Powerful anti-colon tumor effect of targeted gene immunotherapy using folate-modified nanoparticles delivery of CCl19 to activate the immune system

Xiaoxiao Liu,†,§,∇ Bilan Wang,†,∇ Yanyan Li,†,∇ Yuzhu Hu,† xiaoling Li,† Ting Yu,† Yan Ju,† Tao Sun,† Xiang Gao,*,†,# Yuquan Wei†

†Department of Neurosurgery and Institute of Neurosurgery, State Key Laboratory of Biotherapy/Collaborative Innovation Center for Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu, 610041, PR China.

‡Department of Pharmacy, West China Second University Hospital of Sichuan University, Chengdu, 610041, PR China.

§Department of Radiation Oncology, Cancer Center, Affiliated Hospital of Xuzhou Medical University; Jiangsu Center for the Collaboration and Innovation of Cancer Biotherapy, Cancer Institute, Xuzhou Medical University, Xuzhou, 221000, China.

∥Department of radiation oncology, Fudan University Shanghai Cancer Center; Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China

⊥Key Laboratory of Smart Drug Delivery of Ministry of Education, State Key Laboratory of Medical Neurobiology, Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai 200032, PR China

ªWest China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, 610041, PR China

*Correspondence: Xiang Gao
Materials and methods

Materials

Materials were purchased from standard sources: 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) from Avanti Polar Lipids Inc., Alabaster, AL, USA; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma, USA; Dulbecco’s Modified Eagle’s Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 and fetal bovine serum (FBS) from Gibco BRL, USA; methanol and acetic acid (HPLC grade) from Fisher Scientific, UK; and dimethyl sulfoxide (DMSO) and acetone from KeLong Chemicals, China. Antibodies used include: rat anti-mouse CD31 polyclonal antibody (BD PharmingenTM, USA), rabbit anti-mouse Ki67 antibody (Abcam, USA), and rhodamine-conjugated secondary antibody (Abcam, USA).

The MPEG(2000)-PLA(3000) diblock copolymer with a designed molecular weight of 3,000 Da was synthesized by opening the L-lactide ring, initiated by MPEG. MPEG (5.0 g) was melted in a 50-mL flask following the addition of anhydrous L-lactide (7.5 g) and Sn(Oct)2 (1 mL) under nitrogen. The reactant mixture was maintained at 125°C for 24 hours. The crude product was dissolved in tetrahydrofuran and then purified by precipitation in ice-cold diethyl ether followed by filtration. This process was performed in triplicate, and the resulting product was vacuum dried at ambient temperature. The number average molecular weight (Mn) of MPEG-PLA was
5,010 Da (data not shown). MPEG (with a molecular weight of 2,000 Da) (Sigma-Aldrich Co.) was dried in a one-necked flask under vacuum and stirred at 105°C for 90 minutes before use.

FA-MPEG(2000)-PLA(3000) was synthesized in two steps. First, COOH-PEG(2000)-PLA(3000) diblock copolymer with a designed molecular weight of 3,000 Da was synthesized by opening the L-lactide ring, initiated by COOH-PEG. COOH-PEG (5.0 g) was melted in a 50-mL flask following the addition of anhydrous L-lactide (7.5 g) and Sn(Oct)₂ (1 mL) under nitrogen. The reactant mixture was maintained at 125°C for 24 hours. The crude product was dissolved in tetrahydrofuran and then purified by precipitation in ice-cold diethyl ether followed by filtration. This process was performed in triplicate, and the resulting product was vacuum dried at ambient temperature. The number average molecular weight (Mn) of FA-MPEG-PLA copolymer was 5,010 Da (data not shown). COOH-MPEG (with a molecular weight of 2,000 Da) (Sigma-Aldrich Co.) was dried in a one-necked flask under vacuum and stirred at 105°C for 90 minutes before use.

Second, folic acid was linked with ethylenediamine, and then coupled with PLA-PEG-COOH under DCC/NHS conditions to obtain the crude product. The final targeting polymer was further purified by dialysis to give TM as a white powder. The FA-PEG-PLA was characterized by ¹H NMR.

**Molecular Dynamics**

DOTAP was built at first with Marvin Sketch (http://www.chemaxon.com) and optimized at the molecular mechanical level with the MMFF94 method [1]. Then it
was further optimized at the semiempirical level using the AM1 method \[^1\] with the Fletcher-Reeves algorithm by employing Hyperchem software (HyperChem, Professional 8.0, Hypercube, Inc., 1115 NW 4th Street, Gainesville, Florida 32601, USA.). The structures of FA-PEG-PLA and DOTAP were constructed, optimized and simulated according to a strategy described in a published document \[^1\].

To understand in detail the mechanism by which DOTAP passively targets tumor tissue, interactions between the bi-block copolymer FA-PEG-PLA and DOTAP in different circumstances were investigated with molecular dynamics simulation. DOTAP was docked randomly to the simulated bi-block copolymer FA-PEG-PLA at first by merging it to the FA-PEG-PLA in the workspace of HyperChem to obtain the initial structure of complex composed of DOTAP and FA-PEG-PLA. Then two stages of Langevin dynamics simulations were performed in an effort to explore interactions between the two components of the complex in water. In the process of simulation, the temperature, friction coefficient and random seed were set to 300K, 0.05 ps-1 and 0, respectively. CHARMM27, was chosen as the force field. At the first stage of simulation, interactions in water were simulated and the solvation effect was considered implicitly by setting the scale factor for the dielectric permittivity to 80. At the subsequent simulation stage, interactions near the tumor tissue were simulated by setting the scale factor. At each stage, the run time was set to 500 ps.

**Preparation of F-DMA/CCL19**

Fifteen milligrams of DOTAP, 15mg Fa-PEG-PLA and 70mg MPEG-PLA were dissolved in acetone (2 mL). The mixture was then placed into a round-bottom flask, and acetone was removed using a water bath (55°C) under negative pressure conditions (20min). Then, a 5% glucose solution (GS) (5 mL) was added to prepare the F-DMA gene carrier. At last, the CCL19 plasmid was added into F-DMA solution and F-DMA/CCL19 was obtained.

**Characterization of F-DMA/CCL19**

The morphological characteristics of F-DMA/CCL19 were observed using
transmission electron microscopy (TEM; FEI Tecnai G² F20, Hillsboro, OR, US). The samples were diluted with distilled water, placed on a copper grid and negatively stained with molybdophosphoric acid for 1 min. The grid was then allowed to dry at room temperature and examined using TEM. The mean particle size and zeta potential of F-DMA/CCL19 were determined by a Zetasizer NanoZS ZEN 3600 (Malvern Instruments, Ltd., Malvern, Worcestershire, U.K.).

**Agarose gel electrophoresis of naked plasmid DNA and F-DMA/CCL19 complexes**

After F-DMA/CCL19 was prepared, agarose gel electrophoresis was conducted in pH 7.4 TAE buffer containing the nucleic acid stain Gold View. Briefly, F-DMA was mixed with CCL19 at increasing ratios (0:1, 5:1, 10:1, 50:1) to form complexes. A gel retardation assay was conducted on a 1% agarose gel (Invitrogen Corp., Carlsbad, CA, U.S.) in Tris-acetate running buffer containing Gold View at 120 V for 20 min. The electrophoresis gels were digitally photographed using a gel documentation system (Bio-Rad Laboratories, Hercules, CA, U.S.).

**In vitro immune cell stimulation test**

The murine colon cancer cell line CT26 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and routinely cultured in RPMI 1640 medium (Gibco-BRL, Rockville, IN, USA), containing 10% FBS (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin and 100μg/ml streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

CT26 cells were seeded on a Costar 6-well plate (Corning Incorporated, Corning, NY, USA) at a density of 1.5*10⁵ cells/well in 2 mL of complete RPMI-1640 culturing medium. The cells were washed and cultured for another 24 h with 800 μL serum-free RPMI-1640 medium per well. GS, F-DMA, F-DMA/pVax, DMA/CCL19, and F-DMA/CCL19 in a final volume of 200 μL and containing 4 μg plasmid DNA were subsequently added to designated wells and allowed to incubate for 5 h.
The supernatants in each group were then collected to culture spleen derived T lymphocytes and DCs, and peritoneal derived macrophages. The activation of T lymphocytes, maturation of DCs, and the polarization of macrophage were measured by flow cytometry. The supernatants were also applied to culture peritoneal macrophages to test the IFN-γ and TNF-α expression levels by ELISA.

In another test, the supernatants were further applied to culture lymphocytes derived from the spleen, and the lymphocyte proliferation after 24 h of culture was tested by the CCK-8 method and labeled with EdU, CD4 and CD8 to identify the cell type. In addition, the subsequent lymphocytes culture supernatants at 24h in each group were collected for the measurement of its cytotoxicity to CT26 tumor cell with CCK-8, and IFN-γ and TNF-α expression by ELISA.

**Imaging In vivo**

In vivo colon cancer mouse models were established by intraperitoneal injection of CT26 cells (5×10⁵cells/0.2 mL serum-free RPMI-1640). After 10 days, the experimental group mice (F-DMA/pDNA-toto-3) were administered by intraperitoneal injection with F-DMA/pDNA-toto-3 (20µg) in 200 µL glucose solution, while the control group received the corresponding volume of DMA/pDNA-toto-3. At last, images were taken on the IVIS Lumina imaging system (Caliper, USA).

**Murine colon cancer model establishment**

All animal experiments were performed in accordance with guidelines and approved by the Animal Care Committee of Sichuan University (Chengdu, China). Female BALB/c mice (6–8 weeks old) were purchased from Vital River (Beijing, China) and housed in a specific-pathogen-free (SPF) environment with a consistent room temperature and humidity and handled in strict accordance with good animal practice.
In vivo colon cancer mouse models were established by the subcutaneous (s.c.) injection of CT26 cells (1\times10^6 cells/0.2 mL serum-free RPMI-1640) or intra-peritoneal injection of 5\times10^5 CT26 cells. Mice were randomly allocated into five groups (GS, F-DMA, F-DMA/pVax, DMA/CCL19, F-DMA/CCL19).

To assess the tumor growth of the subcutaneous model, treatment began six days after inoculation. The experimental group mice (F-DMA/CCL19) were administered via tail vein injection every day 15 times with liposomal plasmid DNA (20 μg) in 200 μL GS, while the other control groups received the corresponding volume of GS, F-DMA, F-DMA/pVax or DMA/CCL19.

To assess the tumor growth in the abdominal metastatic tumor model, treatment began three days after inoculation. The experimental group mice (F-DMA/CCL19) were administered an intra-peritoneal injection every two days for 6 times total with liposomal plasmid DNA (20 μg) in 200 μL GS, while the other control groups received same volume of the corresponding GS, F-DMA, F-DMA/pVax or DMA/CCL19.

Mice were monitored for adverse therapeutic effects, mouse weight and tumor volume. At the time of sacrifice (48 h after the final dose), the tumor tissues were harvested and the tumor weights were recorded. Tumor tissues, peritoneal fluid and vital organs of the mice were also harvested for further ELISA (CCL-19, IFN-γ and TNF-α) and flow cytometry analysis.

**Phenotypical analysis of immune cells by flow cytometry**

APC anti-mouse CD8a, FITC anti-mouse CD4, PE or APC anti-mouse CD69, PE or APC anti-mouse IFN-γ, FITC anti-mouse MHC-II, PE anti-mouse CD86, Percp-Cy 5.5 anti-mouse CD45, FITC anti-mouse CD11b, PE anti-mouse CD206, APC anti-mouse F4/80, PE anti-mouse Foxp3, FITC or PE anti-mouse Gr-1 and
isotype-matched mAbs were purchased from BD Biosciences.

Tumor tissue and spleen derived immune cells from all the treatment groups of the intraperitoneal model were harvested, ground with a pestle and filtered with a cell strainer (BD Biosciences, San Jose, CA, US) to form a single cell suspension. Peritoneal fluid was also collected for macrophage isolation and MDSCs. For cell surface staining, cells were directly stained with either IgG control or fluorescence conjugated antibodies; for intracellular cytokine staining, cells were permeabilized and fixed after surface staining, and stained with fluorescence-conjugated antibodies or IgG controls. Analysis was carried out on a FACS-Calibur flow cytometer (BD Biosciences).

**Proliferation, apoptosis, microvessel density and immune microenvironment assays of tumor tissues**

Ki67 staining was performed using immunofluorescence method. In brief, hydrated subcutaneous tumor section underwent antigen retrieval with high pressure. Primary antibody rabbit anti-mouse Ki67 antibody (1: 50, Abcam, USA) and secondary antibody RH123-conjugated secondary antibody (1:500, Abcam, USA) was used for staining of Ki67. Stained sections were observed under a fluorescent microscope (×200, Olympus, Japan).

Tumor apoptotic levels were determined using a terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) immunofluorescence kit (Promega, Madison, WI, US) according to the manufacturer’s instructions.

The quantification of MVD was estimated by counting the number of microvessels in five random fields at 200× magnification. A single microvessel was defined as a discrete cluster or single cell stained positive for CD31. Paraffin embedded sections from each group were treated for antigen retrieval and peroxidase inactivation, blocked by normal serum (ZSGB Bioscience, China), then incubated with Rat anti-mouse CD31 antibody (Abcam, USA) at 4°C overnight. Subsequently, HRP-conjugated secondary antibodies (Servicebio,
China) were added and incubated for 50 minutes at 37°C, and DAB was then used to render color. The microvessel density (MVD) was assessed by counting the number of microvessels per high-power field in each section under a microscope (Olympus, Japan). All the above sections were observed or counted by two investigators or pathologists in a blinded fashion.

Tumor immune microenvironment were determined using Immunofluorescence analysis using a rabbit anti-mouse CD8 and F4/80 antibody (Abcam) with a secondary antibody labeled with FITC detection method, according to the manufacturer’s instructions.

**Toxicity assessment**

To evaluate the potential F-DMA/CCL19 treatment associated toxicity, the vital organs tissues (heart, liver, spleen, lung and kidney) of the treated mice were harvested, fixed in 4% paraformaldehyde solution, embedded in paraffin and sectioned at 5 μm. The sections were hydrated and stained with hematoxylin and eosin (H&E) for histomorphometric analysis and observed by two pathologists in a blinded manner with a 4-point scoring system (Supplementary Table 1)[2]. In addition, blood and serum were harvested for routine blood tests and biochemical parameter tests. Blood cell counts were analyzed using an automated veterinary haematological analyzer with a preprogrammed murine calibration mode (Hemavet 950FS; Drew Scientific, Waterbury, CT). Hepatic and renal function was analyzed in a Hitachi Automatic Analyzer (Boehringer, Indianapolis, IN).

**Statistical analysis**

All data were analyzed using GRAPHPAD PRISM software (GraphPad, San Diego, CA). Data from multiple groups were analyzed using ANOVA. Tumor volumes [(smaller diameter)^2(larger diameter)*0.52] and assessment of vessel density were analyzed using the Student’s t test. All values were presented as the mean±the standard error of measurement. p<0.05 was considered to be statistically significant for all experiments.
Safety statement

No unexpected or unusually high safety hazards were encountered.

Reference:

1. Gao X.; Wang S.; Wang B.; Deng S.; Liu X.; Zhang X.; Luo L.; Fan R.; Xiang M.; You C. Improving the anti-ovarian cancer activity of docetaxel with biodegradable self-assembly micelles through various evaluations. *Biomaterials* 2015, *53*, *646-658*.

2. Mann P.C.; Vahle J.; Keenan C. M.; Baker J. F.; Bradley A. E.; Goodman D.G.; Harada T.; Herbert R.; Kaufmann W.; Kellner R. International harmonization of toxicologic pathology nomenclature: an overview and review of basic principles.*Toxicol Pathol* 2012, *40*(4 Suppl), 7S-13S.

| Numerical Score | Description | Definition |
|-----------------|-------------|------------|
| 0               | Minimal     | The amount of change present barely exceeds that which is considered to be within normal limits. |
| 1               | Slight      | In general, the lesion is easily identified but of limited severity. |
| 2               | Moderate    | The lesion is prominent but there is significant potential for increased severity. |
| 3               | Severe      | The degree of change is as complete as possible (occupies the majority of the organ). |

**Supplementary Table 1: Illustration of a 4-point scoring system**
Supplementary figure 1  Preparation and Characterization of FA-MPEG-PLA.

HOOC-PEG-PLGA was synthesized by a classic ring-opening reaction of lactide induced by PEG-COOH catalyzed by Sn(Oct)2. Folic acid was first linked with ethylenediamine, then coupled with PEG-COOH under DCC/NHS condition to obtain the crude product. The final targeting polymer was further purified by dialysis to give TM as a white powder.
Supplementary figure 2 Nuclear magnetic Resonance characterization

MPEG-PLA and FA-MPEG-PLA were characterized by $^1$H NMR, where the peaks were carefully distributed to the protons.
Supplementary figure 3 Analysis of T cell proliferation after the treatment of tumor supernatant.

When CT26 cells were transfected with GS, F-DMA, F-DMA-pVax, DMA/CCL19 and F-DMA/CCL19 for 72h, the supernatant from different treatments were added into spleen derived lymphocytes and treated for 24h. The subset of CD4+EdU A),
CD8+EdU B) lymphocytes were test by flow cytometry. (mean ± SEM, n=3; *p < 0.05, **p<0.01; F-DMA/CCL19 versus DMA/CCL19; F-DMA/CCL19, DMA/CCL19 versus GS, DMA, DMA/pVax) Results represent three independent experiments.

Supplementary figure 4 Imaging in vivo and CCL19 Elisa Assay
A) Mouse were treated with DMA/pDNA and F-DMA/pDNA, then the organs were harvested for imaging in vivo; B) CCL19 elisa assay was performed in the organ tissues after different treatment.
Supplementary figure 5. Analysis of T cell response after the treatment of F-DMA/CCL19.

T cells isolated from spleen tissue in different groups of mice were subjected to flow cytometry analysis. Total number of 30000 cells was analyzed. T cells were subjected to CD4^+CD69^+ A), CD8^+CD69^+ B), CD4^+IFN-γ^+ C), CD8^+IFN-γ^+ D) and CD4^+Foxp3^+ E). (mean ± SEM, n=3; *p < 0.05, **p<0.01; F-DMA/CCL19 versus DMA/CCL19; F-DMA/CCL19, DMA/CCL19 versus GS, DMA, DMA/pVax) Results represent three independent experiments.
Supplementary figure 6. Treatment with F-DMA/CCL19 programs macrophage phenotype to M1 in dose-dependent manner.

After the administration of GS, F-DMA, F-DMA/pVax, DMA-CCL19 and F-DMA/CCL19, ascites A) and tumor tissue B) cells were harvested and stained with CD45 antibody, CD11b antibody, F4/80 antibody and CD206 antibody. The percentage CD45⁻CD11b⁺F4/80⁻CD206⁹ M2 macrophage cells is shown. (mean ± SEM, n=3; *p < 0.05, **p<0.01; F-DMA/CCL19 versus DMA/CCL19; F-DMA/CCL19, DMA/CCL19 versus GS, DMA, DMA/pVax) Results represent three independent experiments.
Supplementary figure 7. Treatment with F-DMA/CCL19 reduced MDSCs.
After the administration of GS, F-DMA, F-DMA/pVax, DMA/CCL19 and F-DMA/CCL19, spleen and peritoneal cells were harvested and stained with Gr1 and CD11b antibodies for MDSC identification.  A) Gr1^+CD11b^+ MDSC in spleen cells; B) Gr1^+CD11b^+ MDSC in peritoneal cells. (mean ± SEM, n=3; *p < 0.05, **p<0.01; F-DMA/CCL19 versus DMA/CCL19; F-DMA/CCL19, DMA/CCL19 versus GS, DMA, DMA/pVax) Results represent three independent experiments.
Supplementary figure 8 Immune microenvironment assays of tumor tissues

Representative tumor tissue sections following the CD8 staining (Blue: DAPI; Green: CD8) A) and F4/80 staining (Blue: DAPI; Green: F4/80) analysis B). (mean ± SEM, n=3; *p < 0.05, **p<0.01; F-DMA/CCL19 versus DMA/CCL19; F-DMA/CCL19, DMA/CCL19 versus GS, DMA, DMA/pVax)
Supplementary figure 9. Antitumor mechanisms of FDMA/CCL19.

Representative tumor tissue sections following the Ki67 staining (Red: Ki67; Blue: DAPI) A), CD31 analysis (Tawny:CD31) B) and TUNNEL assay (Green: Tunnel; Blue: DAPI) C). Tumor cell proliferation, tumor vessels and tumor cell apoptosis were assessed by counting the number of the Ki67-positive index rate CD31-positive vessels per field and TUNNEL-positive cells index rate (three high power fields per slide). FDMA/CCL19 was superior to the controls in increasing tumor apoptosis, inhibiting tumor cell proliferation and angiogenesis. Scale bars is 50μm. (mean ± SEM, n=3; *p < 0.05, **p<0.01; F-DMA/CCL19 versus DMA/CCL19; F-DMA/CCL19, DMA/CCL19 versus GS, DMA, DMA/pVax)
Supplementary figure 10. Toxicity assessment in vivo with pathological section. Histological examinations of HE-stained vital organ sections including A) heart, B) liver, C) lung, D) kidney and E) spleen. No significant pathological changes were detected. Scale bars is 50μm.
Supplementary figure 11. Biochemical index examination

Biochemical index examination of GS., F-DMA, F-DMA/pVax, DMA/CCL19 and F-DMA/CCL19 treated mice including A) albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), Aspartate transaminase (AST); B) blood urine nitrogen (BUN), cholesterol (CHOL), creatine kinase (CK), creatinine (CREA); C) glucose (GLU), high density lipoprotein (HDL), lactate dehydrogenase (LDH), low density lipoprotein (LDL); D) total bilirubin (TBIL), triglyceride (TG), total protein (TP), uric acid (UA); E) amylase (XAMY). No significant different was
observed among the groups.

Supplementary figure 12. Blood hematological index examination

Blood hematological index examination A) white blood cell (WBC), B) hemoglobin (HGB), C) red blood cell (RBC) and D) platelet (PLT) of GS, F-DMA, F-DMA/pVax, DMA/CCL19 and F-DMA/CCL19 treated mice. No significant different was observed among the groups.