) were used for obtaining BMDCs and BMDMs.

Protein purification

Plasmids containing different sizes of CARS1 were constructed in the pET-28a vector containing N, and C-terminal 6X his tag. All constructs were transformed into BL21-codon plus-RIPL cells, and colonies were further used for inoculation. A large scale of cells was grown in LB until OD 600 reaches 0.5, and protein expression was induced using 0.5 mM of IPTG (EMD Millipore) for 16 h at 4 °C. Cell pellets were obtained from centrifugation and disrupted by sonication with Tris buffer containing 300 mM NaCl. After obtaining supernatant from centrifugation at 20,000 *g* for 30 min, it was poured over a column containing Ni-NTA resin (Thermo Fisher Scientific). The washing step was performed with 50 mM Tris, pH7.5, containing 300 mM NaCl, 5 % glycerol, and 15 mM imidazole. After eluting proteins with 10 ml of elution buffer (50 mM Tris pH 7.5, 300 mM NaCl, Glycerol 5%, 300 mM imidazole), endotoxin was

Cho S, *et al. J Immunother Cancer* 2020; 8:e000277. doi: 10.1136/jitc-2019-000277

removed using Triton X-114 (Sigma-Aldrich). Qualified proteins below 0.04 EU/mg from LAL assay (Thermo Fisher Scientific) were used for all experiments.

ELISA

RAW264.7, PMA-differentiated THP-1, BMDC, and BMDC cells were seeded at 5 × 10^5 cells/ml in 24 well plates for O/N, and the medium was changed into serum-free media for 2 h before the treatment. In the case of RAW264.7 and PMA-differentiated THP-1, 100 nM of proteins were treated for 4 h. PMA-differentiated THP-1 cells were pre-incubated with anti-TLR2 (Invivogen) or anti-TLR4 (Invivogen) for an hour before treating CARS1 or UNE-C1. To rule out LPS contamination, CARS1 or UNE-C1 was pre-treated with proteinase K (Thermo Fisher Scientific) or boiled for an hour. Before treating proteins, some cells were pre-incubated with polymyxin B (Sigma-Aldrich) for an hour. For primary cells, the same molar concentration of proteins was incubated for 24 h. Supernatants were obtained from centrifugation at 500 g for 10 min and subjected for ELISA using mouse IL-6, TNF-α, and IL-12p70 ELISA set (BD).

Circular dichroism (CD) spectroscopy

CD spectroscopy was used to investigate thermal stability of UNE-C1. To compare structural conformation of UNE-C1 and boiled UNE-C1, the samples in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT were subjected to far-UV CD measurements at 20 °C using a 1-mm path length quartz cuvette in a Jasco J-815 CD spectrometer (Jasco, Tokyo, Japan). CD spectra were acquired over the wavelength range of 195–260 nm with 1.0 nm bandwidth and converted into mean

residue ellipticity (degree cm^2 $dmol^{-1}$). Blank spectra obtained using buffer without protein were subtracted. The thermal unfolding spectra of the UNE-C1 and boiled UNE-C1 were monitored at 222 nm wavelength using the same settings as described above. The samples were heated from 10 ºC to 90 ºC at a rate of 1ºC min^{-1}. The melting temperatures (T_m) were determined from a nonlinear regression model (Sigmoidal, Sigmoid, 4 Parmeter; SigmaPlot 10.0.0.54). The CD spectra were measured in triplicate.

Aminoacylation activity

Full-length CARS1 and its fragments, including ΔUNE-C1, ΔUNE-C2, and UNE-C1 were tested for aminoacylation. The assay was carried out in a buffer containing 50 mM HEPES (pH 7.6), 20 mM KCl, 10 mM $MgCl_2$, 4 mM DTT, 5 mM ATP, 5 mg/ml yeast extractant total tRNA, 60 Ci/mmol [^{35}S] Labeled L-Cysteine (Perkinelmer), and 100 nM of different CARS1 fragments. The reaction was initiated by adding each enzyme into the reaction tube at 37 °C and the reaction samples were taken after 10 minutes. Then, the reaction was quenched by 5 % trichloroacetic acid (TCA) on Whatman filter pads. After the pads were washed with pre-cooled 5 % TCA and 95 % ethanol, aminoacylated cysteine was quantified using a scintillation counter (Perkin Elmer).

RNA isolation and sequencing

BMDCs were treated with UNE-C1 for 24 h before RNA isolation. Total RNA was isolated using Trizol reagent (Invitrogen) and quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, The

Netherlands). Quantification of RNA was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA). For control and UNE-C1 RNAs, the construction of library was performed using QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer's instructions. In brief, each 500ng total RNA were prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5' end was hybridized to the RNA and reverse transcription was performed. After degradation of the RNA template, second strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5' end. The double-stranded library was purified by using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The finished library is purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc., USA).