



Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8⁺ T cell-mediated anti-tumor immunity[☆]

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This paper is dedicated to the memory of Prof. John Samuel, who was a distinguished scientist in the field of cancer immunotherapy. Prof. Samuel supervised this project before he passed away on 17 April 2007.

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ABSTRACT

The purpose of this study was to evaluate the efficacy of poly(lactic-co-glycolic acid) (PLGA)-based vaccines in breaking immunotolerance to cancer-associated self-antigens. Vaccination of mice bearing melanoma B16 tumors with PLGA nanoparticles (NP) co-encapsulating the poorly immunogenic melanoma antigen, tyrosinase-related protein 2 (TRP2), along with Toll-like receptor (TLR) ligand (7-acyl lipid A) was examined. Remarkably, this vaccine was able to induce therapeutic anti-tumor effect. Activated TRP2-specific CD8 T cells were capable of interferon (IFN)- γ secretion at lymph nodes and spleens of the vaccinated mice. More importantly, TRP2/7-acyl lipid A-NP treated group has shown immunostimulatory milieu at the tumor microenvironment, as evidenced by increased level of pro-inflammatory cytokines compared to control group. These results support the potential use of PLGA nanoparticles as competent carriers for future cancer vaccine formulations.

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1. Introduction

The overall objective response rate to current cancer vaccine formulations is only 3.3% [1]. Among the various reasons for this failure in immune response, two important issues stand out: (1) current cancer vaccines can only induce “weak” qualitative and quantitative T cell responses; (2) the immunosuppressive tumor microenvironment inhibits anti-tumor T cell activity at the effector phase [2,3]. The major goal for improvement of response to cancer vaccines

is to develop immunotherapy strategies that can activate robust and lasting immune responses against cancer antigens and, at the same time, be able to reverse the ‘immunosuppressive milieu’ of the tumor microenvironment.

An ideal cancer vaccine formulation comprises three main components; first, an antigen against which the immune responses are induced. The second component is an adjuvant that acts as danger signal to alert the immune system and activate early as well as long-lasting immune responses. The third component is the delivery system that delivers vaccine antigen and adjuvant to the specialized antigen presenting cells (APCs), mainly dendritic cells (DCs), in a targeted and prolonged manner [4]. Among different systems tested, nanoparticles made of poly(D,L-lactic-co-glycolic acid) (PLGA) are of special interest. In addition to their biocompatibility and biodegradability, PLGA nanoparticles (PLGA-NP) offer great flexibility with respect to the manipulation of physicochemical properties of the polymer and the range of antigens and immunomodulators that they can accommodate. PLGA-NP are naturally targeted to DCs through phagocytosis since they have similar size to pathogens. Through phagocytosis particles can efficiently deliver antigens to DCs 100- to 1000-fold higher than what is attainable by non-particulated antigen [5]. Besides, PLGA-encapsulated antigens can be processed by two independent pathways, leading

[☆] BC1-005 (7-acyl lipid A) was recently synthesized by Oncothyreon, Inc. (formerly Biomira, Inc.) as a synthetic analogue of MPLA. Due to the similarity in structure between the two compounds (BC1-005 and MPLA), we had given BC1-005, the name “synthetic MPLA” in our earlier reports [13,14]. However, to avoid any confusion between the natural MPLA “produced and purchased from Galaxo” and the synthetic analogue “synthesized by Biomira”, we have recently decided to use the name “7-acyl lipid A” for BC1-005 (in Ref. [12] and in the current study) instead of synthetic MPLA. An erratum to our earlier publications [13,14] with this information will be submitted soon.

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to presentation by both MHC class I and class II molecules on the surface of DCs, with the final outcome of simultaneous activation of both CD8⁺ and CD4⁺ T cell immune responses, respectively [6].

The discovery of Toll-like receptors (TLRs) and their crucial role in orchestrating both innate and adaptive immune response have led to the development of an entire class of potent immunomodulatory adjuvants, namely TLR ligands. One of the most promising members in this class is monophosphoryl lipid A (MPLA), which has been recently approved for Hepatitis B vaccination in Europe [7] and has been used extensively in a variety of clinical vaccine testing (reviewed in Refs. [8–10]). MPLA is a chemically modified derivative of lipopolysaccharide (LPS), that exhibits potent adjuvant activity, but is up to 10,000-fold less toxic than parent LPS molecule [11]. 7-acyl lipid A, the novel synthetic analogue of lipid A, introduced in collaboration with Oncothyreon, Inc. (formerly Biomira, Inc., Edmonton, AB, Canada) has comparable immunostimulatory effects to MPLA, but harbor additional advantages such as reproducibility, feasibility for large scale production and better control over purity of the final products [12].

The long-term objective of this research is to achieve vaccines based on PLGA-NP that can induce effective T cell immune responses against cancer. Our research group has recently shown that co-delivery of ovalbumin (OVA) as model antigen, and 7-acyl lipid A in PLGA-NP enhances DC maturation and dramatically induces robust OVA-specific primary CD4⁺ and CD8⁺ T cell proliferative responses [13,14]. Moreover, vaccinating healthy C57Bl/6 mice with PLGA-NP co-encapsulating OVA and 7-acyl lipid A dramatically enhanced interferon (IFN)- γ secretion by both OVA-specific CD4⁺ and CD8⁺ T cells in both draining lymph nodes and spleens of vaccinated mice. This activation was superior to what we observed in mice immunized with OVA emulsified in complete Freund's adjuvant (CFA), one of the most powerful adjuvants used in animal studies (our unpublished results). Consistent with these results, in a recent study by Heit et al. [15] a single injection of PLGA microparticles co-encapsulating OVA and TLR9 ligand (CpG-oligodeoxynucleotide (ODN)) efficiently activated OVA-specific T cells and caused complete tumor regression in 80% of animals bearing OVA-expressing B16 melanoma (B16-OVA). These results clearly demonstrate the efficacy of PLGA nano/microparticles in delivering exogenous antigens and adjuvants into DCs and initiating robust T cell responses. However, the vigorous T cell activation can be partially attributed to the fact that chicken OVA is recognized as a foreign antigen (non-self) in mice. As a result, OVA-specific T cells could be easily activated, as they are not subjected to the regulations of central and peripheral tolerance. By contrast, the majority of cancer antigens belong to self. Accordingly, stimulating the immune system against those antigens requires breaking of self-tolerance mechanisms, which is more challenging and difficult to achieve. An elegant study by Bellone et al. [16], had compared the efficacy of three vaccination strategies (naked DNA, peptide-pulsed DCs or mixture of peptide and the *Escherichia coli* toxin LTR72) using either the foreign antigen OVA or the naturally expressed tyrosine related protein 2 (TRP2) tumor antigen in the B16-OVA or B16 melanoma models, respectively. They found similar level of cytotoxic T lymphocytes (CTL) activation and tumor protection against B16-OVA melanoma by all the three vaccines that used OVA antigen. However, when TRP2, a self-tumor-associated antigen, was employed, all vaccines elicited B16-specific CTLs, but only the TRP2-pulsed DCs were able to protect against B16 melanoma. Two conclusions can be made from these results: (1) the induction of *in vitro* CTL activity does not correlate with the *in vivo* anti-tumor activity; (2) foreign (non-self) antigens used in evaluating immunotherapeutic strategies can overestimate the therapeutic outcome and lead to bias in the validation of vaccine efficacy.

In the light of these findings, the purpose of the present study was to rigorously evaluate the efficacy of PLGA-based cancer vaccines using a realistic and clinically relevant tumor antigen, i.e., TRP2. The results of this study showed that co-delivery of TRP2 and 7-acyl lipid A in PLGA-NP is highly effective in inducing TRP2-specific CD8⁺ T cell responses capable of mediating therapeutic anti-tumor response in mouse B16 tumor model. More importantly, our vaccine strategy led to the reversal of immune suppressive milieu of the tumor microenvironment, as evidenced by increase in the level of pro-inflammatory T helper 1 (Th1)-related cytokines and decrease in the level of vascular endothelial growth factor (VEGF), a key factor required for tumor growth [17]. All together, our results support the potential use of PLGA-NP as competent antigen delivery systems, and further illustrated the superior therapeutic outcome of co-delivery 7-acyl lipid, a very promising immunostimulatory adjuvant, for future cancer vaccine trials.

2. Materials and methods

2.1. Reagents

Synthetic 7-acyl lipid A (also called BC1-005), M.Wt., 1955.5 Da was kindly provided by Oncothyreon, Inc. (formerly Biomira, Inc., Edmonton, AB, Canada). TRP2 peptide_{180–188} was purchased from BioSynthesis, Inc. (Lewisville, TX, USA). Polyvinyl alcohol (PVA), M.Wt., 31,000–50,000 Da was obtained from Sigma–Aldrich co. (Oakville, ON, Canada). PLGA co-polymer (monomer ratio 50:50, M.Wt., 7000 Da) was purchased from Absorbable Polymers International (Pelham, AL, USA). Chloroform, methanol, acetonitrile, water (all HPLC grades) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Murine CD8 isolation kit was purchased from StemCell Technologies (Vancouver, BC, Canada). Murine IFN- γ ELISPOT kit was purchased from E-Bioscience (San Diego, CA, USA). DMEM, RPMI media, L-glutamine, and gentamicin were purchased from Gibco-BRL (Burlington, ON, Canada). Fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, UT, USA). Murine IL-6, IL-2, IFN- γ and TNF- α ELISA kits were purchased from E-Bioscience (San Diego, CA, USA). Murine IL-12 and VEGF ELISA kits were purchased from BD Biosciences (Mississauga, ON, Canada) and R&D Systems (Minneapolis, USA), respectively.

2.2. Preparation of PLGA-NP encapsulating TRP2_{180–188} with or without 7-acyl lipid A

Nanoparticles of PLGA containing TRP2 peptide with or without 7-acyl lipid A were prepared by water/oil/water double emulsion/solvent evaporation method. Briefly, TRP2 peptide was dissolved in 75% acetonitrile in water to make 10 mg/mL solution. From this solution 100 μ L was emulsified with PLGA solution in chloroform (300 μ L, 50%, w/v) using a microtip sonicator (Heat systems, Inc., Farmingdale, NY, USA). For the preparation of nanoparticles containing 7-acyl lipid A, 200 μ g of 7-acyl lipid A in 100 μ L of 1:4 methanol–chloroform mixture was added to the polymer–chloroform solution. The resulting primary emulsion (water/oil) was further emulsified in 2 mL of PVA solution (9%, w/v PVA in PBS) by sonication for 45 s at level 4. The secondary emulsion was added drop-wise into 8 mL of stirring PVA solution. Nanoparticles were collected after 3 h of stirring by centrifugation of the emulsion at 40,000 \times g for 10 min at 4 °C. The nanoparticles were washed twice with cold deionized water and lyophilized. The particle size of the nanoparticles was determined by dynamic light scattering technique using a Zetasizer 3000 (Malvern, UK). Quan-

Table 1
Chromatographic gradient program over LC–MS analysis time (15 min)

Time (min)	Mobile phase A ^a (%)	Mobile phase B ^b (%)	Flow rate (mL/min)	Gradient curve
0	0	100	0.2	1
10	70	30	0.2	5
11	0	100	0.2	1

^a Mobile phase A is acetonitrile.

^b Mobile phase B is 0.05% trifluoroacetic acid (TFA)/water.

tification of 7-acyl lipid A content in PLGA-NP was done by liquid chromatography–mass spectrometry (LC–MS) as reported previously [12].

2.3. Quantification of encapsulated TRP2_{180–188} in nanoparticles by LC–MS

2.3.1. Establishment of LC–MS method for the quantification of TRP2_{180–188}

LC–MS analyses were performed using a Waters Micromass ZQ 4000 spectrometer, coupled to a Waters 2795 separations module with an autosampler (Milford, MA, USA). The mass spectrometer was operated in positive ionization mode with selected ion recording (SIR) acquisition. The nebulizer gas was obtained from an in house high purity nitrogen source. The temperature of source was set at 150 °C, and the voltages of the capillary and cone were 3.10 kV and 9 V, respectively. The gas flow of desolvation and the cone were set at 550 and 90 L/h, respectively. Chromatographic separation was achieved using a Waters (Milford, MA, USA) XTerra_{MS}C18 3.5 μm (2.1 mm × 50 mm) as the stationary phase. The mobile phase consisted of two solutions; solution A (acetonitrile) and solution B (0.05% trifluoro acetic acid (TFA)/water). The mobile phase was delivered at a constant flow rate of 0.2 mL/min. The gradient conditions are shown in Table 1. Lansoprazole was used as the internal standard (I.S). Single ion recording (SIR) at *m/z* 1175.2 and 369.9, related to M + H were selected for quantification of TRP2 peptide and the internal standard, respectively (Fig. 1A). Fig. 1B shows the SIR chromatograms of internal standard and TRP2_{180–188}. The peak of TRP2_{180–188} was well separated from that of the internal standard in the established chromatographic condition. The retention times of the internal standard and TRP2_{180–188} were approximately 7 and 8 min, respectively. The analytical run time was 15 min. The regression analysis was constructed by plotting the peak–area ratio of TRP2_{180–188} to I.S (response factor) versus TRP2_{180–188} concentration (μg/mL). The calibration curve was linear within the range of 1.5 μg to 50 μg/mL. The correlation coefficient (*R*²) was always greater than 0.99 (Fig. 1C), indicating a good linearity.

2.3.2. Extraction and quantification of TRP2_{180–188} in PLGA-NP

Extraction of TRP2_{180–188} from PLGA-NP was done by dispersing 5 mg of nanoparticles in 300 μL of 85% acetonitrile in H₂O, followed by centrifugation at 15,000 × *g* for 15 min. The PVA was precipitated and the supernatant (PLGA + peptide) was transferred into eppendorf tube. The solvent (acetonitrile and H₂O) was evaporated using Thermo Savant SpeedVac System. Since the peptide is soluble in methanol (unlike PLGA), the residue was dissolved in 500 μL methanol, followed by centrifugation at 15,000 × *g* for 15 min. The supernatant was then assayed for TRP2_{180–188} by LC–MS. The lowest panel of Fig. 1A shows the mass spectrum of TRP2_{180–188} after extraction from PLGA-NP, which implies that intact peptide was successfully incorporated into the nanoparticle formulation.

2.3.3. Standard and stock solutions

The stock solutions were prepared by dissolving 10 mg of TRP2_{180–188} in 1 mL of 75% acetonitrile in water. The stock solution was stored at –20 °C between experiments. The working solution of TRP2_{180–188} was prepared fresh each day by making a 100-fold dilution of the stock solution in methanol. The calibration standards were then prepared by serial dilution of the working solution. The stock solution of the internal standard was prepared by dissolving 10 mg of lansoprazole A in 1 mL methanol, followed by the preparation of a working solution of 100 μg/mL by a further 100-fold dilution of the stock solution. The stock solution of the internal standard was stored at –20 °C between experiments, and the working solution was prepared fresh at each experiment.

2.4. Animal studies

2.4.1. Mice

Mice (C57Bl/6) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All experiments were performed in accordance to the University of Alberta guidelines for the care and use of laboratory animals. All experiments were performed using 10–16 week old male mice.

2.4.2. Normal mice vaccination experiment

The efficacy of our vaccine formulations were first tested on normal C57Bl/6 mice (with no tumor). Animals (5 mice/group) were subcutaneously (s.c.) vaccinated in right flank region with approximately 10 μg TRP2_{180–188} encapsulated in PLGA-NP with or without ~17 μg 7-acyl lipid A (abbreviated as TRP2/7-acyl lipid A-NP and TRP2-NP, respectively). Control group mice received 10 mg plain PLGA-NP (Empty-NP). Eleven days later all mice received similar booster immunization. Seven days after the second immunization, draining lymph nodes and spleens were isolated and the ELISPOT assay was performed (see details below).

2.4.3. Tumor therapy experiment

B16-F10 cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 100 IU/mL penicillin/streptomycin in 5% CO₂ atmosphere. At day 0, C57Bl/6 mice were injected s.c. at their upper right flank with 0.1 × 10⁶ B16-F10 melanoma cells obtained from 90–95% confluent cultures. Three days later (day 3), animals were randomly assigned to three treatment groups (8–10 mice per group). Similar to normal mice vaccination study, the three groups were s.c. vaccinated (in the lower right flank region) with either Empty-NP, TRP2-NP or TRP2/7-acyl lipid A-NP. Animals were given booster immunization with the same formulations at days 7 and 13. Palpable tumors start to appear between day 7 and day 10. Tumor size was measured with vernier caliper, starting at day 7 and then every 2–3 days until day 21. The longest length and the length perpendicular to the longest length were multiplied to obtain the tumor size (area) in mm². Animals were observed every day, and were euthanized when tumor area exceeded 300 mm² or when ulceration of the tumors was observed. On day 21 all the animals were sacrificed and draining lymph nodes, spleens and tumors were

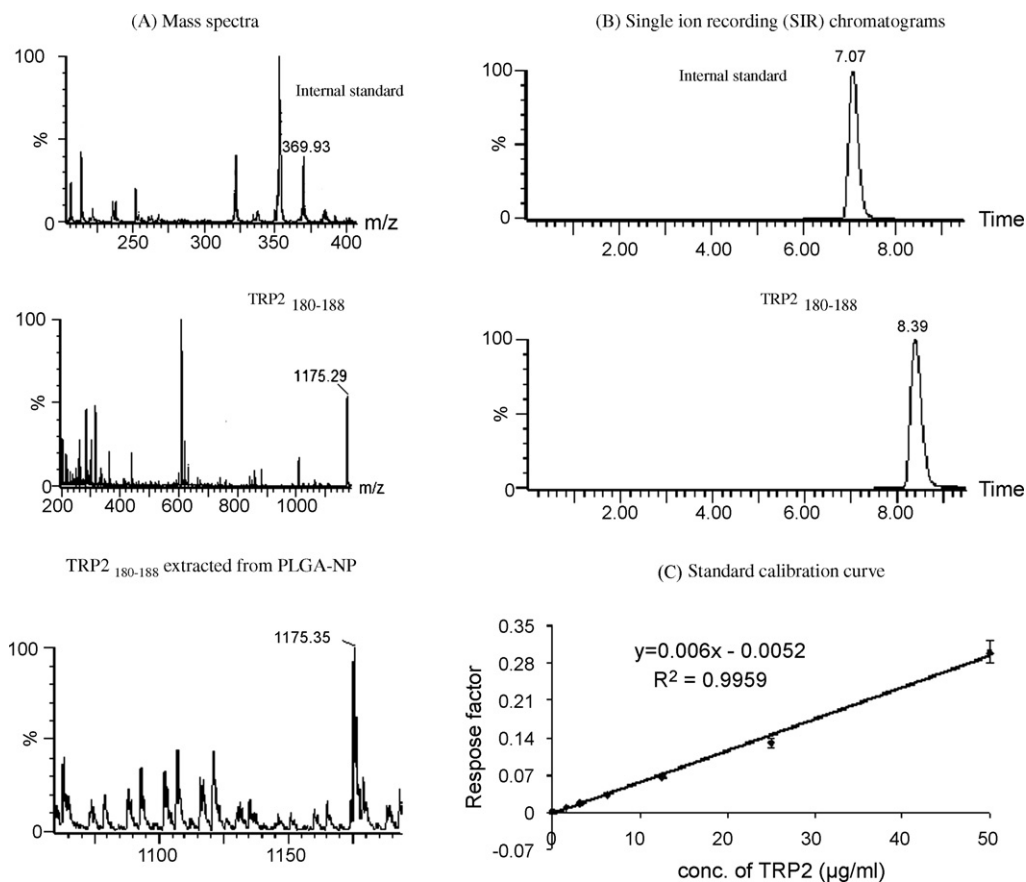


Fig. 1. Liquid chromatography–mass spectrometry (LC–MS)–based method for TRP2_{180–188} quantification. (A) Mass spectra of lansoprazole, the internal standard (I.S.), TRP2_{180–188} solution and TRP2_{180–188} peptide extracted from PLGA-NP. (B) SIR chromatograms of the I.S. and TRP2_{180–188}. Gradient conditions are summarized in Table 1. (C) A representative standard curve for TRP2_{180–188} extending from 1.5 to 50 μg/mL. The calibration curves were constructed by plotting average peak–area ratio of TRP2_{180–188} to the internal standard (response factor ± S.D.) on y-axis vs. TRP2_{180–188} concentration (μg/mL) on x-axis.

isolated for further analysis. Weights of individual tumors were reported and used as a tool to calculate the percentage of mice (for each treatment group) that had controlled tumor growth throughout the study, using the following formula: (number of the mice that have tumor weight less than 0.3 g at day 21/total number of mice in that group) × 100.

2.4.4. Assessment of vaccine-induced immune stimulation

2.4.4.1. Enzyme-linked immunospot (ELISPOT) assay. For the ELISPOT assay, spleens and draining lymph nodes (from either normal or tumor-bearing mice) were isolated and washed three times in PBS. Lymphocytes (from lymph nodes) were suspended at 1×10^7 cells/mL in complete RPMI media with 1% penicillin–streptomycin, 1% L-glutamine, and 5% FCS. On the other hand, splenocyte cell suspensions were divided into two portions; first portion was treated by ACK lysis buffer (156 mM NH₄Cl, 10 mM KHCO₃, 100 μM EDTA) for removal of red blood cells (RBCs). Briefly, 5 mL of ACK lysis buffer was added (per spleen), followed by 1 min incubation at room temperature. Splenocytes were then washed twice in cold plain RPMI media and re-suspended at 1×10^7 splenocytes/mL in complete RPMI. Second part of splenocyte cell suspension underwent CD8⁺ T cell isolation, using EasySep[®] mouse CD8 isolation kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Isolated CD8⁺ T cells were washed twice in PBS and resuspended at 1×10^7 cells/mL in complete RPMI. Lymph nodes, splenocytes and isolated CD8⁺ T cells were then plated in the pre-coated ELISPOT plates, as described below.

Ninety-six-well MultiScreen[™] filter plates (Millipore, Bedford, MA, USA) were coated overnight at 4 °C with 100 μL/well of IFN-γ-specific capture antibody (anti-mouse IFN-γ, clone AN-18, eBioscience, San Diego, CA, USA), diluted in sterile PBS according to the manufacturer's instructions. After the overnight incubation, the coating antibody solution was decanted from the plates. The plates were washed twice with 200 μL/well PBS and then blocked for 1 h at room temperature with 200 μL/well of complete RPMI-1640 for. After blocking, plates were decanted and cells (lymph nodes, splenocytes or isolated CD8⁺ T cells) were added into individual wells (in triplicates) in complete RPMI medium at 1×10^6 cells/well. In addition, using lymphocytes isolated from normal mice (vaccinated from TRP2/7-acyl lipid A-NP), two additional cell numbers were plated; 0.5×10^6 and 2×10^6 cells/well.

Cells were then stimulated by 20 μM of either CD8 irrelevant epitope (SIINFEKL) or positive epitope (TRP2_{180–188}). In negative control wells 100 μL/well of complete RPMI media was added (non-stimulated). The cells were incubated at 37 °C for 18 h in the presence of 5% CO₂ and then washed three times with 0.05% Tween in PBS (PBS-Tween). The detection antibody (Biotin anti-mouse IFN-γ, clone R4-6A2, eBioscience, San Diego, CA, USA), was diluted in 1% bovine serum albumin (BSA) in PBS according to the manufacturer's instructions. Diluted solution was added at a volume of 100 μL per well. After 2 h incubation at room temperature, plates were washed four times with PBS-Tween and incubated for 45 min at room temperature with Streptavidin-HRP (eBioscience, San Diego, CA, USA). Plates were then washed three times with PBS-Tween followed by two times wash with PBS. Spots were developed

by adding 100 μ L/well of freshly prepared AEC (3-amino-9-ethyl carbazole) Substrate Solution (BD Biosciences, Mississauga, ON, Canada). Stopping the substrate reaction was done after 20–30 min by washing three times with distilled water. Plates were then dried and spots were counted in a BioReader 3000 (BioSys, Karben, Germany).

2.4.4.2. Enzyme-linked immunosorbent assay (ELISA). Vaccine-induced alteration in the level of pro-inflammatory cytokines and immuno-suppressive factors in tumor microenvironment were assessed by ELISA. Briefly, isolated tumors were crashed between two slides to form uniform cell suspensions, which were filtered through 70 μ m cell strainers and then counted. Tumor supernatants obtained after centrifugation of approximately 20 million tumor cells were analyzed for the level of TNF- α , IL-12, IFN- γ , IL-2, IL-6 and VEGF by ELISA using the commercially available ELISA kits in a 96 well microplate using a microplate reader (Powerwave with KC Junior software; Bio-Tek, Winooski, VT, USA) at OD of 450 nm according to the manufacturer's directions. The minimum detection levels of the cytokines were: 7, 62, 15, 2, 10 and 7.8 pg/mL for TNF- α , IL-12, IFN- γ , IL-2, IL-6 and VEGF, respectively.

2.4.5. Statistical analysis

The significance of differences among groups was analyzed either by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test for multiple comparisons. Before executing the ANOVA, data were tested for normality and equal variance. If neither of the latter criteria were met, data were compared using a Kruskal–Wallis one-way ANOVA on ranks. *P*-value of ≤ 0.05 was set for the significance of difference among groups. The statistical analysis was performed with SigmaStat software (Systat Software, Inc. San Jose, CA, USA).

3. Results

3.1. Characterization of PLGA-NP

The mean hydrodynamic diameter of nanoparticles ranged between 350 and 410 nm with a polydispersity below 0.2. The efficiency of the method of TRP2_{180–188} extraction from PLGA-NP was 87.6%. Based on LC–MS analysis the encapsulation efficiency for TRP2_{180–188} was $5.2 \pm 0.6\%$ and the loading was $0.94 \pm 0.11 \mu$ g TRP2_{180–188} entrapped (per 1 mg dry weight of NP). The encapsulation efficiency for 7-acyl lipid A was $67.3 \pm 6.9\%$ and the loading was $1.79 \pm 0.18 \mu$ g 7-acyl lipid A entrapped (per 1 mg dry weight of NP).

3.2. Co-delivery of TRP2_{180–188} and 7-acyl lipid A in PLGA-NP induces IFN- γ secretion by TRP2-specific CD8⁺ T cells in lymph nodes and spleens of the vaccinated mice

Despite the low encapsulated levels of TRP2_{180–188} in PLGA-NP, we were interested to see whether vaccination with such low amount of the peptide ($\sim 10 \mu$ g) will induce antigen-specific CD8⁺ T cell activation in healthy C57BL/6 mice. In this experiment, wild-type C57Bl/6 mice were vaccinated twice (11 days apart) and the induction of IFN- γ -producing CD8⁺ T cells was investigated by a single-cell, *ex vivo* ELISPOT assay. In the beginning, we performed a titration study using lymphocytes from the test group (OVA/7-acyl lipid A-NP immunized mice) to optimize cell number per well, that give the best results (higher number of antigen-specific T cells and lowest background in wells stimulated with medium or irrelevant peptide). The result of this titration study is shown in Fig. 2A. Actual number of spots/well \pm standard deviation (S.D.)

is presented in the bar graph in the right panel of Fig. 2A. Number of IFN- γ secreting antigen-specific T cells increased as the number of lymphocytes/well increased. When we used 0.5×10^6 lymphocytes/well, the number of antigen-specific T cells was relatively low. With 1×10^6 lymphocytes/well, there was clear increase in the number of IFN- γ secreting cells with acceptable background. However, the background was very high when we used 2×10^6 lymphocytes/well. Based on these observations, we concluded that the optimum cell number per well is 1×10^6 lymphocytes.

Fig. 2B shows the number of IFN- γ secreting cells/million lymphocytes for the three groups tested. The actual number of spots/well \pm S.D. is presented in the bar graph in the lower panel. Immunization with Empty-NP did not induce any measurable amount of IFN- γ . Immunization with both TRP2-NP and TRP2/7-acyl lipid A-NP induced comparable numbers of IFN- γ secreting T cells (113.5 ± 5 and 145.6 ± 10), respectively. However, TRP2/7-acyl lipid A-NP immunized group induced higher levels of IFN- γ secretion as evidenced by the higher density of the spots (Fig. 2B).

The isolated spleens were treated using either ACK lysis buffer that only removes RBCs or negative selection of only CD8⁺ T cells. Relatively high background was observed in the negative control wells of ACK-lysed spleens of TRP2/7-acyl lipid A-NP immunized mice (Fig. 2C). However, when only CD8⁺ T cells were plated, the background level dropped almost to zero (Fig. 2D). The actual number of spots/well \pm S.D. is presented in the bar graph in the lower panels in Fig. 2C and D. Mice immunized with TRP2/7-acyl lipid A-NP had significantly high numbers of IFN- γ producing TRP2-specific CD8⁺ T cells, compared to Empty-NP immunized mice (60- and 65-fold in Fig. 2C and D, respectively). Unlike lymph nodes, where immunization with either TRP2-NP or TRP2/7-acyl lipid A-NP gave comparable number of TRP2-specific IFN- γ secreting CD8⁺ T cells, in spleens co-delivery of 7-acyl lipid A along with TRP2 in the same NP formulation led to 3- and 12-fold increase in the number of TRP2-specific IFN- γ secreting CD8⁺ T cells (compared to TRP2-NP) using either ACK lysed spleens (Fig. 2C) or isolated CD8 T cells (Fig. 2D), respectively.

3.3. Co-delivery of TRP2_{180–188} and 7-acyl lipid A in PLGA-NP induced potent therapeutic anti-tumor immunity

3.3.1. Tumor growth and size measurements

Three s.c. injections of TRP2-NP appeared to slow down the growth of the tumors compared to what observed in the Empty-NP immunized group (Fig. 3A), although the difference between average tumor sizes of the two groups was not statistically different. Co-delivery of 7-acyl lipid A together with TRP2_{180–188} in the same NP formulation also slowed down tumor development compared to Empty-NP group ($p < 0.05$ in days 17 and 20). Although there was no statistical difference between TRP2-NP and TRP2/7-acyl lipid A-NP immunized groups, the average tumor size of animals immunized with TRP2/7-acyl lipid A-NP were almost half the average size obtained in TRP2-NP immunized animals at all times tested. Large intra-group variability might have impaired the significance of difference for results obtained from these two groups. Conducting the experiment with higher number of mice is needed to clarify the difference between the treatment groups further.

Number of mice/vaccination group ranged from 8 to 10, with 8, 9 and 10 mice immunized with Empty-NP, TRP2-NP and TRP2/7-acyl lipid A-NP, respectively. Whereas all the 10 mice immunized with TRP2/7-acyl lipid A-NP have survived until the end point of the study (day 21), one animal from both Empty-NP and TRP2-NP groups had to be euthanized due to large tumor growth.

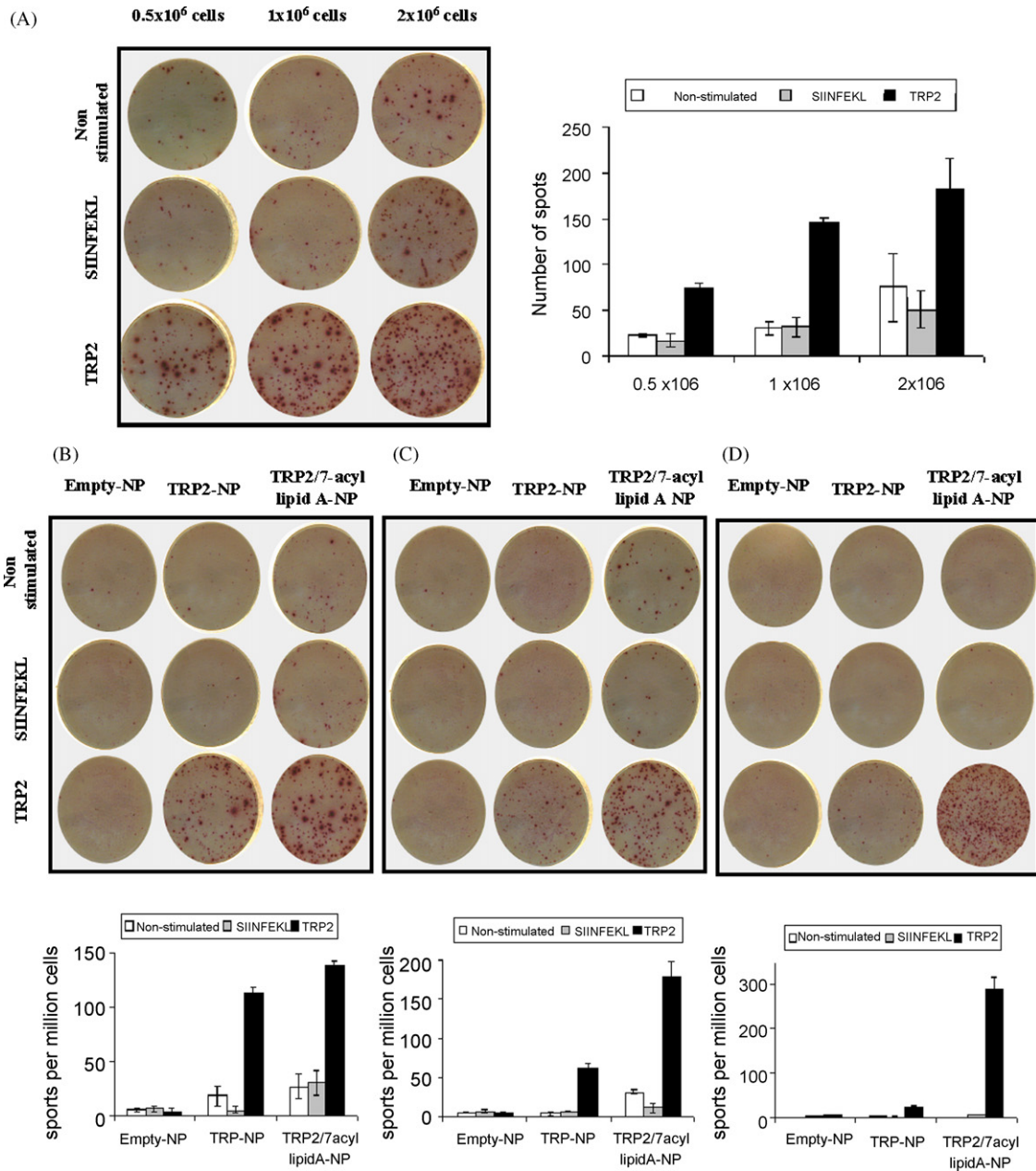


Fig. 2. Vaccination of healthy mice with TRP2_{180–188} containing NP induces IFN- γ secretion by TRP2-specific CD8⁺ T cells in lymph nodes and spleens of the vaccinated mice. C57Bl/6 mice were s.c. vaccinated in right flank region with approximately 10 μ g TRP2_{180–188} peptide encapsulated in PLGA-NP with or without \sim 17 μ g 7-acyl lipid A (TRP2/7-acyl lipid A-NP and TRP2-NP, respectively). Control group mice received 10 mg plain PLGA NP (Empty-NP). Eleven days later all mice received similar booster immunization. Seven days after the second immunization, draining lymph nodes and spleens were isolated and the ELISPOT assay was performed (as described in text). IFN- γ secretion was measured in the vaccinated groups as evidenced by the number of spots/million cells after overnight stimulation with either media alone (non-stimulated) or 20 μ M of either CD8 irrelevant peptide (SIINFEKL) or test peptide (TRP2_{180–188}). (A) Lymphocytes isolated from TRP2/7-acyl lipid A-NP immunized groups were used for optimization of cell number/well. Numbers of spots per different cell numbers/well are shown in the bar graph to the right side. Using 1 million cells/well, IFN- γ secretion was assessed in (B) lymph nodes (C and D) and spleens of the vaccinated mice. Isolated splenocytes were either treated with ACK lysis buffer to lyse red blood cells (C). Alternatively, CD8⁺ T cells were isolated from spleens using CD8⁺ negative isolation kit (D). Numbers of spots/million cells for different treatment groups are shown in the bar graphs below. The values in those graphs are averages of triplicate wells \pm S.D. Data shown are representative of three independent experiments that gave similar results.

3.3.2. Tumor weight measurements

After 21 days from tumor cell injections, the mice were sacrificed, tumors were isolated from each mouse and weighted separately. Results are presented in Fig. 3B as individual tumor weights (in g) for all the mice that survived until the endpoint of the study (7, 8 and 10 mice for Empty-NP, TRP2-NP and TRP2/7-acyl lipid A-NP immunized groups, respectively). In the group immunized with Empty-NP, all the animals developed large tumors (>0.3 g), with the exception of only one mouse that had a smaller

tumor (\sim 0.1 g). On the other hand, among the 8 mice in the TRP2-NP immunized group, only 3 mice developed tumors >0.3 g. Co-delivery of 7-acyl lipid A with TRP2_{180–188} in PLGA-NP decreased the number of mice that develop >0.3 g tumors to only one (out of 10 mice). Fig. 3C illustrates the percentage of mice that had tumor weights <0.3 g for each treatment group averaged from two independent studies showing similar results. Controlled tumor growth was observed in 16, 40 and 85% of animals immunized with Empty-NP, TRP2-NP and TRP2/7-acyl lipid A-NP, respectively. Pictures of

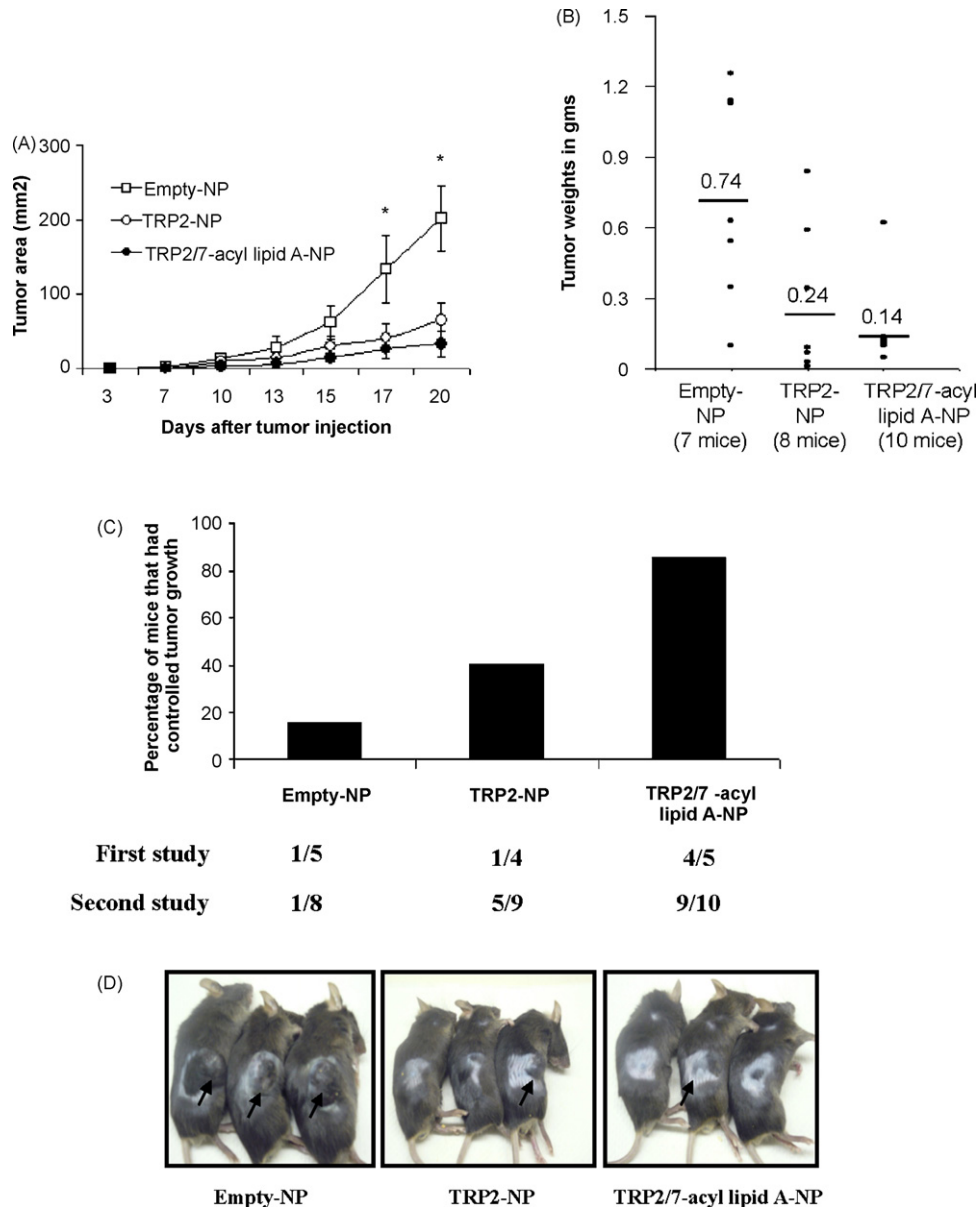


Fig. 3. Therapeutic anti-tumor immunity in mice vaccinated with TRP2_{180–188} containing NP. C57Bl/6 mice were injected s.c. at their upper right flank with 0.1×10^6 B16-F10 melanoma cells (day 0). Three days later, animals were randomly assigned to three treatment groups (8–10 mice per group). Similar to normal mice vaccination study (Fig. 2), the three groups were s.c. vaccinated (in the lower right flank region) with either Empty-NP, TRP2-NP or TRP2/7-acyl lipid A-NP. Animals were given booster immunization with the same formulations at days 7 and 13. Tumor size was measured with vernier caliper every 2–3 days. The longest length and the length perpendicular to it were multiplied to obtain the tumor area in mm². The mean tumor area \pm standard error (S.E.) for each group was plotted vs. time (A). *Indicates significant differences ($p < 0.05$) in tumor area for mice immunized with TRP2/7-acyl lipid A-NP, compared with Empty-NP immunized mice. On day 21 animals were sacrificed and tumors were isolated and weighted separately. Tumor weights of individual mice for each vaccination group are shown as scatter plot (B). Averages of the tumor weights from each group are shown. The experiment was repeated one more time, and similar results were obtained. For each treatment group, the average percentage of mice that had tumor weights less than 0.3 g at the endpoint of the study (day 21) is shown in (C). Numbers below (C) represent the actual numbers of mice that had had tumor weights less than 0.3 g/total number of mice used for each group. Pictures of representative mice in each group at the endpoint of the study are shown in (D). Arrows indicate the position of tumors.

3 mice representative from each treatment group are shown in Fig. 3D.

3.3.3. Ex vivo detection of TRP2-specific CD8⁺ T cells

To examine the underlying mechanism behind controlled tumor development in TRP2/7-acyl lipid A-NP immunized mice, we have isolated lymph nodes and spleens of all the mice and used ELISPOT assay to check the ability of TRP2-specific CD8⁺ T cells to secrete of INF- γ following *in vitro* stimulation. Immunization with Empty-NP did not induce any measurable amount of INF- γ either in draining lymph nodes (Fig. 4A) or spleens (Fig. 4B and C). On the other

hand, among different treatments, mice immunized with TRP2-NP develop strong antigen-specific response in the draining lymph nodes, as evidenced by higher number of INF- γ secreting cells compared to TRP2/7-acyl lipid A-NP immunized group (141.5 ± 10.8 vs. 107.25 ± 8.5 , $p < 0.05$). In contrast, analysis of spleens either by ACK lysis (Fig. 4B) or by CD8⁺ T cell isolation (Fig. 4C) revealed superior TRP2-specific responses in the mice immunized with TRP2/7-acyl lipid A-NP compared to TRP2-NP. Consistent with our observation in the normal mice studies, immunization with TRP2/7-acyl lipid A-NP induced high level of non-specific INF- γ secretion both in draining lymph nodes (Fig. 4A) or in splenocytes treated with ACK

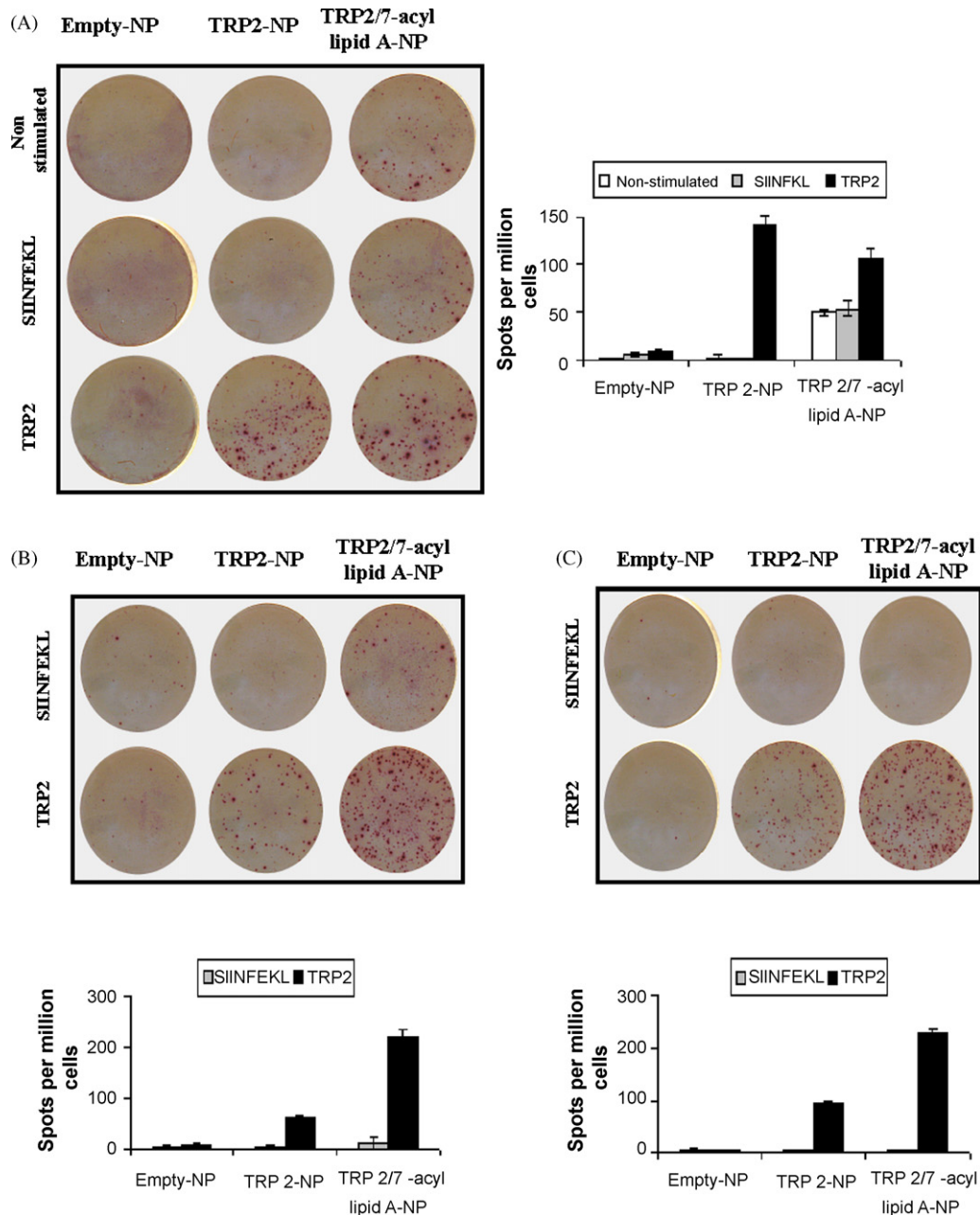


Fig. 4. Ex vivo detection of IFN- γ secretion by TRP2₁₈₀₋₁₈₈-specific CD8⁺ T cells in lymph nodes and spleen of the tumor-bearing vaccinated mice. C57Bl/6 mice were challenged with melanoma cells and vaccinated as described in the text and in Fig. 3. At day 21, animals were sacrificed and ELISPOT assay was performed (as described in text and in Fig. 2) to assess IFN- γ secretion in the (A) draining lymph nodes, and (B and C) spleen. Similar to Fig. 2, spleens were handled by two different ways; splenocytes were (B) either treated with ACK lysis buffer to lyse red blood cells. (C) Alternatively, CD8⁺ T cells were isolated from spleens using CD8⁺ negative isolation kit. Numbers of spots/million cells are presented in bar graphs. The values in those graphs are averages of triplicate wells \pm S.D. Data shown are representative of two independent experiments that gave similar results.

lysis buffer (Fig. 4B). Plating pure CD8⁺ T cell population had eliminated any non-specific INF- γ secretion (Fig. 4C). Average number of spots \pm S.D. ($n=3$) are presented in the bar graphs on the right panel of Fig. 4A (for draining lymph node) and in the lower panels of Fig. 4B and C (for spleens).

3.3.4. Vaccine-induced alteration in the level of pro-inflammatory cytokines and immuno-suppressive factors in tumor microenvironment

Activation of antigen-specific CD8⁺ T cells within lymph nodes and/or spleens may be not enough for complete tumor immunity, as the tumor suppressive microenvironment may hinder migration

of antigen-specific activated T cells into the tumor site or interfere with their anti-tumor activity. So in order to carefully evaluate the effect of our vaccine formulations, it was crucial to detect the level of various immuno-stimulatory versus immuno-suppressive cytokines in the tumor microenvironment. Our results showed that immunization with TRP2-NP slightly increased the level of IFN- γ , TNF- α , IL-6 and IL-2 ($p < 0.05$ compared to Empty-NP). Co-delivery 7-acyl lipid A along with TRP2₁₈₀₋₁₈₈ in the same NP formulation resulted in significantly higher levels of all pro-inflammatory, Th1-related cytokines tested, including IFN- γ , TNF- α , IL-2, IL-6, and IL-12. More importantly, immunization with TRP2/7-acyl lipid A-NP decreased the level of VEGF.

4. Discussion

Induction of potent, specific and lasting T cell responses as well as reversal of immunosuppressive network in the tumor microenvironment are two major challenges in the development of efficacious cancer vaccine strategies. Our objective was to evaluate the potential of PLGA-based cancer vaccine formulations for meeting those challenges in a reliable and realistic tumor model. Murine melanoma B16 is a very aggressive and rapidly growing tumor. These B16 cells possess extensive defects in their MHC class I antigen-processing pathway. As a result, they express very low amounts of MHC I molecules on their surface [18]. Because of their poor immunogenicity and aggressiveness, B16 melanoma represents a challenging tumor model for developing T cell-based immunotherapeutic responses. Despite its poor immunogenicity, B16 contains antigens capable of activating a specific CTL response. Some of melanoma differentiation antigens include MART-1, gp100, tyrosinase, TRP1, and TRP2 [19]. Among those antigens, one epitope of TRP2 (TRP2_{180–188}) is of special interest. Several research groups have shown strong correlation between the presence of TRP2_{180–188}-specific T cells and tumor regression [20–24]. For example, a combination therapy of anti-cytotoxic T lymphocyte antigen (CTLA)-4 monoclonal antibody and granulocyte monocyte-colony stimulating factor (GM-CSF) producing B16 melanoma vaccine revealed that successfully treated mice had elevated level (1.7%) of TRP2_{180–188}-specific T cells circulating in blood [25]. Other studies showed that adoptive transfer of TRP2_{180–188}-specific T cells into C57BL/6 mice reduced the number of experimentally induced B16 lung metastases [26]. Interestingly, TRP2_{180–188} can bind both human HLA-A*0201 and murine MHC class I molecule H2-K^b making it an attractive model antigen that is very relevant to human use.

TRP2_{180–188} was combined with TLR ligands in a wide variety of melanoma immunotherapeutic strategies [27–30]. Development of efficient methods for quantitative analysis of TRP2_{180–188} and TLR ligands in a given vaccine is an essential requirement for better characterization and optimization of different aspects of the vaccine formulation, e.g., encapsulation efficiency, loading and release pattern. We have recently developed a quick, sensitive and reliable LC–MS-based method for the quantification of lipid A analogues in PLGA-NP. Such method had overcome all the problems associated with HPLC analysis of lipid A compounds (e.g., poor UV absorption and the need for pre-column derivatization) [12]. In the current study, we described another LC–MS-based method for the quantification of TRP2_{180–188} encapsulated in PLGA-NP (Fig. 1 and Table 1). Conventional HPLC analysis of TRP2_{180–188} is feasible and not as problematic as that of lipid A compounds. However, LC–MS is preferred due to quick analysis time and high sensitivity.

Our results showed that immunization of normal mice with PLGA-NP encapsulating 10 µg of TRP2_{180–188} with or without 7-acyl lipid A (TRP2/7-acyl lipid A-NP and TRP2-NP, respectively) activated robust TRP2-specific CD8⁺ T cell responses in the draining lymph nodes and spleen of immunized mice and break self-tolerance to TRP2 peptide. Our next challenge was to evaluate the efficacy of our vaccination strategy in tumor-bearing mice. As described in Section 2, therapeutic immunization started 3 days after s.c. inoculation of 10⁵ B16 melanoma cells. Although the tumors were not palpable at this stage (day 3), previous studies have shown that 24 h after s.c. tumor implantation, B16 cells were clearly visible at the site of injection and already started to proliferate (several mitosis) [31]. Our data showed a remarkable reduction in the growth of tumor in the mice immunized with either TRP2-NP or TRP2/7-acyl lipid A-NP as evidenced by decreased tumor area (Fig. 3A) and weight (Fig. 3B), compared to the control group (Empty-NP immunized mice). Due

to large intra-group variability, we could not observe significant differences in tumor size/weights between the two test groups (TRP2-NP and TRP2/7-acyl lipid A-NP). However, there were several clues demonstrating the superior therapeutic effect of TRP2/7-acyl lipid A-NP over TRP2-NP: (1) the average tumor size of animals immunized with TRP2/7-acyl lipid A-NP were almost half the average tumor size obtained in TRP2-NP immunized animals at all times tested (Fig. 3A); (2) none of the mice in the TRP2/7-acyl lipid A NP immunized group had reached the morbid state, where 11% of the TRP2-NP group had to be euthanized before the end of the study; (3) more importantly, 85% of animals immunized with TRP2/7-acyl lipid A-NP had controlled tumor growth compared to 40% in the TRP2-NP immunized group (Fig. 3C).

Data obtained from *ex vivo* analysis of antigen-specific CD8⁺ T cell activation in lymph nodes and spleens of the tumor bearing mice (Fig. 4) was consistent to what we previously observed in healthy mice study (Fig. 2). For instance, immunization with either TRP2-NP or TRP2/7-acyl lipid A-NP overcame self-tolerance mechanisms and activated robust TRP2-specific CD8⁺ T cell responses. In the TRP2-NP immunized group, higher number of the activated CD8⁺ T cells were found in the draining lymph nodes (Fig. 4A), compared to spleens (Fig. 4B and C). The opposite pattern was observed in the TRP2/7-acyl lipid A-NP immunized group, where activated CD8⁺ T cells were found in higher numbers in the spleens (Fig. 4B and C), compared to lymph node (Fig. 4A). This observation may imply that activated CD8⁺ T cells in the TRP2/7-acyl lipid A-NP immunized group have acquired better migratory capacity and were able to leave the lymph node and enter the spleen through the blood stream. Such T cells may also have better accessibility to the tumor site. Finally, we have noticed that s.c. immunization with PLGA-NP co-encapsulating antigens and 7-acyl lipid A always induced strong inflammatory response at the site of injection and in the draining lymph nodes (unpublished observation). In the ELISPOT assay, this inflammation could cause relatively high background readings in the wells that have no stimulation or well stimulated with the irrelevant CD8 peptide (Fig. 2C and Fig. 4B), probably due to the non-specific IFN-γ secretion by innate immune cells in those wells. This background was eliminated when pure CD8⁺ T cells were plated (Fig. 2D and Fig. 4C).

B16 melanoma exhibit severe impairment in multiple components of MHC class I antigen processing machinery, including: the peptide transporter associated with antigen processing (TAP), the proteasome subunits LMP2, LMP7, and LMP10, PA28-α and -β, and the chaperone tapasin [18]. Down regulations or loss of expression and/or functions of those components result in the reduction or loss of MHC class I surface expression, which often leads to immune escape of the tumor cells. Interestingly, earlier studies have shown that all these defects could be corrected by the administration of IFN-γ, which induces the expression of multiple components of the MHC class I antigen processing machinery, and ultimately enhances the surface expression of MHC class I [18]. In the present study, the induction of IFN-γ secretion by host immune cells may have great impact on the success of our vaccination strategy. The elevated level of IFN-γ secretion (either by antigen-specific CD8⁺ T cells or innate cells) could up-regulate MHC class I surface expression on B16 tumor cells, resulting in immune recognition and increased lysis of tumor cells by CTLs.

It is worth mentioning that no anti-tumor therapeutic effect was observed in tumor-bearing mice immunized with 7-acyl lipid A-NP (without antigen) (data not shown). Furthermore, immunization with 7-acyl lipid A-NP alone didn't develop any antigen-specific activation of CD8⁺ T cell responses in either normal or tumor bearing mice as measured by IFN-γ ELISPOT assay (data not shown). Half of the mice in group receiving 7-acyl lipid A-NP (4 out of 8) had to be euthanized before the end point of the study due

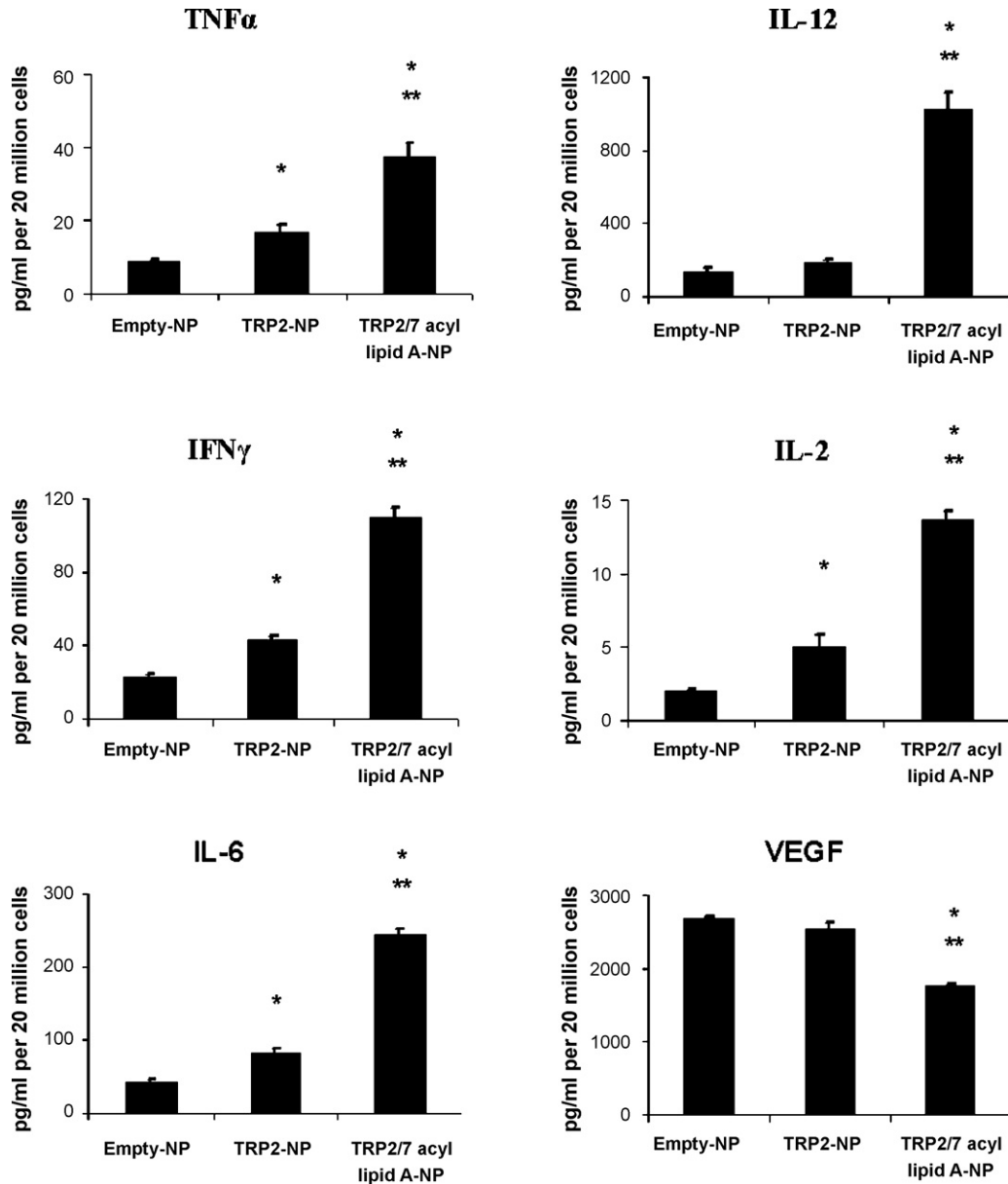


Fig. 5. Assessment of the level of proinflammatory cytokines and immuno-suppressive factors in tumor microenvironment. C57Bl/6 mice were challenged with melanoma cells and vaccinated as described in the text and in Fig. 3. At day 21, animals were sacrificed; tumors were isolated and crushed between two slides to form uniform cell suspension, which were counted and filtered through 70 μ m cell strainers. Tumor supernatants were analyzed for the level of TNF- α , IL-12, IFN- γ , IL-2, IL-6 and VEGF by ELISA. Results are presented as amount of cytokine (pg/mL) per 20 million tumor cells. Each bar represents the mean of triplicate wells \pm S.D. *Indicates significant difference from Empty-NP immunized group ($P < 0.05$). **Indicates significant difference from TRP2-NP immunized group ($P < 0.05$).

to big tumor size and/or ulceration of the tumor. In the experiment described in Fig. 5, we observed up to 2-fold increase in the level of pro-inflammatory cytokines (e.g., IL-6, IL-12 and TNF- α) in the tumors isolated from mice immunized with 7-acetyl lipid A-NP compared to Empty-NP immunized mice (data is not shown). This observation may imply the ability of nanoparticles containing 7-acetyl lipid A to induce DC maturation and activation (even in the absence of antigen). However, the lack of therapeutic effect observed in this group highlights the importance of activating antigen-specific CD8⁺ T cells to provide the full-scale anti-tumor immune responses.

The capability of TRP2/7-acetyl lipid A-NP vaccine strategy to break self-tolerance and to induce superior anti-tumor effect could be further explained through numerous mechanisms. Co-delivery of TLR ligand (7-acetyl lipid A) along with TRP2₁₈₀₋₁₈₈ to the same

DC population provides the three signals required for optimum CTL activation. DC stimulated with TLR ligand increase the expression of peptide/MHC I complex on the cell surface (signal 1), upregulate costimulatory molecules, e.g., CD40, CD80 and CD86 (signal 2), and secrete various cytokines, e.g., IL-12 (signal 3). The three signals combined lead to enhanced activation and proliferation of TRP2-specific CD8⁺ T cell [32]. Another avenue for breaking self-tolerance is through the ability of TLR activated DCs to reverse the T regulatory (Treg) suppressive effects. In fact, it has been recently shown that IL-6 secreted by TLR4-activated DCs renders antigen-specific T cells refractory to the suppressive activity of Treg [33]. Other studies have shown that stimulation of DCs with TLR ligands enhances the proliferation of antigen-specific T cells, making it harder for Treg cells to inhibit them [34,35]. We have recently shown that particulate delivery of 7-acetyl lipid A leads to 1000-fold

increase in the amount of IL-6 secreted by DCs (relative to soluble form) [13]. We have also shown that co-delivery of OVA and 7-acyl lipid A in PLGA-NP to DCs have dramatically enhanced the extent of *in vitro* primary CD4⁺ T cell activation by 1000-fold (compared to soluble formulation) [13]. Vaccine delivery system capable of inducing IL-6 production by DCs, and activation of primary T cell responses to this extent, may assure overcoming Treg-mediated immunosuppression through involvement of activated T cells.

The immunosuppressive environment around the tumor is one of the major elements of compromised anti-tumor immune responses (reviewed in Refs. [36–38]). Several lines of evidence indicate that dominant immunosuppressive cytokines in the tumor microenvironment, e.g., IL-10, TGF- β , and VEGF, significantly inhibit DC maturation/activation, leading to preferential activation of Treg cells on the expense of anti-tumor effector T cells [39–41]. One of the most remarkable finding of our current study is the ability of our vaccine formulation (TRP2/7-acyl lipid A-NP) to shift the balance at the tumor microenvironment towards immune stimulation, as evidenced by the increase in the level of pro-inflammatory/Th1-biased cytokines (IL-2, IL-6, IL-12, TNF- α and IFN- γ) and the decrease in the level of the immunosuppressant VEGF (Fig. 5). These results imply that our vaccine is able to provide immune stimulation and rescue impaired DCs from tumor-induced immune suppression.

TRP2/7-acyl lipid A-NP may also activate innate immune cells through an indirect way. In fact, recent studies have shown that TLR-activated DCs constitute a source of numerous cytokines that induce natural killer (NK) cell activation. In particular, DCs-derived IL-15, IL-12/IL-18 and IFN- α/β could induce NK cell proliferation, IFN- γ secretion and cytotoxic function, respectively [42–44]. A single vaccine strategy capable of activating both CTL and NK cells can act as a double-edged sword, capable of targeting and killing both MHC class I positive and negative tumor cells. One of our next research goals is to directly assess NK cell activation and relative contribution of NK cells to the anti-tumor effects of this vaccine.

5. Conclusion

Our results validated the potential of PLGA-based cancer vaccines to break self-tolerance against cancer antigen, induce potent and specific anti-tumor T cell responses with no concomitant autoimmunity, and activate IFN- γ secretion by antigen-specific CD8⁺ T cells. Remarkably, this vaccine was able to induce therapeutic anti-tumor effect. Activated TRP2-specific CD8 T cells were capable of IFN- γ secretion at lymph nodes and spleens of the vaccinated mice. More importantly, co-delivery of cancer antigen along with 7-acyl lipid A in PLGA-NP have proven to be effective strategy in inducing immunostimulatory milieu at the tumor microenvironment, as evidenced by decreased level of VEGF and elevated levels of IL-2, IL-6, IL-12, IFN- γ and TNF- α . Our results also highlight the immunostimulatory properties of 7-acyl lipid A as a novel adjuvant can potentially serve as a powerful companion to antigens in vaccine formulation. To our knowledge, this is the first report showing that co-delivery of a real cancer antigen along with TLR ligand in PLGA-NP could induce such effects.

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