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# The efficacy of a novel vaccine approach using tumor cells that ectopically express a codon-optimized murine GM-CSF in a murine tumor model

Chu-Chi Lin[a,1], Ching-Chou Tsai[b,1], Jan-Mou Lee[c,1], Chih-Hao Fang[a], Kuo-Shian Chang[a], Kwong-Kwok Wong[d], Cheng-Tao Lin[f], Jiantai Timothy Qiu[a,e,f,*]

[a] Graduate Institute of Biomedical Sciences, Chang Gung University, Taoyuan, Taiwan, ROC
[b] Department of Obstetrics and Gynecology, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung, Taiwan, ROC
[c] Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, ROC
[d] Department of Gynecologic Oncology and Reproductive Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA
[e] Department of Biomedical Sciences, School of Medicine, Chang Gung University, Taoyuan, Taiwan, ROC
[f] Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Taoyuan, Taiwan, ROC

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ABSTRACT

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a potent immunomodulatory cytokine that is known to facilitate vaccine efficacy by promoting the development and prolongation of both humoral and cellular immunity. Here, we investigated a novel vaccine approach using a human papillomavirus (HPV)-16 E6/E7-transformed cell line, TC-1, that ectopically expresses a codon-optimized 26-11-2015 murine GM-CSF (cGM-CSF). Ectopically expressing cGM-CSF in TC-1 (TC-1/cGM) cells significantly increased expression of a GM-CSF that was functionally identical to wt GM-CSF by 9-fold compared with ectopically expressed wild type GM-CSF in TC-1 cells (TC-1/wt). Mice vaccinated with irradiated TC-1/cGM cells exhibited enhanced survival compared with mice vaccinated with TC-1/wt cells when both groups were subsequently injected with live TC-1. Consistently, mice vaccinated with irradiated TC-1/cGM cells exhibited stronger IFN-γ production in HPV E7-specific $CD8^+$ T cells. More dendritic cells were recruited to the draining lymph nodes (dLNs) of mice vaccinated with TC-1/cGM cells than C-1/wt cells. Regarding dLN cell recall responses, both proliferation and IFN-γ production in the HPV E7-specific $CD8^+$ T cells were enhanced in mice that were vaccinated with TC-1/cGM cells. Our results demonstrate that a novel practical molecular strategy utilizing a codon-optimized GM-CSF gene overcomes the limitation and improves the efficacy of tumor cell-based vaccines.



## 1. Introduction

Cervical cancer is the second most frequent gynecological malignancy worldwide and accounts for approximately 12% of all cancers in women [1]. Greater than 99% of cervical cancer patients are carriers of human papillomaviruses (HPV), which makes HPV a major risk factor for cervical cancer [2]. HPVs are double-strand DNA viruses with a genome size of approximately 8 kb that encode 8 or 9 open reading frames consisting of eight early (E1–E9) and two late (L1 and L2) gene products [3]. HPV integration into the genome and high-level E6 and E7 expression are often associated with the manifestation of high-grade cervical neoplasia [2,4,5]. Although virus-like particle (VLP)-based vaccines have been developed with prophylactic activities to prevent most HPV infections [6,7], the therapeutic effect of VLP vaccines has yet to be demonstrated for those who were already infected [8]. Additionally, although prophylactic vaccines induce a humoral immune response against the viral capsid protein L1, a cellular immune response against virus early proteins, E6 and E7, is required to eliminate previously infected cells [9,10]. The viral transforming proteins, E6 and E7, are consistently expressed in cervical cancer cell lines and HPV-associated neoplasms. Thus, E6 and E7 represent true tumor-specific antigens and serve as a target for the development of immunotherapeutic strategies to combat HPV-associated cancers.

Granulocyte macrophage-colony stimulating factor (GM-CSF) was initially characterized as a factor that can support the *in vitro* colony formation of granulocyte-macrophage progeni-

* Corresponding author at: Division of Gyn-Oncology, Department of Obs/Gyn, Chang Gung Memorial Hospital, 5 Fushing Street, Kwei-Shan, Taoyuan 333, Taiwan, ROC. Tel.: +886 3 2118800x3494; fax: +886 3 2118700.
*E-mail address:* jtqiu@adm.cgmh.org.tw (J.T. Qiu).
[1] These authors contributed equally to this work.

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tors [11]. GM-CSF also serves as a growth factor for erythroid, megakaryocyte, and eosinophil progenitors. GM-CSF is produced by various cell types, including T cells, B cells, macrophages, mast cells, endothelial cells, fibroblasts, and adipocytes, in response to cytokine or inflammatory stimuli. In mature hematopoietic cells, GM-CSF is a survival factor and activates the effector functions of granulocytes, monocytes/macrophages, and eosinophils. In recent years, both murine tumor models and human clinical trials revealed that GM-CSF-secreting tumors cells can serve as an option for immunotherapy in several tumor types, including non-small cell lung carcinoma [12], pancreatic [13], prostate [14], melanoma [15], leukemia [16], gliomas [17], cervical [18], and renal cell carcinoma [19]. However, GM-CSF gene expression is highly regulated at multiple levels [20–22]. Native GM-CSF protein is poorly expressed in a tissue-specific and activation-dependent manner. A clinical trial demonstrated that a threshold level of GM-CSF is required for the induction of effective immunity [23]. We previously described that the co-administration of plasmids encoding the codon-optimized murine GM-CSF (cGM) sequence with a DNA vaccine resulted in a strong and protective antibody and cytotoxic T lymphocyte (CTL) immune response against recombinant vaccinia virus challenge [24].

In the present study, we applied a lentiviral-delivered codon-optimized murine GM-CSF into a HPV-16 E6/E7-transformed cell line, TC-1, as a vaccine adjuvant approach. The goal of the present study was to assess whether codon-optimized murine GM-CSF that is transduced into cancer cells as a vaccine would increase (dendritic cell) DC recruitment and result in enhanced antigen (Ag)-specific immune responses [28]. The lentiviral-modified TC-1/cGM cells showed significantly increased GM-CSF protein expression levels as demonstrated by Western blot and (enzyme-linked immunosorbent assay) ELISA assays. The GM-CSF secreted from the lentiviral-modified TC-1/cGM cell culture medium exhibited identical biological functions to the WT GM-CSF in its ability to support NFS-60 cell growth. The infected cells were continuously propagated for more than 30 passages, and stable lentiviral transgene expression was confirmed. We subcutaneously (s.c.) immunized C57BL/6 mice with a lethally irradiated TC-1/cGM cell-based vaccine and subsequently quantified $CD11c^+$ DC and $CD8^+$ by flow cytometry analysis. Vaccination with TC-1/cGM induced strong systemic $CD8^+$ T cell immune responses against lethal TC-1 challenge and specific MHC class I HPV E7 epitopes, as demonstrated by an enzyme-linked immunospot (ELISPOT) assay. Peak DC recruitment was detected at 72 h post-inoculation in dLNs and was significantly increased ($p < 0.05$) in mice that were vaccinated with TC-1/cGM cells compared with control mice or mice inoculated with TC-1/wt cells. These results support the use of this novel codon-optimized GM-CSF for immunotherapeutic cancer vaccine strategies.

## 2. Materials and methods

### 2.1. Mice

Female C57BL/6 (B6) mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) and housed under specific pathogen-free (SPF) conditions at the animal facility of Chang-Gung University. All of the experiments were approved by the Animal Ethics Committee, Chang Gung University. All of the mice that were used were 8–12 weeks in age.

### 2.2. Cell lines

TC-1 cells were generated by co-transformation of primary B6 mouse lung epithelial cells with HPV-16 E6/E7 in combination with an activated *H-ras* oncogene, as described previously [29]. The cell lines are routinely maintained in RPMI 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% heat-inactivated bovine calf serum (Invitrogen, Carlsbad, USA), 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 μM β-mercaptoethanol (complete RMPI-1640) at 37 °C in an atmosphere of 5% $CO_2$. TC-1 cells that stably expressed wt and cGM-CSF were established by infection with lentiviral vectors carrying a wt (LV-wtGM) or a cGM-CSF gene (LV-cGM) [30–32]. A lentiviral vector with an e-GFP reporter gene (LV-eGFP) was used as a negative control. NFS-60 cells (a kind gift from Dr. Tao, Mi-Hua in Academic Sinica, Taipei, Taiwan) are a murine myelogenous leukemia cell line that was derived from an NFS/N mouse and are routinely maintained in complete RPMI 1640 supplemented with 62 ng/ml GM-CSF (Peprotech Inc., Rocky Hill, NJ) [33,34].

### 2.3. Cytokine secretion measurement with ELISA

The levels of GM-CSF, interleukin 4 (IL-4) and interferon gamma (IFN-γ) secreted from the cells were measured by ELISA using commercially available ELISA kits (GM-CSF: R&D, MN, USA; IL-4 and IFN-γ: eBiosicence, CA, USA) following the manufacturers' instructions. The differences between the GM-CSF secretion levels in non-irradiated and irradiated cells (treated with 10,000 rad irradiation) were compared by ELISA. For non-irradiated cells, $1 \times 10^6$ TC-1 and its transgenic clones were cultured, and the cell supernatant was collected after 24 h for ELISA analysis. Irradiated cells ($2 \times 10^5$) were incubated for 4 days. The cell supernatant was collected every 24 h for ELISA analysis.

### 2.4. Immunoblotting

The TC-1 and lentivirus-infected cell lysates (TC-1/null, TC-1/wtGM and TC-1/cGM) were prepared as previously described [35]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard methods. GAPDH was utilized as the loading control in Western blots for protein normalization. Proteins were transferred to nitrocellulose filters (Schleicher and Schuell). The blots were stained with rabbit anti-murine GM-CSF (PeproTech, Rocky Hill, NJ) in a phosphate-buffered saline (PBS) solution with 1% nonfat dried milk. An alkaline phosphatase-conjugated goat anti-rabbit antibody (KPL, Rockville, MD) was utilized as the secondary antibody, and staining was performed with a solution containing 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium that was prepared from chemicals obtained from Sigma (St. Louis, MO).

### 2.5. Determination of the functional activities of the GM-CSF proteins

For the functional assay, $1.25 \times 10^5$ GM-CSF-dependent NFS-60 cells (ATCC CRL-1838) were cultured with 62 ng/ml commercial recombinant GM-CSF proteins (positive control) as ATCC suggested or supernatant that was collected 72 h after culturing TC-1/wtGM or TC-1/cGM cells that were adjusted to the identical concentrations with an ELISA. NFS-60 cells were also cultured in medium alone (negative control) and TC-1 cell supernatant. The NFS-60 cell number was measured with a hemocytometer under a light microscope (Leica DM IL) every 12 h. The supernatants were collected 24 h after culturing, and the corresponding GM-CSF levels were determined with an ELISA.

### 2.6. Vaccine immunization mice model

For the tumor protection experiment, mice ($n = 10$ per group) were subcutaneously injected in the left abdominal region with