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Bacterial spores as particulate carriers for *gene gun* delivery of plasmid DNA

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ABSTRACT

Bacillus subtilis spores represent a suitable platform for the adsorption of proteins, enzymes and viral particles at physiological conditions. In the present work, we demonstrate that purified spores can also adsorb DNA on their surface after treatment with cationic molecules. In addition, we demonstrate that DNA-coated *B. subtilis* spores can be used as particulate carriers and act as an alternative to gold microparticles for the biolistic (*gene gun*) administration of plasmid DNA in mice. Gene gun delivery of spores pre-treated with DODAB (dioctadecyldimethylammonium bromide) allowed efficient plasmid DNA absorption and induced protein expression levels similar to those obtained with gold microparticles. More importantly, we demonstrated that a DNA vaccine adsorbed on spores can be loaded into biolistic cartridges and efficiently delivered into mice, which induced specific cellular and antibody responses. Altogether, these data indicate that *B. subtilis* spores represent a simple and low cost alternative for the in vivo delivery of DNA vaccines by the gene gun technology.

1. Introduction

The functionality of DNA-based vaccines relies on the *in vivo* transfection of cells that express the encoded antigens and, subsequently, lead to activation of antigen presenting cells (APC) that deploy innate and adaptive immune responses (Kutzler and Weiner, 2008; Nguyen et al., 2008). Therefore, experimental strategies to enhance cell transfection efficiency and, consequently, the potency of the induced immune responses are of great relevance. In this context, micro- and nanoparticles carriers have been widely used for the improvement of DNA vaccine strategies as an attempt to overcome their low immunogenicity, particularly in larger mammalian species, such as non-human primates and humans (O'Hagan et al., 2001).

Biolistic administration represents one alternative to increase the performance of DNA vaccines by increasing the number of antigen-transfected cells. This administration method, also known as *gene gun* delivery, introduces the plasmid DNA directly into the epidermis, an anatomical site rich in APCs, particularly Langerhans cells (LHC, immature dendritic cells). Gene gun immunization promotes enhanced DNA transfection and antigen expression leading to DC maturation and improved priming and activation of effector T cell responses (Lin et al., 2010). As a consequence, biolistic immunization requires much lower DNA amounts compared to intradermal or intramuscular immunizations to induce similar immune responses (Rezvan et al., 2011; Nguyen-Hoai et al., 2012; Ginsberg et al., 2010; Kim et al., 2004).

Gene gun delivery uses compressed gas to propel micrometer-sized gold particles coated with plasmid DNA (O'Brien and Lummis, 2006; Woods and Zito, 2008; Gotesman and Williams, 2016). Cationic agents (spermidine and calcium) are used to generate positive charges on the gold particle surface, enabling them to interact electrostatically with the negatively charged nucleic acid molecules. This technology is easily handled and has been largely employed to induce gene expression for different goals in plants (Klein et al., 1988), animals (De Rose et al., 2002; Lambracht-Washington et al., 2011; Nguyen-Hoai et al., 2015) and humans (Sidney, 2016). Several studies have been performed with an optimized process to improve DNA loading and the use of biodegradable and less expensive particles (Svarovsky et al., 2008; Kasturi et al., 2006).

Bacterial spores have different applications in biotechnology and vaccinology (Ricca and Cutting, 2003). The spores of some *Bacillus* sp. strains interact with heavy metals, such as Cd, Zn,

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Mn(II), Cu(II) and Ni (Tebo, 1995; He and Tebo, 1998; Hinc et al., 2010), standing out in ecosystem bioremediation research. Moreover, recent studies demonstrated that *B. subtilis* spores have a negatively charged surface, consisting of a suitable platform to adsorb positively charged molecules, including proteins (Huang et al., 2010), viral particles (Song et al., 2012) and enzymes (Sirec et al., 2012). Additionally, *B. subtilis* spores are safe to handle and their production and purification procedures are simple, quick and inexpensive (Tavares et al., 2013).

In this work, we demonstrated that plasmid DNA can be easily adsorbed on the surface of *B. subtilis* spores after a single step procedure based on the use of cationic agents, particularly dioctadecyldimethylammonium bromide (DODAB). DODAB cationic lipid and its assemblies have been employed in distinct contexts, such as imaging, biosensing, gene and drug delivery and vaccines (Carmona-Ribeiro, 2010). Sonication of DODAB vesicles in water produces small bilayer fragments that have previously been used as antimicrobial agents or as immunoadjuvant carriers for proteins (Rozenfeld et al., 2012). These fragments can activate dendritic cells and stimulate antigen presentation (Lincopan et al., 2009). In this study, we showed that DNA-coated spores can be loaded into gene gun cartridges and efficiently employed to transfect cells under *in vitro* and *in vivo* conditions. In addition, mice immunized with spores loaded with a DNA vaccine showed enhanced antigen-specific cellular and humoral immune responses at similar or higher levels to those achieved by gold microparticles, thus representing a low cost alternative to be used in gene delivery.

2. Material and methods

2.1. Mice

Male C57BL/6 mice at 6–8 weeks of age were supplied by the Animal Breeding Center of the Biomedical Sciences Institute of the University of São Paulo and housed at the Parasitology Department of the University of São Paulo. All the procedures involving animal handling followed the recommendations for the proper use and care of laboratory animals from the University of São Paulo Ethics Committee (protocol number 95-2011).

2.2. DNA plasmid

The pLuc plasmid encodes the luciferase enzyme (*luc2* gene). The gene sequence was cloned in the pcDNA3.0 vector (Invitrogen, CA, USA), which contains a CMV promoter and an ampicillin/neomycin-resistance-encoding gene. The DNA vaccine pgDE7 h encodes the HPV-16 E7 oncoprotein genetically fused near the C-terminal portion of the HSV-1 gD protein, as previously described (Diniz et al., 2013). The chimeric gene sequence was cloned in the pUMVC3 vector (Aldevron, ND, USA), which contains a CMV promoter and a kanamycin-resistance-encoding gene.

2.3. Preparation of spores

The sporulation of *B. subtilis* strain 1012 was performed by the nutrient exhaustion method using the Foerster medium (Foerster and Foster, 1966) and adapted from work by (Tavares et al., 2013). After 7 days of sporulation, the cultures were centrifuged at 10,000 rpm for 10 min and washed three times with distilled water. The spores were suspended in water and incubated for 1 h at 68 °C before quantification. The spores were visualized under a digital microscope EVOS® (AMG) and titrated to determine the number of colony-forming units (CFU/ml), confirmed by Petroff-Hausser chamber quantification (spores/ml). Heat-killed spores were obtained after autoclaving (121 °C for 15 min).

2.4. Particle size and zeta potential assays

Particle size measurements were determined from the mean hydrodynamic diameter. Zeta potentials (ζ) were determined from the electrophoretic mobility μ and the Smoluchowski equation, $\zeta = \mu\eta/\varepsilon$, where η and ε are the viscosity and the dielectric constant of the medium, respectively. Particle size and zeta potential measurements were evaluated before or after adsorption of pDNA to spore surfaces. Each spore sample was concentrated by centrifugation, suspended in 1 ml of water and measured three times with 10 readings for each measurement. Size distribution, zeta-average diameter and zeta-potential for all dispersions were determined by the dynamic light scattering (DLS) technique as described by (Rozenfeld et al., 2012) using a ZetaSizer NanoZS90 Analyzer (Malvern Instruments Ltd., Worcestershire, UK).

2.5. Adsorption of DNA to spores

B. subtilis spores were tested for the ability to adsorb plasmid DNA with different cationic agents. Spores (5×10^8) were suspended in 1 ml of each reagent listed in Table 1 and incubated at room temperature for 1 h with stirring every 15 min. After incubation, the suspension was centrifuged (10,000 rpm/10 min) and the pellet was washed once with distilled water. The spore samples were suspended in 100 μl of a solution containing 1 μg of plasmid DNA according to the conditions described in Table 1. After centrifugation, non-adsorbed DNA was estimated in agarose gels using HindIII-digested λ DNA markers. For biolistic immunization, the adsorption was performed using spores, pDNA and reagent amounts proportionally to the procedure described above.

2.6. Cartridge preparation for gene gun

The spores with adsorbed plasmid DNA were loaded on gene gun cartridges for biolistic administration. First, spores were suspended in a 0.05 mg/ml PVP (polyvinylpyrrolidone) in 100% ethanol and laid in Tefzel® tubes (BioRad) appropriate for the gene gun cartridge preparation. The tubes remained under airtight conditions overnight. Then, after removal of the liquid, the spores bound at the inner tube surface were dried with helium gas. To facilitate the administration, we used 2-fold amounts of pDNA and microparticles per cartridge. For both plasmid DNAs (pLuc and pgDE7 h), adsorption was carried out with 10 mg (1×10^{10}) of spores or gold for the preparation of 10 cartridges containing 2 μg of pLuc or pgDE7 h per cartridge (totalizing 20 μg of pDNA per tube). The cartridges were stored at −20 °C until use. The gold microparticle cartridges were prepared according to the manufacturerís instructions (BioRad) and stored at room temperature.

2.7. In vitro and in vivo gene gun transfection using a luciferase gene reporter

COS-7 cells were cultured in RPMI medium containing 10% (v/v) fetal bovine serum (FBS) and kept at 37 °C at 5% CO_2. Cells were seeded in 60 mm^2 culture plates (10^6 cells per plate) and incubated until 80% confluence. After medium removal, cells were transfected with 1 μg of pLuc using the a biolistic helium particle accelerator (Biomics, Brasília, Brazil) (100 psi of pressure) positioned 3 cm away from the cell plate, followed by the addition of 5 ml of RPMI with 2% FBS. C57BL/6 mice were inoculated using the gene gun device at 500 psi of pressure with 2 μg or 4 μg of pLuc on the shaved abdominal skin. Bioluminescence measurements were carried out 48 h after transfection following incubation for 20 min with 150 μg/ml luciferin solution in the cell plates or 20 min after intraperitoneal injection of 150 mg/kg of body weight of luciferin solution. Image captures and quantification analyses were performed using IVIS®

Table 1
Adsorption of plasmid DNA on the surface of *B. subtilis* spores.

Reagents	No incubation	1 h at 25 °C	6 h at 25 °C	1 h at 4 °C	6 h at 4 °C
Spermidine 0.05 M	–	++	++	–	–
$CaCl_2$ 1 M	–	–	*n/d*	*n/d*	*n/d*
PBS pH 4.0	–	–	*n/d*	*n/d*	*n/d*
PBS pH 7.0	–	–	*n/d*	*n/d*	*n/d*
CTAB 0.5% (wt/vol)	*n/d*	*n/d*	–	*n/d*	–
CTAB 0.01% (wt/vol)	–	+	+	+	+
DODAB BF 2 mM	+++	+++	+++	*n/d*	*n/d*

(–) No adsorption detected; (+) Adsorption of up to 20% of the DNA amount added; (++) Adsorption of up to 50% of the DNA amount added; (+++) Adsorption of 90–100% of the DNA amount added; (n/d) Not determined.

Spectrum equipment (Caliper). The bioluminescence images were shown in the "photon" mode and subsequently analyzed on the same equipment to obtain the total flux (photons/sec) or the radiance values (p/s/cm^2/sr), which refers to the number of photons per second per tissue area in a solid angle ("stearadian", "sr").

2.8. Immunization with a DNA vaccine using a gene gun

C57BL/6 were immunized twice (14 day interval) with pgDE7 h, which encodes the type 16 human papillomavirus (HPV-16) E7 oncoprotein genetically fused to the D glycoprotein of the type 1 human herpes virus (HSV) (Diniz et al., 2013; Aps et al., 2015). Gene gun cartridges were prepared with spores or gold microparticles containing either 4 μg or 2 μg of plasmid DNA, respectively. The shots were applied in the shaved abdominal region, with the equipment touching the skin of the mice. Blood samples were collected two weeks after the first and second dose for antibody and CD8$^+$ T lymphocyte assay. Mice treated with a gene gun with empty cartridges were considered as the negative control group and an additional control group vaccinated with non-coated spores (spores treated with DODAB but not incubated with DNA) was used following the same immunization regimen.

2.9. Enzyme-Linked immunosorbent assay (ELISA)

The anti-E7 and anti-gD antibody titers were measured by ELISA, as previously described (Sbrogio-Almeida et al., 2004). Plates (Polysorp^{TM}, Nunc-Immuno plates) were coated with 250 ng/well of gD recombinant protein obtained as previously described by Porchia and colleagues (Porchia et al., 2011). After incubation for 16 h at 4 °C, the plates were blocked with 3% gelatin in PBS and incubated at 37 °C for 2 h. Samples of individual sera collected 14 days after the first and second doses were added at a 1:25 initial dilution and incubated at 37 °C for 1 h. After incubation, anti-mouse IgG (1:3000), IgG1 (1:10,000) or IgG2c (1:3000) conjugated to peroxidase was added and the reaction was later developed with a solution containing *o*-phenylenediamine dihydrochloride (OPD). The optical densities were determined at 492 nm in an Epoch^{TM} Multi-Volume Spectrophotometer (Bio Tek Instruments) All samples were assayed in duplicate and the absorbance values of the control reactions ("blank", wells without serum addition) were subtracted from the results obtained with the tested serum samples. Dilution curves were drawn for each sample and the titers calculated as the reciprocal values of the highest dilution with an optical density of 0.2 (established as the value between the mean OD of the blank ±3 standard deviations).

2.10. Intracellular cytokine staining (ICS)

Intracellular IFN-γ staining was performed using blood samples collected 14, 21 or 28 days after the vaccine administration, according to previously described procedures (Diniz et al., 2013). The blood samples were treated for lysis of red blood cells and cultured at a concentration of 10^6 cells/well for 6 h at 37 °C in 96-well round bottom microtiter plates with 10 μg/ml of Brefeldin A (GolgiPlug; BD Biosciences, CA, USA) in the presence or not of 3 μg/ml of the E7-specific RAHYNIVTF peptide (amino acids 49-57). After incubation, the cells were stained with FITC-conjugated anti-CD8a antibody and after fixation and permeabilization, with PE-labeled anti-IFN-γ. The buffers and antibodies were purchased from BD Biosciences (CA, USA). The cells were examined by flow cytometry using a FACS Fortessa (BD Biosciences) and the data were analyzed using FlowJo software (TreeStar, OR, USA).

2.11. Statistical analyses

The ANOVA test was applied followed by Tukey's post-test when two or more groups were compared. Student's *t*-test was applied for analyses comparing individual data points from two groups. GraphPad Prism software was used for both analyses.

3. Results

3.1. Adsorption of plasmid DNA on the surface of spores

The adsorption of molecules on *B. subtilis* spores has been achieved with a variety of molecules, including antigens, biologically active enzymes and whole viral particles. In this study, we aimed to test whether these bioparticles can adsorb a DNA plasmid and deliver it into eukaryotic cells. First, we developed an adsorption procedure using different cationic reagents: spermidine, calcium chloride ($CaCl_2$), cetyltrimethylammonium bromide (CTAB), and bilayer fragments of dioctadecyldimethylammonium bromide (DODAB BF) at different concentrations and conditions (Table 1). Treatment of live spores (5×10^8) with the different chemical reagents was performed for 1 h at room temperature, and different incubation periods and temperatures for DNA binding. The adsorption efficiencies were determined by measuring the amount of DNA in the supernatant of the spore-DNA suspension after centrifugation.

Spores treated with 0.05 M spermidine and 0.01% CTAB partially adsorbed DNA after incubation for 1 h at 25 °C. No significant improvement was obtained after a prolonged incubation (6 h) at 25 °C or 4 °C. In contrast, the adsorption of DNA to spores treated with 2 mM DODAB was faster and more efficient. Using this reagent, more than 90% of the total DNA bound to the spores after incubation at 25 °C or immediately after mixing (termed "no incubation"). On the other hand, the incubation of spores at pH 4 or pH 7, used previously for protein adsorption (Huang et al., 2010), did not result in significant binding of DNA on the spore surface, nor did incubating the spores with 1 M $CaCl_2$. The same results were obtained with heat-inactivated spores (data not shown). In conclusion, the adsorption of plasmid DNA on spores treated with 2 mM DODAB was superior to all other tested conditions. Further experiments were carried out using DODAB-treated spores and DNA adsorption for 1 h at 25 °C.

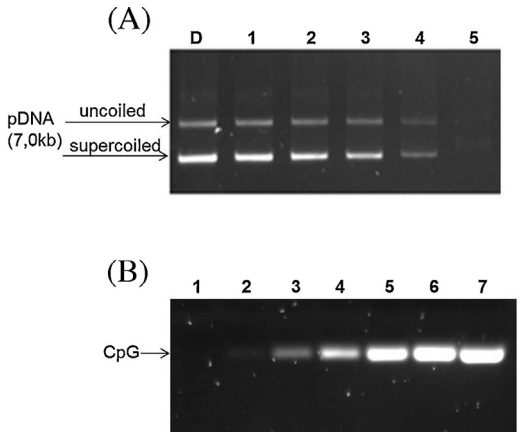

Fig. 1. Determination of molecule loading capacity of DODAB-treated spores. Quantification of non-adsorbed plasmids (pDNA) or linear DNA fragments (CpG) after the adsorption procedure to the spore surface for 1 h at 25 °C. (A) Agarose gel run of the supernatant solution after completing the adsorption protocol with 1 μg of pLuc. Samples: D, 1 μg of pDNA; lane 1, pDNA + 1×10^7 spores; lane 2, pDNA + 5×10^7 spores; lane 3, pDNA + 1×10^8 spores; lane 4: pDNA + 2×10^8 spores; lane 5, pDNA + 5×10^8 spores. (B) Agarose gel run of the supernatant solution after completing the standardized adsorption protocol with 5×10^8 spores and increasing amounts of linear DNA fragments: 1 μg (lane 1), 2 μg (lane 2), 3 μg (lane 3), 4 μg (lane 4), 5 μg (lane 5), 6 μg (lane 6), 10 μg (lane 7).

Next, we determined the minimal amount of spores required to adsorb 1 μg of plasmid DNA. For that purpose, different spore amounts (10^7–5×10^8 spores) were incubated with 1 μg of pLuc for 1 h at 25 °C (Fig. 1). Complete adsorption of DNA was achieved with 5×10^8 spores, corresponding to 0.5 mg of spore dry mass (Fig. 1A). The same results were also obtained with linear DNA fragments (synthetic oligonucleotides of 21 mer) (Fig. 1B), with an adsorption rate of 1 μg to 5×10^8 spores.

Table 2
Polydispersity index of *B. subtilis* spores during the adsorption steps.

Microparticle	Polydispersity index
LS	0.163 ± 0.096
LS-DODAB	0.348 ± 0.041
LS-DODAB-DNA	0.537 ± 0.153
KS	0.160 ± 0.033
KS-DODAB	0.805 ± 0.191
KS-DODAB-DNA	1.000 ± 0.000

3.2. Biophysical characterization of spores adsorbed with DNA

After determining the optimal conditions to adsorb DNA on spores, we next directed our study to investigate biophysical parameters involved in the reaction, such as the size and charge profiles. Purified live or heat inactivated spores of the *B. subtilis* 1012 strain had negative zeta potentials (LS: −35 mV ± 0.8; KS: −30 mV ± 0.4). Treatment with DODAB caused a sharp shift in the spore zeta potential to positive (LS: +33 mV ± 0.2; KS: 29 mV ± 0.7). After the addition of DNA, the zeta potentials of both live and inactivated spores shifted again and became close to neutral (LS: 8 mV ± 0.9; KS: −4 mV ± 0.1) (Fig. 2A), providing evidence for the binding of the plasmid DNA on the spore surface.

In addition, the size (Fig. 2B–C) and polydispersity (PdI) (Table 2) of spore-DODAB-DNA complexes were assessed by the DLS technique. Live and heat-inactivated spores had an average diameter of approximately 1.0 μm and presented similar PdI, approximately 0.16. After treatment with DODAB and DNA, the spore sizes did not change significantly compared to untreated spores, showing no significant difference between these samples (Fig. 2B–C). Nonetheless, DNA coating resulted in a very polydispersed system, with PDI = 1 (Table 2).

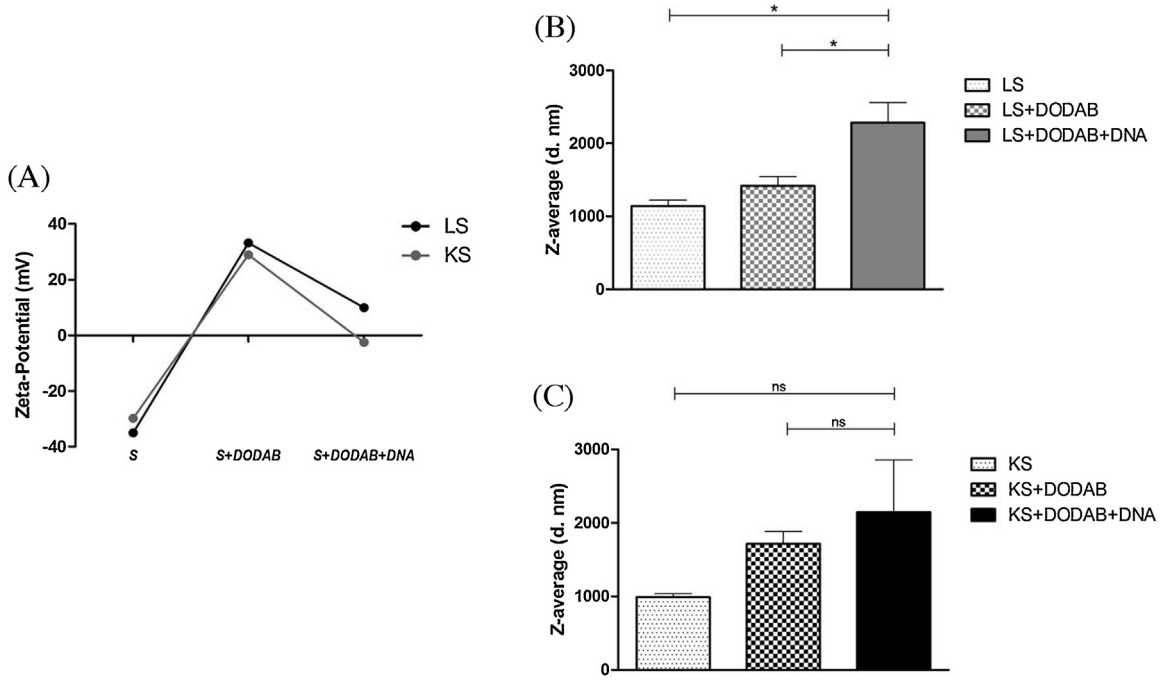

Fig. 2. Zeta potential and diameter of *B. subtilis* spores submitted to different treatments.
(A) Zeta potential (mV) and (B, C) Zeta-average diameter (nm) was obtained from spores (S), live (LS) or heat-killed (KS) under the tested conditions: *S*, purified spores (5×10^8); *S + DODAB*, the same amount of spores treated with 2 mM DODAB; *S + DODAB + DNA*, DODAB-treated spores adsorbed with 1 μg of DNA (pLuc). The data are shown as the mean ± SEM from triplicates in the same experiment. Statistical significance: (*) $p < 0.05$; (ns) non-significant (ANOVA).

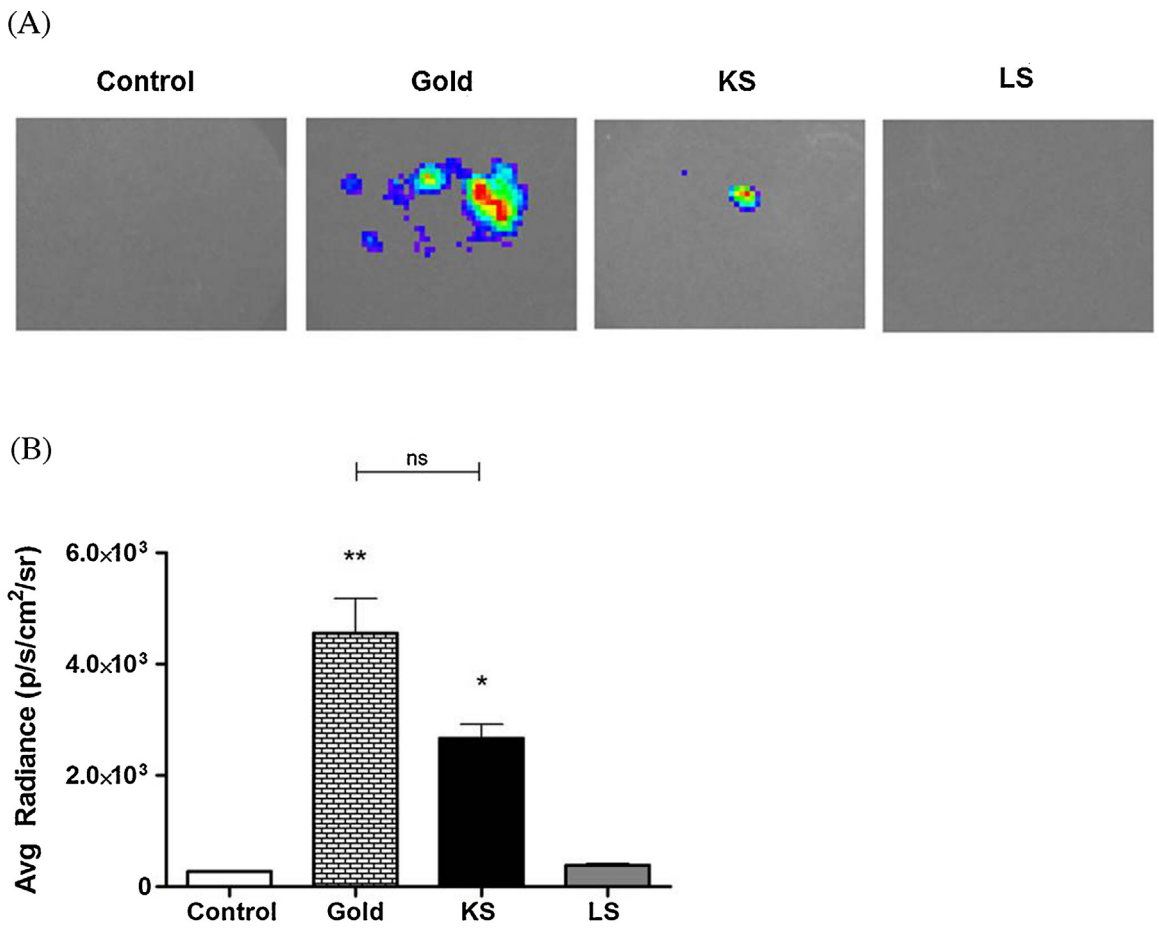

Fig. 3. Transfection of COS7 cells with DNA adsorbed on *B. subtilis* spores and delivered by gene gun delivery.
Transfection cartridges were prepared with 1 μg of pLuc adsorbed to gold particles, heat-inactivated (KS) or live (LS) spores. The luminescence results were obtained 48 h after transfection. (A) Bioluminescent images of one representative transfection plate from two independent experiments with similar results. (B) Average radiance obtained from two independent experiments. All data are shown as the mean ± SD. The control group corresponds to non-transfected cells. Statistical significance: (*) $p < 0.05$, (**) $p < 0.01$ *vs Control* group, (ns) non-significant (ANOVA).

3.3. *Spores as microparticles for gene gun-mediated transfection of eukaryotic cells*

To investigate the use of *B. subtilis* spores as a platform for biolistic administration of plasmid DNA, we first determined the transfection efficiencies in COS-7 cells cultured with a luciferase-encoding plasmid (pLuc). As indicated in Fig. 3, cells submitted to biolistic administration with gold particles were efficiently transfected with pLuc. Similarly, biolistic administration using heat-inactivated spores had similar emission results, with no statistical difference in regard to gold group but a higher luminescence emission compared to live spores (approximately 7-fold) after 24 h (Fig. 3B), indicating that under the tested parameters, inactivated spores can be used for transfection of eukaryotic cells using gene gun delivery of plasmid DNA.

3.4. *Spores as microparticles for* in vivo *delivery of plasmid DNA*

Next, we evaluated if DNA-loaded *B. subtilis* spores could promote *in vivo* transfection of mice after gene gun administration. C57BL/6 mice were inoculated in the abdominal region with pLuc adsorbed on spores (2 or 4 μg/dose) or gold microparticles (2 μg/dose). After 48 h, mice were evaluated for bioluminescence emission using the substrate luciferin (Fig. 4). Using the same amount of DNA for both spores and gold microparticles (one shot of 2 μg of pLuc), live (LS) or killed spores (KS) promoted half the luminescence emission as gold microparticles (Fig. 4B). Notably, after two shots of DNA-coated spores (4 μg of pLuc), killed spores showed similar protein expression levels compared to gold microparticles coated with 2 μg of pLuc, suggesting that heat-inactivated spores could be a suitable carrier for the delivery of DNA vaccines by gene gun devices. The luminescence in mice inoculated with live spores was significantly lower than in animals inoculated with heat-inactivated spores, as previously observed in the *in vitro* assays.

3.5. *Detection of immune responses elicited in mice immunized by gene gun with heat-inactivated* subtilis *spores*

C57BL/6 mice were immunized with spores loaded (4 μg/dose) with a plasmid named pgDE7 h, encoding a chimeric protein derived from the human papillomavirus E7 oncoprotein genetically fused to the herpes virus glycoprotein D. Intramuscular immunization of mice with the same amount of naked DNA did not induce a significant E7-specific $CD8^+$ T cell activation (Diniz et al., 2013) or serum IgG response to the HSV-1 gD protein (Aps et al., 2015). Mice immunized with DNA adsorbed on inactivated *B. subtilis* spores induced the activation of T $CD8^+$ IFN-γ^+ cells targeting the E7 oncoprotein of HPV-16 at numbers equivalent to those achieved in mice immunized with gold particles (Fig. 5A). In addition, mice immunized with pgDE7 h adsorbed on spores showed a high antibody response for the HSV-1 gD component of the hybrid protein at

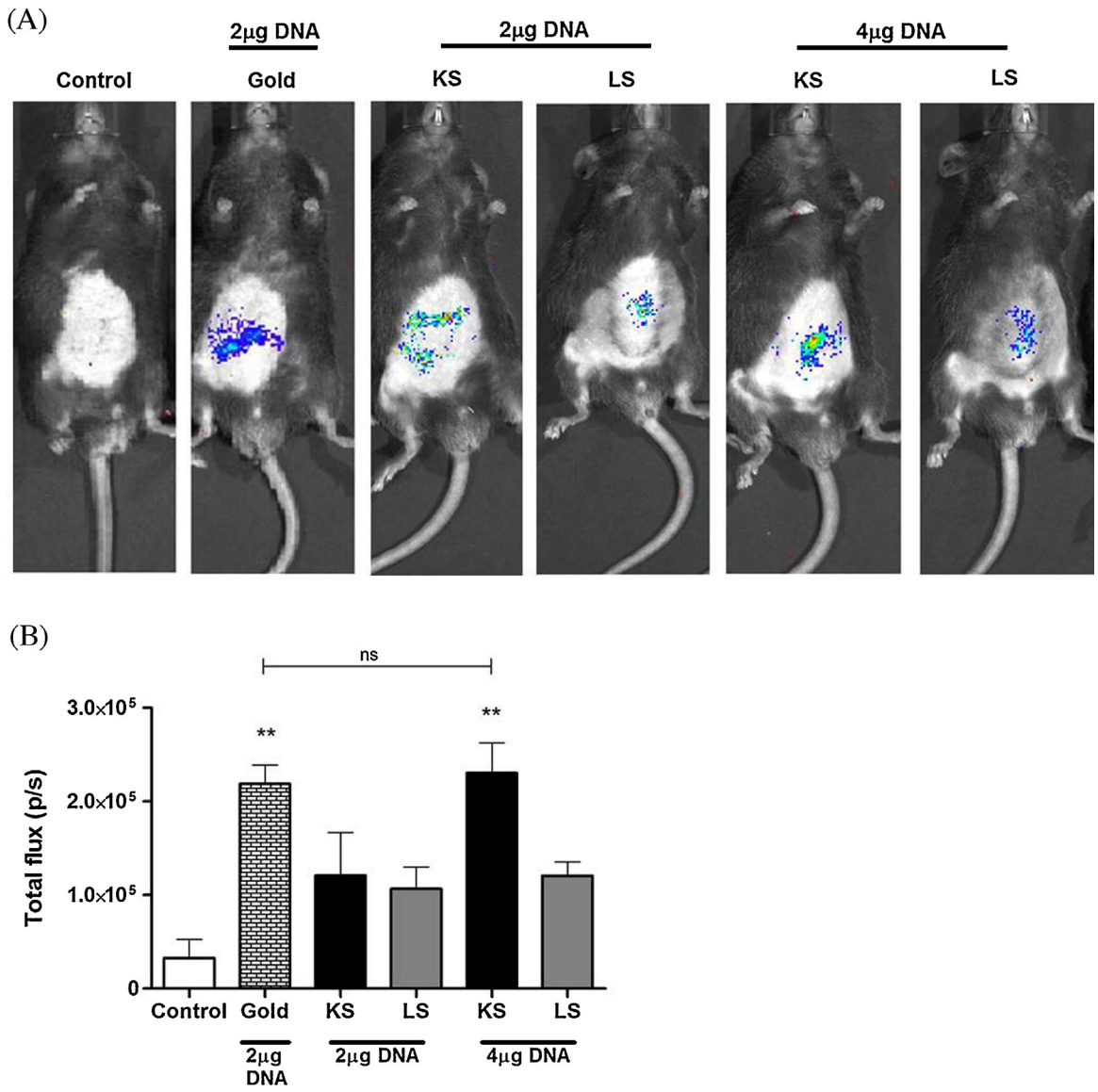

Fig. 4. Administration of plasmid DNA adsorbed on *B. subtilis* spores by gene gun delivery.
Transfection cartridges were prepared with 2 μg of pLuc adsorbed to gold particles, heat-inactivated (KS) or live (LS) spores. Each mouse was treated in the abdominal region with one or two shots of plasmid DNA totaling 2 μg for gold or 4 μg for spore microparticles. The luminescence signal was detected 48 h after biolistic administration. (A) Bioluminescence images are representative of one of two animals per group. (B) The data corresponds to the ± SD of total flux measurements in photons/second of one from two independent experiments with similar results (n = 4). The control group corresponds to untreated animals. Statistical significance: (**) $p < 0.01$ *vs Control* group, (ns) non-significant (ANOVA).

levels similar to those observed in animals immunized with gold particles (Fig. 5B). In addition, gene gun vaccination using spore or gold microcarriers promoted a balanced Th1 and Th2 response with a slight trend to the Th2 profile (Fig. 5C). Collectively, these data show that killed *B. subtilis* spores can be used as an alternative for gold microparticle for the *in vivo* gene gun delivery of DNA vaccines.

4. Discussion

Recently, *B. subtilis* spores have been tested as a platform for loading different molecules with various biotechnological applications. However, to our knowledge, no study has investigated spores coated with plasmid DNA or assessed the potential application of spores as a particulate support for the gene gun delivery of DNA. In this work, we demonstrated that *B. subtilis* spores can adsorb DNA plasmids after a simple procedure using vesicular fragments of DODAB. In addition, our work showed that heat-killed spores can be loaded into biolistic cartridges for gene gun administration and promote *in vitro* and *in vivo* transfections, also inducing specific immune response as achieved with gold microparticles.

In agreement with previous reports (Huang et al., 2010; Kazakov et al., 2008), our work showed that live and heat-killed spores were approximately 1 μm in diameter and had a negative surface charge (Fig. 2), which is not compatible with the negative charge of DNA. To overcome this obstacle, coating the spores with a positively charged intermediate reagent could provide a suitable substrate for DNA adsorption. Nonetheless, the pretreatment of spores employing reagents currently used in plasmid delivery systems, such as spermidine, $CaCl_2$ and CTAB, did not allow efficient DNA binding to spores (Table 1). However, pretreatment of spores with DODAB vesicle fragments promoted a higher adsorption efficiency of plasmid DNA even without extended incubation periods. The adsorption process is faster and simpler than previ-

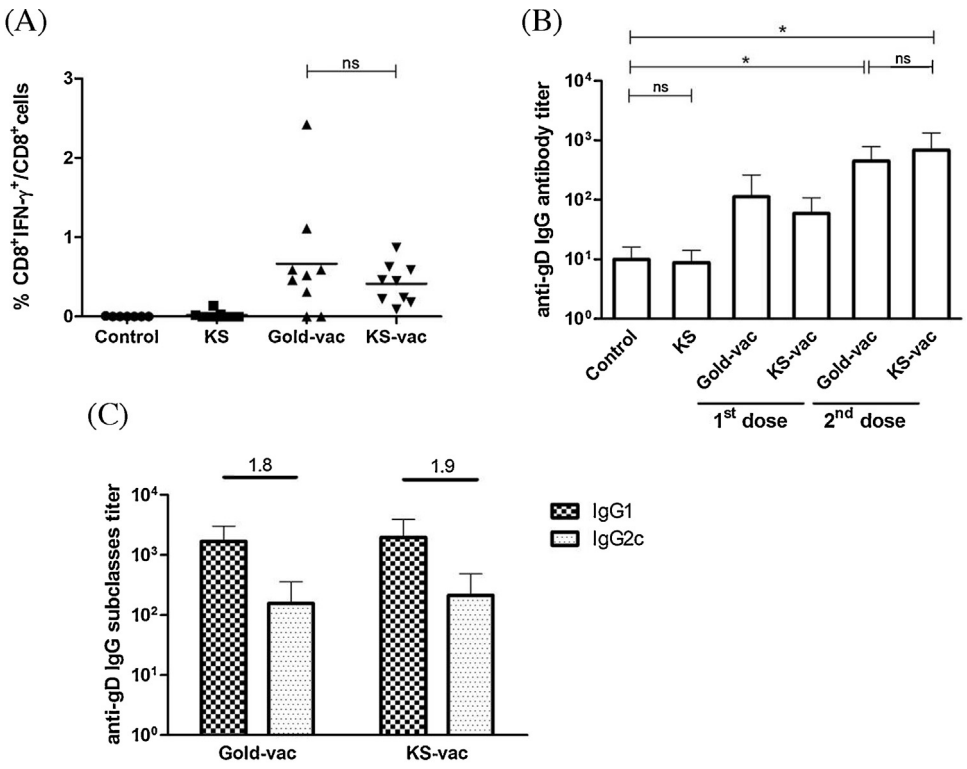

Fig. 5. Antigen specific cellular and humoral responses elicited in mice immunized with a DNA vaccine by the gene gun delivery.
Mice were immunized twice (14 days of interval) with 2 μg or 4 μg per dose of pgDE7 h in the abdominal region using gold (Gold-vac) or killed spore (KS-vac) particles as biolistic carriers. The control group corresponds to non-vaccinated mice and the KS group to mice immunized with non-coated spores. Individual sera were collected 14 days after the first or second immunization for peripheral blood mononuclear cell (PBMCs) analysis. (A) Frequencies of IFN-γ-producing $CD8^+$ T cells after *in vitro* stimulation with the E7-specific peptide (^{49}RAHYNIVTF^{57}) of PBMCs obtained 14 days after the second dose, stained for CD8 (FITC) and intracellular IFN-γ (PE) and monitored by flow cytometry. (B) Specific anti-gD IgG titers determined by ELISA. All data are shown as the mean ± SD based on two independent experiments (n = 8–10). Statistical significance: (*) $p < 0.05$, (ns) non-significant.

ously described procedures for binding supercoiled plasmid DNA on other particulate substrates (Svarovsky et al., 2008). Treatment of spores with DODAB also allowed the binding of oligonucleotides at the same conditions established for double stranded DNA, which is not observed with conventional gold particles (Svarovsky et al., 2008). This property could be particularly useful for immunizations with the immunomodulator molecule CpG. Indeed, Rozenfeld and colleagues (Rozenfeld et al., 2012) reported that the administration of DODAB bilayer fragments combined with CpG oligonucleotides induced enhanced immunological effects after parenteral administration with protein antigens.

Regarding future biotechnology applications, we evaluated the performance of the spore-DODAB-DNA system as a particulate carrier for gene gun delivery. Our results demonstrated that inactivated spores coated with a plasmid encoding the *luc2* reporter gene could transfect eukaryotic cells, emitting similar luminescence levels as those achieved with gold particles, but in clearly reduced areas of transfected cells (Fig. 3). This could be related to the fact that the spores are much lighter than the gold particles, causing a greater loss of material during the gun shot. Besides, DNA-coated spores presented an elevated PdI (reaching 1.0 for killed spores), indicating that the sample has a very broad size distribution and may contain aggregates derived from the agglomeration of the neutral spore-DNA complexes that could influence the transfection efficiency. Based on this result, the next transfection experiments were performed using twice the amount of DNA for the spores cartridges. Interestingly, live *B. subtilis* spores did not promote efficient transfection of cells or mice skin. The germination of the spores in to the cells (Duc et al., 2004; Leser et al., 2008; Ceragioli et al., 2009) causes disassembling of the spore-DNA complexes and protease production during the bacterial cell replication (Prestidge et al., 1971; Nakayama et al., 1977; Takamatsu et al., 2000), which could lead to the degradation of the recombinant protein.

In the vaccinology context, *B. subtilis* spores demonstrated immunostimulatory effects when co-administered with protein antigens (Song et al., 2012; Barnes et al., 2007; Cerovic et al., 2009; Liang et al., 2013). The adjuvant effects of spores have been observed both after administration via mucosal (Huang et al., 2010) and parenteral routes (Aps et al., 2015; Souza et al., 2014). In addition to these observations, our results demonstrated that coating *B. subtilis* spores with plasmid DNA enhanced the cellular and antibody responses induced by a DNA vaccine after intradermal administration through a gene gun device. These results clearly expand the applications of *B. subtilis* spores as a platform for delivery and enhancement of immune responses induced by subunit vaccines, either as purified recombinant proteins or as DNA vaccines.

Gene gun administration has been widely used for the administration of DNA vaccines (Steitz and Tüting, 2013; Bryan et al., 2013; Bergmann-Leitner and Leitner, 2013). It allows a drastic reduction in the vaccine amount, promoting similar immune responses compared to intramuscular administration, which requires around 100-fold more immunogen. Although this technique has been successfully tested in both animals and humans, the high cost of the gold microparticles makes this vaccination strategy unsuitable for large scale immunization programs. For the administration of one thousand doses (500 mg of gold particles) the cost can be up to US$ 400. Meanwhile, the results presented herein demonstrate that the use of inactivated bacterial spores can drastically reduce the costs of gene gun immunization (approximately 25 cents for 1000 doses,

based on the costs of the culture medium components). *B. subtilis* spores can be produced at larger quantities (up to 5 g of dried mass per liter) and at high purity by fairly simple procedures (Tavares et al., 2013). Furthermore, spores of several species of *Bacillus* are considered safe for human consumption, including *B. subtilis* (Hong et al., 2008; Isticato et al., 2001) and can frequently be isolated from soil (Aslim et al., 2002). Moreover, *B. subtilis* spores, in contrast to gold particles, are organic and biodegradable once introduced into mammalian tissues. These experimental evidences, thus, add another application of *B. subtilis* spores in vaccine technologies and shall support further use of the gene gun administration of vaccines.

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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References

Aps, L.R.M.M., Diniz, M.O., Porchia, B.F., Sales, N.S., Moreno, A.C.R., Ferreira, L.C.S., 2015. *Bacillus subtilis* spores as adjuvants for DNA vaccines. Vaccine 33 (20), 2328–2334, http://dx.doi.org/10.1016/j.vaccine.2015.03.043.

Aslim, B., Yuksekdag, Z.N., Beyatli, Y., 2002. Determination of PHB growth quantities of certain *Bacillus* species isolated from soil. Turk. Electron. J. Biotechnol., 24–30, Available at: http://www.biyotekder.hacettepe.edu.tr/5.pdf.

Barnes, A.G., Cerovic, V., Hobson, P.S., Klavinskis, L.S., 2007. *Bacillus subtilis* spores: a novel microparticle adjuvant which can instruct a balanced Th1 and Th2 immune response to specific antigen. Eur. J. Immunol. 37 (6), 1538–1547, http://dx.doi.org/10.1002/eji.200636875.

Bergmann-Leitner, E.S., Leitner, W.W., 2013. Gene gun immunization to combat malaria. Methods Mol. Biol. 940, 269–284, http://dx.doi.org/10.1007/978-1-62703-110-3_21.

Bryan, M., Guyach, S., Norris, K.A., 2013. Biolistic DNA vaccination against *Trypanosoma* infection. Methods Mol. Biol. 940, 305–315, http://dx.doi.org/10.1007/978-1-62703-110-3_24.

Carmona-Ribeiro, A.M., 2010. Biomimetic nanoparticles: preparation, characterization and biomedical applications. Int. J. Nanomed. 5, 249–259, PMCID: PMC2865020.

Ceragioli, M., Cangiano, G., Esin, S., Ghelardi, E., Ricca, E., Senesi, S., 2009. Phagocytosis, germination and killing of *Bacillus subtilis* spores presenting heterologous antigens in human macrophages. Microbiology 155, 338–346, http://dx.doi.org/10.1099/mic.0.022939-0.

Cerovic, V., Jenkins, C.D., Barnes, A.G., Milling, S.W., MacPherson, G.G., Klavinskis, L.S., 2009. Hyporesponsiveness of intestinal dendritic cells to TLR stimulation is limited to TLR4. J. Immunol. 182 (4), 2405–2415, http://dx.doi.org/10.4049/jimmunol.0802318.

De Rose, R., Tennent, J., McWaters, P., Chaplin, P.J., Wood, P.R., Kimpton, W., Cahill, R., Scheerlinck, J.P.Y., 2002. Efficacy of DNA vaccination by different routes of immunisation in sheep. Vet. Immunol. Immunopathol. 90 (1), 55–63, http://dx.doi.org/10.1016/S0165-2427(02)00221-0.

Diniz, M.O., Cariri, F.A.M.O., Aps, L.R.M.M., Ferreira, L.C.S., 2013. Enhanced therapeutic effects conferred by an experimental DNA vaccine targeting human papillomavirus-induced tumors. Hum. Gene Ther. 24 (10), 861–870, http://dx.doi.org/10.1089/hum.2013.102.

Duc, L.H., Hong, H.A., Uyen, N.Q., Cutting, S.M., 2004. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine 22, 1873–1885, http://dx.doi.org/10.1016/j.vaccine.2003.11.021.

Foerster, H.F., Foster, J.W., 1966. Endotrophic calcium, strontium, and barium spores of *Bacillus megaterium* and *Bacillus cereus*. J. Bacteriol. 91 (3), 1333–1344, Available at: http://jb.asm.org/content/91/3/1333.short.

Ginsberg, B.A., Rasalan, T.S., Adamow, M., Mu, Z., Tandon, S., Bewkes, B.B., Roman, R.A., Chapman, P.B., Schwartz, G.K., Carvajal, R.D., Panageas, K.S., Terzulli, S.L., Houghton, A.N., Yuan, J.D., Wolchok, J.D., 2010. Immunologic response to xenogeneic gp100 DNA in melanoma patients: comparison of particle-mediated epidermal delivery with intramuscular injection. Clin. Cancer Res. 16 (15), 4057–4065, http://dx.doi.org/10.1158/1078-0432, CCR-10-1093.

Gotesman, M., Williams, S.A., 2016. Using a handheld gene gun for genetic transformation of *Tetrahymena thermophila*. Methods Mol. Biol., 373–383, http://dx.doi.org/10.1007/978-1-4939-3124-8_22.

He, L.M., Tebo, B.M., 1998. SurfaceSurface charge properties of and Cu(II) adsorption by spores of the marine *Bacillus* sp. strain SG-1. Appl. Environ. Microbiol. 64 (3), 1123–1129, Available at: http://aem.asm.org/content/64/3/1123.short.

Hinc, K., Ghandili, S., Karbalaee, G., Shali, A., Noghabi, K.A., Ricca, E., Ahmadian, G., 2010. Efficient binding of nickel ions to recombinant *Bacillus subtilis* spores. Res. Microbiol. 161 (9), 757–764, http://dx.doi.org/10.1016/j.resmic.2010.07.008.

Hong, H.A., Huang, J.M., Khaneja, R., Hiep, L.V., Urdaci, M.C., Cutting, S.M., 2008. The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. J. Appl. Microbiol. 105 (2), 510–520, http://dx.doi.org/10.1111/j.1365-2672.2008.03773.

Huang, J.M., Hong, H.A., Van Tong, H., Hoang, T.H., Brisson, A., Cutting, S.M., 2010. Mucosal delivery of antigens using adsorption to bacterial spores. Vaccine 28 (4), 1021–1030, http://dx.doi.org/10.1016/j.vaccine.2009.10.127.

Isticato, R., Cangiano, G., Tran, H.T., Ciabattini, A., Medaglini, D., Oggioni, M.R., De Felice, M., Pozzi, G., Ricca, E., 2001. Surface display of recombinant proteins on *Bacillus subtilis* spores. J. Bacteriol. 183 (21), 6294–6301, http://dx.doi.org/10.1128/JB.183.21, 6294-6301.2001.

Kasturi, S.P., Qin, H., Thomson, K.S., El-Bereir, S., Cha, S.C., Neelapu, S., Kwak, L.W., Roy, K., 2006. Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. J. Control. Release 113 (3), 261–270, http://dx.doi.org/10.1016/j.jconrel.2006.04.006.

Kazakov, S., Bonvouloir, E., Gazaryan, I., 2008. Physicochemical characterization of natural ionic microreservoirs: *Bacillus subtilis* dormant spores. J. Phys. Chem. B 112 (7), 2233–2244, http://dx.doi.org/10.1021/jp077188u.

Kim, J.W., Hung, C.F., Juang, J., He, L., Kim, T.W., Armstrong, D.K., Pai, S.I., Chen, P.J., Lin, C.T., Boyd, D.A., Wu, T.C., 2004. Comparison of HPV DNA vaccines employing intracellular targeting strategies. Gene Ther. 11, 1011–1018, http://dx.doi.org/10.1038/sj.gt.3302252.

Klein, T.M., Fromm, M., Weissinger, A., Tomes, D., Schaaf, S., Sletten, M., Sanford, J.C., 1988. Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc. Natl. Acad. Sci. U. S. A. 85 (12), 4305–4309, Available at: http://www.pnas.org/content/85/12/4305.full.pdf.

Kutzler, M.A., Weiner, D.B., 2008. DNA vaccines: ready for prime time? Nat. Rev. Genet. 9 (10), 776–788, http://dx.doi.org/10.1038/nrg2432.

Lambracht-Washington, D., Qu, B.X., Fu, M., Anderson Jr., L.D., Stüve, O., Eagar, T.N., Rosenberg, R.N., 2011. DNA immunization against amyloid beta 42 has high potential as safe therapy for Alzheimer's disease as it diminishes antigen-specific Th1 and Th17 cell proliferation. Cell. Mol. Neurobiol. 31 (6), 867–874, http://dx.doi.org/10.1007/s10571-011-9680-7.

Leser, T.D., Knarreborg, A., Worm, J., 2008. Germination and outgrowth of *Bacillus subtilis* and *Bacillus licheniformis* spores in the gastrointestinal tract of pigs. J. Appl. Microbiol. 104, 1025–1033, http://dx.doi.org/10.1111/j.1365-2672.2007.03633.x.

Liang, J., Fu, J., Kang, H., Lin, J., Yu, Q., Yang, Q., 2013. The stimulatory effect of TLRs ligands on maturation of chicken bone marrow-derived dendritic cells. Vet. Immunol. Immunopathol. 155 (3), 205–210, http://dx.doi.org/10.1016/j.vetimm.2013.06.014.

Lin, K., Roosinovich, E., Ma, B., Hung, C.F., Wu, T.C., 2010. Therapeutic HPV DNA vaccines. Immunol. Res. 47 (1–3), 86–112, http://dx.doi.org/10.1007/s12026-009-8141-6.

Lincopan, N., Santana, M.R., Faquim-Mauro, E., da Costa, M.H., Carmona-Ribeiro, A.M., 2009. Silica-based cationic bilayers as immunoadjuvants. BMC Biotechnol., 9 (1) 5 (19) 10.1186/1472-6750-9-5.

Nakayama, T., Munoz, L., Doi, R.H., 1977. A procedure to remove protease activities from *Bacillus subtilis* sporulating cells and their crude extracts. Anal. Biochem. 78 (1), 165–170, http://dx.doi.org/10.1016/0003-2697(77)90020-3.

Nguyen, D.N., Raghavan, S.S., Tashima, L.M., Lin, E.C., Fredette, S.J., Langer, R.S., Wang, C., 2008. Enhancement of poly(orthoester) microspheres for DNA vaccine delivery by blending with poly(ethylenimine). Biomaterials 29 (18), 2783–2793, http://dx.doi.org/10.1016/j.biomaterials.2008.03.011.

Nguyen-Hoai, T., Kobelt, D., Hohn, O., Vu, M.D., Schlag, P.M., Dörken, B., Norley, S., Lipp, M., Pezzutto, A., Westermann, J., 2012. HER2/neu DNA vaccination by intradermal gene delivery in a mouse tumor model: gene gun is superior to jet injector in inducing CTL responses and protective immunity. Oncoimmunology 1 (9), 1537–1545, http://dx.doi.org/10.4161/onci.22563.

Nguyen-Hoai, T., Pezzutto, A., Westermann, J., 2015. Gene gun her2/neu DNA vaccination: evaluation of vaccine efficacy in a syngeneic her2/neu mouse tumor model. Methods Mol. Biol., 17–37, http://dx.doi.org/10.1007/978-1-4939-2727-2_2.

O'Hagan, D., Singh, M., Ugozzoli, M., Wild, C., Barnett, S., Chen, M., Schaefer, M., Doe, B., Otten, G.R., Ulmer, J.B., 2001. Induction of potent immune responses by cationic microparticles with adsorbed Human Immunodeficiency Virus DNA vaccines. J. Virol. 75 (19), 9037–9043, http://dx.doi.org/10.1128/JVI.75.19.9037-9043.2001.

O'Brien, J.A., Lummis, S.C., 2006. Biolistic transfection of neuronal cultures using a hand-held gene gun. Nat. Protoc. 1 (2), 977–981, http://dx.doi.org/10.1038/nprot.2006.145.

Porchia, B.F., Diniz, M.O., Cariri, F.A., Santana, V.C., Amorim, J.H., Balan, A., Braga, C.J.M., Ferreira, L.C.S., 2011. Purified herpes simplex type 1 glycoprotein D (gD) genetically fused with the Type 16 human papillomavirus E7 oncoprotein enhances antigen-specific CD8+ T cell responses and confers protective antitumor immunity. Mol. Pharm. 8 (6), 2320–2330, http://dx.doi.org/10.1021/mp200194s.

Prestidge, L., Gage, V., Spizizen, J., 1971. Protease activities during the course of sporulation in *Bacillus subtilis*. J. Bacteriol. 107 (3), 815–823, PMCID: PMC247005.

Rezvan, H., Rees, R., Ali, S., 2011. Leishmania mexicana Gp63 cDNA Using gene gun induced higher immunity to L. mexicana infection compared to soluble *Leishmania* antigen in BALB/C. Iran J. Parasitol. 6 (4), 60–75, PMCID: PMC3279899.

Ricca, E., Cutting, S.M., 2003. Emerging applications of bacterial spores in nanobiotechnology. J. Nanobiotechnol. 1 (6), 11, Available at: http://www.jnanobiotechnology.com/content/1/1/6.

Rozenfeld, J.H.K., Silva, S.R., Ranéia, P.A., Faquim-Mauro, E., Carmona-Ribeiro, A.M., 2012. Stable assemblies of cationic bilayer fragments and CpG oligonucleotide with enhanced immunoadjuvant activity *in vivo*. J. Control. Release 160, 367–373, http://dx.doi.org/10.1016/j.jconrel.2011.10.017.

Sbrogio-Almeida, M.E., Mosca, T., Massis, L.M., Abrahamsohn, I.A., Ferreira, L.C.S., 2004. Host and bacterial factors affecting induction of immune responses to flagellin expressed by attenuated salmonella vaccine strains. Infect. Immun. 72 (5), 2546–2555, http://dx.doi.org/10.1128/IAI.72.5, 2546-2555.2004.

Sidney Kimmel Comprehensive Cancer Center. Therapeutic Vaccination for Patients With HPV16+ Cervical Intraepithelial Neoplasia (CIN2/3). Available at: https://clinicaltrials.gov/ct2/show/NCT00988559.

Sirec, T., Strazzulli, A., Isticato, R., De Felice, M., Moracci, M., Ricca, E., 2012. Adsorption of β-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. Microb. Cell Fact. 11 (100), 11, Available at: http://www.microbialcellfactories.com/content/11/1/100.

Song, M., Hong, H.A., Huang, J.M., Colenutt, C., Khang, D.D., Nguyen, T.V.A., Park, S.M., Shim, B.S., Song, H.H., Cheon, I.S., Jang, J.E., Choi, J., Choi, Y.K., Stadler, K., Cutting, S.M., 2012. Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. Vaccine 30, 3266–3277, http://dx.doi.org/10.1016/j.vaccine.2012.03.016.

Souza, R.D., Batista, M.T., Luiz, W.B., Cavalcante, R.C.M., Amorim, J.H., Bizerra, R.S.P., Martins, E.G., Ferreira, L.C.F., 2014. *Bacillus subtilis* spores as vaccine adjuvants: further insights into the mechanisms of action. PLoS One 9 (1), http://dx.doi.org/10.1371/journal.pone.0087454.

Steitz, J., Tüting, T., 2013. Biolistic DNA vaccination against melanoma. Methods Mol. Biol. 940, 317–337, http://dx.doi.org/10.1007/978-1-62703-110-3_24.

Svarovsky, S., Borovkov, A., Sykes, K., 2008. Cationic gold microparticles for biolistic delivery of nucleic acids. Biotechniques 45 (5), 535–539, http://dx.doi.org/10.2144/000112991.

Takamatsu, H., Imamura, A., Kodama, T., Asai, K., Ogasawara, N., Watabe, K., 2000. The yabG gene of *Bacillus subtilis* encodes a sporulation specific protease which is involved in the processing of several spore coat proteins. FEMS Microbiol. Lett. 192 (1), 33–38, http://dx.doi.org/10.1111/j.1574-6968.2000.tb09355.x.

Tavares, M.B., Souza, R.D., Luiz, W.B., Cavalcante, R.C.M., Casaroli, C., Martins, E.G., Ferreira, R.C.C., Ferreira, L.C.S., 2013. *Bacillus subtilis* endospores at high purity and recovery yields: optimization of growth conditions and purification method. Curr. Microbiol. 66, 279–285, http://dx.doi.org/10.1007/s00284-012-0269-2.

Tebo, B.M., 1995. Metal precipitation by marine bacteria: potential for biotechnological applications. Genet. Eng. (N. Y.) 17, 231–263.

Woods, G., Zito, K., 2008. Preparation of gene gun bullets and biolistic transfection of neurons in slice culture. J. Vis. Exp. 12, e675, http://dx.doi.org/10.3791/675.