PLGA-based particulate vaccine delivery systems for immunotherapy of cancer

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Chapter 4

Efficient *ex vivo* induction of T cells with potent anti-tumor activity by protein antigen encapsulated in nanoparticles

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Abstract

Protein antigen (Ag)-based immunotherapies have the advantage to induce T cells with a potentially broad repertoire of specificities. However, soluble protein Ag is generally poorly cross-presented in MHC class I molecules and not efficient in inducing robust cytotoxic $CD8^+$ T cell responses. In the present study, we have applied poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NP) which strongly improve protein Ag presentation by dendritic cells (DC) in the absence of additional TLR ligands or targeting devices. Protein Ag loaded DC were used as antigen presenting cells (APC) to stimulate T cells *in vitro* and subsequently analyzed *in vivo* for their anti-tumor effect via adoptive transfer, a treatment strategy widely studied in clinical trials as a therapy against various malignancies. In a direct comparison with soluble protein Ag, we show that DC presentation of protein encapsulated in plain PLGA-NP results in efficient activation of $CD4^+$ and $CD8^+$ T cells as reflected by high numbers of activated $CD69^+$ and $CD25^+$, interferon (IFN)-γ and interleukin (IL)-2-producing T cells. Adoptive transfer of PLGA-NP-activated $CD8^+$ T cells in tumor-bearing mice displayed good *in vivo* expansion capacity, potent Ag-specific cytotoxicity and IFN-γ cytokine production, resulting in curing mice with established tumors. We conclude that delivery of protein Ag through encapsulation in plain PLGA-NP is a very efficient and simple procedure to stimulate potent anti-tumor T cells.

Keywords: PLGA, OVA, Ag cross-presentation, Adoptive immunotherapy

Précis
This paper shows that DC loaded with protein encapsulated in biodegradable and clinically applied polymer particles efficiently activate $CD8^+$ T cells *in vitro* which upon adoptive transfer *in vivo* show potent anti-tumor immune responses.

1. Introduction

The adaptive immune system plays a major role in anti-tumor control. Induction of a specific immune response against tumor-associated antigen (Ag) is a potential approach for targeted immunotherapy of cancer. The first step in the initiation of an effective anti-tumor response is the uptake of tumor-associated Ag by dendritic cells (DC) and their subsequent presentation to naïve T cells [1,2,3]. DC are highly efficient antigen presenting cells (APC) and play a central role in initiating and regulating adaptive immunity. DC internalize and process exogenous protein Ag and present processed peptide epitopes in the grooves of MHC class I and II molecules to prime $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ helper T (Th) cells, respectively [4]. CTL are capable of direct clearance of malignant cells [5]. Th cells have shown to be vital in CTL priming through CD40-CD40L interactions with DC [6,7]. In addition, activated Th cells secrete cytokines like IL-2 important for CTL proliferation [8,9].

Full-length protein Ag comprise all potential naturally occurring Th and CTL epitopes

and can be clinically applied irrespective of the patient's HLA haplotype. For that reason, protein-based tumor-associated Ag is currently being applied in a variety of immunotherapeutic approaches against cancer [10,11]. However, recent studies have indicated that cross-presentation of protein Ag is an inefficient process leading to poor CTL responses [12,13]. Therefore, improving cross-presentation of protein Ag by DC is essential to further exploit cancer immunotherapy.

Nanoparticles (NP) prepared from the polymer poly(lactic-co-glycolic acid) (PLGA) are promising clinical grade carriers for improving Ag delivery to DC [14,15,16]. PLGA polymers were originally reported for their use as sutures and implants for surgery [17] and since then they have been applied for the preparation of particles for drug delivery purposes, including the delivery of anti-cancer agents [18,19,20]. Internalization of protein Ag-loaded PLGA particles by DC is very efficient, resulting in adequate MHC class I cross-presentation and CTL proliferation *in vitro* [21]. Despite highly efficient DC uptake and cross-presentation *in vitro*, experimental tumor models have shown that the therapeutic effect of PLGA particle-based protein vaccination *in vivo* is strictly dependent on co-encapsulation of Toll like receptor ligands (TLRL) [22]. The necessity for the addition of TLRL for *in vivo* responses is most likely related to observations showing that PLGA-polymers on their own exhibit poor DC or macrophage stimulatory capacity in comparison to TLR4L [23,24]. However TLRL are dispensable for T cell activation *in vitro*, as reported by two previous studies using biodegradable polymer based artificial APC as a method to stimulate T cells *in vitro*. Applying an elegant method to formulate artificial APC using PLGA, T cells were stimulated *in vitro* with efficient proliferative and cytokine producing capacity [25,26]. However, the *in vivo* effector functions of the *in vitro* stimulated T cells were not studied in those reports.

In the present study, the intrinsic capacity of plain protein Ag-loaded PLGA-NP to induce anti-tumor effector T cells with potent functionality *in vivo* is reported. DC, the immune system's natural and most potent APC, express known and yet unknown co-stimulatory molecules and produce various cytokines vital for optimal T cell priming [27,28]. Using murine DC, we performed a detailed analysis of the ability of protein Ag-loaded PLGA-NP, lacking any additional TLRL or targeting moiety, to induce potent tumor-specific effector T cells. For this analysis, we have used a murine model for adoptive T cell transfer therapy, a treatment modality that has been successfully tested in various (pre-)clinical studies against various types of cancer [29,30]. In this murine adoptive T cell transfer therapy model, we show that protein Ag encapsulated in PLGA efficiently and rapidly induces highly activated specific effector $CD8^+$ T cells with a type I cytokine profile that vigorously expand *in vivo* in tumor-bearing mice and have the potency to eradicate established aggressive tumors.

2. Materials and Methods

2.1. Cells

D1 cells, a GM-CSF dependent immature dendritic cell line derived from spleen of WT C57BL/6 (H-2b) mice, were cultured as described previously [35]. Freshly isolated DC (BMDC) were cultured from mouse bone marrow (BM) cells by collecting femurs from WT C57BL/6 strain and cultured as published previously by our group [36]. After 10 days of culture, large numbers of typical DC were obtained which were at least 90% positive for murine DC marker CD11c (data not shown). B3Z $CD8^+$ T-cell hybridoma cell line, specific for the H-2Kb-restricted OVA257–264 CTL epitope SIINFEKL, expressing a β-galactosidase construct under the regulation of the NF-AT element from the IL-2 promoter, was cultured as described before [37] OT-IIZ, a $CD4^+$ T-cell hybridoma cell line, specific for the I-Ab-restricted OVA OVA323–339 Th epitope, expressing the same β-galactosidase construct, was produced in house. The weakly immunogenic and highly aggressive OVA-transfected B16 tumor cell line (B16-OVA), syngeneic to the C57BL/6 strain, was cultured as described [38].

2.2. Preparation and characterization of protein Ag-loaded PLGA-NP

PLGA-NP were prepared using 7-17 kDa PLGA 50:50 (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany) by applying a modified "water-in-oil-in-water" solvent evaporation method as described [39]. In brief, 50 µl of 20 mg/ml pure, endotoxin-free ovalbumin (OVA, Worthington LS003048) dissolved in 25 mM Hepes buffer (pH 7.4) was emulsified with 1 ml of dichloromethane (DCM) containing 25 mg of PLGA with an ultrasonic processor for 15 s at 70 W (Branson Instruments, CT, USA). The secondary emulsion was prepared with 2 ml of 1% (w/v) polyvinyl alcohol (PVA) in water. The double emulsion was then transferred into 25 ml of a 0.3% (w/v) PVA solution, and stirred at 37 °C for 1 h, and the resulting NP were harvested and washed twice with Milli-Q water by centrifugation at 8000 g for 10 min. The NP suspension was aliquoted in cryovials and lyophilized for 24 h. Prior to use, lyophilized NP particle size distribution was determined by means of dynamic light scattering (DLS) using a NanoSizer ZS (Malvern Instruments, Malvern UK) after resuspension of the particles in Milli-Q water. The zeta potential of the particles was also measured with the NanoSizer ZS by laser Doppler velocimetry. The OVA content of the particles was determined with a BCA protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, and encapsulation efficiency (%EE) was determined according to **equation 1**. 500 µg lyophilized PLGA-OVA were resuspended in 350 µl sterile MQ water and endotoxin

content were determined with Bacterial endotoxins methode D. Chromogenic kinetic method' an assay according to European Pharmacopeia 2.6.14 seventh edition.

$$\%\,EE = \frac{\text{protein mass in NP}}{\text{total protein mass}} \times 100 \quad (1)$$

2.2.1. In vitro release study of PLGA-encapsulated protein

For release studies, protein-loaded PLGA NP were prepared as described, but with the addition of 1% (w/w total OVA) of Ovalbumin-Alexa Fluor® 488 (PLGA-OVA-Alexa488) (Invitrogen). Encapsulation of OVA-Alexa488 proceeds with similar efficiency as the regular OVA with no dye conjugated (See **Table 1**).

Table 1: PLGA-OVA NP characteristics

Formulation	Size (nm)	PDI	ZP (mV)	Protein loading (µg OVA/mg PLGA)	OVA encapsulation efficiency (%)	Endotoxin level (IU/ml)
PLGA-OVA	274 ± 19	0.18 ± 0.02	-27 ± 1	25 ± 1	62 ± 2	0.03 ± 0.00
PLGA-OVA Alexa	338 ± 12	0.22 ± 0.10	-27 ± 5	20 ± 1	49 ± 4	0.03 ± 0.03
PLGA empty	311 ± 52	0.14 ± 0.06	-30 ± 7	n/a	n/a	n/a

PLGA-OVA-Alexa488 were resuspended in 1x PBS pH 7.4 at a concentration of 10 mg PLGA/ml and maintained at 37oC in a water bath under constant tangential shaking at 100 rpm in a GFL 1086 shaking water bath (Burgwedel, Germany). At regular time intervals, 250 µl samples of the suspension were taken, centrifuged for 20 min at 18,000×g and supernatants were stored at 4ºC until fluorescence intensity, was determined by fluorescence spectrometry (Tecan, Infinite M 1000). Concentration of OVA-Alexa488 in the supernatant was assessed against a calibration curve containing known concentrations of OVA-Alexa488. Protein release profiles were generated for each NP formulation in terms of cumulative antigen release (%) over time. Release was determined according to **equation 2**.

$$\%\,R = \frac{\text{protein mass in supernatant}}{\text{protein mass in supernatant} + \text{protein mass in NP}} \times 100 \quad (2)$$

2.3. Enzyme-linked Immunosorbent Assay (ELISA)

DC (100,000/well) were plated into a 96-well round bottom plate and incubated for 24 hr with titrated amounts of Ag. Supernatants were harvested and tested for IL-12 p40 using an ELISA assay kit (BD OptEIA™ MOUSE IL-12 Cat. Nr 555165) following manufacturer's instructions.

2.4. MHC class I or class II-restricted Ag presentation and T cell proliferation

DC were incubated for 24 h with soluble OVA (sOVA) or OVA encapsulated in PLGA-NP (PLGA-OVA) at the indicated concentrations. Cells were washed followed by overnight incubation at 37 °C in the presence of either B3Z - to measure MHC class I Ag presentation of SIINFEKL (OVA257-264) in H-2Kb - or OT-IIZ cells - to assess MHC class II Ag presentation of ISQAVHAAHAEINEAGR (OVA323-339) in I-Ab. A colorimetric assay using chlorophenol red-β-D-galactopyranoside (CPRG) as substrate was used to detect IL-2 induced lacZ activity. OVA-specific proliferation of naïve $CD8^+$ and $CD4^+$ T cells was performed by culturing OT-I or OT-II splenocytes in the presence of DC loaded with titrated amounts of PLGA-OVA or sOVA. After 24 h incubated cells were pulsed with [3H]-thymidine and cultured further overnight. Samples were then counted on a TopCountTM microplate scintillation counter (Packard Instrument Co., Meridan, CT, USA).

2.5. Analysis of T cell phenotype and T cell cytokine profile

DC were loaded for 24 h with 0.25 μM OVA in PLGA (PLGA-OVA) or soluble OVA (sOVA), washed extensively and used as APC to stimulate spleen suspensions from OT-I and OT-II mice. DC and splenocytes were co-cultured for 24 h in the presence of 7.5 μg/ml Brefeldin A. Total cells were harvested, washed twice with PBA buffer (0.01 M sodium phosphate, 0.15 M NaCl, 1% (w/v) BSA, and 0.01% (w/v) sodium azide) followed by staining with PerCP rat anti-mouse CD8α monoclonal antibodies (mAb) and AF-conjugated rat anti-mouse CD3 mAb. To assess T cell activation, cells were stained with FITC-conjugated rat anti-mouse CD69 mAb or PeCy7-conjugated rat anti-mouse CD25 mAb. To study the T cell cytokine profile, $CD8^+$ T cells were stained as above and subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Intracellular IFN-γ in the T cells was stained with APC-conjugated rat anti-mouse IFN-γ. Similarly, IL-2 and IL-4 were stained using PE-conjugated rat anti-mouse IL-2, IL-4 respectively. TNF-α was stained with FITC-conjugated rat anti-mouse TNF-α mAb. All antibodies were purchased from BD

Pharmingen. Flow cytometry analysis was performed using a LSRII flow cytometer (BD Pharmingen) and analyzed with FlowJo software (Treestar).

2.6. *In vivo* cytotoxicity

To obtain OVA-specific effector $CD8^+$ T cells, single cell suspensions were prepared from spleen and lymph nodes of OT-I mice, washed twice and resuspended in IMDM supplemented with 10% (v/v) FCS. Whole single cell suspensions were cultured in 6-wells plates with Ag (0.25 μM) loaded DC for 24 h at a ratio of 25:1. DC and splenocyte cultures were incubated for 24 hr at 37ºC. Purified $CD8^+$ T cells were obtained by a negative selection protocol using the Mouse CD8 T Cell Lymphocyte Enrichment Set - DM (BD Biosciences) according to the manufacturer's instructions. This protocol yielded $CD8^+$ T cell purities of at least 90% (data not shown). 2.5 x 106 Purified $CD8^+$ T cells were transferred to syngeneic WT C57BL/6 animals that were rested for 24 h after adoptive cell transfer. To obtain OVA-specific target cells, splenocytes from naïve congeneic C57BL/6 Ly5.1 mice were pulsed for 1 h with 1 μM of SIINFEKL-peptide and co-stained with 10 μM CFSE (CFSE-high) (Molecular Probes, Eugene, OR). As a negative control, 1 μM of the immunodominant ASNENMETM-peptide derived from the influenza virus nucleoprotein co-stained with 0.5 μM CFSE (CFSE-low) was used. Specific and non-specific target cells were mixed 1:1 and injected intravenously (i.v.; 10 x 106 cells of each population). Eighteen hours after cells were transferred, mice were sacrificed and spleen cells were harvested to prepare single cell suspensions that were then subjected to flow cytometric analysis. Injected cells were distinguished by APC-conjugated rat anti-mouse CD45.1 mAb. The percentage specific killing was calculated as follow: 100 - ([(% SIINFEKL-peptide pulsed in treated/% ASNENMETM-pulsed in treated)/(% SIINFEKL-peptide pulsed in non-treated/% ASNENMETM-pulsed in non-treated)] x 100).

2.7. Adoptive transfer OVA-specific T cells in B16-OVA tumor bearing and naïve mice

WT C57BL/6 mice were injected subcutaneously (s.c.) in the right flank with 2 x 105 B16-OVA melanoma cells. Seven days after tumor injection, when tumors were palpable, mice were treated by intravenous infusion of 2.5 x 106 purified effector $CD8^+$ T cells derived from OT-I mice, *ex vivo* stimulated for 24 h in the presence of DC loaded with either PLGA-OVA or sOVA. Tumor growth was measured 1 - 3 times a week and survival was monitored daily. Tumor size (mm2) was calculated by (length) × (width). Mice with tumor sizes that equaled or exceeded 140 mm2 were sacrificed. Tail vein blood samples were collected on day 10, 17 and 31 after $CD8^+$ T cell transfer. Blood samples were prepared by erythrocyte lysis, followed by 2 washing steps with PBA buffer. Transferred $CD8^+$ T cells were analyzed by co-staining with APC-conjugated rat anti-mouse Thy1.1 mAb, FITC-conjugated anti-mouse CD8α mAb and AF-conjugated rat anti-mouse CD3 mAb in combination with APC-Cy7-conjugated anti-mouse CD44

antibody and PB-conjugated anti-mouse CD62L antibody. OVA-specific $CD8^+$ T cell mediated cytokine production was detected by overnight stimulation of peripheral blood cells with SIINFEKL-peptide in the presence of 7.5 µg/ml Brefeldin A. Medium was used as a negative control to correct for baseline cytokine production. Cytokine profile was analyzed by intracellular cytokine staining as described above.

2.8. Statistical analysis

Graph Pad Prism software was used for statistical analysis. Values and percentages of specific $CD8^+$ T cells and secreted cytokine production were analyzed by two-tailed unpaired Student t test. Differences in animal survival between the different groups were calculated using Log-rank (Mantel-Cox) test.

3. Results

3.1. Nanoparticle characterization and protein antigen load and release

We prepared several batches of PLGA-OVA NP with similar characteristics. Particles used in our study had an average size of 327 ± 65 nm (mean ± SD; n = 7) and a polydispersity index (PDI) of 0.19 ± 0.07. Encapsulation efficiency of OVA in NP was determined to be 59 ± 5%. Empty particles used as control particles in this study had a comparable size (311 ± 52 nm) and PDI (0.15 ± 0.05). Endotoxin levels were determined for the prepared batches and was shown to be below 0.04 IU/ml in particle suspensions prepared as described in material and methods. (see **Table 1)**. Release kinetics of OVA from the PLGA-OVA particles were analyzed over a period of 35 days. The validity of using OVA-Alexa 488 fluorescence as a measure of (unlabeled) OVA release was confirmed by measuring the OVA content of the nanoparticles and the total amount released at the end of the release study by BCA assay, which gave very similar values as the fluorescence method (results not shown).

The NP had a burst release of the encapsulated OVA of 28.1 ± 0.2%. At the end of the analysis, we could detect 80.4 ± 2.2% of released OVA in suspension indicating that after 35 days about 20% of the originally encapsulated OVA was still associated with NP showing the slow release character of these NP (see **Supplemental Figure 1**).

3.2. Efficient protein MHC class I and class II Ag presentation by DC loaded with protein encapsulated in PLGA-NP

The efficiency of Ag (cross)-presentation of encapsulated protein Ag in comparison to soluble protein Ag was studied *in vitro*. DC were incubated for 24 h with titrated amounts of Ag, as indicated in μM, either encapsulated in PLGA-NP (PLGA-OVA) or in soluble form (sOVA). Ag presentation by MHC class I or II was assessed using the $CD8^+$ (B3Z) and $CD4^+$ (OT-IIZ) T cell hybridomas. DC loaded with PLGA-OVA very efficiently triggered B3Z T cells (**Figure 1A**). In contrast, DC pulsed with sOVA poorly stimulated B3Z $CD8^+$ T cells unless very high concentrations (≥ 64 μM) of sOVA were used (data not shown). MHC class I cross-presentation of protein Ag was strictly dependent on encapsulation in PLGA-NP, as a mixture of the sOVA with empty PLGA-NP did not induce $CD8^+$ T cell activation (**Figure 1B**). In addition, DC loaded with PLGA-OVA resulted in at least 100-fold enhanced activation of OT-IIZ $CD4^+$ T cells in comparison to DC loaded with sOVA, indicating that also MHC class II presentation was dramatically improved by encapsulation (**Figure 1C**). Next to Ag presentation, we analyzed proliferation of naïve $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells induced by DC loaded with PLGA-OVA or sOVA. Co-culture of Ag pulsed DC with either OT-I or OT-II T cells for 72 h, including overnight incubation in the presence of [3H]-thymidine for the last 18 h showed that PLGA-OVA was at least 1000-fold more efficient than sOVA in inducing OT-I T cell proliferation (**Figure 1D**) and 100-fold better than sOVA in inducing OT-II T cell proliferation (**Figure 1E**). Similar to the used D1 dendritic cells, freshly isolated BMDC loaded with PLGA-OVA were superior in comparison to sOVA-loaded BMDC in the stimulation of OT-I and OT-II T cells resulting in improved T cell proliferation (**Supplemental Figure 2**). In addition, we analyzed DC maturation by surface expression of CD86 and determining the amount of IL-12 in culture supernatants after incubation with the NP after 24 hr incubation. Our data show that the empty or OVA-loaded PLGA NP do not detectably activate and mature DC (**Supplemental Figure 3**) in contrast to LPS (TLR4L) or PolyI:C (TLR3L). This indicates that encapsulation of soluble protein antigen in plain PLGA NP strongly enhances antigen presentation by DC irrespective of DC maturation.

3.3. Activation of T cells by DC loaded with PLGA-NP-encapsulated protein Ag

We analyzed whether PLGA-NP based delivery of protein Ag could induce T cell activation and production of pro-inflammatory cytokines. Naïve OVA-specific $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells were stimulated for 24 h in the presence of PLGA-OVA- or sOVA-loaded DC and analyzed for cells expressing the early activation marker CD69. Both $CD8^+$ (**Figure 2A**) and $CD4^+$ (**Figure 2B**) T cells showed strongly enhanced

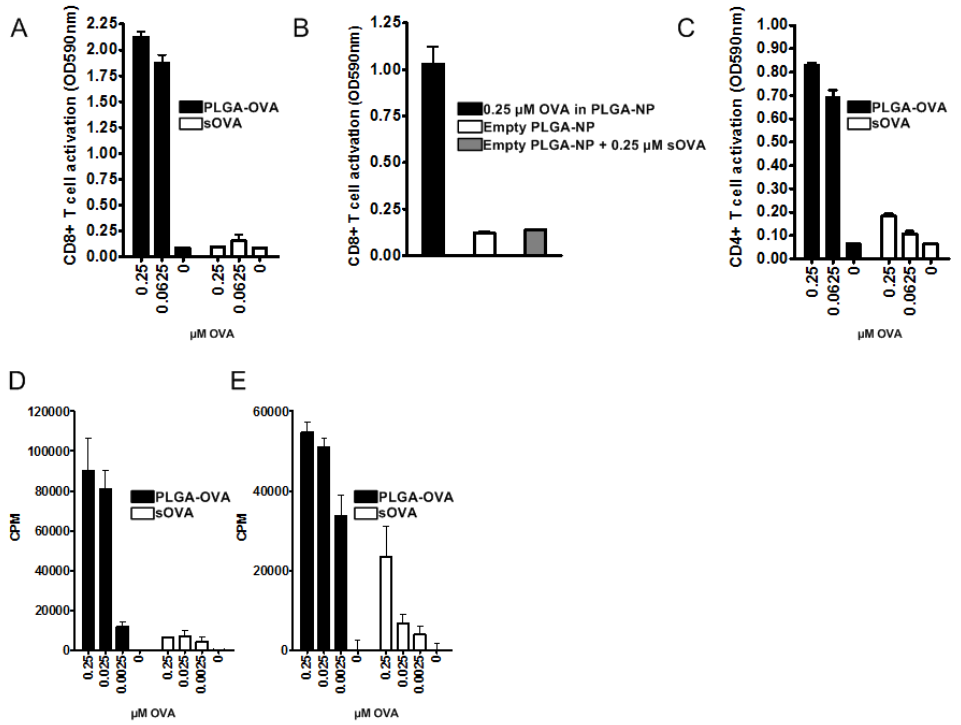

Figure 1: Efficient MHC class I and- class II presentation of OVA Ag incorporated in PLGA-NP. A) D1 cells were pulsed for 24 h with titrated amounts (µM) of OVA, either in soluble form sOVA or encapsulated in PLGA-NP (PLGA-OVA). MHC class I presentation was detected by co-culture with H-2Kb/SIINFEKL-specific B3Z CD8⁺ T cells; **B)** D1 cells were pulsed for 24 h with 0.25 µM OVA in PLGA-OVA, empty PLGA-NP, or a mixture of empty PLGA-NP with 0.25 µM sOVA, washed and co-cultured with B3Z CD8⁺ T cells to assess MHC class I Ag presentation; **C)** D1 cells were pulsed for 24 h with titrated amounts of PLGA-OVA or sOVA, washed, and co-cultured with I-Ab/ISQAVHAAHAEINEAGR-specific OT-IIZ CD4⁺ T cells to assess MHC class II Ag presentation. BMDC were loaded with titrated amounts of PLGA-OVA or sOVA. Ag loaded DC were subsequently used to activate naïve OT-I; **D)** or OT-II T **(E)** cells for 72 h. T cell proliferation was measured in triplicate by 3[H]-thymidine uptake. Data shown are means of triplicate measurements ± SD from one representative example out of at least three independent experiments.

single IFN-γ producers and a relatively smaller population of IL-2 single producers. The cytokines IL-4 and TNF-α could not be detected after *in vitro* stimulation of either CD8⁺ or CD4⁺ T cells with PLGA-OVA pulsed DC (data not shown).

3.4. DC loaded PLGA-NP-encapsulated protein Ag induces CD8⁺ T cells with *in vivo* cytotoxic capacity

To assess their cytotoxic capacity, CD8⁺ T cells stimulated *in vitro* by PLGA-OVA-loaded DC were studied for their ability to lyse Ag-pulsed target cells *in vivo*. Following stimulation, the purified CD8⁺ T cells were transferred into recipient mice. After 24 h SIINFEKL-loaded target and control-target cells were injected and 18 h later mice were sacrificed and spleen single cell suspensions were analyzed by flow cytometry. In line with the observed activation status and cytokine profile, CD8⁺ T cells stimulated with PLGA-OVA-loaded DC demonstrated cytotoxicity against SIINFEKL-loaded target cells. In contrast, CD8⁺ T cells co-cultured in the presence of sOVA-loaded DC were not capable of killing target cells (**Figure 3A** and **B**).

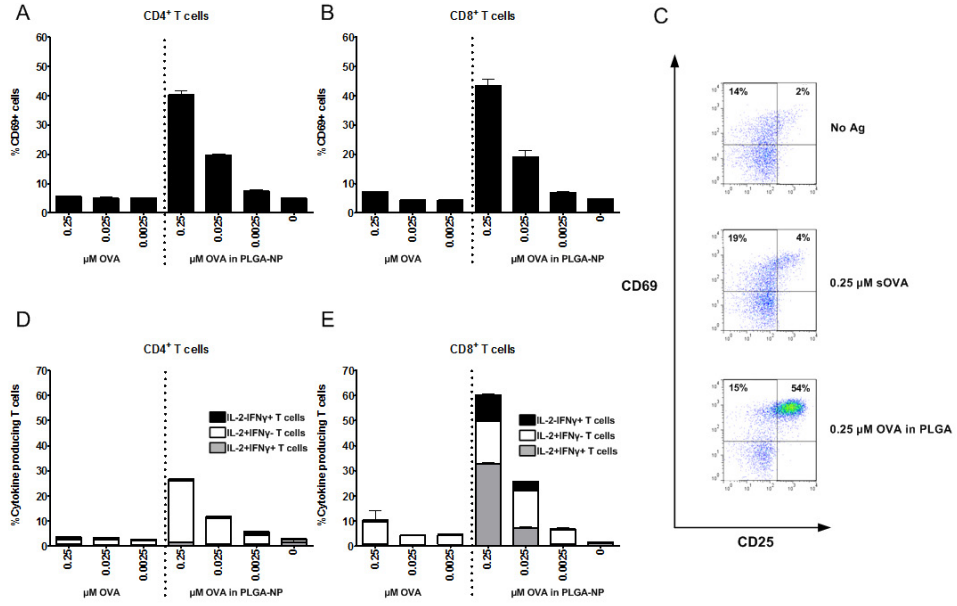

Figure 2: DC pulsed with PLGA-OVA, but not sOVA, induce strong activation of T cells. D1 cells were pulsed for 24 h with titrated amounts of sOVA or PLGA-OVA. Ag loaded DC were washed to remove excess Ag, and co-cultured for an additional 24 h with OT-I or OT-II splenocytes. Cells were harvested and analyzed by flow cytometry for the cell surface expression of A) CD69 on CD8⁺ T cells and B)CD4⁺ T cells ; C) Expression of CD25 and CD69 was analyzed on CD8⁺ T cells which were stimulated for 24 h with DC which were loaded with either PLGA-OVA or sOVA. Immature DC without Ag served as negative control. Intracellular production of IL-2 and IFN-γ by D) CD8⁺ T cells and E) CD4⁺ T cells was analyzed by flow cytometry after 24 hr stimulation with DC pulsed with titrated amounts of PLGA-OVA or sOVA. One representative experiment out of three independent experiments is shown. Data shown are means of triplicate measurements ± SD.

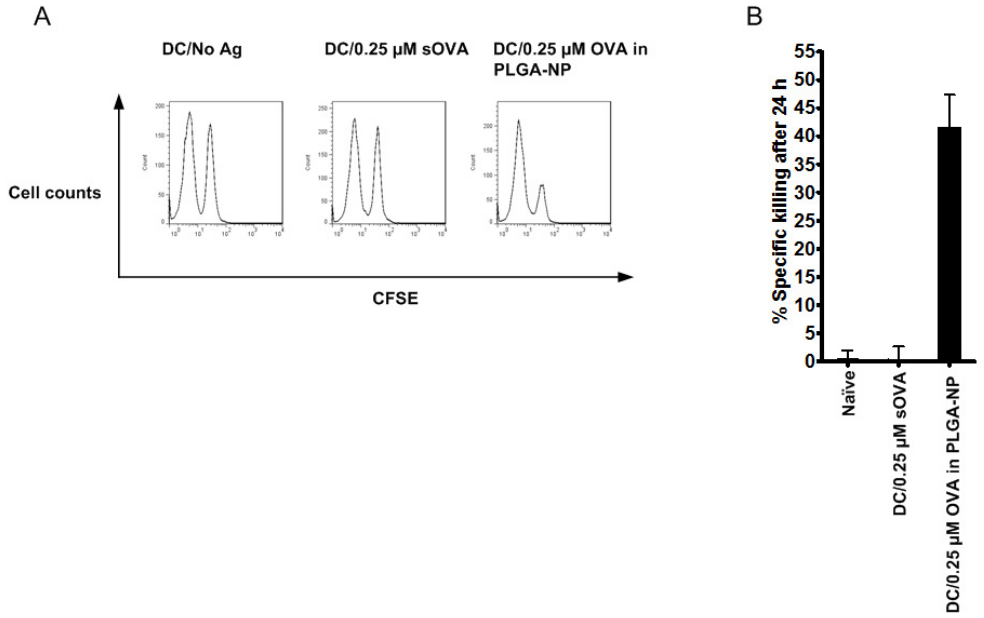

Figure 3: Enhanced *in vivo* **cytotoxicity of** *ex vivo* **PLGA-OVA-stimulated CD8⁺ T cells. A)** Mice were transferred with purified CD8⁺ OT-I T cells which were *in vitro* stimulated with D1 cells loaded with PLGA-OVA and sOVA. Differentially CFSE-labeled SIINFEKL-peptide loaded and control target cells were i.v. administered. After 18 h the spleens from recipient animals were harvested and analyzed by flow cytometry for percentage of specific killing of target cells; **B)** Experiment was performed twice and averages ± SEM of n = 7 mice for each condition are shown in bar graphs.

inoculated s.c. with OVA-expressing B16 melanoma tumor cells. After 7 days, animals were treated by adoptive T cell transfer therapy by a single i.v. injection of 2.5 x 106 purified OVA-specific CD8⁺ T cells stimulated for 24 h *in vitro* in the presence of DC loaded with either PLGA-OVA or sOVA. Tumor growth and animal survival in CD8⁺ T cell transferred mice were compared to those in non-treated animals. Animals were developing palpable tumors within 10 days after s.c. tumor inoculation. In tumor-bearing mice that were treated by adoptive transfer with OVA-specific CD8⁺ T cells stimulated in the presence of PLGA-OVA-loaded DC, we observed a clear therapeutic effect which resulted in delayed tumor growth in comparison to non-treated animals and animals treated with CD8⁺ T cells stimulated with sOVA loaded DC. We observed regression of tumors in the range of 2 - 4 mm2 in some animals by day 14, which were undetectable on day 18 after tumor challenge (insert in **Figure 4A**). By day 22, four animals within this group had tumor recurrences which eventually grew out. Nevertheless, 8 out of 12 tumor-challenged mice treated with PLGA-OVA induced CD8⁺ T cells remained tumor free for the duration of the experiment (**Figure 4A**). By contrast, in tumor challenged animals that received sOVA induced CD8⁺ T cells, tumors reappeared in 11 out of 12 mice and grew out, albeit at a decreased rate when compared to non-treated animals (**Figure 4B**). In all non-treated animals, tumors grew out fast and all mice were sacrificed

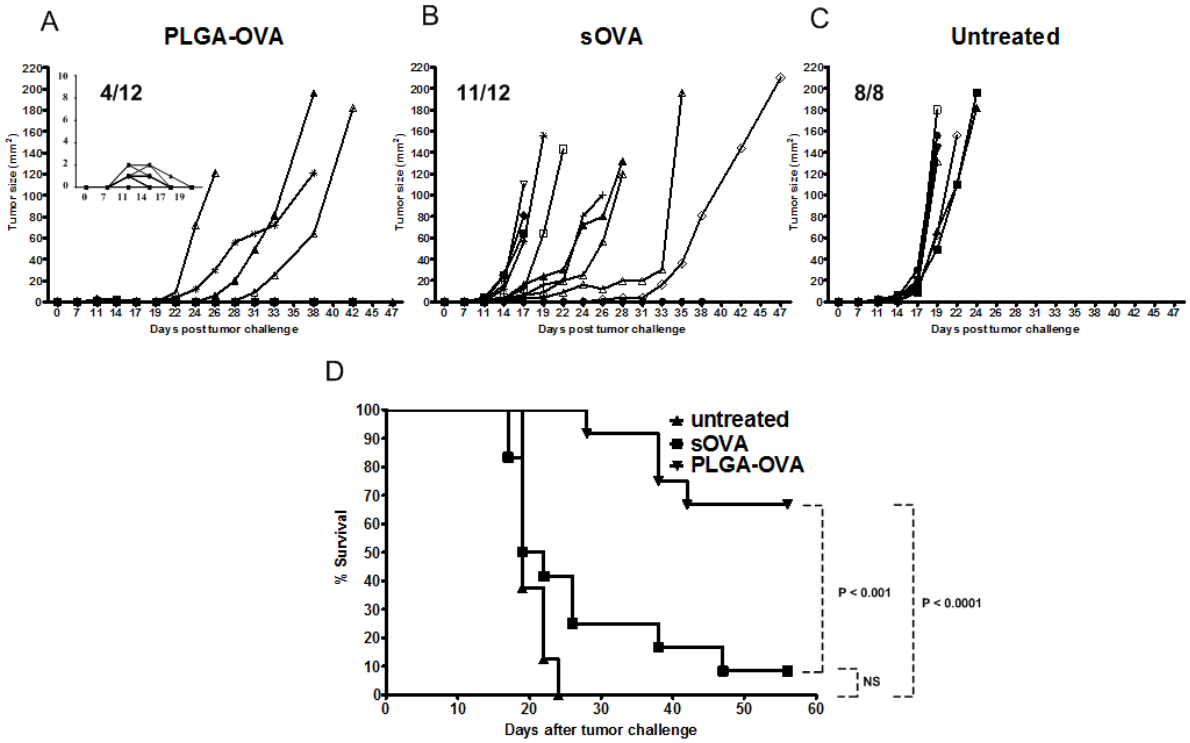

Figure 4: CD8⁺ T cells primed by PLGA-OVA loaded DC eradicate established tumors. Animals were inoculated s.c. on the right flank with 2 x 105 B16-OVA tumor cells and rested for 1 week followed by a single i.v. injection of 2.5 x 106 purified CD8⁺ T cells which were *ex vivo* stimulated with Ag loaded DC as described above. Tumor growth was monitored in individual animals treated with DC/PLGA-OVA induced CD8⁺ T cells (**A**; n = 12 animals), DC/sOVA induced CD8⁺ T cells (**B**; n = 12 animals) or in untreated animals (**C**; n = 8 animals). Insert in A) represents tumor growth curves in the initial 19 days after tumor challenge; **D**) Animal survival per group was assessed and differences between the different groups were calculated using Log-rank (Mantel-Cox) test. $p < 0.001$ for animals treated with DC/PLGA-OVA compared to DC/sOVA induced CD8⁺ T cells. $p < 0.0001$ for animals treated with DC/PLGA-OVA compared to untreated animals. NS = $p > 0.05$ for animals treated with DC/sOVA compared to untreated animals.

by day 24, because of maximum allowed tumor burden (**Figure 4C**). Tumors that did grow in animals that received PLGA-OVA induced $CD8^+$ T cells had a significantly slower average growth rate when compared to tumors from mice that received sOVA induced $CD8^+$ T cells. Consequently, the survival of mice treated with PLGA-OVA induced $CD8^+$ T cells was significantly higher when compared to mice treated with $CD8^+$ T cells stimulated in the presence of sOVA-loaded DC (**Figure 4D**).

3.6. $CD8^+$ T cells stimulated with PLGA-NP-encapsulated protein Ag efficiently expand and produce type I cytokines *in vivo*

We measured by flow cytometry the actual numbers of OVA-specific $CD8^+$ T cells ($CD8^+Thy1.1^+$ OT-I cells) in peripheral blood of tumor challenged mice up to a month after adoptive transfer. Ten days after adoptive transfer of equal amounts of purified OVA-specific $CD8^+$ T cells, mice that had received PLGA-OVA induced cells showed 5-fold higher levels of $CD8^+$ T cells than animals that had received sOVA stimulated $CD8^+$ T -cells (**Figure 5A**). The percentage of PLGA-OVA induced $CD8^+$ T cells remained significantly higher at day 17 and 31 after transfer. (**Figure 5B**). A similar *in vivo* expansion capacity of PLGA-OVA induced $CD8^+$ T cells was observed upon transfer in naïve mice, i.e. not challenged with tumors (data not shown). Furthermore, we analyzed the production of cytokines by $CD8^+$ T cells in peripheral blood by intracellular staining. To this end, peripheral blood mononuclear cells were harvested from mice at day 10, 17 and 31 after adoptive transfer and stimulated overnight in the presence of SIINFEKL-peptide. IFN-γ-, IL-2- and TNF-α-producing $CD8^+$ T cells were detectable by flow cytometry. On day 10 after adoptive transfer, we observed significantly higher percentages of IFN-γ-producing $CD8^+$ T cells in mice that had received PLGA-OVA induced $CD8^+$ T cells when compared to mice that had received sOVA induced $CD8^+$ T cells. We were unable to detect IFN-γ-producing $CD8^+$ T cells in tumor-bearing animals which were not treated using adoptive T cell transfer therapy (data not shown). Although, the percentage of IFN-γ producing $CD8^+$ T cells declined at day 17 and 31 after adoptive transfer, the IFN-γ-producing $CD8^+$ T cells remained significantly higher throughout the analysis period (**Figure 5C**). A trend in increased levels of IL-2- and TNF-α-producing $CD8^+$ T cells could be observed in mice transferred with PLGA-OVA but not with sOVA induced $CD8^+$ T cells (data not shown). In addition, the phenotype of peripheral blood OVA-specific T cells at day 17 and 31 after adoptive transfer of PLGA-OVA induced $CD8^+$ T cells was analyzed. The majority of the cells possessed a central memory phenotype based on high expression of CD62L (L-selectin) and CD44 (**Figure 5D**) showing the superior functionality of the T cells expanded with this simple expansion protocol with NP encapsulated protein antigen.

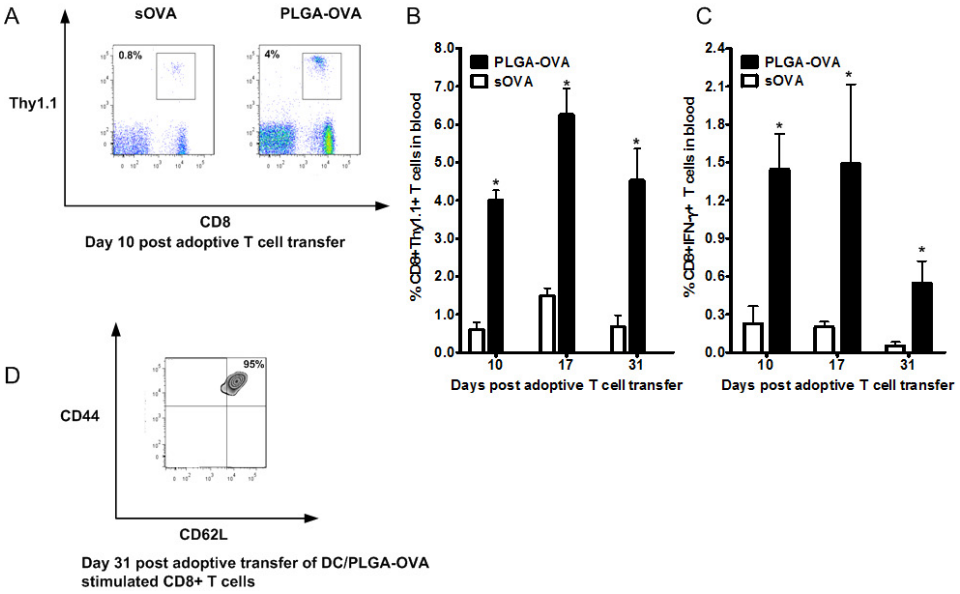

Figure 5: PLGA-OVA stimulated CD8⁺ T cells expand and persist in the peripheral blood and have higher capacity to produce IFN-γ. A) Tumor bearing animals received a single i.v. injection of CD8⁺ T cells stimulated with PLGA-OVA-loaded DC. Tail vein blood samples were taken on day 10 after adoptive transfer of the CD8⁺ T cells and numbers of CD8⁺Thy1.1⁺ T cells were measured by flow cytometry; **B)** *In vivo* persistence of i.v. transferred CD8⁺Thy1.1⁺ T cells in tumor bearing animals was monitored for 4 weeks in blood on day 17 and 31; **C)** Intracellular IFN-γ production by CD8⁺ T cells was analyzed on day 10, 17 and 31 after adoptive transfer; **D)** Memory phenotype of transferred DC/PLGA-OVA *in vitro* stimulated CD8⁺Thy1.1⁺ T cells was determined by analysis of CD44 and CD62L surface expression. Results shown are averages ± SEM from n = 3-12 mice per group, dependent on the number of animals alive at each time-point post tumor challenge. * = $p < 0.05$ for animals treated with DC/PLGA-OVA compared to DC/sOVA induced CD8⁺ T cells using a un-paired student t test.

4. Discussion

In this study we analyzed the phenotype and *in vivo* functionality of T cells stimulated *in vitro* by DC loaded with plain PLGA-NP encapsulated protein Ag with no additional immune stimulatory agent or targeting moiety. We showed that encapsulation of protein Ag in plain PLGA-NP not only enhanced Ag (cross-)presentation by DC but also improved the functionality of the induced T cells to cure animals from tumors upon adoptive T cell transfer. OVA antigen in PLGA-NP was more efficiently processed and presented in MHC class I and II by DC and resulted in potent activation and proliferation of OVA-specific CD8⁺ and CD4⁺ T cells, high production of type I cytokines and tumor

control resulting in an overall survival of 75% of tumor-bearing animals.

PLGA-based particles as vaccine delivery systems were pioneered already more than 30 years ago [14]l lactic-co-glycolic acid. Several studies have shown that efficient anti-tumor immune responses *in vivo* by PLGA-particles require not only encapsulated Ag, but also a co-encapsulated adjuvant such as a TLRL, surface coating of particles with mannan or protamine to stimulate immunity or DC targeting moieties, for example anti-DEC205 antibodies [22,36,37,38].

$CD8^+$ T cells induced in our study were applied for adoptive T cell transfer purposes. Adoptive T cell transfer therapy potency in (pre)clinical setting is enhanced upon efficient *ex vivo/in vitro* stimulation/manipulation of donor T cells [30,39,40]. Using a similar murine model, efficient stimulation of T cells with potent effector functions was reported using artificial APC systems [26]. They constructed PLGA-based artificial APC expressing MHC class I molecules containing a specific CTL short-peptide epitope, which also provides T cell co-stimulation in the form of CD28 and CD3 triggering and releases IL-2 [41,42]. We propose that our simple approach with natural APC is equally efficient and has the advantage that our method is not restricted to the known MHC class I and II-presented T cells epitopes. In addition, the use of natural DC as APC might facilitate priming of T cells via more co-stimulatory pathways [43,44,45] and additional DC-mediated cytokines required for optimal type I pro-inflammatory T cell activation, for example IL-12 [46], and avoids sub-optimal formation of the immunological synapse as has been described for other bead-based artificial APC systems [47].

Adoptive T cell therapy has yielded promising results as a cancer immunotherapy in the last decade [30,48]. Standard adoptive transfer protocols mandate that T cells are cultured for 2 – 14 days in the presence of specific Ag and exogenous cytokines [39,49,50,51] for optimal stimulation and expansion. In contrast, we opted for a short 24 h stimulation of T cells without addition of any exogenous cytokines. We favor short incubation with DC loaded with PLGA-OVA, which potently activates T cells, because longer incubation periods might tip the balance to activation induced cell death (AICD) [52,53]. In addition, our protocol allowed us to transfer T cells that were not skewed based on the cytokines added to the cultures [54,55] nor negatively affected by the added cytokines [42,56,57].

In our culture systems we used two types of DC: D1 cells, a well characterized murine splenic DC cell line, originally isolated from WT C57BL/6 animals [58] and bone marrow derived DC. Both $CD11c^+$ myeloid types of DC were cultured as immature cells in GM-CSF containing media. D1 DC do not exhibit substantial functional differences with BMDC, they possess equal capacity to prime T cells and upon transfer to recipient animals show similar efficiency to induce protective anti-tumor immunity [32]. We compared $CD4^+$ and $CD8^+$ T cell proliferation by PLGA-NP encapsulated protein Ag and we observed similar observations using either D1 cells or WT BMDC as APC. Therefore, easily cultured myeloid types of DC are well suited for the T cell activation protocol with NP encapsulated Ag we describe here.

In this study splenocytes from OT-I mice, which contain high numbers of OVA-specific

T cells, were used to activate and adoptively transfer into recipient animals. We are aware that in clinical settings, majority of patients which were treated with adoptive T cell transfer therapy exhibit lower precursor frequencies of TAA-specific T cells that require stimulation and expansion to yield sufficient numbers for adoptive transfer. Our system still works by DC/PLGA-OVA stimulation of cell cultures containing lower amounts (between 1 - 10%) of OVA-specific T cells regarding T cell activation and cytokine production (data not shown). On the other hand, higher precursor frequencies of both $CD4^+$ and $CD8^+$ Ag-specific T cells have been observed in draining lymph nodes of cervical cancer patients [59,60]. These cells were able to produce type I pro-inflammatory cytokines and proliferate upon specific stimulation suggesting that these cells might be suitable for future adoptive T cell transfer protocols. Indeed, in a melanoma patient case report, it was shown that $CD4^+$ T cells isolated in relatively higher precursor frequencies could be successfully stimulated and transferred to the recipient back to the patient resulting in a clinical response [61].

The PLGA-OVA particles used in our study are devoid of any additional TLRL or immunostimulatory agents. We observed no differences in T-cell proliferation by MyD88 KO BMDC loaded with PLGA-OVA (data not shown), it is therefore unlikely that any unanticipated TLR-stimulation plays a role in our system. The enhanced T cell proliferation and activation is most likely the result of enhanced uptake of Ag available for efficient processing and MHC presentation. Uptake of particulate matter proceeds via phagocytosis [62,63] of protein Ag-loaded PLGA particles by DC resulting in adequate MHC class I cross-presentation to CTL [64]. DC internalize particles and maintain these intracellularly for up to 72 hr [65]. Prolonged presence of Ag inside cells has been shown to result in sustained MHC class I Ag presentation and efficient priming of $CD8^+$ T cells [66].

Transferred DC/PLGA-OVA stimulated $CD8^+$ T cells were still detectable 31 days post adoptive transfer and the majority of these cells possessed a central memory phenotype correlating with tumor control. Numbers of specific T cells *in vivo* have been shown to directly correlate with tumor-regression [67]. Efficient tumor killing is achieved upon efficient $CD8^+$ T cell activation accompanied with high production of type I pro-inflammatory associated cytokines which is dependent on the method of *in vitro* activation [68,69]. Our data are in line with these reports as peripheral $CD8^+$ T cells from mice which received DC/PLGA-OVA stimulated $CD8^+$ T cells were capable of producing type I pro-inflammatory cytokines upon specific peptide stimulation. Indeed, expansion of IFN-γ producing T cells correlates with clinical effect in patients with human papillomavirus type 16 induced vulvar intraepithelial neoplasia [70].

Numbers of cytokine-producing cells decreased with time in all treated animal groups The decrease in numbers of cytokine-producing OVA-specific $CD8^+$ T cells in time might be related to lack of sufficient OVA-specific $CD4^+$ T cells. Co-transfer of DC/PLGA-OVA *in vitro* stimulated $CD4^+$ T cells may prolong and sustain higher numbers of cytokine producing effector $CD8^+$ T cells [71].

We conclude that protein Ag delivery by PLGA-NP might be an attractive and simple strategy to improve *ex vivo* tumor-specific T cell stimulation for clinical adoptive

T transfer therapy. Apparently, the intrinsic characteristics of PLGA-Ag NP to be efficiently internalized and processed by DC is sufficient to induce effector T cells *in vitro* with expansion capacity *in vivo*, and with strong therapeutic effectiveness. So, encapsulation of tumor associated protein Ag in PLGA-NP may serve as a clinically feasible strategy to generate T cells with optimal effector quality for adoptive transfer-based immunotherapy of cancer.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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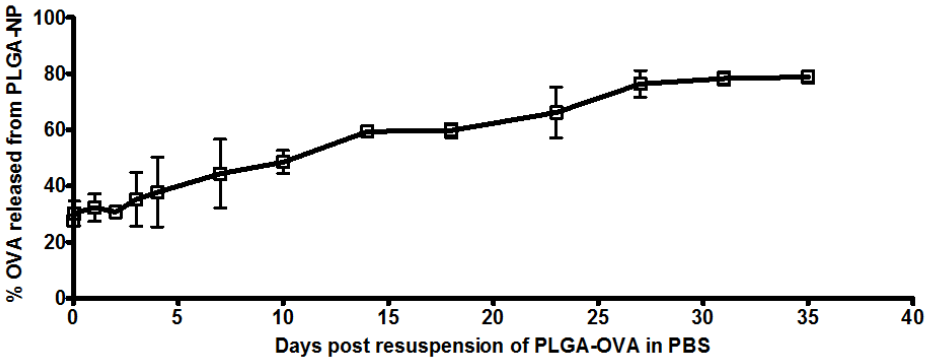
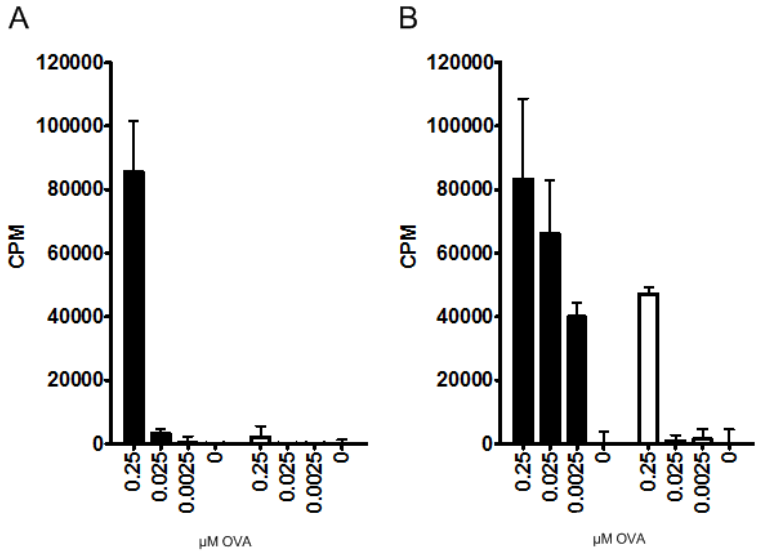
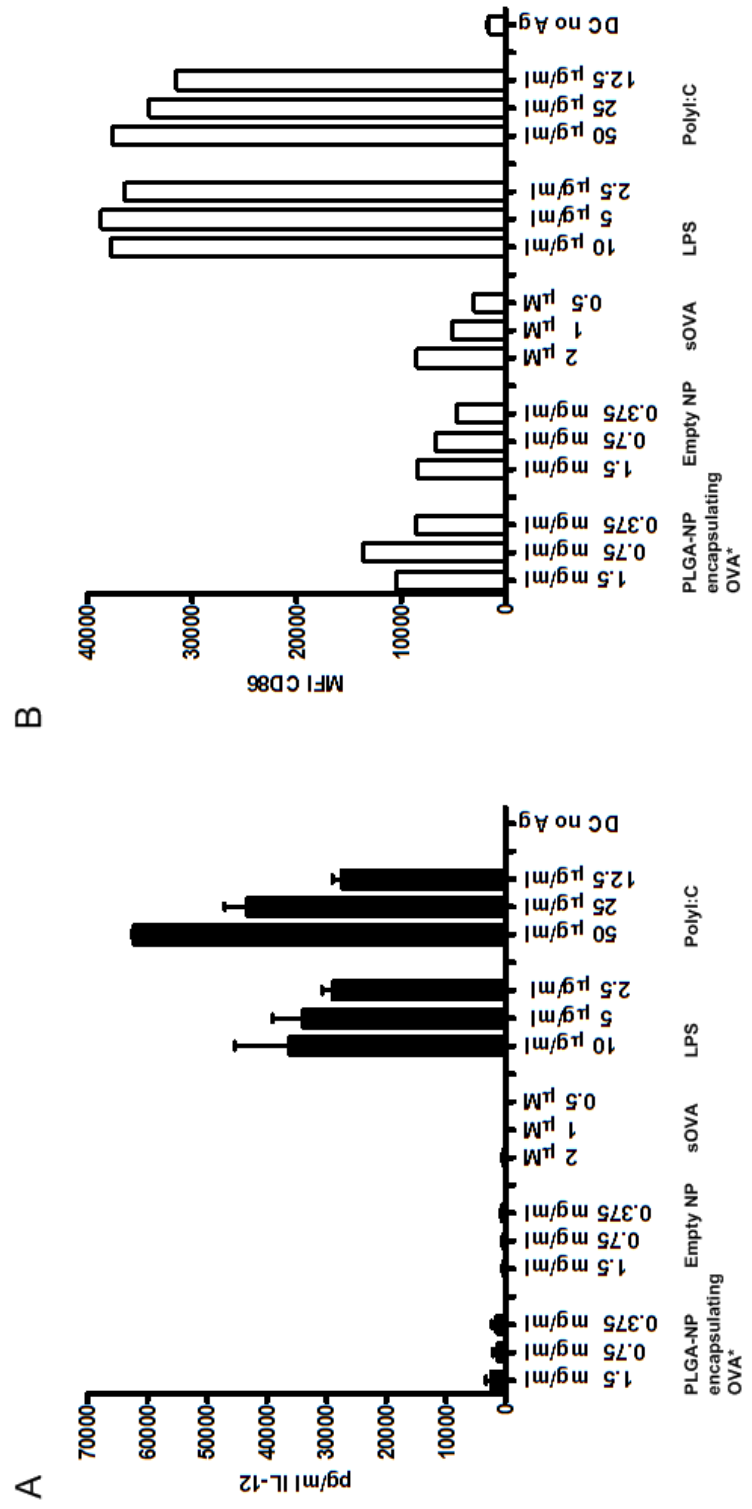
6. Supplemental Material



Supplemental Figure 1. Encapsulated OVA is released gradually from PLGA-NP. PLGA-OVA-Alexa488 with an average OVA content of 14.11 ± 2.89 µg OVA/mg PLGA were re-suspended at a concentration of 10 mg/mL of PBS and incubated at 37ºC under constant shaking. At indicated time points 250 µl samples were collected, centrifuged for 20 min at 18,000xg and the amount of OVA-Alexa488 in the supernatant determined by fluorescence, as described in the material and methods. Average results of two independent release studies with four different batches of PLGA-OVA are shown, mean ± SD.



Supplemental figure 2. Improved CD4⁺ and CD8⁺ T cell proliferation by BMDC loaded with PLGA-OVA in comparison to sOVA-loaded BMDC. WT C57BL/6 BMDC were incubated with titrated amounts of PLGA-OVA or sOVA. Ag loaded BMDC were subsequently used as APC in a co-culture with naïve OT-I **(A)** or OT-II T **(B)** cells for 72 h. T cell proliferation was measured in triplicate by 3[H]-thymidine uptake. Data shown are representative of two independent experiments.



Supplemental Figure 3. PLGA-OVA do not mature DC in comparison to TLRL. DC were incubated in the presence of titrated amounts of PLGA-OVA, empty PLGA-NP, sOVA, a mixture of sOVA and empty PLGA-NP, LPS (Sigma L4130, Escherichia coli 0111:B4) and Poly(I:C (Invivogen, tlrl-picw). After 24 hr supernatants were collected and analyzed via ELISA for IL-12 levels **(A)**. DC were harvested and analyzed by flow cytometry for the expression of CD86 **(B)**. Data shown are representative of three independent experiments. *1.5 mg/ml of PLGA-NP encapsulating OVA (PLGA-OVA) is required to obtain of 0.25 µM OVA