Transdermal nanolipoplex simultaneously inhibits subcutaneous melanoma growth and suppresses systemically metastatic melanoma by activating host immunity

Chia Hung Chen
  Hsinchu MacKay Memorial Hospital

Tzu-Han Weng
  MacKay Memorial Hospital

Cheng-Hsun Chuang
  National Yang Ming Chiao Tung University

Kai-Yao Huang
  Hsinchu MacKay Memorial Hospital

Sih-Cheng Huang
  National Yang Ming Chiao Tung University

Pin-Rong Chen
  National Yang Ming Chiao Tung University

Hsiao-Hsuan Huang
  National Yang Ming Chiao Tung University

Ling-Ya Huang
  National Yang Ming Chiao Tung University

Pei-Chun Shen
  National Yang Ming Chiao Tung University

Po-Ya Chuang
  National Yang Ming Chiao Tung University

Hsiao-Yen Huang
  National Yang Ming Chiao Tung University

Yi-Syuan Wu
  National Yang Ming Chiao Tung University

Shun-Long Weng
  MacKay Medical College

Kuang-Wen Liao (liaonms@nycu.edu.tw)
  National Yang Ming Chiao Tung University
Abstract

**Background:** Melanoma is a malignant cancer with high rates of recurrence, metastasis, and immunosurveillance evasion. Benefit for clinical treatments of melanoma, the transdermal neoadjuvant therapy could reduce surgery region and increase immunotherapy efficacy. Using cationic lipoplex (LPPC) encapsulated doxorubicin (DOX) and carrying CpG oligodeoxynucleotide; the nano-liposomal drug complex (LPPC-DOX-CpG) would have higher cytotoxicity and immunostimulatory activity than the originally unencapsulated DOX and CpG. In addition, CpG adsorption did not interfere with the release of drugs from the complexes. The liposomal-drug complexes were transdermally administered, allowing them to easily penetrate the skin within 12 h. After the application of the treatments, subcutaneous melanoma growth and systemic metastasis were monitored to assess therapeutic efficacy. Then, next-generation sequencing (NGS) data were used to explore the underlying mechanism.

**Results:** Transdermal administration of LPPC-DOX-CpG dramatically suppressed subcutaneous melanoma growth. LPPC-DOX-CpG induced tumor cell apoptosis and recruited immune cells into the tumor area. Interestingly, animal studies also showed that the colonization and growth of spontaneously metastatic melanoma cells in the liver and lung were suppressed by transdermal treatment with LPPC-DOX-CpG. NGS analysis further revealed the activation of the exogenous apoptotic pathway as the major mechanism responsible for the therapeutic effect of LPPC-DOX-CpG. The chemo-attractant, IFN-γ and NF-kB pathways were also triggered to recruit and activate the antigen presenting cells and effector cells.

**Conclusions:** Finally, we have successfully proved transdermal LPPC-DOX-CpG as a promising penetrative carrier to activate anti-tumor immunity against subcutaneous and metastatic tumor.

**Background**

Melanoma is one of the most malignant skin cancers and is characterized by early metastasis, rapid development, poor prognosis, and high lethality [1]. According to online statistics provided by the National Cancer Institute in 2020, more than 100,350 new cases of melanoma were reported in the United States. In addition to surgery, clinical treatments include conventional chemotherapy and radiotherapy as well as emerging biological therapies and immune checkpoint inhibitors (ICIs). The survival rates of melanoma patients with advanced-stage disease can be extensively improved by the use of anti-PD-1/PD-L1 or anti-CTLA4 antibodies, especially when ICIs are used as the first-line treatment [2–5]. However, the clinical response rates of ICI still require improvement [6, 7], and the expensive cost of the treatment is another consideration [8].

In addition, neoadjuvant therapies have improved the prognosis of patients with various types of cancer, including breast, bladder, pancreas, and esophageal cancer [9–12]. These treatments have been shown to improve the clinical outcomes achieved with cancer therapies by mechanisms such as reducing the size of the surgical area, decreasing the risk of tumor recurrence, and improving overall survival [13]. Considering the characteristics of malignant melanoma, such as highly mutated antigens and
immunosuppressive activity [14], ideal treatments should trigger multiple cytotoxic T lymphocytes (CTLs) and antitumor Th cells [15, 16] and induce the production of stimulatory cytokines to attenuate suppressive factors, such as TGF-β [17]. Therefore, many studies on neoadjuvant melanoma treatments have reported encouraging results based on the appropriate regulation of T cells and cytokine release, thereby benefiting melanoma patients [18].

The transdermal administration of neoadjuvants serves as a promising approach to maximize the efficacy of melanoma therapies and minimize adverse side effects [19]. Furthermore, multiple subtypes of dendritic cells (DCs) are highly abundant in the skin, with Langerhans cells populating the epidermis, and CD1a+, CD14+ and CD141+ DCs populating the dermis. CD1a+ dermal DCs induce T cell responses, whereas CD14+ dermal DCs are better equipped to activate humoral responses, and the CD141+ dermal DC subset is considered the most potent cross-presenting DC subset [20]. As such, efficiently activating multiple skin DC subsets simultaneously might elicit broader immune responses.

In this work, we developed a cationic liposome complex (Lipo-PEG-PEI-complex, LPPC) to enhance the transdermal delivery of chemotherapeutic drugs for the noninvasive topical treatment of melanoma and used it combination with an adjuvant to stimulate skin immune cells. Our previous study proved that the LPPC delivery system enhanced the skin penetrability, drug accumulation ability, and efficacy of breast cancer therapies [21]. In addition, LPPC could enhance the cytotoxicity of the drug against different cancer cells [22], and LPPC provided their activities by adsorbing proteins and nucleotides without chemical modification [23, 24]. Moreover, when combined with different adjuvants, the LPPC increased and switched immune responses [25]. Therefore, the LPPC was herein combined with doxorubicin (DOX) and CpG oligodeoxynucleotides (LPPC-DOX-CpG), and the effects of LPPC-DOX-CpG on melanoma were investigated. DOX induced cell death and released melanoma antigens, and the CpG oligodeoxynucleotide (ODN) induced antitumor immunity. In vitro and in vivo studies demonstrated that LPPC-DOX-CpG inhibited tumor growth and activated immune responses to tumors. Furthermore, RNA sequencing (RNA-seq) analysis by next-generation sequencing (NGS) was performed to elucidate the mechanisms underlying the therapeutic effects. These results demonstrate that convenient and efficient LPPC-drug-adjuvant complexes can be developed as a good platform for transdermal neoadjuvant therapies for skin-related cancers.

**Methods**

**Reagents**

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Polyethylene glycol (PEG, MW 8,000), polyethyleneimine (PEI branched, MW 25,000). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and doxorubicin (DOX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Class C CpG oligodeoxynucleotides (CpG ODN) was purchased from InvivoGen (San Diego, CA, USA).
Formation and characterization of LPPC, LPPC-DOX and LPPC-DOX-CpG

LPPC and LPPC-DOX were individually prepared according to procedures outlined in the previous studies [23, 25]. The particle size and the surface charge of LPPC and other complexes were analyzed by Zetasizer (Zetasizer Nano ZS90, Malvern, UK). The encapsulation dosages of LPPC-DOX and LPPC-DOX-CpG were evaluated using a fluorescent spectrophotometer (Hitachi, Tokyo, Japan) at Ex 470 nm/Em 590 nm. In addition, in vitro releases of DOX from LPPC-DOX or LPPC-DOX-CpG were measured as previously described [22]. For CpG ODN adsorption, fifty micrograms of LPPC or LPPC-DOX individually incubated with different amounts of CpG ODN for 30 min and centrifuged to measure the unabsorbed CpG ODN of the supernatant by OD260.

Cytotoxicity of LPPC-DOX or LPPC-DOX-CpG in vitro

B16F10 cells were seeded into a 96-well tissue culture plate at a density of 1×10^3 cells/100 μl/well. Subsequently, the cells were treated with either serial dilutions of non-encapsulated DOX, empty LPPC, LPPC-DOX, or LPPC-DOX-CpG. After 72 h of incubation, the cell viability in each group was evaluated by the MTT colorimetric assay (Sigma-Aldrich). The cell growth inhibition was plotted as a percentage of the untreated control, and the inhibitory concentration at 50% cell survival (IC50) of each reagent was determined from the dose effect curve.

The mRNA expression of cytokines from splenocytes treated with LPPC-CpG

Splenocytes (10^6 cells/well) from C57BL/6J mice were individually treated with LPPC, CpG, or LPPC-CpG for 6 h. Then, total RNA was extracted from cells using TRIzol reagent (Invitrogen, Gaithersburg, MD, USA) in accordance with the manufacturer’s instructions. The mRNA expression levels of IFN-α, TNF-α, IL-6, and actin were measured and analyzed by RT-PCR. Furthermore, the index values were calculated and normalized to that of the control actin by ImageJ software.

Kinetics of LPPC-DOX administered transdermally

Female C57BL/6J mice (4–6 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan), and all animal studies were approved and under the standard procedures by the superintendence of the Institutional Animal Care and Use Committee (IACUC) at National Chiao Tung University (NCTU-IACUC-109013) in Taiwan, ROC. The back skin hairs of the mice were shaved for LPPC-DOX transdermal treatment. After 12 h of treatment, EtOH was used to remove residual and unpermeated LPPC-DOX, and whole-body imaging of the mice was performed at different time points with an IVIS Spectrum System (Caliper Life Sciences Inc., Hopkinton, MA, USA).

Antitumor activity of LPPC-DOX-CpG

Female C57BL/6J mice (n=10) were subcutaneously implanted with 1×10^4 B16F10 cells in 100 μl of PBS. Three days after implantation, the mice bearing B16F10 tumors were treated with LPPC-DOX (containing 0.5 mg/kg DOX), LPPC-CpG (containing 0.05 mg/kg CpG), LPPC-DOX-CpG (containing 0.5 mg/kg both DOX and CpG), LPPC-DOX-CpG (containing 0.1 mg/kg both DOX and CpG), or saline as a control.
mg/kg DOX and 0.05 mg/kg CpG) or vehicle by skin application every day. Tumor size was measured with a caliper, and tumor volume was calculated as $L \times H \times W$. The mice were sacrificed when the tumor size exceeded 2,000 mm$^3$.

**Cytotoxicity of mice splenocytes against B16F10 cells ex vivo**

B16F10 cells were seeded in a 96-well plate ($1 \times 10^3$ cells/well) and incubated overnight. Splenocytes from the mice without tumor growth treated with LPPC-DOX, LPPC-CpG, or LPPC-DOX-CpG were added to the wells as effector cells at E:T ratios of 25:1, 50:1, and 100:1. After incubation for 72 h, the supernatants were removed, the wells were washed six times with PBS buffer, and the cell viabilities were determined by the MTT assay (Sigma-Aldrich).

**Next-generation sequencing analysis**

The mice were sacrificed when the tumor size reached 500 mm$^3$, and RNA was extracted from the tumor mass using a PureLink™ RNA Mini kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then, NGS was processed by NovaSeq 6000 system (Illumina, CA, USA) with paired-end 150 bp, and the raw count was estimated and normalized as fragments per kilobase per million (FPKM). For functional and pathway analysis, the protein-coding genes annotated with the different biological regulations about the immune responses (chemokine, IFN, and NF-kB pathway) or cancer progressions (metastasis, proliferation, and apoptosis) were selected as the signatures, which annotation are based on the GSEA and enrich KEGG in package “clusterProfiler” in R [26-28]. For the expression patterns analysis, these signatures were further observed by tested for the significant difference by $t$-test and ANOVA analysis. Finally, the speculated mechanisms and pathways of transdermal tumor therapy likely regulated by these differential expression genes were investigated by KEGG and published literatures. The supporting data were available to researchers in the Gene Expression Omnibus (GEO) database, and the accession number was GSE197791.

**H&E and immunohistochemistry staining**

The tumors and organs of the mice were dehydrated and embedded in paraffin wax. Thereafter, the tissue sections (4 μm/section) from paraffin-embedded blocks were collected on clean glass slides and dehydrated for 30 min at 60 °C. The tissue slides were further deparaffinized, rehydrated, and stained with Mayer’s hematoxylin and eosin Y solution for 3 min. Finally, the tissue slides were mounted with mounting medium and photographed under a microscope (Olympus, Center Valley, PA, USA). The total number and size of metastases in each lung and liver section were counted and averaged among the animals.

The sections were subjected to ICH staining by using Antigen Retrieval Buffer (Abcam, Cambridge, UK) and a Leica Novolink max polymer detection system (RE7140-K, Leica, Wetzlar, Germany) according to the standard procedures provided by the manufacturer. In addition, the sections were stained and evaluated with a rabbit monoclonal PCNA (1:6,000 dilution, Cell Signaling Tech., MA, USA), cleaved
caspase 3 (1:2,000 dilution, Cell Signaling Tech.), CD8a (1:500 dilution, Cell Signaling Tech.), CD86 (1:500 dilution, Cell Signaling Tech.), CD19 (1:6,000, Cell Signaling Tech.), CD3 (1:300 dilution, Abcam), CD4 (1:500 dilution, Abcam), CD163 (1:500 dilution, Abcam) antibodies; a rabbit polyclonal CD68 antibody (1:5,000 dilution, Abcam). Finally, the sections were observed under a light microscope at a magnification of 400× and photographed.

Statistical analysis

Data were analyzed using the SAS statistical software package (SAS Institute, Inc., Cary, NC, USA). Two independent trials were compared by a t-test, whereas multiple variables were compared by ANOVA. \( P<0.05 \) indicated statistically significant differences. All results are expressed as the mean ± SD.

Results

Characterization of LPPC-DOX, LPPC-CpG and LPPC-DOX-CpG

First, the capacities of LPPC for DOX encapsulation were determined. LPPC (1 mg) exhibited a maximum DOX encapsulation of approximately 0.5 mg (Table 1). In addition, the encapsulation of DOX by the LPPC did not significantly alter the average zeta potential and only slightly increased the particle size by a factor dependent on the DOX increase (Table 1). In conclusion, the LPPC particle size and average zeta potential were approximately 210 nm and 42 mV, respectively.

Sequentially, drug release from LPPC-DOX complexes was assessed. During 120 h of incubation, kinetic analysis of drug release showed that only 20% of the encapsulated DOX was released from LPPC-DOX at 4 °C, while approximately 40% and 98% of the encapsulated DOX was released into the media at 25 °C and 37 °C, respectively (Fig. 1A). The results indicated that the release rate of DOX from the LPPC was temperature dependent.

Second, the CpG ODN absorbing capacity of LPPC or LPPC-DOX complexes was determined. As shown in Fig. 1B, 50 μg of the LPPC adsorbed approximately 9 μg of CpG, which was equal to the absorption ability of LPPC-DOX. Furthermore, CpG absorption led to an increase in the particle size (244.5±9.3 nm) but to a decrease in the zeta potential (29.2±2.9 mV) of LPPC-DOX complexes. Moreover, the drug release kinetics did not significant differ between LPPC-DOX and LPPC-DOX-CpG (Fig. 1C). Together, these results revealed that DOX encapsulation did not interfere with CpG binding and that the binding of CpG to LPPC-DOX did not influence the characteristics of drug release from LPPC particles.

Biofunction of LPPC-DOX-CpG in vitro

Compared to DOX, LPPC-DOX and LPPC-DOX-CpG had higher cytotoxic activities (IC50; 5.5- and 4.8-fold, respectively), and the LPPC obviously increased the cytotoxic effect of DOX on B16F10 cells (Fig. 2A). The cytotoxic kinetics revealed that CpG absorption on LPPC did not influence the cytotoxic activities of LPPC-DOX (Fig. 2A; green and purple lines).
Likewise, the adjuvant effects of LPPC-DOX-CpG complexes on cytokine induction were also examined. As shown in Fig. 2B, LPPC-CpG dramatically increased the expression of the IFN-a, TNF-a, and IL-6 cytokines in splenocytes compared to that induced by CpG alone, which indicated that the LPPC facilitated CpG to activate stronger immune responses. However, the LPPC induced only slight expression of these cytokines, which indicated that the LPPC and CpG had a synergistic effect on cytokine induction. Moreover, quantitative analysis revealed that compared to CpG alone, LPPC-CpG efficiently enhanced the mRNA expression of IFN-a by nearly 4-fold, TNF-a by 13-fold, and IL-6 by 11-fold (Fig. 2C).

LPPC-DOX-CpG administered transdermally exerts antitumor activity in vivo

Subsequently, the transdermal kinetics of LPPC encapsulated DOX were monitored, revealing that transdermal DOX was detectable within 12 h and undetectable after 24 to 36 h (Fig. 3A). According to the kinetic analysis results, the animals were treated once a day for the following animal study.

C57BL/6J mice bearing B16F10 tumors were transdermally treated with empty LPPC, LPPC-DOX, LPPC-CpG, or LPPC-DOX-CpG once every day. B16F10 tumor growth was significantly suppressed in animals treated with LPPC-DOX, LPPC-CpG, and LPPC-DOX-CpG compared with PBS (Fig. 3B). Compared to LPPC-DOX and LPPC-CpG, LPPC-DOX-CpG exerted a better tumor suppressive effect, it could cause significance in decrease of tumor growth from 32<sup>nd</sup> to 34<sup>th</sup> days after treatments. Both LPPC-DOX and LPPC-CpG effectively inhibited tumor growth, and the differences were not statistically significant. Interestingly, although the effect is not weaker, empty LPPC treatment also led to a significant anti-tumor effect on tumor growth comparing to PBS group from 18<sup>th</sup> to 29<sup>th</sup> days. These results showed that LPPC-DOX-CpG inhibited the growth of malignant B16F10 cells more effectively than LPPC-DOX and LPPC-CpG.

Moreover, the mice treated with LPPC-DOX-CpG also exhibited the longest survival time (T<sub>1/2</sub>=47.5 days); LPPC-DOX and LPPC-CpG extended the survival times (T<sub>1/2</sub>=40 days and 40 days) compared to those in the control groups (PBS and LPPC, and T<sub>1/2</sub>=26.3 days and 35 days). Interestingly, 20% of mice treated with LPPC-DOX-CpG were tumor free for more than 60 days, and 10% of mice treated with LPPC-DOX and LPPC-CpG were tumor free (Fig. 3C).

Furthermore, the pathological changes in tumor areas were histologically monitored after treatment with the different agents, revealing that LPPC-DOX-CpG significantly shattered the tumor nucleus and dramatically damaged the tumor tissue. Obviously, LPPC-DOX and LPPC-DOX-CpG also attacked the tumor vessel, resulting in bleeding in the tumor area (Fig. 3D). In addition, while all LPPC-related treatments induced the infiltration of immune cells, LPPC-DOX-CpG recruited the most immune cells to the tumor area (Fig. 3D). These results potentially indicate that the transdermal delivery of this LPPC system inhibited tumor growth by activating the immune response.

Pathologic effects of LPPC-DOX-CpG in vivo

The histopathological tumor-suppressive effects of transdermal LPPC-DOX-CpG treatment were further explored. LPPC-DOX-CpG reduced the expression of PCNA in tumor cells, indicating that tumor
growth may have been partially suppressed by the inhibition of cell proliferation (Fig. 4). Moreover, LPPC-DOX and LPPC-CpG reduced the expression of PCNA in tumor cells, but their effects were weaker than LPPC-DOX-CpG. LPPC-DOX-CpG, LPPC-DOX and LPPC-CpG significantly increased the active caspase 3 compared with that in the LPPC and PBS groups. LPPC-DOX-CpG had the strongest ability to activate caspase 3 and induced the cell apoptosis better than LPPC-DOX and LPPC-CpG.

Moreover, tumor-infiltrating cells were also examined by IHC, revealing that the CD3, CD4 and CD8 antigen levels were increased in the tumor area after LPPC-DOX-CpG, LPPC-DOX and LPPC-CpG treatment. Thus, these treatments induced the infiltration of CD4T or CD8T lymphocytes into tumor areas, and the numbers of CD8T cells were slightly higher than those of CD4T cells (Fig. 4; CD3, CD4, CD8). Similarly, the expression of CD19, a B cell marker, was increased after LPPC-DOX-CpG, LPPC-DOX or LPPC-CpG treatment. Likewise, increased levels of the CD68, CD86 and CD163 antigens were observed in tumor areas, which indicated increased macrophage infiltration into the tumor area and differentiation into both the M1 and M2 subtypes (Fig. 4).

**Systemic effect of transdermally administered LPPC-DOX-CpG**

We next assessed whether the transdermal administered LPPC-DOX-CpG could induce systemic antitumor immunity. In previous in vivo therapy study, tumor-free mice were sacrificed, and the specific cytotoxicity of their splenocytes was examined. As shown in additional file 1, the splenocytes obtained from mice treated for 60 days exhibited antitumor activity, indicating that the transdermal administration of LPPC-DOX-CpG induced systemic immunity against melanoma cells.

In this model, the subcutaneous melanoma cells spontaneously metastasized to colonize the liver or lung and significantly formed colonies (Fig. 5A, PBS and LPPC treatments). These colonies preferred to colonize beside vessel, which suggested that they spread primarily through blood circulation. Transdermal administration of LPPC-DOX-CpG induced systemic immunity against melanoma cells, thereby reducing tumor cell metastasis. The effect of LPPC-DOX-CpG on the distant metastasis of melanoma cells was monitored, revealing that LPPC-DOX-CpG and LPPC-DOX significantly reduced the sizes and numbers of metastatic colonies in the liver and lung (Fig. 5B and 5C). Therefore, these results showed that LPPC-DOX-CpG administered transdermally not only suppressed subcutaneous melanoma growth but also reduced the metastasis of melanoma cells. Systemic antitumor immunity may have contributed to this result.

**Molecular mechanisms underlying the therapeutic tumor-inhibitory effects of transdermal LPPC-DOX-CpG**

Furthermore, the gene expressions in tumor samples of different groups respectively were analyzed by NGS. First, the genes with significantly different expressions in the samples treated with LPPC-DOX-CpG comparing to the treatment of PBS (p < 0.05) were selected. The selected genes with the fold changes (FCs) between 1.1 and 0.9, or the values of expressions (FPKM) less than 0.1 both were further excluded. In total, 468 genes met these criteria, and their names, ID, and parameters (including mean of expression, FC and p value) were displayed in additional file 2, respectively. Then, their functions were annotated.
with the GENECARD online database and divided into 27 categories (Fig. 6A). The gene expression values and FCs were shown in Fig. 6B. The genes were shown to be associated with 4 major functions: immune (22.6%), cell growth (15.4%), metastasis (7.3%) and apoptosis (4.9%), and their values of gene expressions and FC were labeled with color (Fig. 6B). The average expression levels and FCs as well as the distributions were shown in Fig. 6C and 6D. The genes in the metastasis group had the highest expression levels, while those in the immune group had the highest FC values. The major pathways in the four groups were analyzed as follows.

**Molecular mechanisms underlying the therapeutic immune effects of transdermal LPPC-DOX-CpG**

Additional file 3 showed 66 immune-non-enriched differential expression genes (DEGs) in the categorization of immunity were 52 up-regulations and 14 down-regulations for gene expression. And there are another 40 DEGs enriched in certain important pathways according to KEGG, GSEA, and published literature (Additional file 3).

**Chemokine pathways:** As shown in Fig. 7A, Pf4 and CCL2 (FC = 2.5 and 2.2) but not CXCL13 (FC = 0.8) were up-regulated and engaged their receptors including the increasing CCR5, and trigger the cell migration pathways including CCR5 pathway and GPCR pathway. In addition, a macrophage homing marker (CD68) and migration gene (ADGRE5) were increasing (FC = 1.84 and 1.7; Table S2) that indicated the chemo-attracting pathways were activated and cause the macrophages homing to the tumor area. It revealed that LPPC-DOX-CpG treatment could induce chemokine releases and attract the macrophage migration to tumor area through CCR5 and GPCR pathways. As shown in Fig. 7A, LPPC-DOX-CpG caused 10 DEG expressions, but LPPC-DOX or LPPC-CpG only induced 2 significant different expressions (not including CD68) in this pathway (p<0.05). Obviously, only LPPC-DOX-CpG could lead to such effect by inducing the CCL2 and Pf4 releases to attract the migration of immune cell through CCR5 or CXCR3/5 mediated pathway (Fig 7B), but not other treatments.

**Interferon-g pathway:** Although the expression of IFN-g did not significantly differ between the PBS and LPPC-DOX-CpG groups, the expression levels of 11 IFN-g-responsive genes (direct and indirect) were increased (Fig. 7C). Besides, MHC-relative gene expressions obviously were increased, in which 4 DEGs (CTSB, CTSL, CTSS and XBP1: Fc = 1.9, 1.6, 3.6 and 1.3) increased for MHC II expressions and three DEGs (LILRA6, TAP1 and TAPBP: Fc=1.1, 1.7 and 1.6) for MHC I expressions. Among the 11 DEGs related to INF-g (all which were upregulated by LPPC-DOX-CpG treatment), only one (Gbp7) was significantly upregulated by LPPC-CpG, and LPPC-DOX failed to upregulate any genes, indicating that both DOX and CpG were essential for activation of the INF-g pathway (Fig. 7D). These results indicated that LPPC-DOX-CpG, but not LPPC-DOX or LPPC-CpG, activated the INF-g pathway.

**NF-kB pathway:** Moreover, the expression levels of genes associated with the NF-kb pathway were increased after LPPC-DOX-CpG treatment. Ten upregulated DEGs and 1 downregulated DEG were enriched in the inflammatory pathway (Fig. 8A). TLR, IL1R, FcR and LT-BR triggered different signaling pathways to thereby activate NF-kB. Consequently, the levels of TLR-inducible gene ZC3H12A and NF-kB down-steam gene SRGN were also increased by 1.5- and 3.5-fold, respectively,
which indicated that LPPC-DOX-CpG treatment activated the NF-kB pathway. Moreover, the expression of CEBPB, a marker of macrophage activation, was increased by 2.2-fold in the LPPC-DOX-CpG treatment group. In addition, LPPC-DOX and LPPC-CpG failed to activate this pathway, which revealed that both DOX and CpG were essential for its activation (Fig. 8B).

**T- and B- cells:** LPPC-DOX-CpG treatment also enhanced the migration and activation of T- and B-cells by significantly upregulating the expression of 13 DEGs (Fig. 8C). In addition, B-cell migration and activation were potentially activated by the increased expression of the IL-21 receptor pathway. The expression of CD24A, a marker of B-cell activation, was obviously increased in the LPPC-DOX-CpG group (FC = 1.9), indicating that LPPC-DOX-CpG induced B-cell activation. Among these DEGs, LPPC-CpG induced only one DEG and LPPC-DOX induced none, indicating that the LPPC-DOX-CpG-induced activation and migration of T- and B- cells required DOX and CpG (Fig. 8C).

Taken together, these data demonstrated that LPPC-DOX-CpG treatment would induce chemokine releases (Pf4 and CCL2) that would cause macrophage migration to tumor area through CCR5 and GPCR pathways, and activating IFN-g and NF-kB pathways. These signaling would consequently cause the increases in the expressions of MHC I and II, and result in enhancing the presentation and activation of macrophage. Sequentially, the activated macrophages would recruit the lymphocytes to infiltrate into tumor area and activate them.

**Molecular mechanisms underlying the therapeutic effects of transdermal LPPC-DOX-CpG on tumor growth and metastasis**

**Cell proliferation:** Based on the annotation of genes related to cell proliferation in the GENECARD online database, in total 72, there are 51 proliferation non-enriched DEGs (Additional file 4; 29 decreases and 22 increases in the expressions). Other 21 DEGs were analyzed by KEGG, revealing that were enriched in the RAS-related and Wnt pathways (Additional file 4, 9 upregulated and 12 downregulated). LPPC-DOX-CpG treatment mainly inhibited the H-ras/RAF and wnt/b-Catenin/TCF7L1 pathways but also partially enhanced the PI3K/AKT pathway, including the upstream genes EPO and APLN and the downstream gene MTOR (Fig. 9A). In addition, CDK2 expression was increased. Taken together, these results suggested that the inhibition of tumor proliferation by LPPC-DOX-CpG treatment was not the main mechanism influencing tumor growth, which was consistent with the IHC results (Fig. 4). Among the 21 DEGs enriched by LPPC-DOX-CpG treatment, only 9 were significant enriched by LPPC-CpG, and 5 were significantly enriched by LPPC-DOX (p<0.05, Fig. 9B). According to these results, LPPC-DOX-CpG requires both DOX and CpG to exert a minor inhibitory effect on cell proliferation.

**Apoptosis:** Fourteen genes related to apoptosis were upregulated, and 9 were downregulated (Additional file 5). These 23 DEGs were further analyzed, revealing that 16 were significantly enriched in several pathways associated with apoptosis. LPPC-DOX-CpG treatment activated the exogenous apoptosis pathway through the TNF receptor and Fas pathway (Fig. 10A). These pathways were promoted by the increased expression of caspase activation genes and the decreased expression of caspase inhibition
genes. Furthermore, pro-survival genes such as Bcl-2 were downregulated. These results indicated that LPPC-DOX-CpG may have inhibited tumor growth by disrupting the balance between the expression of pro-apoptotic and anti-apoptotic genes to induce apoptosis through the extrinsic apoptotic pathway (Fig. 10A). Furthermore, LPPC-CpG induced the significant differential expression of only 1 gene, and LPPC-DOX induced the significant differential expression of 4 genes (Fig. 10B). These results revealed that LPPC-DOX-CpG required the synergistic effect of DOX and CpG to efficiently trigger apoptotic reactions.

**Metastasis:** Thirty-two DEGs involved in tumor metastasis by LPPC-DOX-CpG treatment, of which 16 were downregulated and 16 were upregulated (Additional file 6). After analyzing their functions, 14 DEGs were found to be enriched in metastasis pathways, suggesting that LPPC-DOX-CpG treatment reduced tumor metastasis by downregulating metastasis-related genes and increasing the expressions of metastasis-inhibitory genes. LPPC-DOX-CpG treatment mainly reduced the expressions of genes associated with cell adhesion to thereby downregulate RhoA-related pathways and increased the gene expressions, including ARHGAP23, ARHGAP25 and RND3, to inhibit RhoA pathways (Fig. 11A). In addition, LPPC-DOX-CpG treatment also down-regulated the Smad2/3-mediated pathway to decrease EMT. LPPC-CpG induced the significant differential expression of 3 genes, while LPPC-DOX-CpG induced the significant differential expression of 14 genes (Fig. 11B) (p<0.05), and LPPC-DOX induced the significant differential expression of only 1 gene. These results indicated that the anti-metastatic ability of LPPC-DOX-CpG required the synergistic effect of CpG and DOX.

Together, these NGS results revealed that the transdermal LPPC-DOX-CpG successfully activated host local immunity to induce tumor cell apoptosis and exerted a systemic antitumor effect to decrease tumor metastasis. Thus, local LPCC treatment simultaneously induces tumor cell death and activates immunity and may be a safe and efficient therapeutic strategy for skin-associated cancers.

**Discussion**

Melanoma originating from melanocytes in the bottom layer of the epidermis is the most aggressive type of skin cancer that has a high mortality and metastasizes to many important organs, including the liver, lung, and even the brain [1, 29]. This study provides an efficient platform for the transdermal delivery of drugs to modulate immune responses to malignant melanoma. The penetrability of drugs across the skin barrier is well known to be difficult, and our previous study has revealed that a cationic complex carrying tamoxifen could easily penetrate the skin to suppress the growth of ER+ breast tumor cells in vivo [21]. Similarly, the LPPC herein promoted the delivery of the antitumor drug DOX across the skin within 12 h (Fig. 3A), confirming its ability to serve as a transdermal vector for the efficient delivery of drugs via the skin penetration route. Interestingly, transdermally administered LPPC-DOX-CpG not only successfully suppressed the growth of subcutaneous malignant B16F10 tumor cells to improve survival (Fig. 3B and 3C) but also induced a systemic immune response to spontaneous metastatic tumor cells in the liver and lung (Additional file 1 and Fig. 5). LPPC-DOX-CpG inhibited malignant melanoma growth via the following mechanisms: (1) it induced the apoptosis of tumor cells by disrupting the balance between pro-apoptotic and pro-survival genes through the exogenous apoptotic pathway but not by inhibiting the cell growth
pathway (Fig. 4 and Fig. 10); (2) it induced the homing of cytokines released by the microenvironment to recruit macrophages and lymphocytes (Fig. 7A); (3) it induced macrophages to preferentially differentiate into the M1 and M2 phenotypes (Fig. 4); and (4) it activated the IFN-γ pathway and downstream molecules (Fig. 7C). Therefore, the transdermal LPPC-DOX-CpG described herein simultaneously suppressed malignant melanoma growth and activated immune responses and could thus be further developed as an immunotherapeutic strategy for melanoma based on the underlying molecular mechanisms.

Transdermal therapies are still limited by the failure of delivered drugs to efficiently penetrate physiological barriers, including cutaneous, cellular, and tumor microenvironmental barriers [30–32]. Previous studies have consistently highlighted the promise of cationic liposomes as a vector for transdermal drug delivery [33, 34]. In our previous study, the drug encapsulated in the LPPC rapidly and deeply penetrated mouse skin within 2 h [21]. In addition, LPPC-encapsulated drugs were more cytotoxicity to cancer cells in vivo than their parental drugs [35, 36]. Therefore, we hypothesized that this therapeutic strategy would suppress subcutaneous tumor growth by enhancing drug cytotoxicity. The results shown in Fig. 3 herein further showed that LPPC-encapsulated DOX penetrated the skin within 12 h and gradually diffused at approximately 24 h. The histopathologic and IHC results also showed that transdermal LPPC-DOX-CpG and LPPC-DOX damaged B16F10 tumor tissues (Fig. 3D) and induced apoptosis (Fig. 4). These results indeed indicated that LPPC-encapsulated DOX not only crossed physiological barriers but also exerted cytotoxic effects on melanoma cells. Compared with LPPC-DOX-CpG and LPPC-DOX, LPPC-DOX-CpG induced more serious tumor tissue damage that was potentially attributed to the CpG-mediated immune attack (Fig. 3D). Several studies have indicated that whole-cell lysates containing a wide variety of tumor-associated antigens (TAAs) can activate broad and efficient immune responses to certain cancers [37], such as colon cancer [38], brain cancer [39], and prostate cancer [40]. However, these TAAs have low immunogenicity, making it difficult to induce an efficient immune response to tumor growth, and an adjuvant is thus required to increase antitumor immunity. In addition, all of the NGS results herein revealed that LPPC-DOX-CpG triggered more efficient antitumor immune responses due to the synergistic effect of DOX and CpG (Fig. 7–11). Therefore, both the cytotoxic activity of DOX and the immunomodulatory activity of CpG are essential for this transdermal tumor therapy platform.

Consequently, LPPC-DOX-CpG also exerted a neoadjuvant therapeutic effect, as it reduced the tumor volume to decrease the size of the surgical area and thereby lowered the risk of tumor recurrence. While other neoadjuvant-like therapies involving the delivery of anticancer drugs were shown to reduce the sizes of tumors, they did not further stimulate antitumor immune responses, and the patients easily relapsed [41–43]. Ideal neoadjuvant therapies will not only decrease the tumor volume but also activate antitumor immune responses to prevent tumor recurrence. LPPC-DOX-CpG also induced systemic immunity and suppressed the metastasis of melanoma cells to the liver and lung (Fig. 5). Thus, LPPC-DOX-CpG could be a good and convenient neoadjuvant therapy for melanoma and may have promising therapeutic efficacy when used in combination with ICI therapy or conventional surgery.
In addition to conventional surgery, chemotherapy and radiotherapy, the clinical treatment options for patients with melanoma include biological therapies and immunotherapies [1, 44]. Currently, immunotherapies, such as PD-1 and CTLA-4 antibodies, are considered promising innovative treatments for melanoma, but the clinical results showed more often than not that only a small subset of patients respond favorably [7]. One major factor potentially accounting for the improved outcomes of some patients is that antitumor T cells are induced prior to treatment [45]. Therefore, successful activation of antitumor T cells could improve the therapeutic efficacy of ICIs. Hence, this transdermal drug delivery platform is suggested to serve as a supplement for ICI therapy. The results in Fig. 4 indicate that all the treatments increased the expression of the T cell markers CD3, CD8 or CD4, indicating that the LPPC system recruited CD4⁺ and CD8⁺ T cells to the tumor area. In addition, antitumor CTL activities were also detectable after the treatments (Fig. S1), and the NGS data revealed that the levels of IFN-γ pathway components, MHC molecules and T-lymphocytes were increased in the tumor area after LPPC-DOX-CpG treatment (Figs. 7 and 8). These data suggest that LPPC-DOX-CpG will enhance the tumor-suppressive effects of ICI therapies. Therefore, this transdermal LPPC-DOX-CpG could be a good supplemental medicine administered prior to ICI treatment.

Clearly, the transdermal LPPC-DOX-CpG induced the release of PF4 and CCL2, which have chemotactic activity for monocytes and induce the differentiation of monocytes into macrophages [46, 47]. As shown in Fig. 7A, only LPPC-DOX-CpG could increase the expressions of CCL2 and Pf4, but not LPPC-DOX or LPPC-CpG. It indicated that synergistic effect of DOX and CpG is necessary for the two chemokine releases. Through chemokine receptors or GPCRs, signals are delivered and result in the migration of immune cells expressing CCR5 or CXCR3/5. The pathologic analysis results showed that LPPC-DOX-CpG treatment enhanced immune cell infiltration, which was consistent with their theoretical function (Fig. 3D). In addition, Pf4 inhibits angiogenesis and can suppress tumor growth and metastasis [48, 49]. Thus, Pf4 expression induced by LPPC-DOX-CpG might not only play an important role in the homing of immune cells but also suppress tumor growth and metastasis.

Moreover, B16F10 cells are malignant cells that are characterized by a low cure rate and a high recurrence rate. The LPPC-related treatments herein not only suppressed the B16F10 tumor growth rate but also resulted in five mice being tumor-free for as long as 90 days (Additional file 1). In accordance with this finding, our previous studies showed that LPPC increased the cytotoxicity of antitumor drugs against different drug-resistant cancer cells and suppressed the growth of breast tumors in a nude mouse model without help from the immune system [21, 22]. Based on different chemotherapies, several published studies have revealed that STAT3 is a potent regulator of melanoma that participates in the processes of cell growth, metastasis, angiogenesis, drug resistance, and even immunoregulation [50, 51]. In addition, B16F10 cells highly express certain genes, such as Pim-3 and BST-2, to promote the activation of STAT3 [52, 53]. The tyrosine kinase receptor/Ras/STAT3 pathway was shown to be responsible for the malignant features of melanoma [54–56]. Here, LPPC-DOX-CpG treatment decreased the expression of numerous genes, including Ras and certain tyrosine kinase receptors, which therefore decreased the malignancy of melanoma (Fig. 9).
Conclusions

In conclusion, the LPPC can both penetrate the skin and exert adjuvant effects as a good transdermal vector. To satisfy the needs of different diseases, convenient and flexible LPPCs can be used in combination with immunomodulators to induce appropriate and correct responses. Finally, this study provides a promising method for treating skin-associated cancers with the LPPC-drug-adjuvant complex.

Abbreviations

ICI: immune checkpoint inhibitors; CTL: cytotoxic T lymphocyte; LPPC: Lipo-PEG-PEI-complex; NGS: next-generation sequencing; DEGs: differential expression genes; TAAs: tumor-associated antigens.

Declarations

Ethics approval and consent to participate

All animal studies were approved by the Institutional Animal Care and Use Committee in National Yang Ming Chiao Tung University (NCTU-IACUC-109013).

Consent for publication

No applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors have declared that no competing interest exists.

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Authors’ contributions

Chia-Hung, SL, and KW designed research. Chia-Hung, CH, KY, SC, PR, HY, HH, LY, and YS performed research. TH, PC, and PY performed and analyzed histopathology. Chia-Hung wrote this paper. SL and
KW reviewed and edited this paper. All authors read and approved the final manuscript.

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References


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Tables

Table 1. The characteristics of LPPC-DOX complexes.

<table>
<thead>
<tr>
<th>LPPC/DOX (w : w)</th>
<th>1 : 0</th>
<th>1 : 0.25</th>
<th>1 : 0.5</th>
<th>1 : 1</th>
<th>1 : 2</th>
<th>1 : 4</th>
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</thead>
<tbody>
<tr>
<td>Particle size (nm)</td>
<td>201.57 ± 8.74</td>
<td>208.53 ± 7.40</td>
<td>206.00 ± 8.14</td>
<td>214.30 ± 10.53</td>
<td>215.60 ± 6.18</td>
<td>215.90 ± 8.51</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>42.03 ± 3.70</td>
<td>42.50 ± 1.83</td>
<td>41.73 ± 2.49</td>
<td>43.47 ± 1.48</td>
<td>39.90 ± 2.23</td>
<td>41.47 ± 2.48</td>
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<tr>
<td>DOX (mg) in 1 mg LPPC</td>
<td>N/A</td>
<td>0.14 ± 0.02</td>
<td>0.27 ± 0.04</td>
<td>0.51 ± 0.01</td>
<td>0.51 ± 0.03</td>
<td>0.52 ± 0.05</td>
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</tbody>
</table>

DOX encapsulated in 1 mg of the LPPC at various weight ratios. The particle size, zeta potential and DOX encapsulation of LPPC-DOX were measured. All values represent the mean ± SD (n = 3).

Figures
Figure 1

The drug release and CpG adsorption capacities of LPPC-DOX and LPPC-DOX-CpG.

(A) The drug doxorubicin (DOX) was released from the LPPC-DOX complexes, and LPPC-DOX complexes were incubated in PBS at 4, 25 or 37 °C. As previously described in the Methods section, the concentration of DOX in each supernatant was measured at different time points of incubation. (B) Various amounts of CpG ODN were added to the LPPC and LPPC-DOX complexes, and the unabsorbed CpG ODN in the
supernatant was measured at OD260. (C) The drug DOX was released from the LPPC-DOX and LPPC-DOX-CpG complexes incubated in PBS at 4, 25 or 37 °C. All values represent the mean ± SD (n = 3).

Figure 2

Cytotoxic effects of the drug and immune activities of CpG from LPPC-DOX-CpG complexes.
(A) B16F10 cells were treated with 0 to 25 mM DOX loaded in the LPPC-DOX-CpG complexes for 72 h. The dosages of LPPC, DOX, and LPPC-DOX were the same as those used for the LPPC-DOX-CpG treatment. The effects of the complexes on cell growth inhibition were assessed by the MTT assay. All values represent the mean ± SD (n = 6) (B) To assess the immune activity of LPPC-CpG, the levels of IFN-α, TNF-α, and IL-6 were evaluated by RT-PCR. A representative gel electrophoresis image was shown. In addition, the cytokine expression levels were calculated and normalized (C). All values represent the mean ± SD (n = 3).
Figure 3

The effects of different treatments on B16F10 tumor growth in vivo.

(A) Images were obtained by IVIS at 12, 16, 24, and 36 h after transdermal treatment with PBS or LPPC-DOX. The photon counts of each mouse were indicated by pseudocolor scales. (B) LPPC-DOX (L-D), LPPC-CpG (L-C) or LPPC-DOX-CpG (L-D-C) was applied to the tumor areas of mice bearing B16F10
tumors every day. The tumor volume was measured with a caliper, and tumor volume was calculated as $L \times H \times W$ (n=8, 9, or 10; the mice without tumor growth are not shown). (C) Mouse survival was monitored daily (n=10). The mice were sacrificed when the tumors exceeded 2,000 mm$^3$ in size. (D) Representative HE staining image of a tumor (200×, scale bar = 100 µm; 400×, scale bar = 25 µm).

<table>
<thead>
<tr>
<th>PCNA</th>
<th>PBS</th>
<th>LPPC</th>
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<th>LPPC-CpG</th>
<th>LPPC-DOX-CpG</th>
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<tr>
<td>Cleaved caspase3</td>
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<td>CD86</td>
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<td>CD163</td>
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Figure 4

Immunohistochemical staining of tumors.

Immunohistochemical staining of PCNA, cleaved caspase 3, CD3, CD4, CD8, CD19, CD68, CD86, and CD163 was performed to analyze their expression in mice of the different treatment groups. Representative photos were selected from the different treatment groups (400×, scale bar = 25 µm).
Figure 5

The antimetastatic effects of different treatments on B16F10 cells in the liver and lung. (A) Representative HE staining images of the liver and lung (200×, scale bar = 100 µm; 400×, scale bar = 25 µm). (B, C) The sizes and numbers of melanoma nodules were calculated and shown as boxplots.
Figure 6

The four major molecular mechanisms underlying the therapeutic effects of transdermal LPPC-DOX-CpG on tumors. (A) Gene functions were annotated with the GENECARD online database and divided into 27 categories. (B) The gene expression and FC values of genes in four major categories. (C, D) The average means and distributions of FPKM and FC values of genes in four major categories.
Figure 7

The molecular pathways underlying the immune effects of LPPC-DOX-CpG treatment as determined by RNA-seq analysis. Significant differences in the RNA expression levels of chemokines (A) and IFN (C) were determined by t-tests and ANOVA, and the results were shown as boxplots. (B, D) The speculated
mechanisms and pathways were created using Biorender (https://biorender.com/). Significantly upregulated genes were shown in red, and significantly downregulated genes were shown in green.

Figure 8
The molecular pathways underlying the immune effects of LPPC-DOX-CpG as determined by RNA-seq analysis. Significant differences in the RNA expression levels of NF-kB (A) and lymphocyte-associated genes (C) were determined by \( t \)-tests and ANOVA, and the results were shown as boxplots. (B, D) The speculated mechanisms and pathways were created using Biorender (https://biorender.com/). Significantly upregulated genes were shown in red, and significantly downregulated genes were shown in green.
Figure 9

The molecular mechanisms underlying the therapeutic effects of transdermal LPPC-DOX-CpG on tumor growth. (A) The speculated mechanisms and pathways were created using Biorender (https://biorender.com/). Significantly upregulated genes were shown in red, and significantly downregulated genes were shown in green. (B) Significant differences in the RNA expression levels of
genes related to cell proliferation were determined by \( t \)-tests and ANOVA, and the results were shown as boxplots.
The molecular mechanisms underlying the therapeutic effects of transdermal LPPC-DOX-CpG on apoptosis. (A) The speculated mechanisms and pathways were created using Biorender (https://biorender.com/). Significantly upregulated genes were shown in red, and significantly downregulated genes were shown in green. (B) Significant differences in the RNA expression levels of genes related to apoptosis were determined by $t$-tests and ANOVA, and the results were shown as boxplots.
Figure 11

The molecular mechanisms underlying the therapeutic effects of transdermal LPPC-DOX-CpG on metastasis. (A) The speculated mechanisms and pathways were created using Biorender (https://biorender.com/). Significantly upregulated genes were shown in red, and significantly downregulated genes were shown in green. (B) Significant differences in the RNA expression levels of
genes related to metastasis were determined by $t$-tests and ANOVA, and the results were shown as boxplots.

**Supplementary Files**

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- Additionalfile4Tablecellproliferation.xlsx
- Additionalfile5TableApoptosis.xlsx
- Additionalfile6TableMetastasis.xlsx