Supplementary Materials for

Potential of *Mycobacterium tuberculosis* chorismate mutase (Rv1885c) as a novel TLR4-mediated adjuvant for dendritic cell-based cancer immunotherapy

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Materials and Methods

Expression and purification of recombinant TBCM protein

The recombinant TBCM protein was purified from *Escherichia coli* as previously described (**32**). Briefly, *E. coli* BL21 strains (RBC Bioscience, Taipei City, Taiwan) were transformed with pET28a-TBCM for expression and purification. Each protein was purified with Ni-NTA His binding resin (Merck, Darmstadt, Germany) and eluted with elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, and 250 mM imidazole) containing 4 M urea. Purified proteins were dialyzed serially against elution buffer to remove imidazole, urea, and residual salts.

Cell culture

For preparation of murine bone marrow-derived dendritic cells (BMDCs), whole bone marrow cells isolated from the tibia and femurs of C57BL/6 mice were lysed with red blood cell lysis buffer and washed with RPMI 1640 medium (Life Technologies, CA, USA). Then, the cells were cultured in IMDM supplemented with 100 units/ml penicillin/streptomycin, 10% fetal bovine serum (FBS), 20 ng/ml GM-CSF and 10 ng/ml IL-4. On Day 6, over 80% of the nonadherent cells expressed CD11c. On Day 6 or 7 of culturing, nonadherent cells and loosely adherent proliferating DC aggregates were harvested for analysis. Human DCs were differentiated after the isolation of $CD14^+$ monocytes from peripheral blood mononuclear cells (PBMCs) isolated from healthy donors according to the approved protocol from the Institutional Review Board (IRB) of Seoul National University (IRB-2109-114-1255). Cells were isolated and cultured in RPMI 1640 supplemented with 100 units/ml penicillin/streptomycin, 10% FBS, 50 ng/ml human GM-CSF and 20 ng/ml human IL-4. Culture medium was changed every two days and the cells were differentiated for five days.

Human monocytic THP-1 cells were cultured in RPMI 1640 (Life Technologies, CA, USA) supplemented with 100 units/ml penicillin/streptomycin and 10% FBS and differentiated by treatment with PMA (200 μg/ml) for 24 h (**33**). Lewis lung carcinoma (LLC) cells and B16F10 murine

melanoma cells were cultured in RPMI medium supplemented with 100 units/ml penicillin/streptomycin and 10% FBS. Splenocytes isolated from tumor-bearing mice were incubated in RPMI medium (Life Technologies, CA, USA) supplemented with 100 units/ml penicillin/streptomycin, 10% FBS and IL-2 for 6 days. EO771 murine breast cancer cells and A549 human lung cancer cells were cultured in DMEM (Life Technologies, CA, USA) supplemented with 100 units/ml penicillin/streptomycin and 10% FBS.

Flow cytometry

To assess BMDC maturation, BMDCs were stained with monoclonal antibodies against CD11c, CD40, CD80, CD86, MHCII, MHCI, or CCR7 (CD197) for 30 min on ice. To assess $CD8^+$ T cell and memory T cell generation *in vivo*, mouse tumor tissues and lymph nodes were dissociated with collagenase IV (0.5 mg/ml, Sigma, USA) and DNase I (20 μg/ml, Sigma, USA). Single tumor cells, lymph nodes and splenocytes were mashed through a 70 μm-pore size cell strainer and stained with mAbs against CD3, CD4, CD8, CD44, CD62L or CD11c for 30 min on ice. For intracellular cytokine staining, cells were cultured for 4 h with PMA (50 ng/ml) and ionomycin (1 μg/ml), and cytokine release was prevented by treatment with brefeldin A. Following fixation/permeabilization, cells were stained with mAbs against IFN-γ, IL-2, IL-4, IL-17A, T-bet, GATA-3, RORγt or IL-12. All antibodies were purchased from BD Biosciences (USA). Fluorescence was measured using FACS Fortessa (BD Biosciences, USA) and FlowJo software (BD Biosciences, USA).

Cell apoptosis assay

Lymphocytes from mouse tumor and lung tissue were isolated by Ficoll-Paque density gradient centrifugation (**34**), and tumor-infiltrating lymphocytes (TILs), lung lymphocytes and lymph node cells were cocultured with CFSE-labeled LLC cancer cells (E:T=5:1) for 24 h at 37℃ in 5% CO_2. Then, the cells were stained with 5 μl 7AAD and 2.5 μl Annexin V-PE for 10 min on ice according to

the manufacturer's guidelines for apoptosis detection kits (BD Biosciences, USA). Flow cytometry was performed by a FACS Fortessa (BD Biosciences, USA). The data were analyzed by FlowJo software (BD Biosciences, USA).

For *in vitro* antigen-specific T cell cytotoxicity assay, human DCs were incubated with A549 lysates (tumor-associated antigens; TAAs) at a ratio of 3:1 tumor cell equivalents in the presence or absence of TBCM (1 μg/ml) or lipopolysaccharide (0.1 μg/ml) for 24 h. Human $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs of healthy donors and $CD8^+$ T cells were cocultured with TAAs-, TBCM- or LPS-treated human DCs in the presence or absence of $CD4^+$ T cells (T cell:DC=10:1) for five days. Thereafter, cells were cocultured with CFSE-labelled A549 cancer cells (E:T=5:1) for 48 h at 37℃ in 5% CO_2. The cells were stained with 7AAD and Annexin V according to the manufacturer's guidelines in Apoptosis detection kit (BD Biosciences, USA) and analyzed by FACs LSRII (BD Biosciences, USA) and FlowJo software (BD Biosciences, USA).

Antigen uptake assay

BMDCs were equilibrated at 37℃ or 4℃ for 30 min and incubated with 50 μg/ml fluorescein isothiocyanate (FITC)-conjugated dextran (60842-46-8, Sigma, USA). Cold staining buffer was added to stop the reaction, and the cells were washed three times, stained with mAb against CD11c, and analyzed using a FACS Fortessa (BD Biosciences, USA). The data were analyzed by FlowJo software (BD Biosciences, USA). Nonspecific binding or uptake of FITC-dextran to BMDCs was detected by incubating BMDCs with FITC-dextran at 4℃, and the resulting background value was subtracted from the specific binding values.

Blocking antibody assay

BMDCs were stimulated with TBCM (1 μg/ml), TLR2 agonist Pam_3CSK_4 (0.1 μg/ml; Sigma, USA) or TLR4 agonist lipopolysaccharide (0.1 μg/ml; *Escherichia coli* serotype 0111:B4, Sigma, USA) for 24 h. For the blocking antibody assay, BMDCs were preincubated with 1 μg/ml anti-TLR2 or anti-

TLR4 for 1 h, followed by stimulation with TBCM or TLR agonists.

In vitro and *in vivo* migration test

Cell migration was detected using 8 μm-pore size Transwell coated with Matrigel (Corning, USA). BMDCs were resuspended in media containing TBCM or LPS, and the cell density was adjusted to 1×10^5 cells/200 μl media in the upper well of the Transwell chamber. The lower compartment was filled with 500 μl media in the presence or absence of 500 ng/ml CCL19 chemoattractant. After 48 h of incubation, the cells in the upper well were wiped using a wet cotton swab, and traversed cells on the lower side of the filter were fixed and stained with crystal violet.

For the *in vivo* migration test, TBCM- or LPS-treated BMDCs were labeled with 1 μM CFSE, and CFSE-DCs (1×10^6 cells/50 μl PBS) were injected into the hind-leg footpad of C57BL/6 mice. 72 h after the injection, inguinal lymph nodes were removed and disaggregated using collagenase I (0.5 mg/ml, Sigma, USA) and DNase I (20 μg/ml, Sigma, USA). CFSE-positive DCs were measured using FACS Fortessa (BD Biosciences, USA) and FlowJo software (BD Biosciences, USA). In the same mouse experiment, one week after DC injection, $CD4^+$ and $CD8^+$ T cells sorted from inguinal lymph nodes were analyzed by RT–qPCR.

Immunofluorescence staining

BMDCs were plated on poly-L-lysine-coated glass coverslips. After treatment with TBCM or TLR agonists, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, and then blocked with 2% bovine albumin serum (BSA) in PBS for 1 h. Thereafter, cells were incubated with mouse anti-NF-kB/p65 polyclonal antibody at 4℃ overnight. After washing with PBS, cells were incubated with FITC-conjugated secondary antibody in a dark room for 1 h and stained with DAPI for 5 min at room temperature. Fluorescence intensity was observed using an FV3000 confocal laser scanning microscope.

For the histological analysis, the fixed tumor tissues were embedded in paraffin and sectioned. After deparaffination and rehydration, each section was permeabilized in 0.5% Triton X-100 in TBST and

blocked in 5% BSA in TBST. Then, the section slides were incubated with primary antibodies against CD8, CXCR3, Perforin-1 and Granzyme (all antibodies purchased from Santa Cruz) at 4℃ overnight, followed by incubation with fluorescent dye-conjugated secondary antibodies in a dark room for 2 h. After staining with DAPI, fluorescence intensity was observed using an FV3000 confocal laser scanning microscope.

Western blot assay

BMDCs and tumor tissues were lysed in radioimmunoprecipitation assay (RIPA, Thermo Fisher Scientific, USA) buffer containing phosphatase inhibitor and protease inhibitor cocktail and homogenized. The protein samples were quantified by Bradford assay, boiled for 5 min at 95℃, separated on a 10% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane. Thereafter, the membranes were blocked in 5% BSA in TBST for 1 h at room temperature and incubated with primary antibodies (all purchased from Cell Signaling Tech, USA) at 4℃ overnight, followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. The protein blots were detected using enhanced chemiluminescence (ECL) reagents (Bio–Rad, USA).

T cell proliferation and polarization assay

$CD4^+$ and $CD8^+$ T cells were isolated from the spleen of C57BL/6 mice by FACSAria III (BD Biosciences, USA), and T cells were stained with 1 μM CFSE. CFSE-labeled $CD4^+$ T cells were cocultured with BMDCs (T cell:DC=10:1) that had been incubated with tumor-associated antigens in the presence or absence of TBCM or LPS. After 24 h incubation, culture supernatants were harvested, and cytokine levels were measured by ELISA (Invitrogen, USA) according to the manufacturer's procedure. For the T cell polarization assay, 96 h after coculture incubation, CFSE-labeled T cells were assessed by flow cytometry. For the T cell polarization assay, 72 h after coculture incubation, intracellular cytokine production and transcription factor expression in T cells were analyzed by intracellular cytokine staining. In the same manner, human DCs were incubated with A549 lysates (tumor-associated antigens; TAAs) at a ratio of 3:1 tumor cell equivalents in the presence or absence

of TBCM (1 μg/ml) or lipopolysaccharide (0.1 μg/ml) for 24 h. Human $CD4^+$ T cells were isolated from PBMCs of healthy donors and cocultured with TAAs-, TBCM- or LPS-treated human DCs (T cell:DC=10:1) for 72 h. Thereafter, intracellular cytokine production and transcription factors expression in T cells were analyzed by intracellular cytokine staining.

RNA extraction and RT–qPCR

Total mRNA was extracted from DCs and tumor tissues using TRIzol reagent (Invitrogen, USA), quantified, and transcribed into complementary DNA by reverse transcriptase. The transcription level of target genes was detected using a CFX Connect real-time system (Bio–Rad, USA) and analyzed by RT–qPCR with sets of primers (**Supplementary Table S1**). The housekeeping gene GAPDH was used as an internal control. The fold change was calculated using the delta-delta CT method (**35**) and normalized by the average delta CT value of the PBS group or all groups.

LPS decontamination test

To confirm that BMDC maturation induced by TBCM was not due to contaminating endotoxins such as LPS, pretreatment with polymycin B (Sigma, USA), heat denaturation, and digestion with proteinase K (Sigma, USA) were conducted. BMDCs were preincubated with 10 μg/ml polymycin for 20 min at room temperature prior to treatment with TBCM or LPS. For heat denaturation, TBCM and LPS were boiled at 100℃ for 30 min. For digestion with proteinase K, TBCM and LPS were incubated with 10 μg/ml proteinase K at 37℃ for 1 h, followed by enzyme denaturation at 100℃ for 15 min, and then added to BMDC cultures. After 24 h incubation, cytokine levels in BMDC culture supernatants were detected by ELISA (Invitrogen, USA) according to the manufacturer's procedure.

Statistical analysis

Data are shown as the mean$\pm$SD and were analyzed using GraphPad Prism 9 statistical software (GraphPad, CA, USA). Significant differences among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A p value of < 0.05

was considered to denote statistical significance. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Supplementary Table S1. Primer sets used for RT–qPCR

Target gene	foward (5'→3')	reverse (5'→3')
CXCL9	GCCATGAAGTCCGCTGTTCT	GGGTTCCTCGAACTCCACACT
CXCL10	GACGGTCCGCTGCAACTG	GCTTCCCTATGGCCCTCATT
CXCR3	GAGGTTAGTGAACGTCAAGTG	GGGGTCCCTGCGGTAGATCTG
IFNg	TGGCATAGATGTGGAAGAAAAGAG	TGCAGGATTTTCATGTCACCAT
Gzmb	ATGCTGCTAAAGCTGAAGAGT	TTCCCCAACCAGCCACATAG
Prf	TTGGTGGGACTTCAGCTTTCC	CCATACACCTGGCACGAACT
FasL	CTAGAGGGCCGGACCAAAGGAGACC	AGGTGGAAGAGCTGATACATTCCTAATCCC
Trail	ATGGTGATTTGCATAGTGCTCC	GCAAGCAGGGTCTGTTCAAGA
IL-12	GGAAGCACGGCAGCAGAAT	GGCGGGTCTGGTTTGATG
E-Cadherin	AATGGCGGCAATGCAATCCCAAGA	TGCCACAGACCGATTGTGGAGATA
N-Cadherin	TGGAGAACCCCATTGACATT	TGATCCCTCAGGAACTGTCC
TSP-1	GCAGCACACACAGAAGCATT	CAATCAGCTCTCACCAGCAG
Fibronectin1	TAGCAGGCTACCGACTGACCG	CACCCAGCTTGAAGCCAATCC
Snail1	CCACTGCAACCGTGCTTTT	CACATCCGAGTGGGTTTGG
Vimentin	TTCTCTGGCACGTCTTGACC	CTCCTGGAGGTTCTTGGCAG
ZEB1	ACAAGACACCGCCGTCATTT	GCAGGTGAGCAACTGGGAAA
p38 MAPK	GACGAATGGAAGAGCCTGAC	AGATACATGGACAAACGGACA
Slug	CATTGCCTTGTGTCTGCA	AGAAAGGCTTTTCCCCAGTG
ERK1	GCTCGACCACACTGGCTTTC	GATCAACTCCTTCAGCCGCTC
Cyclin D1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
Cyclin D2	GAGTGGGAACTGGTAGTGTTG	CGCACAGAGCGATGAAGGT
Bcl2	TGAGTACCTGAACCGGCATCT	GCATCCCAGCCTCCGTTAT
BAX	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG
BAD	GCCCTAGGCTTGAGGAAGTC	CAAACTCTGGGATCTGGAACA
p53	CACGTACTCTCCTCCCCTCAAT	AACTGCACAGGGCACGTCTT
Rac1	GAGACGGAGCTGTTGGTAAAA	ATAGGCCCAGATTCACTGGTT
HIF-1α	GAAATGGCCCAGTGAGAAAA	CTTCCACGTTGCTGACTTGA
IL-8	ATGGCTGCTCAAGGCTGGTC	AGGCTTTTCATGCTCAACACTAT

MMP-2	GAGTTGGCAGTGCAATACCT	GCCGTCCTTCTCAAAGTTGT
MMP-9	AGTTTGGTGTCGCGGAGCAC	TACATGAGCGCTTCCGGCAC
CD28	TTTACCTATCAGCCCCAGTTTC	AGTTCCATTGCTCCTCTCGTT
CD40L	CGGCAAATACCCACAGTTCCT	AGCACCAGCTTGTAATTCAAACA
CD44	GAATGTAACCTCCGCTACG	GGAGGTGTTGGACGTGAC
CD69	GGGCTGTGTTAATAGTGGTCCTC	CTTGCAGGTAGCAACATGGTGG
CXCL11	CATTTTGACGGCTTTCATC	AAGGTCACAGCCATAGCCCT
Smad2	CCCACTCCATTCCAGAAAAC	GAGCCTGTGTCCATACTTTG
Flt3L	GAGGACGTCAACACCGAGAT	AGGTGGGAGATGTTGGTCTG
VEGF	TGCTCACTTCCAGAAACACG	GGAAGGGTAAGCCACTCACA

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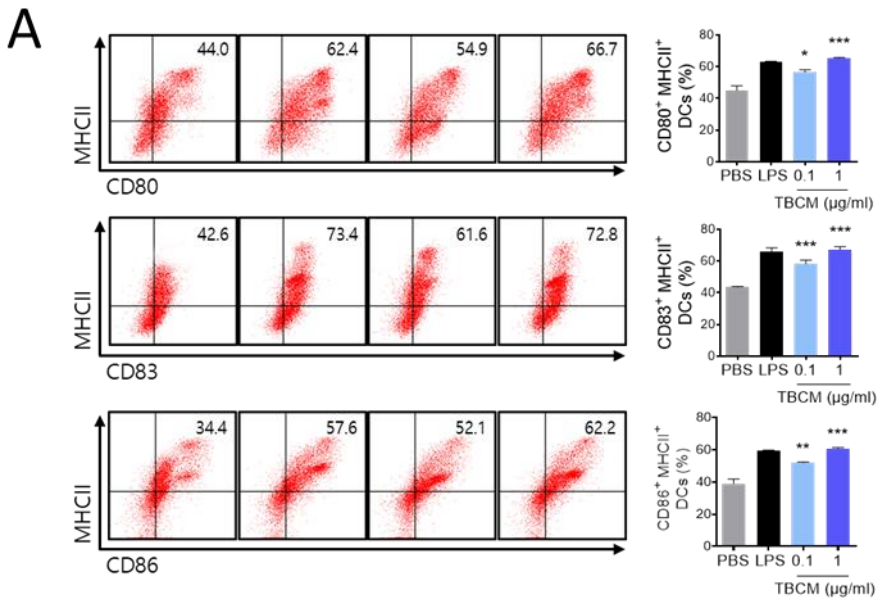

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185 **Supplementary Figure S1. TBCM induces elevated co-expression of MHCII and CD80, CD83 or**
186 **CD86 in DCs.** BMDCs were incubated with TBCM (0.1 or 1 μg/ml) or LPS (0.1 μg/ml) for 24 h, and
187 then assessed for co-expression of surface maturation marker MHCII and CD80, CD83 or CD86 by
188 flow cytometry. (**A**) Population of $MHCII^+$ $CD80^+$, $MHCII^+$ $CD83^+$, and $MHCII^+$ $CD86^+$ DCs.
189 Significant differences ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$) among the different groups are
190 shown in the related figures, and the data are presented as the means $\pm$ s.e.m. of four independent
191 experiments.

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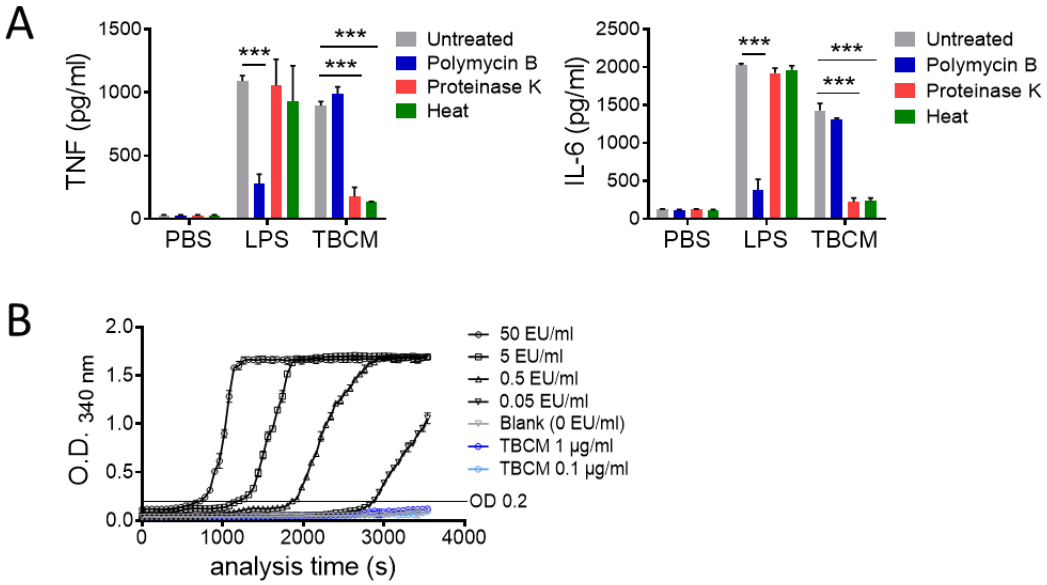
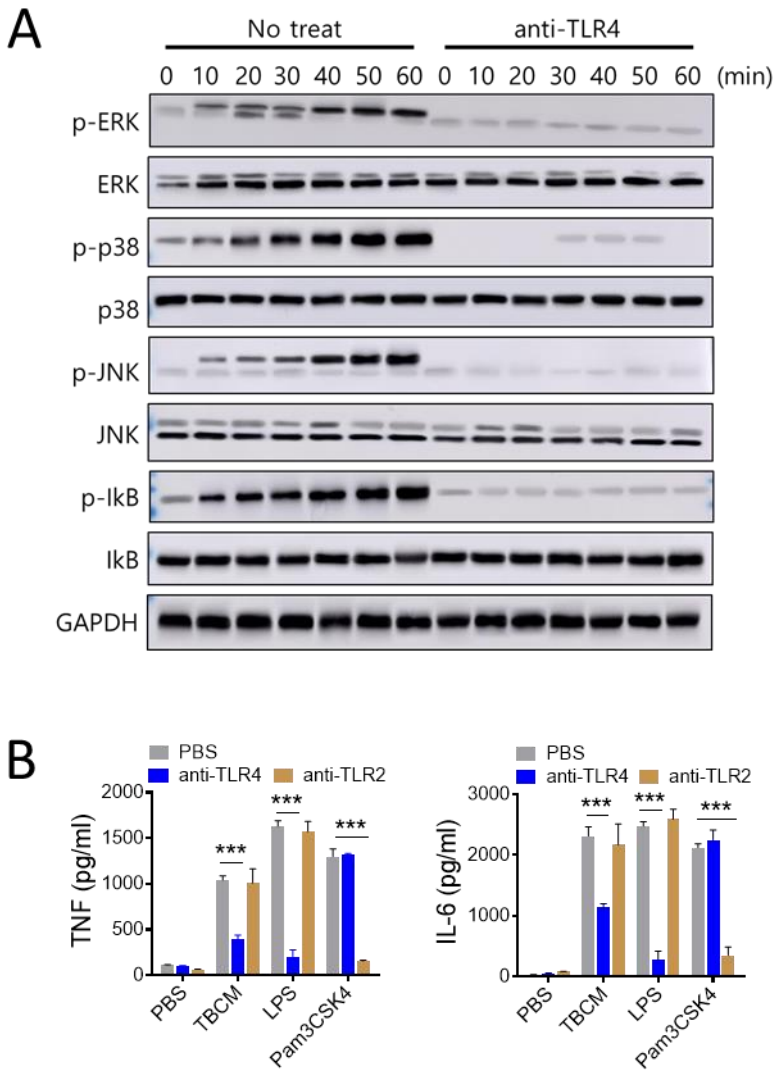
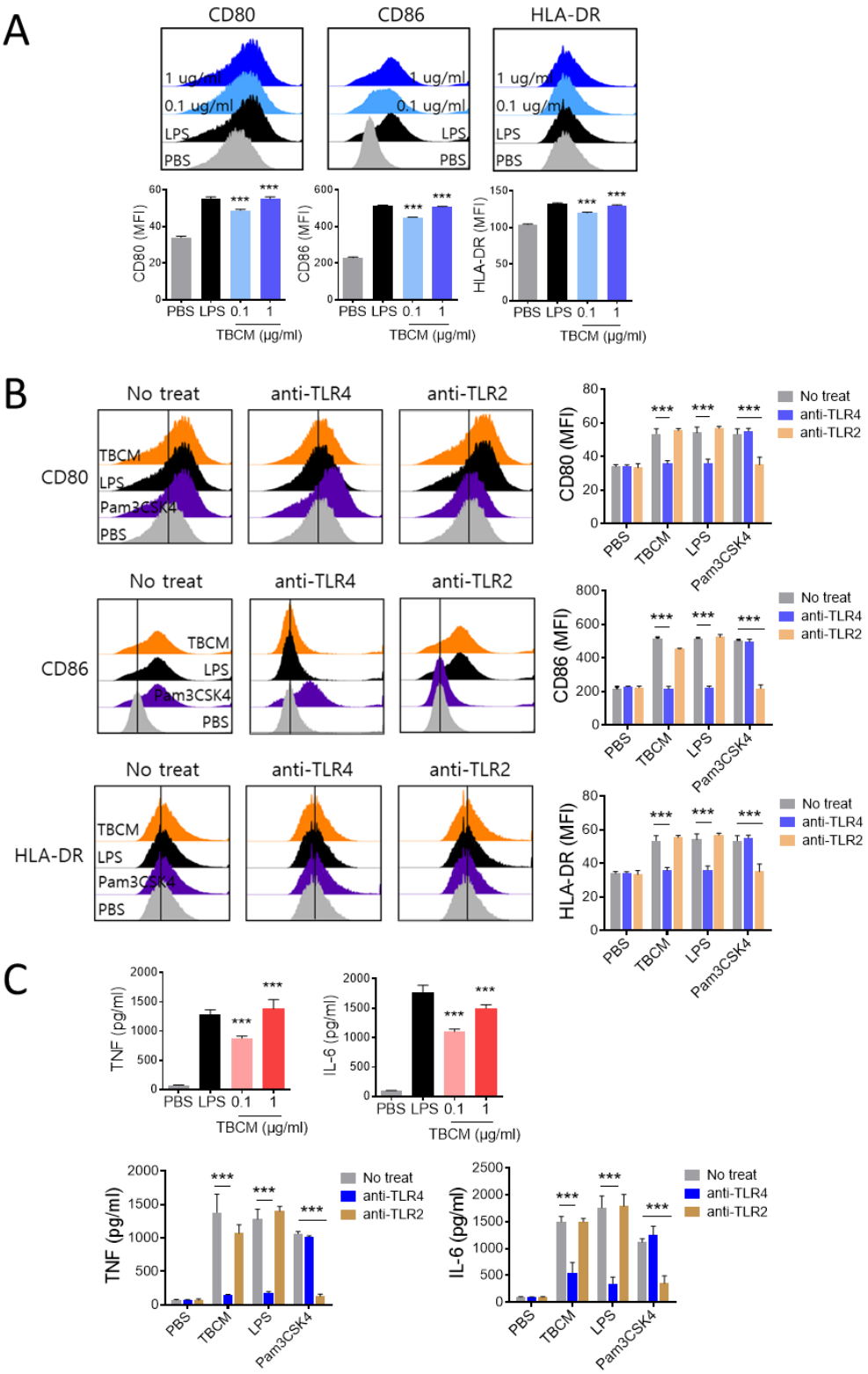


Supplementary Figure S2. DC activation after treatment with polymycin B, proteinase K and heat denaturation. (**A**) For the endotoxin contamination test, TBCM and LPS were preincubated with polymycin B (10 μg/ml), proteinase K (10 μg/ml) and heat denaturation (boiling at 100℃) and then added to BMDC cultures. 24 h after incubation, cytokine levels in culture supernatants were assessed by ELISA. (**B**) LPS contamination of purified TBCM was assessed by LAL assay (Lonza). Significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) among the different groups are shown in the related figures, and the data are presented as the means ± s.e.m. of three independent experiments.



Supplementary Figure S3. TBCM induces DC activation through interaction with TLR4. BMDCs were incubated with anti-TLR2 IgG or anti-TLR4 IgG prior to incubation with TBCM (1 μg/ml), LPS (0.1 μg/ml) or Pam_3CSK_4 (0.1 μg/ml). (**A**) DCs were harvested at the indicated time points, the DC lysates were then subjected to SDS-polyacrylamide gel electrophoresis, and an immunoblot analysis was conducted. (**B**) Cytokines in culture supernatant were measured by ELISA. Significant differences ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$) among the different groups are shown in the related figures, and the data are presented as the means ± s.e.m. of three independent experiments.



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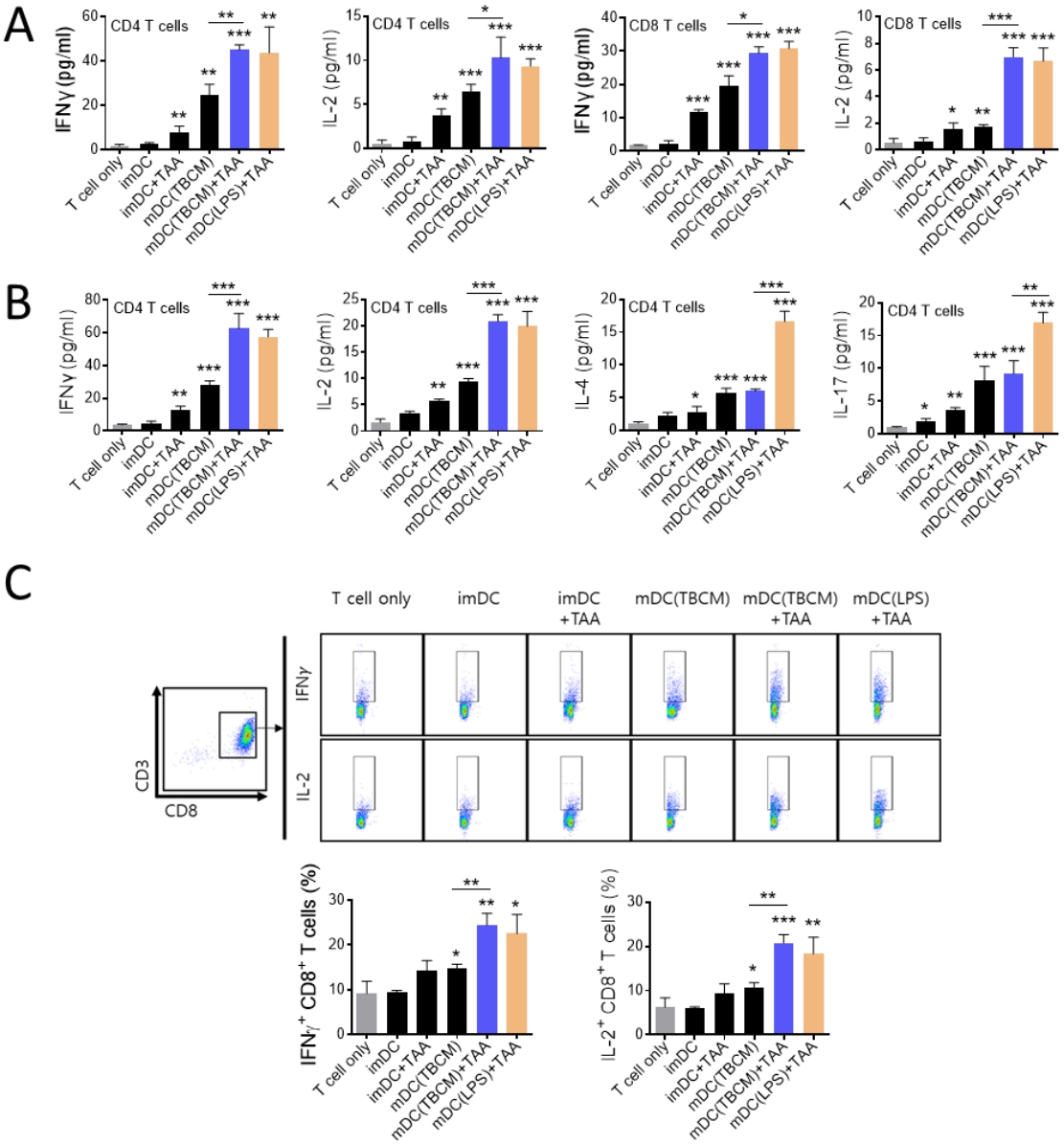
214 **Supplementary Figure S4. TBCM induces human monocytic THP-1 activation through**

215 **interaction with TLR4.** Human monocytic THP-1 cells were differentiated by treatment with PMA

(200 μg/ml) for 24 h and then incubated with anti-human TLR4 IgG and anti-human TLR2 IgA prior to incubation with TBCM (1 μg/ml) or LPS (0.1 μg/ml). (**A,B**) THP-1 cells were harvested 24 h after incubation, and the surface expression of CD80, CD86 and HLA-DR was determined by flow cytometry. (**C**) Cytokines in the culture supernatants were detected by ELISA. Significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) among the different groups are shown in the related figures, and the data are presented as the means ± s.e.m. of three independent experiments.



223

224 **Supplementary Figure S5. TBCM-induced DCs induce cytokine production in CD8$^+$ T cells.**
225 BMDCs were incubated with LLC lysates (tumor-associated antigens; TAAs) at a ratio of 3:1 tumor
226 cell equivalents in the presence or absence of TBCM (1 μg/ml) or lipopolysaccharide (0.1 μg/ml) for
227 24 h. CD4$^+$ and CD8$^+$ T cells isolated from mouse spleen were stained with CFSE and cocultured with
228 TAA-, TBCM- or LPS-treated DCs (T cell:DC=10:1). (**A**) 24 h after coculture, cytokine production in
229 the culture supernatant was measured by ELISA. (**B**) 72 h after coculture, cytokine production in
230 culture supernatant was measured by ELISA. (**C**) 72 h after coculture, intracellular cytokine

231 production in $CD8^+$ T cells was assessed by flow cytometry. Significant differences (*$p < 0.05$, **p

232 < 0.01, and ***$p < 0.001$) among the different groups are shown in the related figures, and the data

233 are presented as the means $\pm$ s.e.m. of four independent experiments.

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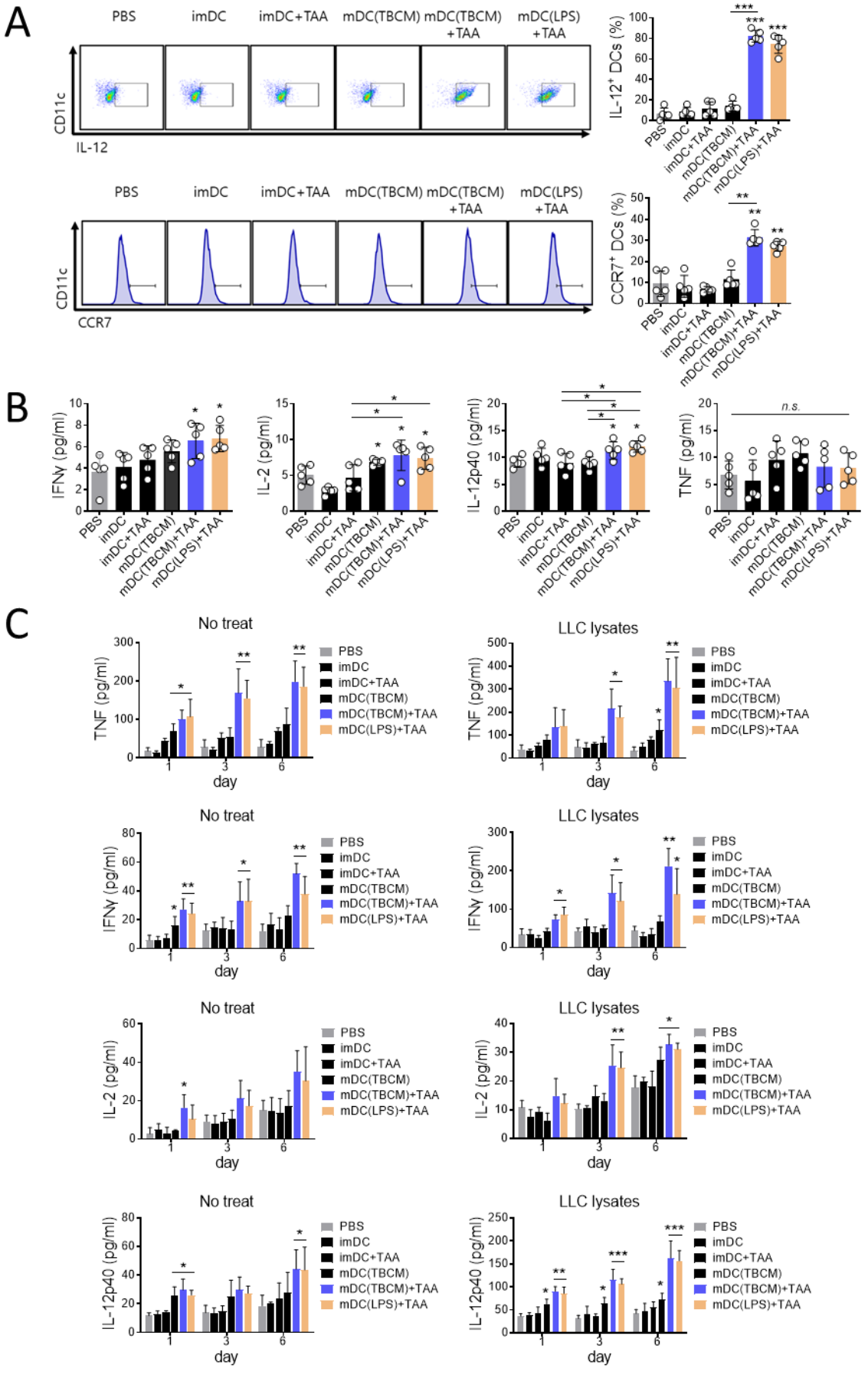
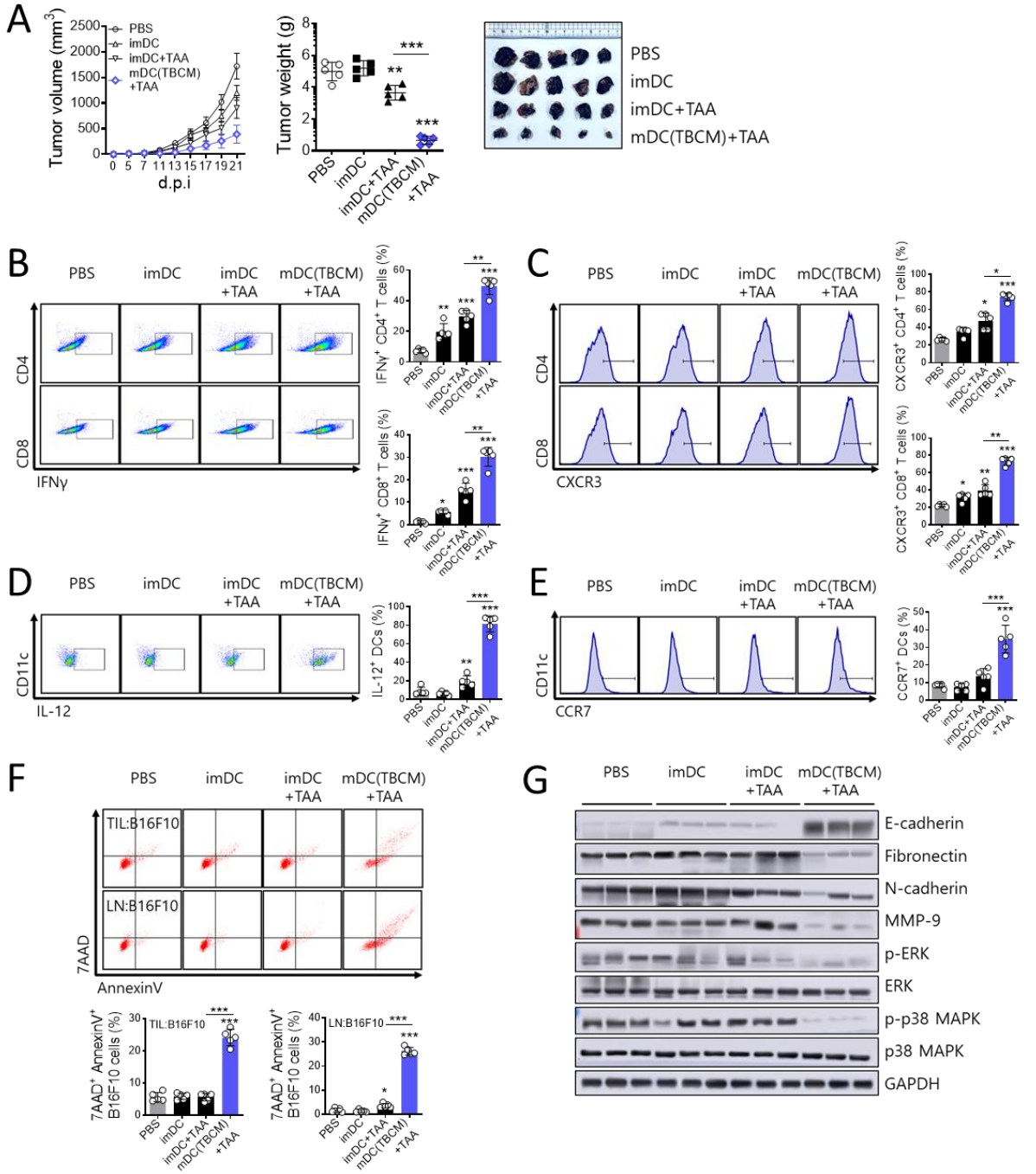

A
PBS
imDC
imDC+TAA
mDC(TBCM)
mDC(TBCM) +TAA
mDC(LPS) +TAA
CD11c
IL-12
IL-12+ DCs (%)
CCR7
CCR7+ DCs (%)
B
IFNγ (pg/ml)
IL-2 (pg/ml)
IL-12p40 (pg/ml)
TNF (pg/ml)
n.s.
C
No treat
LLC lysates
TNF (pg/ml)
IFNγ (pg/ml)
IL-2 (pg/ml)
IL-12p40 (pg/ml)
day

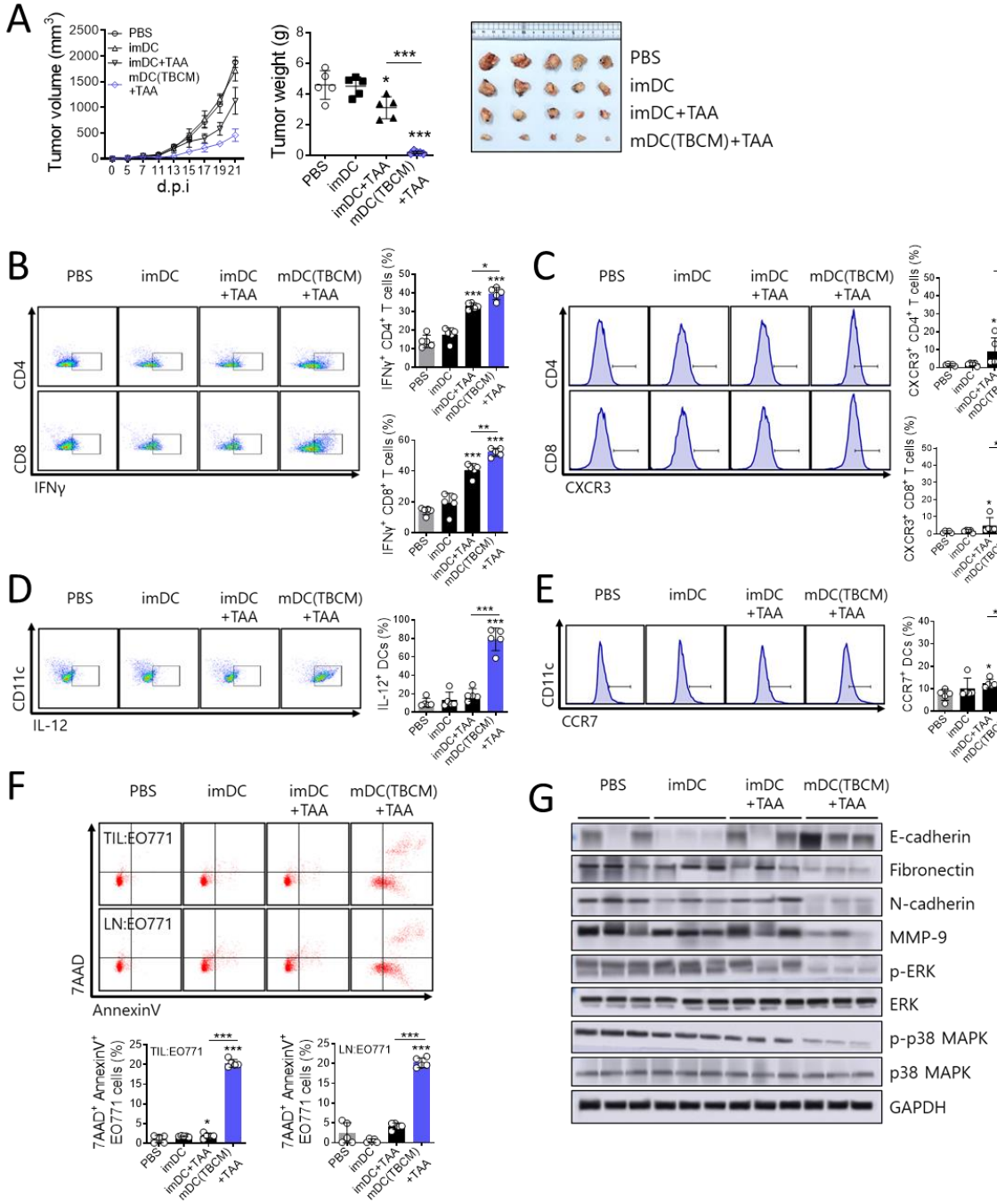
Supplementary Figure S6. TBCM-induced DCs enhanced inflammatory cytokine production in response to LLC lysate stimulation. C57BL/6 mice were subcutaneously injected with LLC cancer cells and vaccinated with DCs via footpad injection twice at intervals of one week. (**A**) On day 25 after the cancer cell injection, tumor-infiltrating DCs were assessed by flow cytometry. (**B**) On day 25 after the cancer cell injection, systemically produced cytokines in serum were measured by ELISA. (**C**) On Day 25 after cancer cell injection, splenocytes were incubated with LLC lysates for 6 days. Significant differences (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) among the different groups are shown in the related figures, and the data are presented as the means $\pm$ s.e.m. of five independent experiments.



246

247 **Supplementary figure S7. TBCM-treated DCs exerts therapeutic effects in B16F10 cancer via**
248 **induction of antigen-specific immune response.** (**A**) C57BL/6 mice were subcutaneously injected
249 with B16F10 cancer cells and vaccinated with DCs via footpad injection twice at intervals of one
250 week. Representative pictures shows excised tumor on day 24 after cancer cell injection. Growth

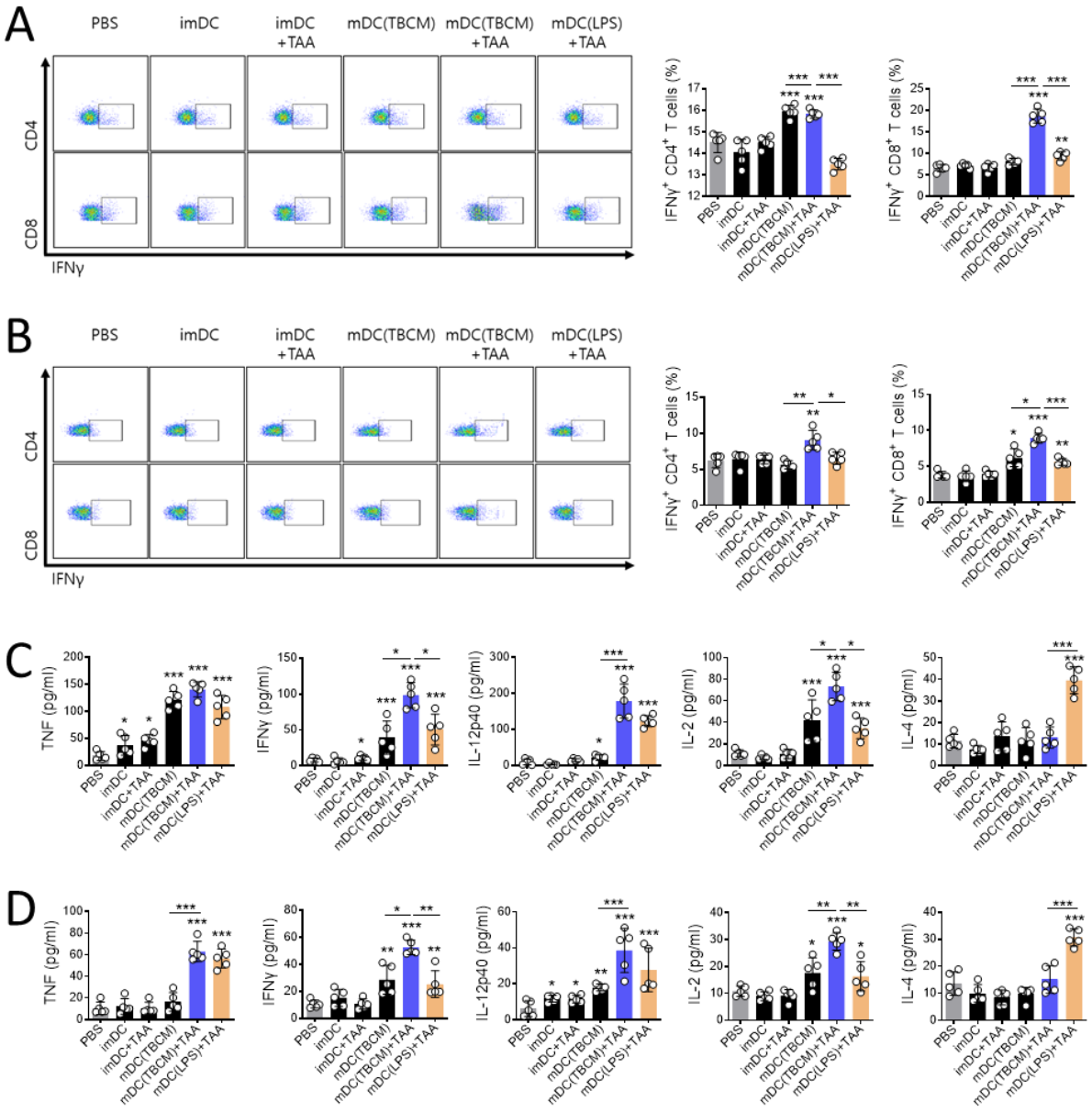
curve shows tumor volume as the mean±SD at the indicated time points, and tumor weight measured on day 25 after the cancer cell injection. (**B-E**) On day 24 after the cancer cell injection, tumor-infiltrating lymphocytes (TILs) and dendritic cells were assessed by flow cytometry. (**F**) Lymph nodes cells and TILs were isolated and cocultured with CFSE-labelled B16F10 cells for 24 h, and cytotoxicity of the immune cells were assessed by cell apoptosis assay. (**G**) Protein expression of ERK, epithelial-mesenchymal transition (EMT)-related proteins, and matrix metalloproteinases in primary tumor cells was assessed by immunoblotting assay. Significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) among the different groups are shown in the related figures, and the data are presented as the means ± s.e.m. of five independent experiments.



261

262 **Supplementary figure S8. TBCM-treated DCs exerts therapeutic effects in EO771 cancer via**
263 **induction of antigen-specific immune response.** (**A**) C57BL/6 mice were subcutaneously injected
264 with EO771 cancer cells and vaccinated with DCs via footpad injection twice at intervals of one week.
265 Growth curve shows tumor volume as the mean ± SD at the indicated time points, and tumor weight
266 measured on day 25 after the cancer cell injection. Representative pictures shows excised tumor on

day 24 after cancer cell injection. (**B-E**) On day 24 after the cancer cell injection, tumor-infiltrating lymphocytes (TILs) and dendritic cells were assessed by flow cytometry. (**F**) Lymph nodes cells and TILs were isolated and cocultured with CFSE-labelled EO771 cells for 24 h, and cytotoxicity of the immune cells were assessed by cell apoptosis assay. (**G**) Protein expression of ERK and EMT-related proteins in primary tumor cells was assessed by immunoblotting assay. Significant differences (*$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$) among the different groups are shown in the related figures, and the data are presented as the means $\pm$ s.e.m. of five independent experiments.



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276 **Supplementary figure S9. TBCM-induced DCs exerts sustained tumor prevention via T cell**
277 **activation.** C57BL/6 mice were vaccinated with BMDCs twice at intervals of one week. Seven weeks
278 after the last DC injection, LLC cancer cells were injected intravenously. (**A,B**) Seven days after the
279 cancer cell injection, cytokine-releasing T cells in lymph nodes and lung were assessed by flow
280 cytometry, respectively. (**C,D**) Seven days after the cancer cell injection, lymph nodes and lung
281 lymphocytes were cocultured with LLC cancer cells for 24 h, and then cytokines in culture
282 supernatants were measured by ELISA. Significant differences (*$p < 0.05$, **$p < 0.01$, ***$p <$

283 0.001) among the different groups are shown in the related figures, and the data are presented as the
284 means ± s.e.m. of five independent experiments.

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