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A Tumor Mitochondria Vaccine Protects against Experimental Renal Cell Carcinoma

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Mitochondria provide energy for cells via oxidative phosphorylation. Reactive oxygen species, a byproduct of this mitochondrial respiration, can damage mitochondrial DNA (mtDNA), and somatic mtDNA mutations have been found in all colorectal, ovarian, breast, urinary bladder, kidney, lung, and pancreatic tumors studied. The resulting altered mitochondrial proteins or tumor-associated mitochondrial Ags (TAMAs) are potentially immunogenic, suggesting that they may be targetable Ags for cancer immunotherapy. In this article, we show that the RENCA tumor cell line harbors TAMAs that can drive an antitumor immune response. We generated a cellular tumor vaccine by pulsing dendritic cells with enriched mitochondrial proteins from RENCA cells. Our dendritic cell-based RENCA mitochondrial lysate vaccine elicited a cytotoxic T cell response in vivo and conferred durable protection against challenge with RENCA cells when used in a prophylactic or therapeutic setting. By sequencing mtDNA from RENCA cells, we identified two mutated molecules: COX1 and ND5. Peptide vaccines generated from mitochondrial-encoded COX1 but not from ND5 had therapeutic properties similar to RENCA mitochondrial protein preparation. Thus, TAMAs can elicit effective antitumor immune responses, potentially providing a new immunotherapeutic strategy to treat cancer. *The Journal of Immunology*, 2015, 195: 4020–4027.

Recent research advances in cancer immunotherapy have progressed rapidly, culminating in durable remission for hematological malignancies using T cells that express a chimeric AgR (CAR) targeted against CD19 (1). Manipulation of immune responses as cancer therapy is very different compared with direct pharmacological attack on the tumor, so it is not surprising that immunotherapy is still generating unexpected and often conflicting results, as well as occasional adverse events (2). Breakthroughs in therapeutically targeting solid malignancies remain elusive, and the identification of novel tumor associated Ags (TAAs) continues to be a critically important requirement. In this article, we describe tumor mitochondria-derived peptides as a source of novel TAAs.

The typical human cell contains ~100 mitochondria, each of which has an average of five mitochondrial DNA (mtDNA) molecules, yielding ~500 copies of mtDNA/cell (3). Interestingly,

mtDNA lacks repair proteins and protective histones found in nuclear DNA and, at the same time, reactive oxygen species (ROS) produced by oxidative phosphorylation (4) result in mutations that are more abundant in mtDNA than in nuclear DNA (5, 6). During physiological development, mtDNA mutations exist in a heteroplasmic state: within a single cell, some mitochondrial genomes harbor mutations, whereas others do not. In contrast, human tumors have a high frequency of homoplasmic mtDNA. This observation has been interpreted as reflecting a growth advantage or other advantage for tumor cells containing certain homoplasmic mtDNA mutations (5, 7).

Mitochondrial Ags are presented to and recognized by the immune system, and there is evidence that mitochondrial peptides may participate in the induction of autoimmune diseases (8, 9). For example, autoreactive T cell responses to peptides derived from the pyruvate dehydrogenase complex are found in almost all cirrhosis patients (10). Antimitochondrial immune responses also occur in cancer, as illustrated by the presence of CD4⁺ T cells specific for cytochrome B among tumor-infiltrating lymphocytes isolated and expanded from melanoma biopsies (11). Somatic mtDNA mutations are present in up to 100% of kidney, colorectal, ovarian, breast, urinary bladder, lung, and pancreas tumors (12, 13). These features suggest that the mitochondria may be a source of novel tumor-associated mitochondrial Ags (TAMAs) that could be used to develop therapeutic vaccines to treat cancer.

In this article, we describe a vaccine strategy that uses dendritic cells (DCs) pulsed with mitochondrial lysate, including TAMAs isolated from tumor cells. We selected murine kidney tumor cells as the model system because previous studies characterized renal cell carcinoma as being highly sensitive to immunotherapy (14–16) and having a high incidence of mtDNA mutations (17, 18). We generated the DC-based vaccine using mitochondria-enriched lysate from RENCA cells, a spontaneously arising renal adenocarcinoma (19, 20), and vaccinated mice both prophylactically and therapeutically. Protective immunity was observed when the TAMA

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Abbreviations used in this article: bmDC, bone marrow-derived DC; CAR, chimeric AgR; DC, dendritic cell; DC/Kidney mito, DC pulsed with healthy kidney mitochondria; DC/RENCA mito, DC pulsed with RENCA mitochondria; DC/Solid Tumor mito, DC pulsed with RENCA tumor-derived mitochondria; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; TAA, tumor-associated Ag; TAMA, tumor-associated mitochondrial Ag.

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vaccine was used prophylactically; it also was effective in delaying tumor growth in the therapeutic setting. We sequenced and aligned the 13 coding sequences included in the mtDNA from RENCA cells or kidneys of BALB/c mice (which are syngeneic with RENCA cells) and identified two TAMA candidates within the sequences of the mitochondrial-encoded COX1 and ND5 genes of the RENCA cells. To confirm that the T cell response against these TAMAs generated effective immunity, we immunized mice with peptides derived from these mutated genes. The resulting immune responses impaired RENCA tumor growth in a CD8⁺ T cell-specific manner. We conclude that targeting TAMAs is a novel immunotherapy approach that is effective, safe, and has durable responses.

Materials and Methods

Mice and cell lines

BALB/c (H-2^d) mice (8-wk-old) were purchased from The Jackson Laboratory. Murine renal carcinoma RENCA and colon carcinoma line CT26 (H-2^d) were purchased from the American Type Culture Collection.

Purification of mitochondria and mtDNA extraction

Mitochondria were purified from RENCA cell lines and tissues (RENCA solid tumor and kidney) using the discontinuous sucrose gradient method described previously (21). Preparations were frozen (liquid nitrogen) and thawed (37°C, water bath) for three cycles, sonicated, and irradiated at 5000 rad for sterilization. Quantification was carried out with the BCA assay. mtDNA was isolated from cell lines using a Mitochondrial DNA Isolation Kit from BIOVISION.

Tumor lysate preparation

Cultured RENCA cells were detached from tissue flasks with trypsin and washed twice with PBS. Cells were resuspended in PBS, frozen/thawed six times, and quantified by BCA assay.

Western blot

Western blot was performed with mitochondrial extract proteins with Abs specific for COX4 (clone 1D6E1A8), GAPDH (clone 71.1), or histone H3 (Sigma; polyclonal). Membranes were incubated with a 1:3000 dilution of HRP-conjugated Abs and developed with the ECL system.

Mouse bone marrow-derived DC generation and maturation

Bone marrow cells were isolated from hind leg femurs and tibias of mice, and DCs were isolated, cultured, and matured as previously described (22).

DC uptake

Intact mitochondria and live tumor cells were stained for 10 min at 37°C in 0.5 μM CellTrace CFSE. Stained preparations were divided into aliquots that were frozen/thawed. Frozen/thawed CFSE cells and frozen/thawed CFSE mitochondria were cocultured with normal DCs (100 μg/ml) for 3 and 6 h at 37°C. DC uptake was evaluated using flow cytometry with a FACSCanto. For microscopic analysis, immature DCs and CFSE-stained mitochondria were cocultured for 4 h, cytocentrifuged, fixed in 4% paraformaldehyde, and mounted with DAPI Fluoromount-G. Slides were examined using a confocal microscope (Leica). Adherent cells at the bottom of the plate after coculture were washed in PBS, fixed, and mounted with DAPI Fluoromount-G. DC uptake was monitored using a fluorescence microscope (ECLIPSE Ti; Nikon).

Synthetic peptides and peptide immunization

Mutated COX1_{291–306} (MFTVGLDVDTRTYFT), COX1_{295–310} (GLDVDTRTYFTSATM), and COX1_{299–314} (DTRTYFTSATMIIAI) peptides were purchased from Eunoia Biotech. Mutated ND5_{516–535} (SFSTLLGGFTSIIHR), ND5_{520–539} (LLGGFTSIIHRITPM), and ND5_{524–543} (FTSIIHRITPMKSLN) peptides were purchased from Mimotopes (23). For prophylactic peptide vaccination, mice were divided into different groups and immunized three times with 100 μg of either the combination of the three mutated COX1 peptides or the combination of the three mutated ND5 peptides; each individual peptide was distributed in an equimolar manner in the multi-peptide preparations. The vaccination consisted of three injections at weekly intervals in CFA and IFA. One week after the last immunization, animals were challenged with a s.c. injection of 1 × 10⁵ RENCA cells and monitored for tumor growth every other day.

DC-based immunotherapy

Mitochondria from RENCA solid tumors and RENCA cell lines, as well as control mitochondria, were purified, cocultured with BALB/c-derived DCs, and matured as described above. In prophylactic settings, DC vaccine pulsed with 100 μg/ml protein lysate/million cells was administered intradermally (1 × 10⁶ DCs/dose in 100 μl PBS) once weekly for 3 wk. Two weeks after the last injection, mice were inoculated s.c. with 1 × 10⁶ live RENCA cells. In the therapeutic protocol, mice were injected s.c. with 1 × 10⁶ RENCA cells and, starting 1 wk later, they were given three injections of 1 × 10⁶ DCs at weekly intervals. Tumor progression was monitored every other day from the tumor challenge.

Adoptive cell transfer

Tumor-free BALB/c mice were vaccinated three times with mitochondria-pulsed DCs or control DCs and sacrificed 1 wk later. CD3⁺, CD4⁺, and CD8⁺ T cells were magnetically isolated from the spleens, and serum was collected by intracardiac bleeding. Isolated T cells were injected i.v. into RENCA tumor-bearing mice (challenged 7 d before transfer) that had been sublethally irradiated (4–5 Gy) 8 d before adoptive transfer. Serum was administered i.p. twice weekly for a total of six injections into RENCA tumor-bearing mice.

Immunohistochemistry

CD3⁺ cells were detected by immunohistochemistry in solid tumors. Tumors were embedded in OCT and immediately snap-frozen in dry ice. Sections (6 μm thick) were stained for CD3, as previously described (24). Slides were analyzed in a blinded fashion by two independent observers.

ELISPOT for IFN-γ

Isolated CD3⁺ T cells from the spleen of tumor-bearing mice were cocultured for 16 h with RENCA cells at a 10:1 ratio. After incubation, plates were washed with PBS and Tween 20, ELISPOT was performed, and spots were counted using an automated ELISPOT reader (Autoimmun Diagnostika) (24).

Cytokine analysis and flow cytometry

In a 24-well plate, 1 × 10⁶ immature DCs were exposed to mitochondria or whole-tumor lysate (100 ng/ml) in DC complete media for 12 h. Cocultures were matured overnight with LPS (1 μg/ml) and IFN-γ (100 ng/ml). Cells were harvested, and supernatant was collected and analyzed for IL-2, IL-5, and MCP-1 by the Human Immunology Core, University of Pennsylvania. Cells were labeled with mAbs from eBioscience (CD11b [clone M1/70], CD11c [clone N418], PD-L1 [clone MH5]), BD Biosciences (CD86 [clone GL1], CD80 [clone 16-10A1]), and BioLegend (CD40 [clone 3/23], CD45 [clone 30-F11]). Labeled cells were evaluated with a FACSCanto, and the data were analyzed using FlowJo software (TreeStar). DCs were gated as CD11b⁺/CD11c⁺.

Intracellular cytokine staining for IFN-γ

A total of 1–3 × 10⁶ mouse splenocytes was incubated with the indicated peptides (final concentration of each peptide, 10 μg/ml) and 1 μg/ml Golgi Stop (BD Biosciences, Pharmingen) at 37°C for 12–16 h and labeled with CD3⁺/CD4⁺ or CD8⁺ and IFN-γ, as previously described (25). Cells were analyzed on a FACSCanto flow cytometer using FlowJo software.

Urine total protein and creatinine quantification

Urine and serum from mice that received three doses of DC-based vaccine (DC alone, DC pulsed with RENCA-derived mitochondria [DC/RENCA mito], and DC pulsed with healthy kidney mitochondria [DC/Kidney mito]), as well as from control animals, were collected and assessed for total protein and creatinine levels. Samples were analyzed by the core service at the Ryan Veterinary Hospital at the University of Pennsylvania.

Histological evaluation of kidney in vaccinated mice

Kidneys of DC-vaccinated mice were collected to assess for possible treatment toxicity. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Histopathologic evaluation was performed on standard H&E-stained sections. Microscopic analysis was performed separately and in a blinded fashion by two independent observers.

mtDNA amplification and sequencing

Protein-coding sequences were amplified using PCR with 22 pairs of overlapping primers (Supplemental Table I). PCRs were carried out in

25- μ l reactions using Phusion DNA polymerase (New England Biolabs) and 10 ng mtDNA template. Prior to sequencing, PCR products were cleaned up using a PureLink Quick PCR Purification Kit (Invitrogen; Life Technologies), and DNA concentration was determined by NanoDrop. Sanger Sequencing was performed on an ABI 3770 (Applied Biosystems) at the Penn Genomics Analysis Core of the University of Pennsylvania. Sequencing was performed with the same primers used for PCR.

Data analysis

For CD3⁺ T cell quantification in tumor sections and ELISPOT, statistical analyses for the indicated comparisons were performed using the pairwise Student *t* test. All *p* values presented are two sided. The log-rank test was performed for the survival curves. Single-step multiple-comparison procedure Tukey test was performed for repeated measurements at different time points. All experiments were performed a minimum of three times; in the case of in vivo studies, each group consisted of 10 animals, unless otherwise noted. All figures portray one representative experiment.

Study approval

All animal studies were approved by the Institutional Animal Care and Use Committee and University Laboratory Animal Resources at the University of Pennsylvania. Mice were treated in accordance with University of Pennsylvania guidelines.

Results

Mitochondria enriched from tumor cells are taken up by DCs but do not induce maturation

For all experiments, mitochondria were prepared from tissues or cell culture by dounce homogenization, low-speed centrifugation to remove nuclei, and high-speed centrifugation to enrich for mitochondria. Functional mitochondria were isolated using a discontinuous density sucrose gradient. Enrichment was confirmed by Western blot (Fig. 1A) using specific Abs for mitochondria protein (26) (COX4; *top row*), the cytosolic compartment protein (27) (GAPDH; *middle row*), and nuclei (histone H3; *bottom row*).

Danger-associated molecular pattern mediators, such as mtDNA, ATP, and formylated peptides, which are released during apoptosis or necrosis, enhance the inflammatory response (28, 29). Because DCs also express receptors for mtDNA (30), we investigated the response of bone marrow-derived DCs (bmDCs) from BALB/c mice. bmDCs were cultured in GM-CSF and IL-4 and then exposed to RENCA lysate enriched for mitochondria, RENCA whole-cell lysate, or LPS plus IFN- γ for 12 h (31). Mitochondria lysate did not induce any significant upregulation of the maturation markers CD80, CD86, CD40, or PD-L1 (Fig. 1B). Activated DCs secrete Th1 or Th2 and other polarizing cytokines (32); therefore, bmDCs were pulsed with mitochondrial lysate or cell lysate, and supernatants were analyzed for IL-2 (33), IL-5 (34), and MCP1. We measured a modest increase in IL-2 and IL-5 that did not reach statistical significance (Fig. 1C) and concluded that mitochondrial lysate does not activate bmDCs in vitro.

One hallmark of Ag presentation is the active engulfment of extracellular material (35). To demonstrate that mitochondria lysate was engulfed by bmDCs, freshly isolated intact mitochondria were labeled with CFSE, centrifuged, and frozen/thawed to rupture the mitochondria. When the CFSE-labeled mitochondria lysate was fed to immature bmDCs, both the cell lysate and mitochondrial lysate were actively engulfed by immature DCs (Fig. 1D).

DC/RENCA mito lysate suppress tumor growth and induce long-term protection

To examine the effects of mitochondria-based DC vaccines on renal tumor growth, we initially performed prophylactic immunizations. BALB/c mice were immunized intradermally with BALB/c-derived bmDCs pulsed with mitochondrial lysate derived from RENCA tumors extracted from tumor-bearing mice, from RENCA cells grown in vitro, or from kidneys from healthy mice.

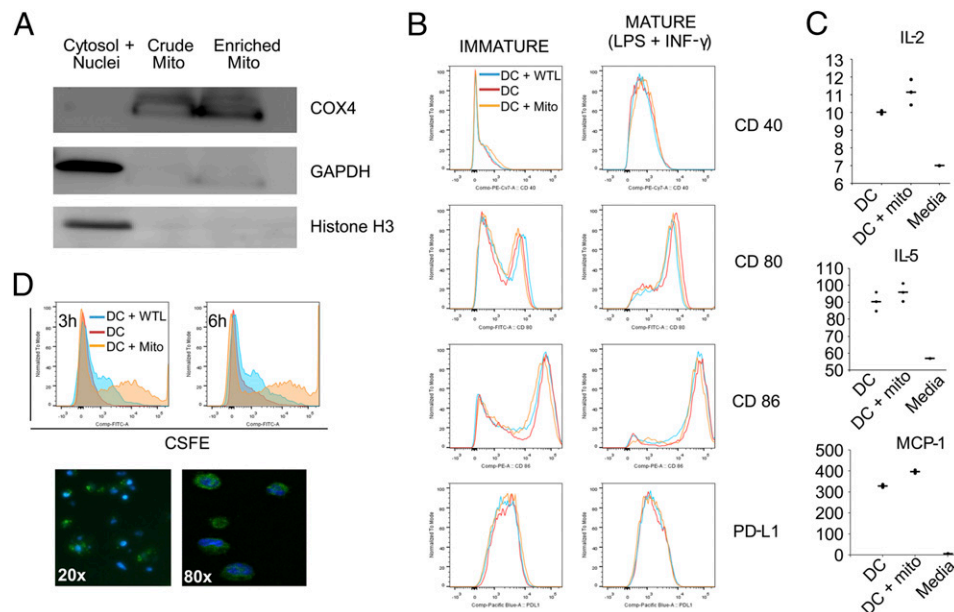


FIGURE 1. Mitochondria lysate is engulfed by in vitro-derived bmDCs but does not induce maturation. **(A)** Mitochondrial enrichment of tumor lysate was verified by Western blot analysis using mitochondria-specific COX4, cytosolic GAPDH, and nuclei-specific histone H3 Abs. **(B)** Immature DCs were pulsed with mitochondrial preparation (Mito) or whole-tumor lysate (WTL) and then matured with IFN- γ and LPS (*right panels*) or left untreated (*left panels*). CD11c, CD11b double-positive cells were analyzed for CD40, CD80, CD86, and PD-L1 markers by flow cytometry. **(C)** Cytokine production (IL-2, IL-5, and MCP1) was measured from supernatants of immature DCs cocultured with mitochondria (DC + mito) or immature unpulsed DCs (DC) (*p* = not significant between pulsed and unpulsed samples for all cytokines examined, all data pg/ml). **(D)** DCs are able to engulf mitochondria. Mitochondria and live tumor cells were stained with CFSE. CFSE-stained preparations were cocultured with immature DCs for 3 and 6 h (*upper left panel* and *upper right panel*, respectively) and analyzed by flow cytometry. Fluorescence microscopy (*lower left panel*) and confocal microscopy (*lower right panel*) revealed DCs (blue nuclei) and CFSE mitochondria (green)-overlapping pixels, indicating active uptake of mitochondria by immature DCs.

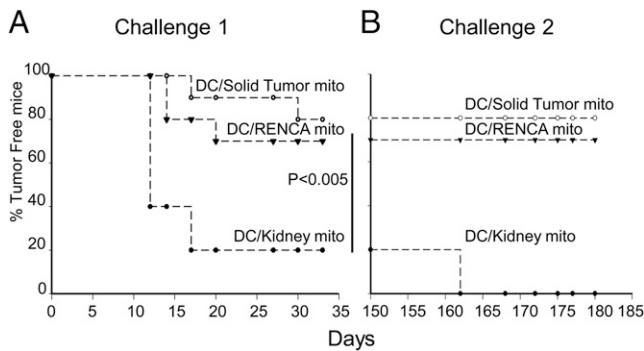


FIGURE 2. Prophylactic vaccination using DCs loaded with RENCA mitochondria lysate suppresses tumor growth and induces long-term protection. **(A)** BALB/c lysate derived from RENCA solid tumors (DC/Solid Tumor mito) resected from 20-d-old tumor-bearing mice, from RENCA cells grown in vitro (DC/RENCA mito), or from kidneys (DC/Kidney mito) of healthy mice. Two weeks after the last immunization, animals were injected (s.c.) with 1×10^6 RENCA cells and monitored for tumor growth. Data are mean ($n = 10$ mice/group for each experiment). $p < 0.05$, Tumor mito or RENCA mito versus Kidney mito, log-rank test. **(B)** Animals that rejected tumors after the first challenge in **(A)** were rechallenged with RENCA cells 3 mo later. All of the animals previously immunized with DC/RENCA mito and DC/Solid Tumor mito were protected after a second challenge.

Immunization was performed weekly for a total of three immunizations. Mice immunized with DCs pulsed with RENCA tumor-derived mitochondria (DC/Solid Tumor mito) rejected 80% of tumors, whereas the animals immunized with in vitro-cultured DC/RENCA mito rejected 70% of tumors, indicating that these lysates induce protective immune responses. Mitochondria enriched from normal kidney did not elicit a highly protective response (Fig. 2A).

Adaptive immunotherapy can generate long-lasting memory that protects the host against tumor persistence or recurrence (36, 37). Mice that rejected tumor after the first challenge were rechallenged with RENCA cells 3 mo later. All of the animals previ-

ously immunized with DC/RENCA mito were protected against the second tumor challenge. The few surviving animals from the group that had been immunized with DC/Kidney mito (Fig. 2B, ●) were rechallenged with RENCA and succumbed after 10 d ($p < 0.05$, log-rank test).

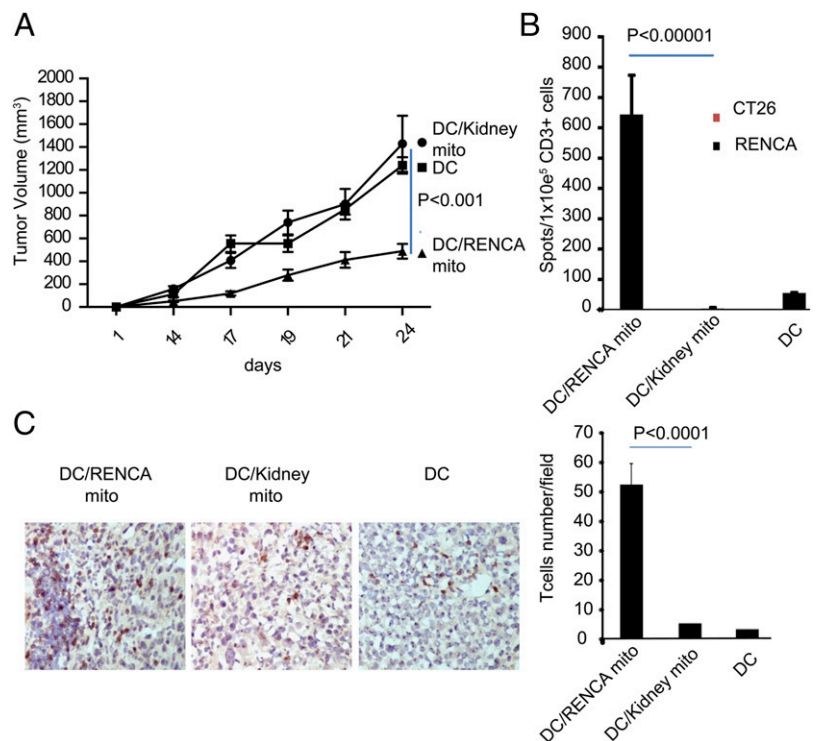
DC/RENCA mito lysate protect mice when used therapeutically, promote T cell infiltration of tumors, and induce IFN- γ production by T cells

We first challenged mice with RENCA tumor cells, followed by injection of bmDCs pulsed with mitochondrial lysate derived from RENCA cells grown in vitro or from healthy kidney cells. DCs alone or pulsed with healthy kidney mitochondria had no therapeutic effect, whereas DC/RENCA mito significantly delayed tumor development (Fig. 3A, Supplemental Fig. 2) ($p < 0.01$). $CD3^+$ infiltration into the tumors of mice treated with DC/RENCA mito was observed, whereas DC/Kidney mito proteins or DCs alone did not elicit significant $CD3^+$ cell infiltration (Fig. 3C) ($p < 0.0001$, DC/RENCA mito versus DC/Kidney mito, t test). We next tested the capacity of T cells purified from animals that had been immunized with DCs to recognize RENCA cells in vitro, using IFN- γ secretion as an indication of recognition. Purified T cells derived from DC/RENCA mito-immunized animals vigorously responded to RENCA cells in vitro, whereas T cells from DC/Kidney mito-immunized animals did not respond (Fig. 3B) ($p < 0.00001$, DC/RENCA mito versus DC/Kidney mito, t test). CT26 cells used as a tumor cell line control did not elicit significant IFN- γ by T cells derived from DC/RENCA mito-immunized animals, demonstrating the specificity of the T cell response (Fig. 3B).

RENCA mitochondria vaccine drives a T cell-specific immune response against tumors

To determine whether the antitumor response by mitochondria-enriched RENCA vaccine is cellular or humoral in nature, we performed adoptive transfer of total $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, or serum from mitochondria-enriched immunized

FIGURE 3. Therapeutic vaccination with DCs loaded with RENCA mitochondria lysate controls tumor progression. **(A)** In a therapeutic setting, mice were challenged s.c. with 1×10^6 RENCA cells and were vaccinated 1 wk later with bmDCs pulsed with DC/RENCA mito, DC/Kidney mito, or DCs alone (DC). Data are mean \pm SEM ($n = 10$ mice/group for each experiment, except DC group [$n = 5$]). All p values are pairwise multiple comparisons. $p < 0.01$, DC/RENCA mito versus DC/Kidney mito or versus DCs, Tukey test. **(B)** T cells isolated from DC/RENCA mito-vaccinated mice are reactive against RENCA cells. Purified T cells (1×10^5) from DC/RENCA mito-vaccinated mice were cocultured with stimulator RENCA cells or control CT26 cells (10:1 ratio), and IFN- γ ELISPOT was performed. **(C)** Immunohistochemistry (left panels) was performed, which quantitatively demonstrated heavy $CD3^+$ cell infiltration into RENCA tumors of animals treated with DC/RENCA mito (right panel). Original magnification $\times 20$.



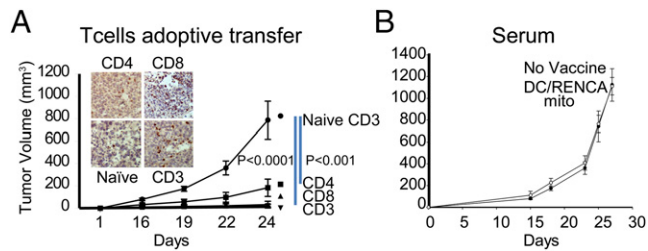


FIGURE 4. Adoptive transfer demonstrates a T cell-mediated antitumor effect upon vaccination using DCs loaded with RENCA mitochondria lysate. Tumor-free mice (BALB/c) were vaccinated with DCs pulsed with RENCA mitochondria lysate as previously described; mice were sacrificed 1 wk after the last vaccination, and lymphocyte populations (CD3⁺, CD4⁺, and CD8⁺ T cells) were isolated from splenocytes. Serum was also collected from vaccinated and naive mice (nonimmunized). **(A)** Isolated CD3⁺, CD4⁺, and CD8⁺ T cells and naive CD3⁺ T cells were injected i.v. into RENCA tumor-bearing mice (challenged 1 wk before the adoptive transfer) that were sublethally irradiated (4–5 Gy) 8 d before adoptive transfer. Data are mean \pm SEM ($n = 10$ mice/group for each experiment). Adoptively transferred CD3⁺, CD4⁺, or CD8⁺ T cells control tumor progression and result in CD3⁺ T cell infiltration. $p < 0.0001$, CD8 and CD3 versus naive CD3; $p < 0.001$, CD4 versus naive CD3. **(B)** Serum, injected i.p. into another group of mice, did not control tumor progression ($p =$ not significant).

mice into lymphodepleted (irradiated) RENCA tumor-bearing mice. Serum and CD3⁺ cells from nonimmunized mice were in-

cluded as additional controls (24). Fig. 4 demonstrates that the antitumor response generated by the DC/RENCA mito vaccine was mediated by T cells (Fig. 4A), whereas serum (Fig. 4B) lacked any therapeutic effect. The cell-transfer experiments also illustrate the central role played by CD8⁺ T cells in comparison with CD4⁺ T cells, because adoptive transfer of CD8⁺ or total CD3⁺ T cells results in complete rejection of tumors. As shown in Fig. 2, heavy T cell infiltration was observed in each of the groups adoptively transferred with CD3⁺ and CD8⁺ cells, whereas minimal CD3⁺ T cell infiltration was observed in mice that were adoptively transferred with CD3⁺ T cells from naive (nonimmunized) mice.

Sequencing of the 13 coding sequences included in the mtDNA of RENCA cells reveals two TAMA candidates

mtDNA is tens to hundreds times more likely to acquire mutations compared with nuclear DNA. Mechanisms that facilitate mtDNA mutations include the generation of ROS in close proximity to the mtDNA, the lack of histones, less extensive repair mechanisms than nuclear DNA, and a nucleotide imbalance that leads to decreased polymerase γ fidelity (4, 6). Given that some components of the mitochondrial lysate from RENCA contained the TAMAs, we chose to sequence the 13 coding sequences of mtDNA. mtDNA was purified from several RENCA cell lines and amplified by PCR with 22 pairs of overlapping primers (Supplemental Table I). Two point mutations generating amino acid alterations were

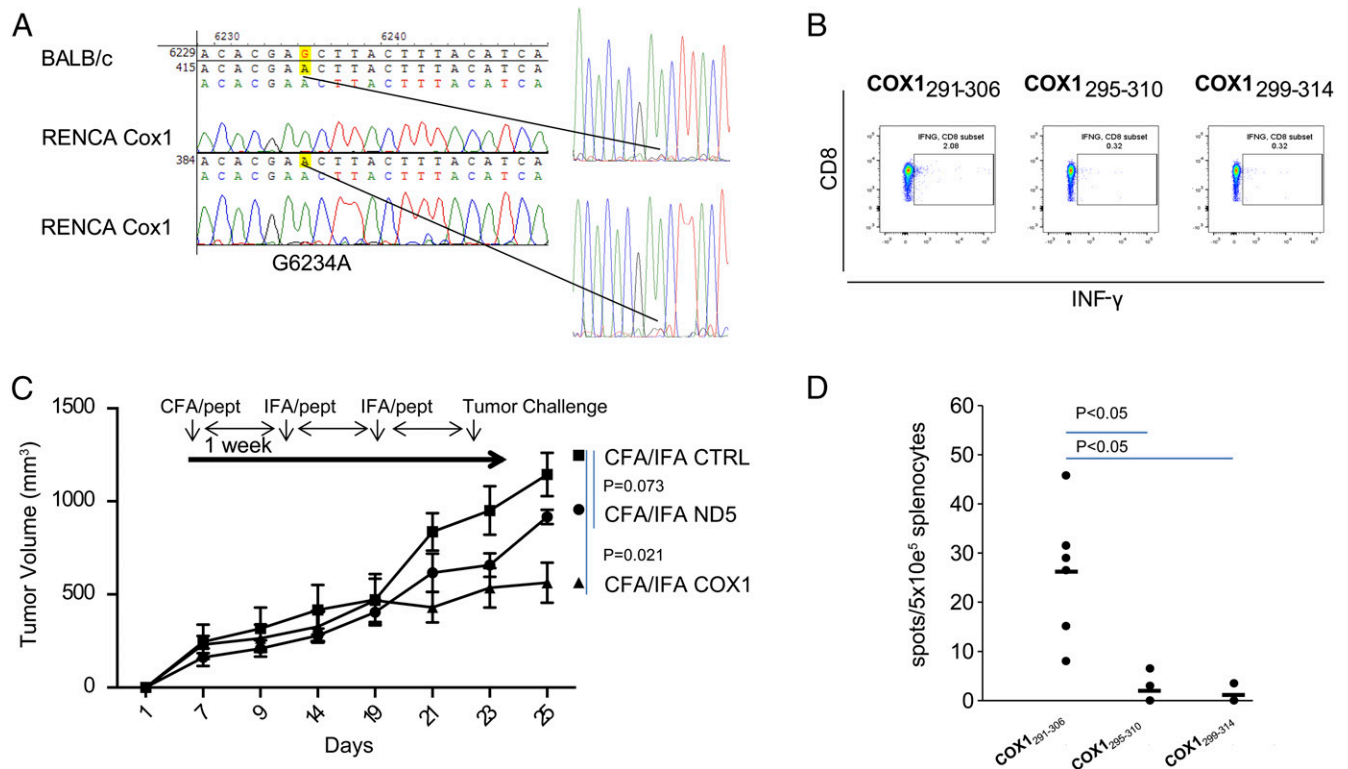
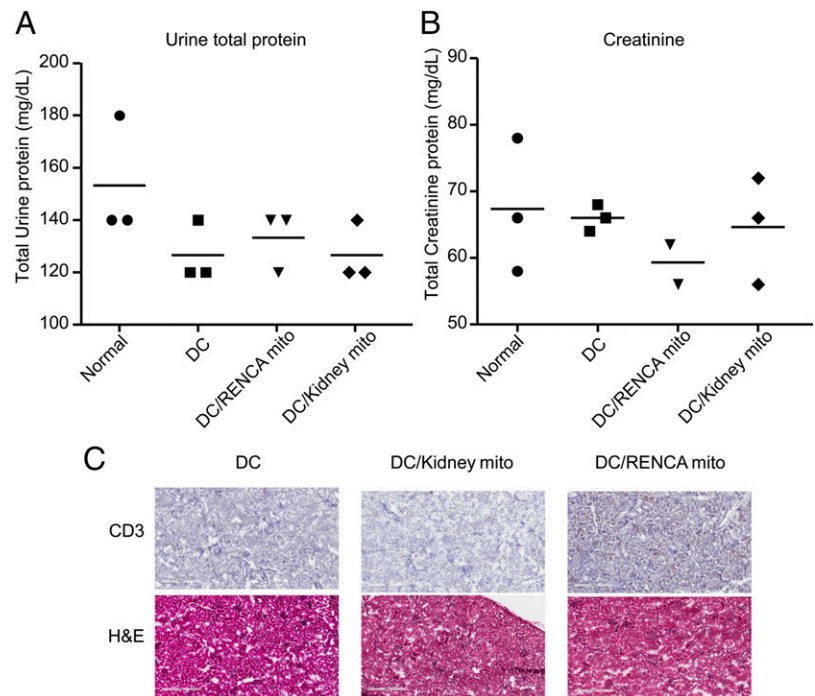


FIGURE 5. Screening of RENCA mtDNA reveals a mutation in COX1 protein that generates a neo-“nonself” epitope. mtDNA was amplified by PCR with 22 pairs of overlapping primers. **(A)** RENCA COX1 alignment to the reference sequence (EF108333.1, *Mus musculus* strain BALB/cByJ mitochondrion, complete genome) revealed the mutation G6234A; the enlarged chromatogram shows the probable homoplasmic nature of the mutation (COX1-II F6 and R6 PCR primers; Supplemental Table I). **(B)** Mutated COX1₂₉₁₋₃₀₆ peptide reacts to DC/RENCA mito-immunized CD8⁺ T cells. Three COX1 peptides were generated and used for overnight *in vitro* restimulation of splenocytes obtained from DC/RENCA mito-immunized mice. Specific CD3⁺/CD8⁺ T cell reactivity against COX1₂₉₁₋₃₀₆, but not against the other COX1 peptides or ND5 mutated control peptides (Supplemental Fig. 1B), was measured for IFN- γ by intracellular cytokine staining. **(C)** Multipptide COX1 immunization results in RENCA tumor growth control. A minimum of five mice/group was immunized with the mutated COX1 peptide pools, mutated ND5 peptide pools, or control preparation. One week later, mice were injected s.c. with 1×10^5 RENCA cells, and tumor progression was monitored, showing a protection of COX1-immunized animals. $p = 0.021$, versus control group, or $p = 0.036$, two-tailed ANOVA). **(D)** At the end point of the experiment, splenocytes of these mice were isolated and tested *in vitro* for reactivity against the mutated COX1 peptides by IFN- γ ELISPOT assay. $p < 0.05$, peptide 291–306 versus 295–310 or 299–314.

FIGURE 6. Kidneys are unaffected by DC/RENCA mito immunization. Urine and serum from vaccinated mice (DC, DC/RENCA mito, and DC/Kidney mito) and control animals (Normal) were collected and measured for total protein (A) and creatinine (B) levels. p = not significant between normal versus any treatment in either group. (C) Kidneys from vaccinated animals were collected to assess for possible treatment toxicity. Labeling of CD3⁺ T cells showed no differences in the number of T cells infiltrating the kidneys of DC-vaccinated animals (upper panels). H&E staining of kidneys of DC-vaccinated mice showed no abnormalities detected for either group (lower panels). Original magnification $\times 10$.



identified: one mutation was in position G6234A, which converted Ala to Thr in COX1 cytochrome oxidase I (complex IV) (Fig. 5A), and the other was in position C13330A (Pro to Thr) in the ND5 subunit of NADH (protein complex I) (Supplemental Fig. 1A).

To verify the functional relevance of the identified TAMA candidates, we designed peptides for vaccination or for in vitro restimulation and included these mutations (38). For each TAMA, three peptides were generated, each 15 aa in length and overlapping by 5 aa, including the mutation (39). These peptides were used for overnight in vitro restimulation of splenocytes obtained from RENCA mitochondria protein lysate-pulsed DC-immunized mice. Splenocytes (CD8⁺ T cells) from mice immunized with DC/RENCA mito specifically recognized the first mutated COX1_{291–306} peptide (MFTVGLDVDTRTYFT) (Fig. 5B) but not any of the ND5-mutated peptides (Supplemental Fig. 1B). Mice were then immunized with the COX1_{291–306} peptide in CFA and then boosted twice with the same peptide in IFA, followed by RENCA challenge. In agreement with the IFN- γ results, the COX1-mutated peptides induced a significant decrease in tumor burden, whereas ND5-mutated peptides also attenuated tumor growth, but it did not reach statistical significance (Fig. 5C). Splenocytes (CD8⁺) from COX1 peptide-immunized animals responded to the first COX1_{291–306} mutant peptide but not control, confirming the inclusion of the immune dominant epitope of the TAMA within the COX1_{291–306} peptide (Fig. 5D, Supplemental Fig. 1C).

RENCA-derived mitochondrial vaccine does not affect kidney functions

To evaluate whether our treatment had an impact on renal function, we collected urine and serum from treated and control animals and tested them for total protein and creatinine, a metabolic waste product that is removed from the blood chiefly by the kidneys. If kidney filtration is deficient, creatinine blood levels rise accordingly. Because serum proteins are readily reabsorbed from urine, the presence of excess protein indicates either an insufficiency of absorption or impaired filtration (40). We found no indication of toxicity or dysfunction in the mice receiving DC vaccines against RENCA targets, confirming the safety of our approach (Fig. 6A,

6B). Finally, because the vaccines target kidney tumor cells via T cell-mediated immune responses, we examined the kidneys of treated and untreated animals (control) by H&E and CD3⁺ staining, with a pathologist blindly scoring the slides. No gross differences between the groups were identified, demonstrating that there are no off-target effects (Fig. 6C).

Discussion

DCs, the professional APCs of the immune system, can be exploited for cancer immunotherapy (41). DCs activate effector mechanisms, including Ag-specific cytotoxic T lymphocytes, Abs, and NK cells, and generate long-lasting immune memory (42). Preclinical and early clinical data confirm that DC vaccines induce potent immune responses (43). Given that each patient may express his/her own set of TAMAs and a specific MHC haplotype, the isolation of tumor mitochondria for the generation of a DC-based vaccine represents an innovative approach to personalized immunotherapy. Moreover, the use of mitochondria lysate provides the entire possible repertoire of full-length TAMAs included in the specimen, bypassing the major limitation of epitope/peptide-based cancer vaccines (i.e., MHC restriction). In this article, we present evidence that mitochondrial protein lysates are taken up by DCs and presented to T cells, eliciting a CD8⁺ IFN- γ ⁺ T cell response specific for TAMAs. DC/RENCA mito lysate protect mice in both a therapeutic and prophylactic setting. Prophylactic vaccination of RENCA tumor-bearing mice using peptides that included the COX1 mutation, but not the ND5 mutation, conferred protection. Overall, our data suggest that mitochondria from tumors provide a source of TAMAs that, in a model of renal cancer, provide a safe and effective target for immunotherapy.

Renal cancer (as well as other cancer types) harbors multiple mtDNA mutations and is particularly immunosensitive (16, 18, 44). In addition, immunotherapeutic strategies have an important place in the management of advanced clear cell renal cell carcinomas (15, 16, 45). We used the well-established RENCA mouse model and initiated our studies by purifying lysate for mitochondria and establishing that immature bmDCs could efficiently engulf the lysate in vitro. Because mitochondrial danger-associated

molecular patterns released into the circulation during trauma activate human peripheral polymorphonuclear neutrophils, leading to degranulation, and DCs also express receptors for mitochondrial danger-associated molecular patterns (28, 29, 46), we hypothesized that mitochondrial lysate would activate bmDCs; however, we found that lysate did not activate or change the phenotype of IFN- γ - or LPS-matured bmDCs. T cells isolated from immunized mice actively secrete IFN- γ in the presence of RENCA target cells, whereas they do not respond to syngeneic CT26 colon cancer cells, demonstrating the specificity of the immune response.

Tumor control of DC/RENCA mito vaccine is mediated primarily by CD8⁺ T cells, whereas serum from vaccinated donors did not play a therapeutic role in our adoptive-transfer experiments. However, we did not directly measure anti-mitochondrial Abs generated by our vaccine; therefore, we cannot entirely rule out the possibility that mitochondrial vaccine-generated Abs contribute to the observed efficacy of vaccine. Previous studies correlated tumor progression and survival of cancer patients with the number of tumor-infiltrating lymphocytes (47). In the mitochondria-vaccinated mice there was marked T cell infiltration that was specific to the DC/RENCA mito-immunized group; the control group immunized with a vaccine prepared using healthy kidney mitochondria showed no response.

Much of the ongoing efforts in the development of vaccines for cancer and chronic infectious diseases is aimed at creating effective memory responses (48). Mice that were vaccinated with DC/RENCA mito maintained their tumor-free status after being rechallenged with RENCA tumors 3 mo later, illustrating the effective generation of immune memory.

To identify the source of immunogenicity in the mitochondrial lysate, we sequenced the 13 coding sequences of RENCA mtDNA. This revealed two putative TAMAs within the COX1 and ND5 sequences. When we used mutated peptides from COX1 and ND5 for in vitro restimulation of splenocytes isolated from DC/RENCA mito-vaccinated mice, only COX1 stimulation resulted in IFN- γ production. We then evaluated whether these peptides were directly involved in the mechanism of tumor rejection by using the peptides as vaccines. The results confirmed our in vitro findings because the COX1 peptide had prophylactic properties, whereas the ND5 peptide did not. This finding indicates that TAMAs, although not always immunogenic, can also serve as a source of synthesized, peptide-based immunization strategies in our system.

The improvement in next-generation sequencing, as well as other techniques, helped to underline the heterogeneity of tumor nuclear mutations both within a single patient and across patients (48). To account for tumor heterogeneity and mitochondrial homoplasmism in our study, we sequenced mitochondria from RENCA cells of various passages and at different confluence levels. In our model, the identified TAMAs were consistent between all of the cell lines analyzed and the in vivo-derived cells (data not shown). However, probably as a result of the heterogeneity in a given tumor, a single type of immune therapy may only be effective in a subpopulation of cancer patients; therefore, we claim that further mitochondrial analysis is required to cover a greater repertoire of cancer types at different stages. However, other immunotherapies targeting specific TAAs (such as CAR T cell therapy and DC-based vaccines) have been successful, despite the heterogeneity of the tumors. This could be explained, in part, by mechanisms of “cross-priming” induced by direct or indirect killing of tumor cells expressing the target Ags and harboring other mutations.

Promising immunotherapy approaches, such as CAR T cell therapy, DC therapy, and peptide vaccination, are only as effective as the selection of the TAA (49). Potential TAA candidates must meet a number of criteria: they must be immunogenic,

abundantly expressed by tumor cells, not be expressed in normal tissue to limit off-target effects, and functionally important for the tumor, so that expression is unlikely to be lost. Several cancers’ histology harbor mutations in mtDNA and TAMAs that meet most, if not all, of the criteria of an ideal TAA (11, 12). Lastly, immunotherapies based on CAR depend on the specificity of the TAA but often have toxic side effects, such as cytokine release syndrome, which vary from patient to patient and can be fatal in some cases (2, 50, 51). In addition, selection of a TAA for CAR therapy requires the availability of high-affinity Abs (scFv), which limits the pool of TAAs. Our data suggest that TAMAs may be uniquely tumor specific; they do not generate off-target effects and, when used with an effective immunotherapy platform, such as DCs, can elicit tumor-specific T cell responses and durable antitumor responses. To our knowledge, our study is the first to demonstrate that tumor mtDNA mutations can be used to generate a specific immune response upon active immunization in a murine model of renal cancer, resulting in durable tumor rejection. Further exploration and characterization of TAMAs may expand the pool of suitable TAAs for inclusion in immunotherapy protocols for different histological cancers.

Disclosures

The authors have no financial conflicts of interest.

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