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PII: S0304-3835(18)30229-5

DOI: 10.1016/j.canlet.2018.03.032

Reference: CAN 13822

To appear in: *Cancer Letters*

Received Date: 14 August 2017

Revised Date: 20 March 2018

Accepted Date: 22 March 2018

Please cite this article as: Y.-L. Chen, M.-C. Chang, Y.-C. Chiang, H.-W. Lin, N.-Y. Sun, C.-A. Chen, W.-Z. Sun, W.-F. Cheng, Immuno-modulators enhance antigen-specific immunity and anti-tumor effects of mesothelin-specific chimeric DNA vaccine through promoting DC maturation, *Cancer Letters* (2018), doi: 10.1016/j.canlet.2018.03.032.

Abstract

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Immuno-modulators Enhance Antigen-specific Immunity and Anti-tumor Effects of Mesothelin-specific Chimeric DNA Vaccine through Promoting DC Maturation

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Abstract

As a tumor antigen, mesothelin (MSLN) can be identified in various malignancies. MSLN is potential for antigen-specific cancer vaccines. We generated a novel chimeric DNA vaccine using antigen-specific connective tissue growth factor lined with MSLN (CTGF/MSLN). The anti-tumor effects of the CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and toll-like receptor 3 ligand–poly(I:C) were validated in an MSLN-expressing model. CTGF/MSLN DNA with anti-CD40Ab and poly(I:C) vaccinated mice demonstrated potent anti-tumor effects with longer survival and less tumor volumes. An increase in MSLN-specific $CD8^+$ T cells and anti-MSLN Ab titers was also noted in CTGF/MSLN DNA with anti-CD40Ab and poly(I:C) vaccinated mice. The CTGF/MSLN DNA vaccine combined with immuno-modulator EGCG also generated potent anti-tumor effects. Immuno-modulators could enhance the antigen-specific anti-tumor effects of CTGF/MSLN DNA vaccine through promoting the DC maturation. In addition, MSLN-specific cell-based vaccine with AAV-IL-12 and the CTGF/MSLN DNA vaccine with anti-CD40Ab/polyp(I:C) generated more potent anti-tumor effects than the other combinational regimens. The results indicate that an MSLN-specific DNA vaccine combined with immuno-modulators may be an effective immunotherapeutic strategy to control MSLN-expressing tumors including ovarian and pancreastic cancers, and malignant mesothelioma.

Keywords: mesothelin, dendritic cell, anti-tumor effect, mesothelin-expressing tumor, immunotherapy

Abbreviations: MSLN, mesothelin; CTGF, connective tissue growth factor; TAA, tumor-associated antigen; CSF1R, colony stimulating factor 1 receptor; TME, tumor microenvironment; EGCG, epigallocatechin-3-gallate; AAV, adeno-associated virus; IL-12, interleukin-12; IFN-γ, interferon-gamma; PE, phycoerythrin; FITC, fluorescein isothiocyanate; Luc, luciferase; poly(I:C), polyinosinic:polycytidylic acid; PARP, poly ADP ribose polymerase; BMM, bone marrow monocyte; LPS, lipopolysaccharide; DC, dendritic cell; TLR-3, toll-like receptor 3

1. Introduction

Conventional modalities for dealing with malignancies include surgery, chemotherapy, and radiation therapy. One of the reasons for treatment failure is the tumor microenvironment (TME), which is both a cause and a consequence of tumorigenesis [1]. Many hallmarks of cancer are related to this microenvironment, including the ability to induce proliferation and angiogenesis and avoid apoptosis, hypoxia, and immune detection [2]. Currently, efforts to develop potent treatment modalities are being employed to overcome the obstacles of the TME.

An effective therapeutic method should only attack tumors, without destroying normal tissue and avoiding drug resistance. From the standpoint of immunology, immune manipulation may be an attractive alternative approach because it has the ability to discriminate between neoplastic and non-neoplastic cells [3]. Malignant tumors are immunogenic at some cancer sites. Therefore, identification of the distinctive tumor-associated antigens (TAAs) to generate various TAA-containing preparations for stimulating host immunity is an essential step in the development of a cancer vaccine [4-6].

Mesothelin (MSLN) is a secreted protein anchored to the cell membrane by a glycosylphosphatidylinositol linkage. This molecule is highly expressed on the surfaces of cancer cells, such as pancreatic, gastric, endometrial, and ovarian

carcinomas, and thought to be an immunogenic TAA [7-11]. Through the phosphoinositide 3-kinase (PI3K)/Akt-dependent pathway, MSLN can reduce chemotherapeutic sensitivity [12]. Furthermore, MSLN has been reported to be a poor prognostic factor for gastric and ovarian cancers and is detected in triple-negative breast cancer patients [13-15]. Consequently, MSLN could represent both a prognostic tumor marker for predicting the clinical outcome of cancer patients and a therapeutic TAA for developing antigen-specific immunotherapies to treat MSLN-expressing tumors.

Connective tissue growth factor (CTGF) is a cysteine-rich protein originally identified in a conditioned medium of human umbilical vein endothelial cells [16]. CTGF has been reported to possess the ability to promote the proliferation and survival of endothelial cells [17,18]. In our previous study, a chimeric E7-specific CTGF/E7 DNA vaccine demonstrated a potent anti-tumor immune response by extending the survival of antigen presenting cells (APCs) [19].

Like our previous report [19], CTGF was linked to MSLN DNA to develop a novel MSLN-specific DNA vaccine against MSLN-expressing tumors in this study. However, tumor-bearing hosts receiving CTGF/MSLN DNA vaccine treatment alone could not have potent anti-tumor effects. In consideration of dealing with the endogenous TAA, MSLN-related immune tolerance during carcinogenesis, induction

of more mature dendritic cells (DCs) was needed [20,21], even though CTGF could prolong the survival of transduced DCs [19]. Therefore, combination of anti-CD40 Ab and toll-like receptor 3 (TLR-3) ligand–polyinosinic:polycytidylic acid [poly(I:C)] was applied as adjuvant of CTGF/MSLN DNA vaccine for DC induction [22]. Anti-tumor effect of this combinational treatment modality was investigated in an MSLN-expressing tumor model [23]. Consequently, we showed that MSLN-specific DNA vaccine combined with immuno-modulators is a potentially innovative approach for immunotherapy of MSLN-expressing cancer such as ovarian and pancreastic cancers, and malignant peritoneal mesothelioma in the survey.

2. Materials and Methods

2.1 Mice

Female C57BL/6J mice (6-8 weeks old) were purchased and maintained in the animal facility of the School of Medicine, National Taiwan University. All of the animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. In all of the following experiments, the mice were divided into groups of five.

2.2 Cell lines

WF-0 and WF-3 tumor cells were generated as described previously [23]. The cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 50 U/mL penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM non-essential amino acids, and 0.4 mg/mL G418 at 37°C in a 5% carbon dioxide atmosphere [24]. The cell lines were tested for mycoplasma contamination.

WF-3/Luc tumor cells were generated by transducing WF-3 tumor cells with the lentiviral vector [21]. Briefly, luciferase cDNA was amplified by PCR from pGL2-basic (Promega, Madison, WI) and cloned into the pLKO/AS3.1.EGFP3 lentiviral vector (Academia Sinica, Taiwan) to generate pLKO/luciferase/AS3.1.EGFP3, which was transfected into 293T cells with pCMVΔR8.91 (Academia Sinica, Taiwan) and pMDG (Academia Sinica, Taiwan) to assemble the lentivirus. The lentivirus was collected 48 h after transfection. The WF-3 cells were further infected with lentivirus with 8 μg/mL

polybrene (Sigma Chemicals Co., St Louis, MO) for 48 h. A single clone was isolated and cultured for further studies.

2.3 Plasmid DNA constructs and DNA preparation

The generation of pcDNA-CTGF and MSLN was described previously [19,21]. Briefly, to generate pcDNA-MSLN, MSLN was amplified by PCR using human ovarian cancer cell line OVCAR-3 cDNA as the template and the following primers: 5′-CCGGGAATTCCCTCCCTGGGATCTACACAG-3′ and 5′-CGCAAGCTTCAGGACGGTGAGAACAGGTC-3′. To generate pcDNA3-CTGF/MSLN, the PCR product of MSLN was cloned into the EcoRI/HindIII sites of pcDNA3-CTGF vector. All of the constructs were verified by restriction analysis and DNA sequencing.

2.4 Generation of MSLN peptide and WF-3 lysates

The generation of MSLN peptide and WF-3 lysates was described previously [21]. Briefly, the H-2D^{b}-restricted MSLN peptide-specific cytotoxic T-lymphocyte epitope (amino acid [aa] 406-414, GQKMNAQAI) was synthesized (Kelowna Inc., Taipei, Taiwan) [25].

Whole MSLN protein was prepared from WF-3 tumor cell lysates. Cells were suspended in PBS (0.5 mL) and lysed by five freeze (liquid nitrogen)/thaw (room temperature) cycles. The lysate was centrifuged at 15,000 *g* (30 min, 4°C) before

collecting the supernatant. The protein extracts were quantified using the BCA Protein Assay Kit (Pierce).

2.5 Preparation of CTGF/MSLN DNA vaccine

DNA-coated gold particles and gene gun particle-mediated DNA vaccines were prepared using a helium-driven gene gun according to a previously published protocol [19]. The gold particles (BioRad, Hercules, CA) were weighed and suspended in 70% ethanol. This suspension was vortexed vigorously and then centrifuged to collect the particles. After washing with distilled water three times, the collected particles were resuspended in DNA solution (1 μg DNA per mg gold particles), vortexed, and sonicated for a few seconds prior to adding 2.5 M $CaCl_2$ and 0.05 M spermidine solution with vortexing. This solution was kept on ice for 10 min and the DNA-coated gold particles collected and washed three times with 100% ethanol. Finally, the particles were resuspended in 100% ethanol at an appropriate concentration and used to make bullets. DNA-coated gold particles were delivered to the shaved abdominal region of mice using a low pressure-accelerated helium Gene Gun (BioWare Technologies Co. Ltd., Taipei, Taiwan) with a 50 psi discharge pressure.

2.6 Vaccination with CTGF/MSLN DNA with or without anti-CD40Ab and/or poly(I:C)

The vaccination protocols for DNA vaccines with anti-CD40 Ab and/or

poly(I:C) are presented in **Figure 1A (prevention experiment) and Figure 3A (therapeutic experiment).** Mice were immunized with 2 μg/mouse of DNA vaccine (pcDNA3 alone, pcDNA3-CTGF, pcDNA3-MSLN, or pcDNA3-CTGF/MSLN) twice per week for 2 weeks with a total amount of 4 times/experiment as described above. One day after the first and third DNA vaccination, anti-CD40 Ab (50 μg/mouse; FGK4.5, BioExpress) and/or poly(I:C) (100 μg/mouse; Invitrogen, Carlsbad, CA) was administered with a total amount of 2 times/experiment. PBS was used as the negative control.

2.7 Intracellular cytokine staining and flow cytometry analysis

Mice received either no vaccination or DNA-coated gold particles with or without anti-CD40 Ab and/or poly(I:C) as described above. Mice were sacrificed 1 week after the last DNA vaccination and splenocytes harvested. Splenocytes from each group were incubated for 16 h with either 1 μg/mL of MSLN peptide (aa 406-414, GQKMNAQAI) containing an MHC class I epitope for detecting MSLN-specific $CD8^+$ cytotoxic T cell precursors, or 100 μg/mL of WF-3 cell lysates for detecting MSLN-specific $CD4^+$ helper T cell precursors [21]. Golgistop (Pharmingen, San Diego, CA) was added 6 h before harvesting the cells, which were then stained with phycoerythrin (PE)-conjugated CD4 or CD8 Ab (Pharmingen, Heidelberg, Germany) and subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the

manufacturer's instructions (Pharmingen). Next, intracellular cytokine staining for fluorescein isothiocyanate (FITC)-conjugated interferon-gamma (IFN-γ) (Pharmingen) or immunoglobulin isotype control Ab (rat IgG1) (Pharmingen) was performed [19]. Data were collected using a FACS Calibur flow cytometer and analyzed by CellQuest software (BD Pharmingen, Heidelberg, Germany) as described elsewhere [19].

2.8 Detection of anti-MSLN Abs by enzyme-linked immunosorbent assay (ELISA)

Sera were prepared from immunized mice 14 days after their last DNA immunization. MSLN-specific Abs were detected in the sera using direct ELISA as described previously [21]. Briefly, each well of a 96-microwell plate was coated with 0.05 μg of mouse MSLN recombinant protein (Abnova, Taipei, Taiwan) and incubated at 4°C overnight. The wells were then blocked with PBS containing 20% fetal bovine serum. Serum was serially diluted in PBS, added to the ELISA wells, and incubated at 37°C for 2 h. After washing with PBS containing 0.05% Tween 20, the plate was incubated with a 1:2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Biosource, Camarillo, CA) at room temperature for 1 h. The plate was washed, developed with 1-Step Turbo TMB-ELISA (Clinical Science Lab, Mansfield, MA), and the reaction stopped with 1 M H_2SO_4. The ELISA plate was read using a standard ELISA reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

2.9 In vivo tumor protection experiments

One week after the last DNA vaccination, immunized mice (10 per group) were intraperitoneally challenged with $1x10^5$ WF-3/Luc tumor cells. Bioluminescence tumor images were used to detect the tumor growth measured using the IVIS Imaging System Series 200 (Xenogen, Alameda, CA) twice a week until they died, sacrificed at the indicated day, or 90 days after tumor challenge. To detect bioluminescence signals, mice were injected intraperitoneally with 300 μL of 15 mg/mL luciferin (Promega, Madison, WI) and imaged after 10 min. Bioluminescence signals were then acquired for 3 min and recorded.

2.10 In vivo antibody depletion experiments

In vivo antibody depletion experiments were performed as described previously [21]. Briefly, mice immunized with pcDNA3-CTGF/MSLN DNA vaccine with or without anti-CD40 Ab and/or poly(I:C) as described above were challenged with $1x10^5$ WF-3/Luc tumor cells 7 days after the last vaccination. Depletion was started 1 week before tumor challenge using 100 μg/mouse of purified monoclonal antibodies GK1.5, 2.43, PK136, and AFS98 to deplete CD4, CD8, NK1.1, colony stimulating factor 1 receptor (CSF1R, for DC depletion), respectively (Bio X cell, West Lebanon, NH) [21,25,26]. Depletion was terminated 70 days after the tumor challenge. The mice were monitored for evidence of tumor growth using the IVIS twice a week until they died or were sacrificed on day 100.

2.11 Preparation of MSLN-specific cell-based vaccine Meso-VAX

MSLN-specific cell-based vaccine Meso-VAX was prepared as described previously [21]. Briefly, pcDNA3-hMSLN was transfected into WF-0 cells to create WF-0/hMSLN cells. The WF-0/hMSLN tumor cells were further irradiated and defined as the Meso-VAX vaccine.

2.12 Preparation and generation of adeno-associated virus (AAV) containing IL-12

The AAV containing mouse interleukin-12 cDNA (AAV-IL-12) was prepared as described previously [21].

2.13 In vivo tumor treatment

For the *in vivo* tumor treatment experiments, the mice were intraperitoneally injected with $1x10^5$ WF-3/Luc tumor cells on day 0 as described previously [21]. Three days after tumor challenge, the mice were immunized intraperitoneally with DNA vaccine with or without anti-CD40 Ab and/or poly(I:C) as described above. Mice receiving PBS were used as a negative control. Bioluminescence tumor images were used to detect the tumor growth as described above.

In experiments combining DNA vaccine and AG490, mice were injected intraperitoneally with $1x10^5$ WF-3/Luc tumor cells on day 0 as described above. Two days after tumor challenge, the mice were immunized with DNA vaccine and 50 mg/kg of AG490 via intraperitoneal injection three times per week for 4 weeks.

Bioluminescence tumor images were used to detect the tumor growth as described above.

In experiments combining DNA vaccine and EGCG, mice were intraperitoneally injected with $1x10^5$ WF-3/Luc tumor cells on day 0 as described above. The mice were then immunized with DNA vaccine and 10 mg/kg of EGCG via oral administration three times per week for 4 weeks. Bioluminescence tumor images were used to detect the tumor growth as described above.

In experiments combining DNA vaccine with anti-CD40 Ab, poly(I:C) and EGCG, mice were intraperitoneally injected with $1x10^5$ WF-3/Luc tumor cells on day 0. Three days after tumor challenge, the mice received DNA vaccine with anti-CD40 Ab, poly(I:C) and EGCG. Bioluminescence tumor images were used to detect the tumor growth. All the procedures were described as above.

To compare the therapeutic effects of cell- and DNA-based vaccines combined with various immune modulators, mice were intraperitoneally injected with $1x10^5$ WF-3/Luc tumor cells on day 0 and then given Meso-VAX with or without AAV-IL-12 as described previously [21] or the pcDNA3-CTGF/MSLN DNA vaccine with or without anti-CD40 Ab and/or poly(I:C) as described earlier. The mice received a booster with the same regiment every 7 days for 4 weeks. Mice receiving PBS were used as a negative control. Tumor images were measured using the IVIS Imaging

System Series 200 as described above.

2.14 Complement-dependent cell-mediated cytotoxicity assays

To evaluate whether the DNA-based vaccine combined with anti-CD40 Ab and/or poly(I:C) can also generate complement-dependent cell-mediated cytotoxicity to tumor cells, the complement-dependent toxicity assay was performed as described previously [21]. Briefly, WF-3/Luc tumor cells were seeded onto a 96-well plate (5×10^3/well) overnight. Sera collected from various vaccinated groups were added into the well in the following amounts: 0, 20, and 50 μL. Naive rabbit serum in culture medium at a final dilution of 1:5 was used as complement (Sigma-Aldrich, St Louis, MO) in a total volume of 100 μL. After incubation for 18 h, cell viability was measured based on bioluminescent activity using the IVIS Imaging System Series 200.

2.15 Western blot analysis for detecting the apoptosis of tumor cells

Western immunoblotting were performed with some modifications [27]. Briefly, the WF-3/Luc (5×10^3/well) were seeded overnight and treated with sera from various groups for 12 h. These cells were then lysed and analyzed. The protein extracts were quantified with a BCA Protein Assay Kit (Pierce, Rockford, IL). Then, 60 μg of each cell lysate was resolved by SDS/PAGE (12% gel), transferred onto a PVDF/nylon membrane (Millipore), and probed with antibodies specific to poly ADP ribose polymerase (PARP) or caspase 3 (Cell Signaling Technology, Danvers, MA). The

membrane was then probed with either horseradish peroxidase-conjugated goat anti-rabbit antibody. The specific bands were visualized by an ECL® (enhanced chemiluminescence) Western blotting system (GE Healthcare).

2.16 Generation and analysis of bone marrow monocyte (BMM)-derived immature DCs

BMM-derived immature DCs were generated by culturing bone marrow mononuclear cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech, Rocky Hill, NJ) as described previously [22,28]. To generate mature BMM-derived DCs, lipopolysaccharide (LPS, Sigma-Aldrich Chemie GmbH) was added at indicated time points and the cells collected after 24 h.

Whether anti-CD40 Ab combined with poly(I:C) can stimulate DC maturation has been investigated previously [30]. For the anti-CD40 Ab combined with poly(I:C) experiments, 1 μg/mL anti-CD40 Ab and 2 μg/mL poly(I:C) was added on the first day of culture and the anti-CD40 Ab and 2 μg/mL poly(I:C)-containing medium replaced every 2 days. A PBS-treated group was used as a control. LPS (50 ng/mL) was used as an exogenous stimulus to activate DCs at day 6 and the cells collected 24 h later for further study and analysis.

To investigate the potential DC maturation agents that could be used in combination with CTGF/MSLN DNA vaccination, two potential DC maturation stimulators, EGCG [29] and AG490 [30] were analyzed. Briefly, 25 μM of EGCG or

AG490 (Sigma-Aldrich, St Louis, MO) was added into the DC culture medium after the first day of culture and replaced every other day until analysis.

2.17 Flow cytometric analysis of surface markers of BMM-derived DCs

Immature and mature BMM-derived DCs were cultured as described above. The cells were stained with FITC-conjugated anti-CD11c (eBioscience) and PE-conjugated anti-CD86 (BioLegend) and analyzed by flow cytometry as described previously [22,28].

2.18 Surface marker staining and flow cytometry of splenocytes and intratumoral lymphocytes and monocytes

The splenocytes and intratumoral lymphocytes and monocytes were obtained from mice with various treatment modalities on day 28 after tumor challenge and prepared as previously described [31]. The treatment protocols were described previously. To evaluate the activation status of $CD8^+$ lymphocytes of splenocytes and TME (tumor microenvironment), the surface marker expression of CD223 (activation marker of T lymphocytes) was detected [32,33]. The cells were stained with FITC-conjugated anti-mouse CD3 (BioLegend, San Diego, CA), PE/Cy5-conjugated anti-mouse CD8 (BioLegend), and PE-conjugated anti-mouse CD223 (eBioscience, San Diego, CA). To detect the maturation status of DCs in TME, the cells were stained with FITC-conjugated anti-mouse CD11c (eBioscience) and PE-conjugated anti-mouse

CD86 (BioLegend) [22,28]. Flow cytometry assays and analyses were performed as previously described [19].

2.19 Measurement of intratumoral IFN-γ secretion by ELISA

The expression level of intratumoral IFN-γ was measured as previously described with some modification [34]. Tumors (100 μg) were obtained from mice with various treatment modalities on day 28 after tumor challenge. The treatment protocols were described above.These samples were minced and placed in RPMI-1640 (1 mL) at 37°C for 24 h, and then centrifugated at 900 g for 15 mins to collect the supernatants for further cytokine analysis. Concentrations of IFN-γ in supernatants were determined by ELISA kits (Invitrogen, Vienna, Austria).

2.20 Statistical analysis

All data were expressed as mean±SEM and are representative of at least two different experiments. The data from surface marker staining in flow cytometric analysis and tumor treatment experiments were evaluated by analysis of variance (ANOVA). Comparisons between individual data points were made using the Student's t-test. Survival curves were generated using the Kaplan-Meier method and differences in survival curves calculated using the log-rank test. $P<0.05$ was considered significant.

3. Results

3.1 CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and TLR-3 ligand–poly(I:C) generated tumor protection effects in mice challenged with MSLN-expressing tumor cells

First, we performed *in vivo* prevention experiments to evaluate the anti-tumor effect of the chimeric CTGF/MSLN DNA vaccine. The experimental protocols were shown in Figure 1A. As shown in Figure 1B, the mice vaccinated with no insert, MSLN, CTGF, or CTGF/MSLN DNA alone survived less than 63 days after tumor challenge without significant difference (P=0.58, log-rank test). All of the mice immunized with CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) were still alive after tumor challenge during the experiment (Fig. 1C). Compared with the group receiving CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C), none of the mice immunized with CTGF/MSLN DNA vaccine alone, or CTGF/MSLN DNA vaccine combined with anti-CD40 Ab or poly(I:C) were alive after 63 days of tumor challenge (P<0.001, log-rank test, Fig. 1C).

Thus, CTGF/MSLN DNA vaccine combined with anti-CD40Ab and poly(I:C), but not CTGF/MSLN DNA vaccine alone, protected mice against lethal MSLN-expressing tumor cells.

3.2 Vaccination with CTGF/MSLN combined with anti-CD40 Ab and poly(I:C) enhanced the MSLN-specific immunologic profiles

The number of MSLN-specific IFN-γ-secreting $CD4^+$ helper T lymphocytes in mice immunized with CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) ($19.0\pm5.0/3.5\times10^5$ splenocytes) was similar to the other groups (no insert: 17.0±5.8, CTGF: 27.1±5.1, MSLN: 19.0±5.7, CTGF/MSLN alone: 15.3±3.4, anti-CD40 Ab with poly(I:C) only: 24.1±6.4, CTGF/MSLN with anti-CD40 Ab: 17.7±3.3, CTGF/MSLN with poly(I:C): 12.3±3.8; P=0.62, one-way ANOVA, Fig. 2A).

Representative figures of flow cytometric analysis for MSLN-specific IFN-γ-secreting $CD8^+$ cytotoxic T cell precursors are shown in Figure 2B. Mice vaccinated with the CTGF/MSLN DNA vaccine and anti-CD40 Ab with poly(I:C) ($337.3\pm23.6/3.5\times10^5$ splenocytes) had significantly more MSLN-specific IFN-γ-secreting $CD8^+$ T cell precursors than the other groups (no insert: 20.0±3.4, CTGF: 31.4±6.5, MSLN: 30.0±2.7, CTGF/MSLN alone: 83.1±6.0, anti-CD40 Ab with poly(I:C) alone: 25.3±2.3, CTGF/MSLN with anti-CD40 Ab: 27.5±3.1, and CTGF/MSLN with poly(I:C): 28.7±1.7; P=0.004, one-way ANOVA, Fig. 2C).

The antigen-specific humoral immunity of various groups was further evaluated by detecting the anti-MSLN Abs. The CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) (2.21±0.51) resulted in the highest titers of anti-MSLN Ab among the groups (no insert: 0.30±0.04, CTGF: 0.35±0.03, MSLN: 0.26±0.02, CTGF/MSLN alone: 0.67±0.07, anti-CD40 Ab with poly(I:C): 0.29±0.03,

CTGF/MSLN with anti-CD40 Ab: 0.34±0.04, and CTGF/MSLN with poly(I:C): 0.35±0.04; OD450 in 1:100 dilution, $P<0.001$, one-way ANOVA, Fig. 2D).

These results demonstrate that the combination of the pcDNA3-CTGF/MSLN DNA vaccine with anti-CD40 Ab and poly(I:C) enhanced the MSLN-specific cell-mediated and humoral immunities.

3.3 CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) significantly reduced peritoneal tumors in therapeutic experiments

The protocols for *in vivo* therapeutic experiments were shown in Figure 3A. Mice receiving the CTGF/MSLN DNA vaccine and anti-CD40 Ab with poly(I:C) (378.0±6.1/3.5×10^5 splenocytes) had significantly more MSLN-specific IFN-γ-secreting $CD8^+$ T cell precursors than the other groups (no insert: 30.3±2.4, CTGF/MSLN alone: 68.7±1.8, anti-CD40 Ab with poly(I:C) alone: 33.0±2.6, CTGF/MSLN with anti-CD40 Ab: 57.0±2.1, and CTGF/MSLN with poly(I:C): 57.7±2.3; $P<0.001$, one-way ANOVA, Fig. 3B).

The luciferase activities of WF-3/Luc tumor-bearing mice in various groups as detected by the IVIS system were shown in Figure 3C. The CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) ($4.8\pm0.5\times10^6$) exhibited the least luminescence of all the groups after 28 days of tumor inoculation (PBS: $1.4\pm0.2\times10^7$, CTGF/MSLN alone: $1.3\pm0.1\times10^7$, anti-CD40 Ab with poly(I:C): $1.2\pm0.1\times10^7$,

CTGF/MSLN with anti-CD40 Ab: $1.0\pm0.1\times10^7$, and CTGF/MSLN with poly(I:C): $1.2\pm0.2\times10^7$; $P<0.001$, one-way ANOVA, Fig. 3D). Seventy percent of the mice that received the CTGF/MSLN DNA vaccine with anti-CD40 Ab and poly(I:C) were alive 90 days after challenge with WF-3/Luc tumor cells. Whereas, none of the mice in the other groups survived more than 63 days ($P<0.001$, log-rank test, Fig. 3E).

Thus, our data indicate that the CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) generates potent therapeutic effects against lethal MSLN-expressing tumor cells.

3.4 CD8$^+$cytotoxic T cells and DCs are essential for the anti-tumor effects of CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C)

To determine the impacts of lymphocyte subsets and DCs on the anti-tumor effects of CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C), *in vivo* antibody depletion experiments were performed. Luminal analysis of Ab depletion is shown in Figure 4A. Mice with CD8 ($1.8\pm0.1\times10^7$) and CSF1R ($2.0\pm0.1\times10^7$) Ab depletion exhibited greater luminescence than those without Ab depletion ($1.0\pm0.1\times10^6$), with CD4 Ab depletion ($1.6\pm0.1\times10^6$), or with NK1.1 Ab depletion ($1.7\pm0.1\times10^6$) after 28 days of tumor challenge ($P<0.001$, one-way ANOVA, Fig. 4B). The luminescence between CD8 and CSF1R Ab depletion groups did not show difference ($P=0.26$, Student's t-test, Fig. 4B). Furthermore, none of the vaccinated mice depleted of

$CD8^+$cytotoxic T cells and DCs survived after 60 days of tumor challenge (Fig. 4C). Compared with the CD8 cell-depleted and DC-depleted groups, all of the non-depleted, NK cell-depleted, and 80% of the CD4 cell-depleted mice were still alive 100 days after tumor challenge (P<0.001, log-rank test, Fig. 4C).

These results revealed that $CD8^+$cytotoxic T cells and DCs are essential for the anti-tumor effects generated by the CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C).

3.5 Post-vaccination sera could generate MSLN-specific complement-dependent cell-mediated cytotoxicity

Next, we determined whether anti-MSLN Ab in the sera of vaccinated mice could generate antigen-specific complement-dependent cell-mediated cytotoxicity. The luminescence measured in MSLN-specific complement-dependent cell-mediated cytotoxicity assays in various groups are shown in Figure 5A. The CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) ($2.1\pm0.0\times10^5$) had the lowest luminescence among the groups (naïve: $6.7\pm0.6\times10^6$, CTGF/MSLN alone: $6.6\pm0.6\times10^6$, anti-CD40 Ab with poly(I:C): $5.8\pm0.7\times10^6$, CTGF/MSLN with anti-CD40 Ab: $5.6\pm0.6\times10^6$, CTGF/MSLN with poly(I:C): $5.5\pm0.3\times10^6$; in 50 μL sera, P<0.001, one-way ANOVA, Fig. 5B). As shown in Figure 5C, the expression levels of cleaved PARP and caspase 3 were highest in WF-3/Luc cells treated with sera from the

CTGF/MSLN combined with anti-CD40Ab and poly(I:C) mice, when detected by western blotting as compared with the other groups.

These results demonstrated that MSLN-specific Abs in the sera of mice vaccinated with CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) can enhance complement-dependent cell-mediated cytotoxicity to kill WF-3/Luc tumor cells.

3.6 CTGF/MSLN DNA vaccination combined with DC maturation agent EGCG can have enhanced anti-tumor effects

Our previous study demonstrated that combination of anti-CD40 Ab and toll-like receptor (TLR)-3 agonists can reverse the effects of morphine on the maturation and function of DCs, thereby recovering host immunity and generating potent anti-tumor effects [22]. We determined whether other potential DC maturation-stimulating agents, specifically AG490 and EGCG, can enhance the anti-tumor effects by stimulating DC maturation. The number of $CD11c^{+}CD86^{+}$ cells detected by flow cytometry is shown in Fig. 6A. After stimulated by LPS, the anti-CD40 Ab with poly(I:C) group had the highest percentage of $CD11c^{+}CD86^{+}$ cells (50.2±4.1%) among the groups. The EGCG-treated group also had a higher percentage of $CD11c^{+}CD86^{+}$ cells (47.0±4.8%) than the naïve group (38.5±4.7%, P=0.01, one-way ANOVA, Fig. 6B). However, the AG490-treated group had the

lowest percentage of $CD11c^+CD86^+$ cells among the groups (34.6±3.58%, Fig. 6B).

We determined whether AG490 or EGCG combined with the CTGF/MSLN DNA vaccine can enhance the anti-tumor effects of tumor-bearing mice *in vivo*. The luminescence of WF-3/Luc tumor-bearing mice as detected by the IVIS imaging system is shown in Figure 6C. In Figure 6D, the CTGF/MSLN vaccine combined with anti-CD40 Ab and poly(I:C) group exhibited the least luminescence ($7.6±0.5×10^6$) of all the groups. The CTGF/MSLN vaccine combined with EGCG-treated group exhibited less luminescence ($9.0±0.4×10^6$) than the naïve group ($2.6±0.1×10^7$, $P<0.001$, Student's t-test), and the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG-treated group also exhibited less luminescence ($8.5±0.4×10^6$) than the naïve group ($P<0.001$, Student's t-test). The luminescence activities of CTGF/MSLN vaccine combined with anti-CD40 Ab and poly(I:C), CTGF/MSLN vaccine combined with EGCG, and the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG groups did not show significant difference ($P=0.14$, one-way ANOVA). The CTGF/MSLN vaccine combined with AG490 ($2.4±0.1×10^7$)-treated and naïve groups exhibited similar luminescenceto the naïve group ($P=0.20$, Student's t-test).

Mice receiving the CTGF/MSLN vaccine combined with anti-CD40 Ab and poly(I:C) (8.5±0.4%), CTGF/MSLN vaccine combined with EGCG (7.0±0.4%), and

the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG (8.3±0.3%) had significantly higher percentages of $CD11c^{+}CD86^{+}$ cells (mature DCs) in TME than the other groups (tumor only: 1.2±0.1%, CTGF/MSLN alone: 1.3±0.0%, and CTGF/MSLN combined with AG490: 2.7±0.3%; $P<0.001$, one-way ANOVA, Fig. 6E). However, the percentages of mature intratumoral DCs in CTGF/MSLN vaccine combined with anti-CD40 Ab and poly(I:C), CTGF/MSLN vaccine combined with EGCG, and the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG groups did not show difference ($P=0.06$, one-way ANOVA, Fig. 6E).

Therefore, our data indicate that the CTGF/MSLN DNA vaccine combined with DC maturation agents could generate potent therapeutic effects against lethal MSLN-expressing tumor cells.

3.7 Meso-VAX cell-based or CTGF/MSLN DNA vaccine combined with different immuno-modulators to generate more potent anti-tumor effects

Finally, we evaluated whether different MSLN-specific vaccines combined with different immuno-modulators can generate similar or different anti-tumor effects. The results of the analysis of mice immunized with Meso-VAX cell-based or CTGF/MSLN DNA vaccine with AAV-IL-12 or anti-CD40 Ab and poly(I:C) are shown in Figure 7A. As shown in Figure 7B, mice that received Meso-VAX cell-based vaccine with AAV-IL-12 **(**$3.7\pm0.1\times10^{6}$**)** exhibited less luminescence than

those that received anti-CD40Ab and poly(I:C) (8.4±1.1×10^6, P=0.01, one-way ANOVA). In contrast, mice that received the CTGF/MSLN DNA vaccine with anti-CD40 Ab and poly(I:C) (5.4±0.3×10^6) exhibited less luminescence than those that received AAV-IL-12 (7.6±0.2×10^6, P=0.01, one-way ANOVA).

Our results indicate that different antigen-specific vaccines, even those targeting the same antigen, need to combine with different immuno-modulators to generate more potent anti-tumor effects.

4. Discussion

In the present study, we developed a chimeric antigen-specific CTGF/MSLN DNA vaccine combined with immune modulator anti-CD40 Ab and TLR-3 ligand–poly(I:C) to target endogenous MSLN-expressing tumor cells. The anti-tumor effects were enhanced by increasing the number of MSLN-specific $CD8^+$ T cells and the complement-dependent toxicity activity using this combinational strategy. In addition to anti-CD40 Ab and poly(I:C), we also demonstrated that the CTGF/MSLN DNA vaccine combined with other DC immuno-modulators, such as EGCG, can enhance MSLN-specific anti-tumor effects. Finally, we showed that different types of antigen-specific cancer vaccines should be combined with various immuno-modulators to achieve more potent anti-tumor effects.

Compared to normal cells, MSLN is overexpressed by cancer cells and is immunogenic to induce MSLN-specific immunity [7-11]. Therefore, developing MSlN-based antibodies, vaccines and T-cell therapies could be an attractive strategy for MSLN-associated cancer immunotherapy [35]. Our previous study revealed that an MSLN-specific cell-based vaccine (Meso-VAX) combined with AAV-IL-12 can generate potent MSLN-specific immunities and anti-tumor effects in an animal model [21]. The CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) also generated potent MSLN-specific immunities and anti-tumor effects in the animal

model used in this survey.

Similar to other therapeutic vaccines targeting MSLN [21,25], MSLN-specific DNA vaccine alone could not generate effective anti-MSLN immunological responses and anti-tumor effects. However, DNA vaccine alone, for example CTGF/E7 or CRT/E7 targeting exogenous antigens such as human papillomavirus E7 oncogene, could generate potent enough E7-specific immunities and anti-tumor effects in E7-expressing tumor cells [19,36]. A possible explanation is host immune tolerance to endogenous antigens, such as MSLN, during carcinogenesis, which is mainly caused by the suppression of APCs, including DCs, in the TME [37,38].

DCs could capture, process and present the TAAs on major histocompatibility complex (MHC) class I and class II molecules and migrate to draining lymph nodes to activate effector T lymphocytes [38]. If capture and presentation occurs in the presence of immunogenic maturation signals, DCs could elicit anticancer protective T-cell responses in the lymphoid organs [39]. In addition to effector T cells, DCs may trigger antibody and natural killer or natural killer T cell responses, which also contribute to tumor immunity. These maturation signals could be supplied endogenously by, for example, dying or necrotic tumor cells releasing factors, or exogenously by, for example, TLR ligands or agonist antibodies against activating receptors, including CD40 [38]. Without such maturation stimuli, DCs could induce tolerance leading to

T-cell anergy or the production of regulatory T cells [40-43].

Therefore, combined administration of TLR ligands and CD40 agonists as essential adjuvants for DC maturation is an efficient strategy for optimizing vaccines to enhance protective or therapeutic immunity [44]. In this survey, the CTGF/MSLN DNA vaccine generated more potent MSLN-specific immunity against MSLN-expressing tumor cells when combined with anti-CD40 Ab and poly(I:C). In addition to enhancing MSLN-specific immunologic profiles, mice treated with this combinational therapy could have more activating $CD8^+$ T lymphocytes in spleen and TME and higher intratumoral IFN-γ secreting levels (Supplementary Fig.1). Such regimens also resulted in higher percentages of mature DCs in TME than CTGF/MSLN DNA vaccine alone. In addition to anti-CD40 Ab and poly(I:C), EGCG could also stimulate the maturation of intratumoral DCs and enhance the anti-tumor effects against MSLN-expressing tumor cells when combined with the CTGF/MSLN DNA vaccine.

Cancer vaccines could generate anti-tumor effects through both cell-mediated and humoral immunity. Several cancer vaccines have focused on MHC class I-restricted peptides, which can elicit cytotoxic T lymphocyte activity [45,46]. In addition, humoral immunity generated from cancer vaccines can contribute to the anti-tumor effects through complement- or antibody-dependent cellular cytotoxicity [46]. As shown in the

present study, the CTGF/MSLN chimeric DNA vaccine could generate MSLN-specific Abs, which could induce MSLN-specific complement-dependent cell-mediated cytotoxicity. The CTGF/MSLN chimeric DNA vaccine combined with anti-CD40 Ab and poly(I:C) generated anti-tumor effects through humoral and cell-mediated immunities; these anti-tumor mechanisms may be the result of combinational therapy.

Rational combinations of distinct treatment modalities have been developed to target distinct elements of tumor biology to achieve synergistic antitumor effects [47]. The goal of cancer immunotherapy is to establish a durable population of highly active, tumor-specific T cells [47]. Despite promising developments in cancer immunotherapy, immunotherapeutic approaches in most tumor types have met with failure [48], primarily because cancer cells can apply a variety of pathways to evade immune surveillance [38,47]. To overcome the obstacles of immunotherapy, strategically combining immunotherapies with other immuno-modulators to harness potential synergies is critical for maximizing the benefits for cancer patients [49]. As shown in this and previous studies [21], treatment of tumors with MSLN-specific vaccines alone cannot generate potent anti-tumor effects. Only when antigen-specific vaccines are combined with different immuno-modulator(s) can generate potent anti-tumor effects be generated.

There are some limitations of this study. There was no another alternative

MSLN-expressing tumor model available to validate our results. Therefore, the combinational regimens might be currently considered as an effective modality for treating malignant peritoneal mesothelioma based on the nature of our tumor model. Delivery systems, immunostimulants, and combinations could be classified as vaccine adjuvants of cancer immunotherapeutics [50]. However, not all types of the adjuvants were applied to investigate the mechanism of cancer vaccine in this study. In addition, $CD8^+$ cytotoxic T cells, DCs and complement-dependent cell-mediated cytotoxicity could contribute to anti-tumor effects of this combinational therapeutics. But, the relationships among various immunocytes (such as effectors and suppressors), related cytokine expression levels, and complement system were not completely explored. Therefore, further studies are needed to evaluate the underlying immunological regulation to elucidate the real functional components.

In conclusion, a chimeric MSLN-specific DNA vaccine, CTGF/MSLN, combined with immuno-modulators (e.g., anti-CD40 Ab and TLR-3 ligand–poly(I:C), or ECGC) could be an effective strategy for cancer immunotherapy to control endogenous tumor antigen, such as in MSLN-specific tumors. This strategy could generate both humoral and cell-mediated anti-tumor immunity by inducing the maturation of DCs. Application of this strategy to the endogenous MSLN-expressing tumors including ovarian and pancreastic cancers, and malignant peritoneal

mesothelioma may be indicated.

Acknowledgement

This work was supported, in part, by Instrumentation Center, College of Science, National Taiwan University, the 2nd and 7th core laboratory facilities of the Department of Medical Research of National Taiwan University Hospital and granted by the Ministry of Science and Technology (100-2325-B-002 -003, 101-2325-B-002 -004, 102-2325-B-002 -004, and 103-2314-B-002-191-MY3) and National Taiwan University Hospital (NTUH 106-S3495). The lentiviral vector used to construct WF-3/Luc were obtained from the National RNAi Core Facility Platform located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, supported by the National Core Facility Program for Biotechnology Grants of National Science Committee of Taiwan (100-2319-B-001-002).

Conflict of interests

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. ***CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and TLR-3 ligand–poly(I:C) generated tumor protection effects against MSLN-expressing tumor cells.*** (**A**) Diagrammatic representation of various prevention protocols using DNA vaccination with or without anti-CD40 Ab and/or poly(I:C). **(B)** Overall survival of mice treated with various DNA vaccines. None of the mice vaccinated with no insert, MSLN, CTGF, or CTGF/MSLN DNA alone could survive longer than 63 days after tumor challenge (P=0.58, log-rank test). **(C)** Overall survival of mice treated with the CTGF/MSLN DNA vaccine and anti-CD40Ab and/or poly(I:C). Compared with the group receiving CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C), all the mice receiving CTGF/MSLN DNA vaccine alone, or CTGF/MSLN DNA vaccine combined with anti-CD40 Ab or poly(I:C) could not survive after 63 days of tumor challenge (P<0.001, log-rank test). Data are from at least two experiments.

Figure 2. CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) enhanced the MSLN-specific immunologic profiles. (A) Number of mesothelin-specific IFN-γ-secreting $CD4^+$ helper T cell precursors/3.5×10^5 splenocytes by flow cytometry (mean±SEM). The number of MSLN-specific IFN-γ-secreting $CD4^+$ helper T lymphocytes of mice immunized with CTGF/MSLN

DNA vaccine combined with anti-CD40 Ab and poly(I:C) was similar to the other groups (*P*=0.62, one-way ANOVA). *Note:* Column 1: no insert, 2: MSLN, 3: CTGF, 4: CTGF/MSLN alone, 5: CTGF/MSLN with anti-CD40 Ab, 6: CTGF/MSLN with poly(I:C), 7: anti-CD40 Ab with poly(I:C), 8: CTGF/MSLN with anti-CD40 Ab and poly(I:C). **(B)** Representative figures of mesothelin-specific IFN-γ-secreting $CD8^+$ cytotoxic T cell precursors in various vaccinated groups by flow cytometry. *Note:* Column B1: no insert, B2: MSLN, B3: CTGF, B4: CTGF/MSLN alone, B5: CTGF/MSLN with anti-CD40 Ab, B6: CTGF/MSLN with poly(I:C), B7: anti-CD40 Ab with poly(I:C), B8: CTGF/MSLN with anti-CD40 Ab and poly(I:C). **(C)** Numbers of mesothelin-specific IFN-γ-secreting $CD8^+$ cytotoxic T cell precursors/3.5×10^5 splenocytes by flow cytometry (mean±SEM). Mice vaccinated with the CTGF/MSLN DNA vaccine and anti-CD40 Ab with poly(I:C) had significantly more MSLN-specific IFN-γ-secreting $CD8^+$ T cell precursors than the other groups (*P*=0.004, one-way ANOVA). *Note:* Column1: no insert, 2: MSLN, 3: CTGF, 4: CTGF/MSLN alone, 5: CTGF/MSLN with anti-CD40 Ab, 6: CTGF/MSLN with poly(I:C), 7: anti-CD40 Ab with poly(I:C), 8: CTGF/MSLN with anti-CD40 Ab and poly(I:C). **(D)** Mesothelin-specific Abs detected by ELISA in various vaccinated groups (mean±SEM). The CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) resulted in the highest titers of anti-MSLN Ab among the groups

(OD450 in 1:100 dilution, $P<0.001$, one-way ANOVA). Data are from at least two experiments.

Figure 3. ***CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) could significantly reduce peritoneal tumors.*** **(A)** Diagrammatic representation of different therapeutic protocols using CTGF/MSLN DNA vaccine with or without anti-CD40 Ab and/or poly(I:C). **(B)** Numbers of mesothelin-specific IFN-γ-secreting $CD8^+$ cytotoxic T cell precursors/3.5×10^5 splenocytes 7 days after last vaccination (=on day 21 after tumor challenge) by flow cytometry (mean±SEM). Compared with the other groups, mice receiving the CTGF/MSLN DNA vaccine and anti-CD40 Ab with poly(I:C) had significantly more MSLN-specific IFN-γ-secreting $CD8^+$ T cell precursors ($P<0.001$, one-way ANOVA). *Note:* Column1: no insert, 2: CTGF/MSLN alone, 3: anti-CD40 Ab with poly(I:C) alone, 4: CTGF/MSLN with anti-CD40 Ab, 5: CTGF/MSLN with poly(I:C), 6: CTGF/MSLN with anti-CD40 Ab and poly(I:C). **(C)** Representative luminescence images of mice in various groups using the IVIS system at the indicated intervals. **(D)** Luminescence of tumor-bearing mice in various vaccinated groups (mean±SEM). Mice immunized with the CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) exhibited the least luminescence ($P<0.001$, one-way ANOVA). **(E)** Survival analysis of mice in the various vaccinated groups. Seventy percent of mice that received CTGF/MSLN DNA vaccination

combined with anti-CD40 Ab and poly(I:C) were alive 90 days after WF-3/Luc tumor challenge and none of the mice in the other groups could survive more than 63 days (P<0.001, log-rank test). Data are from at least two experiments.

Figure 4. CD8$^+$ cytotoxic T cells and DCs were essential for the anti-tumor effects of CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C). (A) Representative luminescence images of the mice in various Ab depletion groups. Mice immunized with CTGF/MSLN DNA vaccine with or without anti-CD40 Ab and/or poly(I:C) were challenged with 1x10^5 WF-3/Luc tumor cells 7 days after the last vaccination. One week before tumor challenge, 100 μg/mouse of purified monoclonal antibodies GK1.5, 2.43, and PK136, and AFS98 were applied to deplete CD4+, CD8+, and NK1.1, CSF1R (DC depletion), respectively. Depletion was performed every other day for 1 week and every week onwards and terminated 70 days after the tumor challenge. The tumor growth was monitored by the IVIS twice a week until the mice died or were sacrificed on day 100. **(B)** Luminescence of mice in various Ab depletion groups (mean±SEM). Mice with CD8 and CSF1R Ab depletion exhibited greater luminescence than those with CD4 or NK Ab depletion (P<0.001, one-way ANOVA). **(C)** Survival analysis of mice in various Ab depletion groups. None of the mice with depleted CD8$^+$ cytotoxic T lymphocytes and DCs were alive more than 60 days after WF-3/Luc tumor challenge. Compared with the CD8 cell- and

DC-depleted groups, all of the mice without Ab depletion, with depleted NK cells, and 80% of the mice with depleted $CD4^+$ cells remained alive 100 days after tumor challenge (P<0.001, log-rank test). Data are from at least two experiments.

Figure 5. ***CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) could enhance MSLN-specific complement-dependent cell-mediated cytotoxicity***

(A) Representative luminescence images in various groups. **(B)** Quantification of luminescence in mesothelin-specific complement-dependent cell-mediated cytotoxicity assays (mean±SEM). Sera collected from various groups were added into the well having WF-3/Luc tumor cells (5×10^3/well). Then, naïve rabbit serum in culture medium at a final dilution of 1:5 was used as complement in a total volume of 100 μL. After incubation for 18 h, cell viability was measured. Sera from mice vaccinated with CTGF/MSLN combined with anti-CD40Ab and poly(I:C) exhibited the least luminescence among the vaccinated groups (P<0.001, one-way ANOVA for both 20 and 50 μL of sera). **(C)** Detection of cleaved PARP and caspase 3 in various groups by western blotting. The expression levels of cleaved PARP and caspase 3 of WF-3/Luc cells treated with sera from the CTGF/MSLN combined with anti-CD40Ab and poly(I:C) mice were highest as compared with the other groups. Data are from at least two experiments.

Figure 6. ***CTGF/MSLN DNA vaccine combined with various DC maturation agents***

could generate potent anti-tumor effects. **(A)** Representative flow cytometric analysis of the maturation status of BMM-derived DCs treated with immuno-modulator. (**B)** Maturation status of BMM-derived DCs treated with anti-CD40 Ab and poly(I:C), EGCG, or AG490 *in vitro* (mean±SEM). After the BMM-derived DCs were stimulated by LPS, the anti-CD40 Ab and poly(I:C)-treated group had the highest percentage of $CD11c^{+}CD86^{+}$ cells. The EGCG-treated group also had a higher percentage of $CD11c^{+}CD86^{+}$ cells than the AG490-treated and naïve groups (P=0.01, one-way ANOVA). **(C)** Representative luminescence images for various groups. **(D)** Luminescence of tumor-bearing mice in various vaccinated groups (mean±SEM). CTGF/MSLN combined with anti-CD40 Ab with poly(I:C) group exhibited the least luminescence. The CTGF/MSLN vaccine combined with EGCG-treated group exhibited less luminescence than the naïve group (P<0.001, Student's t-test), and the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG-treated group also exhibited less luminescence than the naïve group (P<0.001, Student's t-test). The luminescence activities of CTGF/MSLN vaccine combined with anti-CD40 Ab and poly(I:C)-, CTGF/MSLN vaccine combined with EGCG, and the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG groups did not show significant difference (P=0.14, one-way ANOVA). The CTGF/MSLN vaccine combined with AG490 group exhibited the similar luminescence to the naïve group (P=0.20, Student's

t-test). **(E)** Percentages of $CD11c^{+}CD86^{+}$ cells (mature DCs) in tumor sites on day 28 after tumor challenge by flow cytometry (mean±SEM). Mice receiving the CTGF/MSLN vaccine combined with anti-CD40 Ab and poly(I:C), CTGF/MSLN vaccine combined with EGCG, and the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG had significantly higher percentages of mature DCs in TME than the other groups (P<0.001, one-way ANOVA). However, the percentages of mature intratumoral DCs in CTGF/MSLN vaccine combined with anti-CD40 Ab and poly(I:C)-, CTGF/MSLN vaccine combined with EGCG-, and the CTGF/MSLN vaccine combined with anti-CD40 Ab, poly(I:C) and EGCG-treated groups did not show significant difference (P=0.06, one-way ANOVA). Data are from at least two experiments.

Figure 7. Different MSLN-specific vaccines could generate potent anti-tumor effects in combination with different immuno-modulators. (A) Representative luminescence images of mice vaccinated with Meso-VAX or CTGF/MSLN DNA vaccine combined with AAV-IL-12 or anti-CD40 Ab and poly(I:C). **(B)** Luminescence of mice in various groups (mean±SEM). The mice that received Meso-VAX cell-based vaccine and AAV-IL-12 exhibited less luminescence than mice that received Meso-VAX cell-based vaccine and anti-CD40Ab and poly(I:C) (P=0.01, one-way ANOVA). In contrast, mice that received CTGF/MSLN DNA vaccine with

anti-CD40 Ab and poly(I:C) exhibited less luminescence than mice that received CTGF/MSLN DNA vaccine and AAV-IL-12 (P=0.01, one-way ANOVA). Data are from at least two experiments.

Supplementary Figure 1. Mice treated with CTGF/MSLN DNA vaccine combined with anti-CD40 Ab and poly(I:C) had higher numbers of activated $CD8^+$ T lymphocytes in spleen and TME, and intratumoral IFN-γ secretion.

(A) Percentages of activated $CD8^+$ T lymphocytes in spleen on day 28 after tumor challenge by flow cytometry (mean±SEM). CD 223 was considered as activation marker of T lymphocytes. Mice vaccinated with the CTGF/MSLN DNA vaccine and anti-CD40 Ab with poly(I:C) (2.8±0.1%) had significantly higher percentages of $CD223^+CD8^+$ T lymphocytes than the other groups (no insert: 1.0±0.0%, CTGF: 1.2±0.0%, MSLN: 1.0±0.0%, CTGF/MSLN alone: 1.3±0.0%, anti-CD40 Ab with poly(I:C) alone: 1.2±0.0%, CTGF/MSLN with anti-CD40 Ab: 1.1±0.0%, and CTGF/MSLN with poly(I:C): 1.1±0.0%; P<0.001, one-way ANOVA). **(B)** Percentages of activated $CD8^+$ T lymphocytes in TME on day 28 after tumor challenge by flow cytometry (mean±SEM). Mice vaccinated with the CTGF/MSLN DNA vaccine and anti-CD40 Ab with poly(I:C) (5.6±0.2%) had significantly higher percentages of $CD223^+CD8^+$ T lymphocytes than the other groups (no insert: 1.6±0.1%, CTGF: 1.5±0.1%, MSLN: 1.5±0.0%, CTGF/MSLN alone: 1.9±0.1%, anti-CD40 Ab with poly(I:C) alone: 1.4±0.1%, CTGF/MSLN with anti-CD40 Ab: 1.4±0.1%, and CTGF/MSLN with poly(I:C): 1.4±0.1%; P<0.001, one-way ANOVA). **(C)** Expression levels of IFN-γ in TME/100 μg samples on day 28 after tumor

challenge by ELISA (mean±SEM). Mice vaccinated with the CTGF/MSLN DNA vaccine and anti-CD40 Ab with poly(I:C) (274.3±4.9 pg/mL) had significantly higher intratumoral IFN-γ levels than the other groups (no insert: 24.6±1.6 pg/mL, CTGF: 23.3±2.5 pg/mL, MSLN: 23.1±3.5 pg/mL, CTGF/MSLN alone: 71.0±2.2 pg/mL, anti-CD40 Ab with poly(I:C) alone: 62.1±3.7 pg/mL, CTGF/MSLN with anti-CD40 Ab: 61.3±3.9 pg/mL, and CTGF/MSLN with poly(I:C): 54.0±2.7 pg/mL; $P<0.001$, one-way ANOVA). *Note:* Column1: no insert, 2: MSLN, 3: CTGF, 4: CTGF/MSLN alone, 5: CTGF/MSLN with anti-CD40 Ab, 6: CTGF/MSLN with poly(I:C), 7: anti-CD40 Ab with poly(I:C), 8: CTGF/MSLN with anti-CD40 Ab and poly(I:C). All data are from at least two experiments.

Figure 1

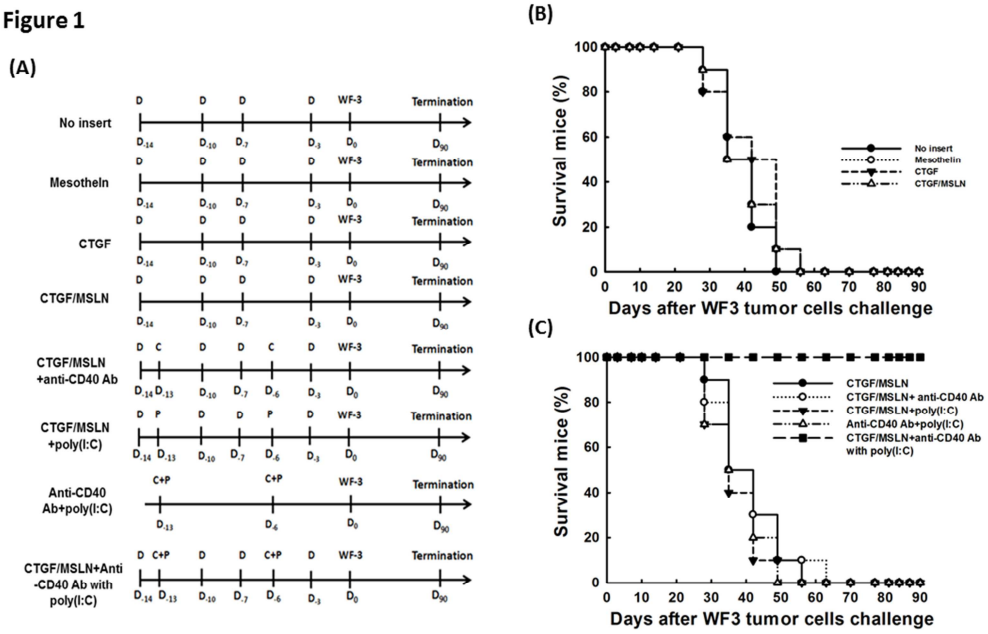
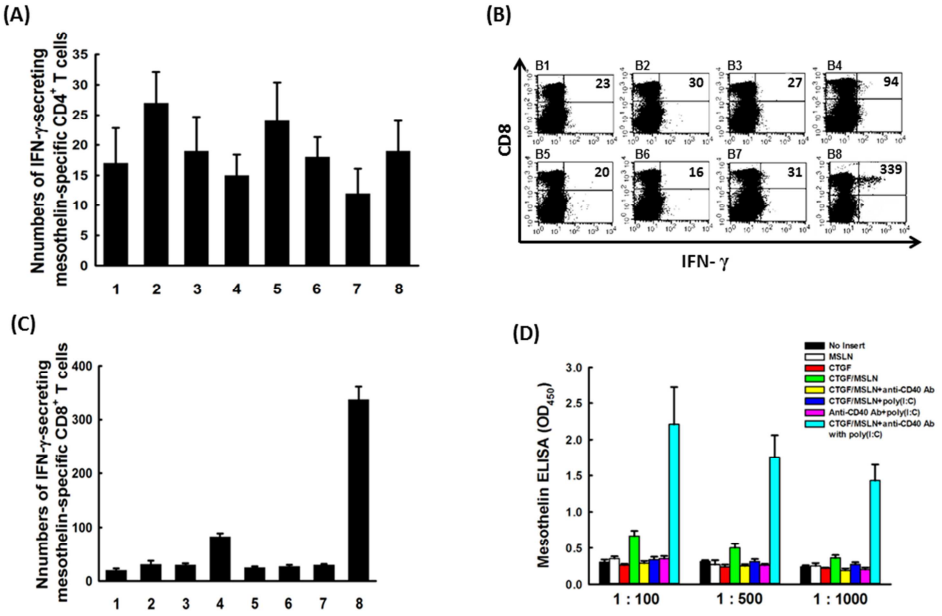


Figure 2



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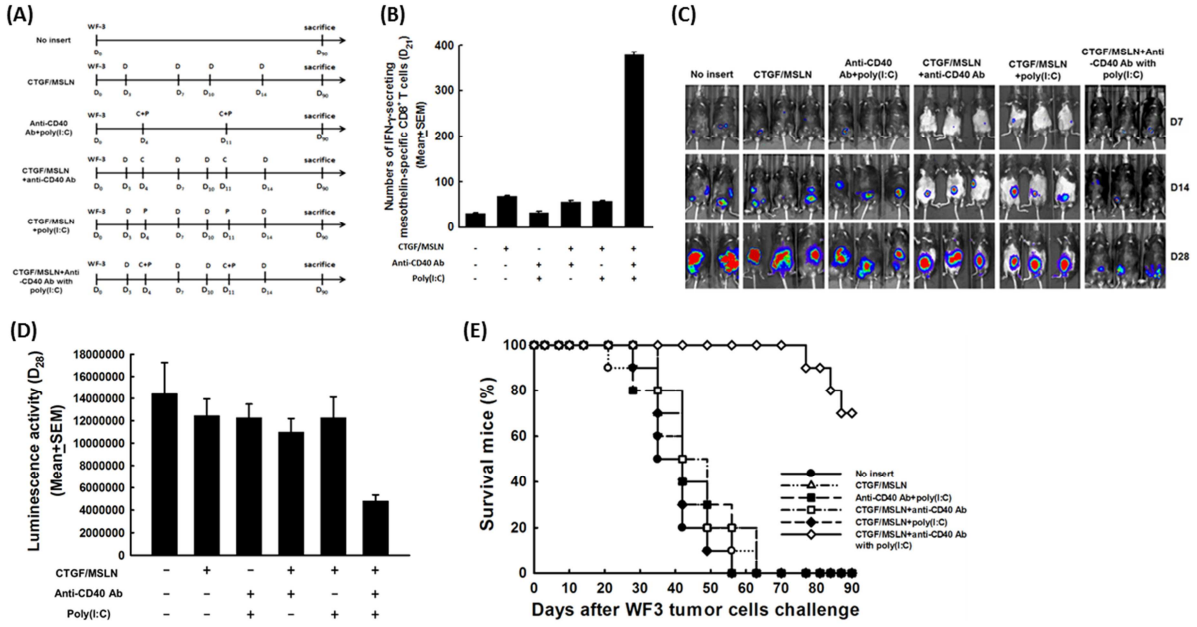

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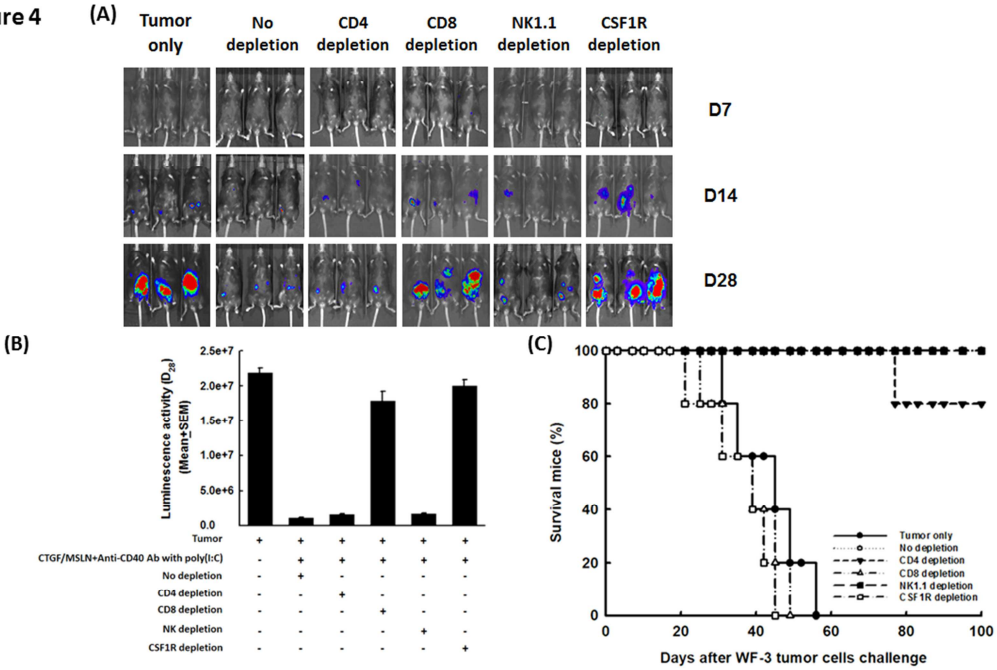
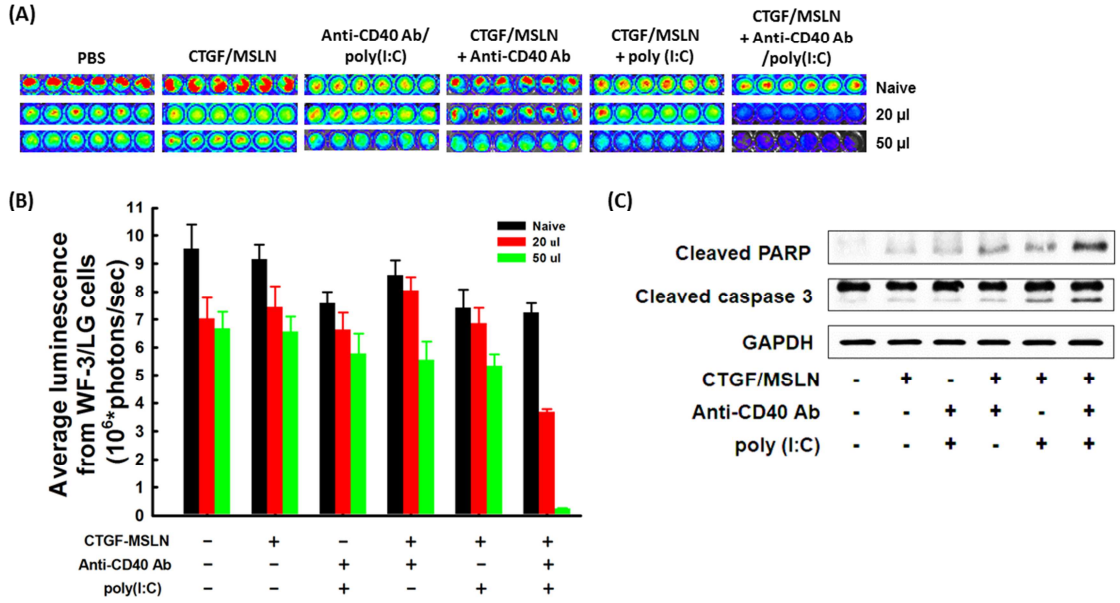


Figure 5



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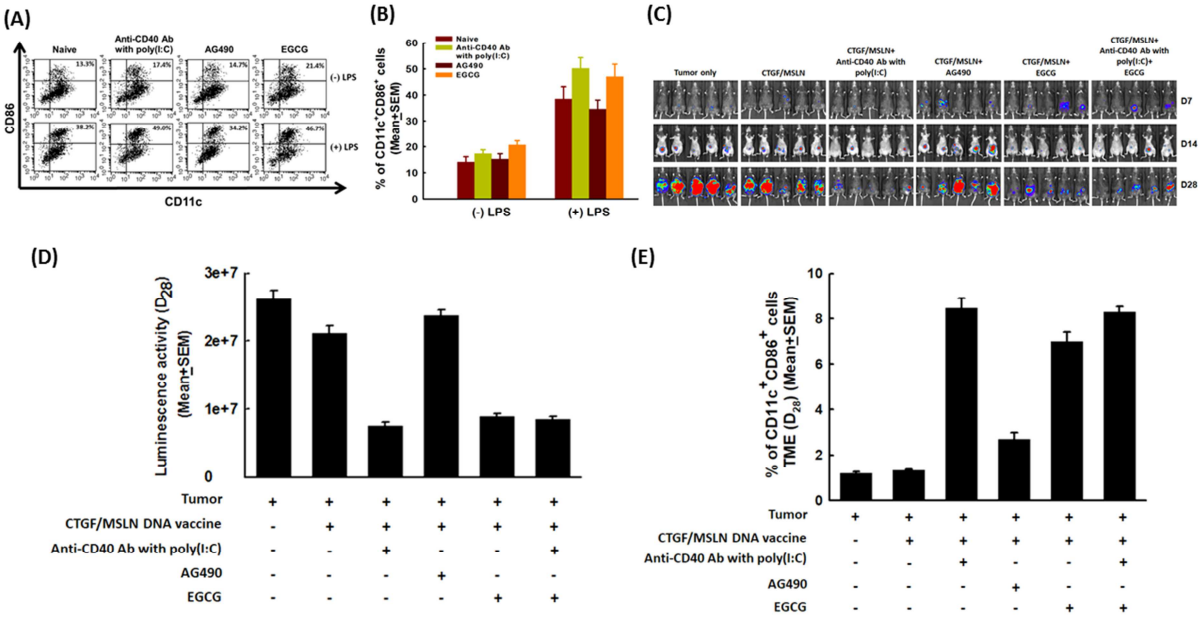
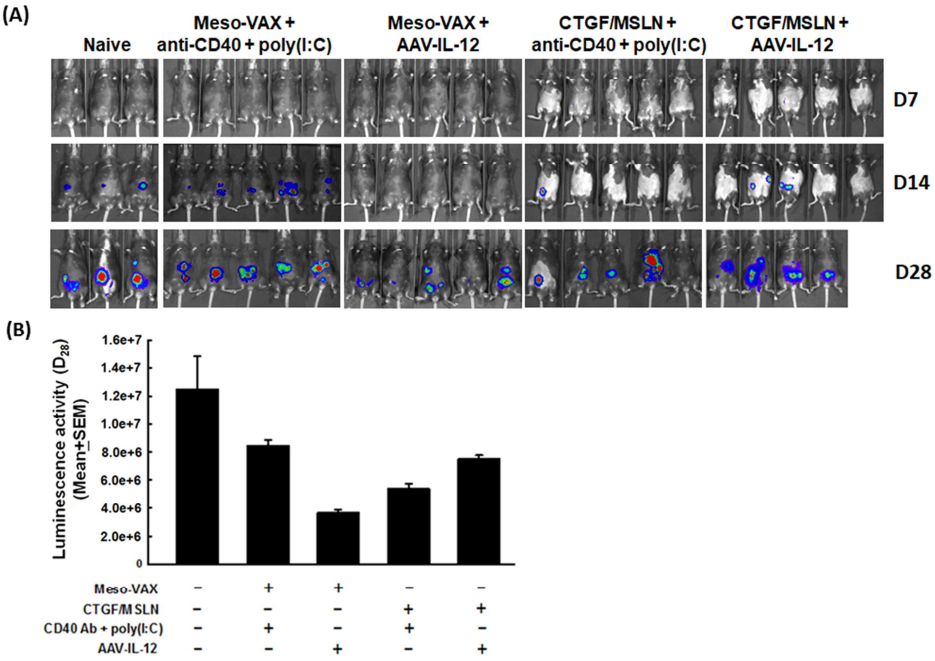


Figure 7



Highlights

An MSLN-specific DNA vaccine combined with immuno-modulators can be an effective strategy for cancer immunotherapy to control MSLN-expressing tumors.

Immuno-modulators could enhance the antigen-specific anti-tumor effects of CTGF/MSLN DNA vaccine through promoting the maturation of DCs.

Conflict of interest

The authors declare that they have no conflicts of interest.