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Mannosylated T/Tn with Freund's adjuvant induces cellular immunity

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Abstract

Inducing cancer-specific cellular immune responses has become an attractive strategy in cancer treatment. In this study, we investigated the role of several adjuvants in eliciting T/Tn-specific cellular immunity and protection against T/Tn expressing tumor challenge. T/Tn (9:1) antigen was purified from blood type "O" erythrocytes donated from healthy Korean volunteers. Immunization was performed using: T/Tn only, T/Tn mixed with Freund's adjuvant (T/Tn + FA), keyhole limpet hemocyanin (KLH)-conjugated T/Tn mixed with FA (KLH-T/Tn + FA), and oxidized mannan-conjugated T/Tn mixed with FA (ox-M-T/Tn + FA). Mice immunized with ox-M-T/Tn + FA generated T/Tn-specific CD3, helper T (Th) cells, major histocompatibility complex (MHC) II, and MHC I; T/Tn presentation was significantly high and tolerogenic $CD11b^+$ was the lowest among the tumor models. To verify Th type, we stained intracellular cytokines (interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, and IL-10) using CD3 co-staining. Th1 (IFN-γ and GM-CSF) cytokines were highly expressed and showed high FasL/Fas ratios, cytotoxic T lymphocyte (CTL) activity, and cytotoxic T lymphocyte precursor (CTLp) activity in mice immunized with ox-M-T/Tn + FA. Lymphocyte infiltration was highest in mice immunized with ox-M-T/Tn + FA. Additionally, we monitored FasL, MHC I, CD301, and T/Tn expression levels using immunohistochemistry (IHC) on macrophage and tumor sites. The expression of all markers was highest in the ox-M-T/Tn + FA group. Furthermore, tumor retardation and survival rate were highest in the ox-M-T/Tn + FA group. These results demonstrate that a vaccine formulation of T/Tn conjugated with ox-M and mixed with FA-induced cellular immunity and sustained a humoral immune response without over-activating the immune system, thus effectively inhibiting tumor growth.

Keywords

CTL, mannosylated T/Tn, T/Tn-specific cellular immunity

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Introduction

Immunoreactive T (Thomsen-Friedenreich) and Tn antigens are immediate precursors of human blood group MN- and O-glycosidic-linked carbohydrates. T and Tn antigens are nearly always found on human carcinomas (CAs), whereas they are blocked in healthy or non-cancer-diseased tissues.[1–3] Attachments of mucins to truncated carbohydrate side chains can prove the role of mucins in cancer mechanisms and recognition by the immune system.[4] There is incomplete glycosylation on cancer cells, which results in new carbohydrate and peptide backbone epitopes not normally present on normal cells.[3,5] Thus, these new cancer-associated

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antigenic epitopes are distinguishable from normal epitopes in antibody detection.[5,6]

T, Tn, and sTn antigens were examined in 72 consecutive primary breast CAs by immunohistochemistry using well-defined monoclonal antibodies, and their semiquantitative values were correlated with established clinicopathologic prognostic parameters of the disease to determine their relationship with long-term clinical outcome.[7] Likewise, high expression of T, Tn, and sTn antigens are noted on human malignant hematopoietic cells.[8] In a long-term human clinical trial spanning 19 years, using T/Tn vaccine intradermally in patients with advanced breast cancer was proven to be effective with regards to long-term survival.[8] Of the 19 vaccinated patients, all survived after 5 years, 11 survived between 10 and 18 years, 5 were alive but did not reach 10 years, 3 died before reaching 10 years, and 3 were disease-free. Delayed-type hypersensitivity (DTH) reactions were induced with significant inflammation, increased T helper cells, and decreased T regulatory/cytotoxic cell ratio;[8] hence, targeting T/Tn antigen is a promising strategy for cancer immunotherapy studies. In phase I/II human clinical trials, patients with advanced adenocarcinoma immunized with MUC1 immunogens showed induction of humoral and cellular immunity.[9] In one particular study, 30% of patients produced cellular immunity and 50% unexpectedly generated strong IgG1 antibody responses.[10] Numerous approaches to stimulate immunity against antigens have emerged in the last decade, such as nanoparticles, virus-like particles, dendrimers, viral vectors, DNA delivery, cell-penetrating peptides, bacterial toxins, and targeting cell-surface receptors on dendritic cells.[11] Dendritic cells loaded with tumor peptides can induce immune responses, preventing the growth of tumors in mice;[12,13] such an approach rapidly moved into human clinical trials with strong immune response induction.[14] These approaches aim to stimulate antigen-specific T cell responses and show promising effects; however, the efficacy of future immunotherapeutic treatments should rely on the stimulation of both humoral and cellular anti-tumor immune responses.[15]

In recent years, there has been increased interest in targeting T/Tn antigens for cancer therapy, and in particular, porphyrin conjugated to the plant lectin Moringa G, which recognizes T/Tn, was shown to be toxic to leukemia (Tn-positive) and healthy (Tn-negative) cells.[16] In addition, conjugates of tetanus toxoid with synthetic glycopeptide tandem-repeat sequences of MUC1 and T antigen induce strong antibody responses that recognize the MCF-7 breast cancer cell line;[17] the inclusion of 1–5 tandem-repeat domains induces antibodies with high selectivity for human breast cancer tumor tissues.[18] Furthermore, we demonstrated novel synthesis of self-assembling and self-adjuvanting glycopeptide vaccines containing MUC1 tandem-repeat glycopeptide-containing T antigen, the universal helper peptide epitope PADRE, and the adjuvant Pam_2Cys. In mice, the vaccine was able to induce robust cytotoxic T lymphocyte (CTL) responses that recognized a MUC1-positive cancer cell line.[19,20]

We previously showed that carbohydrate polymer—mannan (mannose complex)—in its oxidized form (comprising aldehydes and Schiff bases), when conjugated to the tumor-associated antigen MUC1, induces Th1 cytokines by macrophages, dendritic cells, and CTLs and protects mice against a MUC1+ tumor challenge.[21–27] Interestingly, oxidized mannan binds to toll-like receptor 4, inducing activation and maturation of dendritic cells.[28] In rhesus macaques, immunization with oxidized mannan-MUC1 induced both humoral and cellular immune responses.[29] In humans, MUC1 conjugated to oxidized mannan induces specific MUC1 antibody and T cell responses[25,30,31] and long-term protection against breast cancer recurrence.[32–34] In addition, ex vivo–generated dendritic cells pulsing with oxidized mannan-MUC1 lead to tumor protection in mice and immune response induction and clinical responses in patients with adenocarcinoma.[35,36] Furthermore, in bovine models, interleukin (IL)-1, IL-6, IL-12, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), Inducible nitric oxide synthase (iNOS), and tumor necrosis factor alpha (TNF-α) were enhanced by monocyte-derived macrophages after stimulation with oxidized mannan.[27] It is clear that oxidized mannan has the ability to induce cellular (and humoral) immunity in a number of models. Moreover, weakly immunogenic antigens or peptides conjugated to keyhole limpet hemocyanin (KLH) are able to convert the peptide to being highly immunogenic, partly due to its strong immunogenicity and its numerous available lysines. In addition, KLH can act as a linker between the peptide and a

carrier. Indeed, we have demonstrated that peptides from myelin basic protein conjugated to KLH and then to reduced mannan formed a strong conjugate between the peptide and the carrier (reduced mannan) and induced immune responses in mice; without KLH, no conjugation is noted.[37,38] In addition, mixing peptide conjugates with adjuvants (such as Freund's adjuvant (FA)) has been shown to further increase their immunogenicity, particularly for induction of antibody responses. Based on this information, we determined the roles of immune activators that could elicit effective cellular and Th1 immune responses for a T/Tn vaccine by evasion of immune deviation. We previously demonstrated that oxidized mannan conjugated to glycoproteins bearing T/Tn and mixed with incomplete FA-induced anti-T/Tn cellular immune responses that significantly inhibited tumor growth in mice.[39] In contrast, KLH-conjugated T/Tn immunogen mixed with FA could not inhibit T/Tn expressing tumor growth and merely induced T/Tn-specific cellular immunity. In this study, we compared several adjuvants in order to elicit robust T/Tn-specific cellular immunity. We used glycoproteins bearing T/Tn carbohydrates (T/Tn).

Materials and methods

Human O red blood cell–derived T antigen

T/Tn antigen was prepared from blood group O red blood cells (RBCs) as previously described.[1–3,40] Briefly, the MN glycoprotein was extracted from blood group O RBC stroma at room temperature with 45% aqueous phenol plus electrolytes and purified by fractional centrifugation and ethanol fractionation.[41] T epitopes were uncovered on the MN glycoprotein by specific removal of *N*-Acetylneuraminic acid using *Vibrio cholerae* neuraminidase.[42,43] The physical, chemical, and biological characteristics of the T antigens were reported previously.[44]

Expression of T/Tn antigen on tumor cell lines

The anti-T/Tn antibody, the rat monoclonal ascites anti-T Ca3114 (IgM) antibody, donated from Dr GF Springer's laboratory of the Chicago Medical School (North Chicago, IL, USA) was used to detect T/Tn in murine cell lines.[45] Rat ascitic monoclonal anti-T (Ca3114) was also reactive with ovarian and breast cancer cells. Cultures of 5×10^5 cells from murine tumor cell lines (CTLL-2I, SP2/0, Raw264.7, and TA3HA) were incubated with anti-T/Tn antibody for 30 min at 4°C; isotype-matched antibodies were used as a negative control. After washing, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Igs (PharMingen, San Diego, CA, USA) for 30 min at 4°C. Cells were fixed with 2% paraformaldehyde-phosphate-buffered saline (PBS) (PFA-PBS)[46] until FACScan analysis.

Conjugation of KLH or mannan to T/Tn

The conjugation of KLH to T/Tn carbohydrates was conducted using Imject Immunogen EDC Conjugation Kits (Thermo Fisher Scientific, Waltham, MA, USA). The conjugate was purified by gel filtration using the columns provided. The purified conjugate was collected, and conjugation was confirmed by absorbance at 280 nm.[43] Methods of conjugation of mannan to antigens have previously been reported,[22,23,26] and a similar method was used. Briefly, mannan (Sigma-Aldrich, St. Louis, MO, USA) was oxidized to poly-aldehydes by treating 14 mg mannan in 1 mL 0.1 M phosphate buffer (pH 6.0) with the addition of 100 μL 0.1 M sodium periodate in phosphate buffer for 1 h at 4°C to enable oxidation. Ethanediol (10 μL) was added to the mixture and incubated for a further 30 min at 4°C, after which the entire mixture was passed through a PD-10 column (Sephadex G-25 M column; Pharmacia Biotech, Uppsala, Sweden) and equilibrated in 0.1 M bicarbonate buffer (pH 9.0), and the oxidized mannan fraction was collected. T/Tn (180 μg) was added to oxidized mannan and allowed to conjugate overnight at room temperature. For gel electrophoresis and western blot analysis, samples to be tested were mixed with or without sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and loaded onto 5% SDS or native gels. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were subjected to periodic acid-Schiff base (PAS; carbohydrate) staining, Coomassie (protein) staining, or western blot analysis.

PAS staining

After SDS-PAGE or native gel electrophoresis, gels were incubated with 10% HAc and 90% Me-OH for overnight. Afterward, the gels were incubated in periodate solution (0.7% periodic acid

and 5% HAc) for 1 h, then rinsed with double distilled water (ddW). A meta-bisulfate solution (0.2% sodium meta-bisulfate and 5% HAc) was added for 10 min, and the gels were incubated with Schiff's reagent for 1 h. The gels were destained for 1 h in ddW and then dried.

Surface expression

The expression of CD22, CD3, CD11b, major histocompatibility complex (MHC) I, MHC II, T/Tn, CD95 (Fas), and CD95L (FasL) were determined using splenocytes from immunized mice. Splenocytes (5×10^5) were incubated with purified antibodies for 30 min at 4°C; isotype-matched antibodies were used as a negative control. After washing with 0.1% BSA-PBS Phosphate buffered saline (PBS) pH 7.4, contains bovine serum albumin (BSA), cells were incubated with fluorescence (FITC; PharMingen) conjugates for 30 min at 4°C. After washing with 0.1% BSA-PBS, cells expressing CD95 (Fas) or CD95L (FasL) were co-stained with anti-mouse CD3-PE (PharMingen) for 30 min at 4°C. Cells were fixed with 2% PFA-PBS[46] until FACScan analysis. All antibodies were commercially purchased (PharMingen) except for anti-T/Tn antibody which monoclonal ascites anti-T antibody (Ca3114, IgM) raised in rat was a gift from Dr GF Springer[45] and used in the study.

Intracellular cytokine assay

Red cell–depleted splenocytes (10^7) were suspended with 10% fetal bovine serum (FBS)-RPMI 1640 and re-stimulated with 25-μg/mL T/Tn (β-D-Gal-[1-3]-D-GalNAc) or control oligosaccharide, *N*-Acetyllactosamine (β-D-Gal-[1-4]-D-GlcNAc; Sigma-Aldrich), in 6-well plates overnight at 37°C. Splenocytes were washed in PBS and fixed in 4% PFA-PBS for 5 min at 37°C. RPMI (Roswell Park Memorial Institute) Medium 1640 Cells (1×10^6) were resuspended with 0.1% saponin/5% skim-milk-PBS (S/M-PBS) and incubated with primary antibodies for 30 min at 18°C. Splenocytes were stained with CD3. Fluorescence-conjugated IL-4, interferon gamma (IFN-γ), and biotinylated GM-CSF and IL-10 (PharMingen) were used as primary antibodies. Isotype-matched antibody was used as a control. Cells were washed twice with S/M-PBS and resuspended in 0.1% BSA-PBS. For GM-CSF or IL-10 staining, cells were incubated with avidin-fluorescence (PharMingen) for 30 min at room temperature, washed, and analyzed.[46] Staining was analyzed using a Coulter EPICS XL Flow Cytometry (Beckman Coulter, Miami, FL, USA).

Cell proliferation assay

Ten days following the final immunization, MACS (Miltenyi Biotec Korea Co., Ltd, Seoul, Republic of Korea)-sorted $CD3^+$ T cells (10^6) were suspended with 10% FBS-RPMI 1640 and re-stimulated with 25-μg/mL T/Tn (β-D-Gal-[1-3]-D-GalNAc, Sigma, Missouri) or control oligosaccharide, *N*-Acetyllactosamine (β-D-Gal-[1-4]-D-GlcNAc; Sigma, Missouri), in 6-well plates for 3 days at 37°C. Cells were washed with PBS, and their viability was assayed by trypan blue staining.

In vitro CTL assay

The target cells (1×10^6) were resuspended in 150 μL and labeled with 150 μCi (150 μL of 1 mCi/mL) of sodium chromate (^{51}Cr) (PerkinElmer, Waltham, MA, USA) for a final concentration of 3.33×10^6 cells/mL. The cells were then incubated in a humidified chamber at 37°C with 5% CO_2 for 1 h to allow uptake of ^{51}Cr. They were gently resuspended once during incubation to increase uptake. The labeled target cells were washed three times and resuspended to a final concentration of 1×10^5 cells/mL. Cells were counted by trypan blue staining, and 1.35×10^6 $CD3^+$ cells from spleens were resuspended in 450 μL. In a 96-well round-bottom plate, 150 μL of $CD3^+$ cell suspension was transferred to three wells for the first effector: target ratio. The next three wells were loaded with 100 μL of media. Using a multichannel pipette, a threefold serial dilution was performed by transferring 50 μL across the six wells in triplicate and discarding the final 50 μL. Hence, the E:T ratios were 50:1, 25:1, and 10:1. To determine minimal or maximal release, 100 μL of media or 100 μL of 1% Triton X-100 was added to each well in triplicate. Then, 100 μL of ^{51}Cr-labeled target cells were added to each well and incubated in a humidified chamber at 37°C with 5% CO_2 for 4 h. The plates were sealed and centrifuged at 1500 r/min for 5 min at 4°C to pellet the cells. Using a multichannel pipette and proceeding from replicates of low-level to high-level ^{51}Cr, 100 μL of supernatant was aspirated without disturbing the pellet into Titretube micro tubes (Bio-Rad, Des Plaines, IL, USA) for counting on a Packard-Cobra Auto Gamma counter (PerkinElmer).

The following equation was used to calculate the mean percentage specific lysis: % Specific lysis = ((Sample cpm − minimal release cpm)/(Maximal cpm − minimal release cpm)) × 100%

In vitro expansion of precursor CTL

Single-cell suspensions were prepared from spleens of immunized mice, and RBCs were lysed using lysis buffer (Sigma-Aldrich) at room temperature for 1 min. Spleen cells (8×10^6 cells/mL) were added into 24-well flat-bottom plates in a total volume of 2 mL. Recombinant murine IL-2 (BD Biosciences, Franklin Lakes, NJ, USA) and 25-μg/mL T/Tn per well were added for activation. Cells were incubated in a humidified chamber at 37°C with 5% CO_2 for 5 days. Target cells (1.6×10^5; 1:50) were labeled with 150 μCi (^{51}Cr) (PerkinElmer). Then, 100 μL of ^{51}Cr-labeled target cells were added to each well and incubated at 37°C with 5% CO_2 for 5 h. The mean percentage specific lysis was calculated as: % Specific lysis = ((sample cpm − minimal release cpm)/(maximal cpm − minimal release cpm)) × 100%

Immunization and tumor challenge

Specific pathogen-free A/J mice (females aged 6–8 weeks) were obtained from SLC Inc. (Hamamatsu, Japan). Mice were handled under specific pathogen-free conditions according to the guidelines issued by the Seoul National University Animal Research Committee. Mice were immunized intraperitoneally (i.p.) in four groups: PBS, 5-μg T/Tn mixed with incomplete FA (Sigma-Aldrich) (T/Tn + FA), 5-μg KLH-conjugated T/Tn mixed with FA (KLH-T/Tn + FA), and 5-μg oxidized mannan-conjugated T/Tn mixed with FA (ox-M-T/Tn + FA). All mice were boosted on day 10 and day 17 with the same immunogens; the immunization schedule was previously described.[22,23,46] Five days after the last immunization, mice were challenged subcutaneously with 10^3 TA3HA tumor cells. For cellular analysis, 7–10 days following the last immunization, mice were culled and immune responses analyzed.

Immunohistochemistry

Tumor masses were blocked after the sacrifice o f tumor-bearing mice. Tumor-infiltrating lymphocytes were indicated by hematoxylin & eosin (H&E) staining. For immunohistochemical staining, all of the paraffin-embedded sections were cut to 5-μm thickness, deparaffinized with xylene, and dehydrated with graded ethanol. Antigen recovery was performed in heat-activated antigen retrieval pH 9 (Dako, Carpinteria, CA, USA), after which the specimens were incubated with 3% H_2O_2 for 15 min. Non-specific binding was blocked with protein block (Dako) for 20 min at room temperature. The sections were incubated with FasL (PharMingen), MHC I (PharMingen), CD301 (PharMingen), and T/Tn (Chicago Medical School) at 1:50 dilution for 2 h. Subsequently, the sections were incubated with EnVision+ Dual Link System-HRP (Dako) for 30 min, visualized with 3,3-diaminobenzadine for 10 min, and washed and counterstained with hematoxylin. Appropriate negative controls were concurrently performed. All of the slides were reviewed by a pathologist.

Results

Characterization of T/Tn

T/Tn was separated by SDS-PAGE, and gels were subjected to PAS (carbohydrate) stain (Figure 1(a)), Coomassie (protein) staining (Figure 1(b)), and western blot analysis using anti-T/Tn antibody (Figure 1(c)). T/Tn was highly glycosylated (Figure 1(a) left, lane 5) compared with ovalbumin (OVA) (Figure 1(a) left, lane 6), another glycosylated protein. However, in our system, the degree of glycosylation of OVA was not enough for PAS staining to detect its carbohydrate portion. T/Tn in our system was shown to have multiple bands in SDS-PAGE (Figure 1(a) left, lane 5), which means that T/Tn has multiple S-S bonds at 120 and 66 kD. When T/Tn was run in a native gel, 1 band was shown (Figure 1(a) right, lane 5) and compared BSA (Figure 1(a) right, lane 7). The protein backbone of T/Tn was stained by Coomassie (Figure 1(b), lane 5) at approximately 120 and 60 kD. It was slightly lighter than the MN molecule (Figure 1(b), lane 4), and different from the glycophorin A (Figure 1(b), lane 2) and stroma (Figure 1(b), lane 3). T/Tn was detected in a western blot with anti-T/Tn Ab (Ca3114, donated from Dr GF Springer's laboratory) (Figure 1(c), lane 5). The antibody was specific for T/Tn and did not detect OVA (Figure 1(c), lane 6). Hence, we can conclude that T/Tn is a highly glycosylated antigen with a peptide backbone.

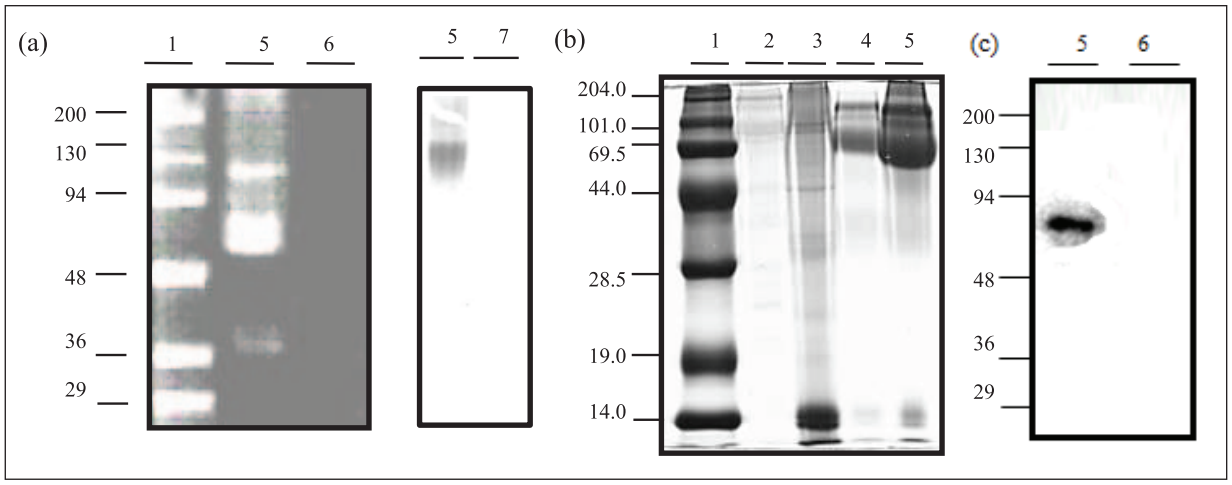


Figure 1. Characterization of T/Tn glycoprotein. T/Tn was separated by SDS-PAGE and gels were subjected to (a) PAS (carbohydrate) stain, (b) Coomassie (protein) stain, and (c) western blot analysis using anti-T/Tn antibody. Lane 1 = molecular weight marker, lane 2 = glycophorin A, lane 3 = stroma, lane 4 = MN antigen (precursor of T/Tn), lane 5 = purified T/Tn, lane 6 = OVA (negative control), and lane 7 = BSA (negative control). Same batches of T/Tn were used in (a–c).

Screening a suitable dose of T/Tn in mice against tumor challenge

To establish a T/Tn tumor model, several murine tumor cell lines were tested for T/Tn expression using flow cytometry. TA3HA (97.1%) expressed highest levels of T/Tn antigen, whereas CTLL-2I (49.4%), SP2/0 (25.9%), and Raw264.7 (35.5%) expressed moderate to weak levels. TA3HA mammary adenocarcinoma expressed the highest level and was therefore used for in vivo studies (Figure 2). To characterize the dose-response characteristics, different amounts of T/Tn (5 or 25 μg) were injected intraperitoneally (i.p.) three times, and mice received 10^3 TA3HA cells subcutaneously (s.c.) 5 days after the final injection. Mice immunized with 5-μg T/Tn exhibited significant retardation of tumor growth (Figure 3(a)). Tumor protection was less significant in the 25-μg T/Tn-immunized group, even though anti-T/Tn antibody levels were equivalent to those in the 5-μg immunization group; this suggests that immune deviation was induced by 25-μg T/Tn immunization (Figure 3(b)). Based on these tumor-protective properties, all further experiments were conducted using the immunization dose of 5-μg T/Tn.

T/Tn-specific immune repertoires

Ten days after the final injection and 1 month following tumor challenge, whole splenocytes were prepared and analyzed for specific immune cell repertoire. All splenocytes were gated. CD3 levels were lowest in the ox-M-conjugated T/Tn group 10 days after the final injection (Figure 4(a)). In contrast, the population of $CD4^+$ was highest in the ox-M-conjugated T/Tn group in the $CD3^+$ portion (Figure 4(b)). To predict antigen presentation power, we monitored MHC class II (MHC II) levels (Figure 4(d)) 10 days after final immunization and 1 month after TA3HA tumor cell challenge (Figure 4(i) and (j)); MHC II levels were highest in the ox-M-T/Tn + FA group. In particular, in the tumor challenge group, MHC II levels were extremely high when the cells were gated by macrophage population (Figure 4(j)). In addition, 1 month after tumor challenge (Figure 4(h)), the expression levels of MHC I were highest in the ox-M-conjugated T/Tn group but lowest in the same group 10 days after the last immunization (Figure 4(c)). We also monitored CD3, CD11b, and T/Tn expression; CD3 levels were highest in the ox-M-conjugated T/Tn group (Figure 4(e)), but tolerogenic $CD11b^+$ cells were lowest in the ox-M-conjugated T/Tn group (Figure 4(f)). T/Tn presentation was highest in the ox-M-conjugated T/Tn group (Figure 4(g)).

Mice immunized with T/Tn conjugated to oxidized mannan mixed with FA exhibit strong cellular immune responses

To detect T cell activation, we determined the expression of CD95L (FasL) or CD95 (Fas) in T cells, and we divided percentages of $FasL^+$ T

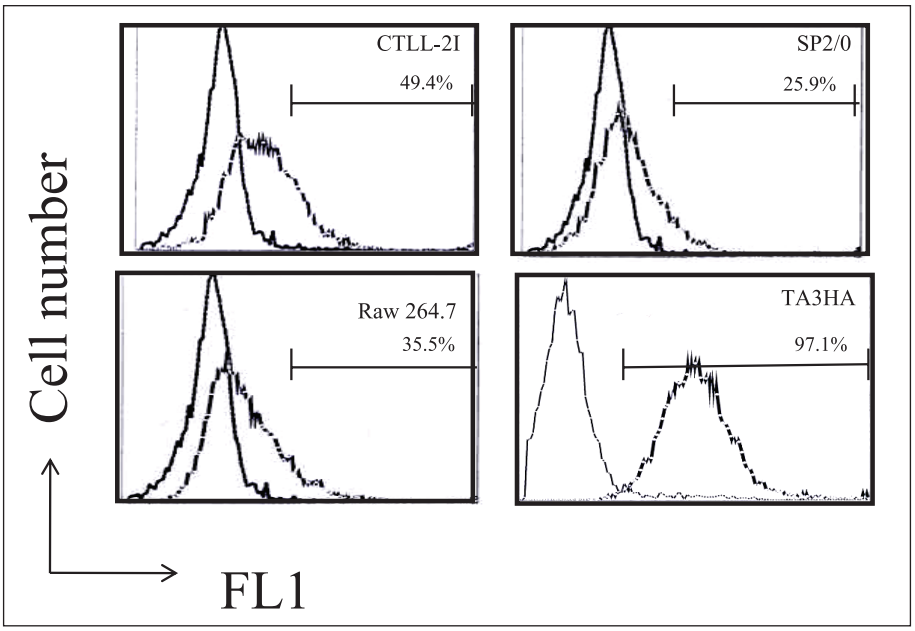


Figure 2. T/Tn expression in tumor cell lines. T/Tn expression was determined in murine cancer cells using flow cytometry. The cell lines tested are shown. CTLL-2I (IL-2 independent murine T cell), SP2/0 (B lymphoblast), Raw264.7 (Abelson murine leukemia virus–induced tumor), and TA3HA (mammary adenocarcinoma). Isotype-matched antibody was used as a negative control.

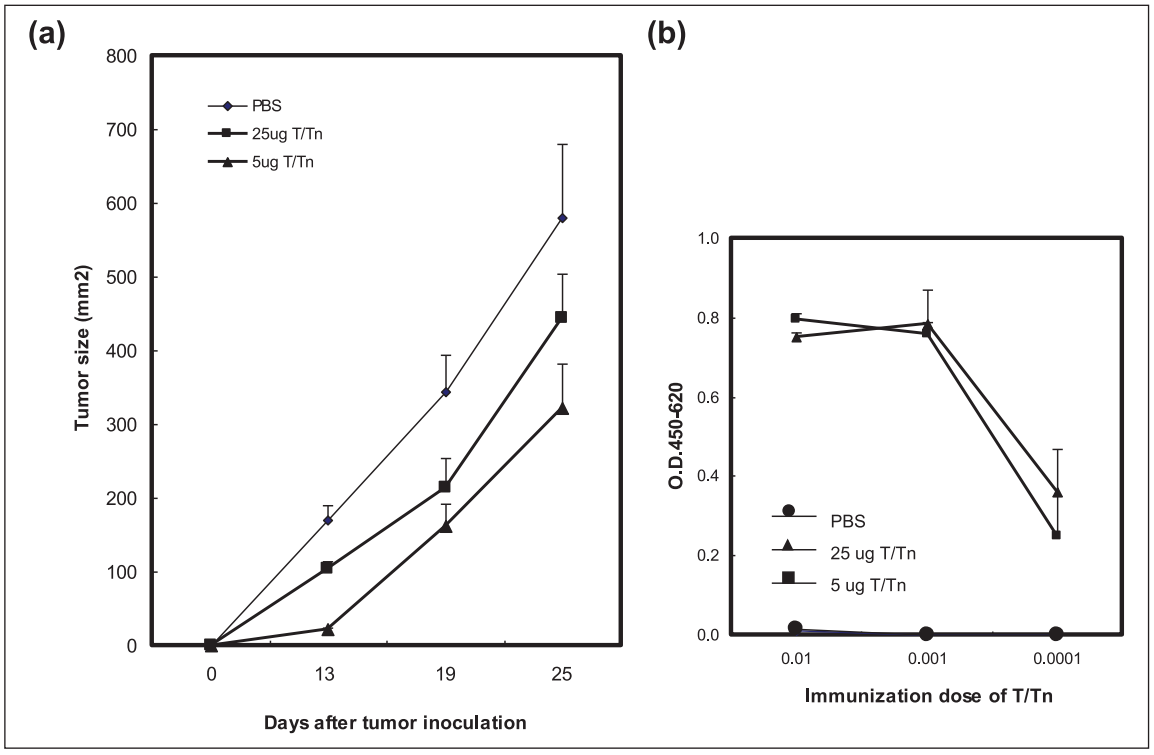


Figure 3. Effect of antigen dose on T/Tn immunization: (a) effect of T/Tn vaccination on subcutaneous growth of TA3HA tumor cells in Strain A mice. Strain A mice were immunized with 5- or 25-μg T/Tn twice and challenged by s.c. injection of 10^3 TA3HA cells 5 days after the last vaccination into the left flank of each mouse. Tumor size was determined every 2 days by caliper measurement. Each value represents the mean ± SD (n = 8 per group). (b) T/Tn-specific antibody response. Three days after the final injection with 5 or 25 μg of T/Tn, mice were sacrificed by bleeding. Three different dilutions were tested, from $1/10^2$ to $1/10^4$, and the absorbance was read at 450 nm referred at 620 nm using Multiskan EX/RC (Lab Systems, Vantaa, Finland). n = 5 in each group, and all experiments were done in triplicate.

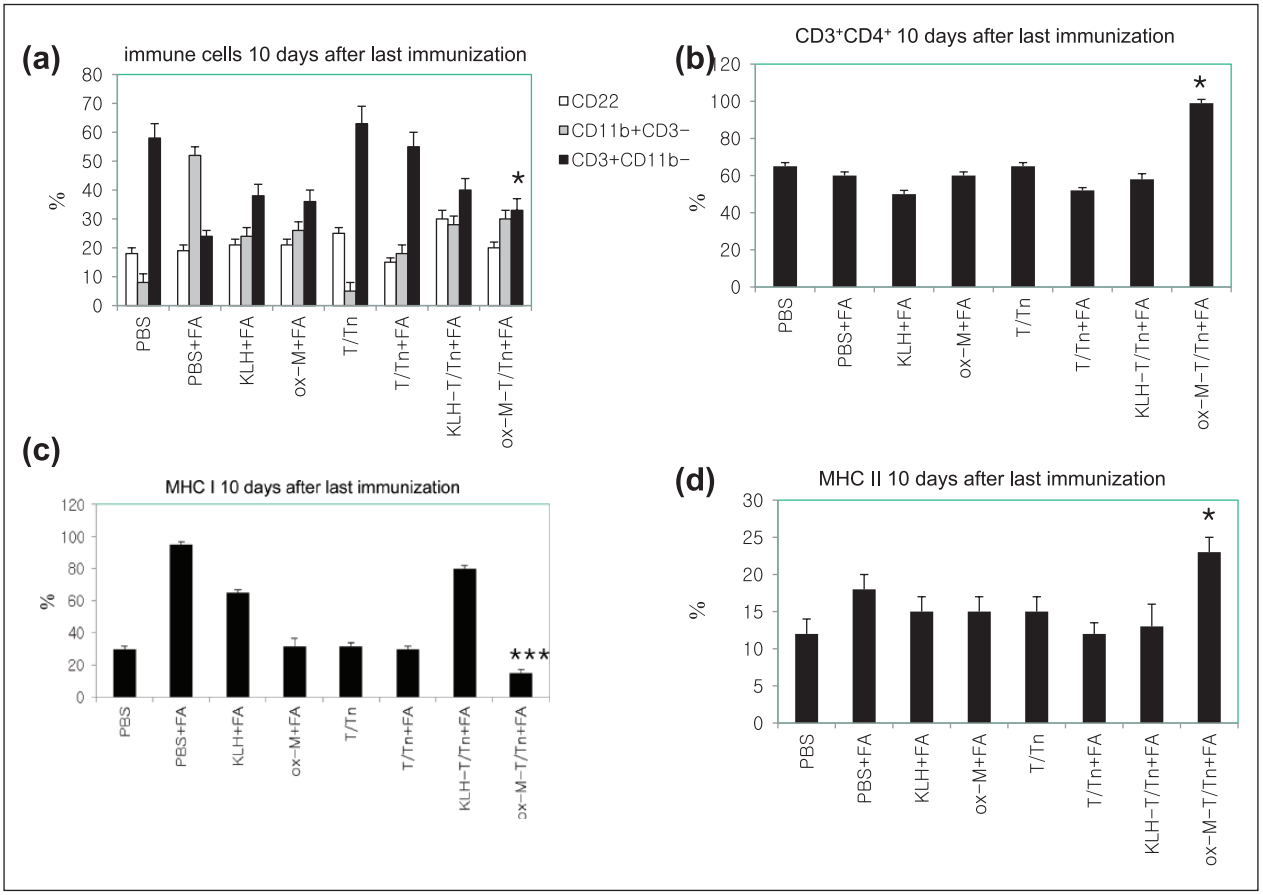
cells by percentages of Fas+ (FasL/Fas ratio). FasL/Fas ratio was highest in the ox-M-T/Tn + FA-immunized group at either 10 days after the final injection (Figure 5(a)) or 1 month following tumor challenge (Figure 5(b)). Interestingly, intracellular cytokine staining (Figure 5(c)), IFN-γ expressing, and GM-CSF-expressing T cells were highly expressed in the ox-M-T/Tn + FA-immunized group. In contrast, IL-4-expressing T cells were dominant in both the T/Tn- and T/Tn + FA-immunized groups, and IL-10-expressing T cells were not significantly different among the groups. T/Tn-specific $CD3^+$ T cell proliferation was also the highest in the ox-M-T/Tn + FA-immunized group (Figure 5(d)). CTL activity (Figure 6(a)) and cytotoxic T lymphocyte precursor (CTLp) activity (Figure 6(b)) of T cells isolated from spleens of immunized mice were determined using T/Tn+ tumor target TA3HA cells. CTL and CTLp tumor target-specific lysis by both methods was highest in the ox-M-T/Tn + FA-immunized mice group according to T/Tn expression level.

Tumor protection by immunization with ox-M-T/Tn and FA

We monitored tumor retardation after various T/Tn immunizations. In the ox-M-T/Tn + FA-immunized group, tumor growth was significantly retarded (Figure 7(a)). The survival rate was also highest in the ox-M-T/Tn + FA group (Figure 7(b)). H&E staining was used to monitor immune cell recruitment. As shown in Figure 8, immune cell recruitment was highest in the ox-M-T/Tn + FA group. FasL, MHC I, and CD301 on macrophages were also densest in the ox-M-T/Tn + FA group (Supplementary Table 1).

Discussion

T/Tn antigen has been shown to be immunogenic, effective,[47] and safe in preventing recurrence of advanced human breast cancer.[48–50] It has been reported that immunization with T/Tn alone generates humoral and DTH responses but no tumor protection.[1,2] Numerous studies have improved T/Tn cancer vaccines using conjugation with carriers,



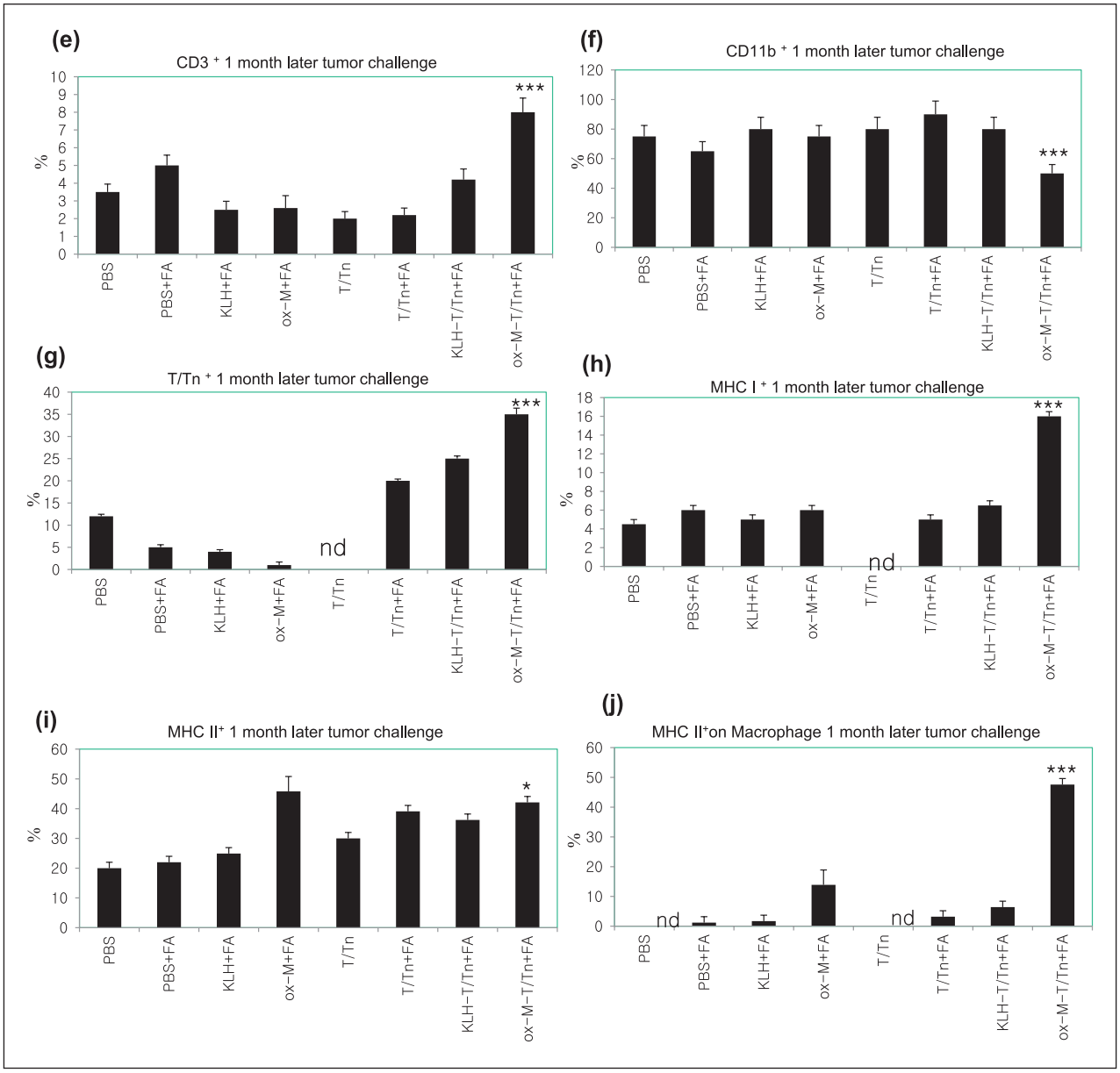


Figure 4. Immune cell repertoire after T/Tn immunization and after tumor challenge. Ten days after the final immunization with 5 μg of T/Tn, spleen cells were prepared. (a) CD22, CD3, and CD11b cells were monitored. To monitor antigen presentation power, spleen cells were stained with (b) $CD3^+CD4^+$ cells (Th cells). (c) MHC I and (d) MHC II at 10 days after final immunization. Spleen cells were prepared and monitored to determine (e) $CD3^+$ cells, (f) tolerogenic $CD11b^+$ cells, (g) T/Tn presentation, (h) MHC I, (i) MHC II, and (j) MHC II on macrophages 1 month after tumor challenge.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; nd: not detected.

antigen modification, and application of antigen-presenting cells.[51–54]

In this study, we determined a tumor vaccine formula to elicit cellular immune responses. To that end, we monitored target cells highly expressing T/Tn and noted the high expression of T/Tn in the TA3HA mammary adenocarcinoma cell line syngeneic in A/J mice. We then determined a suitable dose of T/Tn immunization which yielded the highest tumor retardation. Tumor protection was more significant in the 5-μg T/Tn-immunized group than in the 25-μg group, suggesting that 5 μg can overcome immune deviation.[39] To monitor the immune repertoire, we determined T, B, and $CD11b^+$ cell by flow cytometry in each immunized group, and the $CD3^+$ proportion was lowest in the ox-M-conjugated T/Tn group, but highest in the same group 1 month later. In the ox-M-T/

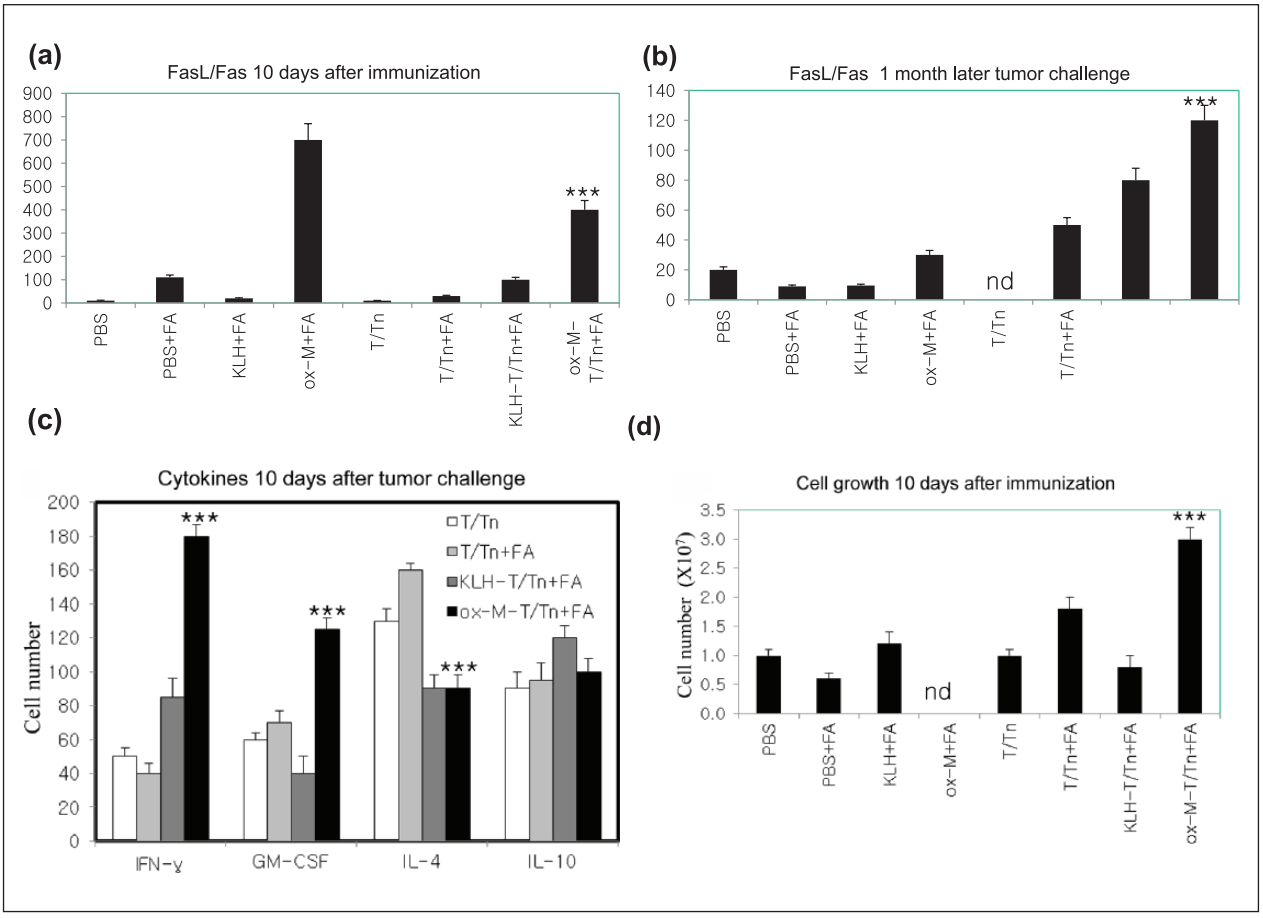


Figure 5. Cellular immunity after T/Tn immunization. Splenocytes from immunized mice were monitored for expression of Fas or FasL ($CD3^+$ double staining) 10 days after the final injection (a) and 1 month following tumor challenge (b). Cells were stained and analyzed by flow cytometry. The percentage of $FasL^+$ splenocytes was divided by the percentage of Fas^+ splenocytes (FasL/Fas ratio), indicative of activation of T cells. (c) Splenocytes (10^7) from immunized mice were incubated in vitro for 16–18 h in the presence of 25-µg T/Tn or *N*-Acetyllactosamine (negative control). Cytokine expressions (IFN-γ, GM-CSF, IL-4, and IL-10) were double-stained with CD3 and determined using intracellular cytokine staining. Specific cytokines were calculated as follows: percentage of cytokine-positive cells stimulated with 25-µg T/Tn divided by cells stimulated with 25-µg control antigen. (d) T/Tn-specific T cell proliferation. $CD3^+$ T cells (10^6) 10 days after last immunization were incubated in vitro for 3 days in the presence of 25-µg T/Tn or *N*-Acetyllactosamine (negative control). Cells were counted by trypan blue staining. n = 5 in each group and in triplicate.
$^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; nd: not detected.

Tn + FA group, the number of $CD4^+$ T cells was highest, indicating that the majority of the T cells were helper T (Th) cells following immunization. These Th cells were CTL-priming and cytokine-regulating; indeed, MHC I and II expression were highest in the ox-M-T/Tn + FA group, suggesting antigen presentation ability and likelihood to stimulate $CD4^+$ and $CD8^+$ T cells. Following tumor challenge, the proportion of $CD3^+$ cells was highest in the ox-M-T/Tn + FA group, but tolerogenic $CD11b^+$ cells were the lowest in this group. Strikingly, T/Tn presentation was highest in the ox-M-T/Tn + FA group. In addition, the FasL/Fas ratio of T cells was high in the ox-M-T/Tn + FA group, suggesting that cell viability was highest in these mice. Intracellular cytokine analysis using flow cytometry demonstrated high levels of IFN-γ and GM-CSF in mice immunized with ox-M-T/Tn + FA, although IL-4 was high in T/Tn- and T/Tn + FA-immunized groups. T/Tn-specific T cell proliferation was only noted in ox-M-T/Tn + FA. Furthermore, tumor protection was shown only in ox-M-T/Tn + FA-immunized mice,[39] as well as significant T/Tn-specific CTL activity and CTLp activity. Taken together, immunization with ox-M-T/Tn + FA clearly elicits T/Tn-specific T cell immunity, Th1 response, and CTL activity in addition to humoral response.[29]

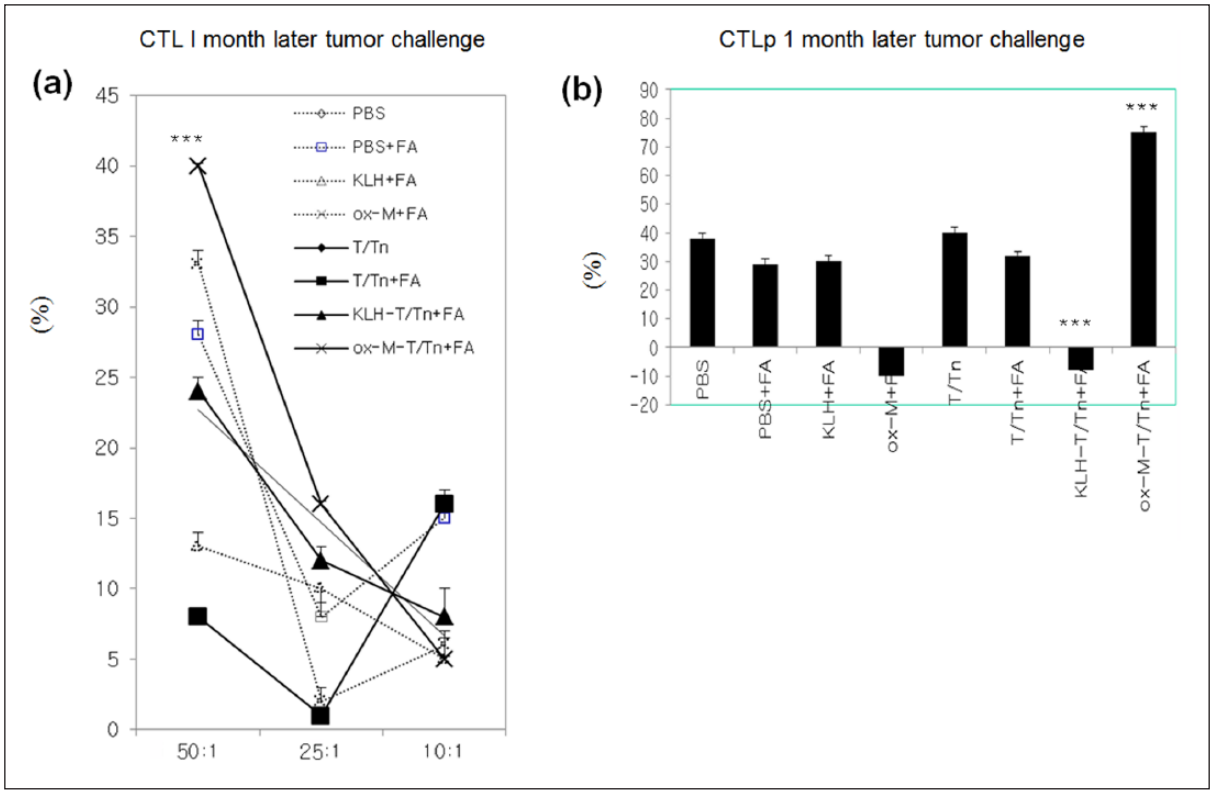


Figure 6. T/Tn-specific CTL activity. Splenocytes were prepared 1 month after tumor challenge, re-stimulated in vitro (with 25-μg T/Tn), cultured with ^{51}Cr-labeled TA3HA cells, and used to measure either CTL activity (a) or CTLp expansion (b). The specific lysis was determined as: ((Sample cpm − minimal release cpm)/(Maximal cpm − minimal release cpm)) × 100%. n = 5 in each group, and in triplicate.
*P < 0.05; **P < 0.01; ***P < 0.001.

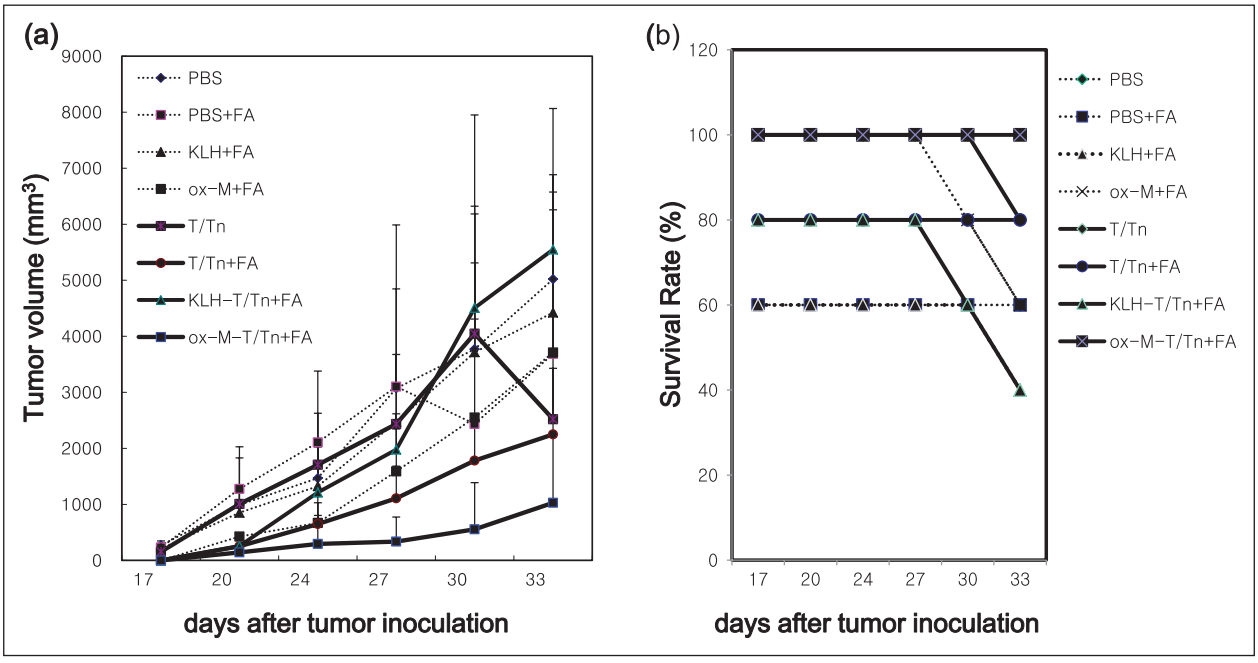


Figure 7. Tumor growth after immunization. Tumor protection following immunization of mice with T/Tn. Tumor growth (a) and survival rate (b) of T/Tn-immunized mice. Two weeks after the third injection of various T/Tn immunogens, mice were injected subcutaneously with 1 × 10^3 TA3HA tumor cells. Tumor volume was evaluated 15 days after tumor challenge every other day. Tumor volume (mm^3) was calculated as follows: horizontal (mm) × vertical (mm) × depth (mm).

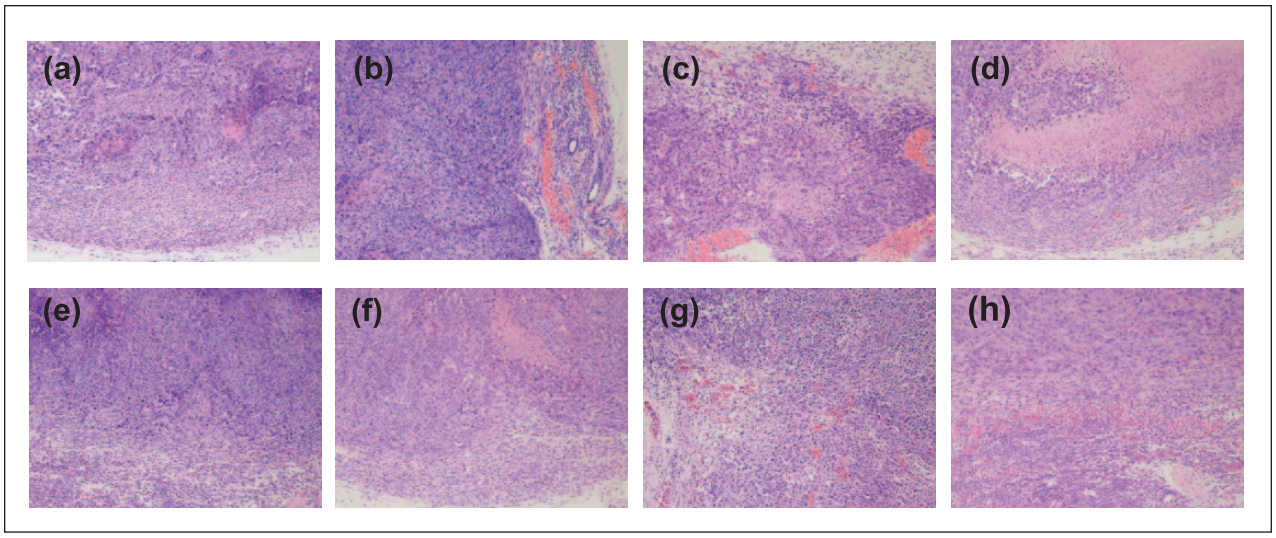


Figure 8. Hematoxylin & eosin staining. Tumor masses were prepared in 4% paraformaldehyde for one overnight. Tissue blocks were sectioned into 5-µm diameter slices. Slides were prepared by hematoxylin and eosin staining and observed at 100×: (a) PBS, (b) PBS + FA, (c) PBS + FA, (d) ox-M + FA, (e) T/Tn, (f) T/Tn + FA, (g) KLH-T/Tn + FA, and (h) ox-M-T/Tn + FA.

Interestingly, H&E staining of tumor masses showed a high degree of tumor-infiltrating lymphocytes in the ox-M-T/Tn + FA group, but not in KLH-T/Tn + FA-immunized mice. Moreover, the expression of surface markers suggestive of immune activation (MHC I, FasL) was not significant in the KLH-T/Tn + FA group. When TA3HA tumor cells were incubated with sera from immunized mice, in vitro cell growth was only apparent in KLH-T/Tn + FA-immunized mice by MTT assay (data not shown). It is clear that ox-M-T/Tn + FA was the most efficient T/Tn vaccine formula to induce cellular immunity among those tested in this study, and it induced tumor protection.[39] These findings are consistent with observations previously described for the cancer antigen MUC1 using oxidized mannan as a carrier.[21–24,55] We are currently determining epitopes recognized by antibodies[39] and CTL.

Only the T and Tn antigens seem to be associated with malignant transformation of mammary gland cells and to be of potential value as diagnostic markers.[56] Therefore, T/Tn Ag has very good tumor specificity. Mo and colleagues[57] reported that after hepatocyte growth factor/scatter factor (HGF) treatment, Huh7 cells lost epithelial characteristics and obtained mesenchymal markers. Lectin microarray analysis identified a decreased affinity implying that glycan-containing T/Tn antigen structures were reduced. Cell surface glycan alterations in the epithelial–mesenchymal transition (EMT) process may coincide with the expression of glycosyltransferase.[57]

Till date, tumor therapy against T/Tn was focused on phototoxicity.[13] In addition, the mechanism by which T/Tn activates the immune system is not clear. In this study, we demonstrated a mechanism for T/Tn to induce immune activation and proved the efficacy of a T/Tn antigen-based approach. Mannosylated T/Tn can induce increased expression levels of MHC I and MHC II on antigen-presenting cells (macrophages), increase $CD3^+CD4^+$ T helper cell populations, induce strong cellular immune responses, increase Th1 cytokines, increase Fas/FasL activation, and induce tumor retardation. Tumor protection and survival rate were highest in the ox-M-T/Tn + FA group due to superior immune cell infiltration. Hence, oxidized mannan-conjugated to T/Tn antigen and mixed with FA is a strong immune stimulant against T/Tn and further studies should be conducted investigating this formula as a potential immunotherapeutic in humans with adenocarcinoma.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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