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Mol Pharm. Author manuscript; available in PMC 2013 February 6.

Published in final edited form as:
Mol Pharm. 2012 February 6; 9(2): 261–268. doi:10.1021/mp200350n.

Trp2 peptide vaccine adjuvanted with (R)-DOTAP inhibits tumor growth in an advanced melanoma model

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Abstract

Previously we have shown cationic lipid (R)-DOTAP as the immunologically active enantiomer of the DOTAP racemic mixture, initiating complete tumor regression in an exogenous antigen model (murine cervical cancer model). Here, we investigate the use of (R)-DOTAP as an efficacious adjuvant delivering an *endogenous* antigen in an aggressive murine solid tumor melanoma model. (R)-DOTAP/Trp2 peptide complexes showed decreasing size and charge with increasing peptide concentration, taking a rod-shape at highest concentrations. The particles were stable for at 2 weeks at 4°C. A dose of 75nmol Trp2 (formulated in (R)-DOTAP) was able to show statistically significant tumor growth delay compared to lower doses of 5 and 25nmol which were no different than untreated tumors. (R)-DOTAP/Trp2 (75nmol) treated mice also showed increased T cell IFN-γ secretion after restimulation with Trp2, as well as CTL activity *in vivo*. This vaccination group also showed the highest population of functionally active tumor-infiltrating lymphocytes, indicated by IFN-γ secretion after restimulation with Trp2. Thus, (R)-DOTAP has shown the ability to break tolerance as an adjuvant. Its activity to enhance immunogenicity of other tumor associated antigens should be studied further.

Keywords

(R)-DOTAP; peptide vaccine; melanoma; immunotherapy

Introduction

Recent clinical advances for the treatment of melanoma have great potential to revolutionalize the field, however not without associated toxicity. The recent FDA approval of the anti-CTLA4 antibody ipilimumab for unresectable and metastatic melanoma shows a step forward for biological drugs and immunotherapy for melanoma However, the approval also came with a boxed warning for immue-related adverse events (irAE) which can range from mild to severe.[1] Interestingly, the severity of irAE seemed to correlate with a positive response to therapy.[2]

Intensive research on the genetics of melanoma have led to advances in small molecule inhibitors as well.[3] Several companies are investigating drugs for melanomas with oncogene mutations, most notably, BRAF V600E which occurs in almost 50% of melanoma cases.[4] The recent approval of vemurafenib showed up to an 81% response rates in trials.[5] Other small molecule BRAF inhibitors are still in the clinical trials and include RAF-265, XL281, and GSK2118436. However, even with promising BRAF inhibitors selective for patients

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with the V600E mutation, side effects are seen, such as resectable cutaenous carcinoma, and more importantly, induction of resistance.[1, 5, 6] With side effects produced by the new frontier of both biological and small molecule drugs for melanoma, there is still space to innovate a treatment that is able to treat aggressive tumors with fewer off-target effects.

As a treatment option, peptide vaccines offer the safety and specificity of a restricted epitope. Specific antigen in the form of peptide sequences (8–10 amino acids long) can be delivered to antigen presenting cells (APCs). Restricting the sequence choice to bind to major histocompatibility complex (MHC) class I ensures $CD8^+$ T cell engagement. Activation of APCs and presentation of the peptide can stimulate a corresponding cytotoxic T lymphocyte (CTL) response against the epitope. As an antigen, peptides are easy to manufacture and specific, however, they are rarely immunostimulatory alone. Dendritic cells pulsed with melanoma tumor-specific peptide were unable to stimulate a therapeutic response when injected on the same day as the tumor.[7] Delivery of a peptide antigen with an adjuvant is required for potent activation of APCs, and associated CTL activity.

In the case of melanoma, sensitizing CTLs against the corresponding endogenous, or self-antigen poses yet another challenge. As T cells develop in the thymus, self-reactive T cells are deleted, and regulatory T cells develop in circulation and particularly in the presence of tumors. Some have taken the approach to genetically modify T cell receptors to be specific for tumors, instead of efforts to vaccinate and allow a response to develop in an aided fashion.[8] A strong adjuvant would be required to break through this tolerance and stimulate a powerful anti-tumor response.

While many melanoma peptide antigens are well characterized, tyrosinase-related protein 2 (Trp2) is particularly attractive as an antigen. Trp2 peptide (amino acids 180–188 of the Trp2 protein, sequence: SVYDFFVWL) is MHC class I $H\text{-}2K^b$ restricted in mouse, allowing for examination of a $CD8^+$ CTL response. The sequence is also HLA-A2 restricted, thus the potential exists for the same formulation to be tested both in mouse and in humans. Additionally, since melanoma and glioma cells are both neuroectodermal in origin, Trp2 is also expressed in this additional cancer line, allowing for a natural secondary application of any developed formulation [7, 9]. However, as delivery cargo, Trp2 peptide is hydrophobic and difficult to formulate in an aqueous solution or delivery vehicle. The most successful work with Trp2 peptide as an antigen has been with preventative models.[10–13] Few studies examine the treatment of established tumors, and none to date have shown any effect in aggressive solid tumor models.[14–16]

Previously, our group has studied DOTAP lipid as an adjuvant in peptide and protein vaccine formulations. A DOTAP/peptide vaccine has shown complete tumor regression in an exogenous antigen model.[17, 18] Further investigation of the DOTAP lipid, showed chiral specificity of the adjuvant activity.[18] These studies led us to discover (R)-DOTAP as the active immune-stimulating enantiomer. (R)-DOTAP (or even the DOTAP racemic mixture) has never been tested as an adjuvant in an endogenous tumor model, and indeed the challenges of breaking tolerance are not slight. Particularly, treating an established tumor model in melanoma is rarely tested due to the difficulty of generating an adequate immune response to cause tumor growth delay due to the suppressive effects of an established tumor. In work presented here, we address the formulation challenges of delivering an adequate dose of the innately hydrophobic Trp2 peptide and utilize (R)-DOTAP/Trp2 complexes in an advanced melanoma model to break tolerance and show statistically significant tumor growth delay.

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Experimental Section

Materials

(R)-DOTAP [(R)-1,2-dioleoyl-3-trimethylammonium-propane] was obtained from Merck KGaA (Darmstadt, Germany). H-2K^b restricted peptides Trp2 (H-SVYDFFVWL-OH, amino acids 180–188 from the tyrosinase-related protein 2 over-expressed in human and murine melanoma, MW 1175) and Ova (H-SIINFEKL-OH, amino acids 257–264 from the ovalbumin, MW 1773) were obtained from Peptide 2.0 (Chantilly, VA), supplied as trifluoracetate salts. Molecular biology grade water was obtained from Cellgro (Manassas, VA). Sodium methoxide, pure titrant (0.5M in methanol) and HPLC grade methanol were obtained from Fisher Scientific (Pittsburgh, PA). Absolute ethanol was obtained from Decon Labs, Inc. (King of Prussia, PA).

Trp2 peptide preparation

Specific weights of Trp2 peptide were dissolved in a 1:1 solution of methanol:ethanol. Serial dilution of Trp2 peptide was measured on a Shimadzu UV-Vis spectrophotometer (model UV-2501 PC, Columbia, MD), and absorbance recorded. The measurements were made in triplicate, and standard curve created utilizing the Beer-Lambert law. The extinction coefficient, $\varepsilon = 5{,}367.7$ ($M^{-1}cm^{-1}$), was found with the measured absorbance at 280nm wavelength to estimate Trp2 peptide concentration and encapsulation.

Trp2 peptide was formulated as a disodium salt for delivery. Peptide arrived as a trifluoroacetate salt and was dissolved in HPLC grade methanol in a glass vial and a magnetic stir bar added. The peptide was stirred in an ice bath, as two equivalents of sodium methoxide were added drop wise. Titrated peptide was allowed to stir for 10min as the solution became clear. Aliquots of the peptide solution were added to water and pH measured (pH 9). Methanol was dried off using a rotoevaporator, and peptide was resuspended as a 1.5mM working concentration in molecular biology grade water.

Preparation and Evaluation of Vaccine Formulations

Briefly, 6,000nmol of (R)-DOTAP dissolved in chloroform was dried in a glass vial under a stream of nitrogen and stored under vacuum in a desiccator overnight to ensure removal of any remaining chloroform. Lipid/peptide complexes were formed by thin film hydration, using a 1mL solution of Trp2 peptide at different concentrations in molecular grade water (Cellgro, Manassas, VA) and subsequent vortexing. After 1h at room temperature, the suspensions were extruded ten times through two 100nm polycarbonate membranes (Fisher Healthcare, Houston, TX).

Particle size and zeta potential were measured with a Malvern Zetasizer Nano ZS (Worcestershire, United Kingdom). Samples were prepared for size and charge characterization by adding 10μL 6mM (R)-DOTAP/(1.5 to 0mM) Trp2 complexes to 990μL of molecular biology grade water. Trp2 peptide encapsulation efficiency was measured by adding 50μL of extruded lipid/peptide complexes to a Microcon 100kDa molecular weight cut off centrifugal filtrate device (Millipore, Bedford, MA). The column was centrifuged at 10,000g for 10min at 25°C. Flow-through was analyzed using 2μL of sample on a Nanodrop Spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE), measuring the absorbance at 280nm wavelength. Concentration of peptide in flow-through (unencapsulated) was calculated using Beer-Lambert law ($A_{280} = \varepsilon * b * c$), with $\varepsilon = 5{,}367.7$ ($M^{-1}cm^{-1}$), calculated from a standard curve of Trp2 peptide. Final encapsulated concentration was calculated by subtracting the concentration in flow-through from the known concentration used for hydration. Trp2 peptide loss in the column was $< 5\%$.

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Transmission electron microscope (TEM) images of the (R)-DOTAP/Trp2 complexes were acquired by the use of a JEOL 100CX II TEM (Tokyo, Japan). Briefly, 4μl of nanoparticle solution was dropped on to a 300 mesh carbon coated copper grid (Ted Pella, Inc., Redding, CA) for 2min. Excess fluid was removed with filter paper, and copper grid was dried before the observation with TEM. A total of 210 particles from 3 different fields were measured. Particle dimensions were calculated by measuring the longest dimension and the perpendicular diameter.

Tumor cell culture

B16F10 cells, a murine melanoma model (syngeneic with C57BL/6) were originally obtained from American Type Culture Collection (ATCC) (Manassas, VA). Previously, in collaboration with Dr. Pilar Blancafort at the University of North Carolina at Chapel Hill (UNC), B16F10 was stably transfected with GL3 firefly luciferase gene using a retroviral vector to create B16F10-luc.[19, 20] B16F10-luc cells were used in all studies presented here and maintained in Dulbecco's Modified Eagle Medium (Gibco (Invitrogen), Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 100U/mL penicillin (Invitrogen) and 100μg/mL streptomycin (Invitrogen).

Mice and Immunizations

Six week old female C57BL/6 mice obtained from the National Cancer Institute, were used in all studies. On day 0, $3x10^5$ B16F10-luc cells in 50μL of PBS were injected subcutaneously into the hair-trimmed abdomen. On day 6, formulations were subcutaneously injected into the contralateral side of the abdomen in 100μL of 5% dextrose solution. In all experiments, 300nmol (R)-DOTAP per mouse was the therapeutic dose of adjuvant used, as shown by our lab previously.[18] Tumors were measured every 2–3 days with calipers, recording the longest diameter as length and the perpendicular dimension as width. Area was calculated as (length)×(width). Humane sacrifice of mice was performed after tumors reached 20mm in one dimension. All animal protocols were approved by the University of North Carolina at Chapel Hill's Institutional Animal Care and Use Committee.

Multi-organ interferon gamma (IFN-γ) production by CD8+ T cells

Mice were inoculated with $3x10^5$ B16F10-luc cells on day 0. On day 6, mice were vaccinated with varying doses of (R)-DOTAP/Trp2 (with R-DOTAP at a constant dose of 300nmol/mouse). On day 13, mice were sacrificed and spleen, vaccine draining lymph node (right inguinal) and tumor draining lymph node (left inguinal) were removed from each mouse in a sterile hood. Organs were processed into a single cell suspension by crushing through a 70μm filter (BD Biosciences, San Jose, CA). After removal, cells were incubated in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with FBS, non-essential amino acids (NEAA) (Cellgro), antibiotic/antimycotic (Cellgro), 1μL/mL GolgiStop™ (BD Biosciences) and 5μM Trp2 or Ova peptide for 6h at 37°C. Cells were removed, washed with staining buffer (BD Pharmingen, San Diego, CA) and stained with anti-CD8 (clone 53-6.7). Cells were then treated with Cytofix/Cytoperm™ according to kit instructions (BD Pharmingen) and stained intracellularly with anti-IFN-γ (clone XMG1.2), washed and analyzed with a FACS Canto flow cytometer (BD Biosciences) and analyzed with FACS Diva software (BD Biosciences).

In vivo Cytotoxic T Lymphocyte (CTL) Assay

The *in vivo* CTL assay has been described previously and was performed here with slight modifications.[18, 21] Briefly, mice were inoculated with $3x10^5$ B16F10-luc cells on day 0 and vaccinated with (R)-DOTAP/Trp2 formulations on day 6. Seven days later, naïve mice were sacrificed and splenocytes removed. Splenocytes were pulsed with either 10μM Trp2 or Ova

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peptide for 1–2h in complete media at 37°C. Cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich, St. Louis, MO), with Ova peptide-pulsed and Trp2 peptide-pulsed cells stained with 0.4 and 4μM, respectively in serum free RMPI 1640 media for 15min. Cells were then washed with complete media (RMPI 1640 with FBS, NEAA and antibiotic/antimycotic) and counted. Equal numbers of $CFSE^{high}$ (Trp2-pulsed cells) and $CFSE^{low}$ (Ova-pulsed cells) were mixed together and stained with 8μM PKH-26 (Sigma-Aldrich) according to manufacturer's instructions. Vaccinated mice were injected with 10^7 labeled cells and *in vivo* killing of the targets was allowed to take place for 22h. After that time, spleens from treated mice were removed and processed into a single cell suspension and red blood cells lysed with ACK lysing buffer (Invitrogen). Cells were washed in complete media and resuspended in phosphate buffered saline (Sigma-Aldrich). The cells were analyzed with a FACS Canto flow cytometer (BD Biosciences) and FACS Diva software (BD Biosciences), first gating for the lymphocyte population, then for the PKH-26 positive cells, to determine the amount of specific lysis of the $CFSE^{high}$ Trp2-pulsed cells. The following equation from [21], describes *Ova* and *Trp2* representing the number of peptide-pulsed cells present after the *in vivo* killing time allotment.

$$\%\ \text{specific lysis} = \frac{(Ova*x - Trp2)}{(Ova*x)} * 100\%$$

$$\text{where } x = \frac{Trp2}{Ova} \text{ from naïve mice}$$

Tumor Infiltrating Lymphocyte Analysis

B16F10-luc tumors from mice vaccinated on day 6 were removed on day 13 in a sterile environment. Tumors were minced and processed through a 70μm strainer to form a single cell suspension. Cells were blocked with Fc block (anti CD16/CD32 (clone 2.4G2)) for 15 min, then stained with anti-CD3 (clone 145-2C11), anti-CD8 (clone 53-6.7) and anti-CD4 (clone RM4-5) and analyzed by flow cytometry.

Functionality of Tumor Infiltrating Lymphocytes

Alternately, lymphocytes were isolated from the tumors. Tumors were prepared as a single cell suspension (described above). The cells were washed and resuspended in complete media. Tumor infiltrating lymphocytes (TIL) were separated from the tumor using Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden), a density gradient method. Briefly, the single cell suspension of tumor cells were resuspended in 8mL of complete media (RPMI 1640 with 10% FBS, NEAA and antibiotic/antimycotic). This volume was layered over 4mL of Ficoll-Paque™ PLUS in a 15mL conical centrifuge tube and centrifuged at 2,500rpm for 30min at 25°C. After centrifugation, the cells at the density interface were removed, and washed with complete media. To assay for $CD8^+$ cell activity, tumor infiltrating lymphocytes were plated at a concentration of $2x10^6$ cells/mL and stimulated with 5μM Trp2 or Ova peptide for 6h at 37°C. The TIL were incubated with Fc Block, anti-CD8, stained intracellularly for IFN-γ and analyzed by flow cytometry as described above.

Statistical Analysis

Statistical analysis was completed by using a two-tailed Student's t-test. Data was qualified as statistically significant if the *P* value was less than 0.05.

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Results

Formulation Characterization

Maximum solubility of Trp2 peptide in water was measured at 1.825mM after conversion to a disodium salt, compared to 0.06mM of the trifluoracetate salt in water, an increase of 30.4 fold.

Our method of preparing (R)-DOTAP/Trp2 complexes by thin film hydration and extrusion formed stable nano-sized complexes. We observed decreasing size with increasing concentration of Trp2 incorporated, ranging from 120 to 87nm, for 0 to 1.5mM Trp2 used for hydration, respectively (Figure 1A). All particles had a polydispersity index measurement less than 0.1 (data not shown). Likewise, we observed a decrease in zeta potential with increasing Trp2 dose incorporated, as the Trp2 peptide formulated as a disodium salt, contains two exposed negative charges (on the aspartic acid and C terminus) after conversion from a trifluoroacetate salt (pH 4) to a disodium salt (pH 9). Percent entrapment was consistently high over a range of Trp2 doses (Figure 1B). Stability of the complexes was measured over two weeks at 4°C, showing consistent size (no aggregation or flocculation) (Figure 1C). TEM images of (R)-DOTAP/Trp2 (75nmol) show rod-like particles with an average length and width of 78.8 ±29.8 and 32.9 ±6.8nm, respectively (Figure 1D).

Tumor Growth Delay by Therapeutic Vaccination

Mice were inoculated with tumors on day 0, treated with (R)-DOTAP/Trp2 vaccines on day 6 with varying concentrations of Trp2 peptide, and tumor growth was followed for 20 days (Figure 2). While (R)-DOTAP/Trp2 nanoparticles delivering 5nmol and 25nmol Trp2 had no effect on tumor growth delay, delivery of 75nmol Trp2 adjuvanted by (R)-DOTAP was able to show statistically significant tumor growth delay, with $P<0.005$ compared to control mice. Shown in the Figure 2 inset, body weight of these tumor-bearing mice was also monitored, and while no statistical difference exists, there was observable weight gain (due to tumor size) in control, (R)-DOTAP/Trp2 (5nmol), and (R)-DOTAP/Trp2 (25nmol) groups, that was not seen in the (R)-DOTAP/Trp2 (75nmol) group.

Multi-Organ IFN-γ Production by $CD8^+$ T cells from Vaccinated Mice

Seven days after vaccination with various (R)-DOTAP/Trp2 nanoparticles, mice were sacrificed and lymph organs of interest extracted and processed to single cell suspensions to analyze Trp2 responsiveness in $CD8^+$ populations. Cells from the spleen, tumor-draining lymph node and vaccine draining lymph node (inguinal for both) were treated with Trp2 or a non-specific peptide (Ova), as well as GolgiStop™ to prevent protein secretion for 6h and then stained for $CD8^+$ cells as well as intracellular IFN-γ accumulation (Figure 3). There was no background from Ova-pulsed cells secreting IFN-γ for any group in any organ. Naïve mice, tumor-bearing mice, 75nmol Trp2 peptide alone, and the lowest dose of (R)-DOTAP/Trp2 (5nmol) showed no response to Trp2 pulse in any lymph organ.

Of the organs examined, splenocytes showed the highest population of responding cells, with mice receiving the (R)-DOTAP/Trp2 (75nmol) vaccination producing the greatest response at 0.3% of $CD8^+$ cells (Figure 3A). (R)-DOTAP/Trp2 (25nmol) also showed some response in $CD8^+$ splenocytes, but at a much lower level (0.1%). Vaccine-draining lymph node samples, showed an increased response in 75 and 25nmol Trp2 deliverable dose (0.1%) (Figure 3B). Tumor-draining lymph nodes also showed the same response for 75 and 25nmol Trp2 deliverable dose (0.1%), which possibly indicates the contribution of the tumor close to the skin's surface, dampening the strong response of the 75nmol formulation to the 25nmol level at those locations (Figure 3B, C).

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In vivo Cytotoxic T Lymphocyte Assay

Seven days after vaccination, tumor-bearing mice were injected with Trp2- and Ova-pulsed targets labeled with different levels of CFSE for 22h before sacrificing the mice and removing the remaining cells (by processing the spleen). The purpose of this assay is to evaluate if a vaccinated mouse can show specific lysis of targets presenting the antigen from vaccination (Trp2 peptide). Unvaccinated tumor-bearing mice showed similar amounts of specific lysis as 75nmol Trp2 peptide alone, and (R)-DOTAP/Trp2 (5nmol) and (25nmol) doses (Figure 4). Seventy five nmol of peptide alone (without (R)-DOTAP), also showed negligible amounts of specific lysis. However, (R)-DOTAP/Trp2 (75nmol), showed significant specific lysis after 22h of *in vivo* incubation of Trp2- and Ova-pulsed targets. This indicates that not only the dose of Trp2, but also the inclusion of (R)-DOTAP is crucial to elicit a CTL response.

Tumor infiltrating lymphocytes

Seven days after vaccination, tumors were removed, minced and prepared as a single cell suspension to detect the infiltrating $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes (Figure 5). Twenty-five nmol and 75nmol Trp2 formulated in (R)-DOTAP were able to elicit recruitment of both $CD4^+$ and $CD8^+$ T cells into the solid tumor (Figure 5). Nearly 1% of the cells in the tumor mass of (R)-DOTAP/Trp2 (75nmol) vaccinated mice were $CD3^+CD8^+$ cells; 0.2% were identified as $CD3^+CD4^+$ cells. (R)-DOTAP/Trp2 (25nmol) had a less profound effect, but quantifiable amounts of $CD8^+$ and $CD4^+$ T cells (0.3% and 0.1%, respectively). Other groups (untreated tumor, 75nmol Trp2 peptide alone, and (R)-DOTAP/Trp2 (5nmol)), showed lower than 0.1% $CD4^+$ or $CD8^+$ T cells out of the tumor mass.

When lymphocytes were isolated from tumors of vaccinated mice, reactivity to Trp2 was examined. After a 6h pulse with Trp2 or Ova peptide (and GolgiStop™), cells were examined for IFN-γ secretion (Figure 6). (R)-DOTAP/Trp2 (75nmol) showed statistically significant response to Trp2 compared to all other groups, nearly 6% of all $CD8^+$ TIL, with $P<0.005$. There was some background IFN-γ secretion by Ova-pulsed cells in tumors of (R)-DOTAP/Trp2 (75nmol) treated mice. Lower deliverable doses of Trp2 (25nmol and 5nmol), and unvaccinated mice showed Trp2 reactivity by less than 1% of $CD8^+$ TIL.

Discussion

Even with current advances in melanoma therapy (anti-CTLA4 mAb and small molecule BRAF inhibitors), there still exists an opportunity to innovate and further refine therapeutic treatment for aggressive melanoma decreasing side effects and honing specificity. Data presented here has shown that peptide-based vaccines offer a safe and significant advancement against an aggressive murine melanoma solid tumor model.

Overcoming formulation difficulty of the Trp2 peptide

Others have made efforts to combat the difficulty in formulating Trp2 peptide in an aqueous solution, ranging from looking at modifying the epitope to conjugation to larger proteins to enhance delivery.

Tang et. al. investigated increasing Trp2 binding efficacy to HLA-A201 by modifying the anchor residues.[10] Modifying SVYDFFVWL to S**M**YDFFVWL (or a Trp2 2M modification) was delivered in comparable doses to what we've shown in this study. When 100μg (63.8nmol) was delivered with 50μg of the PADRE T helper epitope (AKFVAAWTLKAAA) in incomplete Freund's adjuvant (IFA) to a transgenic HLA-A2.1/K^b model, higher amounts of IFN-γ was elicited compared to vaccination with wild type peptide. Trp2 has also been modified into (S**I**YDFFVWL), called "deltaV" which showed

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increased T cell responsiveness to Trp2 by intracellular IFN-γ staining. However, when deltaV was delivered with poly(I:C) and anti-CD40 and later boosted with these two adjuvants, no increased growth delay was seen with in a prophylactic B16F10 model compared to wild type peptide.[11] Other inventive ways to deliver Trp2 to APCs have been published. Kou et. al. developed a ScFv-peptide conjugate to target Trp2 to $CD11c^+$ dendritic cells *in vitro*.[12] This conjugate has shown stimulation of T cell proliferation, but Trp2 peptide alone showed comparable levels in the study. As a self-antigen, suppression mechanisms also exist to prevent the expansion of T cells in a tumor environment, thus the difficulty of low doses of Trp2 in (R)-DOTAP/Trp2 (5nmol) and (25nmol) in eliciting a IFN-γ response (Figure 3A).

Common models for murine melanoma rarely include therapeutic treatments in advanced solid tumors

An advanced solid tumor model is rarely investigated in Trp2 peptide-based vaccines; more often, a prophylactic model or CTL characterization is tested.[10–13] However, it is unlikely that a prophylactic melanoma vaccine would ever be commercially developed. The potential side effect of vitiligo is not likely to be traded for the possibility of protection, particularly as Trp2 peptide reactivity has been tied to this autoimmune depigmentation.[22]

Trp2 peptide encapsulated in liposomes with p53 peptide, PADRE T helper peptide, CpG oligos together to was able to prevent tumor establishment when delivered six days after inoculation of $1x10^4$ B16F10 cells.[14] However, as a measure of tumor challenge, only two-thirds of the control group ever established tumors. Another liposomal treatment required CpG to show a therapeutic response, with two doses of liposomal Trp2 and a separate formulation of liposomal CpG delivered in a $1x10^5$ B16F10 subcutaneous model.[15] However it is not clear from the data published from either study, that tumors were palpable or even visible when treated. Our choice of an advanced tumor model, $3x10^5$ B16F10-luc cells for subcutaneous inoculation, was based on all mice having measurable tumors on day 6, compared to inoculation with fewer cells (data not shown). Also, while CpG is a well characterized TLR9 agonist, the elegance of liposomal (non-viral) delivery is not aptly executed by the above two studies, with multiple adjuvants and multiple delivery devices needed. Our adjuvant, (R)-DOTAP also acts as the carrier for Trp2 peptide, simplifying the vaccine to active ingredients only, one stable and versatile particle that can show response against an established tumor (Figure 1, Figure 2).

Viral delivery of Trp2 peptide or protein has shown non-robust immunity compared to the statistically significant tumor growth delay we see with (R)-DOTAP/Trp2 nanoparticles delivering 75nmol of antigen (Figure 2) in an aggressive solid tumor model. Recombinant adeno-associated virus (rAAV) carrying Trp2 cDNA delivered 22 days before tumor challenge was unable to induce tumor growth delay.[16] Delivered at a dose of 10^{10} or 10^{11} rAAV viral particles were unable to protect against a tumor challenge of $3x10^5$ B16F10, even with the addition of other adjuvants, including CpG oligonucleotides and imiquimod. Interestingly, like (R)-DOTAP/Trp2 (25nmol), the rAAV-Trp2+CpG treatment showed an IFN-γ response from splenocytes, however, was not able to break tolerance and show any tumor growth delay. Multiple doses of Trp2 peptide conjugated to exposed lysines on the tobacco mosaic virus (TMV) was delivered with CpG oligonucleotides prior to inoculation with $5x10^4$ B16F1, showing no statistical difference compared to control or TMV with a different melanoma antigen p15e.[13] Additionally, in this challenge model, tumor penetration was 11 out of 12.

Dendritic cells pulsed with Trp2 peptide have also shown ineffective responses in both prophylactic and simultaneous tumor models.[7] However, when the HIV TAT protein transduction domain was ligated to a 472 amino acid sequence from the Trp2 protein or to

the Trp2 peptide (180–188 amino acids); both showed better tumor prevention compared to dendritic cells pulsed with Trp2 peptide only.[23, 24] This supports the need for an intracellular delivery mechanism like (R)-DOTAP/Trp2 formulations to ensure Trp2 peptide is presented on MHC class I. An interesting result of the above mentioned study showed *in vitro* CTL reactivity to B16 cells correlated with tumor size. Our *in vivo* CTL results on day 13, when all groups had statistically indistinguishable tumors, is an early indicator of the ability of a formulation to cause statistically significant tumor growth delay (Figure 4, Figure 2).

Our choice to use an aggressive subcutaneous model versus a lung metastatic model was to utilize the existence of a suppressive tumor microenvironment. Many types of suppressive immune cell exist in the tumor that tolerize the immune system to the tumor's presence, and combating these cell types with possible therapies should be the next frontier of immunotherapy.[25] With a larger tumor mass existing in the solid tumor model versus the metastatic model, the bar is higher for a vaccine to break this tolerance. In a model of transgenic Trp2 peptide specific T cells, the adoptive therapy of T cell transfer was able to cause statistically significant decrease in lung nodules (P>0.0001), however, had no effect whatsoever on any tumor growth delay in a solid tumor model.[26] The adoptive cell transfer was able to show nearly 98% specific lysis of Trp2 pulsed targets in an *in vivo* CTL assay, however, not able to reject the $2x10^5$ B16F10 solid tumor inoculation. Even with the addition of a Trp2-pulsed dendritic cell vaccine, no significant tumor growth delay was observed in the solid tumor model. While Trp2 specific T cells were detected in the solid tumor, they did not affect tumor growth, unlike our detection of infiltrating T cells that correlated with tumor growth delay (Figure 5, Figure 6).

Future directions in melanoma immunotherapies should tackle established tumors with an existing suppressive microenvironment. As shown by the ipilimumab approval and increasing numbers of biologic drugs; targeting suppressive mechanisms in and around the tumor, shutting down this protective barrier could empower immunotherapy to become the standard of care over conventional chemotherapy. Anti-CTLA4 antibodies are already being tested in a wide range of tumors, expanding the application beyond melanoma.[27] The ability and strength of (R)-DOTAP to break tolerance in this model after one immunization and the flexibility of the adjuvant to deliver an adequate dose of antigen in a stable formulation pushes it forward to be tested in other aggressive tumor models and possibly into clinical development.

Abbreviations

APCs	antigen presenting cells
CFSE	carboxyfluorescein succinimidyl ester
CTL	cytotoxic T lymphocyte
IFA	incomplete Freund's adjuvant
irAE	immune-related adverse events
MHC	major histocompatibility complex
rAAV	recombinant adeno-associated virus
(R)-DOTAP	(R)-1,2-dioleoyl-3-trimethylammonium-propane
TIL	tumor infiltrating lymphocytes
TMV	tobacco mosaic virus
Trp2	tyrosinase-related protein 2

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Acknowledgments

The authors graciously thank the NIH for funding (grant CA129421), and PDS Biotechnology for access to (R)-DOTAP. Elizabeth A. Vasievich is supported by a Pre-Doctoral Fellowship in Pharmaceutics from the PhRMA Foundation. Younjee Chung is thanked for her assistance in processing samples. Arlene Bridges and the ADME Mass Spectrometry Center in the Department of Pathology and Laboratory Medicine at UNC is thanked for the use of their Mass Spectrometry instrument. Feng Liu and Wei-Yun Sheng are thanked for thoughtful discussion with the authors surrounding the manuscript.

Literature Cited

1. Flaherty KT. Narrative review: BRAF opens the door for therapeutic advances in melanoma. Ann Intern Med. 2010; 153:587–591. [PubMed: 21041578]
2. Hersey P. Immunotherapy of melanoma. Asia Pac J Clin Oncol. 2010; 6 Suppl 1:S2–S8. [PubMed: 20482529]
3. Ko JM, Fisher DE. A new era: melanoma genetics and therapeutics. J Pathol. 2011; 223:241–250. [PubMed: 21125678]
4. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417:949–954. [PubMed: 12068308]
5. Puzanov I, Flaherty KT. Targeted molecular therapy in melanoma. Semin Cutan Med Surg. 2010; 29:196–201. [PubMed: 21051014]
6. Fedorenko IV, Paraiso KH, Smalley KS. Acquired and intrinsic BRAF inhibitor resistance in BRAF V600E mutant melanoma. Biochem Pharmacol. 82:201–209. [PubMed: 21635872]
7. Grauer OM, Sutmuller RP, van Maren W, et al. Elimination of regulatory T cells is essential for an effective vaccination with tumor lysate-pulsed dendritic cells in a murine glioma model. Int J Cancer. 2008; 122:1794–1802. [PubMed: 18076066]
8. Jakobisiak M, Golab J. Genetic modification of T cells improves the effectiveness of adoptive tumor immunotherapy. Arch Immunol Ther Exp (Warsz). 2010; 58:347–354. [PubMed: 20694520]
9. Liu G, Khong HT, Wheeler CJ, Yu JS, Black KL, Ying H. Molecular and functional analysis of tyrosinase-related protein (TRP)-2 as a cytotoxic T lymphocyte target in patients with malignant glioma. J Immunother. 2003; 26:301–312. [PubMed: 12843792]
10. Tang Y, Lin Z, Ni B, et al. An altered peptide ligand for naive cytotoxic T lymphocyte epitope of TRP-2(180-188) enhanced immunogenicity. Cancer Immunol Immunother. 2007; 56:319–329. [PubMed: 16906394]
11. McWilliams JA, McGurran SM, Dow SW, Slansky JE, Kedl RM. A modified tyrosinase-related protein 2 epitope generates high-affinity tumor-specific T cells but does not mediate therapeutic efficacy in an intradermal tumor model. J Immunol. 2006; 177:155–161. [PubMed: 16785510]
12. Kou G, Shi S, Wang H, et al. Preparation and characterization of recombinant protein ScFv(CD11c)-TRP2 for tumor therapy from inclusion bodies in Escherichia coli. Protein Expr Purif. 2007; 52:131–138. [PubMed: 17027283]
13. McCormick AA, Corbo TA, Wykoff-Clary S, Palmer KE, Pogue GP. Chemical conjugate TMV-peptide bivalent fusion vaccines improve cellular immunity and tumor protection. Bioconjug Chem. 2006; 17:1330–1338. [PubMed: 16984144]
14. Mansour M, Pohajdak B, Kast WM, et al. Therapy of established B16-F10 melanoma tumors by a single vaccination of CTL/T helper peptides in VacciMax. J Transl Med. 2007; 5:20. [PubMed: 17451606]
15. Jerome V, Graser A, Muller R, Kontermann RE, Konur A. Cytotoxic T lymphocytes responding to low dose TRP2 antigen are induced against B16 melanoma by liposome-encapsulated TRP2 peptide and CpG DNA adjuvant. J Immunother. 2006; 29:294–305. [PubMed: 16699372]
16. Triozzi PL, Aldrich W, Ponnazhagan S. Regulation of the activity of an adeno-associated virus vector cancer vaccine administered with synthetic Toll-like receptor agonists. Vaccine. 2010; 28:7837–7843. [PubMed: 20937315]
17. Chen W, Yan W, Huang L. A simple but effective cancer vaccine consisting of an antigen and a cationic lipid. Cancer Immunol Immunother. 2008; 57:517–530. [PubMed: 17724588]

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18. Vasievich EA, Chen W, Huang L. Enantiospecific adjuvant activity of cationic lipid DOTAP in cancer vaccine. Cancer Immunol Immunother. 2011
19. Li SD, Chono S, Huang L. Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles. J Control Release. 2008; 126:77–84. [PubMed: 18083264]
20. Chono S, Li SD, Conwell CC, Huang L. An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor. J Control Release. 2008; 131:64–69. [PubMed: 18674578]
21. Byers AM, Kemball CC, Moser JM, Lukacher AE. Cutting edge: rapid in vivo CTL activity by polyoma virus-specific effector and memory CD8+ T cells. J Immunol. 2003; 171:17–21. [PubMed: 12816977]
22. Rosenberg SA, White DE. Vitiligo in patients with melanoma: normal tissue antigens can be targets for cancer immunotherapy. J Immunother Emphasis Tumor Immunol. 1996; 19:81–84. [PubMed: 8859727]
23. Wang HY, Fu T, Wang G, et al. Induction of CD4(+) T cell-dependent antitumor immunity by TAT-mediated tumor antigen delivery into dendritic cells. J Clin Invest. 2002; 109:1463–1470. [PubMed: 12045260]
24. Shibagaki N, Udey MC. Dendritic cells transduced with TAT protein transduction domain-containing tyrosinase-related protein 2 vaccinate against murine melanoma. Eur J Immunol. 2003; 33:850–860. [PubMed: 12672050]
25. Vasievich EA, Huang L. The Suppressive Tumor Microenvironment: A Challenge in Cancer Immunotherapy. Mol Pharm. 2011
26. Singh V, Ji Q, Feigenbaum L, Leighty RM, Hurwitz AA. Melanoma progression despite infiltration by in vivo-primed TRP-2-specific T cells. J Immunother. 2009; 32:129–139. [PubMed: 19238011]
27. Calabro L, Danielli R, Sigalotti L, Maio M. Clinical studies with anti-CTLA-4 antibodies in non-melanoma indications. Semin Oncol. 2010; 37:460–467. [PubMed: 21074061]

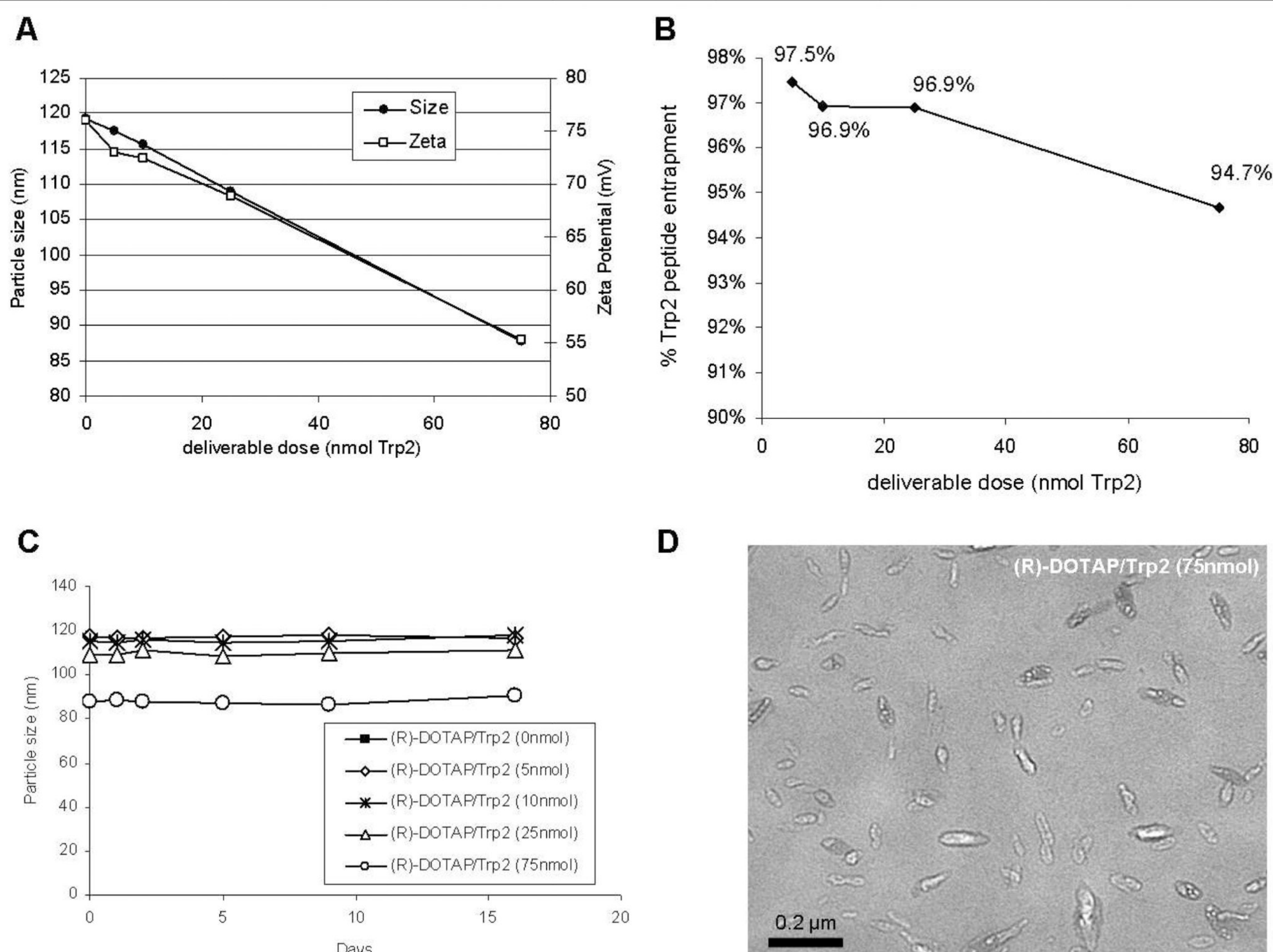

Figure 1. R-DOTAP/Trp2 characterization and stability

(A) Size, charge and (B) entrapment of Trp2 was evaluated on (R)-DOTAP/Trp2 complexes with varying concentrations of Trp2, n=3. (R)-DOTAP/Trp2 nanoparticles were stored at 4°C and (C) size measured over a period of two weeks, n=3. Experiment was repeated twice. In (C), the size measured from 0nmol Trp2 was the same as that with 5nmol Trp2, overlaying completely at every time point. (D) TEM image of (R)-DOTAP/Trp2 (75nmol) complexes. A total of 210 particles from 3 different fields were measured. Particle dimensions were calculated by measuring the longest dimension and the perpendicular diameter.

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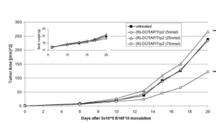

Figure 2. B16F10-luc tumor growth inhibition by (R)-DOTAP/Trp2 complexes *in vivo*
Six week old female C57BL/6 mice were inoculated with $3x10^5$ B16F10-luc cells s.c. in the abdomen on day 0. On day 6, treatments were s.c. injected into the opposite side of the abdomen. Lipid formulations delivered contained 300nmol (R)-DOTAP and varying doses of Trp2 peptide. Tumors were measured with calipers with area calculated as length times width. The inset represents body weight tracked over time. Seven mice were used per group. Experiment was repeated twice. *: $P<0.005$.

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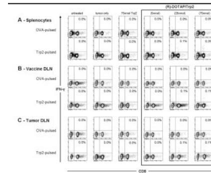

Figure 3. Multi-organ IFN-γ production from (R)-DOTAP/Trp2 vaccinated mice

(A) Splenocytes, (B) vaccine draining lymph nodes, or (C) tumor draining lymph nodes were isolated (on day 13) from tumor-bearing mice treated with (R)-DOTAP/Trp2 (300nmol (R)-DOTAP/ varying Trp2 doses) (on day 6). Resulting cells were pulsed with Trp2 or an irrelevant peptide (Ova) for 6h, washed, and stained for CD8 and IFN-γ. The numbers on the contour plots indicate percentage of IFN-γ^{+}CD8^{+} T cells out of all CD8^{+} T cells. Data from one representative mouse is shown, with five to seven total animals per group. Experiment was repeated twice.

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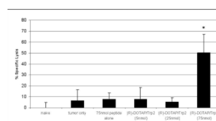

Figure 4. *In vivo* CTL shows specific lysis of Trp2-pulsed targets elicited by (R)-DOTAP/Trp2 (75nmol) vaccination

Targets pulsed with Trp2 or an irrelevant peptide (Ova) were stained with high (Trp2) or low (Ova) concentrations of CFSE, injected into (R)-DOTAP/Trp2 vaccinated mice and *in vivo* killing was allowed for 22h, then spleens removed and analyzed by flow cytometry. Percent specific lysis, $P<0.05$ between (R)-DOTAP/Trp2 (75nmol) and other groups. Three to five mice were used per group. Experiment was repeated twice.

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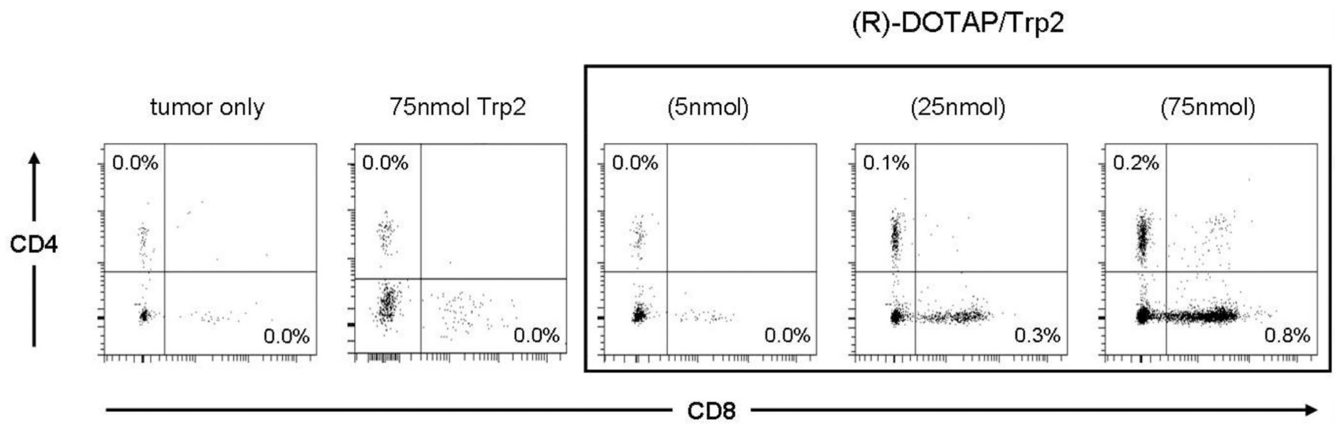

Figure 5. $CD3^+$ Tumor Infiltrating lymphocytes are higher numbers with higher vaccination doses

$CD4^+$ and $CD8^+$ tumor infiltrating T cells assayed from tumors of mice treated with varying doses of (R)-DOTAP/Trp2. Cells were gated on $CD3^+$, and percentages represent $CD3^+CD4^+$ or $CD3^+CD8^+$ out of total numbers of cells in the tumors. Data from one representative animal is shown, three to four mice were used per group. Experiment was repeated twice.

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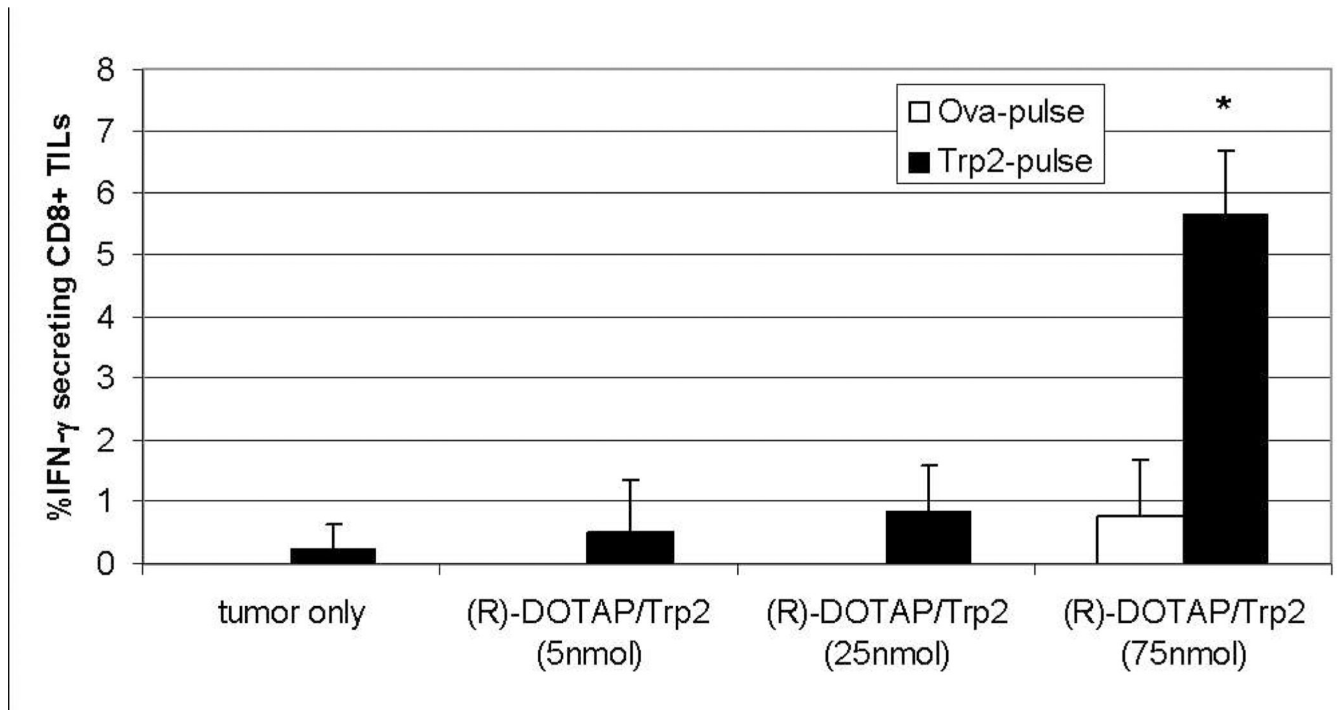

Figure 6. Tumor infiltrating lymphocytes respond to Trp2 after a high vaccination dose

TIL were isolated from tumors of mice vaccinated with varying doses of (R)-DOTAP/Trp2, pulsed for 6h with Trp2 or an irrelevant peptide (Ova) and analyzed for intracellular IFN-γ in $CD8^+$ cells. Percentages are IFN-γ^+CD8^+ cells out of all $CD8^+$ cells., *: $P<0.005$. Three mice were used per group. Experiment was repeated twice.