Regular Article

Use of Iontophoresis Technology for Transdermal Delivery of a Minimal mRNA Vaccine as a Potential Melanoma Therapeutic

Rabab A. Husseini,[a,b] Naoko Abe,[c] Tomoaki Hara,[c] Hiroshi Abe,[c] and Kentaro Kogure*[,d]

[a] *Department of Pharmaceutics, Faculty of Pharmacy, Zagazig University, Zagazig 44519, Egypt:* [b] *Department of Pharmaceutical Health Chemistry, Graduate School of Pharmaceutical Sciences, Tokushima University, 1 Shomachi, Tokushima 770–8505, Japan:* [c] *Department of Chemistry, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464–8602, Japan: and* [d] *Department of Pharmaceutical Health Chemistry, Graduate School of Biomedical Sciences, Tokushima University, 1 Shomachi, Tokushima 770–8505, Japan.*
Received October 18, 2022; accepted November 17, 2022

mRNA vaccines have attracted considerable attention as a result of the 2019 coronavirus pandemic; however, challenges remain regarding use of mRNA vaccines, including insufficient delivery owing to the high molecular weights and high negative charges associated with mRNA. These characteristics of mRNA vaccines impair intracellular uptake and subsequent protein translation. In the current study, we prepared a minimal mRNA vaccine encoding a tumor associated antigen human gp100$_{25–33}$ peptide (KVPRNQDWL), as a potential treatment for melanoma. Minimal mRNA vaccines have recently shown promise at improving the translational process, and can be prepared *via* a simple production method. Moreover, we previously reported the successful use of iontophoresis (IP) technology in the delivery of hydrophilic macromolecules into skin layers, as well as intracellular delivery of small interfering RNA (siRNA). We hypothesized that combining IP technology with a newly synthesized minimal mRNA vaccine can improve both transdermal and intracellular delivery of mRNA. Following IP-induced delivery of a mRNA vaccine, an immune response is elicited resulting in activation of skin resident immune cells. As expected, combining both technologies led to potent stimulation of the immune system, which was observed *via* potent tumor inhibition in mice bearing melanoma. Additionally, there was an elevation in mRNA expression levels of various cytokines, mainly interferon (IFN)-γ, as well as infiltration of cytotoxic CD8$^+$ T cells in the tumor tissue, which are responsible for tumor clearance. This is the first report demonstrating the application of IP for delivery of a minimal mRNA vaccine as a potential melanoma therapeutic.

Key words iontophoresis, minimal mRNA vaccine, melanoma

INTRODUCTION

Vaccination is considered the most effective means for controlling and preventing the prevalence of infectious diseases.[1)] In 2020, the United States Food and Drug Administration (FDA) approved two mRNA vaccines to combat the 2019 coronavirus (COVID-19) pandemic.[2)] In fact, the accelerated development of these COVID-19 mRNA vaccines relied on years of preclinical and clinical research aimed at enhancing the use of mRNA vaccines for cancer therapy.[3)] As a result the COVID-19 pandemic, considerable attention has been focused on the development and use of mRNA vaccines and therapeutics for a wide variety of human diseases, including for heart disease, autoimmune disorders, rare genetic diseases, human immunodeficiency virus (HIV), and has also provoked a breakthrough in the area of cancer vaccines.[4–6)] Cancer vaccines mostly encode for different tumor-associated or tumor-specific antigens (TAAs or TSAs) aimed at stimulating cell-mediated immune responses, such as cytotoxic T lymphocytes (CTLs), which have the potential effect of attacking malignant cells.[4,7)]

Over the past decade, nucleic acid-based cancer vaccines, including mRNA vaccines and DNA vaccines, have been suggested as a promising platform over other conventional vaccines (*e.g.*, peptide vaccines and viral vector-based vaccines) for various reasons, including improved safety, efficacy and the potential to elicit humoral- and cell-mediated immune responses.[2,8)] However, nucleic acid-based vaccines are preferred over other traditional vaccines, while mRNA vaccines offer advantages over DNA vaccines.[9)] Unlike DNA vaccines, mRNA vaccines lack the possibility for genomic integration, resulting in minimal concerns for gene disruption, mutagenesis and tumorigenesis.[8–10)] Moreover, mRNA vaccines are distinguished by their short half-life and well-tolerated safety profile, as well as their rapid, high-yield, safe, and cost-effective production.[1,3,7–10)] On the other hand, mRNA vaccines also have some disadvantages that limit their application, such as high molecular weights, instability, immunogenic properties, and insufficient *in vivo* delivery, which can lead to degradation of mRNA vaccines, inhibition of antigen expression, and consequently weak immune cell activation.[4,7,10–12)] Hence, researchers and mRNA vaccine manufacturers are focusing their efforts on overcoming these difficulties *via* different approaches, such as designing various delivery vehicles for improving cellular uptake and cytoplasmic translation and improving the purification methods to eliminate double-stranded contamination that provokes innate immunity to result in mRNA vaccine degradation.[4,8,13)] Furthermore, modifications in the backbone of mRNA vaccines, including the poly(A) tail, the 5′ cap, un-translated regions, the sequence patterns in the open reading frame (ORF), and also incorporation of modified nucleotides, can contribute to overcoming the above-mentioned hurdles associated with mRNA vaccines.[8)]

In the current work, a minimal mRNA based vaccine

* To whom correspondence should be addressed. e-mail: kogure@tokushima-u.ac.jp

encoding TAA human gp100$_{(25–33)}$ (KVPRNQDWL) as a potential melanoma therapeutic was prepared by introducing a short poly(A) tail 20-nt (M.W.: 20460). Despite the resultant increase in length of the mRNA sequence, the poly(A) tail is crucial for protecting the mRNA vaccine from de-capping and degradation, as recent studies have shown that shortening the poly(A) tail sequence could enhance the translation process in addition to simplifying the synthesis process.[4,7,8,14] While the negative charges and high molecular weights (10^5–10^6) of naked non-formulated mRNA vaccines can impair their intracellular uptake and efficacy, clinical trials have demonstrated success in inducing a potent anti-tumor immune response after intranodal, intradermal, and subcutaneous (s.c.) injection of naked mRNA cancer vaccines.[1,3,7,8] However, intranodal injection involves complicated procedures, and achieving high efficiency of injected naked mRNA vaccines is related to the high frequency of dendritic cells in the draining lymph nodes.[2,7,9] Additionally, various antigen presenting cells (APCs) reside in the skin, making it an ideal immunocompetent site to augment the vaccine response.[4,7] As a result, the intradermal route has been widely used for vaccine delivery.[3,4,7] In addition to the need for special training, intradermal injection can also increase the risk of undesirable side effects at the injection site, such as swelling, infection, erythema, and pain.[2,15–17] Accordingly, our work focused on using iontophoretic transdermal technology, in which a weak electric current (0.3–0.5 mA/cm^2) is applied for non-invasive delivery of hydrophilic macromolecules *via* the skin.[15–19] Iontophoresis (IP) relies on various mechanisms, including electrorepulsion, electroosmosis and recently, intercellular cleavage of both tight and gap junctions.[15–19] In the present study, we utilize two unique features related to IP: the first is the ability to deliver hydrophilic macromolecules *via* the stratum corneum of the skin; and the second is the potential to improve cellular uptake of negatively-charged macromolecules, as reported previously for small interfering RNA (siRNA).[20,21] However, In fact, IP and electroporation are used previously for the delivery of charged compounds like DNA, RNA and peptides. Despite the used voltage in electroporation is extremely higher and reach up to 100 Volt compared to IP (10 V or less), it has been reported that electroporation succeeded in the delivery of mRNA mouse zygotes and facilitated clustered regularly interspaced short palindromic repeats CRISPR-associated protein 9 (CRISPR/Cas9)-based genome editing.[22] Also, electroporation has been shown to enhance the delivery efficiency of large, self-amplifying mRNA *in vivo*, upon measuring reporter gene expression and immunogenicity of genes encoding HIV envelope proteins.[23] Taken together, this proved that mRNA is generally stable electrically not only to the low voltage of the IP but also, to the high voltage of electroporation. Herein, we investigated the potential effect of combining a newly synthesized minimal mRNA vaccine with non-invasive transdermal IP technology to enhance the transdermal delivery of naked non-formulated minimal mRNA vaccine as a potential melanoma therapeutic. We investigated the potential effect on reducing tumor volume in mice bearing melanoma and the mRNA expression levels of various cytokines by RT-PCR. Finally, we confirmed the stimulated immune response through immunohistochemistry analysis for the infiltrated cytotoxic CD8$^+$ and CD4$^+$ T cells in the tumor tissue and systemically by detecting serum interferon (IFN)-γ.

MATERIALS AND METHODS

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

Materials The fluorescein isothiocyanate (FITC)-labeled oligonucleotide GGAGCCACCATGAAGGTGCCCCGG AACCAGGACTGGCTGTGAAAAAAAAAAAAAAAA AAAAA (M.W.: 19800) was purchased from Hokkaido System Science Co., Ltd. (Hokkaido, 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, U.S.A.) and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), respectively. An Ag-AgCl electrode was purchased from 3M 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 enzyme-linked immunosorbent assay (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 available.

Iontophoresis Technology for Delivery of FITC-Labeled Oligonucleotide FITC-labeled oligonucleotide was used as a model of mRNA to investigate transdermal delivery by IP. IP was conducted according to our previous report with some modifications.[19] In brief, after anesthetizing the mice with chloral hydrate (400 mg/kg mouse) dissolved in phosphate buffered saline (PBS) intraperitoneally, their dorsal skin was shaved for IP application. Then, moistened nonwoven fabric (0.5 cm^2) containing FITC-labeled oligonucleotide (10 μg/50 μL) was attached to the shaved dorsal skin, and another wetted nonwoven fabric (0.5 cm^2) containing PBS (50 μL) was placed 1 cm away. Each piece of the wetted nonwoven fabric was connected to Ag-AgCl electrodes. The Ag-AgCl electrodes with nonwoven fabric containing labeled oligonucleotide or PBS (7.4) 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 0.17 mA/0.5 cm^2 for 1 h. Finally, mice were incubated for 3 h, followed by excision of their skin for cross-sectioning.

Intradermal Distribution of FITC-Labeled Oligonucleotide after Applying Iontophoresis Mice were euthanized after the incubation time (3 h), and their skin was excised, em-

bedded in OCT compound, and then frozen with dry ice/ethanol. Sections (10 μm thick) were cut from the frozen blocks using a cryostat (CM3050S; Leica Biosystems, Tokyo, Japan). Dako Fluorescence Mounting Medium was used for mounting the 10 μm thick frozen sections onto MAS-coated glass slides. Finally, the skin sections were stored in the dark until dry for subsequent observation of intradermal distribution of the labeled oligonucleotide using a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany).

Preparation of Minimal Naked mRNA Vaccine as a Potential Melanoma Therapeutic Capped 62-nt mRNA encoding decapeptide MKVPRNQDWL, which is shown as 5′_m^7G-(5′)ppp(5′)_G_m_G_m_AGCCACCAUGAAGGUGCCCCG GAACCAGGACUGGCUGUGAAAAAAAAAAAAAAAA AAAAAA_3′ (m^7G, 7-methyl-G; G_m, 2′-*O*-methyl-G), was synthesized according to a previous report.[14] Briefly, 5′ phosphorylated 62-nt RNA was synthesized using standard phosphoramidite chemistry on an automated DNA/RNA synthesizer. RNA was deprotected in a 1 : 1 mixture of 40% aqueous methylamine and 28% ammonium solution at 65 °C for 20 min, followed by overnight treatment with 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) solution at room temperature. Deprotected RNA was then purified by reversed-phase HPLC (RP-HPLC). The synthesized 5′ phosphorylated RNA was chemically capped using 7-methylguanosine diphosphate imidazolide (Im-m^7GDP) in a reaction mixture of 20 μM RNA, 10 mM Im-m^7GDP, 10 mM $CaCl_2$, and 10 mM 2-nitroimidazole in dimethyl sulfoxide (DMSO). First, aqueous solutions of RNA and $CaCl_2$ were mixed and lyophilized. After being suspended in DMSO, DMSO solutions of Im-m^7GDP and 2-nitroimidazole were added. The mixture was heated at 55 °C for 3 h, followed by RNA recovery *via* alcohol precipitation. Capped RNA was further purified by RP-HPLC. After purification, capped RNA was alcohol-precipitated and re-dissolved with water. The RNA concentration was measured based on absorbance at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo). Capped mRNA was analyzed by LC-MS using an Agilent 1260 Infinity II LC/MSD system, and the mass was found to match the theoretical mass: Calcd, 20732.63; Found 20735.64 (+3.01).

Therapeutic Effect of Iontophoretic Delivery of a Minimal mRNA Vaccine on Tumor Regression Male C57BL/6J mice (6 weeks old) were subcutaneously challenged into their flank with B16F1 cells (10^5 cells/mouse) suspended in PBS (day 0). The mRNA vaccine therapeutic was initiated on day 5 after tumor inoculation. Five doses of vaccine were administered every 3 d for a total of 13 d. Mice were immunized by a fixed volume (30 μg/50 μL/dose mouse) of mRNA vaccine, which was administered *via* one of two routes, namely s.c. injection or IP. IP was applied above the tumor tissue and was carried out at 0.17 mA/0.5 cm^2 for 1 h as described above. Previously, sham-IP did not show a significant effect on tumor regression so non-treated control group (-IP) was used.[16] Tumor burden was determined every other day using a digital caliper according to the following equation: T_{vol} (mm^3) = length × $width^2$ × 0.5. At day 22 after tumor inoculation mice were euthanized and the skin, spleen and tumor tissues were harvested and stored at −80 °C for further analysis.

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

RT-PCR for Quantitative Analysis of Various Cytokines in Different Tissues 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 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.

Investigation of Infiltrated Cytotoxic $CD8^+$ and $CD4^+$ T Cells in the Tumor and Spleen Tissues by Immunohistochemistry Analysis At day 22 post tumor inoculation in immunized mice, the tumor and spleen tissues were 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. Tissue 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. Tissue sections were then incubated with the diluted rabbit anti-mouse CD8 and CD4 primary antibodies separately for 18 h at 4 °C. Then, the 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,

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

tumor sections were mounted with Dako Fluorescence Mounting Medium and allowed to dry. The sections were observed using a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany).

ELISA Male C57BL/6J mice (6 weeks old) were subcutaneously challenged into their flank with B16F1 cells (10^5 cells/mouse) on day 0. Mice were immunized with mRNA vaccine *via* one of two routes, namely s.c. injection or IP as mentioned above. Blood was collected on days 6, 9, 10 and 22 after tumor inoculation. Blood samples were stored at 4 °C for 3 h followed by centrifugation (Tomy, MX-160, Tokyo, Japan) at 2490 g/30 min for separation of serum, which was stored at −80 °C until assayed. Finally, serum IFN-γ concentration was 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. Data are presented as mean ± standard deviation (S.D.).

RESULTS AND DISCUSSION

In the present study, the negatively charged, high molecular weight (M.W.: 19800) FITC-labeled oligonucleotide (green signal) was used as a model of mRNA to investigate the potential effect of IP technology on mRNA delivery into skin layers. Non-treated healthy skin was used as a control, which showed the absence of any fluorescence signal (Fig. 1a). On the other hand, Fig. 1b shows the homogenous distribution of the FITC-labeled oligonucleotide in the skin layers to a depth of about 100 μm after IP application. Copious amounts of immune cells are known to reside in the skin, including epidermal Langerhans cells (LCs) and dendritic cells (DCs).[16,24–26] The most distinguished immune cells in the skin are epidermal LCs, which are known for their ability to extend their dendrites and elicit a strong immune response.[15,16,27] Penetration of the FITC-labeled oligonucleotide to depths of 100 μm after IP application indicates its accumulation within the epidermal layer, which ranges from 100 to 200 μm in thickness.[15] Consequently, the FITC-labeled oligonucleotide can be engulfed by the epidermal LCs for subsequent activation of an immune response. Based on these results, we can conclude that IP application was successful at inducing a homogenous distribution of the high molecular weight FITC-labeled oligonucleotide in the epidermal layer of the skin, which was achieved *via* various mechanisms including electrorepulsion, electroosmosis and intercellular cleavage of both tight and gap junctions.[15–17,24]

We subsequently investigated therapeutic outcomes following immunization of mice bearing melanoma with a non-formulated naked mRNA vaccine encoding TAA human $gp100_{(25–33)}$ (KVPRNQDWL) using IP. It has been reported that inclusion of a short poly(A) tail can simplify mRNA preparation as well as improve its stability and enhance intracellular translation.[4,7,8,14] We therefore prepared a minimal mRNA vaccine with a short poly(A) tail 20-nt (M.W.: 20460) against melanoma challenge. Furthermore, mRNA is known to be highly negatively charged, which impairs its intracellular uptake.[1,3,7,8] Consequently, in this study we anticipated that IP application would not only improve the delivery of minimal mRNA into skin layers, but also enhance intracellular uptake

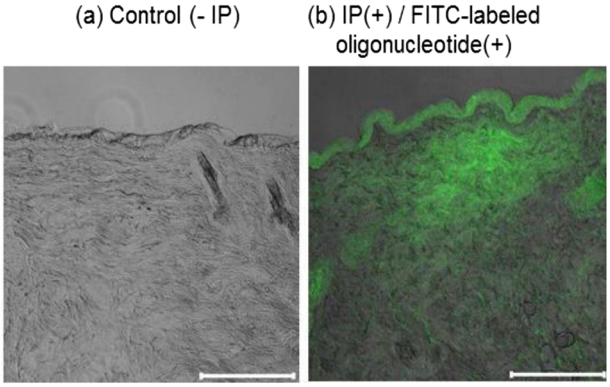

Fig. 1. Intradermal Distribution of FITC-Labeled Oligonucleotide (green) after IP Application

10 μm thick skin sections were prepared for observation by confocal microscopy. (a) control group (-IP). (b) Intradermal distribution of FITC-labeled oligonucleotide after IP application (0.17 mA/0.5 cm^2, 1 h) followed by 3-h incubation. Scale bars = 100 μm.

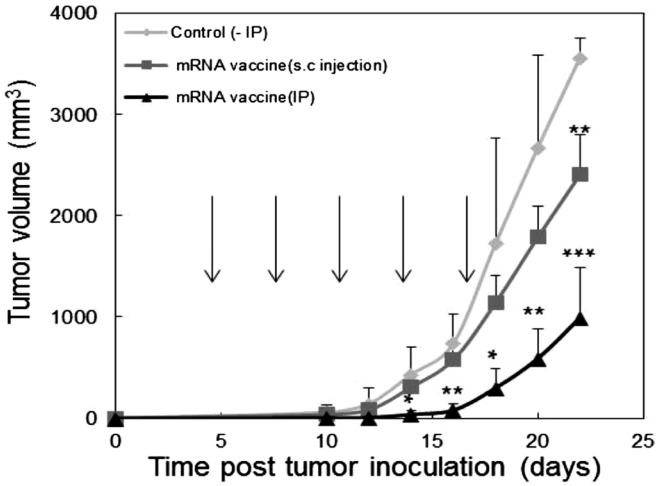

Fig. 2. Effect of Immunization with mRNA Vaccines on Tumor Inhibition

After challenging mice with B16F1 cells (day 0), 5 doses of therapeutic mRNA vaccine were administered every 3 d (indicated by arrows) by different routes (either s.c. injection or IP). Tumor volume was measured every other day using a digital caliper. Values represent the mean ± S.D. ($n = 4$). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

by APCs as reported previously for IP delivery of siRNA.[20,21] In a more detail the effect of various endocytosis inhibitors (*e.g.*, amiloride, filipin, sucrose, and low temperature exposure) was studied and after visualization by confocal microscopy, the IP-induced cellular uptake pathway of siRNA (M.W.: 12000) was found to be due to the activation of many specific signaling molecules related to endocytosis.[20] Additionally, endosomes were found to leak macromolecules exhibiting molecular weights <70000.[21] Thereby, we have prepared a minimal mRNA with a lower molecular weight (M.W.: 20460) that can achieve an endosomal escape. In addition, previous reports suggest that inclusion of a short poly(A) tail can enhance mRNA translation. A potent immune response was therefore expected by combining the mRNA therapeutic with IP technology.

Mice bearing melanoma were treated with the minimal naked mRNA vaccine by different routes (either s.c. injection or IP). As predicted, a significant regression in tumor volume was observed at each day after IP application compared to non-vaccinated mice, with an exception at days 10 and 12, in

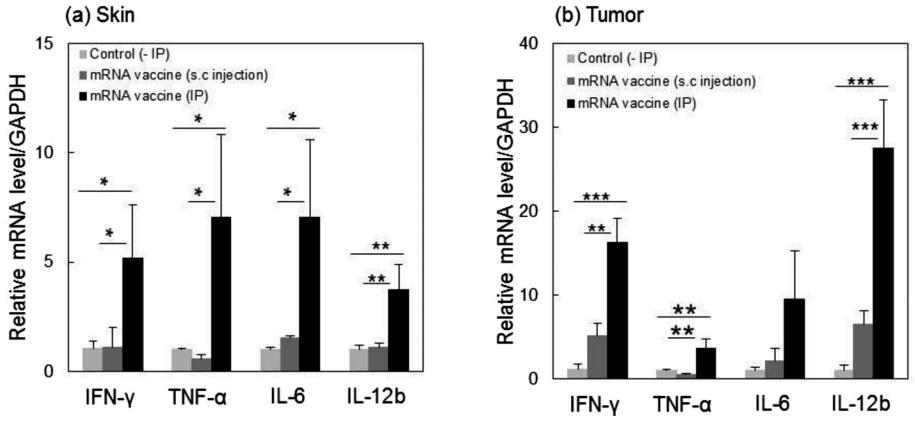

Fig. 3. Quantitative Analysis of mRNA Expression Levels of Inflammatory Cytokines in Different Tissues of mRNA-Vaccinated Mice

(a) Skin tissue and (b) tumor tissue. Mice were immunized with the mRNA vaccine *via* IP or s.c. injection as mentioned previously. At day 22 after tumor 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. ($n = 3$). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

which no significant difference was observed (Fig. 2). On the other hand, s.c. injection of the minimal mRNA vaccine did not show a significant reduction in tumor volume at all days except for the last day (day 22), in which a slight significant difference was noted compared to non-vaccinated mice. Further, when comparing IP application and s.c. injection, there was a non-significant difference noted at all days, with the exception of days 16, 20, and 22, in which slight significant differences were noted (Fig. 2). Taken together, these findings demonstrate the successful delivery of a minimal mRNA vaccine into skin layers by IP application, resulting in subsequent stimulation of an immune response as demonstrated by a reduction in tumor burden after immunization.

Following IP application, the mRNA vaccine was likely distributed homogenously into skin layers, where epidermal immune cells reside, mainly LCs that can extend their dendrites and survey the skin to capture the vaccine, resulting in subsequent cytoplasmic delivery of the vaccine *via* cellular uptake.[16,28,29] Reduction in tumor volume was likely due to infiltration of cytotoxic tumor-specific $CD8^+$ T cells into the tumor microenvironment, in addition to secretion of the most critical inflammatory cytokine, IFN-γ, for tumor regression.[30–34] To confirm these hypotheses, mRNA expression levels of various cytokines, including IFN-γ, TNF-α, IL-6 and IL-12b, were detected by RT-PCR. Previously, sham-IP did not show a significant effect on mRNA expression levels of various cytokines so non-treated control group (-IP) was used.[16] Results revealed a significant difference in mRNA expression levels of all cytokines in both the skin and tumor tissues in the IP-vaccinated group compared to either the non-vaccinated group or the s.c-vaccinated group, with an exception only for IL-6 in the tumor tissue, in which no significant difference was observed (Figs. 3a, b). Furthermore, the s.c-vaccinated group did not show any significant difference compared to the non-vaccinated group (Figs. 3a, b). The cytokines likely contributed to fighting melanoma growth *via* various mechanisms, including activation of adaptive immunity.[16,35–37] IL-12b plays a role in up-regulating the secretion of IFN-γ, which is considered one of the key factors for tumor regression based on its ability to: inhibit angiogenesis, increase intra-tumoral levels of major histocompatibility molecules (MHC I and II), and promote apoptosis.[30,33,34,38–40] Moreover, IFN-γ augments the differentiation of cytotoxic $CD8^+$ T cells, which are known to have a lethal effect on tumor cells.[28] Consequently, both IFN-γ and IL-12b are considered crucial cytokines for activating adaptive immunity. Moreover, other pro-inflammatory cytokines, particularly TNF-α and IL-6, also play a critical role in eliciting an immune response by enhancing the trafficking of DCs toward the draining lymph nodes (DLNs) and the infiltration of cytotoxic $CD8^+$ T cells in the tumor tissue.[28,30,34,41,42] Based on these previous findings, we analyzed tumor and spleen tissues in the present study for infiltrated cytotoxic $CD8^+$ and $CD4^+$ T cells after immunization with the minimal mRNA vaccine by IP application.

Figures 4a and b show infiltration of both cytotoxic $CD8^+$ and $CD4^+$ T cells (green signals) in both tumor and spleen tissues in the IP-vaccinated group compared with the non-vaccinated group. Infiltration of cytotoxic $CD8^+$ and $CD4^+$ T cells in the tumor microenvironment is critical for tumor regression, as previously noted,[43–46] and these results demonstrate the ability of our IP-administered minimal mRNA vaccine to elicit cell-mediated adaptive immunity, which is considered a main element required for tumor clearance. Moreover, based on the unique features of the mRNA vaccines in stimulating both the cellular and humoral immunity we are expecting the ability of our minimal mRNA vaccine on stimulating the humoral immunity.

Finally, to evaluate the potency of our minimal mRNA vaccine at inducing a systemic immune response, serum IFN-γ levels were detected at various days following vaccination. Serum IFN-γ levels were significantly different in the s.c- and IP vaccinated groups compared with the non-vaccinated group, with an exception at day 9 for the s.c-injected group (Fig. 5). However, there was a non-significant difference between the s.c- and IP- vaccinated groups at days 6, 9, and 10, while serum IFN-γ levels in the IP-vaccinated group were slightly higher than those in the s.c-vaccinated group. Moreover, at day 22 following the full 5-dose treatment, IFN-γ serum levels in the IP-vaccinated group were strongly up-regulated (11.83 ± 0.87 pg/mL) compared to the s.c-vaccinated group (1.49 ± 0.18 pg/mL). These results demonstrate the ability of our minimal mRNA vaccine to induce and maintain a systemic immune response *via* permanent stimulation of skin resident immune cells.[16] Moreover, delivery of the minimal

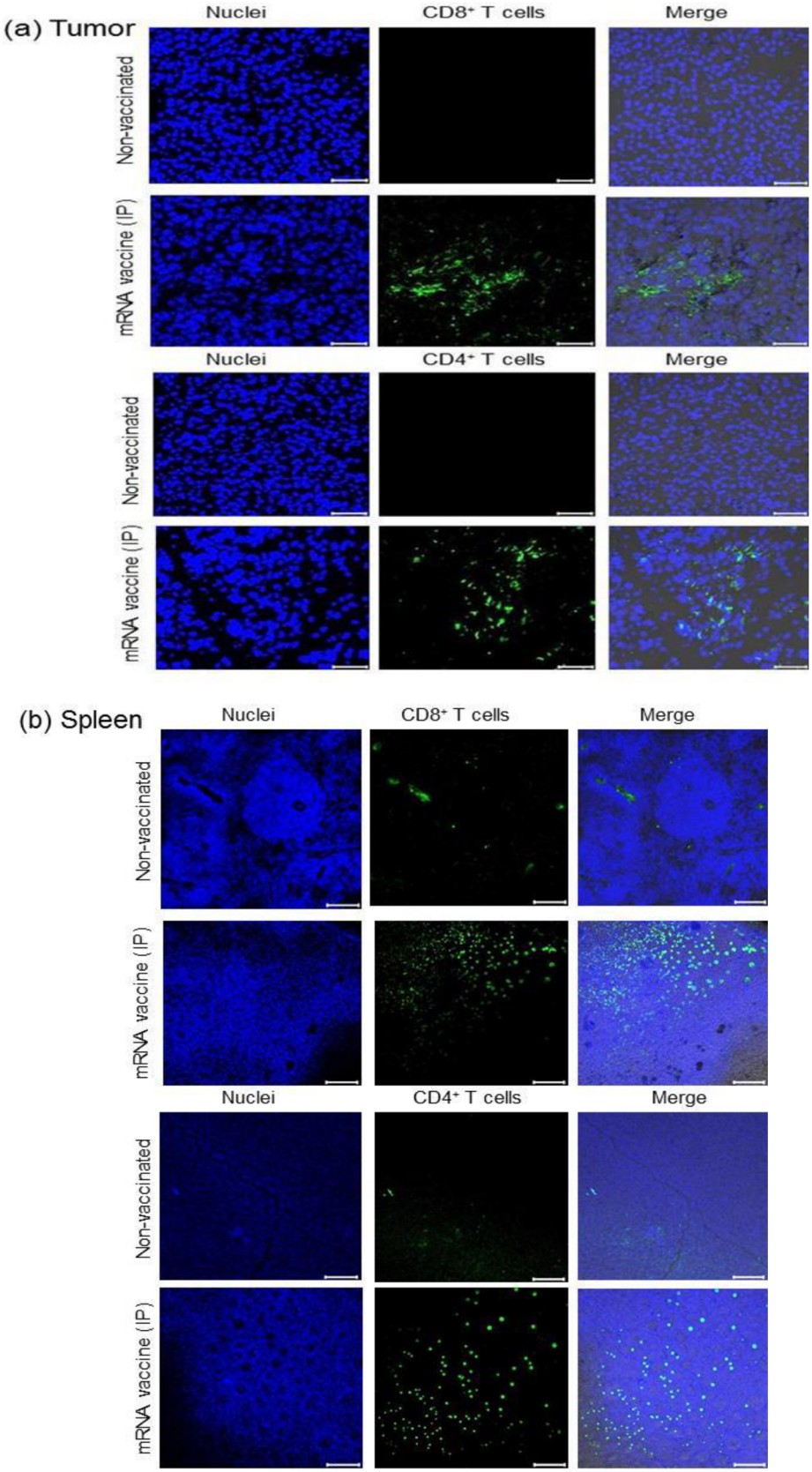

Fig. 4. Immunohistochemical Detection of Infiltrated Cytotoxic $CD8^+$ and $CD4^+$ T Cells in Tumor and Spleen Tissues Collected at Day 22 after Tumor Inoculation in Mice Immunized with the mRNA Vaccine Using IP

(a) Tumor tissue, (b) spleen tissue. $CD8^+$ and $CD4^+$ T cells are represented by the green (Alexa 488) fluorescence. Nuclei are stained blue (DAPI). Scale bars = 50 μm.

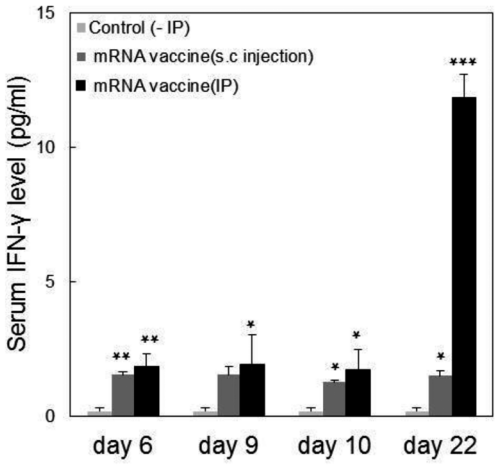

Fig. 5. Detection of Serum IFN-γ Levels at Different Time Intervals in Mice Bearing Melanoma Treated with the mRNA Vaccine

After challenging mice with B16F1 cells (day 0), mice were treated with the mRNA vaccine. At days 6, 9, 10, and 22 post tumor inoculation, blood was drawn for determination of IFN-γ serum levels. Data are mean ± S.D. ($n = 3$). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$).

mRNA vaccine *via* IP showed a significantly potent induction of systemic and localized immunity, melanoma regression and elevation in cytokine expression compared to s.c. injection, which showed a weak (and non-significant) effect. These findings are likely driven by the unique features of IP technology in being able to homogeneously distribute and accumulate the minimal mRNA vaccine into skin layers, and subsequently expose it for engulfment by skin-abundant immune cells in addition to improving intracellular uptake of the vaccine. On the other hand, the mRNA vaccine is expected to quickly diffuse after s.c. injection, which can impair the activation of APCs. Taken together, results of the present study demonstrate successful immune system activation and melanoma regression by combining a newly synthesized minimal mRNA with IP technology, and offer a new step in the field of mRNA vaccination. Moreover, we are planning in our next step to include our minimal mRNA vaccine in the form of nanoparticles to provide a complete protection against skin nucleases and to obtain a higher effect.

CONCLUSION

In conclusion, we demonstrated the efficiency of IP technology in overcoming challenges associated with the stratum corneum for delivery of hydrophilic macromolecules using a non-formulated minimal mRNA vaccine encoding for TAA against melanoma challenge. As expected, a potent immune response was induced after combining IP technology with the minimal mRNA vaccine, which resulted in tumor inhibition and up-regulation of cytokine expression, as well as infiltration of cytotoxic $CD8^+$ and $CD4^+$ T cells into the tumor microenvironment. To our knowledge, this is the first report demonstrating the successful transdermal delivery of a minimal mRNA vaccine using IP technology.

Acknowledgments This study was supported by the Egyptian Ministry of Higher Education, the Honjo International Scholarship Foundation, and by a research program for the development of intelligent Tokushima artificial exosome (iTEX) from Tokushima University, Japan.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Chaudhary N, Weissman D, Whitehead KA. mRNA vaccines for infectious diseases: principles, delivery and clinical translation. *Nat. Rev. Drug Discov.*, **20**, 817–838 (2021).
2) He Q, Gao H, Tan D, Zhang H, Wang J. mRNA cancer vaccines: Advances, trends and challenges. *Acta Pharm. Sin. B.*, **12**, 2969–2989 (2022).
3) Lorentzen CL, Haanen JB, Met Ö, Svane IM. Clinical advances and ongoing trials on Mrna vaccines for cancer treatment. *Lancet Oncol.*, **23**, e450–e458 (2022).
4) Miao L, Zhang Y, Huang L. mRNA vaccine for cancer immunotherapy. *Mol. Cancer*, **20**, 41 (2021).
5) Kariko K, Whitehead K, van der Meel R. What does the success of mRNA vaccines tell us about the future of biological therapeutics? *Cell Syst.*, **12**, 757–758 (2021).
6) *Strategy to Achieve Global Covid-19 Vaccination by mid-2022*. WHO, Geneve (2022).
7) Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines—a new era in vaccinology. *Nat. Rev. Drug Discov.*, **17**, 261–279 (2018).
8) Wadhwa A, Aljabbari A, Lokras A, Foged C, Thakur A. Opportunities and challenges in the delivery of mRNA-based vaccines. *Pharmaceutics.*, **12**, 102 (2020).
9) Bidram M, Zhao Y, Shebardina NG, Baldin AV, Bazhin AV, Ganjalikhany MR, Zamyatnin AA Jr, Ganjalikhani-hakemi J. mRNA-based cancer vaccines: a therapeutic strategy for the treatment of melanoma patients. *Vaccines.*, **9**, 1060 (2021).
10) Duan LJ, Wang Q, Zhang C, Yang D, Zhang X. Potentialities and challenges of mRNA vaccine in cancer immunotherapy. *Front. Immunol.*, **13**, 923647 (2022).
11) Zhou WZ, Hoon DSP, Huang SKS, Fujii S, Hashimoto K, Morishita R, Kaneda Y. RNA melanoma vaccine: induction of antitumor immunity by human glycoprotein 100 mRNA immunization. *Hum. Gene Ther.*, **10**, 2719–2724 (1999).
12) Miao L, Li L, Huang Y, Delcassian D, Chahal J, Han J, Shi Y, Sadtler K, Gao W, Lin J, Doloff JC, Langer R, Anderson DG. Delivery of mRNA vaccines with heterocyclic lipids increases anti-tumor efficacy by STING-mediated immune cell activation. *Nat. Biotechnol.*, **37**, 1174–1185 (2019).
13) Huff AL, Jaffee EM, Zaidi N. Messenger RNA vaccines for cancer immunotherapy: progress promotes promise. *J. Clin. Invest.*, **132**, e156211 (2022).
14) Abe N, Imaeda A, Inagaki M, Li Z, Kawaguchi D, Onda K, Nakashima Y, Uchida S, Hashiya F, Kimura Y, Abe H. Complete chemical synthesis of minimal messenger RNA by efficient chemical capping reaction. *ACS Chem. Biol.*, **17**, 1308–1314 (2022).
15) Toyoda M, Hama S, Ikeda Y, Nagasaki Y, Kogure K. Anti-cancer vaccination by transdermal delivery of antigen peptide-loaded nanogels *via* iontophoresis. *Int. J. Pharm.*, **483**, 110–114 (2015).
16) Kigasawa K, Kajimoto K, Nakamura T, Hama S, Kanamura K, Harashima H, Kogure K. Noninvasive and efficient transdermal delivery of CpG-oligodeoxynucleotide for cancer immunotherapy. *J. Control. Release*, **150**, 256–265 (2011).
17) Fukuta T, Oshima Y, Michiue K, Tanaka D, Kogure K. Non-invasive delivery of biological macromolecular drugs into the skin by iontophoresis and its application to psoriasis treatment. *J. Control. Release*, **323**, 323–332 (2020).
18) Labala S, Jose A, Chawla SR, Khan MS, Bhatnagar S, Kulkarni OP, Venuganti VVK. Effective melanoma cancer suppression by iontophoretic co-delivery of STAT3 siRNA and imatinib using gold nanoparticles. *Int. J. Pharm.*, **525**, 407–417 (2017).

19) Hasan M, Fukuta T, Inoue S, Mori H, Kagawa M, Kogure K. Iontophoresis-mediated direct delivery of nucleic acid therapeutics, without use of carriers, to internal organs *via* non-blood circulatory pathways. *J. Control. Release*, **343**, 392–399 (2022).
20) Hasan M, Hama S, Kogure K. Low electric treatment activates Rho GTPase *via* heat shock protein 90 and protein kinase C for intracellular delivery of siRNA. *Sci. Rep.*, **9**, 4114 (2019).
21) Hasan M, Tarashima N, Fujikawa K, Ohgita T, Hama S, Tanaka T, Saito H, Minakawa N, Kogure K. The novel functional nucleic acid iRed effectively regulates target genes following cytoplasmic delivery by faint electric treatment. *Sci. Technol. Adv. Mater.*, **17**, 554–562 (2016).
22) Hashimoto M, Takemoto T. Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing. *Sci. Rep.*, **5**, 1–7 (2015).
23) Wadhwa A, Aljabbari A, Lokras A, Foged C, Thakur A. Opportunities and challenges in the delivery of mRNA-based vaccines. *Pharmaceutics.*, **12**, 102 (2020).
24) Hama S, Kimura Y, Mikami A, Shiota K, Toyoda M, Tamura A, Nagasaki Y, Kanamura K, Kajimoto K, Kogure K. Electric stimulus opens intercellular spaces in skin. *J. Biol. Chem.*, **289**, 2450–2456 (2014).
25) Hettinga J, Carlisle R. Vaccination into the dermal compartment: techniques, challenges, and prospects. *Vaccines.*, **8**, 534 (2020).
26) Celluzzi CM, Falo LD Jr. Epidermal dendritic cells induce potent antigen-specific CTL-mediated immunity. *J. Invest. Dermatol.*, **108**, 716–720 (1997).
27) Kubo A, Nagao K, Yokouchi M, Sasaki H, Amagai M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J. Exp. Med.*, **206**, 2937–2946 (2009).
28) Cheng R, Fontana F, Xiao J, Liu Z, Figueiredo P, Shahbazi MA, Wang S, Jin J, Torrieri G, Hirvonen JT, Zhang H, Chen T, Cui W, Lu Y, Santos HA. Recombination monophosphoryl lipid A-derived vacosome for the development of preventive cancer vaccines. *ACS Appl. Mater. Interfaces*, **12**, 44554–44562 (2020).
29) Belyakov IM, Hammond SA, Ahlers JD, Glenn GM, Berzofsky JA. Transcutaneous immunization induces mucosal CTLs and protective immunity by migration of primed skin dendritic cells. *J. Clin. Invest.*, **113**, 998–1007 (2004).
30) Liu J, Fu M, Wang M, Wan D, Wei Y, Wei X. Cancer vaccines as promising immuno-therapeutics: platforms and current progress. *J. Hematol. Oncol.*, **15**, 28 (2022).
31) Jaini R, Kesaraju P, Johnson JM, Altuntas CZ, Jane-wit D, Tuohy VK. An autoimmune-mediated strategy for prophylactic breast cancer vaccination. *Nat. Med.*, **16**, 799–803 (2010).
32) Scott BA, Yarchoan M, Jaffee EM. Prophylactic vaccines for nonviral cancers. *Annu. Rev. Can. Biol.*, **2**, 195–211 (2018).
33) Nanni P, Nicoletti G, Palladini A, Croci S, Murgo A, Antognoli A, Landuzzi L, Fabbi M, Ferrini S, Musiani P, Iezzi M, De Giovanni C, Lollini P. Antimetastatic activity of a preventive cancer vaccine. *Cancer Res.*, **67**, 11037–11044 (2007).
34) Garcia-Hernandez ML, Gray A, Hubby B, Klinger OJ, Kast WM. Prostate stem cell antigen vaccination induces a long-term protective immune response against prostate cancer in the absence of autoimmunity. *Cancer Res.*, **68**, 861–869 (2008).
35) Inoue J, Yotsumoto S, Sakamoto T, Tsuchiya S, Aramaki Y. Changes in immune responses to antigen applied to tape-stripped skin with CpG-oligodeoxynucleotide in mice. *J. Control. Release*, **108**, 294–305 (2005).
36) Nickoloff BJ, Turka LA, Mitra RS, Nestle FO. Direct and indirect control of T cell activation by keratinocytes. *J. Invest. Dermatol.*, **105** (Suppl.), 25S–29S (1995).
37) Ezepchuk YV, Leung DYM, Middleton MH, Bina P, Reiser R, Norris DA. Staphylococcal toxins and protein A differentially induce cytotoxicity and release of tumor necrosis factor-α from human keratinocytes. *J. Invest. Dermatol.*, **107**, 603–609 (1996).
38) Clausen BE, Stoitzner P. Functional specialization of skin dendritic cell subsets in regulating T cell responses. *Front. Immunol.*, **6**, 534 (2015).
39) Cheng EM, Tsarovsky NW, Sondel PM, Rakhmilevich AL. Interleukin-12 as an *in situ* cancer vaccine component: a review. *Cancer Immunol. Immunother.*, **71**, 2057– 2065 (2022).
40) Schmitz-Winnenthal FH, Escobedo LVG, Beckhove P, Schirrmacher V, Bucur M, Ziouta Y, Volk C, Schmied B, Koch M, Antolovic D, Weitz J, Büchler MW, Z'Graggen K. Specific immune recognition of pancreatic carcinoma by patient-derived CD4 and CD8 T cells and its improvement by interferon-gamma. *Int. J. Oncol.*, **28**, 1419–1428 (2006).
41) Heine A, Juranek S, Brossart P. Clinical and immunological effects of mRNA vaccines in malignant diseases. *Mol. Cancer*, **20**, 52 (2021).
42) Zhang Y, Guan X, Jiang P. Cytokine and chemokine signals of t cell exclusion in tumors. *Front. Immunol.*, **11**, 594609 (2020).
43) Bialkowski L, van Weijnen A, Van der Jeught K, Renmans D, Daszkiewicz L, Heirman C, Stangé G, Breckpot K, Aerts JL, Thielemans K. Intralymphatic mRNA vaccine induces CD8 T-cell responses that inhibit the growth of mucosally located tumours. *Sci. Rep.*, **6**, 22509 (2016).
44) Chen J, Ye Z, Huang C, Qiu M, Song D, Li Y, Xu Q. Lipid nanoparticle-mediated lymph node–targeting delivery of mRNA cancer vaccine elicits robust CD8+ T cell response. *Proc. Natl. Acad. Sci. U.S.A.*, **119**, e2207841119 (2022).
45) Haabeth OAW, Blake TR, McKinlay CJ, Waymouth RM, Wender PA, Levy R. mRNA vaccination with charge-altering releasable transporters elicits human T cell responses and cures established tumors in mice. *Proc. Natl. Acad. Sci. U.S.A.*, **115**, E9153–E9161 (2018).
46) Laczkó D, Hogan MJ, Toulmin SA, *et al.* A single immunization with nucleoside-modified mRNA vaccines elicits strong cellular and humoral immune responses against SARS-CoV-2 in mice. *Immunity*, **53**, 724–732.e7 (2020).