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## Co-delivery of human cancer-testis antigens with adjuvant in protein nanoparticles induces higher cell-mediated immune responses

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### Abstract

Nanoparticles have attracted considerable interest as cancer vaccine delivery vehicles for inducing sufficient CD8<sup>+</sup> T cell-mediated immune responses to overcome the low immunogenicity of tumor microenvironments. Our studies described here are the first to examine the effects of clinically-tested human cancer-testis (CT) peptide epitopes within a synthetic nanoparticle. Specifically, we focused on two significant clinical CT targets, the HLA-A2 restricted epitopes of NY-ESO-1 and MAGE-A3, using a viral-mimetic packaging strategy. Our data shows that simultaneous delivery of a NY-ESO-1 epitope (SLLMWITQV) and CpG using the E2 subunit assembly of pyruvate dehydrogenase (E2 nanoparticle), resulted in a 25-fold increase in specific IFN- $\gamma$  secretion in HLA-A2 transgenic mice. This translated to a 15-fold increase in lytic activity toward target cancer cells expressing the antigen. Immunization with a MAGE-A3 epitope (FLWGPRALV) delivered with CpG in E2 nanoparticles yielded an increase in specific IFN- $\gamma$  secretion and cell lysis by 6-fold and 9-fold, respectively. Furthermore, combined delivery of NY-ESO-1 and MAGE-A3 antigens in E2 nanoparticles yielded an additive effect that increased lytic activity towards cells bearing NY-ESO-1<sup>+</sup> and MAGE-A3<sup>+</sup>. Our investigations demonstrate that formulation of CT antigens within a nanoparticle can significantly enhance antigen-specific cell-mediated responses, and the combination of the two antigens in a vaccine can preserve the increased individual responses that are observed for each antigen alone.

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## Keywords

Nanoparticle; Cancer-testis antigen (CT); NY-ESO-1; MAGE-A3; HLA-A2; Cancer vaccine

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## 1. Introduction

Boosting a patient's immune system by immunotherapy represents a promising approach in cancer treatment,<sup>1</sup> and cancer vaccines in particular enable the recognition of tumor-associated antigens for targeted destruction.<sup>2</sup> Although these cancer vaccines have been shown to elicit CD8 T cell immune responses, the typical response levels generated are usually clinically insufficient to overcome the low immunogenicity and immunosuppressive microenvironment of tumors.<sup>3,4</sup> For example, in a review of clinical studies, an overall objective response rate (50% tumor size reduction) of only 2.6% was observed with peptide vaccines derived from gp100, MART-1, TRP-2, cancer testis NY-ESO-1, MAGE-A12, or HER2 antigens (alone or in combination with adjuvant); these results suggest the need for development of more effective strategies and therapies.<sup>1</sup>

The application of nanotechnology has shown an exceptional promise towards improvement of cancer diagnosis and treatment in recent years.<sup>5–7</sup> Antigen uptake by dendritic cells (DCs) depends on the antigen properties such as geometry,<sup>8</sup> surface charge,<sup>9</sup> and importantly, size.<sup>10,11</sup> Some nanoparticles have the advantage of being in the optimal size for DCs uptake and passive transport to the lymphatic system, with prior research demonstrating that particles between approximately 20–45 nm are taken up more effectively by the DCs residing in the lymph-nodes.<sup>8,12,13</sup> Therefore, delivery of vaccine components with these nanoparticles may facilitate and increase DC interaction, resulting in a more effective immune responses.<sup>14–15</sup>

One nanoparticle with favorable DC uptake properties is the E2 nanoparticle.<sup>15–17</sup> E2 is a 25-nm non-viral protein nanoparticle derived from the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*.<sup>18</sup> It is composed of 60 identical monomer subunits that self assemble into a highly thermostable dodecahedral caged structure with a hollow 12-nm cavity,<sup>19</sup> and it can be engineered at the internal, external, and inter-subunit interfaces to change the properties and functionality of the nanoparticle.<sup>20–23</sup> Our research group has also previously demonstrated that the viral-mimetic, simultaneous delivery of adjuvant (internally packaged) and MHC-I restricted antigens (bound on the surface) via E2 nanoparticles resulted in an increase in DCs activation and antigen cross-presentation.<sup>16</sup> This was associated with a significant increase in antigen-specific IFN- $\gamma$  secretion, tumor cell lysis, delayed B16-F10 tumor growth, and increased survival time in C57Bl/6 mice.<sup>15</sup> Cell uptake by immune cells and biodistribution of E2 have also been reported.<sup>17</sup>

The target epitopes in this current study are HLA-A2 restricted peptide sequences from New York esophageal squamous cell carcinoma-1 (NY-ESO-1) and melanoma antigen family A, 3 (MAGE-A3).<sup>24</sup> While other tumor-associated antigens (TAAs) are often expressed at low levels in healthy tissue, expression of CTs is restricted only to cancer cells and the immune-privileged cells in the testis. Furthermore, CT antigens exist in a high proportion of different human tumors such as melanoma, bladder, lung, prostate, and breast cancers.<sup>25,26</sup> In particular, NY-ESO-1 is expressed in 82% of neuroblastomas and 46% melanomas<sup>27</sup> while

MAGE-A3 is also expressed in 76% of melanoma cancers.<sup>28</sup> A phase II clinical trial of NY-ESO-1/ISCOMATRIX vaccine which was recently completed in June 2017 (NCT00518206, [ClinicalTrials.gov](https://clinicaltrials.gov)) resulted in 4% partial response (based on a standard of 30% reduction in tumor size), 48% stable disease, and 48% progressive disease; this result highlights the generation of response to NY-ESO-1, but also the need and potential for development of alternative strategies that will yield more effective therapies.

Given the wide range of tumors that express CT antigens, their relatively high expression level in cancer, their restricted expression, and their potential for vaccine improvement, the CT class of antigens is an important and significant clinical target. In this study, we examined the feasibility of using the E2 nanoparticle to induce cell-mediated immune responses against NY-ESO-1 and MAGE-A3 in a mouse model that is transgenic for the human major histocompatibility complex, HLA-A2. While our prior work examined gp100, an antigen that has mouse and human analogues, this current study focuses on antigens that are specifically expressed in humans.

We also investigate the extent of cell-mediated and cytolytic responses by simultaneous delivery of NY-ESO-1-and MAGE-A3-containing nanoparticles. Tumor escape after single-epitope vaccination is common since cancers often lose expression of the targeted antigen to evade the immune system.<sup>29</sup> Immunization with combined antigens can possibly decrease the risk of tumor escape resulting from antigen loss.<sup>30,31</sup> Furthermore, increasing the number of different antigen targets in a vaccine can induce a broader range of T cell responses simultaneously, which could be effective in a higher number of patients. Because there is a lack of immune-competent murine tumor models expressing these CT antigens to examine *in vivo* anti-tumor efficacy in the most physiologically relevant way possible, we examined lytic ability *ex vivo* using human cancer cell lines expressing both NY-ESO-1 and MAGE-A3. To our knowledge, our study is the first to test the efficacy of cell-mediated responses to clinically-relevant CT peptide epitopes formulated as a nanoparticle, and it examines these human epitopes in nanoparticles both individually and in combination.

## 2. Methods

### 2.1. Materials

Reagents were purchased from Fisher Scientific unless otherwise noted. Complete RPMI used in this study for splenocytes was comprised of RPMI 1640 (Mediatech) with 10% heat-inactivated FBS (Hyclone), 1mM sodium pyruvate (Hyclone), 100 µg/ml of streptomycin (Hyclone), 0.1 mM nonessential amino acids (Lonza), 2 mM L-glutamine (Lonza), and 100 units/ml penicillin. Cancer cell lines used in this study were cultured in DMEM media (Sigma) supplemented with 10% heat-inactivated FBS (Hyclone).

### 2.2. Peptides and CpG

CpG 1826, a bacterial DNA ligand for TLR9, was purchased from Invivogen, and 5' benzaldehyde-modified CpG 1826 with a phosphorothioated backbone was synthesized by Trilink. The NY-ESO-1 and MAGE-A3 peptide epitopes were synthesized by Genscript or Genemed Synthesis (Table 1). Peptides were synthesized both with and without an N-

terminal cysteine; the functionalized thiol on the cysteine-modified peptides was used for conjugation to E2, whereas peptides with no cysteine were used as controls. In this study, the abbreviation (e.g., NYESO, MAGE) refers to the peptide, while the names NY-ESO-1 and MAGE-A3 refer to the whole protein.

### 2.3. E2 purification and characterization

In this study, we used the D381C mutant of the E2 nanoparticle, which has an aspartic acid-to-cysteine mutation at position 381 in the internal hollow cavity of the nanoparticle. The cysteine of D381C can be used for site-directed conjugation, and this nanoparticle is abbreviated as "E2" in this study. Expression, purification, and characterization of E2 (D381C mutant) were performed as previously described.<sup>19</sup> In summary, *E. coli* strain BL21 (DE3) containing the E2 gene was cultured in Luria-Bertani medium containing 100 µg/ml ampicillin. Expression was induced by adding 1mM of IPTG when the culture reached the optical density of 0.7–0.9 measured at 600 nm. Cells were harvested and stored at –80°C. Cells were lysed using a French pressure cell (Thermo Scientific), and the insoluble fraction was removed by centrifugation. The soluble fraction heated at 70°C for 20 min. Denatured native *E. coli* protein aggregates were removed by centrifugation. The recovered supernatant was loaded to a HiPrep Q Sepharose anion exchange column followed by a Superose 6 size exclusion column.<sup>19</sup> Purity and the molecular weight of purified E2 were confirmed with SDS-PAGE and electrospray ionization mass spectrometry. Dynamic light scattering and transmission electron microscopy were used to check the size, assembly, and monodispersity of the particles. As previously described, lipopolysaccharide was removed using Triton X-114 (Sigma) extraction, and endotoxin levels were evaluated using an LAL ToxinSensor kit (Genscript).<sup>16</sup>

### 2.4. CpG and peptides conjugation

CpG 1826 modified with a 5'-benzaldehyde was attached to the TCEP-reduced cysteines in the internal cavity of E2 nanoparticles using a N-β-maleimidopropionic acid hydrazide (BMPH) linker. The average number of CpG molecules conjugated to the internal cavity of E2 nanoparticle was estimated with intensity analysis in ImageJ software, using standardized concentrations.<sup>16</sup> Peptides with N-terminal cysteines were conjugated to the native lysines on the surface of the E2 nanoparticle by mixing the nanoparticle with a sulfo-SMCC linker in the presence of a 10-fold excess of TCEP-reduced peptides (relative to E2 monomer), and incubating overnight at 4°C. HPLC was used for peptide quantification as previously described,<sup>15</sup> and details are in Supplementary Materials.

### 2.5. Zeta potential measurements

To measure zeta potential, the diffusion barrier method with monomodal analysis were used because they are compatible with the amounts and physiological ionic strengths of our samples. Malvern capillary cells were filled with buffer (50 mM potassium phosphate at pH 7.4 with 100 mM NaCl), and 150 µl of nanoparticles (at 1.3 mg/ml) were gently injected to the bottom of the buffer-filled capillary cells. Zeta potential was measured with a Malvern Zetasizer (Nano ZS).

## 2.6. Mice

Transgenic mice expressing the human HLA-A2 gene were obtained from Jackson Laboratory. All animal studies were carried out in accordance with protocols approved by the Institute for Animal Care and Use Committee (IACUC) at the University of California, Irvine. Briefly, 6–8 week old female HLA-A2 transgenic C57BL/6 mice were immunized subcutaneously at the base of the tail at Day 0. A priming immunization was followed by a booster after 14 days. Injections were 120  $\mu$ l and contained specific amounts of peptide, E2, and CpG, based on the formulations tested. Seven days after the last immunization, mice were sacrificed and spleens were isolated.

## 2.7. IFN- $\gamma$ ELISpot

For IFN- $\gamma$  ELISpot, we used the Ready-Set-Go kit (eBioscience). Single-cell suspensions in RPMI were prepared from the spleens isolated from immunized mice, and added at  $5 \times 10^5$  and  $10^6$  cells/well to PVDF ELISpot plates that were pre-coated with an anti-mouse IFN- $\gamma$  antibody. Cell were incubated with either 10  $\mu$ g/ml of the relevant peptide or an irrelevant peptide (SIINFEKL) for 24 hrs at 37°C. U nstimulated cells in RPMI were plated and served as a negative control. Positive control wells contained 2% PHA-M (Gibco). IFN- $\gamma$  spots were developed following the manufacturer's protocol. Plates were scanned and quantified using an ELISpot reader (Cellular Technology) and immunospot analysis software (Immunospot Analysis Pack).

## 2.8. Cell lines

A375, a human malignant melanoma cell line, and MCF-7, a human breast cancer cell line, were purchased from ATCC. A375 was cultured in DMEM supplemented with 10% FBS, and MCF-7 was cultured in DMEM supplemented with 10% FBS and 0.01 mg/ml human recombinant insulin. Cells were incubated at 37°C, under 5% CO<sub>2</sub>, and were passaged 2–3 times a week.

## 2.9. Cell lysis assay

Single cell suspensions prepared from splenocytes isolated from immunized mice were cultured in RPMI at  $5 \times 10^6$  cell/ml and incubated overnight at 37°C. On day 1, 10  $\mu$ g/ml of target peptide was added to the cells, incubated for 24 hrs at 37°C, and washed twice with PBS to remove the unbound peptide. On day 3, culture was supplemented with 0.4 ng/ml of IL-2. Peptide stimulated splenocytes were collected on day 5 to perform the cytotoxicity assay. LDH release was measured with a calorimetric assay, CytoTox 96 (Promega), to examine the specific lysis of target cancer cell lines expressing NY-ESO-1 and MAGE-A3 antigens. A375 expresses both NY-ESO-1<sup>37</sup> and MAGE-A3,<sup>38</sup> while MCF7 has low expression of these antigens.<sup>39,40</sup> Splenocytes were counted with a hemocytometer, co-cultured with the target cancer cell lines at effector-to-target ratios of 100:1, 50:1, and 25:1, and evaluated for LDH release following the manufacturer's protocol. Data is reported as "% Lysis", calculated as

$$\% \text{Lysis} = \frac{\text{Experimental LDH Release}}{\text{Maximum LDH Release}} \times 100$$

where "experimental LDH release" is the LDH release from cancer cells co-cultured with splenocytes minus the sum of LDH release from the cancer cells and the splenocytes cultured separately. "Maximum LDH release" is from lysed cancer cells using the kit's lysis buffer minus background LDH release from cancer cells.

## 2.10. Statistical analysis

Statistical analysis was carried out using GraphPad Prism. Data are presented as mean  $\pm$  standard error of the mean (S.E.M.) of at least three independent experiments ( $n = 3$ ). Statistical analysis was determined by a two-way ANOVA over all groups followed by a Tukey's multiple comparison test, unless otherwise noted. P-values less than 0.05 were considered significant.

## 3. Results and Discussion

### 3.1. Conjugation of CpG and cancer-testis peptides to E2 nanoparticle yielded intact nanoparticles

Both CpG and peptides were successfully conjugated to the E2 nanoparticle, and the results are consistent with those previously described for conjugation of other epitopes to the E2 nanoparticle.<sup>15,16</sup> The CpG-conjugated nanoparticle (CpG-E2) displayed two distinct bands on SDS-PAGE [Fig. 1A]; the lower band at  $\sim 28$  kDa is the unconjugated E2 monomer and coincides with  $28118 \pm 13$  Da measured by mass spectrometry, and the band at  $\sim 35$  kDa is indicative of the conjugation of one CpG molecule attached to one 28-kDa E2 monomer. By analyzing band intensities, we estimate a conjugation ratio of  $21 \pm 4$  CpG molecules encapsulated per (60-mer) protein particle, similar to prior studies.<sup>16</sup>

Simultaneous conjugation of CpG to the internal cavity and peptides to external surface of E2 resulted in two distinct broad bands [Fig. 1A]. The broad band between 30–35 kDa shows the heterogeneous conjugation of peptides (from Table 1) to the surface of E2 monomers (peptide-E2), and the band between 35–40 kDa confirms the simultaneous conjugation of peptide and CpG to the E2 monomers (CpG-peptide-E2). We quantified the number of peptides conjugated to the E2 nanoparticle with HPLC,<sup>15</sup> and found that on average,  $140 \pm 16$  NYESO or  $155 \pm 21$  MAGE peptides were attached on each protein nanoparticle. Conjugation of CpG and peptides to the E2 nanoparticle resulted in a 1:1 mass ratio. This ratio of CpG and peptide has also been used in other studies to successfully induce CD8 T cell responses.<sup>41,42</sup>

Dynamic light scattering revealed a hydrodynamic diameter of  $28.4 \pm 0.7$ ,  $30 \pm 1.3$ , and  $30 \pm 0.9$  nm for E2, CpG-NYESO-E2, and CpG-MAGE-E2 respectively [Fig. 1B]. DLS data confirmed that particles remained unaggregated and within the optimal reported size for lymphatic drainage (20–45 nm),<sup>5,12,13</sup> even after conjugation. This data verifies that attachment of short peptides (9 amino acid length) on the surface of the nanoparticle does not result in a dramatic change in size. TEM analysis further confirmed intact, non-aggregated CpG-NYESO-E2 [Fig. 1C] and CpG-MAGE-E2 [Fig. 1D] nanoparticles. The zeta potential of E2, CpG-NYESO-E2, and CpG-MAGE-E2 nanoparticles were  $-11.7 \pm 1$  mV,  $-12.8 \pm 1$  mV, and  $-11.1 \pm 1.8$  mV, respectively. This data confirmed that conjugation



of NYESO or MAGE peptides on the surface and CpG inside of the nanoparticles did not change the overall surface charge compared to the E2 nanoparticle itself.

### 3.2. Immunization with CpG-NYESO-E2 nanoparticles yielded increased antigen-specific IFN- $\gamma$ secretion

Different vaccine formulations of NY-ESO-1 antigen peptide, CpG, and E2 were investigated, and ELISpot results are presented in Figure 2. Immunization with the CpG-NYESO-E2 nanoparticle significantly increased the NY-ESO-1 epitope-specific IFN- $\gamma$  secretion by 25-fold, compared to immunization with unbound CpG and NYESO, at an equivalent amount of antigen and adjuvant [Fig. 2B,C]. In contrast, we observed negligible IFN- $\gamma$  response by the cells pulsed with an irrelevant SIINFEKL peptide [Fig. 2C], confirming that the immune response generated from immunization was specific to the NYESO epitope. Splenocytes isolated from mice immunized with bare E2 nanoparticle also lacked significant amounts of NYESO-specific IFN- $\gamma$  secretion [Fig. 2C], confirming that higher IFN- $\gamma$  secretion resulting from CpG-NYESO-E2 immunization was not a result of non-specific immune responses to the E2 delivery platform itself.

We previously observed higher DC activation towards CpG-E2 nanoparticles and also an elevated antigen cross-presentation when a model ovalbumin peptide and CpG were delivered within an E2 nanoparticle, relative to unbound antigen and adjuvant.<sup>16</sup> This suggests that our observed increase in IFN- $\gamma$  secretion in this experiment results from higher DCs activation and more efficient cross-presentation of the NYESO epitope. This mechanism of improving T cell immunity by increasing DC uptake and activation is supported by Dhodapkar et al., who observed higher antigen-specific CD8 T cell responses with a vaccine that targeted NYESO-1 antigen to the DEC205 receptor of DCs.<sup>43</sup> The high IFN- $\gamma$  secretion specific to NYESO epitope (aa 157–165) in our study is consistent with ELISpot/IFN- $\gamma$  data previously reported on a NY-ESO-1 protein with ISCOMATRIX vaccine<sup>44</sup> and a lentiviral vector encoding the NY-ESO-1 gene.<sup>45</sup> However, in contrast to those cases, our vaccine formulation contains only a single NYESO-1 epitope rather than the whole protein or gene, respectively, used in the previous studies.

We also observed higher peptide-specific IFN- $\gamma$  secretion for the group receiving immunizations of 50  $\mu$ g CpG-NYESO-E2 compared to 25  $\mu$ g CpG-NYESO-E2 [Fig. 2C; groups e, f]. This demonstrates dose dependency of the generated cell-mediated immune response to the NYESO epitope. However, immunization with 100  $\mu$ g of CpG-NYESO-E2 did not increase the IFN- $\gamma$  secretion compared to 50  $\mu$ g, and in fact showed a significant decrease across the cohort of mice [Fig. S1]. This could be due to increased levels of suppressive T cells,<sup>46</sup> T cell exhaustion,<sup>47</sup> or high antigen doses leading to increase tolerance.<sup>48</sup>

In addition to the NY-ESO-1 peptide epitope examined in Figure 2 (NYESO), we examined another epitope [NYESO(p2)] (see Table 1) that had been previously reported to yield a relatively low lysis of NYESO(p2)-pulsed T2 cells when incubated with T lymphocytes isolated from a patient.<sup>34</sup> In contrast to that study, however, our data showed that immunization with nanoparticle formulations of the NYESO(p2) epitope [CpG-NYESO(p2)-E2] did not result in any significant increase in the IFN- $\gamma$  secretion [Fig. S2-



B]. To our knowledge, there have been no additional reports regarding the immunogenicity of this peptide. One explanation for this apparent discrepancy is that the *in vitro* data using T2 cells<sup>34</sup> may not recapitulate what happens *in vivo*.

### 3.3. Higher lysis activity toward NY-ESO-1+ cancer cells was observed for the group immunized with CpG-NYESO-E2 nanoparticles

Lytic capacity of splenocytes isolated from immunized mice was tested on a human melanoma cell line expressing NY-ESO-1 (A375)<sup>49–50</sup> and on a human breast cancer cell line negative for NY-ESO-1 expression (MCF-7)<sup>39</sup>, both positive for HLA-A2.<sup>39,49</sup> The increase in antigen-specific IFN- $\gamma$  levels that resulted from CpG-NYESO-E2 immunization [Fig. 2] translated to a 15-fold increase in the lysis activity towards A375, compared to unbound peptide and CpG [Fig. 3A]. In contrast, no significant lysis was observed for the control cell line, MCF-7 [Fig. 3A]. This data supports the specificity of the immune response for the NY-ESO-1 antigen.

Our data also confirms that lysis activity towards the target cancer cell line is dose-dependent; higher activity toward A375 can be achieved by increasing the effector-to-target ratio [Fig. 3B], where the effector cells are the splenocytes isolated from the immunized mice and the target cells are the cancer cells. Our lysis data is comparable to those previously reported on the NY-ESO-1/ISCOMATRIX vaccine by which the entire NY-ESO-1 protein was delivered.<sup>44</sup> Remarkably, we observed almost the same extent of lysis by delivering a single NYESO-1 epitope rather than the whole antigen with multiple epitopes, albeit toward a different target cell line.

Splenocytes isolated from mice immunized with bare E2 nanoparticles or antigen/CpG alone did not increase specific lysis [Fig. 3A]; this confirms that the significant increase in lysis resulting from the CpG-NYESO-E2 immunization is due to the complete nanoparticle-adjuvant-antigen delivery system, and not the effect of E2 or antigen alone. The E2 data is consistent with prior work by Perham and De Berardinis, which previously demonstrated a negligible lysis activity towards the mouse lymphoma RMA-S cell line by splenocytes isolated from mice immunized with bare E2 nanoparticle.<sup>51</sup> Taken together, our data show that conjugation of the NY-ESO-1 epitope and CpG adjuvant to the nanoparticle is an effective delivery strategy for increasing INF- $\gamma$  response, and it results in a functional lysis activity that is specific towards the NY-ESO-1 epitope.

### 3.4. Immunization with CpG-MAGE-E2 nanoparticles yielded increased antigen specific IFN- $\gamma$ secretion

Peptide epitopes for MAGE-A3 were conjugated to the E2 nanoparticle bearing the CpG adjuvant. The ELISpot results of immunizations with the different MAGE formulations are presented in Figure 4. Vaccination with the CpG-MAGE-E2 nanoparticles increased the number of IFN- $\gamma$  spots, relative to free CpG and MAGE [Fig. 4B]. We also observed a dose-dependent response, where significantly higher IFN- $\gamma$  secretion was obtained for mice immunized with the 50  $\mu$ g dose compared to the 25  $\mu$ g dose. Statistical analyses show that IFN- $\gamma$  levels resulting from the irrelevant SIINFPEKL control peptide for mice immunized with 50  $\mu$ g CpG-MAGE-E2 (group e) is not significantly different compared to the other

vaccine formulations in Figure 4B. Similar to CpG-NYESO-E2, CpG-MAGE-E2 increased the specific IFN- $\gamma$  secretion compared to the free peptide/CpG control group, but to a lower extent (6-fold for MAGE vs. 25-fold for NYESO for the comparable 50  $\mu$ g dose). High IFN- $\gamma$  secretion to this MAGE epitope was also observed in a previous study, where HLA-A2 mice were immunized with DNA vaccine encoding 5 epitopes, followed by a booster of peptide epitopes in solution plus a hepatitis B virus core containing a T helper peptide.<sup>35</sup> In contrast to the previous study, however, we obtained high IFN- $\gamma$  secretion to the MAGE epitope without a need for T helper peptides or a separate DNA vaccine.

The second MAGE-A3 epitope in this study, MAGE(p2), did not affect the splenocyte IFN- $\gamma$  secretion when conjugated to E2-CpG compared to unbound peptide and CpG [Fig. S2-C]. The apparent discrepancy between our study compared to a previous investigation<sup>52</sup> could be due to the different experimental conditions. In the prior study, much higher doses of peptide MAGE(p2) was used (200  $\mu$ g total) compared to ours (10  $\mu$ g total), together with an additional 120  $\mu$ g of hepatitis B virus core containing a T helper epitope.<sup>52</sup>

### **3.5. Higher lysis activity toward MAGE-A3<sup>+</sup> cancer cells was observed for the group immunized with CpG-MAGE-E2 nanoparticles**

MAGE-specific lytic activity of splenocytes isolated from immunized mice was tested using A375 and MCF-7, which are positive<sup>38,53</sup> and negative<sup>40</sup> for MAGE-A3, respectively, and results are presented in Figure 5. Splenocytes isolated from mice immunized with CpG-MAGEE2 nanoparticles significantly enhanced the lytic activity toward A375 by 9-fold at a 100:1 effector-to-target ratio [Fig. 5A], compared to free peptide and CpG. As expected, lytic activity observed for the mice immunized with CpG-MAGE-E2 is specific to the cell line expressing MAGE, where no significant lysis was observed for the control cell line MCF-7. As with the results for the NY-ESO-1 antigen on our nanoparticle, our data confirms dose dependency of lysis activity toward target cell line [Fig. 5B]. Our data is also consistent with results previously reporting that the MAGE-specific CD8 T cells generated from immunization of MAGE epitope within bacteriophage are capable of recognizing and lysis of the MAGE-A3<sup>+</sup> cell line.<sup>54</sup> This data, together with the data generated for NYESO epitope, confirms that the E2 nanoparticle can be used as an effective platform to simultaneously deliver clinically-tested CT antigens and CpG, resulting in an increase in the cellular-mediated immune responses generated to the target epitope.

### **3.6. Co-immunization with nanoparticles bearing both NYESO and MAGE epitopes yielded an additive IFN- $\gamma$ effect and increased the lysis activity**

Prior studies have demonstrated that targeting a tumor through multiple antigen sources can decrease the possibility of tumor escape. For example, Banchereau et al. demonstrated clinical benefit for patients who received vaccines composed of four different melanoma antigens if at least two out of four antigen peptides were immunogenic in patients, rather than one peptide alone.<sup>55-56</sup> Similar observations have been made for renal cell cancer.<sup>57</sup> Therefore, we investigated the effects of simultaneous immunization with CpG-NYESO-E2 and CpG-MAGE-E2 nanoparticles.

We observed that immunization with the CpG-NYESO-E2 and CpG-MAGE-E2 nanoparticles together (25  $\mu\text{g}$  each, 50  $\mu\text{g}$  total per dose) significantly increased the NYESO and MAGE epitope-specific IFN- $\gamma$  secretion, compared to immunization with simultaneous vaccination of unbound CpG, NYESO, and MAGE peptides [Fig. 6B]. As expected, we observed negligible IFN- $\gamma$  for the cells pulsed with an irrelevant SIINFEKL peptide [Fig. 6B], confirming the specificity of immune response generated from immunization.

We also obtained the same levels of IFN- $\gamma$  secretion to the individual NYESO and MAGE epitopes when CpG-NYESO-E2 and CpG-MAGE-E2 nanoparticles were co-administered, relative to each nanoparticle formulation separately [Fig. 6B]; this shows that the specific IFN- $\gamma$  responses to individual epitopes were preserved after co-immunization. The result is an additive effect to IFN- $\gamma$  secretion, with the total IFN- $\gamma$  frequencies of group f (sum of NYESO- and MAGE-specific IFN- $\gamma$ ) being approximately equal to the sum of individual NYESO-specific IFN- $\gamma$  (group b) and MAGE-specific IFN- $\gamma$  (group d). Furthermore, this additive effect to IFN- $\gamma$  secretion from the simultaneous immunization is entirely antigen-specific and is not due to any adjuvant effect due the CpG-E2 nanoparticle itself, since the addition of CpG-E2 alone did not promote further specific IFN- $\gamma$  secretion (compare formulations a to b, and c to d; Fig. 6B).

Consistent with the ELISpot data, splenocytes isolated from mice simultaneously immunized with CpG-NYESO-E2 and CpG-MAGE-E2 nanoparticles (25  $\mu\text{g}$  each, 50  $\mu\text{g}$  total per dose) significantly enhanced the lytic activity toward A375 [Fig. 6C], in a dose dependent manner [Fig. 6D], relative to unbound CpG, NYESO, and MAGE epitopes. As expected, the lytic activity was specific to A375, with no lysis observed for the control cell line MCF-7 [Fig. 6C]. Furthermore, an elevated and additive lysis activity was observed for the mice that were coimmunized with CpG-NYESO-E2 and CpG-MAGE-E2 formulations compared to each formulation separately [Fig. 6C].

However, co-immunization with higher doses of CpG-NYESO-E2 and CpG-MAGE-E2 nanoparticles (50  $\mu\text{g}$  each, 100  $\mu\text{g}$  total per dose) did not amplify the specific-IFN- $\gamma$  secretion [Fig. S3] or lysis activity [Fig. S4], relative to each formulation separately; in fact, both IFN- $\gamma$  and lysis effects were lower than the effects of each individual antigen-nanoparticle alone. This observation could be due to T cell exhaustion<sup>47</sup> or peptide competition on the MHC of the antigen presenting cells (e.g., DCs) or on the T cell receptors.<sup>58,59</sup> This data, together with our data for the 100  $\mu\text{g}$  CpG-NYESO-E2 results [Fig. S1], shows that for our current immunization schedule, the optimal dose for maximal immunogenic response is 50  $\mu\text{g}$  total nanoparticle (either 50  $\mu\text{g}$  for one antigen, or 25  $\mu\text{g}$  for two antigens).

Overall, our results show that co-delivery of a multi-epitope nanoparticle vaccine can elicit higher cell-mediated immune responses than single-epitope formulations. Although dosing multiple (unconjugated) peptides in solution has shown to increase immune responses in the clinic,<sup>39-41</sup> and others have investigated other classes of antigen combinations,<sup>60,61</sup> our study is the first investigation, to our knowledge, that has examined the efficacy of administering multiple cancer-testis epitopes peptides within the context of nanoparticles. Our data show that (1) coimmunization of our E2 nanoparticle vaccines can preserve the

individual cell-mediated effects against cancer-testis antigens and (2) that these IFN- $\gamma$  and lytic effects are additive.

Vaccination of cytotoxic T-cell epitopes in combination with helper epitopes have also been investigated. While some studies show this to be a successful strategy,<sup>62</sup> other evidence has demonstrated that the CD8 T cell response can actually be reduced after co-immunization with T helper epitopes.<sup>62,63</sup> Thus, using T helper epitopes within our nanoparticle vaccines could be another important approach to examine towards increasing efficacy.

## 4. Conclusion

In this study, we have investigated the effects of incorporating clinically-relevant human cancer-testis antigens into a nanoparticle platform that had been previously shown to increase cancer vaccine efficacy. We examined whether higher cell-mediated immune responses can be achieved by simultaneously packaging of CT antigens and CpG adjuvant to the E2 nanoparticle. In a HLA-A2 transgenic mouse model, we found that immunization with nanoparticle formulations containing CpG and CT antigens resulted in a significantly higher specific IFN- $\gamma$  frequencies compared to unbound antigen and CpG. Further, we observed an elevated lysis activity towards a target cancer cell line (A375). Additionally, simultaneous delivery of CpG-NYESO-E2 and CpG-MAGE-E2 nanoparticles preserved the effects of the individual antigen nanoparticles, and resulted in an additive IFN- $\gamma$  secretion and lysis activity relative to each separate nanoparticle formulation. Altogether, this work shows the advantages of using the E2 nanoparticle as an effective vaccine platform to deliver cancer-testis antigens for higher cell-mediated activation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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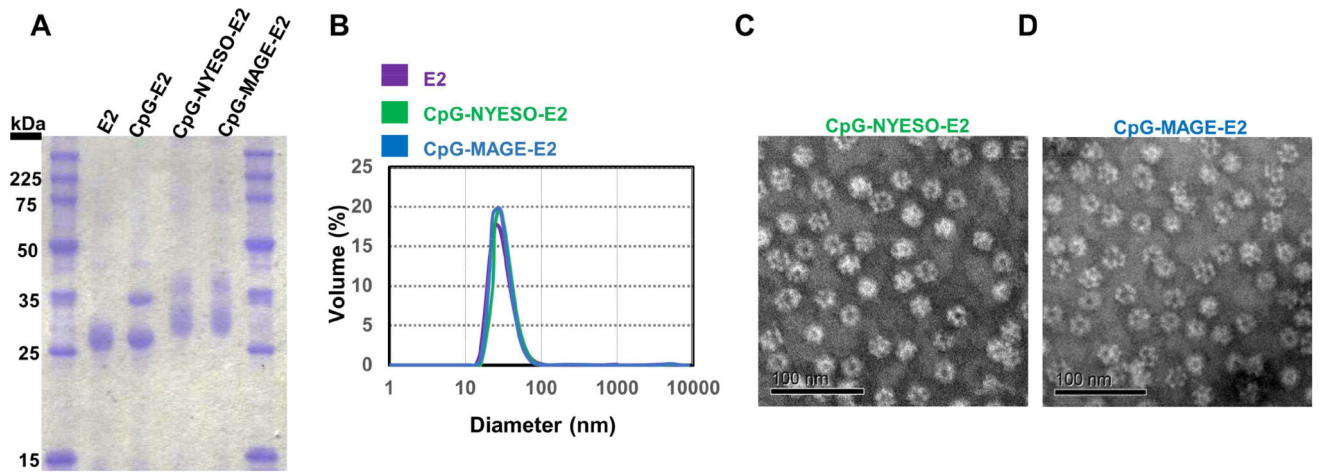
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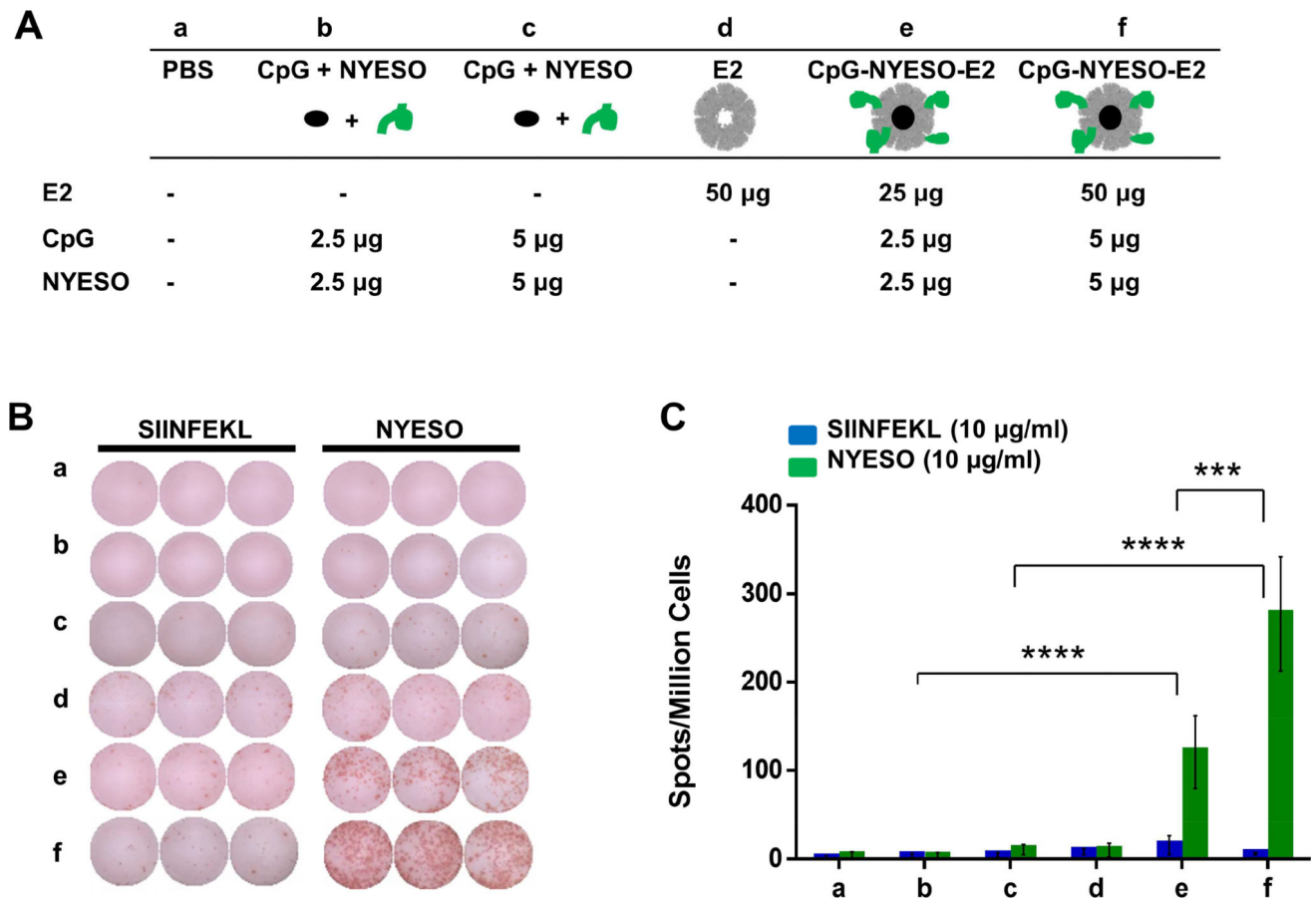


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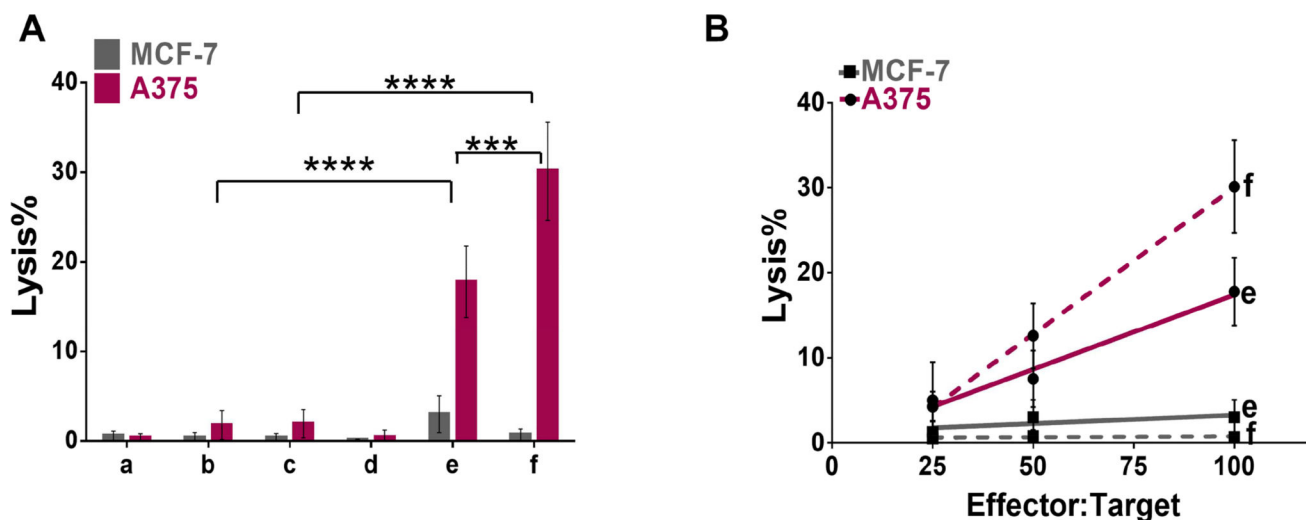


**Figure 1. Characterization of functionalized nanoparticles**

**A)** SDS-PAGE shows successful conjugation of CpG and peptides (NYESO, MAGE) to E2 nanoparticles. **B)** DLS reveals nanoparticle sizes of about 30 nm, before and after conjugation. **C)** TEM of CpG-NYESO-E2 nanoparticles. **D)** TEM of CpG-MAGE-E2 nanoparticles. Scale bars are 100 nm.

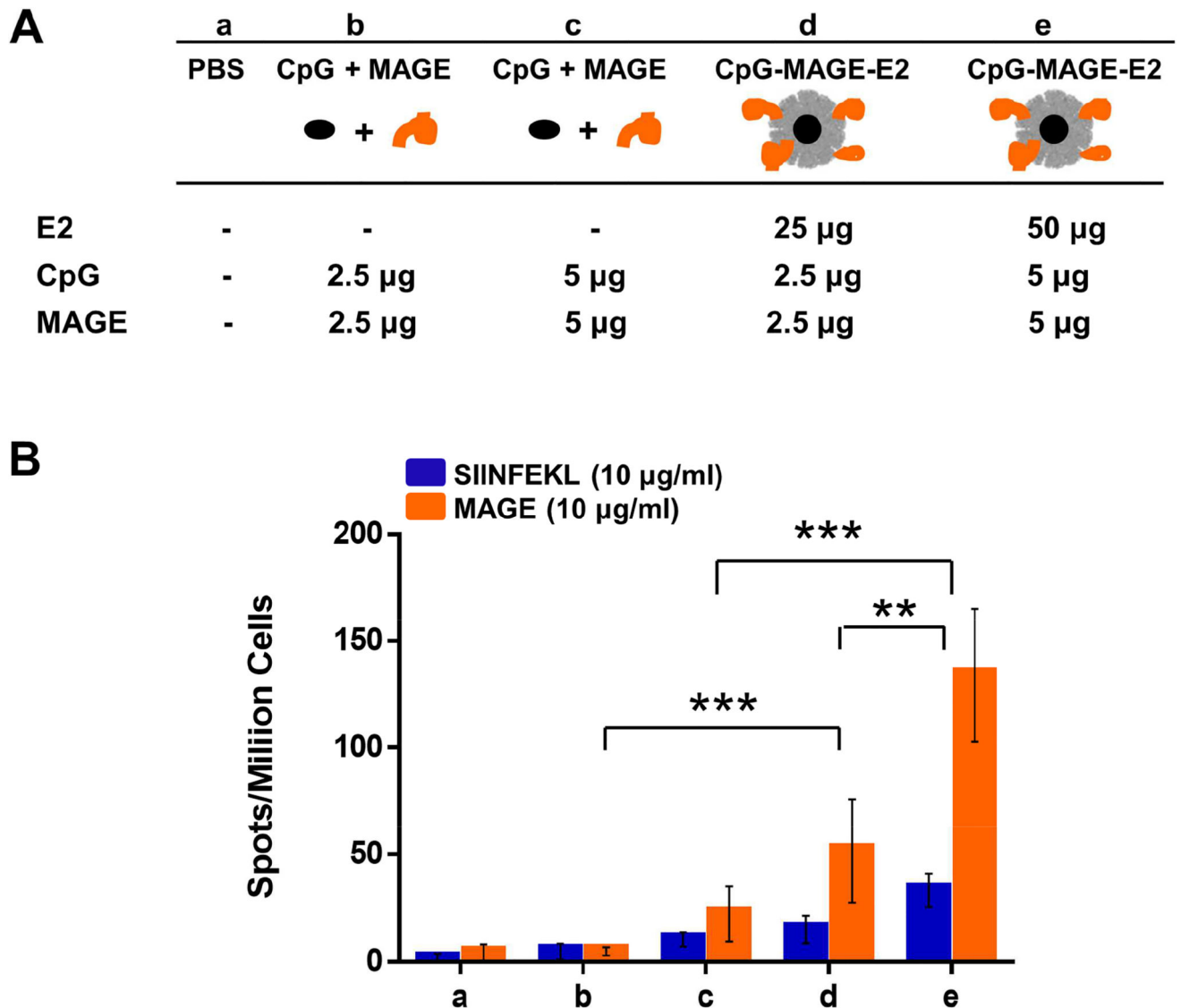


**Figure 2. ELISpot analysis of splenocytes from immunization with NY-ESO-1 formulations**  
**A)** Vaccine components per dose of different formulation groups (a–f). **B)** Representative ELISpot data from splenocytes of immunized group, pulsed with irrelevant peptide (SIINFEKL) or relevant epitope peptide (NYESO). **C)** Summary of averaged ELISpot data, which evaluated antigen-specific IFN- $\gamma$  secretion. HLA-A2 mice were immunized with different formulations (a–f), and splenocytes were pulsed *ex vivo* in the presence of relevant peptide (NYESO) or irrelevant peptide (SIINFEKL) and analyzed for specific IFN- $\gamma$  secretion. Higher NY-ESO-1 epitope-specific IFN- $\gamma$  secretion was observed for the group that received CpG-NYESO-E2. Data is presented as average  $\pm$  S.E.M. of at least 3 independent experiments ( $n = 3$ ). Statistical significance was determined by two-way ANOVA followed by a Tukey's test (\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

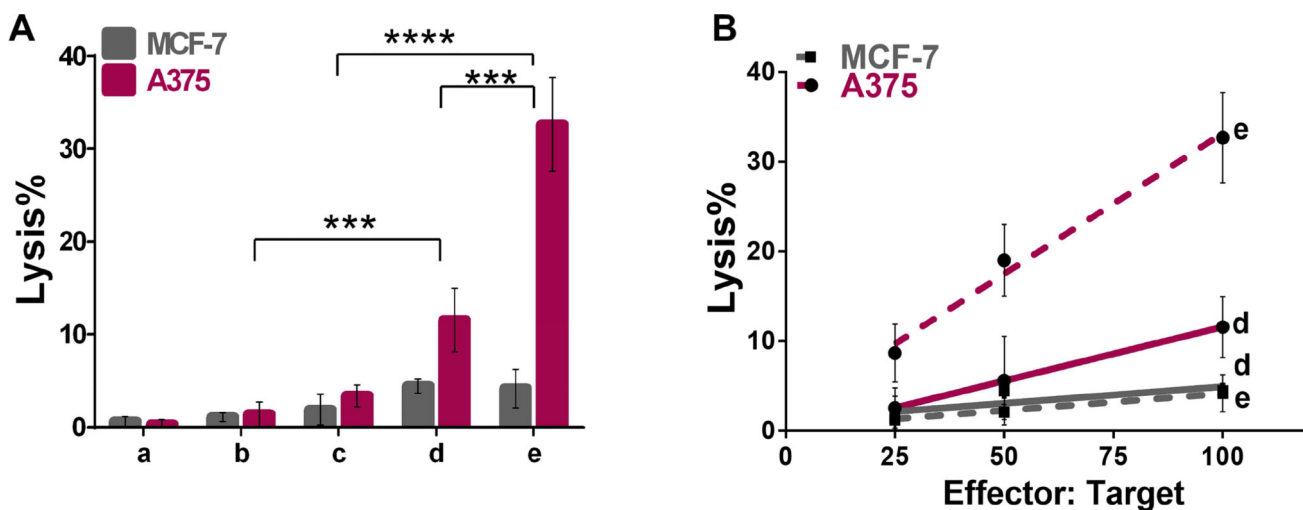


**Figure 3. Results of cell lysis assays using splenocytes from immunization with NY-ESO-1 formulations**

**A)** Summary of average lysis data at 100:1 effector:target ratio. HLA-A2 mice were immunized with different formulations (a–f; see Fig. 2A for schematic), and splenocytes were pulsed *ex vivo* in the presence of NYESO peptide. Splenocytes isolated from mice immunized with CpG-NYESO-E2 nanoparticles enhanced lysis activity toward A375, relative to free peptide and CpG. **B)** Cell lysis results at different effector:target ratios. HLA-A2 mice were immunized with different formulations (a–f; see Fig. 2A for schematic), and splenocytes were pulsed *ex vivo* in the presence of NYESO peptide. Lytic ability toward A375 cell line resulted from CpG-NYESO-E2 nanoparticles immunization is dose dependent. Data is presented as average  $\pm$  S.E.M. of at least 3 independent experiments. Statistical significance was determined by two-way ANOVA followed by a Tukey's test (\*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

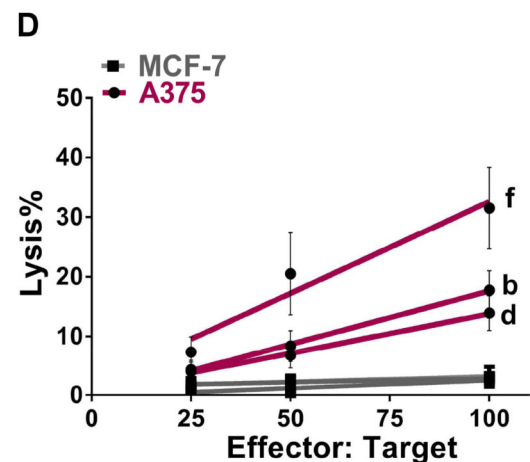
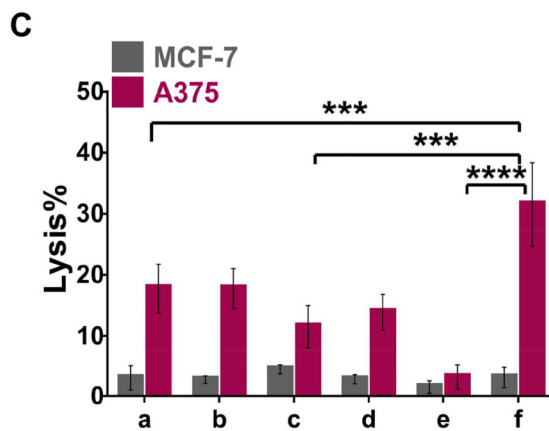
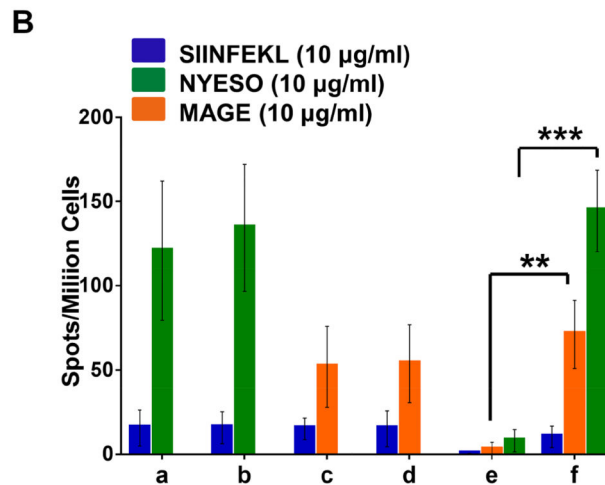
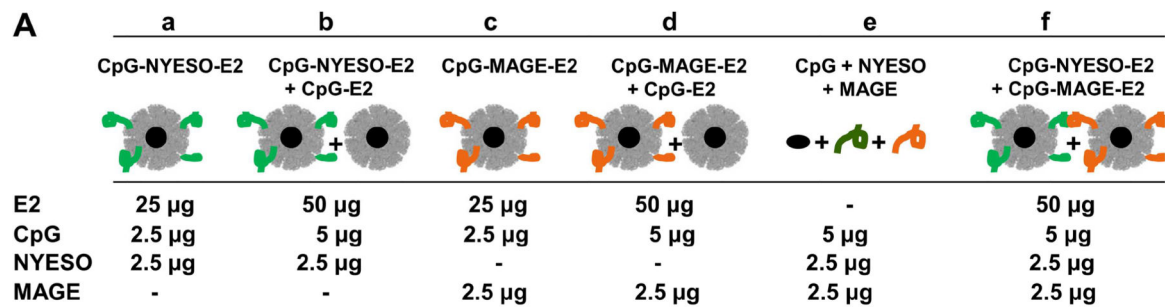


**Figure 4. ELISpot analysis of splenocytes from immunization with MAGE-A3 formulations**  
**A)** Vaccine components per dose of different formulation groups (a–e). **B)** Summary of averaged ELISpot data, which evaluated antigen-specific IFN- $\gamma$  secretion. HLA-A2 mice were immunized with different formulations (a–e), and splenocytes were pulsed *ex vivo* in the presence of relevant peptide (MAGE) or irrelevant peptide (SIINFEKL) and analyzed for specific IFN- $\gamma$  secretion. Higher MAGE-A3 epitope-specific IFN- $\gamma$  secretion was observed for the group that received CpG-MAGE-E2. Data is presented as average  $\pm$  S.E.M. of 3 independent experiments ( $n = 3$ ). Statistical significance was determined by two-way ANOVA followed by a Tukey's test (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).



**Figure 5. Results of cell lysis assays using splenocytes from immunization with MAGE-A3 formulations**

**A)** Summary of average lysis data at 100:1 effector:target ratio. HLA-A2 mice were immunized with different formulations (a–e; see Fig. 4A for schematic), and splenocytes were pulsed *ex vivo* in the presence of MAGE. Splenocytes isolated from mice immunized with CpG-MAGE-E2 nanoparticles enhanced lysis activity toward A375, relative to free peptide and CpG. **B)** Cell lysis results at different effector:target ratios. HLA-A2 mice were immunized with different formulations (a–e; see Fig. 4A for schematic), and splenocytes were pulsed *ex vivo* in the presence of MAGE peptide. Lytic ability toward A375 cell line resulted from CpG-MAGE-E2 nanoparticles immunization is dose dependent. Data is presented as average  $\pm$  S.E.M. of at least 3 independent experiments. Statistical significance was determined by two-way ANOVA followed by a Tukey's test (\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).



**Figure 6. Cell-mediated immune responses using splenocytes from co-immunization with NYESO-1 and MAGE-A3 formulations**

A) Vaccine components per dose of different formulation groups (a–f). B) Summary of averaged ELISpot data, which evaluated antigen-specific IFN- $\gamma$  secretion. HLA-A2 mice were immunized with different formulations (a–f). Splenocytes of immunized mice were pulsed *ex vivo* in the presence of relevant peptide (NYESO or MAGE) or irrelevant peptide (SIINFEKL) and analyzed for specific IFN- $\gamma$  secretion. Data is presented as average  $\pm$  S.E.M. of at least 3 independent experiments. Statistical significance was determined by two-way ANOVA followed by a Bonferroni's test (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). C) Summary of average lysis activity at 100:1 effector: target ratio. Mice received immunizations with



different formulations (a–f), and splenocytes were incubated with target cells (A375 or MCF-7). Co-immunization with CpG-NYESO-E2 and CpG-MAGE-E2 nanoparticles significantly enhanced the lytic activity toward HLA-A2<sup>+</sup> and MAGE-A3<sup>+</sup> cell line (A375), relative to immunization with one antigen. **D)** Cell lysis results at different effector:target ratios. Data show dose-dependent cell lysis for both individual and combined antigens when bound to E2 and CpG, relative to unconjugated peptides and CpG. Data is presented as average  $\pm$  S.E.M. of at least 3 independent experiments. Statistical significance was determined by two-way ANOVA followed by a Tukey's test (\*\*p < 0.01; \*\*\*p < 0.001).

**Table 1**

List of peptide epitopes sequences and their respective abbreviations in this study. Conventional single-letter abbreviations for amino acids (aa) are used in the peptide epitope sequence.

Abbreviation	Antigen source	Sequence	Serotype	Reference
NYESO	NY-ESO-1	C-SLLMWITQV (aa 157–165)	HLA-A2	32, 33
NYESO(p2)	NY-ESO-1	C-ILTIRLTAA (aa 132–140)	HLA-A2	34
MAGE	MAGE-A3	C-FLWGPRALV (aa 271–279)	HLA-A2	35, 36
MAGE(p2)	MAGE-A3	C-KVAELVHFL (aa 112–120)	HLA-A2	36