Nanoparticulate vaccine inhibits tumor growth via improved T cell recruitment into melanoma and huHER2 breast cancer

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Abstract

Nanoparticulate vaccines are promising tools to overcome cancer immune evasion. However, a deeper understanding on nanoparticle–immune cell interactions and treatments regime is required for optimal efficacy. We provide a comprehensive study of treatment schedules and mode of antigen-association to nanovaccines on the modulation of T cell immunity in vivo, under steady-state and tumor-bearing mice. The coordinated delivery of antigen and two adjuvants (Monophosphoryl lipid A, oligodeoxynucleotide cytosine-phosphate-guanine motifs (CpG)) by nanoparticles was crucial for dendritic cell activation. A single vaccination dictated a 3-fold increase on cytotoxic memory-T cells and raised antigen-specific immune responses against B16.M05 melanoma. It generated at least a 5-fold increase on IFN-γ cytokine production, and presented over 50% higher lymphocyte count in the tumor microenvironment, compared to the control. The number of lymphocytes at the tumor site doubled with triple immunization. This lymphocyte infiltration pattern was confirmed in mammary huHER2 carcinoma, with significant tumor reduction.

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Key words: Cancer vaccine; Cytotoxic T cells; Melanoma; Breast cancer; Tumor-infiltrating lymphocytes

One of the fundamental roles of the immune system is the maintenance of tissue homeostasis and immunosurveillance including the detection and destruction of foreign or altered invaders, such as pathogens or tumors. This process actively involves the coordination of innate and adaptive immune reactions and the discrimination between self and non-self-antigens.1,2

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The cancer immunotherapy approach uses these specificities of the immune system to provide a more efficacious and better tolerated therapy in contrast to conventional chemotherapeutic and radiotherapy. Several vaccination strategies have been previously tested in vivo, including whole tumor cells, cell lysates, proteins, or specific peptide fragments, leading to the generation of tumor-specific T cell responses. Nevertheless, the therapeutic benefit of these approaches stays unclear and further well-designed studies are needed to clarify the potential of each approach.

Nanoparticle (NP)-based vaccines offer several advantages over antigens and adjuvants in solution, such as safety, cost efficacy, and versatile platforms that can improve the targeted delivery of tumor antigens. By prolonging antigen release, these biomaterial-based particulate carriers can extend antigen exposure to professional antigen-presenting cells (APC), boosting the proliferation of tumor-specific cytotoxic T lymphocytes (CTL) and thus augmenting specific anti-cancer efficacy compared to soluble antigens. These particulate delivery systems, in fact, mimic pathogens that are commonly recognized, phagocytosed, and processed by APC. Dendritic cells (DC) are the most important APC and play an important role in bridging innate and adaptive immune responses. When APC encounter NP, they are activated and further migrate to lymph nodes where they present the antigen to T cells, in an immunostimulatory context as major histocompatibility complex (MHC) class I (MHC-I) or II (MHC-II)-peptide complex to the CD8+ cytotoxic T lymphocytes (CTL) or CD4+ T helper (Th) cells, respectively. For T cells to become activated, next to the antigen presentation through MHC-peptide complex, two additional signals need to be guaranteed, namely co-stimulation and cytokine signals. Accordingly, efficient immunity requires the simultaneous co-delivery of antigens and adjuvants, such as the Toll-like receptor (TLR) ligands, to DC.

Our group has been devoted to the application of NP as antigen delivery carriers for cancer immunotherapy through the development and study of biodegradable polymeric NP. We recently developed two novel nanovaccines, which had antigen adsorbed onto the NP surface (AdsNP), or entrapped within the polymeric matrix (EntrapNP). Two polymers glycol chitosan (gCS) and poly(lactic-co-glycolic acid) (PLGA) or poly(ethylene glycol) (PEG)-PLGA block copolymers were selected for the NP formulations. When developing these carriers, we aimed to produce AdsNP and EntrapNP with highly similar NP physicochemical properties, such as average size and polydispersity index (PDI), were inferred by Dynamic Light Scattering (DLS), while ζ potential was assessed by Laser Doppler Velocimetry (LDV). NP morphology was investigated by tapping mode with Atomic Force Microscopy (AFM) (Supplementary material).

Results

Our group recently developed two nanoparticulate vaccines, one presenting the antigen adsorbed onto the NP surface (AdsNP), while the other delivered the antigen entrapped within the polymeric matrix (EntrapNP). Their physicochemical properties were previously characterized, including antigen-NP association. Here, we explored the ability of these distinct carriers to modulate the activation and functionality of
DC-mediated antigen-specific T cell immune responses in vivo under physiological conditions and in two different tumor-bearing mice, upon different vaccination schedules.

Both AdsNP and EntrapNP allowed high co-entrapment of the antigen and two TLR adjuvants (CpG and MPLA) (Table 1). EntrapOVA NP and AdsOVA NP presented a mean size diameter below 195 nm, with a narrow particle size distribution (Table 2). NP size and PDI did not significantly change after inclusion of the two adjuvants (CpG and MPLA) (Table 2), regardless of the mode of antigen association, thus confirming NP batch stability, reproducibility and homogeneity. The ζ-potential measured in Phosphate Buffered Saline (PBS) (pH 7.4) that mimics the intracellular cytosolic environment, was for all formulations close to neutrality (Table 2).

EntrapOVA NP showed 19.56 μgOVA/mg polymer, while the adsorption efficiency of AdsOVA NP was 19.72 μg/mg. We utilized gCS polymer in all formulations, due to its high viscosity and positive charge (+30 mV)\(^{14-16}\) that led to higher polymer-protein physical and chemical interactions, augmenting antigen loading in both NPs.\(^{16,17}\) Antigen loading was considerably higher compared to other recently published studies.\(^{18,19}\)

AFM images (Figure 1, A-B) show that both NP presented a smooth surface and spherical topography with uniform size distribution, which correlated with average size distribution data obtained by DLS. However, section analysis of EntrapOVA NP demonstrated an average diameter 30 nm smaller than the hydrodynamic diameter evidenced by DLS, due to the hydrated PEG chains that extend and form corona. This specific phenomenon was not detected on AFM images of AdsOVA NP.

In vivo uptake of antigen-loaded NP by APC

We first studied the in vivo Rho-labeled NP internalization by CD11b\(^+\)CD11c\(^-\), CD11c\(^+\)CD11b\(^-\) and CD11c\(^-\)CD11b\(^+\) cells,\(^{20}\) as representatives of the myeloid popliteal and inguinal lymph node compartment at 16 h p.i., with high throughput and spatial resolution ImageStreamX (Figure 1; Suppl. Figure S1). In Figure 1, we presented the percentage of each APC population with internalized NP from the total population of cells that was interacting with NP.

CD11c\(^+\)CD11b\(^+\) classical DC presented approximately 50% uptake for AdsNP and EntrapNP. DC internalized EntrapNP and AdsNP with similar efficiencies, without significant preference. CD11c\(^+\)CD11b\(^-\) classical DC presented the highest internalization of EntrapOVA NP. Only a minor amount (~10%) of NP was internalized by CD11b\(^+\)CD11c\(^-\) cells, i.e., macrophages (Figure 1, C-E).

Intriguingly, animals vaccinated with AdsOVA NP also displayed 38% plasmacytoid DC (pDC), defined as PDCA-1\(^+\) cells in the draining lymph nodes that were positive for NP, which were significantly higher (*P < 0.05) compared to EntrapOVA NP (Figure 1, E).

In vivo T cell activation and proliferation at 6, 16, 24 and 48 h after NP immunization

EntrapOVA NP and AdsOVA NP were used in combination with the engraftment of antigen-specific CD4\(^+\) (OT-II) and CD8\(^+\) (OT-I) T cells, carrying a transgenic OVA-responding T cell receptor (TCR), to better trace and characterize the kinetics of the in vivo activation and proliferation of antigen-specific immune response upon vaccination. The phenotypic changes in the expression of surface molecules (CD25, CD69, and CD44) were measured in combination with CFSE dilution (Suppl. Figure S2). CD25 and CD69 are early activation markers,\(^{21,22}\) while CD44 is a detection marker of early T cell memory.\(^{23,24}\) After immunization with EntrapOVA NP and AdsOVA NP, we observed an increase in the absolute number of activated T

### Table 1

<table>
<thead>
<tr>
<th>NP formulation</th>
<th>Lipophilic phase</th>
<th>Entrapped aqueous phase</th>
<th>Surfactant IP(^a)</th>
<th>Surfactant EP(^b)</th>
<th>Protein adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>EntrapOVA</td>
<td>PLGA-PEG</td>
<td>gCS, 10% PVA, OVA</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EntrapOVA-Adjsc(^c)</td>
<td>PLGA-PEG + MPLA(^d)</td>
<td>gCS, 10% PVA, OVA, CpG</td>
<td>2% (w/v) PVA</td>
<td>0.3% (w/v) PVA</td>
<td>–</td>
</tr>
<tr>
<td>AdsOVA</td>
<td>PLGA</td>
<td>gCS, 10% PVA</td>
<td>–</td>
<td>–</td>
<td>OVA</td>
</tr>
<tr>
<td>AdsOVA-Adjsc(^c)</td>
<td>PLGA, MPLA(^d)</td>
<td>gCS, 10% PVA, CpG</td>
<td>–</td>
<td>–</td>
<td>OVA</td>
</tr>
</tbody>
</table>

\(^a\) Internal phase.

\(^b\) External phase.

\(^c\) Adjuvants.

\(^d\) Dissolved in DCM.

### Table 2

Physicochemical characterization of OVA-loaded NP. Mean ± SD; n ≥ 5.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (nm)</th>
<th>PDI(^a)</th>
<th>ζ-potential (mV)</th>
<th>Protein loading (μg/mg)</th>
<th>Loading capacity</th>
<th>Surface adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>EntrapOVA</td>
<td>188 ± 8.0</td>
<td>0.160 ± 0.05</td>
<td>0.41 ± 0.20</td>
<td>19.56</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>EntrapOVA-Adjsc(^b)</td>
<td>198 ± 7.0</td>
<td>0.180 ± 0.03</td>
<td>0.66 ± 0.30</td>
<td>18.97</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AdsOVA</td>
<td>195 ± 14</td>
<td>0.190 ± 0.10</td>
<td>−1.15 ± 0.50</td>
<td>-</td>
<td>19.72</td>
<td></td>
</tr>
<tr>
<td>AdsOVA-Adjsc(^b)</td>
<td>197 ± 12</td>
<td>0.200 ± 0.04</td>
<td>−1.32 ± 0.40</td>
<td>-</td>
<td>21.17</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Polydispersity Index.

\(^b\) Adjuvants.
cells, as shown by the serial dilution of CFSE dye indicating new generation of activated T cells (Figure 2, A-B). The percentages of CD4⁺ and CD8⁺ T cells expressing CD25 and CD69 increased exponentially over time. The number of CD25⁺/CD69⁺ CD4⁺ and CD25⁺/CD69⁺ CD8⁺ T cells increased 2- or 3-fold respectively, within 16 h p.i. Consistent with the results observed for CD25⁺ and CD69⁺ T cells, the percentage of CD4⁺ and CD8⁺ T cells expressing CD44 activation marker was gradually upregulated over time with either NP. However, AdsOVA NP showed 20% lower ability (**P < 0.001) to induce CD44 at 48 h.

In vivo cytotoxicity assay

Upon immunizations with NP formulations, with or without adjuvants, efficient in vivo killing activity towards the SIINFEKL-pulsed cells was generated, as shown by the disappearance of the CFSE⁺ peak of cells (Figure 3, A, Suppl. Figure S3). The positive control (OVA and Adjs in solution) presented the same trend, while the negative control group without OVA antigen did not display detectable killing activity. Similar data were obtained for the cytotoxic ability of T cells in the lymph nodes (Figure 3, A) and spleens (data not shown).

Establishment of memory CD8⁺ T cells

Long-lasting and stable immunological memory is essential for successful vaccination, guaranteeing rapid and effective responses to foreign antigens previously encountered. Here we report the establishment of a long-lasting effector memory CTL 8 weeks upon single immunization of a nanoparticulate vaccine.
Antigen recall induced a remarkable proliferation of CD8+ (OT-I) in mice immunized with EntrapOVA-Adjs NP 8 weeks earlier (Figure 3, B-C; gating strategy in Suppl. Figure S4). The CD8+ T cell population obtained following the immunization of animals with the mixture of OVA and adjuvants in solution was 3-fold smaller (***P < 0.001) than that induced following the immunization with AdsOVA-Adjs NP (Figure 3, C). Interestingly, significantly lower re-stimulation was detected after the immunization with AdsOVA-Adj NP or OVA & Adjs in solution demonstrated a similar proliferation (0.38% and 0.29%, respectively) (Figure 3, C) and thus a statistically non-significant difference between the amount of cytotoxic memory T cells.

Two groups, challenged with PBS and EntrapOVA-Adjs NP without re-stimulation, were used as controls and did not show expansion of CD8+ (OT-I) T cells. As expected, animals immunized with vaccines free of any adjuvant (AdsOVA NP, EntrapOVA NP or OVA in solution) failed to generate memory CD8+ (OT-I) T cells.

Nanovaccines’ therapeutic efficacy against B16.MO5 melanoma challenge

We evaluated the impact of two treatment schedules, next to NP antigen-association method (Figure 4, A). Both parameters might be pivotal for tumor regression and infiltration of lymphocytes in the TME.

The groups immunized with PBS and Empty NP served as controls and presented similar tumor growth, indicating that the nanoparticulate-vehicle itself did not possess an immunotherapeutic effect. All the other treated groups showed a significant reduction in tumor growth (***P < 0.001) upon the administration of nanovaccines (Figure 4, B). The slowest tumor growth was achieved after the 3-dose immunization schedule with EntrapOVA-Adjs NP, followed by the animals treated with 3-times AdsOVA-Adjs NP and a single dose of EntrapOVA-Adjs NP. This can be also appreciated in the representative images of melanoma tumor mass removed at the end of the assay (Figure 4, C). The mean mass of tumors per 100 g of final body weight (Figure 4, E) was in accordance with the obtained mean tumor growth curves. The EntrapOVA-Adjs NP 3-times immunized group presented the lowest mean tumor mass per 100 g of final body weight, even if no statistically significant differences were observed for the tumor mass of animals immunized with our nanoparticulate formulations. Finally, no abrupt weight loss was observed throughout the assay (Figure 4, D).

As nanovaccine efficacy could be attributed to the adoptive transfer of OT-I (CD8+) and OT-II (CD4+) T cells, we addressed this hypothesis by excluding the graft prior triggering the immune response and administering only the developed nanoparticulate vaccine. This study provides additional insights into the NP-mediated immune response induced in one of the most severe melanoma phenotypes.

A single vaccination with EntrapOVA-Adjs NP or AdsOVA-Adjs NP was able to significantly decrease mean tumor development and growth, compared to control group (**P < 0.001, Supp. Figure 5A-C), even in the absence of OT-I cells. The strongest effect was observed in animals treated with EntrapOVA-Adjs NP. Therefore, the anti-tumor immunity was induced following the development of an adaptive immune response starting with the endogenous pool of T cells.

T cell infiltration within tumor microenvironment

TIL have been previously reported to confer a positive prognostic value in the efficacy of immunotherapeutic approaches in tumor patients.26–28
Increased levels of antigen-specific TIL (Figure 5, A-B; Suppl. Figure S6) were observed in tumors of all treated groups compared to PBS and Empty NP by flow cytometry. The vaccination with EntrapOVA-Adjs NP presented 8-fold higher count for CD8⁺ and 3-fold for CD4⁺ T cells, compared to the control group, indicating high success rate in tumor reduction (***P < 0.001 and **P < 0.01, respectively). On the other side, single or 3-time dose of AdsOVA-Adjs NP was less effective in T cell infiltration, particularly regarding CD4⁺ T cells, while recruitment of CD8⁺ TIL was statistically significant (*P < 0.05), when compared to control (Figure 5, B-C). An extensive increase (10-fold) of intra-tumoral T cells was also detected in the absolute number of TILs after 3-time vaccination (Figure 5, A), which can be also observed in the hematoxylin and eosin (H&E)-stained sections (Figure 5, D).

H&E corroborate with previous evidences, showing that upon vaccination with the nanovaccines, the infiltration and proliferation of TIL were raised, compared to PBS or empty NP groups. CD8 and CD4-stainings (Figure 5, D) presented a general increase of CD8⁺ and CD4⁺ TIL after the administration of a single dose of the EntrapOVA-Adjs nanovaccines. Finally, 3-time immunization with nanovaccines led to the highest infiltration of CD8⁺ and CD4⁺ TIL.

Antigen-specific T cell activation in melanoma-bearing animals

To characterize the activation of antigen-specific T cell in the response to tumor, we measured the cell surface expression of: i) CD107a (also known as lysosomal-associated membrane glycoproteins; LAMP-1) on antigen-specific CD8⁺ (OT-I) T
cells, ii) cytotoxic T-lymphocyte-associated-antigen 4 (CTLA-4) and iii) programmed cell death protein 1 (PD-1) cell surface receptors on T cell populations in different organs, namely spleen, lymph nodes and tumor (Supp. Figure S7).

The results showed upregulation of CD107a after immunization with AdsOVA-Adjs NP or EntrapOVA-Adjs NP (***P < 0.001), compared to controls, in which CD107a levels remained below 5% (Figure 6, A-C). Comparing single and 3-time vaccination, all treatments provoked significant upregulation levels of CD107a. However, animals vaccinated only with a single dose of AdsNP presented a 2-fold decrease of CD8+ T cell activation in the draining lymph nodes compared to the animals immunized with EntrapNP. This suggests a more favorable activation of CD8+ T cells in case of EntrapNP, potentially enhancing memory effect against cancer.

On the other side, we detected PD-1 upregulated expression on CD4+ T cell upon the 3-time vaccination protocol with AdsOVA-Adjs NP or EntrapOVA-Adjs NP in lymph nodes (Figure 6, D-E), but not in spleens of the treated animals. A 40% upregulation was observed especially in the group vaccinated three-times with AdsOVA-Adjs NP, while mice immunized with EntrapOVA-Adjs (3×) presented a 20% increase on the expression of PD-1 on CD4+ T cell, suggesting better T cell priming and activation.

Cytokine quantification in tumors and spleens of treated melanoma-bearing mice

Cytokines are responsible for regulation, initiation and promotion of cell-to-cell communication. In order to understand the type of immune response that nanovaccines and tested schedules were driving, we determined the levels of inflammatory cytokines (IFN-γ, TNF-α, IL-4) by intracellular staining (Suppl. Figures S8-S9). CD8+ T cells in the spleens presented upregulated secretion of IFN-γ and TNF-α cytokines after a single immunization with the nanovaccine (AdsNP or EntrapNP), but not after a multiple vaccination schedule (***P < 0.001, Suppl. Figure S9, A-B). On
the other side, the tumor infiltrating CD8+ T cells secreted only significantly higher levels of IFN-γ upon a single immunization with EntrapOVA-Adjs or AdsOVA-Adjs NP (**Pb0.001, Suppl. Figure S9, D–E). Interestingly, all the vaccines failed to induce secretion of IL-4 (data not shown). In addition, serum levels of those pro-inflammatory cytokines were not different from those detected in the control groups (Suppl. Figure S10).

**Therapeutic efficacy of the MHCI and MHCII HER2 peptide NP against mammary huHER2 tumor**

EntrapNP and the multivaccination approach presented the most potent immunomodulatory effect in healthy and melanoma-bearing mice. Therefore, in this final proof of concept study, we co-entrapped MHCI and MHCII HER2 peptides with the previously used adjuvants (CpG and MPLA) in EntrapNP. Once the orthotopic tumor reached 3-5 mm in diameter, animals received three doses of the vaccines, 7 days apart (Figure 7, D). The control groups (PBS and Empty NP) presented similar tumor growth, while the group treated with specific tumor antigens showed a significant delayed tumor growth (*Pb0.05, **Pb0.01 and ***Pb0.001).

Importantly, the group treated with EntrapHER2-Adjs NP presented lower incidence of metastatic lesions into other mammary glands.

Treatment with the nanovaccine, upon tumor challenge, resulted in the recruitment of TIL into the TME (Figure 7, E). Representative histology sections of H&E staining show a significant infiltration of TIL in tumor-bearing mice treated with EntrapHER2-Adjs NP, compared to the control groups. Particularly, from immunohistochemistry, we can appreciate that the vaccination with EntrapHER2-Adjs NP recruited mainly CD8+ T lymphocytes with the increase support of CD4+ T cells (Figure 7, E).

**Discussion**

AdsNP or EntrapNP was composed of gCS and PLGA or PLGA-PEG polymers, which have been used and characterized over a decade for the development of nanomedicines.30–34 For the EntrapNP, we utilized PLGA-PEG polymer to best attain the hydrophilic surface of the nanovaccine, which was granted in the AdsNP through the adsorption of the protein.
presence of PEG chains shielded the negative charge of PLGA at NP surface in EntrapOVA NP. PLGA and PLGA-PEG polymers possess hydroxyl functional groups, while gCS has both hydroxyl and amine functional groups, which facilitate NP cytosolic translocation from acidifying endocytic vesicles, via so-called “proton sponge effect”. Subsequent NP decomposition in the cytosol will favor the cross-presentation of exogenous antigens through MHCI pathway, leading to an enhanced activation and proliferation of CTL that are pivotal in destroying cancer cells.

Both delivery systems were efficiently internalized by APC, particularly DC. However, analysis showed that DC subsets presented different preferential and internalization profiles for both types of nanovaccines. EntrapNP was mainly internalized by CD11c+CD11b− followed by CD11c+CD11b+ DC, 56% and 41%, respectively, while AdsNP was internalized by different

Figure 7. Therapeutic efficacy of the HER2 antigen-loaded nanoparticles (NP) tested on orthotopic huHER2 breast cancer model mice. (A) Mean tumor growth curves. (B) Representative images of mammary tumors removed at the end of the assay. (C) Mean final tumor weight/100 g of final body weight. (D) Assay schedule. (E) Representative histology sections of H&E staining and immunohistochemistry staining for CD8 and CD4 (multiple fields and magnifications, N = 3). Bars, 200 μm. Graph values are presented as Mean and all the error bars are standard deviations of the mean. Number of independent assays that were conducted for this experiment is 2; for each group we used 6-7 mice. Significant differences in tumor growth reduction compared to the control groups PBS and Empty NP were presented as *P < 0.05, **P < 0.01 and ***P < 0.001.
APC (CD11c⁺CD11b⁻, CD11c⁺CD11b⁺ and PDCA-1⁺ cells) in similar proportion (48%, 49% and 40%, statistically non-significant). We assume that this resulted from the pathogen-like appearance of AdsNP. Overall, these findings may imply that variations in antigen association influence DC subsets internalization profile, resulting in particular T cell responses and thus, have a different impact on the control of tumor evasion, which we further investigated in this paper. This study indeed opens new lines for further investigations into the molecular mechanisms and interactions of NP with non-classical subpopulations of DC. Yet this is beyond the scope of this paper.

Our main focus was on the activation of adaptive immune responses. Thus, we used OT-I (CD8⁺) and OT-II (CD4⁺) T cell transfer to get a clean readout system for the antigen-specific T cell response. We found that within the first 48 h p.i., differences in the overall antigen-specific CD4⁺ and CD8⁺ T cell activation were significant for both nanovaccines. However, we detected 20% lower (**p < 0.001) levels of CD44, an early memory marker, 48 h upon AdsNP immunization, suggesting a lower capacity of AdsNP in inducting effective CD8⁺ memory response. Yet, the 48 h period is too short to support this assumption. Therefore, we performed an additional study to better investigate the induction of the long-lasting and robust memory of CTL in 8 weeks' timeframe upon the immunization with AdsNP or EntrapNP. We found that the in vivo cytotoxicity assay, due to the low sensitivity inherent to this technique, could not provide quantitative information about the higher efficacy and selectivity of the vaccine in killing SIINFEKL-pulsed target cells. However, we could confirm qualitatively that the efficacy of AdsNP and EntrapNP, with or without adjuvants, was at least as good as our positive control, OVA & Adjs in solution. Nevertheless, the efficient selective killing of SIINFEKL-pulsed target cells does not necessary guaranty long-lasting effective responses upon a second encounter with the foreign antigen. Therefore, we performed additional experiments to provide deeper insights in memory of antigen-specific cells.

The activation of CD8⁺ T cells upon EntrapOVA NP was more prominent compared to the one quantified in animals immunized with AdsOVA NP. These data suggested that EntrapOVA NP generated more robust antigen-specific CD8⁺ immune responses towards effective memory T cells, even if no differences were previously observed in the in vivo killing assay for both EntrapNP and AdsNP. This observation may be explained by an absent or delayed TLR signal in AdsNP. While the adjuvants (CpG and MPLA) were dispersed into the matrix of the nanoparticulate vehicle, OVA was adsorbed onto NP surface through the weak electrostatic interactions. Therefore, OVA could be desorbed from NP surface prior to the release of CpG and MPLA, which would be rather controlled by the digestion and erosion of NP matrix. However, it should be emphasized that the co-delivery of OVA and adjuvants following the administration of AdsOVA-Adjs NP did occur during the short-term experiment. Therefore, this long-term study shows that the administration of AdsOVA-Adjs NP may not guarantee the co-stimulatory signal essential for an efficient generation of activated and long-lasting memory CD8⁺ T cells. With this study we show the potential and strength of our developed EntrapOVA-Adjs nanovaccine. Therefore, this long-term memory study provides important insights into the effect of antigen delivery on the overall nanovaccine efficacy.

Having in consideration the promising effects of our nanovaccine on immune cells under physiological steady-state conditions, B16.MO5 melanoma model was used for a comprehensive characterization of the anti-tumor antigen-specific T immunomodulation. B16.MO5 cells are characterized by a constitutive production of OVA protein, which render them targetable by immune cells instructed to recognize OVA as antigen. Authors are aware of the severity of its epigenetic phenotype, high enzymatic activity and multiple immune escape tumor-mediated mechanisms. However, an important advantage of this system is mostly related to the xenograft nature of the OVA antigen, constituting a clean and accurate read-out model that allows detailed and controlled characterization of the antigen-specific nature of the overall immune responses. Thus it may support a possible correlation of the data obtained under steady-state conditions.

This study showed the exceptional ability of the EntrapNP nanovaccine in promoting CD8⁺ and CD4⁺ TIL recruitment into TME. After a single vaccination with EntrapNP, an 8-fold higher count for CD8⁺ and 3-fold for CD4⁺ T cells, compared to the control group, was obtained, indicating high success rate in tumor reduction (**p < 0.001). On the other side, single or 3-time dose of AdsNP was not effective in T cell infiltration. Thus, this finding is important for tumor development and recurrence, which is in agreement with the long memory assay, PD-1 expression and cytokine release.

Interestingly, repeated immunization with this EntrapOVA-Adjs NP led to 5-fold increase in the recruitment of CD8⁺ and 3-fold increase in the infiltration of CD4⁺ T cells at the tumor site. As a consequence of the higher frequency of immunization in the 3-time vaccinated animals, the levels of antigens and adjuvants available were most probably higher and at a more constant rate than those obtained following a single administration of these vaccines. This may explain the better anti-tumor protection and higher infiltration of both CD8⁺ and CD4⁺ T cells at the tumor site of animals treated with the EntrapOVA-Adjs NP. Therefore, these findings must be related to the treatment schedule used. On the other side, 3-fold increase in CD8⁺ T cells was detected after a single and 3-time vaccination with AdsOVA-Adjs NP. However, infiltration of CD4⁺ T cells was not statistically significant. However, taking into account all the above results related to long-lasting protective memory effect and tumor recurrence incidence, EntrapOVA-Adjs NP was the most efficient vaccine.

Moreover, single vaccination with EntrapOVA-Adjs and AdsOVA-Adjs NP presented the highest levels of IFN-γ-producing (**p < 0.001) and TNF-α-producing (**p < 0.001) CD8⁺ T cells, in the spleens, suggesting single vaccination as the most beneficial treatment to keep the tumor growth under control, as previously shown by the mean tumor growth curves, while in the TME CD8⁺ lymphocytes were the most efficient in the IFN-γ release. In line with the previous assay, it is the observation that, following the stimulation with EntrapNP, the pro-inflammatory cytokines IFN-γ and TNF-α did not inhibit the suppression of T-cell activation or proliferation, as it happened upon 3-time vaccination with Ads
or EntrapNP. The draining lymph nodes activated by the 3-time vaccination with AdsOVA-Adjs or EntrapOVA-Adjs NP showed a 40% and 20% upregulation of PD-1 expression on CD4+ T cells, respectively. This suggests lower CD4+ T cell priming and thus possible lymphocyte exhaustion over time. This CD4+ helper mediated immune response is important for the establishment of memory CD8+ T cell responses, which has been optimally ensured upon the treatment with single vaccination. However, EntrapOVA-Adjs NP drove a more efficient pro-inflammatory Th1 immune response (2-fold lower upregulation of PD-1 expression on CD4+ lymphocytes). Overall, we did not detect any upregulation of PD-1 or CTLA-4 on antigen-specific CD8+ T cells. CTL are the ones with the killing capacity towards tumor cells and thus, these results are also in line with those previously reported mean tumor growth curves and final tumor weight. The 3-time immunized animals presented no alterations in IFN-γ and TNF-α secretion by splenic CD8+ T cells or increased serum levels of those pro-inflammatory cytokines compared to the control groups (Suppl. Figure S10), suggesting the absence of systemic inflammation and enhancement of animals’ immunity, which kept the tumor growth under control.

The multiple vaccination treatment with EntrapNP vaccine containing HER2 tumor antigens resulted in lymphocyte recruitment into the TME in a mammary huHER2 cancer model. It is clear that our treatment approach with EntrapNP induced infiltration of TIL compared to the control groups and thus demonstrated statistically significant (**P < 0.01) tumor reduction and, most importantly, limited tumor cell dissemination and metastasis. In addition, the obtained success rate was much higher than the previously reported data obtained using liposomes as a delivery carrier. Altogether, with this final additional study, we confirmed the efficacy of the developed nanovaccine for anti-tumor immunotherapeutic purposes against multiple types of solid tumors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2017.12.011.

References


