Am J Cancer Res 2019;9(4):740-751
www.ajcr.us /ISSN:2156-6976/ajcr0093357

Original Article

Immune response evoked by tumor-associated NADH oxidase (tNOX) confers potential inhibitory effect on lung carcinoma in a mouse model

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Received March 4, 2019; Accepted March 12, 2019; Epub April 1, 2019; Published April 15, 2019

Abstract: Tumor-associated NADH oxidase (tNOX, ENOX2), which belongs to a family of growth-related NADH oxidases, was originally identified as a plasma membrane protein of rat hepatoma and is inhibited or downregulated by several anti-cancer drugs. The objective of this study was to evaluate the anti-tumor effects of tNOX used as an immunogen against Lewis lung cancer. Human tNOX was expressed in *Escherichia coli*, purified by His-Tag affinity chromatography, and emulsified with the adjuvant, ISA 201 VG. Immunological analyses of the generated tNOX vaccine were performed in mice. The results of ELISA and ELISpot were significantly higher in tNOX vaccine group compared to the control group. *In vivo*, we examined the anti-tumor effects of mice that received the tNOX vaccine via the intraperitoneal or subcutaneous routes. Mice were vaccinated three times at 2-week intervals, challenged at 2 weeks after the final vaccination, and terminated at 34 days post-challenge. Antibody titers, tumor volume and histopathological scores were used to evaluate the anti-tumor effects of the tNOX vaccine. Our results revealed that tNOX-vaccinated mice had significantly higher antibody titers than negative control (NC) and challenge control (CC) mice. When compared to the corresponding CC groups, the intraperitoneal and subcutaneous vaccination with tNOX showed a significantly smaller tumor mass volume ($P < 0.05$) and a significantly lower histological lesion score ($P < 0.05$), respectively. Our results demonstrate that the use of a xenogeneic tNOX as an immunogen in mice activates immune responses and anti-tumor effects against Lewis lung cancer.

Keywords: Tumor-associated NADH oxidase (tNOX, ENOX2), tumor-associated antigen (TAA), biomarker, cancer immunotherapy

Introduction

A tumor-associated NADH oxidase (tNOX, ENOX2) belonging to a family of growth-related NADH oxidases was originally identified as a plasma membrane protein in rat hepatoma; tNOX is constitutively activated, whereas its normal counterpart is responsive to hormones and growth factors [1, 2]. tNOX oxidizes the reduced NADH or hydroquinones to the oxidized form, NAD^+, and is correlated with cell growth [1, 3]. tNOX activity has been found in the sera of patients with various types of cancer but absence in healthy volunteers [4, 5]. tNOX protein expression was recently identified in several human tumor cell lines, including those arising from gastric, lung and colon cancer [6, 7], and validated as a cancer-specific cell surface protein to cancer-drug therapies [8, 9]. A number of anti-cancer drugs, including capsaicin [10], green tea catechin (-)-epigallocatechin-3-gallate (EGCg) [11], isoflavone [12], doxorubicin [13] and oxaliplatin [14], have been shown to attenuate cancer cell growth by inducing apoptosis via the inhibition or downregulation of tNOX. Furthermore, an *in vivo* study confirmed that the RNA interference (RNAi)-induced knockdown of tNOX reduced the growth of

HCT116 (human colon colorectal carcinoma) cell-derived tumors [6].

Cancer has been a growing global threat to public health and leading causes of morbidity and mortality worldwide. In 2019, more than 1,762,000 new cancer cases and 606,000 cancer deaths are predicted to occur in the United States [15]. The conventional anti-cancer treatments include surgery, chemotherapy and/or radiation. The cancer immunotherapy using body's own immune system to fight cancer cells is one of the novel anti-cancer treatments and has attracted a lot of attention. Many cancer immunotherapies have been approved by the United States (US) Food and Drug Administration (FDA), therefore the anticancer immunotherapy has become a real choice for patients in clinical cases [16]. The anticancer immunotherapy, such as the cancer vaccines, which comprise a type of cancer biological immunotherapeutic, can act in a prophylactic or therapeutic manner. The first approved prophylactic cancer vaccine is specific for use in women: GARDASIL® can prevent cervical cancer by protecting against human papilloma virus (HPV) infection [17]. The licensed DNA-based cancer vaccine, ONCEPT™, is a therapeutic cancer vaccine for melanoma in dogs [18]. This ONCEPT™ encoding xenogeneic human tyrosinase exploits the immune system to recognize canine melanoma cells and elicit an antigen-specific immune response to eliminate these cancer cells.

The key mechanism of the anticancer immunotherapies is to activate the immune system by introducing tumor-associated antigens (TAAs) as immunogens to induce immunotherapy. Therefore, the selection of a proper TAA is the critical step in the development of an efficacious anticancer immunotherapy [19]. Due to the properties involving in cell tumorigenesis, many TAAs have been identified and tested for their efficacy in cancer immunotherapies. Some proteins, such as glycoprotein 100 and tyrosinase of melanoma or mucin-1 of non-small cell lung cancer, are characterized as TAAs and involved in cell proliferation, migration and tumor progression [20, 21]. The current evidences all suggested that tNOX is characterized as a potential tumor biomarker, and is also believed to be an appropriate TAA for anticancer immunotherapy but has not yet been proved.

In the present study, we constructed and prepared a human tNOX protein as prophylactic immunotherapeutic agent, and examined its immunogenicity and anti-tumor effects in a mouse model. Our results reveal that injection with the xenogenic tNOX protein in a prophylactic strategy could induce an anti-tumor response and significantly inhibit lung tumor growth in an *in vivo* model.

Materials and methods

Materials

Cell culture media, fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). The anti-rabbit IgG and anti-mouse IgG antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-actin antibody was from Chemicon International, Inc. (Tamecula, CA, USA). The anti-tNOX polyclonal antibody and anti-His tag antibody were purchased from Protein Tech Group, Inc. (Chicago, IL, USA). The restriction enzymes were from New England Biolabs, Inc. (Ipswich, MA, USA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified.

Cell culture

NIH3T3 (mouse fibroblast), LLC (Lewis lung carcinoma), MRC-5 (human lung fibroblast), A549 (human lung carcinoma) and A375 (human malignant melanoma) cells were grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 50 μg/mL streptomycin. B16F10 (mouse melanoma) cells were grown in MEM and supplemented as described above. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Construction and analysis of tNOX recombinant plasmids

The reference sequences for the human and mouse tNOX genes were obtained from National Center for Biotechnology Information (NCBI; GenBank accession numbers AF207881.2 and BC025915.1). Human and mouse tNOX complementary DNAs (cDNAs) were amplified from total RNAs of HCT116 cells and B16F10 cells by reverse-transcription polymerase chain reaction (RT-PCR) using the primer pairs:

5'-ATGCAAAGAGATTTTAGATGG-3'/5'-TGAGGTCAGCTTCAAGCCCTCGAA-3' and 5'-CTATGACGCTGCCTGTGTC-3'/5'-AGGTCAGCTTCAAGCCCTC-3', respectively. The obtained fragments were sequenced, and the encoding amino acids were analyzed with the MegAlign software (DNASTAR, Madison, WI, USA). The amplified fragments of human and mouse tNOX were cloned into the *Nco*I/*Pml*I sites of pTriEx-3 (Novagen, Merck KGaA, Darmstadt, Germany) to generate pTri/tNOX and pTri/MtNOX, respectively. The amplified fragment of human tNOX was also cloned into the *Nde*I/*Not*I sites of pET-28 (Novagen) to generate pET/tNOX.

Preparation and analysis of cell extracts

Cell extracts were prepared in lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 2 mM PMSF, 10 ng/mL leupeptin and 10 μg/mL aprotinin). Aliquots containing 40 μg of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The tNOX protein was determined by western blotting.

Overexpression of tNOX protein in non-cancerous cells

NIH3T3 cells were transiently transfected with pTri/tNOX, pTri/MtNOX or pTriEx-3 using the jet-PEI transfection reagent (Polyplus-transfection SA, Illkirch Cedex, France) according to the manufacturer's protocol. The overexpression of tNOX protein was validated in cell extracts by SDS-PAGE and western blotting.

Continuous monitoring of cells by the xCELLigence system

To continuously monitor changes in cell growth or cell invasion and migration (CIM), we used the xCELLigence System (Roche, Mannheim, Germany) as described previously [22]. In brief, approximately 1×10^4 cells/well were seeded to E-plates or the top chamber of a CIM plate. The plates were incubated for 30 min at room temperature and then placed onto the Real-Time Cell Analyzer (RTCA) station (Roche). Cell growth was measured by hourly assessment of the impedance, and cell migration was measured as a change in impedance at the electrode/cell interface, which occurred as cells migrated from the top chamber to the bottom chamber.

Expression and purification of human tNOX recombinant proteins

The recombinant construct, pET/tNOX, was transformed into *Escherichia coli* (*E. coli*) BL21 cells, and the transformed *E coli* cells were cultured in Luria-Bertani (LB) broth containing 50 μg/ml kanamycin at 37°C. The expression of recombinant tNOX was induced with 1 mM IPTG at 30°C with shaking (150 rpm) for 4 hours. The tNOX protein was purified on a nickel column packed with complete His-Tag Purification Resin (Roche) according to the manufacturer's protocol. The eluted protein was dialyzed into 1X phosphate-buffered saline (PBS) and quantitated using the Protein Assay Dye Reagent Concentrate (BioRad, Hercules, CA, USA). The purified and desalted tNOX protein was verified by SDS-PAGE and western blotting, and used as an immunogen for the following experiments.

Preparation of tNOX as a subunit vaccine

MONTANIDE ISA 201 VG (SEPPIC, Paris, France) was used as an adjuvant in this study. To prepare a tNOX subunit vaccine, the purified tNOX protein was emulsified with the adjuvant at a weight ratio of 1:1 at 30°C with agitation at a low shear rate. Each vaccination dose contained 18.75 μg immunogen in a total volume of 0.5 ml. 1X PBS was emulsified with adjuvant as the blank vaccine control. The emulsified solutions were stored at 4°C.

Animal study

Specific pathogen free (SPF) female C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The animal use protocol was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University (Taichung, Taiwan).

For immunological analysis, 6-week-old mice were randomly divided into two groups (n = 3 per group): group-1 mice were left untreated as a negative control (NC), while group-2 mice were vaccinated with the tNOX subunit vaccine. The schedule of the animal study is depicted in **Figure 1**. Vaccination was performed

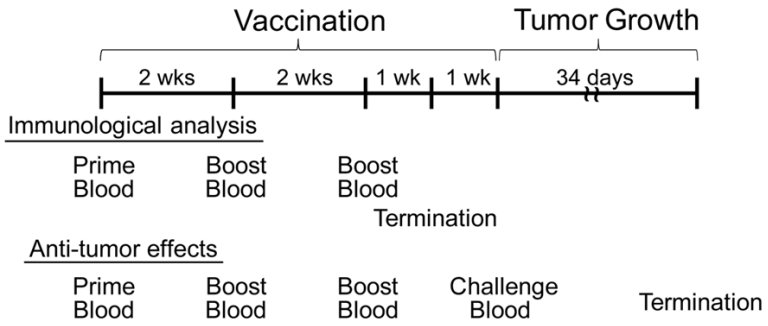

Figure 1. Schedules of the animal studies used for immunological analysis and evaluation of anti-tumor effects. Six-week-old mice were vaccinated three times at intervals of 2 weeks (wks). Blood samples were collected on the priming day and at 2 weeks after each vaccination. For immunological analysis, mice were terminated 1 week after the final boost. For our evaluation of anti-tumor effects, mice were challenged with LLC cells at 2 weeks after the final boost and monitored for 34 days thereafter.

Table 1. Animal study information

Groups[a]	Inoculums	Administration route[b]	
		Study-1	Study-2
1. NC	NA	NA	NA
2. CC	1X PBS; emulsified ISA 201	IP	SC
3. tNOX	tNOX; emulsified ISA 201	IP	SC

[a]: NC: negative control (no treatment), CC: challenge control (blank vaccination/tumor challenge), tNOX: tNOX vaccine group (tNOX subunit vaccine/tumor challenge).
[b]: NA: non-applicable, IP: intraperitoneal, SC: subcutaneous.

three times at intervals of 2 weeks. Blood samples were collected on the priming day and at 2 weeks after each vaccination. One week after the final boost, mice were sacrificed and spleens were collected for analysis of immune responses.

To evaluate the anti-tumor effect of tNOX subunit vaccine, we randomly divided 6-week-old mice into 3 groups (n = 5 per group) (**Table 1**): group-1 mice were left untreated as a negative control (NC group); group-2 mice were subjected to blank vaccination/tumor challenge as the challenge control (CC group); and group-3 mice were injected with the tNOX subunit vaccine/tumor challenge (tNOX vaccine group). The schedule of the animal study is depicted in **Figure 1**. Two weeks after the final boost, mice were subcutaneously injected with 150 μl of LLC cell mixture, which contained 5×10^5 cells mixed with Matrix gel (BD, Franklin Lakes, NJ, USA) at a volume ratio of 1:1. The tumor size was recorded at 7, 17, 24 and 34 days after challenge and the tumor volume was calculated using the formula (Length × Width^2 × 0.5) [23]. Thirty-four days after tumor cell injection, mice were euthanized. The tumor mass, heart, lung, liver and spleen were quickly removed and formalin-fixed, and sections were subjected to hematoxylin and eosin (H&E) staining. Tumor masses were recorded and the lesion scores were graded according to the severity of the affected cells in each H&E-stained section, as follows: 1 = minimal (< 1%); 2 = slight (1-25%); 3 = moderate (26-50%); 4 = moderate/severe (51-75%); and 5 = severe/high (76-100%) [24].

Evaluation of humoral immune response after administration of the tNOX subunit vaccine

The humoral immune responses triggered by the tNOX subunit vaccine to induce anti-tNOX antibody in mice were evaluated by enzyme-linked immunosorbent assay (ELISA). The conditions for ELISA were optimized in our lab (data not shown). In brief, 96-well immunoplates (Nunc, St. Louis, MO, USA) were coated with 0.05 μg/well of purified tNOX protein overnight at 4°C. The plates were blocked with 1% BSA, loaded with 100 μl/well of diluted sera (1:32,000) and incubated at room temperature for 1 hour. The plates were washed five times, loaded with 100 μl/well of secondary antibody (1:5000) and incubated at room temperature for 1 hour. The plates were washed five times and loaded with 100 μl/well of NeA-Blue solution (Clinical Science Products, Inc., Mansfield, MA, USA) for color development. Absorbance was measured at 655 nm with ELISA reader (Model 680; BioRad). The relative anti-tNOX antibody titer was calculated using the formula: Antibody titer = Average absorbance of the group at a specific time point/Average absorbance of the group on the priming day.

Enzyme-linked immunospot (ELISpot) assay

ELISpot assays were performed using a Mouse IFN-γ ELISpot Development Module (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, approxi-

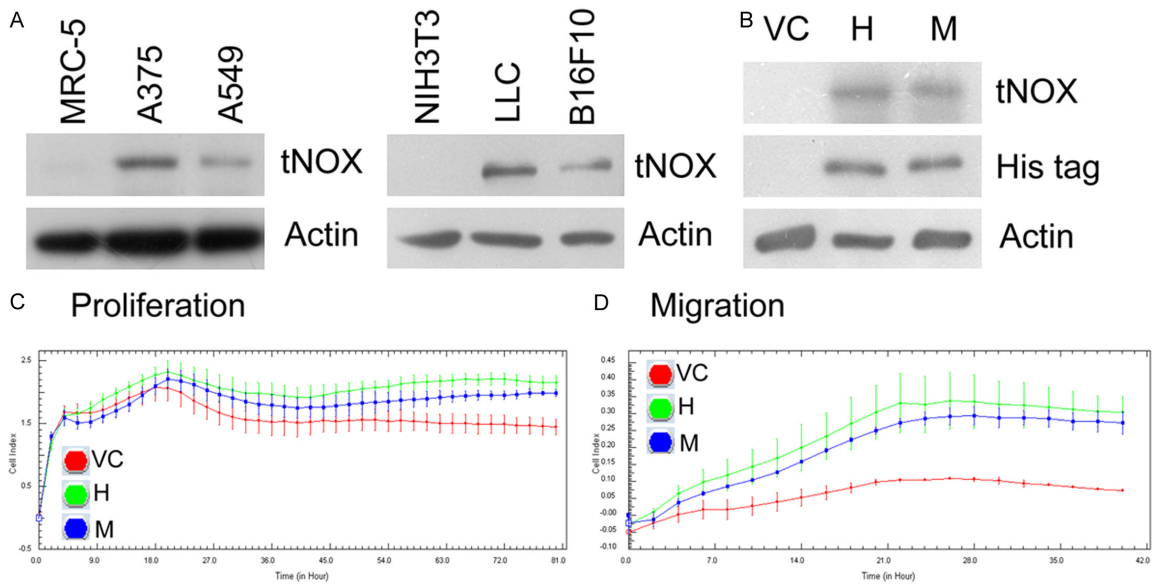

Figure 2. Determination of tNOX expression and the effects of tNOX in mouse cell lines. (A) Mouse and human cell extracts were prepared and analyzed by western blotting. The presence of endogenous tNOX was detected with an anti-tNOX polyclonal antibody. Actin was used as an internal control. (B) NIH3T3 cells were transfected with pTriEx-3 (vector control, VC), pTri/tNOX (human tNOX, H) or pTri/MtNOX (mouse tNOX, M) for 2 days, and overexpression of tNOX in transfected cells was analyzed by western blotting. The effects of tNOX expression in non-cancerous mouse cells were determined by dynamic monitoring of cell proliferation (C) and migration (D) using impedance technology, as described in the Materials and Methods.

mately 4 × 10^5 splenocytes/well were stimulated with RPMI medium supplemented with 10% FBS, tNOX protein (5 μg/ml) or Concanavalin A (2.5 μg/ml, Con A; Sigma) for 24 hours. After color development, the spots were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The specific interferon-γ (IFN-γ)-secreting T cell response was considered to be positive when the number of spots in the vaccination group were greater than the mean background +3 standard deviations of the negative control [25].

Statistical analysis

Differences between two means were calculated using the Student's t test, and $P < 0.05$ was considered significant. All statistical analyses were performed using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The expression and biofunction of tNOX protein in human and mouse cancer cell lines

The tNOX protein has been found in human tumor tissues and numerous cancer cell lines, and it has been shown to impact tumor cell proliferation and migration. As mouse models are often used as a platform for evaluating cancer immunotherapies, we began by investigating tNOX expression in different mouse and human cell lines. Our BLAST-based amino acid sequence analysis of human and mouse tNOX revealed that the *Mus musculus* tNOX protein was 91.3% similar to that of *Homo sapiens,* suggesting these xenogeneic tNOX proteins may share common characteristics. western blot analysis revealed that the tNOX protein could be detected in various cancer cells, including LLC, B16F10 (mouse), A549 and A375 (human) cells (**Figure 2A**). In contrast, tNOX expression was relatively low in non-cancerous NIH3T3 (mouse) and MRC-5 (human) cells (**Figure 2A**). Next, we evaluated the biological impact of mouse tNOX, which has not been well examined in the literatures. We first confirmed that NIH3T3 cells transfected with pTri/tNOX (human, H) and pTri/MtNOX (mouse, M) expressed tNOX proteins that were recognized by antibodies against both tNOX and His tag (**Figure 2B**). We then performed cell impedance measurements using an xCELLigence System. Our results revealed that overexpression of both forms (human and mouse) of tNOX increased cell proliferation (**Figure 2C**) and migration (**Figure 2D**) compared to the results

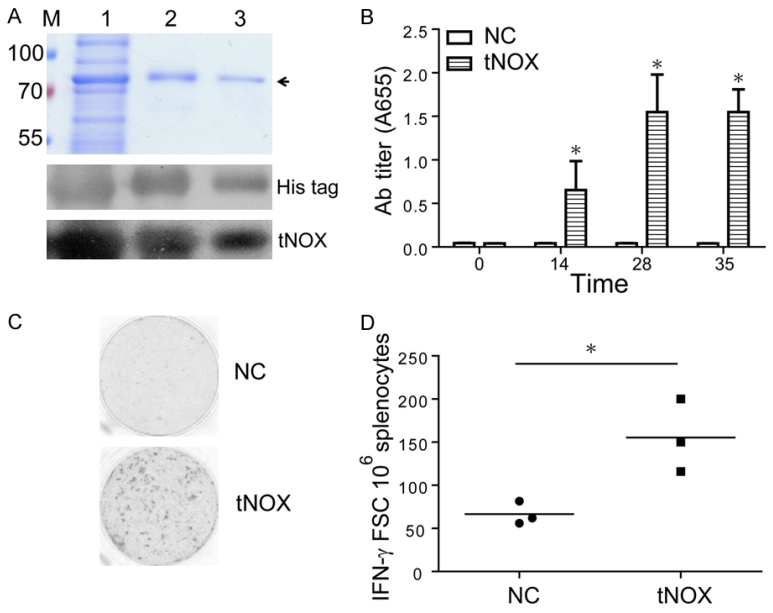

Figure 3. The protein expression and immune responses of the human tNOX recombinant proteins in mice. Recombinant human tNOX was expressed in *E. coli* and purified with His-tag purification resin. A. The expressed, purified and dialyzed proteins were resolved by SDS-PAGE and detected by western blot analysis with anti-His tag and anti-tNOX antibodies, and are shown in Lanes 1, 2 and 3, respectively. The molecular weight (KDa) marker (M) is shown at the left. Arrow indicates the tNOX protein band. B. Blood samples were collected at 0, 14, 28 and 35 days after the first vaccination and antibody titers were measured by ELISA. The specific anti-tNOX antibody response was significantly higher in the tNOX vaccine group compared with unvaccinated controls (*$P < 0.05$). C. Splenocytes of mice were stimulated with human tNOX protein and IFN-γ-secreting T cells were evaluated by ELISpot assay. Representative images show proliferative spots of splenocytes from mice vaccinated with tNOX (tNOX) or without tNOX (NC). D. The frequencies of tNOX-specific IFN-γ-secreting T cells was significantly higher in the tNOX vaccine group compared to the NC group (*$P < 0.05$). The average spot counts of the NC and tNOX vaccine groups were 67 ± 13 and 155 ± 42, respectively.

obtained in control cells. These findings suggest that the human and mouse tNOX protein are highly homologous and have the same biofunction to enhance cell characteristics associated with tumorigenesis.

Immunogenicity and immune responses induced by tNOX protein

A subunit vaccine containing the human tNOX protein was prepared and evaluated for its immunogenicity. The recombinant human tNOX protein was expressed in *E. coli* BL21 cells and purified by affinity chromatography. The purity of the tNOX protein expressed from pET/tNOX was greatly improved by affinity chromatography (**Figure 3A**, lanes 1 and 2). The tNOX protein was then dialyzed in PBS (**Figure 3A**, lane 3) and detected by western blotting, suggesting that tNOX still maintained its original epitopes recognized by the polyclonal antibody against tNOX. The purified and dialyzed tNOX protein was directly emulsified with the adjuvant to prepare a tNOX subunit vaccine. To evaluate the ability of the tNOX subunit vaccine to induce immunogenicity and assess the immune response triggered by vaccination, SPF C57BL/6 mice (n = 3 per group) were intraperitoneally injected three times at intervals of 2 weeks. Blood samples were collected at various time points and isolated sera were assessed for anti-tNOX antibody titer. ELISA revealed that tNOX-vaccinated mice showed a significantly higher antibody response toward tNOX compared to untreated mice ($P < 0.05$) (**Figure 3B**). The highest anti-tNOX antibody titer was observed following the second immunization. Mice were sacrificed 1 week after the final boost, and spleens were collected and used to evaluate the ability of the tNOX subunit vaccine to induce IFN-γ-secreting T cell responses. Our ELISpot assays revealed that tNOX-vaccinated mice exhibited significantly more IFN-γ-secreting splenocytes compared to blank control mice (**Figure 3C**). Indeed, a statistical calculation showed that the frequencies of IFN-γ-secreting splenocytes were more than two-fold higher in tNOX-vaccinated mice than in controls ($P < 0.05$) (**Figure 3D**). These results indicate that vaccination with our xenogeneic tNOX subunit vaccine successfully induced robust immune responses in mice.

Immune response and anti-tumor effects of tNOX subunit vaccine administered by intraperitoneal route in mice

The anti-tumor effects of the prepared tNOX subunit vaccine was evaluated by challenging the immunized mice with LLC cells in a prophylactic setting. SPF C57BL/6 mice (n = 5 per

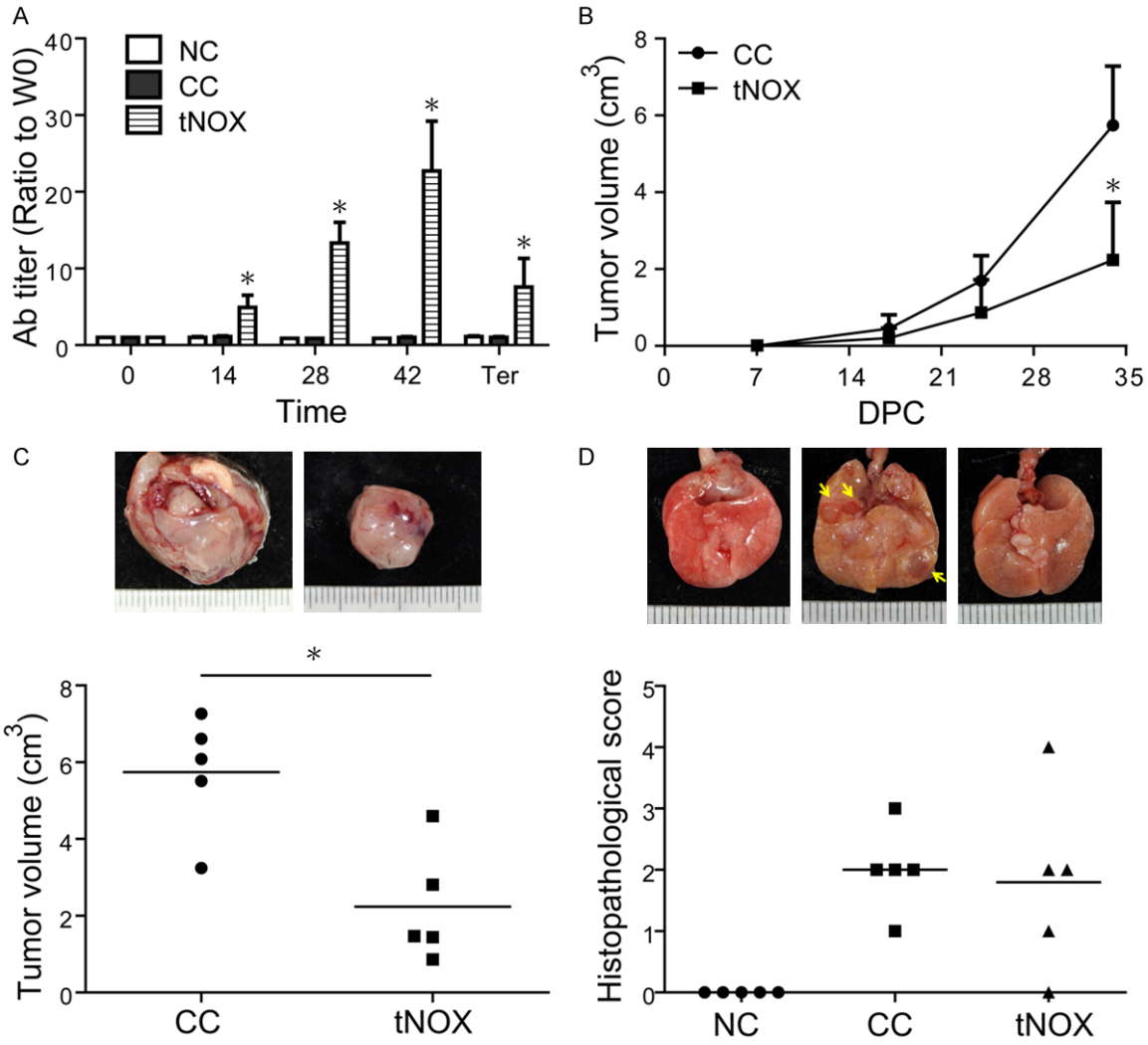

Figure 4. Anti-tumor effects of tNOX subunit vaccine administrated by intraperitoneal route in mice. SPF mice were intraperitoneally vaccinated three times at 2-week intervals, challenged at 2 weeks after the final boost, and terminated at 34 days after the challenge. Serum samples were collected at 0, 14, 28 and 42 days after the first vaccination and on the termination day (Ter), and the immune response was evaluated by ELISA. A. Significantly higher anti-tNOX antibody titers were observed in the tNOX vaccine group versus NC and CC groups at 14, 28 and 42 days after the first vaccination and on the termination day (*$P < 0.05$). B. Tumor volume was measured at 7, 17, 24 and 34 days post-challenge. The tumor mass volume was significantly smaller in the tNOX vaccine group compared to the CC group on the termination day (*$P < 0.05$). C. The tumor mass was removed from each mouse. The average tumor volumes of the CC and tNOX vaccine groups were 5.74 ± 1.54 and 2.24 ± 1.50 cm^3, respectively. The average tumor volume was significantly smaller in the tNOX vaccine group compared to the CC group (*$P < 0.05$). D. Lung tissues were removed and tumor foci were observed (arrow). The histological lesion scores of lungs were not significantly different between the CC and tNOX groups.

group) were intraperitoneally immunized three times at intervals of 2 weeks. During the vaccination period, blood samples were collected and ELISA was used to determine the anti-tNOX antibody titer of isolated sera. Indeed, we observed a significant anti-tNOX antibody titer in the tNOX vaccine group ($P < 0.05$), whereas there was no anti-tNOX antibody response in the NC and CC groups (**Figure 4A**). Two weeks after the final boost, we subcutaneously inoculated the right flanks of mice with 5×10^5 LLC cells. Tumor development was monitored for 34 days. During this period, the tumors of mice that had been vaccinated with the tNOX subunit vaccine were smaller than those of PBS-injected control mice (**Figure 4B**). The mice were terminated, the tumors were removed and the average tumor volumes were investigated. Our results revealed that the average tumor volume was significantly smaller in the tNOX vac-

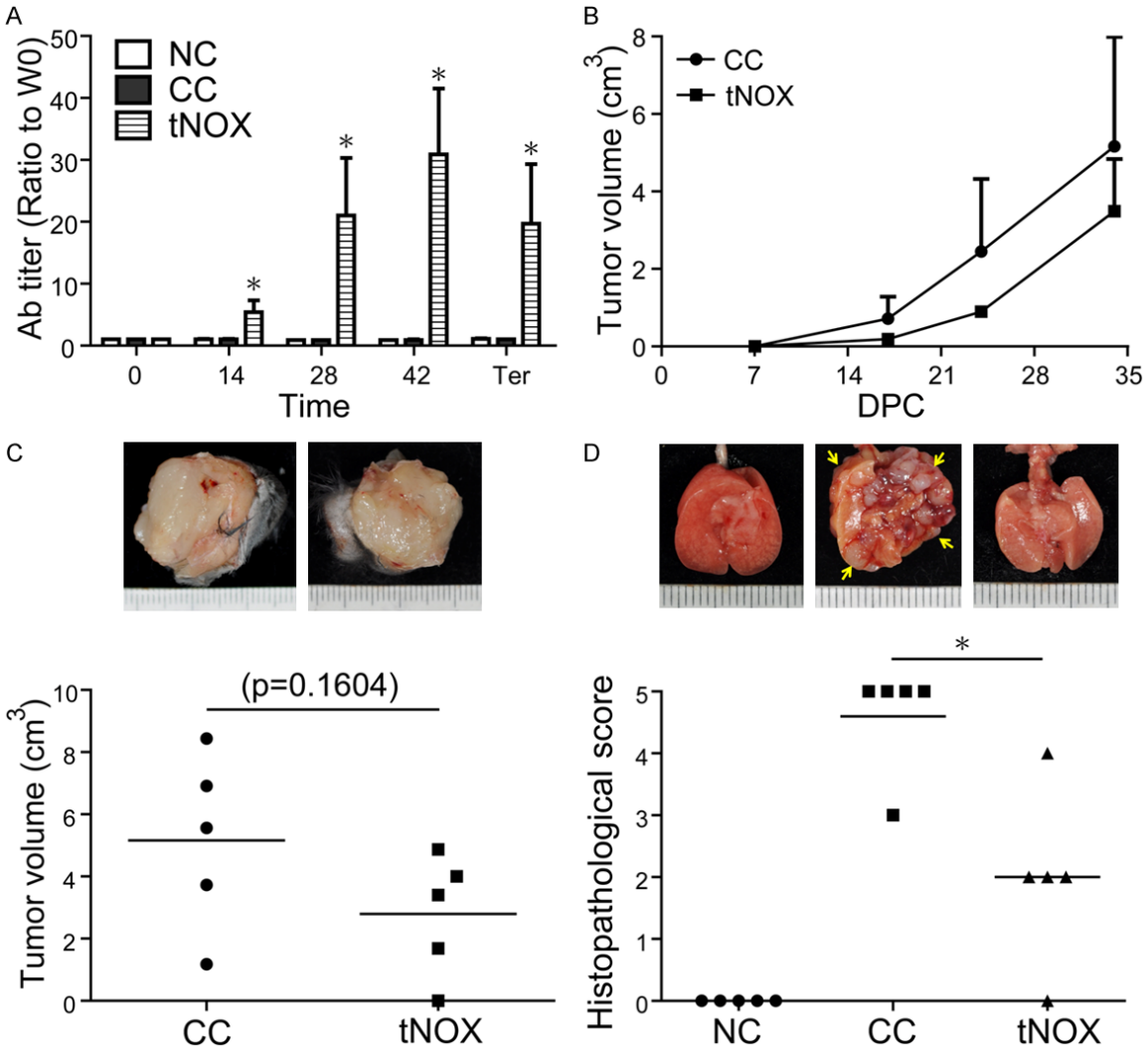

Figure 5. Anti-tumor effects of tNOX subunit vaccine administrated by subcutaneous route in mice. SPF mice were subcutaneously vaccinated three times at 2-week intervals, challenged at 2 weeks after the final boost, and terminated at 34 days post-challenge. Blood samples were collected at 0, 14, 28 and 42 days after the first vaccination and on the termination day (Ter), and the immune response was evaluated by ELISA. A. The anti-tNOX antibody titers were significantly higher in the tNOX vaccine group compared to the NC and CC groups at 14, 28 and 42 days after the first vaccination and on the termination day (*$P < 0.05$). B. Tumor volume was measured at 7, 17, 24 and 34 days post-challenge. The tumor mass volume was smaller in the tNOX vaccine group compared to the CC group at all time points, but the differences were not significant. C. The average tumor volumes of the CC and tNOX vaccine groups were 5.17 ± 2.82 and 3.49 ± 1.35 cm^3, respectively. The average tumor volume was smaller in the tNOX vaccine group than in the CC group, but not to a significant degree ($P = 0.1604$). D. Lung tissues were removed and tumor foci were observed (arrow). The tNOX vaccine group had a significantly lower histological lesion score than the CC group (*$P < 0.05$).

cine group ($P < 0.05$), which exhibited a 61% reduction in tumor size compared to the CC group (**Figure 4C**). Compared to the NC group, the CC and tNOX vaccine groups both exhibited many more tumor masses (arrows) in the lungs (**Figure 4D**), with no difference found in tumor growth in the lungs of mice in the CC and tNOX vaccine groups ($P = 0.7924$). Together, these results indicate that the antibody response induced by tNOX subunit vaccination could target tNOX-overexpressing tumor cells to reduce tumor growth *in vivo*.

Immune response and anti-tumor effects of tNOX subunit vaccine administered by subcutaneous route in mice

As an alternative administration route, subcutaneous vaccination was conducted in this trial. SPF C57BL/6 mice (n = 5 per group) were sub-

cutaneously immunized three times at intervals of 2 weeks. The immune response induced by subcutaneous vaccination was determined by ELISA, and the results showed that the antibody response to tNOX was significantly higher in the tNOX vaccine group compared to the NC and CC groups ($P < 0.05$) (**Figure 5A**). The highest anti-tNOX antibody titer was detected at 2 weeks after the final boost. At that point, the right flanks of the mice were subcutaneously inoculated with 5×10^5 LLC cells. Tumor development was monitored for 34 days. Similar to the results obtained following intraperitoneal vaccination, the tumor size was smaller in mice that had been subcutaneously vaccinated with the tNOX subunit vaccine compared to controls (**Figure 5B**). Analysis of the average tumor volume revealed that the tNOX vaccine group showed a tumor inhibition rate of 32.5% relative to the CC group; this difference did not reach the level of statistical significance ($P = 0.1604$) (**Figure 5C**). H&E staining of lung sections showed that the tNOX vaccine group had significantly fewer lesion scores compared to the CC group ($P < 0.05$) (**Figure 5D**). Collectively, these results indicate that the newly developed tNOX subunit vaccine induces effective immune responses, reduces tumor growth at the lung and/or may decrease lung metastasis.

Discussion

In 2013, the importance of cancer immunotherapy was highlighted by the journal *Science*, which listed it as a Breakthrough of the Year [26]. Moreover, The Nobel Prize in physiology or medicine in 2018 was awarded for the discovery of cancer therapy by inhibition of negative immune regulation. Researchers have been committed to develop more anti-cancer strategies related to the immune system. Among the different types of cancer immunotherapy, cancer vaccines that utilize TAAs to activate antitumor immune responses have yielded some impressive results. However, the potential for inducing immune tolerance against a self-antigen has limited the development of cancer vaccines. One strategy to overcome this tolerance is the use of a xenoantigen, which is an orthologous protein from species other than the target species. In the first such work, researchers vaccinated mice with human glycoprotein 75 and showed that the resulting antibodies induced tumor rejection [27]. Several other studies have shown that xenogeneic cancer vaccines can help break self-antigen tolerance and affect tumor growth [28-31]. Based on this, we herein examined a human tNOX as xenogeneic TAA for the development of prophylactic immunotherapy in mice.

TAAs are usually highly expressed in the tumor masses and involved in tumor progression. tNOX protein is found with unregulated NADH oxidase activity associating with cancer/transformed cell surface and responses to anticancer drugs [3]. Various reports have suggested that tNOX acts as a critical regulator of responses to biological stimuli involved in redox signaling, cell proliferation, cell survival and tumor progression [7, 32-34]. However, there is no precedent for the use of tNOX as an immunogen for cancer immunotherapy. Here, we hypothesized that tNOX may act as a TAA to activate an immune response against cancer cells in an animal model. Therefore, We cloned a human tNOX from HCT116 cells and found that its sequence was 100% identical to the human tNOX gene published in NCBI, suggesting that the encoded protein could maintain similar ability to stimulate cell proliferation and migration as those previously reported by Su et al. [13]. We then cloned a mouse tNOX and found that the encoded amino acid sequence was 100% and 91.3% identical to that of mouse and human tNOX, respectively. The highly homology between these two xenogenic proteins suggests that the mouse tNOX protein may have characteristics similar to those of human tNOX. As expected, our results showed that not only the tNOX protein was expressed in mouse cancerous cells but also overexpression of tNOX impacted cell proliferation and migration in NIH3T3 cells (**Figure 2**). Previous studies also showed that RNAi-mediated knockdown of tNOX leads to significant reductions in cell migration and proliferation *in vitro* and *in vivo* [6, 33]. These characteristics of tNOX supported our hypothesis that it could potentially act as a TAA candidate for immunotherapy. Indeed, our animal trials showed that mice given prophylactic vaccination with xenogeneic tNOX exhibited reductions in both local tumor volume and tumor growth in the lung compared to challenge control animals.

Subunit vaccines are made with antigens, proteins or peptides, prepared with ajuvants and have been applied in cancer immunotherapy [35]. Researches revealed that immunization

with recombinant xenogenic proteins or carrier conjugated proteins could break the self-antigen tolerance, generate antibodies targeting and destroying tumor cells, and exhibit decreased tumor burden [36, 37]. Hence, to evaluate the property of tNOX as TAA in prophylactic immunotherapy, xenogenic tNOX protein was emulsified with ISA201 VG adjuvant to prepare as subunit vaccine. The induced immune responses may vary due to the administration route and the use of an adjuvant. Intraperitoneal administration is a common route for use in laboratory rodents, but it is rarely used in larger mammals. When the intraperitoneal route is used, the injected compounds are mainly absorbed through the portal circulation and undergo hepatic metabolism before they enter systemic circulation [38]. The subcutaneous route is a common parenteral administration method and able to induce systemic absorption for drugs and macromolecules that may be absorbed primarily through the blood capillaries [39]. These different administration routes have resulted in different anti-tumor effects, as seen in our animal study: local tumor growth was significantly inhibited by intraperitoneal administration, while metastasis was significantly inhibited by subcutaneous administration. Since the aim of the present study was to evaluate the potential of tNOX as TAA used in cancer immunotherapy rather than the final design of immunotherapeutic trial, the mechanisms and the suitable route of administration have to be explored in future studies. Furthermore, the ability of an agent to combat tumor growth and metastasis is the key to successful cancer treatment, our results encourage the hope that tNOX could be developed for vaccines aimed at ameliorating tumor growth and metastasis.

Vaccine adjuvants are usually used to enhance the adaptive immune responses and elicit the alternation of humoral or cell-mediated immunity [40]. Montanide™ ISA 201 VG is an adjuvant that can emulsify the recombinant antigen into a water-in-oil-in-water (W/O/W) formulation; it has been shown to rapidly induce protective immune responses and last for a long period of time in both the humoral and cell-mediated immune responses [https://www.seppic.com/montanide-isa-w-o-w]. Here, we successfully expressed/purified the human tNOX protein and emulsified it with ISA 201 VG for use as a xenogeneic subunit vaccine for cancer immunotherapy. After the first vaccination, we detected a significant antibody response, as assessed by ELISA. An antibody response was still detectable at the termination day (48 days after the final injection) (**Figures 4A** and **5A**), revealing that the immunity appeared to be maintained for an extended period. The xenogeneic tNOX subunit vaccine increased the proliferation of IFN-γ-secreting splenocytes, reflecting that it may also activate cell-mediated immunity in mice. Several reports had found that the use of ISA 201 VG as an adjuvant could induce improved immune response and change the direction of immune response to T helper 1 (Th1) cell-mediated responses [41-43]. Collectively, our novel findings show that the xenogenic tNOX protein emulsified with ISA 201 VG could induce robust humoral and specific cell-mediated responses in mice and provide anti-tumor effects when used for cancer immunotherapy.

In conclusion, we herein show that the xenogeneic tNOX prepared as subunit vaccine activates immune responses and affects the growth of Lewis lung cancer in mice. This is the first study to utilize tNOX as a TAA for prophylactic immunotherapy. Our results suggest that tNOX should be viewed as a novel TAA candidate for cancer immunotherapy.

Disclosure of conflict of interest

None.

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