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The Effect of Iontophoretic-Delivered Polyplex Vaccine on Melanoma Regression

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Although the strategy in cancer vaccination is to provide a therapeutic effect against an established tumor, there is an urgent need to develop prophylactic vaccines for non-viral cancers. In this study, we prepared polyplex nanoparticles through electrostatic interactions between a positively-charged modified tumor associated antigen, namely human derived melanoma gp100$_{25–33}$ peptide (KVPRNQDWL-RRRR), and a negatively charged cytosine-phosphate-guanosine motif (CpG-ODN) adjuvant. We previously demonstrated successful transdermal delivery of various hydrophilic macromolecules by iontophoresis (IP) using weak electricity. Herein, we investigated the effectiveness of IP in the transdermal delivery of a prophylactic polyplex vaccine. IP was successful in establishing a homogenous distribution of the vaccine throughout skin. Efficacy of the vaccine was demonstrated against melanoma growth. A significant tumor regression effect was observed, which was confirmed by elevated mRNA expression levels of various cytokines, mainly interferon (IFN)-γ, as well as infiltration of cytotoxic CD8$^+$ T cells. Additionally, we evaluated the therapeutic effect of the vaccine and we found a significant reduction in tumor burden. Stimulation of systemic immunity was confirmed by upregulation of IFN-γ. This is the first report to demonstrate the use of IP in the transdermal delivery of a prophylactic melanoma vaccine.

Key words prophylactic cancer vaccine, iontophoresis, polyplex nanoparticle, antigen peptide, cytosine-phosphate-guanosine motif (CpG-ODN) adjuvant, melanoma

INTRODUCTION

Melanoma is the most serious type of skin cancers because of its aggressiveness and prognostic challenges.[1,2] Various therapeutic approaches to treating melanoma are available, including surgery, radiation, chemotherapy and, recently, immunotherapy and targeted therapy.[3] Vaccines offer promising therapeutic strategies among prevailing infectious diseases.[4–6] In the last 2 decades, the scientific community has benefited from host immune surveillance in developing cancer vaccines.[4,5] However, a cancer vaccine should ideally prevent the disease from occurring, while cancer vaccines have typically relied on therapeutic effects against an established tumor.[4,6–8] There are currently 5 U.S. Food and Drug Administration (FDA)-approved prophylactic vaccines to prevent cancers derived from hepatitis B virus (HBV) and human papilloma virus (HPV).[5,9–11] The success in developing these vaccines offers prophylactic cancer vaccines as a promising strategy in the field of cancer research.[9,12] However, the global cancer burden related to viral etiology represents only $<$20%.[9,10,13] Therefore, various cancer research organizations, such as Cancer Research U.K. and National Cancer Institute (NCI), have recognized the urgent need to develop successful prophylactic cancer vaccines for non-viral cancers, with the aim of reducing the malignancy burden worldwide.[9,10,12,14] Designing prophylactic cancer vaccines is a major challenge, in part due to the need to select safe and immunogenic tumor antigens that are considered appropriate for use.[9,15,16]

Human gp100$_{25–33}$ antigen peptide (KVPRNQDWL) is a tumor associated antigen (TAA) that is significantly expressed in melanoma, as well as in normal melanocytes.[17–19] Despite its expression in normal melanocytes, it is the most commonly used antigen in the development of melanoma vaccines.[18] Previous studies have found that utilizing TAAs allows the immune response to cross-react with self-molecules that present on normal tissues, which can result in autoimmunity.[10] To overcome this issue, human gp100$_{25–33}$ antigen peptide has been combined with H-2Db complex, which belongs to a major histocompatibility complex (MHC) subgroup, with such modification leading to increased specificity for melanoma, as well as improvement in binding affinity to MHC I, stability and immunogenicity.[17–20] Indeed, the safety of the modified peptide on normal melanocytes allowed for its use as a tailored immunotherapy that is currently being investigated in clinical trials for its therapeutic effect.[6,21] In the current study, we relied on the safety point of this peptide for conducting a prophylactic study against non-viral cancer.

In recent years, the skin has been considered as the most tailored target for eliciting an immune response.[6,22–24] The skin is fortified with different antigen presenting cells (APCs) that are located in both the epidermal and dermal layers.[22–24] Although invasive transdermal drug delivery routes, such as intradermal injection and subcutaneous (s.c.) injection, have been recognized as efficient delivery routes, they are also as-

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sociated with undesirable characteristics, such as invasiveness, pain, patient phobia, vagal reflex, and risk of infection, and also require special training.[6,22,25] Hence, many researchers have directed their focus and attention toward utilizing non-invasive transdermal drug delivery routes. The stratum corneum of the skin represents the most formidable hindrance for driving drug molecules across the skin, as it only permits the delivery of small hydrophobic molecules.[3,24,25] To overcome this limitation and improve the permeation of hydrophilic macromolecules, several chemical and physical technologies, such as microneedles, iontophoresis, electroporation and sonophoresis, have been employed.[22,26,27] Efforts in our group have focused on the use of iontophoresis (IP) utilizing weak electric current (0.3–0.5 mA/cm^2), which is considered a simple and a non-invasive technology for delivering charged hydrophilic macromolecules across the skin *via* different mechanisms, such as electrorepulsion, electroosmosis and recently, intercellular cleavage of both tight and gap junctions.[3,6,22,25,27] Indeed, we previously demonstrated the successful delivery of various hydrophilic macromolecules (*e.g.*, small interfering RNA (siRNA), cytosine-phosphate-guanosine motif (CpG-ODN), charged liposomes and antibodies) *via* the skin using IP.[6,25,27] Also, IP has been shown to enhance the delivery efficiency and the therapeutic effect of various nanoparticles, such as doxorubicin solid lipid nanoparticles, co-delivery of signal transducer and activator of transcription 3 (STAT3) siRNA and imatinib using gold nanoparticles, and doxorubicin liposomes.[28–30] These studies prove that the weak electricity of IP cannot affect on the stability and the effect of nanoparticles.

In the present study, we evaluated the delivery of a prophylactic melanoma vaccine comprised of both the CpG-ODN adjuvant and an antigen peptide *via* skin using IP. We also evaluated the effectiveness of this vaccine on inhibiting melanoma growth *in vivo* compared with s.c. injection. We investigated cytokines production in different tissues, as well as infiltration of cytotoxic CD8$^+$ and CD4$^+$ T cells in tumor tissue. Finally, we evaluated the therapeutic effect of this vaccine for reducing tumor growth in mice bearing melanoma.

MATERIALS AND METHODS

Animal and Tumor Cells Male C57BL/6J mice (5 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). B16F1 murine melanoma cells (Dainippon Sumitomo Pharma Biomedical Co., Ltd., Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing both 10% fetal bovine serum (FBS) and 1% (v/v) antibiotics penicillin/streptomycin (100 U/mL), and incubated at 37 °C in 5% CO_2 atmosphere. All animal experiments were performed in accordance with the guidelines for care and use of experimental animals approved by the Animal and Ethics Review Committee of Tokushima University.

Materials CpG-ODN (ODN-1826 sequence: 5′-TCCATG ACGTTCCTGACGTT-3′) and Cy3-labeled CpG-ODN were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). Human gp100$_{25–33}$ antigen peptide modified with tetra-arginine moieties (KVPRNQDWL-RRRR) and fluorescein isothiocyanate (FITC)-labeled KVPRNQDWL-RRRR were synthesized by Peptide Institute, Inc. (Osaka, Japan). Optimal cutting temperature (OCT) compound, Dako Fluorescence Mounting Medium and Cellstain® DAPI Solution (4′, 6-diamidino-2-phenylindole, dihydrochloride) were obtained from Sakura Finetek (Tokyo, Japan), Agilent (Santa Clara, CA 95051, U.S.A.) and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), respectively. An Ag-AgCl electrode was purchased from 3 M Health Care (Minneapolis, MN, U.S.A.). ISOGEN with Spin Column RNA extraction reagent was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). All primers were purchased from Eurofins Genomics (Tokyo, Japan) and their sequences are shown in Table 1. PrimeScript™ RT Master Mix (Perfect Real Time) and TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) were purchased from TaKaRa Bio (Shiga, Japan). Bovine serum albumin (BSA) was purchased from Merck (Tokyo, Japan). Rabbit anti-mouse CD4, CD8 and Quantikine Elisa Kit (MIF00, R&D Systems) were obtained from Funakoshi Co., Ltd. (Tokyo, Japan). Goat anti-rabbit immunoglobulin G (IgG) H&L (Alexa Fluor® 488) was purchased from Abcam (ab150077, Tokyo, Japan). All other reagents used in this study were of the highest grade obtainable.

Preparation of Polyplex Nanoparticles Human gp100$_{25–33}$ antigen peptide was modified with four arginine moieties according to our previous study.[6] After that, polyplex nanoparticles were prepared according to a previous report.[31] First, 3.54 μL (8.91 μg) of positively-charged human gp100$_{25–33}$ antigen peptide (KVPRNQDWL-RRRR) (2.52 mg/mL) was diluted with ribonuclease (RNase)-free water to a total volume of 34.2 μL. Then, a 15.8 μL (10 μg) solution of negatively-charged CpG-ODN (0.63 mg/mL) was slowly added to the above diluted antigen peptide solution, and mixed gently with a micropipette, to yield a final volume of 50 μL. The mixture was then allowed to incubate for 20 min at room temperature to facilitate self-assembly of the polyelectrolytes. The N/P ratio of the antigen peptide to CpG-ODN was optimized to prevent any reduction in the surface charge of the polyplex and also to obtain a small particle size. Particle size, zeta-potential, and polydispersity index (PDI) were measured with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). Fluorescent-labeled polyplex nanoparticles were prepared in a similar way *via* gentle mixing of both FITC-labeled antigen peptide and Cy3-labeled CpG-ODN.

Iontophoresis of Fluorescent-Labeled Polyplex Nanoparticles IP was carried out in mice according to our previous

Table 1. Primer Sequences Used for RT-PCR

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
GAPDH (mouse)	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
IFN-γ (mouse)	ACAGCAAGGCGAAAAAGGATG	TGGTGGACCACTCGGATGA
TNF-α (mouse)	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
IL-6 (mouse)	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-12b (mouse)	CTGGAGCACTCCCCATTCCTA	GCAGACATTCCCGCCTTTG

report with some modulations.[27] Briefly, mice were anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg mouse) dissolved in phosphate buffered saline (PBS). Mice were then shaved to expose their dorsal skin for IP application. For the administration of fluorescent-labeled polyplexes, nonwoven fabric (1 cm^2) moistened with 100 μL of labeled polyplexes (20 μg Cy3-CpG-ODN and 21.62 μg FITC-labeled antigen peptide) was placed on the shaved dorsal skin, and another nonwoven fabric (1 cm^2) wetted with 100 μL of PBS was added 1 cm away. Each piece of nonwoven fabric containing either labeled polyplexes or PBS was connected to Ag-AgCl electrodes. The Ag-AgCl electrodes with nonwoven fabric containing labeled polyplexes or PBS were connected to the cathode and anode, respectively, of a power supply (TTI Ellebeau, Inc., model TCCR-3005, Tokyo, Japan). IP was performed with a fixed current of 0.34 mA/cm^2 for 1 h. Finally, mice were incubated for 3 h, followed by excision of their skin for cross sectioning. Additionally, passive diffusion of fluorescent-labeled polyplexes was also performed for 1 h followed by 3 h incubation.

Effect of Iontophoresis on Intradermal Distribution of Fluorescent-Labeled Polyplexes After 3 h of incubation following IP application, mice were euthanized and their skin was excised, embedded in OCT compound, and then frozen with dry ice/ethanol. The frozen skin sections were cut into 10 μm thick sections using a cryostat (CM3050S; Leica Biosystems, Tokyo, Japan). The 10 μm thick frozen sections were mounted onto MAS-coated glass slides with Dako Fluorescence Mounting Medium and stored in the dark until dry. Finally, a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany) was utilized to observe the distribution of fluorescent-labeled polyplexes in the skin sections exposed to IP or passive diffusion.

***In Vivo* Vaccination for Prophylactic Studies** Healthy male C57BL/6J mice (6 weeks old) were pre-immunized by a fixed volume (50 μL) of different vaccine formulations, which were administered *via* one of two routes, namely s.c. injection or IP. These formulations included pre-immunization with either positively-charged human $gp100_{25-33}$ antigen peptide vaccine (KVPRNQDWL-RRRR) (8.91 μg/dose mouse) or polyplex vaccine containing electrostatically-combined antigen peptide (8.91 μg/dose mouse) and CpG-ODN (10 μg/dose mouse), as described above. IP administration was carried out at 0.17 mA/0.5 cm^2 for 1 h. The anode acted as the active electrode for delivery of the positively-charged antigen peptide formulation, while the cathode acted as the active electrode for delivery of the negatively-charged polyplex formulation. Four groups of mice were vaccinated: i) antigen peptide s.c. injection, ii) antigen peptide IP, iii) polyplex s.c. injection and iv) polyplex IP. For each group, 5 doses were administered every 3 d for a total of 13 d. Five days after the last immunization dose, mice were subcutaneously challenged into their flank (site of IP/s.c. application) with B16F1 cells (8×10^4 cells/mouse) suspended in PBS. Tumor diameters were measured every other day using a digital caliper according to the following equation: T_{vol} (mm^3) = length $\times$ width^2 $\times$ 0.5. Tumor volume in vaccinated mice was compared with that of the control group. Mice were euthanized on day 23 (polyplex groups) and day 22 (antigen peptide groups) after tumor cells inoculation. Tissues (skin, spleen, and tumor) of mice vaccinated with polyplexes (IP or s.c. injection) were harvested and stored at −80 °C for further analysis.

RNA Extraction Tissues (skin, spleen, and tumor) were weighed (45–90 mg) and subsequently homogenized in the presence of 1 mL of ISOGEN Lysis reagent using TissueRuptor II (QIAGEN). The homogenate was then incubated for 5 min at room temperature. Finally, total RNA was purified and extracted with ISOGEN with Spin Column RNA extraction reagent according to the manufacturer's instructions. Total RNA concentration and purity were measured with a Nanodrop 8000 (Thermo Fisher Scientific, DE, U.S.A.).

Quantitative Analysis of mRNA Expression Levels of Inflammatory Cytokines in Different Tissues Using RT-PCR cDNA was prepared from the reverse transcription of 2 μg of total RNA extract using PrimeScript™ RT Master Mix and a MJ Mini Personal Thermal Cycler (BioRad Laboratories, Hercules, CA, U.S.A.). The reverse transcription reaction was conducted at 37 °C for 15 min, while inactivation of reverse transcriptase was conducted at 85 °C for 5 min. RT-PCR analysis was performed using TB Green™ Premix Ex Taq™ II and a Thermal Cycler Dice Real Time System III (TaKaRa Bio). For analysis of the mRNA expression levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12b and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cDNA was denatured at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s for amplification. The sequences of the primers used are shown in Table 1. The mRNA expression levels of IFN-γ, TNF-α, IL-6 and IL-12b were calculated using the $2^{-\Delta\Delta Ct}$ method by normalization relative to GAPDH mRNA.

Immunohistochemistry Analysis of Infiltrated $CD8^+$ and $CD4^+$ T Cells in Tumor Tissue after Pre-immunization with Iontophoretic-Administered Polyplexes At day 23 post tumor cells inoculation in pre-immunized mice, the tumor tissue was collected, embedded in OCT and stored at −80 °C, as described above. Then, frozen blocks of tumor tissue were cut into 10 μm thick sections using a cryostat (CM3050S; Leica Biosystems, Tokyo, Japan), and subsequently immunostained for both $CD8^+$ and $CD4^+$ T cells. The tumor sections were washed first by PBS, followed by blocking with 1.5% BSA dissolved in PBS containing 0.1% Tween-20 for 15 min at room temperature. Tumor sections were incubated with the diluted rabbit anti-mouse CD8 and CD4 primary antibodies separately for 18 h at 4 °C. Then, the tumor sections were incubated with the diluted Alexa 488-labeled goat anti-rabbit IgG for 1 h at room temperature. DAPI was used for staining the nucleus. Finally, tumor sections were mounted with Dako Fluorescence Mounting Medium and left to dry. The tumor sections were observed using a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany).

***In Vivo* Vaccination by Polyplexes for Therapeutic Studies** Male C57BL/6J mice (6 weeks old) were subcutaneously challenged into their flank with B16F1 cells (5×10^5 cells/mouse) suspended in PBS (day 0). Treatment with polyplex vaccine was initiated on day 3 after tumor cells inoculation. Five doses were administered on days 3, 6, 9, 12 and 15. Polyplexes were administered *via* one of two routes, namely s.c. injection (near the tumor site) or IP (above the tumor site). The conditions for IP application and the doses of the polyplexes were the same as those described previously for the *in vivo* prophylactic studies. Tumor volume was determined as previously described, and mice were euthanized on day 21 after tumor cells inoculation.

Table 2. Physicochemical Properties of Polyplex Nanoparticles Prepared at Different N/P Ratios

	N/P (0.5)	N/P (1)	N/P (1.5)	N/P (3)
Particle size (nm)	327.6 + 5.62	253.4 + 3.8	254.23 + 3.97	264.37 + 7.38
Zeta potential (mV)	−35.96 + 0.21	−40.4 + 1.1	+0.61 + 0.01	+9.17 + 0.36
Polydispersity index	0.31 + 0.01	0.29 + 0.02	0.19 + 0.03	0.28 + 0.04

Data are mean ± S.D. ($n = 3$).

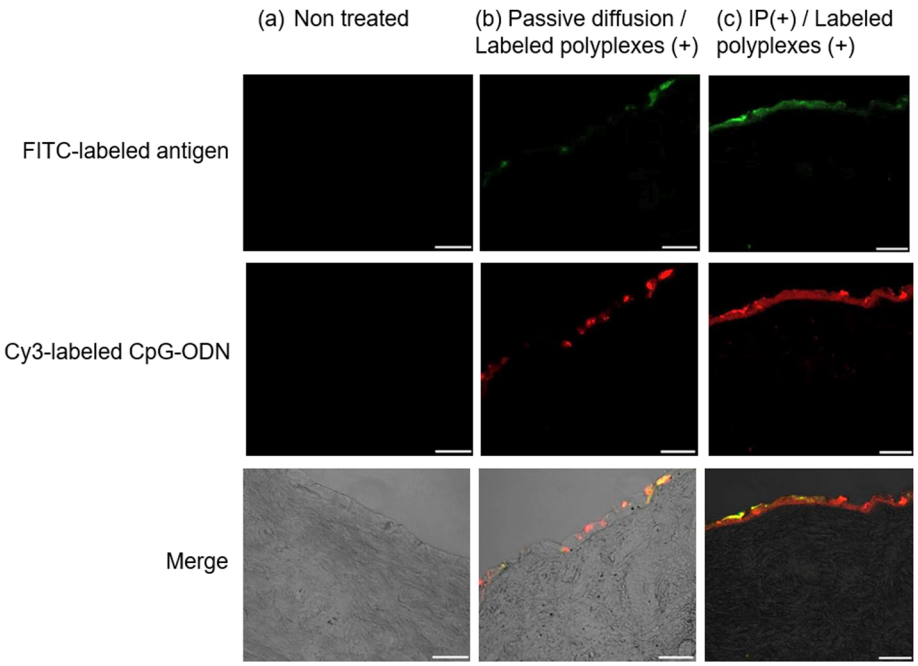

Fig. 1. Distribution of Fluorescent-Labeled Polyplexes in Skin Tissue after IP Application

Labeled polyplexes were prepared by gently mixing FITC-labeled human $gp100_{25\text{-}33}$ (KVPRNQDWL-RRRR) antigen peptide (green) with Cy3-labeled CpG-ODN (red). Cross sections of hairless frozen skin mice (10 μm) were prepared for observation with confocal microscopy. (a) Non-treated. (b) Passive diffusion/labeled polyplexes (+), nonwoven fabric moistened with fluorescent-labeled polyplex solution and attached to the dorsal skin of mice for 1 h followed by 3 h incubation. (c) IP (+)/labeled polyplexes (+), intradermal distribution of fluorescent-labeled polyplexes after IP application (0.34 mA/cm^2, 1 h) followed by 3 h incubation. Merged images of phase contrast, FITC (human $gp100_{25\text{-}33}$ KVPRNQDWL-RRRR; green), and Cy3(CpG-ODN; red) are shown. Scale bars = 50 μm

Skin, spleen and tumor tissues were harvested and stored at −80 °C for further analysis.

Enzyme-Linked Immunosorbent Assay (ELISA) Male C57BL/6J mice (6 weeks old) were subcutaneously challenged into their flank with B16F1 cells (2×10^4 cells/mouse) (day 0). Therapeutic polyplexs were administered *via* one of two routes, namely s.c. injection or IP as mentioned above. Blood was collected on days 4, 7, 8, and 21 after tumor cells inoculation. Blood samples were stored at 4 °C for 3 h followed by centrifugation (Tomy, MX-160, Tokyo, Japan) at $2490 \times \boldsymbol{g}$/30 min for separation of serum, which was stored at −80 °C until assayed. Finally, serum IFN-γ concentrations were determined by sandwich ELISA using Quantikine kit (MIF00, R&D Systems).

Statistical Analysis One-way ANOVA with Tukey *post-hoc* test was used for evaluating statistical differences among 3 groups. Comparisons between 2 groups were determined using unpaired Student's *t*-test. Data are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Preparation of Polyplex Nanoparticles Delivery of nucleic acid-based therapeutics *via* polyplex nanoparticles is considered a versatile strategy for a number of reasons, including the ability of polyplexes to condense nucleic acids into small nanoparticles, cost effectiveness, and ease of production.[32–34] In the present study, we prepared polyplex nanoparticles *via* electrostatic interactions between the negatively charged phosphate groups of the CpG-ODN adjuvant and the positively charged nitrogen atoms of human $gp100_{25–33}$ antigen peptide modified with tetra-arginine moieties (KVPRNQDWL-RRRR). The intracellular uptake of polyplex nanoparticles can be affected by particles size and surface charge.[34] Therefore, we prepared polyplex nanoparticles with different N/P ratios, ranging from 0.5–3, to obtain the most convenient particle size and zeta potential. Polyplex nanoparticles prepared at an N/P ratio of 1 have been shown to exhibit the smallest particle size (253.4 ± 3.8), as well as appropriate zeta potential (−40.4 ± 1.1), suggestive of particle stability,[33] and a PDI of 0.29 ± 0.02 (Table 2), compared to polyplexes prepared at other N/P ratios.

Iontophoresis of Fluorescent-Labeled Polyplex Nanoparticles In the present study, to investigate the effectiveness of IP on the intradermal delivery of polyplex nanoparticles, FITC-labeled antigen peptide (green signal) and Cy3-labeled CpG-ODN adjuvant (red signal) were gently mixed to produce fluorescent-labeled polyplexes. IP was applied for 1 h,

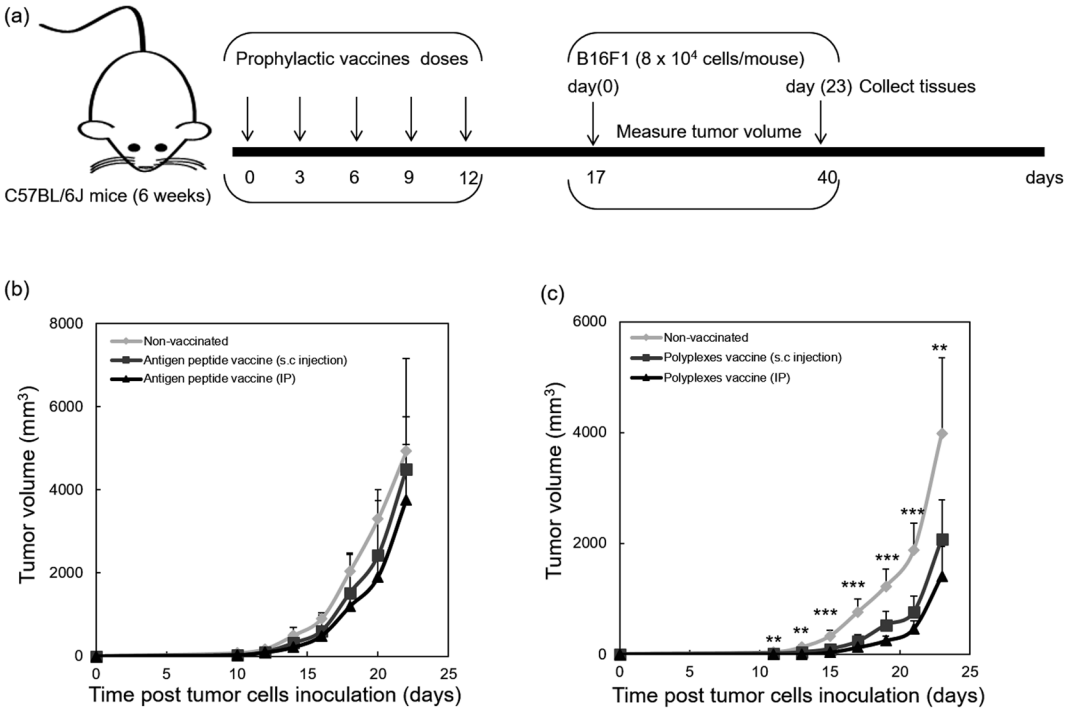

Fig. 2. Effect of Pre-immunization with Prophylactic Vaccines on Tumor Regression

(a) Schematic illustration of pre-immunization regimen with prophylactic vaccines for tumor inhibition. (b) Antigen peptide vaccine. (c) Polyplex vaccine. Male C57BL/6J mice were pre-immunized by different routes (either s.c. injection or IP). Five doses were administered every 3 d. Then, 5 d after the last immunization dose, mice were subcutaneously inoculated with B16F1 cells (site of IP/s.c. application). Tumor burden was measured every other day using a digital caliper. Values represent the mean ± S.D. ($n = 4$). Significant differences (**$p < 0.01$, ***$p < 0.001$) were found in groups of mice vaccinated with polyplex vaccine either by s.c. injection or IP, while there were no significant differences between groups vaccinated with antigen peptide vaccine (either by s.c. injection or IP) compared with the non-vaccinated group.

followed by 3 h incubation, and then observation of the distribution of fluorescent-labeled polyplexes in the skin sections. Fluorescence was not detected in the non-treated healthy skin (Fig. 1a). As shown in Fig. 1b, following passive diffusion of fluorescent-labeled polyplexes, the green fluorescence of FITC-labeled antigen peptide was barely noticeable on the surface of the skin, while the red fluorescence of the Cy3-labeled CpG-ODN adjuvant was slightly distinct. On the other hand, after IP application of fluorescent-labeled polyplexes, both the green fluorescence of FITC-labeled antigen peptide and the red fluorescence of Cy3-labeled CpG-ODN adjuvant showed distinct penetration into the skin to a depth of about 20 μm (Fig. 1c). Co-localization of both green fluorescence and red fluorescence appeared as yellow fluorescence. Fluorescent-labeled polyplexes have shown accumulation within the epidermal layer after IP application and such accumulation exposes the polyplexes to be captured by epidermal immune cells, mainly Langerhans cells (LCs), which distinguished by expanding their dendrites and so, can activate the immune system.[6,22,35] To that end, IP application resulted in intradermal delivery of fluorescent-labeled polyplexes prepared from hydrophilic macromolecules antigen peptide (M.W;1780) and CpG-ODN adjuvant (M.W;6363.01), as predicted based on our previous reports demonstrating transdermal delivery of hydrophilic macromolecules using IP.[6,22,25,27] Surprisingly, we found a very faint red fluorescence for Cy3-labeled CpG-ODN adjuvant distributed through the epidermal layer at depths beyond 50 μm, which may attributed to free Cy3-labeled CpG-ODN adjuvant, while FITC-labeled antigen peptide was distributed to a depth of only about 20 μm (Supplementary Fig. 1c). Additionally, we used the cathode as an active electrode because of the net negative charge of the polyplexes, which may have enhanced the permeation of the free Cy3-labeled CpG-ODN adjuvant through the skin by an electrorepulsion mechanism.

Effect of Iontophoretic-Administered Prophylactic Polyplex Vaccine on Melanoma Inhibition It is known that transcutaneous immunization depends on activation of skin-resident immune cells.[22,36] In this study, we first evaluated the effects of using human $gp100_{25–33}$ antigen peptide (KVPRNQDWL-RRRR) alone as a preventive vaccine in stimulating the immune system and establishing memory cells in tumor-free mice to delay the onset of melanoma, as well as to inhibit tumor growth. Tumor-free male C57BL/6J mice were vaccinated with antigen peptide *via* s.c. injection or IP. Mice were pre-immunized with 5 doses of the antigen peptide, and after 5 d from the last immunization dose they were inoculated with B16F1 cells (Fig. 2a). As predicted, there was a slight reduction in tumor volume in the mice vaccinated by either both s.c. injection and IP compared to non-vaccinated mice; however, this suppression was not significant (Fig. 2b). The insufficiency of this prophylactic antigen peptide vaccine to suppress tumor volume or to even delay its onset is related to the weakness of the antigen immunogenicity.[4,9,37] Low immunogenicity is commonly mediated by numerous mechanisms, and is considered a major challenge in the development of vaccines, alongside the use of antigens alone, which cannot provoke proinflammatory cytokines.[4,37,38] Our results are consistent with previous studies,[4,39] that used different antigens alone for vaccination, which also showed low immunogenicity. As is common in vaccine development, to obtain a potent immunogenic response, inclusion of an appropriate adjuvant is a crucial element.

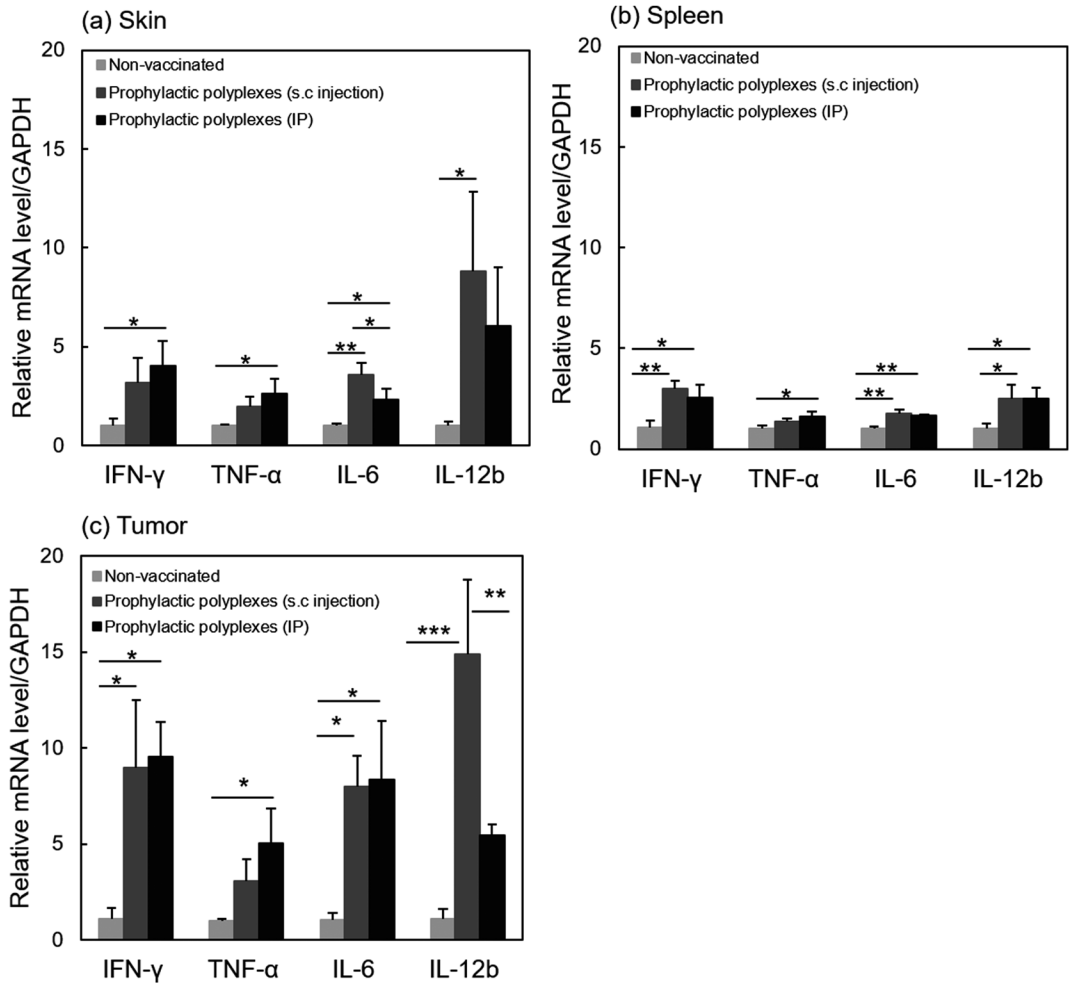

Fig. 3. Quantitative Analysis of mRNA Expression Levels of Inflammatory Cytokines in Different Tissues of Prophylactic Vaccinated Mice (a) Skin, (b) Spleen, and (c) Tumor Tissues

Male C57BL/6J mice were pre-immunized with the polyplex vaccine *via* IP or s.c. injection (the site of tumor cells inoculation) as mentioned in Fig. 2a. At day 23 after tumor cells inoculation, mice were euthanized and tissues were collected. Quantitative evaluation of mRNA expression levels of different cytokines, namely IFN-γ, TNF-α, IL-6 and IL-12b, using RT-PCR was performed. Data are mean ± S.D. ($n = 3$). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Hence, to enhance the efficiency of our prophylactic antigen peptide vaccine, we inserted the CpG-ODN adjuvant into the vaccine to produce polyplex nanoparticles. Next, we examined the potential prophylactic effect of polyplexes on production of immune cells in healthy mice to fight against melanoma growth. As shown in Fig. 2c, there was a significant reduction in tumor volume after pre-immunization with the polyplex vaccine (both s.c. injection and IP) compared to non-vaccinated mice, with an exception only at day 11 in the s.c.-vaccinated group, where a non-significant difference was observed compared to non-vaccinated mice. Also, there was a non-significant difference in tumor reduction between s.c.- and IP- vaccinated groups. In a broader sense, after gathering the adjuvant with the antigen simultaneously, the formed polyplexes promoted activation of the immune system followed by reduction in tumor volume. It has been demonstrated that CpG-ODN adjuvant acts *via* TLR9, which is an intracellular receptor found in various immune cells, mainly LCs, dermal dendritic cells (DCs), B cells and monocytes.[22] Moreover, CpG-ODN adjuvant has been considered as the bridge between innate and adaptive immunity due to its ability to activate innate immune cells as well as APCs, which consequently engulf and process the antigen for presentation to naïve $CD4^+$ and $CD8^+$ T cells (adaptive immunity).[22,40]

The Role of Cytokines Production and Infiltration of Cytotoxic $CD8^+$ T Cells in Inhibiting Melanoma Growth after Pre-immunization with Iontophoretic-Administered Polyplex Vaccine To demonstrate the ability of our polyplex vaccine delivered by IP to activate the immune system against tumor growth, we examined the mRNA expression levels of different cytokines, namely IFN-γ, TNF-α, IL-6 and IL-12b, in various tissues of IP-vaccinated mice compared with mice vaccinated by s.c. injection. Skin, spleen, and tumor tissues were collected at day 23 after tumor cells inoculation in pre-immunized mice (Fig. 2a). Although tumor tissue is the primary target tissue for detection of cytokine levels, we also examined mRNA expression levels in the skin and spleen for the following reasons. The skin is considered a crucial site for eliciting an immune response. Moreover, spleen is a second lymphoid organ which contains naïve adaptive T cells in the white pulp region and these cells are activated after the antigen presentation by APCs.[41]

Results revealed a significant elevation in mRNA expression levels of all analyzed cytokines in the skin tissue compared with the non-vaccinated group, except for IFN-γ and TNF-α in the s.c. injected group. The IP vaccinated group

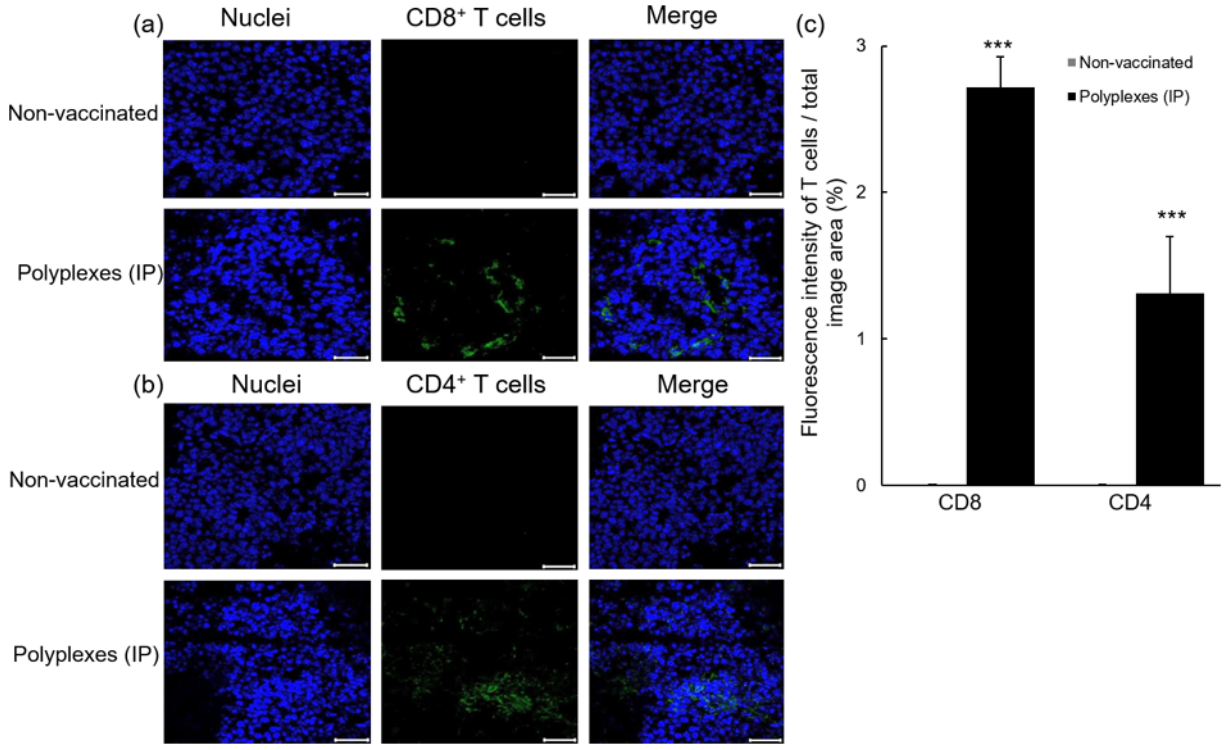

Fig. 4. Immunohistochemical Detection of Infiltrated $CD8^+$ and $CD4^+$ T Cells in Tumor Tissue

Tumor tissues were collected on day 23 after tumor cells inoculation in mice pre-immunized with the polyplex vaccine by IP (the site of tumor cells inoculation). (a) Infiltrated $CD8^+$ T cells. (b) Infiltrated $CD4^+$ T cells. $CD8^+$ and $CD4^+$ T cells are represented by the green (Alexa 488) fluorescence. Nuclei are stained blue (DAPI). Scale bars = 50 μm. (c) Fluorescence intensity of tumor cross sections was quantified by ImageJ software. Data are expressed as mean ± S.D. ($n > 3$). ***$p < 0.0001$.

also showed a non-significant elevation in IL-12b which may be due to the difference in immune response between mice (Fig. 3a). On the other hand, a significant elevation in mRNA expression levels was observed for all analyzed cytokines in the spleen tissue of vaccinated mice compared with non-vaccinated mice, except for TNF-α level in the s.c. injected group (Fig. 3b). mRNA expression levels of all analyzed cytokines in the tumor tissue of vaccinated mice showed a significant elevation compared with non-vaccinated mice, except for TNF-α level in the s.c. injected group and IL-12b level in the IP-vaccinated group (Fig. 3c). There was a non-significant difference in mRNA expression levels of all examined cytokines in all tissues in the s.c. injected group compared with the IP vaccinated group, with the only exceptions being in the skin and tumor tissues, where IL-6 and IL-12b showed significant differences, respectively. Taken together, application of IP to a polyplex vaccine activated the immune system and elicited substantial upregulation of cytokines, which contributed to cell-mediated immunity. Indeed, secretion of proiflammatory cytokines, such as TNF-α and IL-6, or Th-1 cytokines, such as IL-12b and IFN-γ, participate in activating both innate and adaptive immunity, which ultimately fights against melanoma growth.[22,42–44] IL-12b is a proinflammatory type I cytokine that is known for its crucial role in controlling adaptive cell-mediated immunity by eliciting the secretion of IFN-γ by APCs. In addition, IL-12b contributes to the differentiation of both $CD4^+$ and $CD8^+$ T cells by augmenting the secretion of IFN-γ.[4,37,45] Moreover, IFN-γ is considered one of the most important cytokines involved in killing tumor tissue owing to its ability to up-regulate expression levels of MHC I and II molecules in the tumor tissue, which promote the detection of tumor cells by cytotoxic $CD8^+$ T cells to facilitate their clearance.[4,5,46,47] On the other hand, the role of other proinflammatory cytokines, such as TNF-α and IL-6 should also be noted. TNF-α levels are highly correlated to cancer immunity by causing apoptosis and inflammation in addition to contributing to differentiation and maturation of DCs, while IL-6 participates in the enhancement of $CD8^+$ T cell trafficking.[4,5,46,48] Higher levels of all cytokines were present in the tumor tissue compared with the skin and spleen tissues (Fig. 3c). While there were no significant differences observed in either tumor regression or production of cytokines in the IP vaccinated group compared to the s.c. injected group. IP application is also suggested to be superior than s.c. injection based on our previous reports,[22,49] which demonstrated that about half of the applied amount of nucleic acid therapy was retained in the patch following IP application, while the full therapeutic dose was completely delivered following s.c. injection.

Based on these findings, we evaluated the infiltration of both $CD8^+$ and $CD4^+$ T cells in tumor tissue after pre-immunization of healthy mice with our IP-administered polyplex vaccine. We performed immunohistochemical analysis to detect $CD8^+$ and $CD4^+$ T cells in tumor tissue collected on day 23 post tumor cells inoculation in pre-immunized mice. Results showed that the IP-administered prophylactic polyplex vaccine was successful in inducing significant infiltration of both $CD8^+$ and $CD4^+$ T cells (green signals) in the tumor tissue compared with non-vaccinated mice (Figs. 4a, b). Infiltration of cytotoxic $CD8^+$ and $CD4^+$ T cells in the tumor microenvironment is critical for tumor regression.

Effect of Therapeutic Polyplex Vaccine on Cytokines Production and Melanoma Inhibition Next, we examined the therapeutic effect of the vaccine on tumor regression in mice bearing melanoma. We previously determined the

therapeutic efficacy of both an antigen peptide-loaded nanogel and free CpG-ODN adjuvant separately on reducing tumor burden in a melanoma model using IP.[6,22] We demonstrated that while both therapeutic antigen and CpG-ODN adjuvant resulted in a significant reduction in tumor volume, the effects were not very potent. Thus, we sought to combine the antigen peptide and the adjuvant in the form of a polyplex to enhance their therapeutic effects. We found a significant and potent reduction in tumor volume in mice bearing melanoma treated with either both s.c.- and IP-administered polyplex vaccine compared to non-vaccinated group (Fig. 5), confirming the pivotal role of the combination of the adjuvant and antigen on stimulating immunity. Also, there was a non-significant difference between s.c.- and IP- treated groups. As noted earlier, this reduction in tumor volume can be attributed to cytokines secretion (mainly IFN-γ) in addition to infiltration of both $CD8^+$ and $CD4^+$ T cells in the tumor tissue.

Moreover, to confirm the induction of immunity, we determined mRNA expression levels in skin, spleen and tumor tissues. A significant elevation in cytokine levels was found in the skin tissue of vaccinated mice compared with non-vaccinated mice, with the exception of IL-12b in IP-vaccinated group, and IFN-γ and TNF-α in s.c.-vaccinated group (Fig. 6a). There was a significant elevation in the level of cytokines

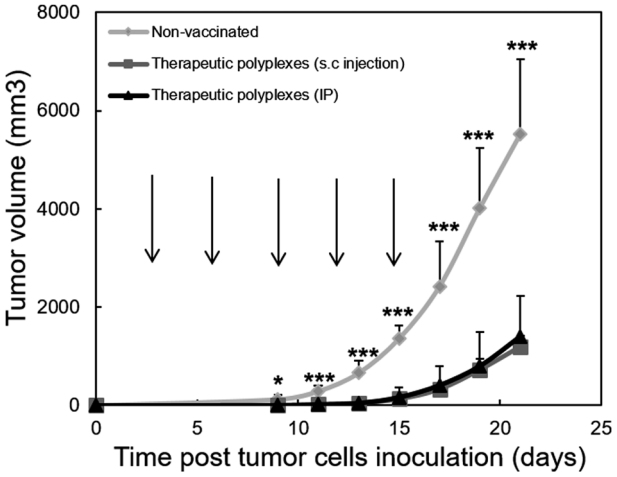

Fig. 5. Anti-tumor Effect of the Therapeutic Polyplex Vaccine in Mice Bearing Melanoma

Male C57BL/6J mice were subcutaneously challenged into their flank with B16F1 cells (day 0). The therapeutic polyplex vaccine was administered *via* IP (above the tumor site) or s.c. injection (near the tumor site) on days 3, 6, 9, 12 and 15 (indicated by arrows). Values represent the mean ± S.D. ($n = 4$). Significant differences ($^*p < 0.05$, $^{***}p < 0.001$) were noted between the vaccinated and non-vaccinated groups.

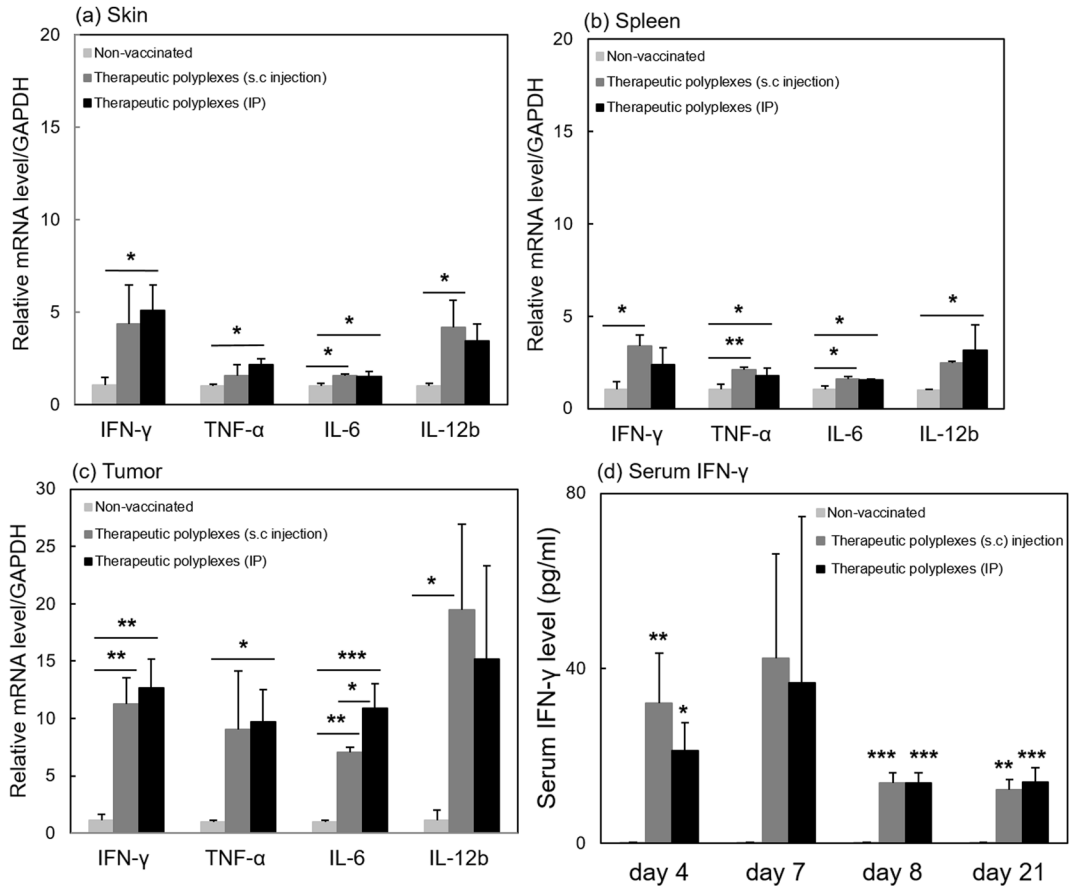

Fig. 6. Quantitative Analysis of Inflammatory Cytokines in Mice Bearing Melanoma Treated with the Polyplex Vaccine

Mice bearing melanoma were treated with the polyplex vaccine *via* IP (above the tumor site) or s.c. injection (near the tumor site). At day 21 after tumor cells inoculation, mice were euthanized and tissues were collected. Quantitative evaluation of mRNA expression levels of different cytokines using RT-PCR was performed (a) skin, (b) spleen, and (c) tumor tissues. d) Detection of IFN-γ levels at different time intervals in the serum of mice bearing melanoma treated with the polyplex vaccine. Male C57BL/6J mice were subcutaneously challenged into their flank with B16F1 cells (day 0). Then, mice were treated with the polyplex vaccine *via* IP (above the tumor site) or s.c. injection (near the tumor site). At days 4, 7, 8 and 21 post tumor cells inoculation, blood was withdrawn for determination of IFN-γ serum levels using ELISA kit. Data are mean ± S.D. ($n = 3$). ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

in the spleen (Fig. 6b), with the exception of IFN-γ and IL-12b levels in IP- and s.c.-vaccinated groups, respectively. Finally, cytokine levels significantly increased in the tumor tissue, with the exception of TNF-α and IL-12b levels in s.c.- and IP-vaccinated groups, respectively (Fig. 6c). Moreover, all examined cytokines showed the highest expression levels in tumor tissue compared with the skin and spleen tissues. Taken together, these results highlight the promising effects of iontophoretic delivery of a therapeutic polyplex vaccine in inhibiting melanoma growth *via* activation of the immune system and mediating the secretion of IFN-γ, which plays a pivotal role in tumor clearance through various mechanisms including activation of cytotoxic $CD8^+$ T cells that are responsible for attacking the tumor.

Interestingly, results of the present study suggest that the polyplex vaccine may be more powerful as a therapeutic vaccine than as a prophylactic vaccine, which may be due to two reasons. In particular, it is known that the action of prophylactic vaccines depends on memory cells, while therapeutic vaccines do not. In addition, Whitmire *et al.* reported that although memory immune cells can recognize a viral infection within a few hours, they start to proliferate and differentiate after about 3 d from the infection, thereby delaying the onset of activation in addition to exhibiting a slower rate of division than naïve cells.[50] Second, in our prophylactic vaccination protocol we challenged the pre-immunized mice with a higher number of B16F1 cells than typically employed in other prophylactic vaccine studies that tend to inoculate mice with a very low number of tumor cells.[7,15,37,46] The above reasons may be responsible for the somewhat lower prophylactic effect of the polyplex vaccine compared to its therapeutic effect on reducing tumor volume. In any event, the polyplex vaccine did demonstrate both prophylactic and therapeutic effects against tumor growth.

Therapeutic Polyplex Vaccine Enhanced Systemic Immunity Finally, to confirm whether the local immune response mediated by the activated skin resident immune cells could gradually initiate a potent systemic immune response, we determined IFN-γ serum levels. After subcutaneously inoculating mice with B16F1 cells (day 0), mice were subsequently treated with polyplex vaccine *via* one of two routes, namely s.c. injection or IP as mentioned above. IFN-γ serum levels were analyzed on days 4, 7, 8 and 21 post tumor cells inoculation. Results showed a significant elevation in IFN-γ serum levels on day 4 in both IP- and s.c.-vaccinated groups compared to the control group. A gradual increase in IFN-γ serum levels was observed on day 7, which was non-significant in both IP- and s.c.-vaccinated groups compared to the control group. Next, there was a down-regulation in IFN-γ serum levels on day 8 compared to days 4 and 7, but the level was still significantly elevated in both IP- and s.c.-vaccinated groups to that of the control group (Fig. 6d). Finally, at day 21 IFN-γ serum levels were nearly similar to the levels at day 8. We can conclude that the polyplex vaccine was able to persistently stimulate the APCs, mainly skin resident LCs and kertinocytes, which participated in inducing a systemic immune response *via* secretion of cytokines.[22] The immune system is known to take up to 3 d to prime the adaptive immune response, which is accomplished by low levels of cytokine secretion after the first immunization dose, followed by a peak in levels of cytokine secretion and the magnitude of the immune response after the second immunization dose, and then a subsequent plateau in cytokine levels.[4] Indeed, we found that IFN-γ serum levels were elevated after the first immunization dose on day 4 followed by tendency toward more elevation which was not significant on day 7 compared to the control group (Fig. 6d). Finally, on day 8, IFN-γ serum levels decreased to 13.79 ± 2.35 and 13.7 ± 2.43 pg/mL for IP- and s.c.-vaccinated groups, respectively. Although levels decreased on day 8, levels were similar to IFN-γ serum levels on day 21, which confirms the ability of therapeutic polyplexes delivered either by IP or s.c. injection to induce and maintain a systemic immune response to fight against melanoma growth. Additionally, there was a non-significant difference between IP- and s.c.-vaccinated groups in all days.

CONCLUSION

In conclusion, the results of our study demonstrate the capability of IP-administered prophylactic polyplex vaccine to act as a safe immunogenic agent to fight against melanoma growth. IP transdermal delivery of hydrophilic macromolecules of a polyplex vaccine was able to overcome the hurdles of the stratum corneum barrier and successfully deliver the vaccine into the epidermal layer for activation of LCs. Moreover, *in vivo* tumor regression in pre-immunized mice was mediated *via* a series of events, including activation of APCs, elevation in mRNA expression levels of various cytokines, and also infiltration of cytotoxic $CD8^+$ and $CD4^+$ T cells in the tumor tissue. Additionally, we demonstrated that our prophylactic cancer vaccine also exhibits a therapeutic effect against mice bearing melanoma. To our knowledge, this is the first report demonstrating successful IP delivery of a prophylactic melanoma vaccine in the form of polyplex nanoparticles.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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