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Key words: DNA vaccine, pCMV-LacZ, pcDNA-hNIS, IgG2a, tumor retardation

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28 **Summary statement**

29 We used plsmid DNA like as LacZ DNA or NIS DNA which are internally translated in the

30 cells, which can be loaded on MHC I, not be secreted. We clarify reversion of immunity from

31 Th2 to Th1 when we repeated intradermal injection of plasmid DNA as a DNA vaccine.

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34 **Running title:** Th1 response after intradermal DNA vaccination

Abstract

In evaluating the effectiveness of DNA vaccines, it is important to (1) monitor the movement of cells transfected with the injected plasmid DNA; and (2) overcome immune deviation, which causes a switch from helper T cell (Th)1 to Th2. Mouse CT26 cells were transfected with the pcDNA-hNIS vector expressing human sodium/iodide symporter (hNIS) gene; the pCMV-LacZ vector expressing β-galactosidase from the cytomegalovirus promoter was used for imaging. Transgene expression was monitored by X-gal staining or γ-ray detection. Whole-body images were obtained by nuclear scintigraphy following intraperitoneal injection of radioactive technetium (^{99m}Tc). Migrating cells expressing hNIS or LacZ were monitored for 2 weeks. Reverse transcription PCR revealed that cells expressing the transgenes had moved out of the injection site. HNIS-expressing cells were observed specifically in peripheral lymphoid tissues, especially in draining lymph nodes and spleen. LacZ DNA was detected with a specific antibody in immunized mice that exhibited Th2-type immunity. IgG2a type was predominant in hNIS-immunized mice, as determined by enzyme-linked immunosorbent assay (ELISA). Moreover, the vaccine caused increases in the IgG2a/IgG1 ratio, number of interferon (IFN)-γ-secreting cells (by enzyme-linked immunospot assay), and IFN-γ level (by cytokine ELISA) in the supernatant of immune cells. Tumor growth was retarded in mice that were immunized with hNIS DNA followed by inoculation with CT26/NIS cells. The movement of mouse cells transfected with plasmid DNA was restricted to immune organs. Transgene expression in these cells was detected for at least 2 weeks post immunization. Repeated intradermal injection of plasmid DNA caused a switch in the humoral immune response to the Th1 type.

Introduction

DNA vaccines can induce both cellular and humoral immunity and are considered as an attractive immunization strategy to protect against infection, autoimmunity, and cancer (Manthorpe et al., 1993; Ulmer et al., 1993; Wolff et al., 1990). The *LacZ* gene encoding β-galactosidase (β-gal) has been used as a reporter for long-term imaging in gene therapy experiments (Boland et al., 2000; Cho et al., 2002; Min et al., 2002). DNA vaccine injected into a target site and internalized by antigen-presenting cells (APCs) without being degraded can elicit a strong immune response in the host (Son et al., 2016a). The fate of injected DNA vaccines can be monitored by examining their distribution or expression in the host (Donnelly, Berry & Ulmer. 2003). For instance, DNA vaccines using *LacZ* injected via various routes has been detected and shown to persist at the injection site as well as in other organs. Biodistribution is primarily investigated by in situ hybridization, immunohistochemistry, and reverse transcription (RT)-PCR, which require that experimental animals be sacrificed and their organs isolated to confirm gene expression levels. In vivo imaging is required in order to analyze gene expression following DNA vaccination in living animals.

Vaccination with the human sodium/iodide symporter (hNIS) gene combined with in vivo monitoring has dual benefits—i.e., targeted immunotherapy against NIS-expressing cancer cells and the ability to evaluate vaccine efficacy by scintigraphic imaging (*Son et al., 20167; Jeon et al., 2007; Jeon et al., 2008*). ^{99m}Technetium (^{99m}Tc) emits γ-rays; hNIS labeled with ^{124}I, ^{125}I, ^{131}I, or ^{99m}Tc has allowed the visualization of various biochemical processes in the tissues of living subjects (Son et al., 2016a).

We used LacZ and NIS as reporter genes to monitor immune responses following DNA immunization. Cytoplasmic LacZ and transmembrane NIS proteins are translated and retained in cells without being secreted. Moreover, they can be loaded onto major histocopatibility complex class (MHC-)I after translation and onto MHC-II after engulfment by APCs, thereby

stimulating helper T cell (Th)1 and Th2 immune responses, and finally avoiding immune deviation (Son et al., 2016b).

Modulating the tumor microenvironment is a critical aspect of cancer immunotherapy (Kuol et al., 2017*)*. hNIS has been used to overcome the challenges posed by the complexity of the tumor microenvironment. Expression of NIS—a specialized active iodide transporter (Chungl, 2002; De La Vieja et al., 2000*)*—results in the accumulation of therapeutic radionucleotides in cancer cells (Chen et al., 2006; Mandell, Mandell & Link 1999*)*. However, the efficacy of targeting cancer cells by hNIS radioiodine gene therapy and thereby modifying antitumor immunity has not been systematically investigated in here.

Normally, intradermal (i.d.) injection (Yu et al., 1999) induces antigen-specific Th2 immune responses. However, in the present study, we performed repeated i.d. injections (Michael et al. 1999; Shedlock & Weiner, 2000) of a naked DNA vaccine consisting of plasmid DNA encoding *hNIS* and the *lacZ* gene as a marker to induce Th1 response. We monitored the distribution and persistence of gene expression and evaluated the capacity for inducing specific Th1 immune responses in the context of a Th2-dominant immune profile. We found that an anti-LacZ humoral and anti-hNIS Th1 immune responses were induced by repeated i.d. immunizations and the use of non-secreted proteins encoding genes that solve immune deviation (Son et al., 2016b).

Materials and Methods

Plasmid DNA

The hNIS-expressing vector pcDNA3.1-FL-hNIS vector (pcDNA-hNIS) expressing hNIS under the control of the cytomegalovirus (CMV) promoter and the neomycin resistance cassette from the simian virus 40 promoter was provided by Dr. S. Jhiang (Ohio State University,

Columbus, OH, USA). The pCMVβ vector expressing β-gal was purchased from Clontech (Mountain View, CA, USA). Plasmids were amplified in *Escherichia coli* DH5α cells and purified using endotoxin-free Giga Prep columns (Qiagen, Valencia, CA, USA).

Detection of enhanced green fluorescent protein plasmid (pEGFP) in CT26 cells

The day before transfection, CT26 cells grown in a 75-cm^2 flask were trypsinized, and 10% of each cell line was mixed in 18 ml of Medium 199 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS); 3 ml of this cell suspension was seeded into one well of a 6-well plate using Transfast reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 2 ml of the reagent and 1 mg of pEGFP plasmid were mixed and incubated at room temperature for 15 min. The mixture was added to the cells and 6 h later, the DNA-transfection mixture was replaced with Medium 199. After 48 h, fluorescence was detected by using flow cytometry on a FACSAria instrument (BD Biosciences, San Jose, CA, USA).

β-gal staining of transfected CT26 cells

The plasmid encoding *lacZ* was transfected as described above. Cells were then washed three times for 5 min at room temperature and then fixed in a solution of 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS; pH 7.6–7.8) for 5 min at room temperature. After rinsing with PBS, substrate solution (1 mg/ml X-gal substrate; Sigma-Aldrich, Deisenhofen, Germany) was added, followed by incubation at 37°C for 6 h. Transfection efficiency was visually confirmed.

Detection of hNIS expression in CT26 cells

The plasmid encoding NIS DNA was transfected as described above. The ability of transfected cells to concentrate ^{99m}Tc or ^{131}I was determined as previously described [14]. Briefly, CT26

cells (5×10^4) were seeded in 24-well plates and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS for 24 h. ^{125}I uptake was determined by incubating cells with 500 μl of Hank's balanced salt solution (bHBSS; Gibco, Grand Island, NY, USA) containing 3.7 kBq of carrier-free ^{125}I and 10 μM sodium iodide (NaI) at 37°C for 30 min to obtain a specific activity of 740 MBq/mmol (20 mCi/mmol). The cells were quickly washed twice with bHBSS and detached using 500 μl trypsin. Radioactivity was measured using a gamma counter (CobraII Packard; PerkinElmer, Waltham, MA, USA).

Immunization

Specific pathogen-free female BALB/c mice (6 weeks old) were obtained from SLC Japan (Hamamatsu, Japan) and were handled according to the guidelines issued by the Seoul National University Animal Research Committee. For in vivo tracking of plasmid vectors, 100 μg of pCMV-LacZ or pcDNA-hNIS resuspended in 50 μl endotoxin-free Tris-ethylenediaminetetraacetic acid buffer (Qiagen) were administered by i.d. injection into the thigh of mice using a 30-G insulin syringe (BD Biosciences, Franklin Lakes, NJ, USA). To identify tumor-protective or antigen-specific cellular immune responses, mice were immunized three times at 2-week intervals in the hind leg with *hNIS* DNA or in the dorsal skin with *lacZ* DNA. Mice were anesthetized by intraperitoneal injection of 0.3 ml of a 1:1:9 solution of rompun, (Parke-Davis, Detroit, MI, USA), ketamine (Bayer, Leverkusen, Germany), and saline (RKS solution).

PCR detection of lacZ and hNIS plasmid DNA

PCR primers were designed to amplify the *lacZ* gene in dorsal skin and *hNIS* gene in various organs, including the draining lymph nodes (dLNs), non-dLNs, spleen, muscle, liver, and heart. The forward and reverse sequences were as follows: *lacZ*, 5'-

TTCACTGGCCGTCGTTTTACAACGTCGTGA-3' and 5'-ATGTGAGCGAGTAACAACCCGTCGGATTCT-3'; and *hNIS*, 5'-AGATGAGCTGACACGGAACAG-3' and 5'-CTGGGGAAAAGTGGGAAAAAGAG-3'. Expression levels were normalized to that of β-actin (5'-CTGTGCTATCCCTGTACGCC-3' and 5'-ATGTGACAGCTCCCCACACA-3'). The 50-μl reaction contained 5 μl PCR buffer, 50 nM each dNTP, 5 nM forward and reverse primers, and 1 U Taq DNA polymerase. The PCR conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 45 s, 63°C for 45 s, and 72°C for 45 s; and72°C for 7 min. PCR products were resolved on a 2% agarose gel and visualized under ultraviolet light. PCR conditions for transgene amplification were as follows: 34 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s.

hNIS reverse transcription (RT)-PCR

Organs were removed from immunized mice and lysed using a homogenizer. Total RNA was extracted from lysates in the presence of RNase inhibitors using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA). Isolated RNA was dissolved in diethyl pyrocarbonate-treated water (Sigma-Aldrich, St. Louis, MO, USA) and used to generate cDNA using a 15-mer poly dT oligonucleotide (Invitrogen, Carlsbad, CA, USA) and Superscript reverse transcriptase (Gibco) with incubation at 37°C for 1 h according to the manufacturer's protocol. Expression of the *hNIS* gene was detected in dLNs, non-dLNs, and spleen by RT-PCR using the primers: 5'-GGCTCCTCGGTGACTCTAGGATGC-3' (forward) and 5'-CATGAATTCTGGGCTCAATTTTCTTGTCC-3' (reverse). To confirm DNA integrity, the mouse β-actin gene (codons 135–223) was amplified with the primers 5'-GGCTCCTCGGTGACTCTAGGATGC-3' (forward) and 5'-CATGAATTCTGGGCTCAATTTTCTTGTCC-3' (reverse) under the following conditions: 34 cycles of 94°C at 60 s, 55°C at 60 s, and 72°C at 60 s.

Whole-body imaging and nuclear scintigraphy of hNIS DNA-immunized mice

At designated times (2, 16, and 24 h and 2, 3, and 11 days) following injection of pcDNA-hNIS, mice were administered 300 μCi of ^{99m}Tc (11.1 MBq) by intraperitoneal injection and anesthetized with 0.3 ml of RKS solution; 30 min later, mice were placed in a prostrate position and scanned with a gamma camera (ON 410; Ohio Nuclear, Solon, OH, USA) equipped with a pinhole collimator. Relative radioactivity was assessed in the entire body over a period of 5 min, and dynamic frames were obtained; 1 h later, LNs, spleen, liver, and skin near the injection site were removed from each mouse and weighed to determine the organ distribution patterns of injected DNA; blood samples were also collected. Tissues were stored at −70°C for 16 h, after which ^{99m}Tc uptake was measured using a gamma counter.

Antibody measurements by enzyme-linked immunosorbent assay (ELISA)

Antibody titers in sera obtained from mice at the end of the experiment were determined by ELISA. Briefly, 96-well microtiter plates were coated overnight with 1 mg β-galatosidase (5 mg/ml) in 0.1 M carbonate buffer (pH 9.5) for detection of anti-β-gal antibodies. To determine anti-NIS antibody titer, 96-well microtiter plates were coated with 1×10^4 irradiated CT26 or CT26/NIS cells in 0.01 M PBS (pH 7.5). After washing with wash buffer (PBS with 0.05% Tween-20 [pH 7.4]), the plates were blocked overnight with assay diluent (BD Pharmingen, San Diego, CA, USA). After washing with wash buffer, eight consecutive 1:3 dilutions of serum sample in assay diluent initially diluted 1:20, 1:40, and 1:100 (for detection of anti-β-gal antibodies) were added to the wells. After 2 h of incubation at room temperature, the plates were washed with wash buffer and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig; Southern Biotechnology Associated, Birmingham, AL, USA). HRP-conjugated goat anti-mouse IgG1 or IgG2a

(Southern Biotechnology Associated) were used to determine the isotype of the antibodies. After washing, 100 µl of substrate solution (tetramethylbenzidine and hydrogen peroxide; BD Pharmingen) were added, and the plate was incubated in the dark for 30 min at room temperature. The reaction was terminated by adding 1 M H_2SO_4 and absorbance was measured at 450 nm on an ELISA plate reader. To calculate anti-NIS and total antibodies titers, purified mouse IgG2a or IgG1 monoclonal antibodies were included in the plates.

Enzyme-linked immunospot (ELISpot) assay

Th1 interferon (IFN)-γ or Th2 interleukin (IL)-4 secretion by stimulated T cells were evaluated with commercially available ELISpot assay kits (Diaclone, Besançon, France) according to the manufacturer's protocol. Briefly, PVDF polyvinylidene difluoride 96-well plates were incubated overnight at 4°C with an anti-mouse IFN-γ or -IL-4 (capture) antibody. The following day, freshly isolated NIS-immunized LN cells (5×10^5 responder cells/well) were washed and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal calf serum and then incubated in anti-INF-γ or -IL-4 antibody-pre-coated 96-well plates at 37°C for 20 h. The cells were removed, and biotinylated anti-mouse IFN-γ or IL-4 (detection) antibodies were added followed by streptavidin-conjugated alkaline phosphatase, which converted the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium to a blue dye. Spots were counted using the Bioreader system (BIO-SYS GmbH, Karben, Germany).

Cytokine ELISA

LNs were removed and single-cell suspensions were obtained by gentle pipetting. Lymphocytes were washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 200 mg/ml streptomycin, 200 U/ml penicillin, and

0.1% 2-mercaptoethanol. Cells were seeded in 24-well tissue culture plates at a final concentration of 2×10^6/ml in enriched RPMI 1640 medium, and stimulated by co-culturing with irradiated CT26 or CT26/NIS cells for 72 h at 37°C in an atmosphere of 5% CO_2. IL-4 and INF-γ levels in culture supernatants of LN cells stimulated with irradiated CT26 or CT26/NIS cells were measured using OptEIA mouse IFN-γ or IL-4 ELISA kits (BD Pharmingen) according to the manufacturer's instructions.

Tumor challenge

At 2 weeks after the final hNIS DNA injection, mice were challenged by subcutaneous injection into the fore leg of 5×10^5 (left) or 1×10^5 (right) CT26/NIS cells resuspended in 100 μl of 10% FBS in DMEM. Tumor dimensions were measured twice a week, and tumor volume was calculated as horizontal length (mm) × vertical length (mm) × depth (mm) = volume (in mm^3).

In vivo tumor imaging

At indicated times after injection of pcDNA NIS into CT26/NIS tumors, 37 MBq of ^{99m}Tc was injected into the tail of mice and static SPECT images were acquired after 10 min using a gamma camera (GE Healthcare, Waukesha, WI, USA) with a low-energy, high-resolution collimator.

Biodistribution study

Mice were sacrificed at the end of the single-photon emission computed tomography (SPECT) scan (90-min time point). Blood, heart, liver, spleen, lung, kidneys, thyroid, stomach, intestine, muscle, bone, and tumor were harvested and weighed. Radioactivity was measured with a gamma counter and the corresponding counts per million/mg tissue was calculated.

Results

Efficiency of plasmid DNA expression

We evaluated the transfection efficiency of each plasmid into CT26 murine colon cancer cells (H-2d; syngeneic in BALB/c mouse strain). EGFP expression was detected by flow cytometry (Fig. 1A). *LacZ* in CT26 cells was visible as a green color (Fig. 1B). The transfection efficiency was confirmed with pcDNA3.1-hNIS by detecting ^{99m}Tc with a gamma counter (Fig. 1C). These results demonstrate that the plasmid vectors are able to transfect cells.

β-gal is expressed in mouse tissues following pCMV-LacZ inoculation

X-gal is an organic compound composed of galactose linked to indole that is commonly used to test for the presence of β-gal according to a color change to blue/green. We monitored β-gal expression in mouse skin following i.d. injection of pCMV-LacZ by X-gal staining. Although there was no spread of the blue dye beyond the injection site (Fig. 2A, left panel), the green color corresponding to X-gal was detected far away from the injection site from 2 h to 30 days post-injection (Fig. 2A, right panel), suggesting that the plasmid encoding the *lacZ* gene was taken up by resident cells (most likely keratinocytes or APCs) that then migrated out of the injection site. We next evaluated the levels of pCMV-LacZ DNA injected into the dorsal skin, which was divided into three sites (Fig. 2B, upper): sections 1, 2, and 3 represent the upper, middle, and lower sites of injection, respectively. The PCR analysis revealed that DNA had moved from the injection site throughout the body up until 10 days after injection (Fig. 2B, lower). *LacZ* DNA was still detected at the injection site at 15 and 30 days.

hNIS gene is expressed in mouse tissues following DNA inoculation

Our data suggested that cells transfected with plasmid DNA and injected into mice could

migrate away from the injection site. We therefore evaluated the expression of *hNIS* DNA in various immune tissues of mice (LN and spleen) and other organs (muscle, heart, and liver) following i.d. injection. hNIS DNA was expressed in lymph nodes as early as 2 h post-injection and in the spleen after 3 days, with expression persisting up to day 18; at 2 h, hNIS DNA was detected in muscle tissues at the injection site (Fig. 3A). An RT-PCR analysis showed that hNIS was expressed as early as 2 h post-injection in the LN and after 1 day in the spleen, with expression persisting up to 18 days; in contrast, mock transfectants showed no *hNIS* expression (Fig. 3B).

To monitor NIS expression, we dissected the organs and detected γ-rays with a gamma counter. At 2 h post-injection, γ-ray levels were highest at the injection site (skin) relative to the mock-injected group (Fig. 4A). Up to 11 days after DNA injection, ^{99m}Tc uptake was highest in lingual LNs (dLN), followed by the LN (non-dLN) and spleen (Fig. 4B, C). To quantify the level of radioactivity in vivo, regions of interest in the scintigraphic images were analyzed at various time points (2, 8, 16, and 24 h and 11 days) after immunization. Whole-body relative radioactivity levels adjacent to the injection site were higher in mice immunized with hNIS plasmid as compared to mock-injected control mice (Fig. 4D). As expected, hNIS gene expression was detected in the LN and spleen for up to 11 days.

Humoral and cellular responses induced by repeated i.d. injections of *lacZ* and *hNIS* DNA

Determining the mechanism by which DNA vaccines stimulate the immune response is critical for identifying the type of immune response that is induced [19, 20]. While i.d. delivery will firstly elicit the humoral response with the release of IgA and IgG1, the intramuscular route has been shown to prime cellular responses by activation of cytotoxic T lymphocytes (CTLs) and production of IgG2a. We therefore evaluated antibody responses against *lacZ*. Anti β-gal antibodies were generated at 10 days, with the levels reaching a peak at 15 days post-

immunization (Fig. 5A). The antibody subclass was assessed by ELISA by coating the wells with NIS-expressing CT26 cells and adding serum from mice immunized with pcDNA or hNIS DNA. The results show that the anti-NIS antibodies generated were of the IgG2a (Th1) isotype (Fig. 5B).

hNIS DNA vaccination induces a strong Th1 cytokine profile

The ELISpot assay was used to evaluate the secretion of IFN-γ and IL-4 by cells isolated from dLNs, non-dLNs, mesenteric (M) LNs and spleen after NIS DNA immunization. To assess hNIS-specific T cell responses, splenocytes and LN cells were isolated 10 days after the final injection. IFN-γ was secreted at a high level by cells from the dLN and to a lesser extent by MLN and non-dLN cells; the spleen also showed a specific anti-NIS IFN-γ response (Fig. 6A). IL-4 was not detected. These data were confirmed by in vitro stimulation of spleen, dLN, and non-dLN cells with irradiated CT26/NIS cells and measurement of cytokine secretion by ELISA. Similar to the results of the ELISpot assay, high levels of IFN-γ were secreted by cells of dLNs relative to non-dLN and spleen, with no IL-4 secretion (Fig. 6B).

Protective tumor immunity induced by DNA immunization

We investigated whether immunization of mice with hNIS DNA conferred protection against tumors expressing NIS. Mice were subcutaneously inoculated with CT26/NIS tumor cells 2 weeks after the final *hNIS* DNA injection (Fig. 7A). Mice immunized with *hNIS* DNA showed significant retardation of tumor growth relative to those injected with pcDNA3 (Fig. 7B). Evaluation of tumor mass by ^{99m}Tc uptake measurements for up to 33 days confirmed these observations.

Discussion

Plasmid DNA injection can lead to transgene expression in vivo (Wolff et al., 1990; Chen et al.,, 2013; Han et al., 2015). However, immune responses induced by DNA vaccines have not been extensively, although they are thought to be similar to the viral infection process. Immediately after injection, plasmid DNA enters cells—mainly keratinocytes and APCs (Tonheim, Bogwald & Dalmo, 2008)—and is transported to the nucleus. The transgene is transcribed and then translated into a protein that is presented by MHC-I or -II to the host immune system. The protein may then be engulfed and degraded, while transgene peptide-loaded APCs can migrate to dLNs to activate naïve T cells (Son et al., 2016).

In this study, we found that the transgene spread far from the site of injection following in vivo inoculation and was detected up to 30 days post-inoculation. The transgene-expressing cells migrated primarily into immune organs such as dLNs, suggesting that the cells are APCs that home to immune organs to activate an inflammatory signal, or else keratinocytes that are targets for the immune response. In the LNs, Th cells activated by transgene-expressing APCs secrete cytokines that can activate B cells to induce an anti-β-gal humoral response and the production of IgG2a anti-NIS antibodies by isotype switching (Boland et al., 2000). Antigen-binding B cells are trapped in the T cell-rich zone of dLNs and are activated by encounters with activated Th cells. Antibody isotype switching is stimulated by multiple cytokines. IL-4 induces a switch to IgG1 and IgE, whereas transforming growth factor (TGF)-β causes a switch to IgG2b and IgA. Th2 cells secrete IL-4 and -5 and TGF-β, which induce IgA. Although Th1 cells are poor initiators of antibody responses, they release IFN-γ for antibody isotype switching to IgG2a and IgG3 (Janeway et al., 2001). In our study, we confirmed IgG2a switching after DNA vaccination, which corresponded to an increase in IFN-γ levels. It is possible that antibody isotype switching ultimately leads to tumor killing by the Th1 response, which activates cytotoxic T cells. Soluble or secreted vaccine antigens may be phagocytosed

by APCs immediately following DNA vaccination and enter the MHC class II exogenous pathway. We therefore used a cellular (LacZ) or transmembrane (NIS) protein to exclude direct APC uptake immediately after translation that could deviate into Th2-type immune responses. We also administered two booster injections to re-activate cellular immunity. The immune response to the first immunization was predominantly humoral; however, memory T cells were subsequently recruited to the injection site, which stimulated a Th1-type immune response (Son et al., 2016b). Repeated immunization (boosting) involves dendritic cells (DCs), but it is unclear how frequently booster injections should be administered. In fact, a homologous prime-boost strategy may not be ideal, as antigen-bearing DCs were shown to be eliminated by effector and memory CTLs in vaccinated mice (Yang et al., 2006).

$CD8^+$ T cells can lyse cells presenting transgenic peptide on MHC-I molecules, resulting in increased antigen release. Furthermore, $CD4^+$ T cells (Th2 response) can activate immature DCs that home to LNs and stimulate B and T cells (Reyes-Sandoval and Ertl, 2001*)*, thereby repeating the cycle of activation. Thus, both humoral and cellular immunity are primed for the next challenge although $CD8^+$ T cells must be induced to lyse tumor cells. It was previously shown that i.d. injection can result in a Th2-type profile (Shedlock and Weiner, 2000), but in this study we observed that humoral immunity could be induced to switch to Th1-type immunity (IgG2a). Furthermore, IFN-γ was significantly induced in the transgene-immunized group relative to controls, which protected the mice from tumor challenge. The presumed mechanism is shown in Figure 8.

Although DNA vaccines can induce cellular responses, the injection route is an important determinant of the type of response. A Th2 response is typically induced by i.d. injection of DNA (*Shedlock & Weiner, 2000*), posing a challenge for the widespread use of DNA vaccines. In this study, we solved this problem by inhibiting the Th2 response through IgG1 to IgG2a switching. We selected an intracellular antigen that can be loaded onto MHC-I

to activate the Th1 response and administered repeated, which could induce memory T cells and can easily stimulate a Th1-type response. Additional studies are needed to determine whether memory T cells are upregulated after repeated i.d. injection of plasmid DNA.

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Competing Interests

The authors declare there are no competing interests.

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Figure legends

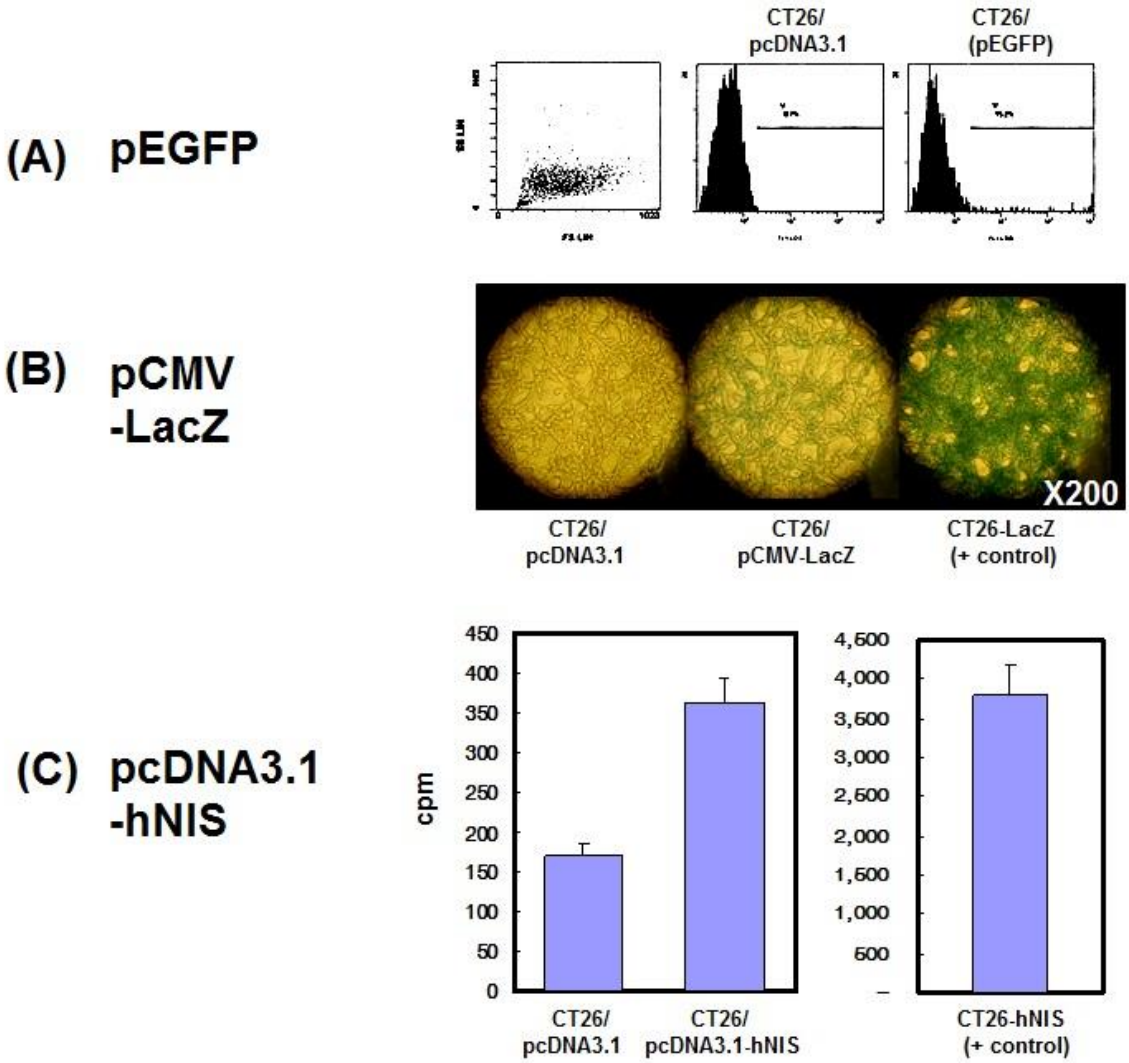


Figure 1. In vitro expression of various plasmid DNAs in CT26 cells. (A) Transfection efficiency and eGFP expression were evaluated by flow cytometry 48 h after transfection. (B) X-gal staining was performed following pCMV-LacZ transfection. (C) ^{99m}Tc uptake was measured using a gamma counter 48 h after transfection of pcDNA-hNIS into the cells. Three independent experiments were performed with one representative example shown.

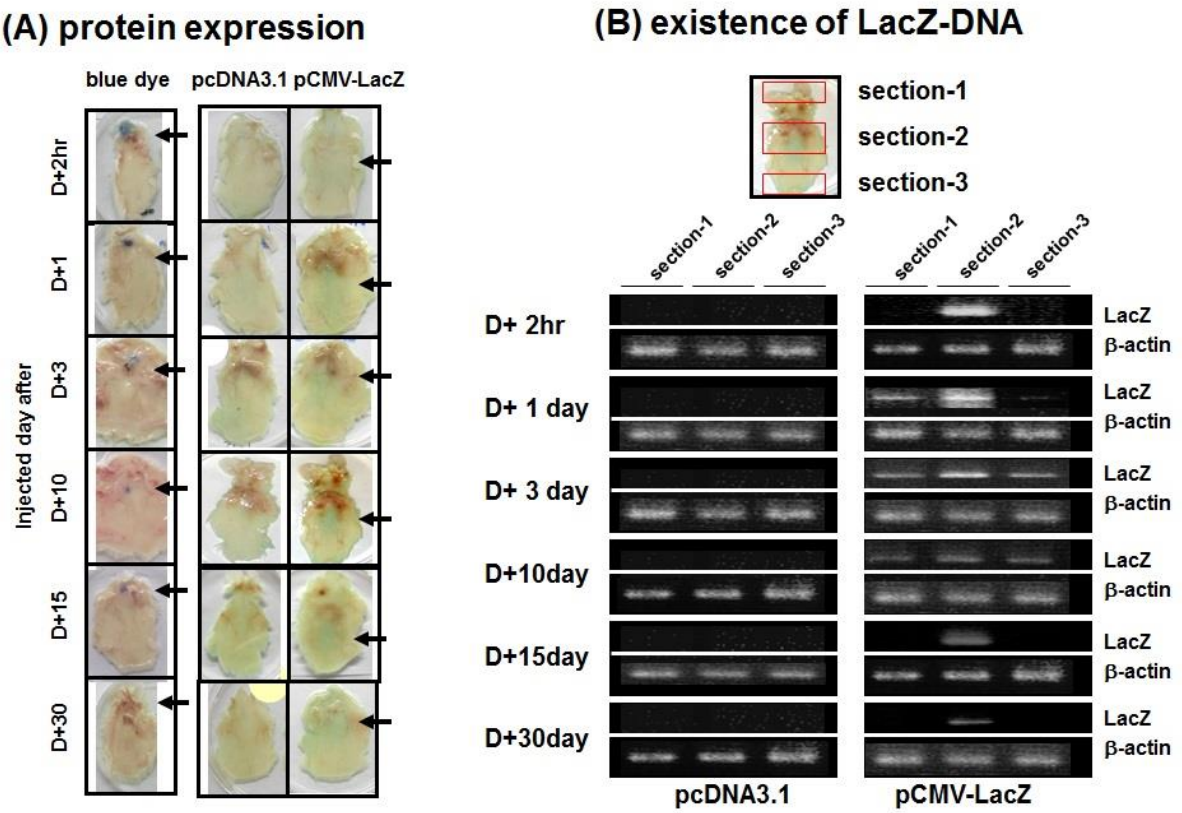
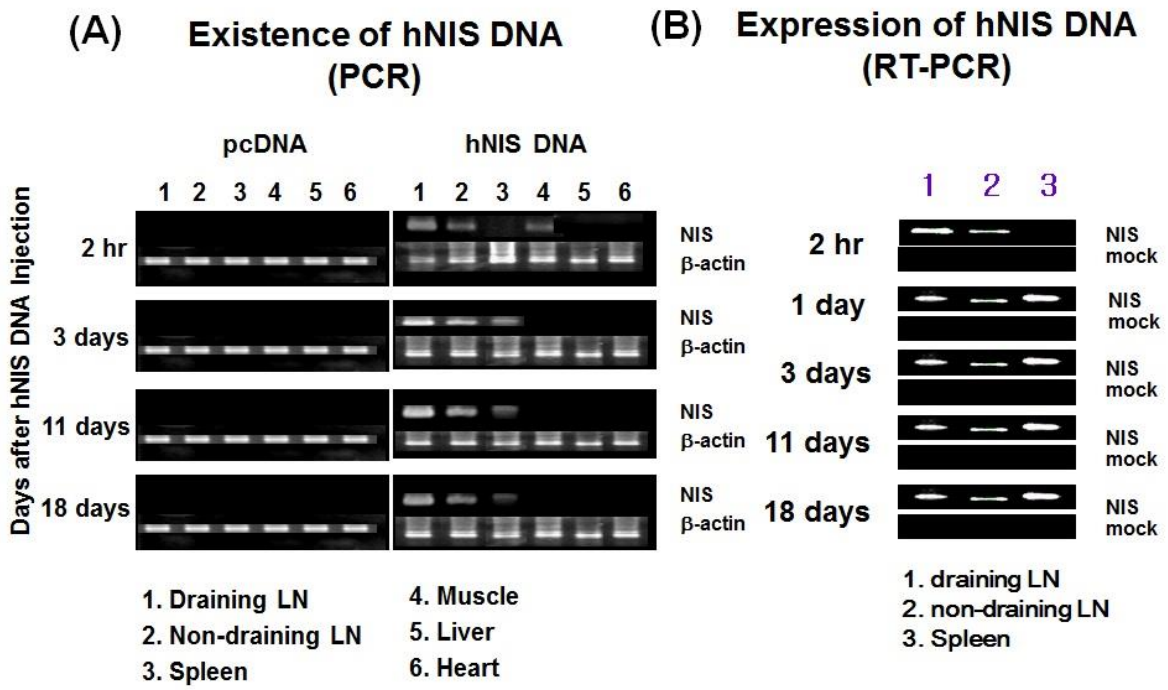
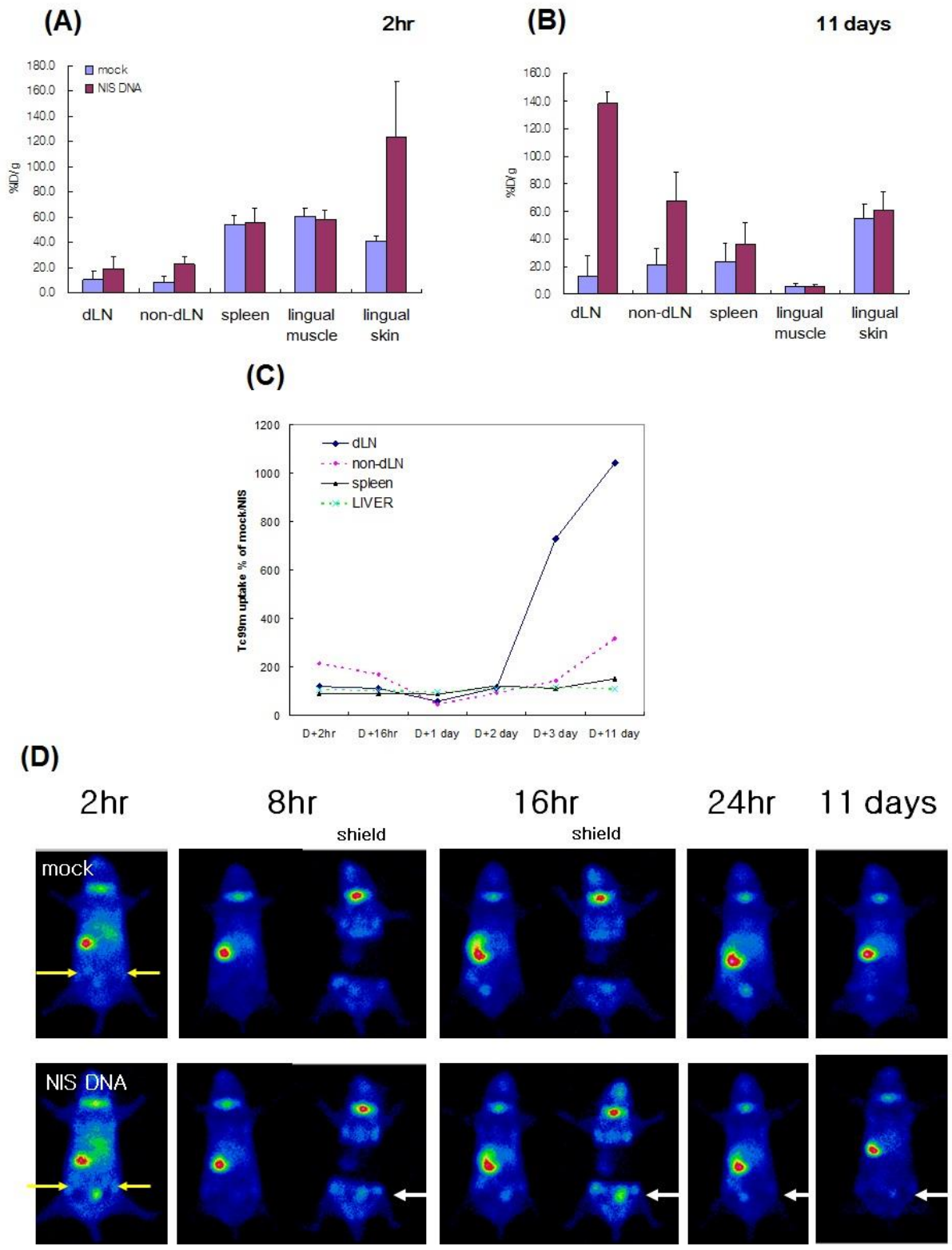


Figure 2. In vivo X-gal staining and detection of *lacZ* DNA uptake by cells. Mice received i.d. injection of 100 μg pCMV-LacZ DNA and in vivo plasmid DNA trafficking was monitored. (A) LacZ protein was detected by X-gal staining at the indicated times. (B) RT-PCR was used to monitor the movement of *lacZ* DNA taken up by cells. The whole dorsal skin was divided into three sections (1, 2, and 3: upper, middle [including injection site], and lower sections, respectively).



499

500 **Figure 3**. **In vivo expression of *hNIS* DNA following injection**. (A) The movement of hNIS-
501 expressing cells and transgene expression was monitored by RT-PCR analysis dLNs, non-dLNs,
502 spleen, lingual muscle, liver, and heart following *hNIS* DNA injection at the indicated time. (B)
503 RT-PCR in immune organs (dLNs, non-dLNs, and spleen) at various time points.



504

505 **Figure 4. Trafficking of plasmid DNA**. γ-Rays were detected using a gamma counter (A) 2 h,

506 (B) 11 days, and (C) 0–11 days after hNIS DNA injection into mice. Immune organs (LNs and

507 spleen), lingual muscle, and lingual skin near the injection site were monitored. (D) Whole-

508 body imaging of immunized mice with nuclear scintigraphy.

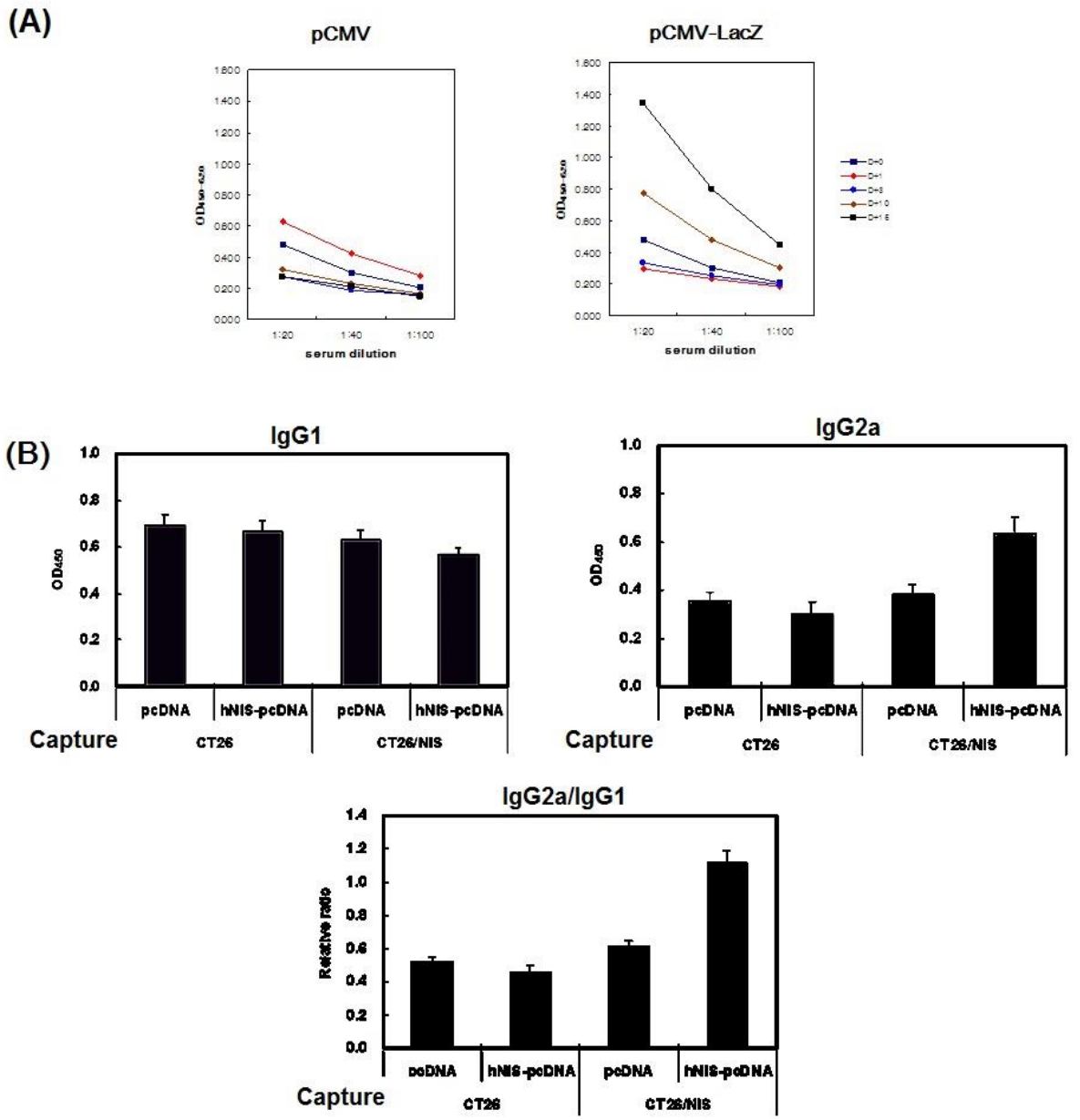


Figure 5. Humoral immunity after DNA vaccination. (A) Serum antibody specific for β-gal. Serum samples were diluted 1/20, 1/40, and 1/100. (B) IgG1 and IgG2a titers against NIS in the sera of *hNIS* DNA-immunized mice. At 10 days after final injection of *hNIS* DNA (6 weeks after the first immunization), serum samples were collected and anti-NIS IgG1 and IgG2a titers were determined by ELISA. IgG1 or IgG2a levels in the serum of pCDNA-NIS- or pCDNA-injected mice were compared to those in the sera (1/100 dilution).

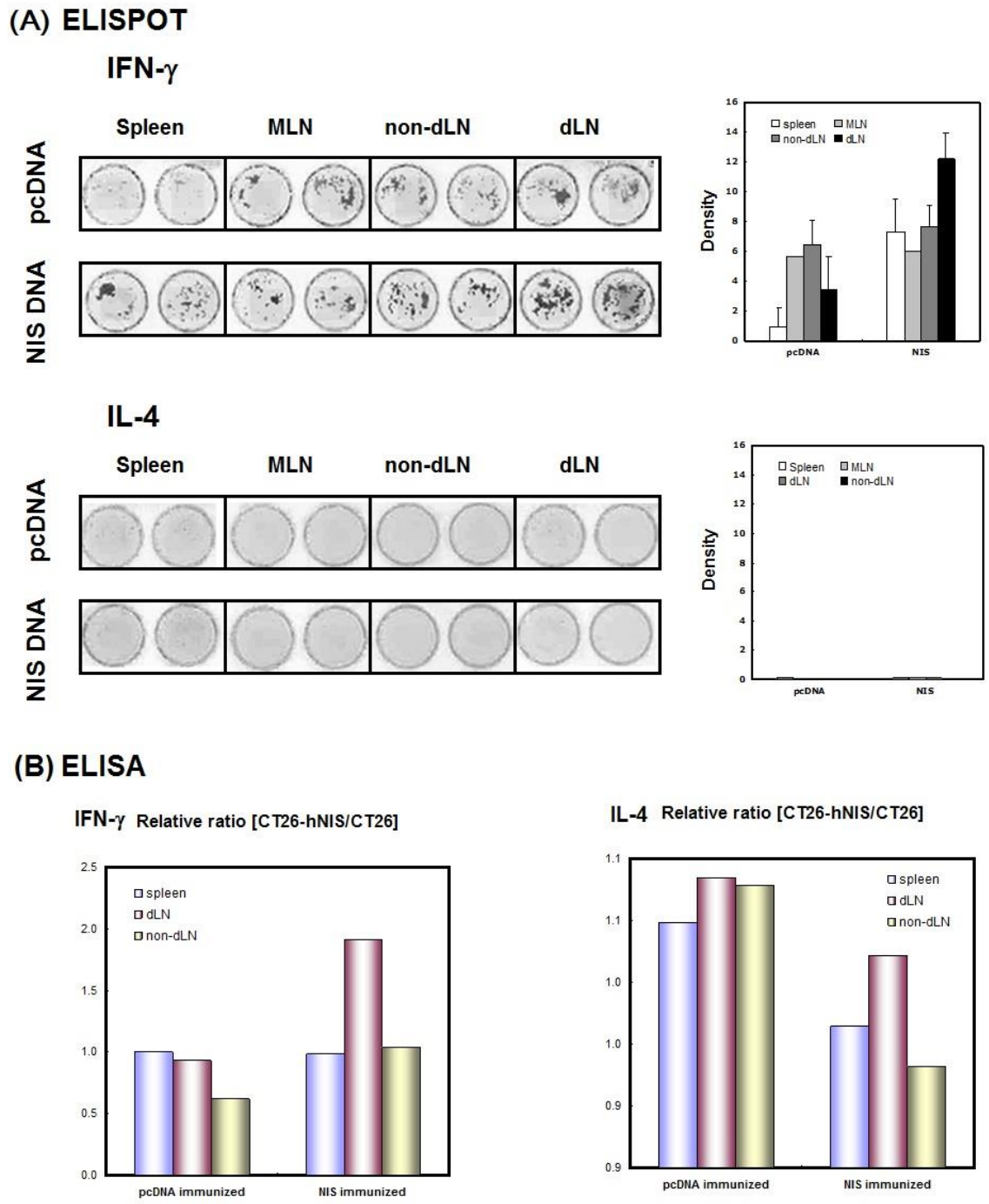
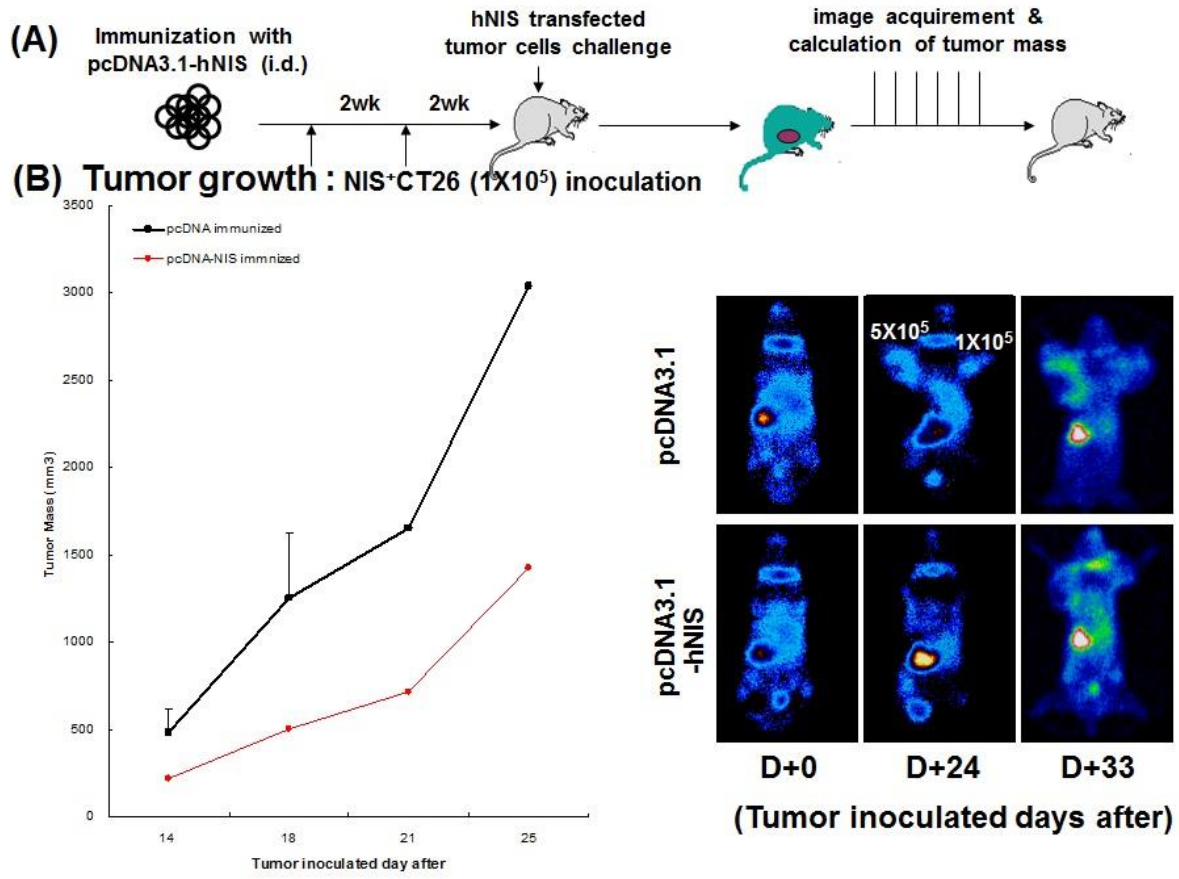
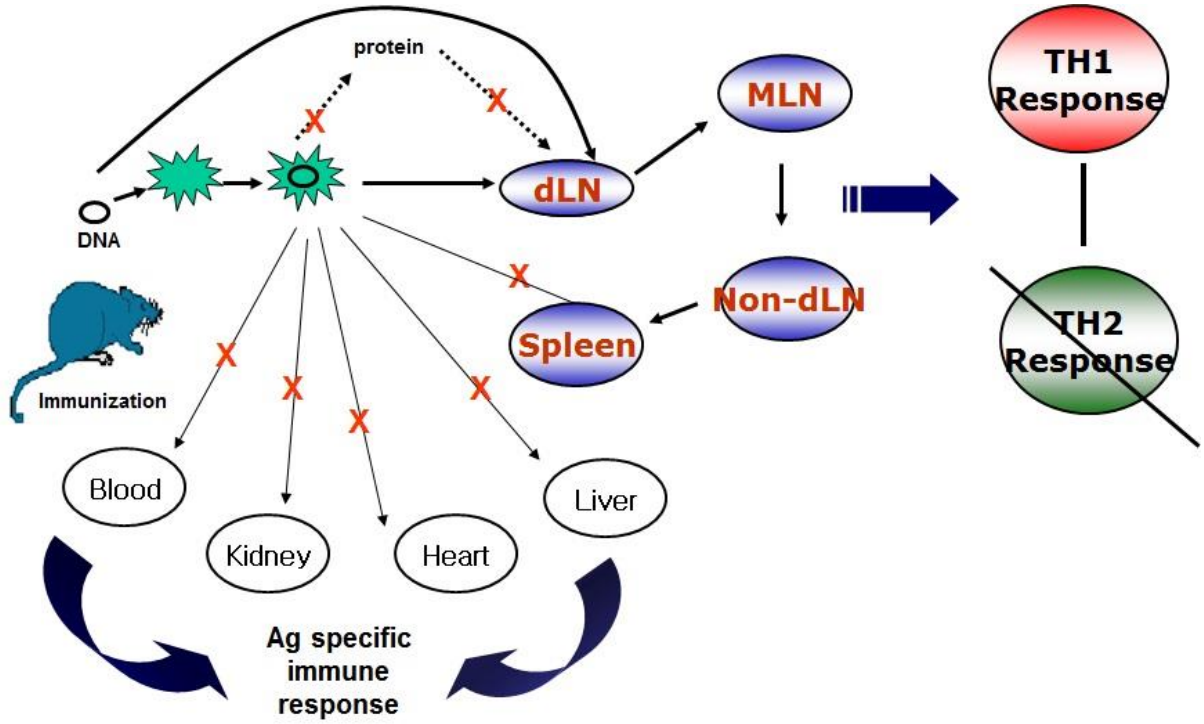


Figure 6. Cellular immunity following DNA vaccination. (A) ELISpot detection of IFN-γ- or IL-4-expressing cells. LNs were collected from *hNIS* DNA-immunized mice and immune cells were isolated with a needle. Cells were cultured for 48 h and IFN-γ or IL-4 levels in the supernatant were determined. (B) Cytokine ELISA detection of IFN-γ or IL-4 in the supernatant of immune cell cultures; the cells were re-activated with irradiated CT26-NIS cells for 48 h before IFN-γ or IL-4 levels in the supernatant were detected.



523

524 **Figure 7. Decrease in tumor growth rate by *hNIS* DNA immunization**. (A) Tumor
525 inoculation scheme. (B) Tumor retardation after NIS immunization. Left panel shows tumor
526 growth following inoculation of the fore leg with 1×10^5 CT26/NIS cells; right panel shows
527 the tumor mass after ^{99m}Tc injection into NIS-immunized mice. Left: 5×10^5; right: 1×10^5.



528

529 **Figure 8. Summary of DNA immunization**. Schematic representation of the mechanism by

530 which DNA immunization results in DNA uptake and cell migration.