# Liposome-Encapsulated Adjuvants are Potent Inducers of Antigen-Specific T-Cells *in Vivo*

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**Abstract:** As shown previously, encapsulation of a peptide derived from tyrosinase-related protein2 (TRP2) into liposomes (artificial virus envelope (AVE) 3) resulted in combination with CpG-oligodeoxynucleotides in the induction of higher numbers of antigen-specific T cells compared to vaccination with free TRP2. Here, we present further data with regard to optimal antigen dose, the relevance of vaccine injection site and on the T cell stimulatory synergism of liposomal adjuvant combinations. Compared to an aqueous solution liposomal TRP2 was more potent in the induction of TRP2-specific T cells at an optimal dose but showed a narrow dose optimum with profoundly impaired T cell responses at higher vaccine doses. Higher T cell numbers were induced when mice were vaccinated into their hint foodpads compared to intradermal vaccination, the site used routinely in murine tumor vaccination models. A synergistic adjuvant effect was observed when CpG-oligodeoxynucleotides were admixed with liposomal monophosphoryl lipid A (MPLA) and the lipopeptide $Pam_3Cys$, respectively. In summary our data demonstrate that liposomes as carriers for peptide-antigen and adjuvant induce a strong antigen-specific T cell response and are superior over vaccine formulations composed of free peptide and adjuvant.

**Keywords:** Liposome, toll like receptor ligand, T-cell response, adjuvant, tumor vaccine.

## INTRODUCTION

Antigen (Ag)-specific immunotherapeutical approaches are attractive treatment modalities to fight cancer. The availability of an increasing number of tumor associated Ags for a variety of different tumor entities has provided the basis for targeting of cytotoxic T-cells specifically to cancer cells [1, 2]. Even though, antigen-specific vaccination strategies had entered clinical stage more than one decade ago, a number of technical challenges to make them a successful treatment have still not been solved [3]. One yet unsolved question is the induction of potent T cell responses against tumors [4-6] It is well accepted that for the initiation of Ag specific immune responses dendritic cells (DCs) play a central role as these cells take up Ags from the periphery and transport them to the secondary lymphoid organs specialized in hosting DC/T-cell interactions [7]. The delivery of Ags to DCs is therefore of key importance for anti-tumor vaccination strategies [8-11]. Liposomes have been shown to be suitable as carriers for improving the delivery of a diversity of drug classes such as antifungals [12], immunosuppressants [13], antibiotics [14], chemotherapeutic anticancer drugs [15-17] as well as viral, bacterial [18] and tumor-Ags to target tissues and cells including DCs [19, 20]. Moreover, they represent a promising pharmaceutical formulation, as they protect the Ag from degradation and form a long-lasting Ag depot at the injection site increasing antigen half life and *in vivo* availability. However, for potent priming of naïve T-cells and induction of sustained immune responses DCs have to mature and undergo a number of phenotypic alterations [21]. DC maturation is achieved very potently by signaling through pattern recognition receptors (PRRs), such as members of the toll-like receptor (TLR) family [22]. Microbial compounds do act as ligands for such receptors, as for example oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN), which are recognized by TLR9 [23]. CpG-ODNs do not only contribute to DC maturation, but also activate host defense mechanisms leading to innate and acquired immune responses [24] and induce TH1-dominated immunity, which is of utmost importance for cancer immunotherapy [8, 25-27]. However, beside the desired effects, TLR agonists particularly CpG-ODNs may also alter morphology and functionality of lymphoid organs in a dose- and time dependent manner [28]. Encapsulation of TLR agonists into liposomal formulations was shown to enhance specificity by reducing these undesired systemic side effects [29]. Based on our recent studies focusing mainly on the effects of liposome encapsulated peptide Ag for the induction of antigen-specific T-cells in the B16 murine melanoma model [19] we now present further data with regard to encapsulation of TLR-triggering adjuvants and combinations thereof, dosing of the vaccine to further optimize antigen-specific stimulation of T-cells and the significance of the site of vaccine administration.

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## MATERIAL AND METHODS

### Peptides and Adjuvants

TRP2 peptide (SVYDFFVWL; corresponding to amino acids 180-188 of tyrosinase-related protein 2 (TRP2)) and ovalbumin (OVA) peptide (SIINFEKL; corresponding to amino acids 257-264 of OVA) were purchased from Bachem (Weil am Rhein, Germany). Both peptides are presented by murine MHC-class I molecule H-2K$^b$. SIINFEKL was stored as a 1 mg/ml stock solution in water, TRP2 peptide was diluted to 20 mg/ml in DMSO and further diluted to 1mg/ml in PBS and stored at -20°C. The immunostimulatory phosphorothioate-stabilized (PTO) oligodeoxynucleotide (ODN) 1826CpG-ODN (5'-TCCATGACGTTC-CTGACGTT-3´) were synthesized by Eurogentec (Köln, Germany) and biomers (Ulm, Germany). The triacyl-lipopeptides *N*-palmitoyl-*S*-[2,3-*bis* (palmitoyloxy)-(2*RS*)-propyl]-(*R*)-cysteine ($Pam_3Cys$) and $Pam_3Cys$-SKKKK were purchased from EMC microcolletions (Tübingen, Germany) and monophosphoryl-lipid A (MPLA) from Avanti Polar Lipids (Alabaster, USA).

### Composition of Different Liposomal Preparations

All lipids were purchased from Avanti (Alabaster, USA) or Lipoid (Ludwigshafen, Germany) and used without further purification. Small unilamellar liposomes were composed of cholesterol (chol), synthetic dioleoyl-phosphatidylserine (DOPS), dilauroylphosphatidyl-ethanolamine (DLPE) and egg phosphatidylcholin (EPC) as describede priviously [19]. In initial binding studies liposomes with different DLPE content were prepared substituting DLPE by EPC giving a lipid concentration of 100 mol% (see Table **1**). AVE3 liposomes are composed of chol, DLPE and DOPS at a molar ratio of 1:1:1, thus AVE3 are negatively charged liposomes (zeta-potential ~ -60 mV). The final amount of lipids was 10 µmol/ml corresponding to 5.9 mg/ml of lipid. Neutral control liposomes (AVE14) were composed of chol, DLPE and EPC at a molar ratio of 1:1:1 thus having DOPS substituted by EPC.

### Binding Studies of Liposomes to DCs

Studies were performed to analyze the binding characteristic of liposomes to murine C57BL/6 splenocytes (for liposome formulation see also Table **1**). Rhodamine-labeled liposomes were prepared as described with the addition of rhodamine-DLPE at a final concentration of 0.3 mol%. A single cell suspension was obtained by smashing and filtering the spleen through a sterile cell strainer (100µm; Falcon, Heidelberg, Germany). Red blood cells were lysed in ACK lysing buffer (0.15 M $NH_4Cl$, 1.0 mM $KHCO_3$, 0.1 mM $Na_2EDTA$) for 5 min at room temperature and thereafter cells were washed three times in HBSS. 5 x $10^5$ cells were incubated in a total volume of 100 µl RPMI for 1 h with 10 nmol (corresponding to 5.9 µg of lipid) with the indicated rhodamine-labeled liposomes, either on ice (4°C) to show binding or at 37°C to analyze beside binding also liposome uptake. To analyze also serum effects, lymphocytes were incubated with liposomes in the presence of 10% fetal calf serum (FCS). Cells were stained with FITC-labeled monoclonal antibodies (mAb) against Thy1.2, B220 and CD11c (all from Pharmingen) to distinguish T- and B cells and DCs, repectively and analyzed by flow cytometry for liposome binding and/or liposome uptake.

### Encapsulation of TRP2 Peptide into AVE3 Liposomes

Encapsulation of TRP2 peptide into AVE3 was conducted as described before [19]. Briefly, TRP2 was admixed with lipids at a molar ratio of 1:20 (TRP2:lipids), 20 g Hepes buffer (pH 7.4, 10 mM) was added and stirred for 30 min at 55°C with an Ultra-Turrax T8 dispersing instrument (IKA Werke, Staufen, Germany) equipped with a S8N-8G dispersing element at approx. 25,000 rpm in a water bath. To obtain a homogeneous suspension and to avoid precipitations the bottle was vortexed several times and evaporated water was replaced. The suspension was transferred to an Emulsiflex C-5 Homogenizer (Avestin Inc., Ottawa, Canada) and homogenization was performed for 30 min at a pressure of 50,000 to 150,000 kPa in front of the homogenization nozzle and a homogenisator pressure of 300 kPa. The liposomal dispersion was filtered for 5 cycles through polycarbonate filters with a pore size of 100 nm resulting in homogenous populations of unilamellar liposomes. The liposome size was determined after storage for at least 24 h at 4°C by photon correlation spectroscopy (Zetasizer 3000 HS; Malvern, Herrenberg, Gemany) and was in the range of 100-150 nm. The TRP2 amount in the liposomes was determined by HPLC (Waters, Eschborn, Germany) using a modified reversed phase HPLC gradient method [30] and was about 100-250 µg/ml liposome preparation (20-50% encapsulation efficiency).

### Encapsulation of CpG-ODN into AVE3-Liposomes

Lipids (40 µmol/ml) were dissolved in chloroform or chloroform:methanol (1:1) and were dried in a rotary evaporator at 30 mbar and 34°C for 15 min. The lipid film was further dried in a vacuum chamber at 10 mbar at room temperature for 1 h, and hydrated in a rotary evaporator with a few glass beads in 1 ml isotonic HEPES-buffer (10 mM; pH 7.4) containing 10 mg/ml ODN. The dispersion was extruded 21 times through polycarbonate membranes (pore size of 50 nm) and free ODNs were removed by size exclusion chromatography using Sepharose CL-4B columns (Pharmacia, Sweden). Liposome fractions were concentrated by ultrafiltration using Vivaspin concen-trators (Sartorius, France; cutoff of 30.000 kDa) and filtered through 0.2 µm sterile filter membranes. The amount of encapsulated ODNs was determined by ion-exchange HPLC. Sizes of liposomes were between 100 to 150 nm and encapsulation efficiency was in the range of 10%.

### Encapsulation of Lipophilic Adjuvants into AVE3-Liposomes

Lipids, the lipophilic adjuvant MPLA and $Pam_3Cys$, respectively were dissolved in chloroform and mixed at a molar ratio of 2.5 and 5%. TRP2 peptide dissolved in DMSO was added to this mixture for incorporation of adjuvant and peptide into the same liposome. The compounds were dried in a rotary evaporator at 10 mbar and 34°C for 1 h. The resulting film was resolved in chloroform and dried again as described before. This step was repeated until a smooth and homogeneous film was obtained, followed by the removal of

residual solvent in a vacuum chamber at 10 mbar. The dried film was hydrated in a rotary evaporator with glass beads in 1ml isotonic Hepes buffer (10 mM; pH 7.4). The dispersion was extruded 21 times through polycarbonate membranes (100 nm pore size) and sterile-filtered.

### *In Vivo* Induction and Restimulation of TRP2-Specific T-Cells

8-week-old female C57BL/6 mice were obtained from Harlan Winkelmann (Borchen, Germany) and were kept under conventional conditions in the animal facilities of vectron therapeutics and the Johannes Gutenberg University of Mainz. All the groups were age- and sex-matched. The study was performed in accordance with German federal regulations of animal experimentation.

Immunization of mice for the induction of Ag-specific T-cells was carried out as was described recently [19]. Briefly, mice (3 to 5 animals per group) were immunized twice at weekly intervals with the indicated vaccine formulations either into the hind food pads or i.d. into the abdominal flank. Seven days after the second immunization a single cell suspension from spleen was prepared and red blood cells lysed by ACK buffer. Splenocytes were restimulated for 16-20 hours either with TRP2, SIINFEKL peptide (unspecific background control; 1 μM each) or without peptide (spontaneous background control). Cells stimulated with anti-α/β TCR (clone H57-597) plus anti-CD28 antibodies (clone 37.51) (1μg/ml each; both Pharmingen, Hamburg, Germany) were used as positive controls.

### IFN-γ Capture Assay for Detection of Antigen Specific T-Cells

To detect antigen-specific IFN-γ secreting cells the mouse IFN-γ-secretion assay was performed (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers instructions. Briefly, cells were labeled with an antibody against IFN-γ/CD45 conjugates for 5 min on ice, then incubated for 45 min at 37°C to allow cytokine secretion. Cross-staining was avoided by keeping the cell density at 1 x $10^5$ cells/ml. Released IFN-γ bound to the capture matrix on cells was stained with secondary phycoerythrin (PE)-conjugated monoclonal antibody against IFN-γ. The cells were counterstained with FITC-labeled anti CD8-Ab, and dead cells were excluded by propidium iodide (1 μg/ml). Cells were analyzed on a FACSCanto using FACSDiVa software (Becton Dickinson, Heidelberg, Germany).

### IFN-γ ELISPOT Assay

The IFN-γ enzyme-linked immunospot (ELISPOT) assay including automated spot evaluation was used in some experiments beside the IFN-γ capture assay for the detection of Ag-specific IFN-γ-secreting cells and was performed as recently described [31, 32]. Briefly, 4 x $10^5$ splenocytes and lymph node cells, respectively were seeded out per well of a ninety-six-well microtiter plate (MAHA S4510; Millipore, Eschborn, Germany) coated overnight at 4°C with 10 μg/ml anti IFN-γ antibody 1-D1K (Mabtech, Stockholm, Sweden) and were stimulated for 16-20 hours with TRP2, control SIINFEKL peptide and as a positive control with phytohemagglutinin (PHA2769; Sigma-Aldrich, Deisenhofen, Germany). Cells were washed off with PBS/0.05% Tween20 (PBS/T). Captured cytokine was detected by incubation for 2 h at 37°C with biotinylated mAb anti-hIFN-γ (7-B6-1; Mabtech) at 2 μg/ml in PBS/0.5% BSA. Spots were vizualized by incubation with Avidin-peroxidase complex (1/100; Vectastain Elite Kit; Vector, Burlingame, CA, USA) and subsequent incubation with peroxidase substrate 3-amino-9-ethyl-carbazole (Sigma, München, Germany) for 4 min and stopped by rinsing the plates under running tap water. Spot numbers were determined with the use of a computer-assisted video image analyser equipped with software KS ELISPOT (Version 4.2.0; Zeiss, Jena, Germany). Results are expressed as means of duplicates analyzed for each experimental setting.

### Statistics

Experiments were repeated at least 3 times. In mouse experiments groups consisted of at least 3 animals/group. Data are presented as mean ± SEM. The statistical significance of differences between test groups was analysed by paired Student's t-test.

## RESULTS

### AVE3 Composition of Liposomes Ensures Specific Binding to DCs but Not to B- and T-Cells

The specific uptake of antigen by professional antigen presenting cells such as DCs and its presentation to naïve T-cells is a prerequisite for induction of an antigen-specific T cell response, whereas presentation by non-professional antigen presenting cells is not desirable. We analyzed the uptake of a range of liposomal preparations differing in their overall DLPE content by DCs (Fig. (**1**); for liposome composition see Table **1**). A single-cell suspension was prepared from murine spleen and cells were incubated with rhodamine-labeled liposomes for 1 h at 4°C and at 37°C in the absence as well as presence of FCS, to distinguish mere binding of liposomes (4°C) from their uptake and phagocytosis (37°C). Cells were stained with antibodies against Thy-1.2, B220 and CD11c to detect T-, B- cells and DCs, respectively and analyzed by flow cytometry. At 4°C approximately 15% of $CD11c^+$- DC stained positive, largely independent of the DLPE content of liposomes, whereas no liposome-binding was observed to T- and B- cells. At 37°C liposome uptake by DCs correlated with liposome DLPE content, and uptake was further increased in the presence of FCS. However, at a DLPE content exceeding 33.3 mol%, liposomes bound to T- and B-cells as well, particularly in the presence of FCS. To avoid such unspecific background, all further experiments were carried out with liposomes having a DLPE content of 33.3 mol% (=AVE3 liposomes) combining optimal specificity and binding characteristics to DCs.

To test how other components within liposomes affect binding and uptake by DCs, we tested also other liposomal formulations. Among others we used one liposome composition (AVE14), in which DOPS was substituted by EPC resulting in liposomes with a neutral charge compared to negatively charged, AVE3. Whereas rhodamine-labeled AVE3 bind to and are engulfed by DCs, no significant uptake of

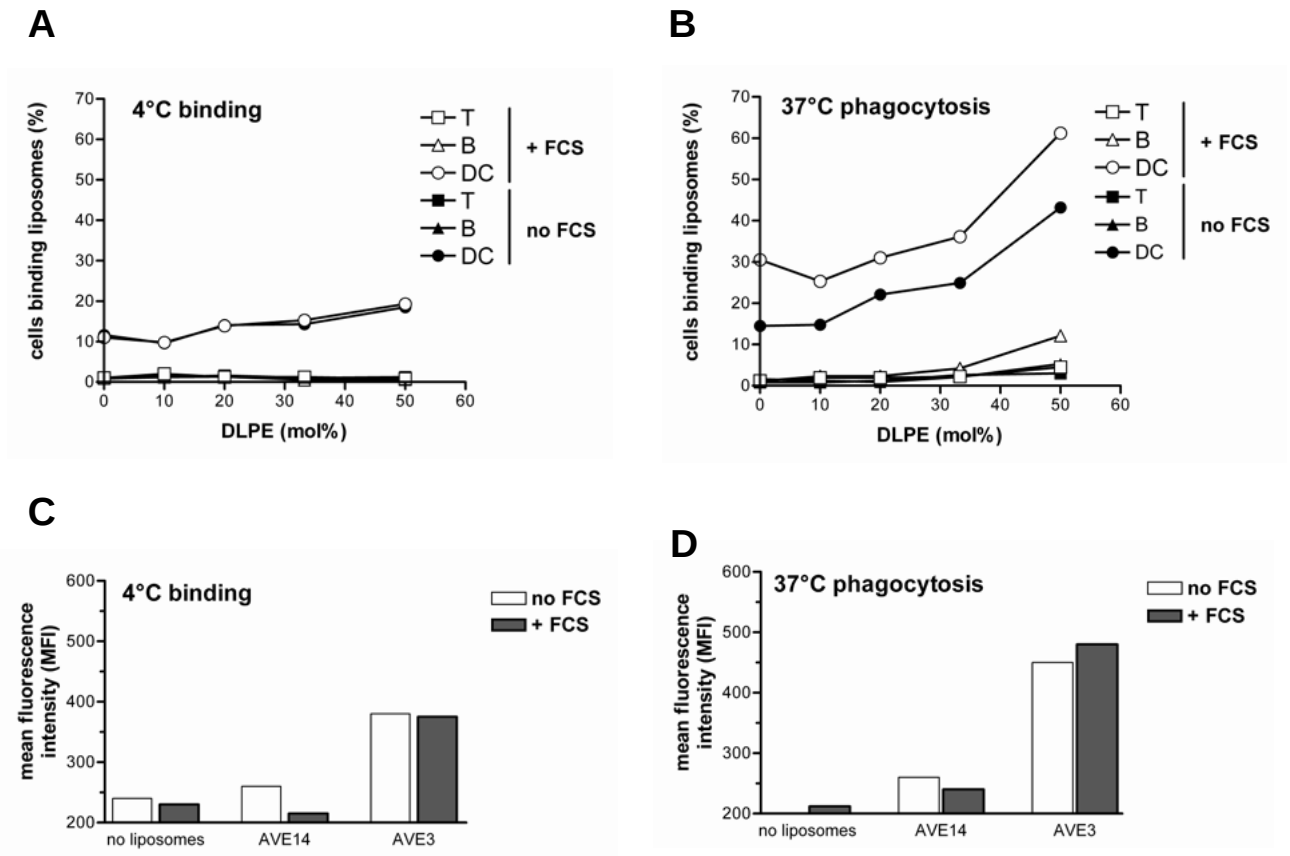


**Fig. (1). Specific binding of liposomes to DCs but not to T and B cells.** Splenocytes from untreated BL6 mice were cultured either in the presence (+FCS) or absence of fetal calf serum (no FCS) with rhodamine labeled liposomes for 1 h at 4°C (**A**,**C**) and 37°C (**B**,**D**), respectively. Binding of liposomes to lymphocyte subpopulations was assessed by flow cytometry after co-staining with CD3, B220 and CD11c to detect specific binding to T- and B-cells and DCs. Gates were set to CD3, B220 and CD11c, respectively and liposome labelled cells are shown. (**A**,**B**) The effect of varying amounts of dilauroylphosphatidyl-ethanolamine (DLPE; for details of liposomal composition see also Table **1**) in liposomes on binding (**A**) and uptake (**B**) of liposomes was measured. (**C**,**D**) The effect of dioleoylphosphatidylserine (DOPS, represented by AVE3) and egg phosphatidylcholin (EPC, represented by AVE14) in rhodamine-labeled liposomes on binding (**C**) and uptake (**D**) by CD11c+ DC. Shown is the mean fluorescence intensity (MFI).

AVE14 liposomes was detected (Fig. **1C**, **D**). Taken together these findings demonstrate a specific binding and uptake of AVE3 liposomes characterized by a DLPE content of 33.3 mol% and DOPS exclusively and efficiently by DCs, not only *in vitro* but also *in vivo* as was demonstrated formerly [19].

**Table 1. Lipid Composition of Liposomes Composed of Varying DLPE Amounts. Lipid Concentration in mol%**

| DLPE Content | Chol | DLPE | POPC | DOPS | EPC |
|---|---|---|---|---|---|
| AVE3-PE0 | 25.0 | - | 50.0 | 25.0 | |
| AVE3-PE10 | 25.0 | 10.0 | 40.0 | 25.0 | |
| AVE3-PE20 | 25.0 | 20.0 | 30.0 | 25.0 | |
| AVE3-PE33 | 25.0 | 33.3 | 16.7 | 25.0 | |
| AVE3-PE50 | 25.0 | 50.0 | - | 25.0 | |
| AVE3 | 33.3 | 33.3 | - | 33.3 | |
| AVE14 | 33.3 | 33.3 | - | - | 33.3 |

### The Route of Administration of AVE3-Encapsulated TRP2 and CpG-ODN Affects the Dose/Response Curve of IFN-γ Release from Antigen-Specific $CD8^+$ T-Cells

In our previous studies, liposomal vaccine and adjuvant were administered to the footpad of mice [19]. To evaluate whether the route of application has an impact on induction of an antigen-specific T-cell response, we compared this to intradermal injection into abdominal flanks of C57BL/6 mice and applied encapsulated TRP2 peptide as well as the free form in the presence of 5 nmol free CpG-ODN. Splenocytes were isolated and TRP2 peptide specific $CD8^+$ T-cells measured in IFN-γ-secretion assay. As described previously, vaccination with liposomal TRP2 peptide had a significantly higher potency to induce Ag-specific $CD8^+$ T-cells as compared to free TRP2 peptide. This effect was more pronounced in mice vaccinated into footpads, where up to 0.5 to 1 log less peptide was needed by the liposomal formulation for the induction of a defined number of antigen-specific T-cells (Fig. **2A**). Dose/response curves obtained by both vaccination forms, however, differed. Owing to the anatomical situation amounts injectable to the footpad were restricted to maximally 100 μg/animal and antigen-specific T-cell stimulation as measured by cytokine release was observed to increase sigmoidally within the respective dose range (Fig.

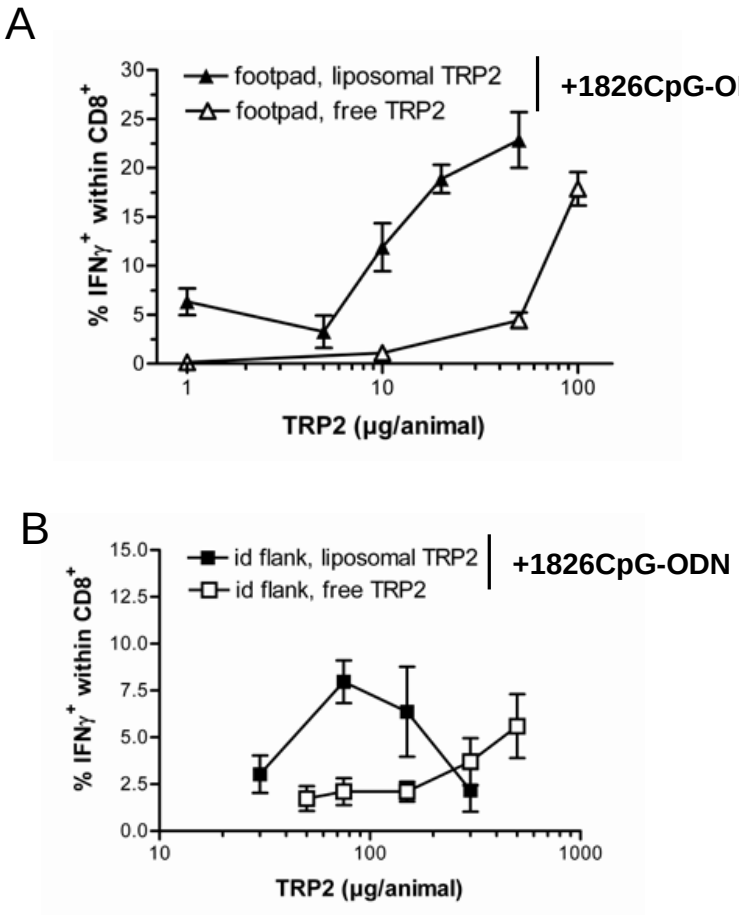


**Fig. (2). Impact of formulation and route of Ag administration on the number of CD8$^+$ T-cells.** Increasing doses of TRP2 peptide together with 5 nmol of 1826CpG-ODN were injected either as aqueous solution or co-encapsulated into liposomes into the hind footpads (n=5 for A) or intradermally (i.d.) into the flanks (n=3 for B) of BL6 mice once weekly for two weeks. 7 days after the second immunization splenocytes were isolated and restimulated with 1 µM TRP2 or control SIINFEKL peptide in the presence of low dose IL2 (30 U/ml) for 16 h. In all experiments IFN-$\gamma^+$ cells with control SIINFEKL peptide were below 0.5%. Results are presented as mean ± SEM.

**2A**). In vaccination studies injecting intradermally into the flank, at least 100 up to 500 µg are generally given and this amount was also required in our system with free TRP2 peptide and adjuvant to obtain an incremental dose/ response curve (Fig. **2B**). For liposomal TRP2 peptide plus CpG-ODN, in contrast, we observed a narrow dose optimum. Doses exceeding 150 µg liposomal TRP2/mouse led to a strong decrease of the antigen-specific T cell response (Fig. **2B**).

### High Doses of AVE3-Encapsulated TRP2 Peptide given Intradermally into Flanks Suppress T-Cell Response and Diminish Lymphocyte Numbers

We wanted to understand the mechanisms underlying impairment of the T cell response consecutive to intra-dermal vaccination of higher doses of liposomal TRP2. To this aim, splenocytes of mice were incubated overnight with antibodies against the T cell receptor (TCR) and CD28 (Fig. **3A**, **B**, **C**). As expected, splenic T-cells from mice vaccinated into the footpad using the liposomal composition (Fig. **3A**), as well as those from mice immunized i.d. into the flanks with free TRP2 peptide and adjuvant (Fig. **3C**) responded well to this pan-T-cell stimulus. T-cells derived from animals vaccinated i.d. with the liposomal composition, however, showed comparable stimulation only if they had been treated with low dose TRP2 peptide. Vaccination with high-dose AVE3/TRP2 plus CpG-ODN however rendered T-cells unresponsive to pan-T-cell stimulation (Fig. **3B**). Only in less than 10% of CD8$^+$ T-cells from mice vaccinated with 300 µg liposomal TRP2 cytokine release was observed, as compared to 80% of CD8$^+$ T-cells from mice vaccinated with 30 µg. Flow-cytometric analysis disclosed that cellular composition in the spleen of immunized animals was also affected. High doses of AVE3/TRP2 but not of free TRP2 peptide plus free CpG-ODN resulted in a significant decrease in total T-cell as well as B-cell numbers yielded from spleen tissue, whereas DC numbers were slightly increased (Fig. **3E**+**F**). Such effects were not seen when AVE3/TRP2 was given into the footpads (Fig. **3A**+**D**).

### Combination of AVE3-Encapsulated Adjuvants with CpG-ODN Exert Synergistic Effects on Antigen-Specific T-Cell Response

The co-application of suitable adjuvants is a prerequisite to mount a strong and robust T cell vaccine response. Triggering different TLRs was recently shown to act synergistically in the induction of DC, which polarize the immune response towards TH1 [33]. Based on this observation, we expected that the

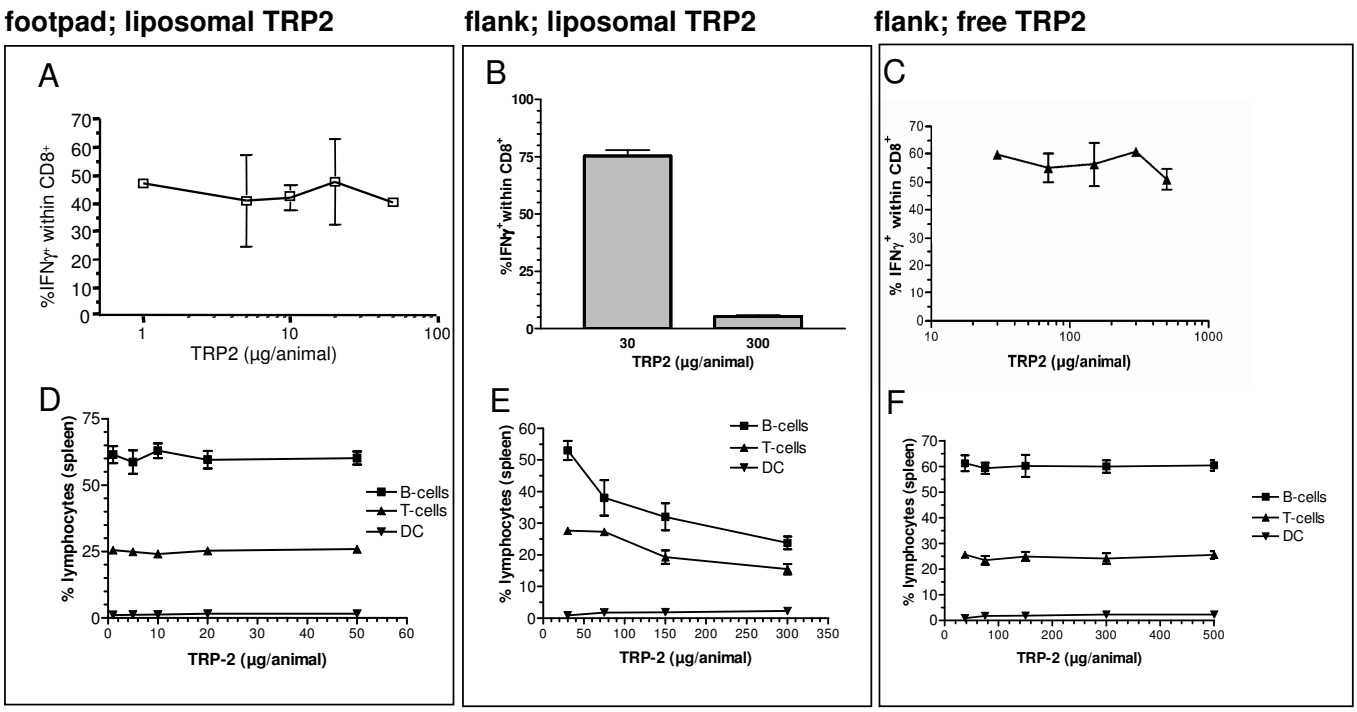


**Fig. (3). Effects of the pharmaceutical form on the cellularity and responsivity of CD8$^+$ T-cells.** BL6 mice were immunized twice with the indicated formulation of TRP2 Ag (liposomal TRP2 and free TRP2) plus 5 nmol free CpG-ODN at weekly intervalls either into the hind footpad or i.d. into the flank. 7 days after the second immunization T-cells from control mice and mice immunized with the indicated TRP2 formulations were stimulated with anti-α/β TCR+anti-CD28 to evaluate their capability to respond to an Ag-independent stimulus (**A, B** and **C**). The cellular composition of spleen cells was evaluated by FACS analysis and the percentage of T- and B-cells and DCs was determined. n=3; results are presented as mean ± SEM.

combination of different TLR ligands could further enhance the outcome of the TRP2 specific T cell response. In addition to CpG-ODN which binds to TLR9, we tested the TLR4 agonist MPLA [34] and the lipopeptide $Pam_3Cys$ known to act through TLR1/2 heterodimers [35]. Whereas for CpG-ODN we have shown previously, that AVE3-encapsulated CpG-ODN is not superior to its free form with regard to the induction of Ag-specific CD8$^+$ T-cells [19] this had not been investigated for $Pam_3Cys$ and MPLA. Therefore, these adjuvants were co-encapsulated with TRP2 into liposomes and administered to mice individually or in combination with free CpG-ODN and antigen-specific T-cell response was measured *ex vivo* by IFN-γ capture assay. Each vaccine formulation containing at least one adjuvant, either MPLA, $Pam_3Cys$ or CpG-ODN induced a significant T cell response (Fig. **4**). However, addition of CpG-ODN as a second adjuvant to $Pam_3Cys$-liposomes (Fig. **4A**) and even more to MPLA (Fig. **4B**) boosted TRP2-specific CD8$^+$ T cell response substantially. Combined adjuvant effects were synergistic rather than additive. For $Pam_3Cys$ we tested whether such synergism is also seen, if the free compound is administered in aqueous solution. Measuring the antigen-specific T-cell response by flowcytometric IFN-γ capture assay (Fig. **5B**) and confirmed independently by ELISPOT technique (Fig. **5A**), we showed that no T-cell response is induced by free $Pam_3Cys$ alone. Most interestingly, the number of antigen-specific T-cells activated by free CpG-ODN was even diminished by addition of free $Pam_3Cys$, in contrast to the synergistic effect we had observed for encapsulated $Pam_3Cys$. It has been reported that such synergistic effects of adjuvants are most prominent if TLR3 and TLR9 agonists are combined [33]. Therefore, we tested the TLR3 ligand poly(*I:C*) in aqueous solution in combination with TLR9 binding CpG-ODN as adjuvant of AVE3-encapsulated TRP2 peptide vaccine. In analogy to our observations with free $Pam_3Cys$, also free poly(*I:C*) alone did not support induction of antigen-specific T-cell responses and suppressed the adjuvant effects of CpG-ODN (Fig. **5C**).

## DISCUSSION

It is well accepted that systemic distribution and persistence of high doses of free peptide results in the presentation of Ag by non-professional APC within and outside of lymphoid organs eventually resulting in T cell energy and functional deletion of tumor-specific T-cells [36]. At the same time, persistent presentation of tumor antigens by DCs is required for efficient expansion and maintenance of effector CTL phenotype [37, 38] even in the chemo- and radiotherapy based cancer treatment [39]. Accordingly, vaccines have to be engineered to balance availability of antigens or loaded DCs for extended time periods and avoidance of loading of non-professional APCs such as T-cells. Liposomal formulations appear to be ideal for complying with these demands as they form a long lasting Ag depot at the injection side for at least one week and are also transported from the intradermal injection site by DCs to the draining LN [19]. We had shown previously, that encapsulation of a peptide antigen into cationic AVE3 liposomes and its application into footpad of mice along with free 1826CpG-ODN as adjuvant allows for induction of an effective antitumor response even to

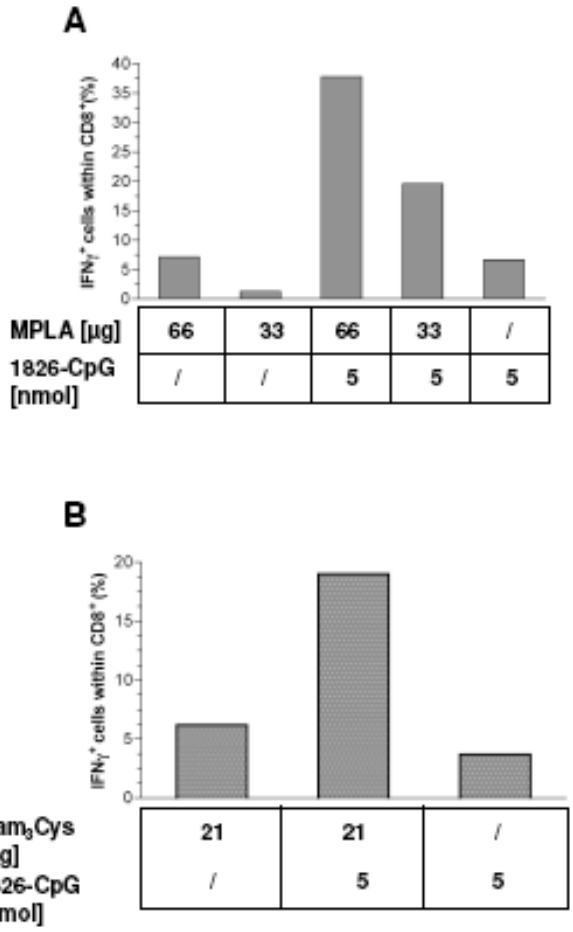


**Fig. (4). Synergistic adjuvant effect between 1826 CpG-ODN and monophosphoryl lipid A (MPLA) and $Pam_3Cys$ in a liposomal formulation.** BL6 mice were immunized twice at weekly interval with a liposomal formulation consisting of MPLA-AVE3/TRP2 or with $Pam_3Cys$-AVE3/TRP2 with or without 1826CpG-ODN (5 nmol) applying 10 µg TRP2/immunization/ mouse. IFN-$\gamma^+$ $CD8^+$ T-cells were determined after restimulation of splenocytes with 1µM TRP2 for 16 h. Shown is one representative experiment out of two. % IFN-$\gamma^+$ cells were always below 0.5% with control SIINFEKL peptide.

nonmutated self antigens of low immunogenicity by generating high avidity CTLs *in vitro*. The aim of this study was to address mandatory issues for optimization of such a vaccine formulation.

Our findings have several important implications:

First, composition of liposomes appears to be of importance for efficient and specific delivery to DCs. Among those tested, we determined AVE3 liposomes as the best formulation with regard to their binding and uptake by DCs. It is not understood yet, which molecular mechanisms are responsible for binding and uptake and whether recognition of liposome components such as phosphatidyl-ethanolamine by specific receptors on the surface of DCs plays a role. A better understanding of these will allow for tailored design of optimized liposome composition and consideration of novel lipophilic components and may even enable targeting of particular DC subpopulations [40].

Second, dose/response relations between liposomal peptide vaccine and the antigen-specific immune response depend on the route of application and as shown for intradermal application in flanks of mice, may also result in unexpected paradoxical effects.

T-cells from mice immunized with 300 µg liposomal TRP2 were unresponsive to a stimulation with TRP2 and to a pan stimulation with anti-TCR + anti-CD28 antibodies. Whether such T cell unresponsiveness is also induced in mice immunized into the footpads can not be definitely answered. Due to limitations in the injectible volume into the footpads we were not able to apply more than 50 µg liposomal TRP2. Coming along with the overall unresponsiveness at high liposomal TRP2 concentrations, we also observed a decline in the number of spleen T and B-cells in a dose-dependent manner. T-cells from mice immunized with free TRP2 did not show any unresponsiveness up to the tested dose of 500 µg TRP2/mouse. Accordingly, no changes in the cellularity of the spleen was observed in these mice. This strong correlation of T cell numbers and their response to TRP2 antigen is obvious and may reflect the fact that due to overstimulation TRP2 specific T cells are indeed depleted. A similar direct effect on T cells was also described recently by others in terms of a strong decrease in OVA-specific proliferation of DO11.10 T cells when DCs were pulsed with high dose of liposomal OVA compared to pulsing with free OVA [41].

The route of vaccine application has a substantial impact on T cell induction, with inoculation into the footpad being superior to intradermal vaccination into the flanks independent of the pharmaceutical formulation of TRP2 peptide (liposomal *vs.* free). This may be due to anatomical and also physical reasons as by the syringe injection pressure, vaccine compounds may be directed from the footpad very efficiently towards the draining popliteal lymph node where Ag presentation by lymph node DCs to $CD8^+$ T-cells can take place [42]. In line with this, popliteal lymph nodes from mice immunized with a CpG-ODN containing vaccine formulation showed a marked increase in size compared to mice immunized i.d. into the flank (data not shown).

As shown formerly AVE3/TRP2 liposomes per se and other cationic liposome formulations do not exhibit an inherent adjuvant effect and at least one adjuvant is necessary to prime and expand TRP2 specific T-cells *in vivo* [19, 43, 44]. Using CpG-ODN solitarily, we did not observe a significant difference between liposomal versus free adjuvant formulations in terms of the number of Ag specific T-cells induced, however, compared to administration of free CpG less liposomal CpG-ODN was necessary [19]. Cationic liposomes are known to enter the endosomal compartment of the cell where beside TLR 7 and 8 also TLR9 is expressed thereby delivering CpG-ODN efficiently to its receptor and this may explain the higher efficacy of liposomal CpG [45, 46].

Another important result of our studies is that AVE3 liposome encapsulation improves adjuvant synergy of some TLR ligands, which is not the case if ligands were given in aqueous solution. Triggering of multiple TLRs may synergistically activate innate immunity as exemplified recently with regard to induction of nitric oxide, IL-12, TNF-α and IL-6 by murine macrophages resulting in enhanced antitumor effects [47]. Therefore we extended the question, whether the combination of different adjuvants could not only

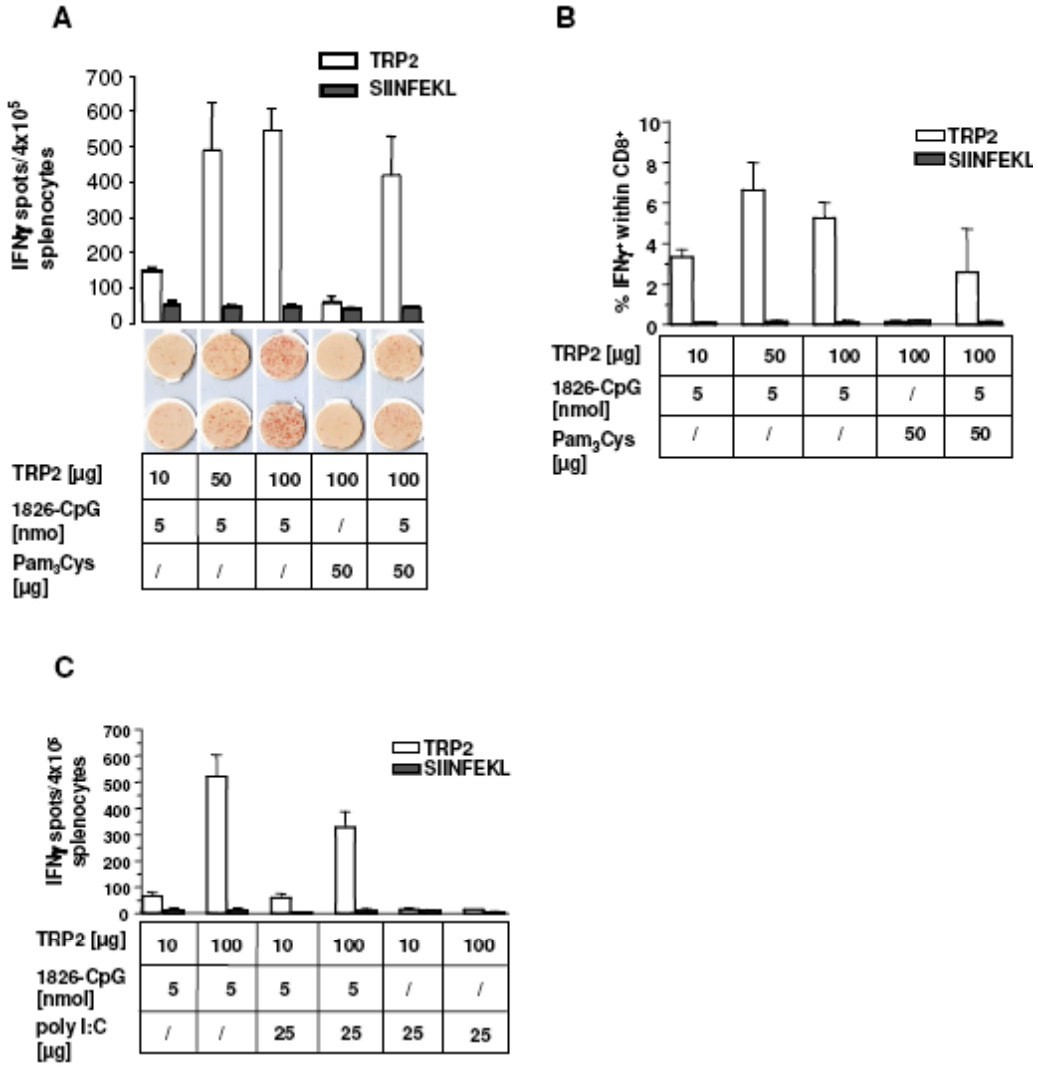


**Fig. (5). Lack of adjuvant effect of Pam3Cys and poly(I:C) with 1826CpG-ODN in a non-liposomal formulation.** BL6 mice were immunized with TRP2 in the presence of the indicated adjuvants according to the immuization scheme as depicted in Fig. **2**. (**A**) The absolute numbers of IFN-γ secreting cells/4x10$^5$ splenocytes after 16h restimulation with TRP2 and control SIINFEKL were quantified by ELISPOT. Shown are duplicates from filters of splenocytes restimulated with TRP2. (**B**) % of CD8+ T-cells were determined by the IFN-γ secretion assay by FACS analysis in the same splenocytes as used for the ELISPOT in (**A**). (**C**) The number of IFN-γ secreting cells were determined in the spleen of mice immunized with the indicated doses of TRP2 and/or 1826 CpG-ODN and poly(I:C). n=3; results are presented as mean ± SEM.

enhance innate but also specific immunity by increasing the number of Ag-specific T-cells. We combined CpG-ODN with the TLR4 ligand MPLA, the TLR1/2 ligand $Pam_3Cys$ and the TLR3 ligand poly(*I:C*) either as a free or a liposomal formulation. When MPLA and $Pam_3Cys$ were used as a liposomal formulation they acted strong synergistically with CpG-ODN, contrariwise even an additive effect was not observed when $Pam_3Cys$ was used in a free form demonstrating the outstanding importance of adjuvant formulation.

The conditions for synergistic action of TLR agonists was recently shown by Napolitani and colleagues with the main focus on DC IL-12p70 release *in vitro* [33]. The triggering of TLRs coupled to the intracellular signalling adapter molecule TRIF (TLR3 and TLR4) and of TLRs localized in endosomal compartments (TLR7/8/9) induced a strong synergistic action. Why the free forms of $Pam_3Cys$ and poly*(I:C)* did not act synergistically in our system may have the following explanations: 1) due to the liposomal formulation the pharmakokinetic is affected in such a way that less adjuvant is needed to act synergistically. As shown previously by our group and others indeed less CpG-ODN is needed when administered as liposomes [19, 29] and this may therefore also be true for MPLA and $Pam_3Cys$. The reason for this may be due to the specific binding to DC thereby increasing the dose of adjuvant at these cells. 2) by the liposomal formulation the adjuvants may be stabilised and protected against degradation resulting in a prolonged bioavailability. 3) in contrast to TLR triggering of pure DC populations *in vitro*, *in vivo* also other cells than DCs like monocytes, macrophages and neutrophils may be triggered by liposomal adjuvants to release cytokines and chemo-kines which may effect the outcome of Ag-specifc T-cells.

Taken together our data underline the significance of the vaccine formulation, both for the Ag and for the adjuvant and demonstrate that liposomal formulations may auxiliary to further boost T cell responses in tumor vaccination strategies. Not only the Ag-formulation but also the route of Ag administration is of significance for the strength and outcome of an Ag-specific T cell response. Taking into account these two aspects may result in enhanced T cell responses and hence in stronger tumor regressions.

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Conflict-of-interest disclosure: A.K. and A.G. hold a patent related to the work described in the present study. I. K., S.K., A.S., M.D., C.H., Ö.T. and U.S. declare no competing financial interests.

## ABBREVIATIONS

| | | |
|---|---|---|
| Ag | = | Antigen |
| TRP2 | = | Tyrosinase related protein 2 |
| AVE | = | Artificial virus envelope |
| AVE3/TRP2 | = | Liposome-encapsulated TRP2-peptide |
| DC | = | Dendritic cell |
| LN | = | Lymphnode |
| ODN | = | Oligodeoxynucleotide |
| i.d. | = | Intradermal |
| TLR | = | Toll like receptor |
| TCR | = | T cell receptor |
| DLPE | = | Dilauroylphosphatidyl-ethanolamine |
| DOPS | = | Dioleoylphosphatidylserine |
| chol | = | Cholesterol |
| EPC | = | Egg phosphatidylcholin |

## REFERENCES

[1] Bernhard H, Karbach J, Wölfel T, *et al.* Cellular immune response to human renal-cell carcinomas: definition of a common antigen recognized by HLA-A2-restricted cytotoxic T-lymphocyte (CTL) clones. Int J Cancer 1994; 59: 837-42.

[2] Sahin U, Tureci O, Pfreundschuh M. Serological identification of human tumor antigens. Curr Opin Immunol 1997; 9: 709-16.

[3] Zhong H, Shurin MR, Han B. Optimizing dendritic cell-based immunotherapy for cancer. Expert Rev Vaccines 2007; 6: 333-45.

[4] Rosenberg SA, Dudley ME. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. Proc Natl Acad Sci USA 2004; 101: 14639-45.

[5] Lucas S, Coulie PG. About human tumor antigens to be used in immunotherapy. Semin Immunol 2008; Apr 3 [Epub ahead of print].

[6] Mocellin S, Nitti D. Therapeutics targeting tumor immune escape: towards the development of new generation anticancer vaccines. Med Res Rev 2008; 28: 413-44.

[7] Allan RS, Waithman J, Bedoui S, *et al.* Villadangos Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity 2006; 25: 153-62.

[8] Zhou Y, Bosch ML, Salgaller ML. Current methods for loading dendritic cells with tumor antigen for the induction of antitumor immunity. J Immunother 2002; 25: 289-303.

[9] Nouri-Shirazi M, Banchereau J, Bell D, *et al.* Dendritic cells capture killed tumor cells and present their antigens to elicit tumor-specific immune responses. J Immunol 2000; 165: 3797-803.

[10] Ignatius R, Mahnke K, Rivera M, *et al.* Presentation of proteins encapsulated in sterically stabilized liposomes by dendritic cells initiates CD8(+) T-cell responses *in vivo*. Blood 2000; 96: 3505-13.

[11] Foged C, Arigita C, Sundblad A, Jiskoot W, Storm G, Frokjaer S. Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. Vaccine 2004; 22: 1903-13.

[12] Subira M, Martino R, Gomez L, Marti JM, Estany C, Sierra J. Low-dose amphotericin B lipid complex *vs.* conventional amphotericin B for empirical antifungal therapy of neutropenic fever in patients with hematologic malignancies-a randomized, controlled trial. Eur J Haematol 2004; 72: 342-7.

[13] Freise CE, Liu T, Hong K, *et al.* The increased efficacy and decreased nephrotoxicity of a cyclosporine liposome. Transplantation 1994; 57: 928-32.

[14] Salem II, Flasher DL, Duzgunes N. Liposome-encapsulated antibiotics. Methods Enzymol 2005; 391: 261-91.

[15] Cheong I, Huang X, Bettegowda C. A bacterial protein enhances the release and efficacy of liposomal cancer drugs. Science 2006; 314: 1308-11.

[16] Goyal P, Goyal K, Vijaya Kumar SG, Singh A, Katare OP, Mishra DN. Liposomal drug delivery systems-clinical applica-tions. Acta Pharm 2005; 55: 1-25.

[17] Pastorino F, Brignole C, Di Paolo D, *et al.* Targeting liposomal chemotherapy *via* both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy. Cancer Res 2006; 66: 10073-82.

[18] Mallick AI, Singha H, Chaudhuri P, *et al.* Liposomised recombinant ribosomal L7/L12 protein protects BALB/c mice against Brucella abortus 544 infection. Vaccine 2007; 25: 3692-704.

[19] Jérôme V, Graser A, Müller 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.

[20] Ludewig B, Barchiesi F, Pericin M, Zinkernagel RM, Hengartner H, Schwendener RA. *In vivo* antigen loading and activation of dendritic cells via a liposomal peptide vaccine mediates protective antiviral and anti-tumour immunity. Vaccine 2000; 19: 23-32.

[21] Bancherau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392: 245-52.

[22] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4: 499-511.

[23] Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408: 740-5.

[24] Hartmann G, Weiner GJ, Krieg AM. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci USA 1999; 96: 9305-10.

[25] Miconnet I, Koenig S, Speiser D, *et al.* CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide. J Immunol 2002; 168: 1212-18.

[26] Vabulas RM, Pircher H, Lipford GB, Hacker H, Wagner H. CpG-DNA activates *in vivo* T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. J Immunol 2000; 164: 2372-8.

[27] Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med 1997; 186: 1623-31.

[28] Heikenwalder M, Polymenidou M, Junt T, *et al.* Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxy-nucleotide administration. Nat Med 2004; 10: 187-92.

[29] Storni T, Ruedl C, Schwarz K, Schwendener RA, Renner WA, Bachmann MF. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. J Immunol 2004; 172: 1777-85.

[30] Gyongyossy-Issa MI, Muller W, Devine DV. The covalent coupling of Arg-Gly-Asp-containing peptides to liposomes: purification and biochemical function of the lipopeptide. Arch Biochem Biophys 1998; 353: 101-8.

[31] Herr W, Linn B, Leister N, Wandel E, Meyer zum Büschenfelde KH, Wölfel T. The use of computer-assisted video image analysis for the quantification of CD8+ T lymphocytes producing tumor necrosis factor alpha spots in response to peptide antigens. J Immunol Methods 1997; 203: 141-52.

[32] Britten CM, Meyer RG, Frankenberg N, Huber C, Wolfel T. The use of clonal mRNA as an antigenic format for the detection of antigen-specific T lymphocytes in IFN-gamma ELISPOT assays. J Immunol Methods 2004; 287: 125-36.

[33] Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 2005; 6: 769-76.

[34] Evans JT, Cluff CW, Johnson DA, Lacy MJ, Persing DH, Baldridge JR. Enhancement of antigen-specific immunity *via* the TLR4 ligands MPL adjuvant and Ribi 529. Expert Rev Vaccines 2003; 2: 219-29.

[35] Takeuchi O, Sato S, Horiuchi T, *et al.* Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002; 169: 10-14.

[36] Toes RE, Offringa R, Blom RJ, Melief CJ, Kast WM. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. Proc Natl Acad Sci USA 1996; 93: 7855-60.

[37] Elamanchili P, Lutsiak CM, Hamdy S, Diwan M, Samuel J. "Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. J Immunother 2007; 30(4): 378-95.

[38] Uto T, Wang X, Sato K, *et al.* Targeting of antigen to dendritic cells with poly(gamma-glutamic acid) nanoparticles induces antigen-specific humoral and cellular immunity. J Immunol 2007; 178: 2979-86.

[39] Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008; 8: 59-73.

[40] van Broekhoven CL, Parish CR, Demangel C, Britton WJ, Altin JG. Targeting dendritic cells with antigen-containing liposomes: a highly effective procedure for induction of antitumor immunity and for tumor immunotherapy. Cancer Res 2004; 64: 4357-65.

[41] Korsholm KS, Agger EM, Foged C, *et al.* The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. Immunology 2007; 121: 216-26.

[42] Grover A, Kim GJ, Lizee G, *et al.* Intralymphatic dendritic cell vaccination induces tumor antigen-specific, skin-homing T lymphocytes. Clin Cancer Res 2006; 12: 5801-8.

[43] Moingeon P, Haensler J, Lindberg A. Towards the rational design of Th1 adjuvants. Vaccine 2001; 19: 4363-72.

[44] White K, Kearns P, Toth I, Hook S. Increased adjuvant activity of minimal CD8 T cell peptides incorporated into lipid-core-peptides. Immunol Cell Biol 2004; 82: 517-22.

[45] Zaks K, Jordan M, Guth A, *et al.* Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. J Immunol 2006; 176: 7335-45.

[46] de Jong S, Chikh G, Sekirov L, *et al.* Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN. Cancer Immunol Immunother 2007; 56: 1251-64.

[47] Whitmore MM, DeVeer MJ, Edling A, *et al.* Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. Cancer Res 2004; 64: 5850-60.

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