Supplementary Materials for

A melanin-mediated cancer immunotherapy patch

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Other Supplementary Material for this manuscript includes the following:
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Materials and Methods

Cell culture.

The mouse melanoma cell line B16F10 and mouse mammary carcinoma cell line 4T1 were purchased from the American Type Culture Collection. B16F10-luc and BRAFV600E/PTEN-/- Duke-clone 6 cell line (BPD6 or BP) (generated by Dr. Brent A. Hanks’s lab at Duke) cells were obtained from Dr. Leaf Huang at University of North Carolina at Chapel Hill. B16F10 cells were maintained in the Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin (Invitrogen) and 100 U/mL streptomycin (Invitrogen). 4T1 and BP cells were maintained in the Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin (Invitrogen) and 100 U/mL streptomycin (Invitrogen). Cells were cultured in an incubator (Thermo Scientific) at 37°C under an atmosphere of 5% CO₂ and 90% relative humidity and were sub-cultivated approximately every 3 days at 80% confluence using 0.25 wt% trypsin at a split ratio of 1:3.

Protein and melanin quantification of tumor lysates.

Primary tumors were collected from the B16F10 melanoma-bearing mice. For the quantification of tumor lysate protein, 3 mL T-PER Tissue Protein Extraction Reagent was added to 1.5 g tumor tissue and homogenized using sonication. After incubation on ice for 2 hrs, lysates were centrifuged for 20 min at 10,000 × g at 4 °C to pellet tissue debris and the protein concentration of the resulting supernatants was determined using a Coomassie Plus™ (Bradford) Assay Kit. For the quantification of melanin, homogenized tumor tissue was resuspended in 1 M sodium
hydroxide and heated to 99°C for 10 min. Melanin concentration was determined by measuring the absorbance at 475 nm and calculated based on a standard curve. For preparation of the microneedles (MNs), 3 mL phosphate-buffered saline (PBS) medium buffer was added to 1.5 g tumor tissue and homogenized by sonication.

**Mechanical strength test.**

Mechanical strength of microneedles with a stress-strain gauge was determined by pressing a stainless steel plate against MNs on an MTS 30G tensile testing machine. Initial gauge was 2.00 mm between the tips of MN and the plate, with 10.00 N as the load cell capacity. Speed of the plate approaching MNs was set as 0.1 mm/s. Failure force of the MN was recorded as the force at which the needle began to buckle.

**Skin penetration test.**

To evaluate the skin penetrating capacity of MNs, they were inserted into the skin of mice for 10 min. Mice were euthanized and skin samples were stained with trypan blue for 10 min before imaging by an optical microscopy (Leica EZ4 D stereomicroscope). In separate experiments, skin samples were stained with hematoxylin and eosin (H&E) (Histology Laboratory at NC State College of Veterinary Medicine).

**Granulocyte-macrophage colony-stimulating factor (GM-CSF) release profile.**

The amount of GM-CSF released from MNs was detected by ELISA (eBioscience Mouse GM-CSF ELISA Kit) according to the manufacturer’s protocol. The release of GM-CSF from MNs
was monitored by immersing the tips of MNs into PBS medium buffer in 37°C under moderate shaking. Near infrared (NIR) irradiation was performed on the MNs for 10 min per day for 3 days. At pre-determined time points, 100 µL of the medium was collected for analysis and additional 100 µL of the fresh medium was added. The absorption intensity of GM-CSF was determined at 450 nm by a microplate reader (Infinite M200 PRO, Tecan). The concentration of tumor lysate proteins were also analyzed by Bradford assay at the same time. The percentage of bioactive GM-CSF was assessed by an *in vitro* bioactivity assay and calculated by normalizing the amount of GM-CSF bioactivity to the amount of GM-CSF protein detected by ELISA. The bioactivity assay utilized the bone marrow derived dendritic cells (DCs) isolated from C57BL/6J mice (female, aged 6-8 weeks; Jackson Laboratories). 10% v/v of AlamarBlue reagent was added to each cell culture well, and after incubation for 4 hrs at 37 °C, plates were read at the absorbance of 490 nm. All standards and samples were normalized to the blank controls, and the bioactivity of the experimental samples was determined relative to the standard curve.

**Cytotoxicity study.**

Cytotoxicity studies toward blank MN formulations with various dissolved concentrations were performed using B16F10 cells. Cells were seeded into 96-well plates at a density of 5×10³ cells per well and cultivated in 100 µL of DMEM (25 mM glucose) with 10% FBS, 1× penicillin-streptomycin (Pen-Strep), 1× L-Glutamine and 2.5 µL of Beta Mercaptoethanol per 500 mL media. Plates were then incubated in 5% CO₂ at 37°C to reach 70-80% confluence before adding serial dilutions of the released media incubated with MN solution. After incubation with samples for 24 hrs, cells were washed with PBS solution and incubated with 100 µL of fresh FBS-free DMEM and 20 µL of freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide solution (MTT solution, 5.0 mg/mL). Plates were incubated for additional 4 hrs at 37°C in the dark. Afterwards, the solution was carefully removed and 100 µL dimethyl sulfoxide was added. Absorbance of the plates was read at 590 nm and a reference wavelength of 630 nm using a microplate reader (Infinite M200 Pro, Tecan, Morrisville, NC, USA) within 10 min.

In vivo bioluminescence and imaging.

Bioluminescence images were collected with a Xenogen IVIS Spectrum Imaging System. Living Image software (Xenogen) was used to acquire the data 10 min after intraperitoneal injection of D-luciferin (Pierce) in Dulbecco’s phosphate-buffered saline (DPBS) (15 mg/mL) into animals (10 µL/g of body weight).

Confocal microscopy.

Regional skin and tumors were dissected and fixed in 4% paraformaldehyde at 4°C and then embedded in OCT compound (Sakura Finetek) and flash-frozen in an isopentane bath on dry ice. Frozen samples were sectioned (5 µm thickness), mounted on microscope slides, and stored at −20°C. For staining of CD3 (Thermo Fisher Scientific, A18644), CD4 (Thermo Fisher Scientific, A18667), CD8 (Thermo Fisher Scientific, Cat A18609), CD11c (Biolegend, 117309), CD49b (Biolegend, 108909), heat shock proteins 70 (HSP70) (Abcam, ab2787) and HSP90 (Abcam, ab1429) by immunofluorescence, the slides were washed twice, permeabilized for 30 min using a 0.1% Triton X100 solution, and subsequently blocked for one hour using a 1% bovine serum albumin (BSA) solution. After blocking, primary monoclonal antibody at 1/200 dilution was applied overnight at 4°C, followed by washing and incubation with secondary
antibody at 1/400 dilution. Secondary antibodies were added to some samples, including Goat anti-Rat IgG Secondary Antibody (Thermo Fisher A18866), Rabbit anti-Rat IgG (H+L) Secondary Antibody (Thermo Fisher Scientific, A18920). Slides were washed thrice, applied with Hoechst 33342 or 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) to stain the cell nucleus and covered with coverslips. For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis staining, the fixed tumor sections were stained by the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer’s protocol. Hoechst 33342 was used for nuclear staining. Samples were imaged using the Olympus IX70 multi-parameter fluorescence microscope and a confocal microscope (Zeiss). Images were processed using the ImageJ software.

**Isolation of tumor-infiltrating cells and lymphoid tissue cells.**

B16F10 tumor cell samples were isolated by grinding tumors through 40 µm nylon cell strainer (Corning) in RPMI. BP and 4T1 tumor samples were minced with scissors before incubation with trypsin/EDTA (0.25%/0.1%) for 20 min. Tumor cells were homogenized by repeated pipetting and filtered through a 40 µm cell strainer in RPMI to generate single-cell suspensions. Cell suspensions were washed once with complete RPMI. Lymphoid tissue cells were isolated from mouse skin into single cell suspension as described. All samples were re-suspended in fluorescence-activated cell sorting (FACS) buffer (PBS/0.5% albumin) before testing.
Cytotoxic T lymphocyte activity.

Splenocytes harvested from mice were stimulated *in vitro* with tumor lysate. Cells were washed through the strainer 3 times with excess PBS and then cultured together with B16F10 target cells in 96-well culture plates at the effective target cell ratio of 500:1. After 24 hrs, the supernatants were collected to detect the lactate dehydrogenase (LDH) leakage level with a nonradioactive cytotoxicity assay (Thermo Scientific), which indicated the level of specific lysis of target cells by effective cells. The percentage of specific lysis was calculated according to % specific lysis = ((experimental LDH release – effective cell LDH release)/(maximum LDH release – spontaneous LDH release)) × 100%.
Fig. S1. Mechanical property of the MN. Data points show one representative MN. The experiments were repeated three times and similar results were obtained.
Fig. S2. Characteristics of tumor lysate solution and synthetic melanin. (A) Absorption spectrum of B16F10 whole tumor lysate solution, synthetic melanin and PBS. (B) Fourier transform infrared spectroscopy of melanin treated with or without 1.0 W/cm² NIR irradiation for 10 min.
Fig. S3. Heating behavior of MN patches by repetitive NIR irradiation. (A) Pink zone indicates the NIR light on, white zone indicates the NIR light off. (B) Surface temperature changes of the MN patch when exposed to 1.0 W/cm² NIR light or no NIR light. Data points represent mean ± SD (n = 3). Error bars indicate SD.
Fig. S4. Surface temperature of MN patches with various loadings of tumor lysates upon NIR irradiation. MN patches were exposed to 1.0 W/cm$^2$ NIR light. (A) The surface temperature changes of MN patches with different concentration of extracted tumor lysate proteins. Data points represent mean ± SD ($n = 3$). Error bars indicate SD. (B) The surface temperature change of MN patches with different thickness of MN backing. MN patches with different backing thickness were used: (1) 169 µm, (2) 175 µm, (3) 179 µm, (4) 181 µm and (5) 202 µm.
Fig. S5. In vitro release profiles of GM-CSF and tumor lysate proteins. Profiles of (A) in vitro collective release and (B) bioactivity of GM-CSF from MN patches over time. The collective release of (C) GM-CSF and (D) tumor lysate proteins in buffer solution with 10 min/day NIR treatment of MN patch over time. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (NS, not significant; P > 0.05).
Fig. S6. Scanning electron microscopy images of MN patch after insertion into the mouse skin over time.
Fig. S7. DC function evaluation after in vitro activation. (A) DC viability measured by Live/Dead assay. (B) Cytokine interleukin 12 (IL-12) concentration in cell supernatants. The DCs were treated with MNs loaded with tumor lysate or lipopolysaccharide (LPS) and GM-CSF and exposed to different time periods of NIR irradiation. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05).
Fig. S8. Cytotoxicity study of the blank MNs. MN patches were re-dissolved and added to B16F10 cells for 24 hrs of incubation. Error bars indicate SD (n = 6). Statistical significance was calculated by Student’s t test (NS. P > 0.05).
Fig. S9. Characterization of the skin after MN insertion. (A) Image of the trypan blue and (B) H&E staining showing the penetration of the MNs into the mouse skin (scale bar: 200 µm). The region where the MN insertion took place is indicated by the black dashed line.
Fig. S10. Melanin-loaded MNs confer protective immunity in vivo. (A) Surface temperature changes of individual animal in real time after MN insertion into the skin and NIR (1.0 W/cm²) irradiation measured by an infrared thermal camera. (B) Average tumor volumes for treated and control mice. (C) Kaplan-Meier survival curves for treated and control mice. Data points represent mean ± SD (n = 6). Error bars indicate SD. Statistical significance was calculated by Student’s t test and log-rank test (* P < 0.05; ** P < 0.01).
Fig. S11. Tumor growth in control and treated mice. (A) Average and (B) individual B16F10 tumor growth curves for mice after tumor inoculation and the indicated treatments. The tumor growth curve was plotted 10 days after tumor cell inoculation. Data points represent mean ± SD (n = 8). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05; **P < 0.01; ***P < 0.001).
Fig. S12. Quantified B16F10 bioluminescent tumor signals in control and treated mice. Error bars indicate SD ($n = 3$). Statistical significance was calculated by Student’s $t$ test (NS, $P > 0.05$; $*P < 0.05$; **$P < 0.01$).
**Fig. S13. Tumor weights in control and treated mice.** Data points represent mean ± SD ($n = 8$). Error bars indicate SD. Statistical significance was calculated by Student’s $t$ test ($**P < 0.01$).
Fig. S14. **Histology and apoptosis analysis of the tumor sections.** (A) H&E staining and quantitative analysis of melanin content in tumor sections. (B) Fluorescence images and quantitative analysis of *in situ* TUNEL-positive nuclei of tumors collected from mice after each indicated treatments. The tumor sections were stained with the fluorescein-dUTP (green) for apoptosis and DAPI for nuclei (blue) (scale bar: 100 µm). Data points represent mean ± SD (*n* = 3). Error bars indicate SD. Statistical significance was calculated by Student’s *t* test (*P* < 0.05; **P** < 0.01; ***P*** < 0.001).
Fig. S15. Tumor growth of mice receiving the transdermal cancer immunotherapy. Average tumor volumes in mice after tumor inoculation and treatments of vaccine MNs and NIR irradiation under conditions of CD8 T cell depletion (CD8), CD4 T cell depletion (CD4), natural killer (NK) cell depletion (NK) and B cell depletion (B). Controls are naive mice treated with blank MN (blank) and vaccine MN with NIR irradiation (MN + NIR). Data points represent mean ± SD (n = 8). Error bars indicate SD. Statistical significance was calculated by Student’s t test (NS. P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001).
Fig. S16. Measurement of local microcirculatory blood perfusion of mice. Data were collected right after treatment of different conditions using the laser Doppler flowmetry. Mice were treated with: (A) blank MN, (B) vaccine MN and (C) vaccine MN and NIR irradiation. The experiments were repeated three times and quantitative analysis is summarized in Table S2.
Fig. S17. Immunologic responses after the transdermal cancer immunotherapy. (A) Representative plots and (B) quantitative analysis of tetramer GP100 staining in CD8+ T cells from tumors in treated mice analyzed by flow cytometry. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05).
Fig. S18. Quantification of IgG1 subtypes in serum after treatment with blank, MN, or MN + NIR. Serum was collected at day 15 after treatment with blank, MN or MN + NIR. Data points represent mean ± SD (n = 8). Error bars indicate SD. Statistical significance was calculated by Student’s t test (* P < 0.05; ** P < 0.01).
Fig. S19. **ROS detection by flow cytometry in tumor sections.** (A) Normalized mean ROS signal intensity in tumors collected from different experimental groups. (B) Quantitative mean ROS signal intensity ($n = 6$). Error bars indicate SD. Statistical significance was calculated by Student’s $t$ test ($*P < 0.05$).
Fig. S20. HSP90 expression after the transdermal cancer immunotherapy. (A) Immunofluorescent staining of HSP90 (green) in the skin collected after the indicated treatments. Actin filaments were visualized by phalloidin (red) and cell nuclei stained with Hoechst (blue) (scale bar: 100 µm). (B) Quantitative analysis of HSP 90 signal intensity in the immunofluorescent staining images. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05).
Fig. S21. Cytokine kinetics after the transdermal cancer immunotherapy. Cytokine concentration was measured in extracted patch and surrounding tissue in B16F10 tumor model at the indicated time points. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05; **P < 0.01).
Fig. S22. Histology analysis after the transdermal cancer immunotherapy. (A) H&E staining of organs collected from mice after combinational treatments and control mice with lung metastasis as well as healthy mice. The dark blue arrows indicate the metastatic tumors in the lung. (B) Pictures of lungs collected (scale bar: 100 µm) and quantification of the lung nodules from mice receiving different treatments. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (***P < 0.001).
Fig. S23. Antitumor effect of the transdermal cancer immunotherapy toward different tumor models. (A) Average and (B) individual B16F10 and BP tumor growth curves for mice receiving the indicated treatments. Data points represent mean ± SD (n = 8). Error bars indicate SD. Statistical significance was calculated by Student’s t test (***P < 0.001).
Fig. S24. Surface temperature changes of the melanin-loaded MNs. Images were measured by an infrared thermal camera in real time after insertion into mouse skin and 1.0 W/cm² NIR irradiation. MNs were loaded with melanoma BP lysate or breast cancer 4T1 lysate with melanin.
**Fig. S25. Antitumor effect of the transdermal cancer immunotherapy.** (A) Tumor growth of vaccinated C57BL/6J mice after BP tumor cell challenge. Mice were pretreated with blank MN (blank), MN loaded with BP tumor lysate and melanin (MN) or loaded MN combined with NIR irradiation (MN + NIR). (B) Tumor growth of C57BL/6J mice bearing established BP tumors before therapeutic treatment with blank MN (blank), MN loaded with BP tumor lysate and melanin (MN) or loaded MN combined with NIR irradiation (MN + NIR) (C) Tumor growth of vaccinated BALB/cJ mice after 4T1 tumor cell challenge. Mice were pretreated with blank MN (blank), MN loaded with 4T1 tumor lysate and melanin (MN) or loaded MN combined with NIR
irradiation (MN + NIR). (D) Tumor growth of BALB/cJ mice bearing established 4T1 tumors before therapeutic treatment with blank MN (blank), MN loaded with 4T1 tumor lysate and melanin (MN) or loaded MN combined with NIR irradiation (MN + NIR). Data points represent mean ± SD (n = 8). Statistical significance was calculated by Student’s t test (*P < 0.05; **P < 0.01; ***P < 0.001). Asterisks indicate statistically significant differences between MN + NIR group and all other treatment groups.
Fig. S26. HSP70 expression after the transdermal cancer immunotherapy. (A) Immunofluorescent staining of HSP70 (green) in the regional skin with actin filaments visualized by phalloidin (red) and cell nuclei stained with Hoechst (blue) in BP and 4T1 tumor models respectively (scale bar: 100 µm). (B) Quantitative intensity of HSP70 in the regional skin samples. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05; **P < 0.01).
Fig. S27. Representative quantitative analysis of the DC activation. Activated DCs (CD86+, CD80+) were collected in the draining lymph nodes in (A) BP and (B) 4T1 tumor models. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05).
Fig. S28. Cytokine kinetics after the transdermal cancer immunotherapy. Cytokine concentrations were measured in the extracted patch and surrounding tissue in (A) BP and (B) 4T1 tumor models. Data points represent mean ± SD (n = 3). Error bars indicate SD. Statistical significance was calculated by Student’s t test (*P < 0.05).
Fig. S29. Average weights of mice after the transdermal cancer immunotherapy in control and treated mice. (A) Average weight of vaccinated C57BL/6J mice after BP tumor cell challenge. (B) Average weight of C57BL/6J mice bearing established BP tumors before therapeutic treatment. (C) Average weight of vaccinated BALB/cJ mice after 4T1 tumor cell challenge. (D) Average weight of BALB/cJ mice bearing established 4T1 tumors before therapeutic treatment. Data points represent mean ± SD (n = 8).
Fig. S30. H&E staining of organs collected after the transdermal cancer immunotherapy in (A) C57BL/6J mice bearing BP tumors and (B) BALB/cJ mice bearing 4T1 tumors after vaccinations (scale bar: 100 µm).
Table S1. Melanin content of tumors excised from tumor-bearing mice.

<table>
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<th>Tumor model</th>
<th>B16F10</th>
<th>BP</th>
<th>4T1</th>
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<tbody>
<tr>
<td>Melanin content (µg/mg tumor)</td>
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<td>0.00 ± 0.01</td>
<td>0.00 ± 0.01</td>
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</table>
Table S2. Measurement of total local microcirculatory blood perfusion of mice receiving different treatments using the laser Doppler flowmetry.

<table>
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<tr>
<th>Perfusion unit (PU)</th>
<th>Mean</th>
<th>S.D.</th>
<th>Min</th>
<th>Max</th>
<th>Area</th>
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<tbody>
<tr>
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<td>27.4</td>
<td>45.5</td>
<td>150.2</td>
<td>1196.7</td>
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<tr>
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<td>35.2</td>
<td>49.5</td>
<td>228.0</td>
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