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Effectiveness, against tuberculosis, of pseudo-ternary complexes: Peptide-DNA-cationic liposome

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ABSTRACT

We report the effects of a synthetic peptide designed to act as a nuclear localization signal on the treatment of tuberculosis. The peptide contains 21 amino acid residues with the following specific domains: nuclear localization signal from SV 40T, cationic shuttle sequence, and cysteamide group at the C-terminus. The peptide was complexed with the plasmid DNAhsp65 and incorporated into cationic liposomes, forming a pseudo-ternary complex. The same cationic liposomes, composed of egg chicken L-α-phosphatidylcholine, 1,2-dioleoyl-3-trimethylammonium-propane, and 1,2-dioleoyl-3-trimethylammonium-propane (2:1:1 M), were previously evaluated as a gene carrier for tuberculosis immunization protocols with DNAhsp65. The pseudo-ternary complex presented a controlled size (250 nm), spherical-like shape, and various lamellae in liposomes as evaluated by transmission electron microscopy. An assay of fluorescence probe accessibility confirmed insertion of the peptide/DNA into the liposome structure. Peptide addition conferred no cytotoxicity *in vitro*, and similar therapeutic effects against tuberculosis were seen with four times less DNA compared with naked DNA treatment. Taken together, the results indicate that the pseudo-ternary complex is a promising gene vaccine for tuberculosis treatment. This work contributes to the development of multifunctional nanostructures in the search for strategies for *in vivo* DNA delivery.

1. Introduction

DNA vaccines have become a promising alternative for the treatment and prevention of different infectious diseases [1–3], such as tuberculosis [4]. Typically, this kind of vaccine consists of a modified bacterial plasmid DNA coupled to a gene sequence that encodes a desired protein. Regarding the several methods of *in vitro* or *in vivo* DNA administration, the DNA will reach the cell nucleus, be transcribed into mRNA, and translated to the corresponding protein. If this DNA is captured by specialized antigen presenting cells (APCs), the endogenously produced protein will be processed to small peptides and presented on the cell surface, stimulating the T cell adaptive immune response. Also, the entire protein could be secreted from the cell and recognized by B cells, stimulating specific antibody production [5]. This versatile and broad range of immune response activation makes plasmid DNA a very useful tool.

However, naked DNA is rapidly degraded *in vivo* by extracellular deoxyribonucleases and exhibits poor cellular uptake. An alternative to overcoming this difficulty is the development of safe and efficient gene carriers [6]. In this context, cationic liposomes can be used as non-viral carriers, protecting and directing gene material to the cells [7,8]. The mechanism of DNA transfection by cationic liposomes has been studied by many authors, and these gene carriers are well known to deliver DNA efficiently into the cytosol [9–12]. After DNA release into the cytosol, transfection success still depends on DNA trafficking toward the nucleus, and its transport across the nuclear envelope still remains one of the most important barriers [13].

During cell division, eukaryotic cells have a disordered nuclear envelope, facilitating DNA entrance. However, if the cell is not dividing, the nuclear envelope is an important barrier for macromolecules, such as plasmid DNA [13,14]. The nuclear envelope consists of a highly ordered membrane containing pores that allow the

Abbreviations: NLS, nuclear localization signals; EPC, L-α-phosphatidylcholine (egg, chicken); DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMB-Chol, cholesteryl-3 β-carboxybutylene-N-dimethylamine; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinases; IL, interleukin; MTT, 3-(4,5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide.

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passive diffusion of small molecules [15,16]. Larger molecules can be transported through the nuclear pore by specialized cargo proteins, and one well-known transport mechanism is through nuclear localization signals (NLS). NLS basically consist of a short peptide sequence within proteins that allows association of the molecule with specific cargo proteins that provide nuclear transport [17]. In this context, NLS represents an additional strategy and can be incorporated into non-viral gene carriers, such as liposomes, focusing the improvement of plasmid DNA delivery inside the nucleus to overcome nuclear macromolecule exclusion. Different research groups have designed cationic synthetic peptides containing NLS, increasing *in vitro* and *in vivo* transfection rates [18]. The overall cationic nature of peptides allows DNA condensation. Byrnes and colleagues covalently coupled the M9 sequence to the cationic peptide (scrambled sequence of the cationic SV40 T antigen—KCRGKVPGKYGKG) [19]. The binary DNA/peptide complex was associated to lipofectamine (cationic lipid carrier) and *in vitro* transfection increased 20-fold. Recent studies demonstrated that lipofectamine (commercial cationic lipid vector) associated with DNA/peptide containing the minimal SV40 T-antigen NLS (EGPKKKRKVG) and a scrambled version of SV40 (EKRGKVKPKG) successfully transfected cells. Relative to lipid alone, peptide–lipoplexes enhanced *in vitro* transfection by up to 4.6-fold. The presence of the peptide in the lipoplex increased the internalization efficiency up to 4.5-fold, decreased the percentage of lysosomal DNA by 2.1-fold, and increased the efficiency of nuclear accumulation by 3.0-fold [20]. In an *in vivo* model, Schirmbeck and colleagues demonstrated that mice immunized with DNA encoding the hepatitis B surface antigen (HBsAg) coupled to NLS exhibited a 10–15-fold increase in HBsAg-specific antibody production compared with uncoupled vector [21]. Moreover, in *Leishmania major* challenged mice, immunization with linear DNA coupled with NLS offered greater protection than a prime-boost administration protocol [22].

We developed a DNA vaccine encoding the 65-kDa heat shock protein gene from *Mycobacterium leprae* (DNAhsp65), which exhibits prophylactic and therapeutic effects on mice with tuberculosis (TB) [23–26]. This vaccine elicits robust CD4 and CD8 memory T cells, with CD8 being the prominent cell population, resulting in cytotoxic activity and IFN (interferon)-γ cytokine production [27,28], fundamental elements for killing mycobacteria. Furthermore, DNAhsp65 immunotherapy suppressed TH2 cytokine levels (which oppose IFN-γ effects) and controlled the intensity of local inflammation [29,30]. In this sense, the fine-tuning of the host immune system induced by DNAhsp65 administration is the key for optimal results in combating the *Mycobacterium tuberculosis* pathogen.

Although these results were encouraging, the amount of plasmid DNA necessary for this goal was high. Thus, our research group developed a cationic liposome containing DNAhsp65 containing the lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and L-α-phosphatidylcholine (EPC) to sustain protection against TB and decrease the administered dose of DNA. The lipid composition was the same as originally developed by Perrie and colleagues [31]; therefore, the DNA content was increased in the lipid structures and then complexed with preformed dehydrated–hydrated vesicles (DRV). Preclinical results demonstrated the prophylactic potential of liposome genetic vaccine, with low cytotoxicity added to the advantage of being a single-dose intranasal vaccine with advantageous results for DNA complexed with DRV liposomes instead of conventional encapsulation [32,33]. Thus, in the present study, our aim was to design a non-viral gene carrier with multifunctional domains that combines a new synthetic peptide containing a NLS with DNAhsp65/cationic liposome (previously developed [32,33]) useful for tuberculosis treatment. The pseudo-ternary (NLS/DNA/cationic liposome) complex was characterized according to its physical–chemical properties, including average diameter, zeta potential, gel retardation assay, and accessibility to DNA fluorescent probe, and it was also evaluated for cytotoxicity *in vitro* and therapeutic effect *in vivo*.

2. Materials and methods

2.1. Materials

Plasmid pVAX1 encoding no gene (DNAmock) or pVAX-hsp65 encoding a 65 KDa heat shock protein from *M. leprae* (DNAhsp65) was designed for a tuberculosis gene vaccine and supplied by the Center for Tuberculosis Research, Medical School of São Paulo University at Ribeirão Preto [4]. EPC, DOPE, and DOTAP chloride salt were purchased from Avanti Lipids and used without further purification.

2.2. Peptide synthesis and purification

The KCRGKVPGKYGKGPKKKRKVC-amide, the NLS, was synthesized as described previously [34]. The crude peptide was submitted to O_2 oxidation in aqueous solution at neutral pH for 20 h. Peptide purification was carried out on a Waters 510 HPLC instrument using a Vydac C18 preparative column (22-mm internal diameter, 250-mm length, 70-Å pore size, 10-μm particle size). The peptide was eluted with a linear gradient using H_2O containing 0.1% TFA (solvent A) and 60% acetonitrile in H_2O containing 0.1% TFA (solvent B). A linear gradient (25–55% B) over 90 min with a flow rate of 10 mL/min and UV detection at 220 nm was used. The fractions were screened under isocratic conditions in a Chromolit C18 analytical column. Pure fractions of the S-S cyclized peptide were pooled, lyophilized, and characterized for homogeneity by analytical HPLC (Waters Associates, Milford, MA, USA), mass spectrometry on RP-HPLC/MS (Micromass, Manchester, UK), and amino acid analysis (Biochrom 20 Plus, Amersham Biosciences, Uppsala, Sweden).

The 21 amino acid residues of the synthesized peptide had the following specific domains and characteristics: (i) NLS from SV 40 T (simian virus), 7 residues (PKKKRKV) [35], (ii) cationic amino acid sequence derived from SV 40 T, shuttle sequence with 13 residues (KCRGKVPGKYGKG) [19,36] with the purpose of improving electrostatic interactions with DNA, (iii) a cysteamide group at the C-terminus [37,38], which was also acetylated at the N-terminus to increase transfection efficiency, and (iv) because this peptide contains two cysteine residues, we promoted cyclization to avoid any uncontrolled disulfide bridge formation during complexation with DNA.

2.3. Preparation of binary (NLS/DNA) and pseudo-ternary (NLS/DNA/cationic liposomes) complexes

The binary NLS/DNA complex was formed by adding the appropriate amount of peptide to DNA aqueous solution while vortexing for 40 s. The binary complex was incubated at 4 °C for 10 min before use. The peptide/DNA proportion was established in terms of molar charge ratio (+/−) between the peptide positive charges (NH_4^+) and DNA negative molar charges (PO_4^{3-} groups) ($R_{NLS+/DNA-}$). This balance was based on the physiological pH. In this special case, 1 mol of peptide (Mw = 3484.97) corresponds to 10 mols of positive charge and 1 μg of DNA corresponds to 3 nmols of negative charge [39].

The pseudo-ternary complex was obtained by adding the binary complex to the cationic liposomes while vortexing for 40 s. This complex was kept at room temperature for at least 30 min before physico-chemical and *in vivo* characterization. This formulation

was at physiological NaCl concentration (0.9%). In this study, the proportion of cationic liposome to DNA was kept the same as in our previous study (molar charge ratio between cationic lipid charge and DNA negative molar charge, $R_{LIP+/DNA-}$, of 10) [32]. The cationic liposomes were prepared as described previously [32,33]. Briefly, the lipid stock solutions in chloroform were added in a round flask, and the thin film was obtained under a vacuum. After hydration at 16 mM in water, the liposomes were extruded in two polycarbonate membranes at 12 kgf/cm^2 (15 times). The extruded liposomes were freeze-dried for 24 h and rehydrated at 64 mM (NaCl 1.44%) to obtain "empty" dehydrated–hydrated vesicles (DRVs). This NaCl concentration was selected for the final pseudo-ternary complex to achieve 0.9% NaCl. Fig. 1 presents a schematic diagram of pseudo-ternary complex formation.

2.4. Physico-chemical characterization

2.4.1. Average diameter and zeta potential for the binary (NLS/DNA) and pseudo-ternary (NLS/DNA/cationic liposome) complexes

The average hydrodynamic diameter and size distribution for the pseudo-ternary complex were determined by dynamic light scattering (Zetasizer 3000 – Malvern) using a Ne–He laser and measuring a scattering angle of 173°. The average diameter of the binary DNA/peptide complex was obtained by optical microscopy (Leica model DMLM) with image acquisition (Leica Qwin500 software). In this case, the average diameter and standard deviation were estimated after acquiring different images and measuring at least 300 particles. The zeta potential was measured using Zetasizer 3000—Malvern by diluting the binary or pseudo-ternary complex in an appropriate volume of saline solution (0.9% NaCl) at pH 6.4 and 25 °C.

2.4.2. Gel retardation assay for determining the molar charge ratio for complete DNA complexation with NLS ($R_{NLS+/DNA-}$)

Determination of the molar charge ratio for complete DNA complexation with NLS ($R_{NLS+/DNA-}$) was based on Eastman et al.'s method for DNA/liposome complex [40] adapted for peptide/DNA. Basically, two micrograms of DNA was complexed with the NLS at different molar charge ratios ($R_{NLS+/DNA-}$) and incubated at room temperature for 10 min. Six microliters of bromophenol blue (15% Ficoll 400, 0.1% cyanol xylene, 0.1% bromophenol blue) was added to the samples, which were electrophoresed in a 0.75% agarose gel containing 0.25 μg/ml ethidium bromide in 40 mM Tris–acetate buffer solution, 1 mM EDTA TAE 1× at 70 V for 40 min. The EtBr-stained DNA bands were visualized and photographed on an ultraviolet transilluminator.

2.4.3. Plasmid DNA accessibility

DNA accessibility to the fluorescence probe (Pico Green) in the binary and pseudo-ternary complex was determined at different molar NLS/DNA ratios ($R_{NLS+/DNA-}$). A working solution was prepared by diluting the Pico Green stock solution 200 times in TE buffer (10 mM Tris–HCl/1 mM EDTA, pH 7.5). For the NLS/DNA binary complex, 50 μl of DNAhsp65 solution (1 μg/ml) was complexed with 50 μl of NLS at different concentrations to form complexes at different $R_{NLS+/DNA-}$. For the NLS/DNA/cationic liposome pseudo-ternary complex, 33 μl of DNAhsp65 solution (1 μg/ml) was complexed with 33 μl of NLS at different concentrations to form complexes at different $R_{NLS+/DNA-}$. Binary complex was added to 34 μl of liposome dispersion at the same DNA/cationic liposome molar charge ratio ($R_{LIP+/DNA-}$ of 10, according to [32]). The binary or pseudo-ternary complex was incubated at room temperature for 10 min. One hundred microliters of the working solution was added to the complexed mixture and incubated for 2–4 min. The intensity of the fluorescence was then measured using a plate fluorimeter (Gemini XS, Molecular Device) using excitation and emission wavelengths of 485 and 525 nm, respectively, according to the manufacturer's specifications. The fluorescence intensity profiles were expressed as fluorescence intensity (absolute units) and plotted as a function of the NLS/DNA molar charge ratio ($R_{NLS+/DNA-}$). This methodology was adapted from Ferrari and colleagues [41].

2.4.4. Morphology

Transmission electron microscopy (TEM) and the negative staining method were used to visualize the morphology of the pseudo-ternary NLS/DNA/cationic liposome complex based on our previous work [32]. Briefly, carbon-coated 200 mesh copper grids with collodion film were used. The pseudo-ternary complex was diluted to 1 mM (based on the total lipid content) and then applied to the carbon grid. After incubation for 5 min at room temperature, the excess was blotted. One drop of uranyl acetate (1% w/w in saline solution) was added to the carbon grid and incubated for 1 min at room temperature before the excess was blotted and air-dried. A Carl Zeiss CEM 902 microscope equipped with a Castaing–Henry–Ottensmeyer energy filter was used and the images obtained using a CCD camera (Proscan).

2.5. In vitro cytotoxicity assay

Prior to biological evaluation, all formulations were tested for endotoxin levels with QCL-1000 Limulus amebocyte lysate. The detected levels were under 0.01 EU/ml, suitable for *in vitro* and *in vivo* assays. The standard 3-(4,5-diethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric cytotoxicity assay was used for *in vitro* cell cytotoxicity [42]. The J774-macrophage cell line was grown in RPMI medium at 10^6 cells/ml and added to 96-well cell culture plates at 10^5 cells/well. The cells were incubated for 24 h at 37 °C with 5% CO_2. Serial dilutions of the binary or pseudo-ternary complexes or naked DNA in RPMI medium were added as described

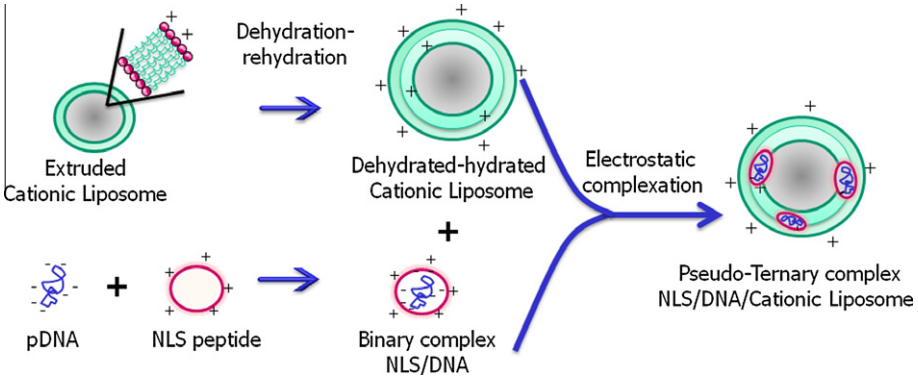

Fig. 1. Schematic representation of the pseudo-ternary complex.

below. Cells were incubated with vaccine preparations for 24 h, and then, 100 μl MTT reagent (5 mg/ml in RPMI) was added to each well. MTT was allowed to incubate with the cells for 4 h. The supernatant was aspirated, and 100 μl of isopropanol and 0.1 mol/l HCl were added to each well. After plate agitation, the solubilized compounds were read with a spectrophotometer set at 570 nm. Cell survival at the end of treatment was calculated as a ratio of the percentage of control cells (macrophages incubated with only media). All assays were performed in quadruplicate.

2.6. In vivo M. tuberculosis infection

2.6.1. Culturing and infection

The H37Rv strain of *M. tuberculosis* (American Type Culture Collection, Rockville, MD) was grown in 7H9 Middlebrook broth (Difco Laboratories, Detroit, MI) for 7 days. The culture was harvested by centrifugation and the cell pellet re-suspended in sterile phosphate buffered saline (PBS) and vigorously agitated. The homogeneous suspension was filtered through a 2-μm filter (Millipore, Bedford, MA). Because CFU determination takes 4–6 weeks, we used fluorescein diacetate (Sigma, Saint Louis, MO) and ethidium bromide staining [43] to rapidly assess the viability of *M. tuberculosis* cultures upon infection. An anterior midline incision was made to expose the trachea. A 30-gauge needle attached to a tuberculin syringe was inserted into the trachea and intratracheal dispersion used to introduce 10^5 viable CFU of *M. tuberculosis* H37Rv in 100 μl of PBS into the lungs [44]. Control mice received only intratracheal PBS.

2.6.2. Gene therapy

DNA treatment was initiated 30 days after TB induction by injecting 50 μg of plasmid DNA in 50 μl of saline into the quadriceps muscle of each hind leg on four occasions at 10-day intervals. Alternatively, separate groups of mice were treated with the binary or pseudo-ternary complex ($R_{NLS+/DNA-}$ and $R_{LIP+/DNA-}$ of 10 in the case of the pseudo-ternary complex) via the intranasal route at the same time point. Ten days after the last dose, mice were killed and bacterial growth assessed in the lungs after tissue homogenization.

2.6.3. Determination of M. tuberculosis colony forming units (CFU) in the lungs

Recovery of *M. tuberculosis* was performed as described previously [24]. Briefly, the number of live bacteria recovered from the lungs was determined as CFU by plating 10-fold serial dilutions of homogenized tissue on Middlebrook 7H11 agar (Difco) incubated at 37 °C. Colonies were counted after 28 days. Results are expressed as $\log_{10}$ of CFU/g lung.

3. Results

3.1. Binary NLS/DNA complexation

The DNA complex with the NLS peptide was evaluated by gel retardation assay. The peptide/DNA molar charge ratio ($R_{NLS+/DNA-}$) that completely retains the DNA in the binary complex was 0.5 (Fig. 2).

DNA accessibility to the fluorescence probe was determined at different molar charge ratios ($R_{NLS+/DNA-}$) for the binary NLS/DNA complexes (Fig. 3).

All complexations were carried out with the same amount of DNA, only changing the amount of peptide. Increasing the amount of peptide (NLS) decreased the fluorescence (Fig. 3), even after complete DNA incorporation into the complex at $R_{NLS+/DNA-}$ 0.5 (Fig. 2). However, two main regions, one with small decay ($R_{NLS+/DNA-}$ = 0.1–0.5) and a second with sharp decay ($R_{NLS+/DNA-}$ > 0.5)

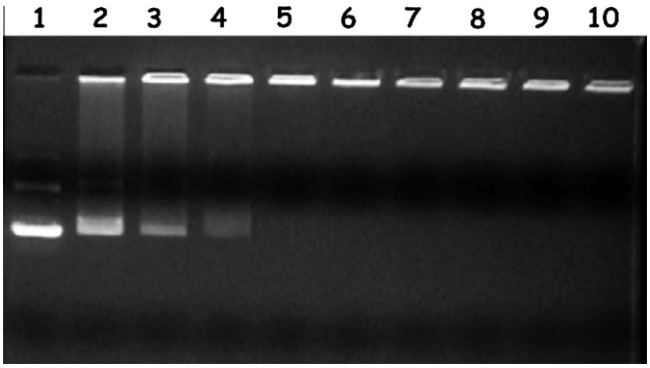

Fig. 2. Gel retardation assay for NLA/DNA complexation. Two micrograms of DNAhsp65 was complexed at different NLS/DNA molar charge ratios ($R_{PEP+/DNA-}$) in 0.9% saline solution for 10 min at room temperature. Lane 1, DNA (pVAX-hsp65); lanes 2–10, increasing NLS/DNA molar charge ratios ($R_{NLS+/DNA-}$): 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, respectively.

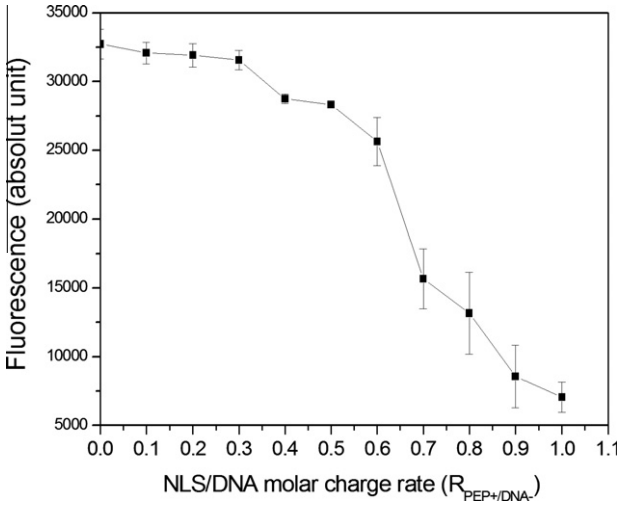

Fig. 3. Fluorescence profile as a function of peptide/DNA molar charge ratio ($R_{NLS+/DNA-}$) using the PicoGreen probe in ultra pure water. The complexation reactions were carried out at room temperature in water. Error bars correspond to SD of three independent experiments (n = 3).

are observed in Fig. 3. Interestingly, 0.5 is the NLS/DNA molar charge ratio shown to promote complete DNA incorporation into the peptide complex in the gel retardation assay. The sharp fluorescent decay profile observed for $R_{NLS+/DNA-}$ higher than 0.5 indicates that the increased peptide content decreases DNA intercalation by the fluorescent probe, suggesting increased DNA condensation.

3.2. Pseudo-ternary NLS/DNA/cationic liposome complex

In order to investigate the influence of liposome addition on the binary NLS/DNA complex, we performed similar fluorescent experiments as described in Fig. 3. In this case, the pseudo-ternary complexes were obtained at the same molar charge ratio for cationic lipid/DNA ($R_{LIP+/DNA-}$) of 10 [32,33] but varying the NLS_+/DNA_- molar charge ratio ($R_{NLS+/DNA-}$). In other words, we fixed the amount of DNA and cationic liposome and changed the amount of peptide. Fig. 4 presents the fluorescent profile, indicating the fluorescence decay with increasing NLS/DNA molar charge ratio. This behavior indicates a higher $R_{NLS+/DNA-}$ (increased peptide) correlates with decreased fluorescence as a consequence of lower probe accessibility to the DNA, even in the presence of cationic liposome. This behavior suggests that both the peptide and cationic liposomes protect DNA from fluorescent probe intercalation.

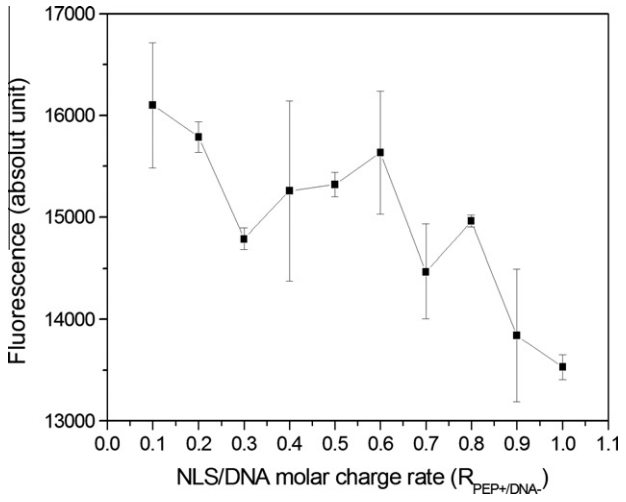

Fig. 4. Fluorescence profile as a function of the NLS(+)/DNA(−) molar charge ratio ($R_{NLS+/DNA-}$) using a PicoGreen probe for the pseudo-ternary NLS/DNA/cationic liposome complex. The complex reactions were carried out at the same DNA and cationic lipid concentration ($R_{LIP+/DNA-}$ = 10). Error bars correspond to SD of three independent experiments (n = 3).

We selected $R_{NLS+/DNA-}$ = 1 as the condition that promoted the highest level of fluorescent decay (Figs. 3 and 4) to study the formation of the pseudo-ternary complex (NLS/DNA/cationic liposome). The corresponding average diameters and zeta potentials are presented in Table 1. We used the binary complex (NLS/DNA) as a control.

Our protocol to obtain the pseudo-ternary complex was based on two steps: the first was related to the peptide/DNA complexation and the second involves the complexation reaction between the binary NLS/DNA complex with cationic liposomes. Interestingly, when we obtained the binary complex, we were able to identify the presence of large particles (heterogeneous distribution) that could be observed by optical microscopy. We estimated the average diameter as 31 μm after acquiring different images and measurements of various particles (Leica Qwin500 software). The zeta potential of the binary complex was 1.5 mV (Table 1).

Surprisingly, the larger particles disappeared after the addition of the binary complex to the cationic liposome. We observed that the pseudo-ternary complex presented as two populations, the most relevant being approximately 250 nm (95.23% in terms of the number of particles) with a zeta potential of 11.3 mV (Table 1).

Table 1
Physico-chemical properties of the pseudo-ternary complex containing the peptide with nuclear localization signal (NLS), DNAhsp65, and cationic liposomes.[a]

Complex type	Mean diameter nm ± SD[b] (%)[c]	Zeta potential mV ± SD[d]
NLS/DNA/cationic liposome (Pseudo-ternary)	248.9 ± 37.02 (95.23) 975.7 ± 195.9 (4.77)	11.3 ± 3.4
NLS/DNA (Binary) – control	31,000 ± 17,000 [e]	1.5 ± 8.25

[a] Liposome composition—EPC:DOTAP:DOPE 50:25:25 M. Pseudo-ternary complexes—NLS/DNA/cationic liposome were prepared at molar cationic lipid/DNA charge ratio ($R_{LIP+/DNA-}$) of 10 and peptide/DNA molar charge ratio ($R_{NLS+/DNA-}$) of 1.
[b] Mean ± standard deviation (SD). Diameter measurements were performed in three independent samples.
[c] Percentages refer to distribution of number-weighted particles.
[d] Mean ± standard deviation (SD). Zeta potential measurements were performed in three independent samples.
[e] Average diameter estimated by optical microscopy using Leica Qwin500 software after the acquisition of different images and measurement of at least 300 particles.

The morphology of the pseudo-ternary complex was evaluated by transmission electron microscopy as presented in Fig. 5.

The pseudo-ternary complex assumed spherical-like geometry (Fig. 5A). The results confirmed our previous evaluation of the mean diameter and size distribution. Multi-lamellarity was also identified in Fig. 5B–D. We found no evidence of larger particles as identified for the binary DNA/peptide complex, confirming the light scattering measurements (Table 1).

3.3. In vitro cytotoxicity evaluation

With the characterized formulations, the next step was to evaluate the *in vitro* cytotoxicity on the J774 cell line. Addition of the NLS to the liposomes did not induce cytotoxicity (Fig. 6). Interestingly, cell proliferation was augmented when the cells were incubated with liposome plus NLS compared with control (Fig. 6), which could be related to the adjuvant properties of liposomes [45]. Increasing the formulation concentration led to re-establishment of the cytotoxicity pattern similar to cells treated only with NLS. This behavior could be associated with an over-stimulation of cells, suppressing cell proliferation. Similarly, this phenomenon was observed in the J774 cell line over-stimulated with lipopolysaccharide (LPS), leading to extracellular signal-regulated kinase (ERK) hyperactivation and interleukin (IL)-12 suppression, culminating with cell proliferation decrease [46]. Because all formulations presented in this work were endotoxin free, the properties observed were related to NLS *per se*.

3.4. Therapeutic effect conferred by NLS addition to the DNAhsp65/cationic liposome vaccine against TB

Considering that the binary DNAhsp65/cationic liposome complex was effective against TB [47], we further evaluated its potential with NLS addition in a therapeutic protocol. Infected mice treated with control formulations carrying plasmid backbone without a gene insert (DNAmock) or NLS did not confer significant CFU reduction (Fig. 7, insert). Though the lack of a therapeutic effect was observed for DNAhsp65/cationic liposome treatment, the presence of NLS in this formulation circumvents the inefficiency given the comparable results to the DNAhsp65-treated group (Fig. 7).

4. Discussion

In previous studies, we characterized and evaluated the efficacy of immunization with DNAhsp65 carried by cationic liposomes (DNAhsp65/cationic liposome) in animals challenged with *M. tuberculosis*, reducing the administered dose with maintenance of protection [48]. In addition to DNAhsp65 delivery in the cell cytosol being optimized by liposomes, we focused on increasing the transfection ability of internalized DNA, aiming to improve the overall process. Therefore, we developed and used a small peptide sequence with properties that drive other molecules in the cytosol to the nucleus through classical transportation using α and β importins [49]. The cationic nature of the peptide used in this work allowed electrostatic complexation with DNA and further incorporation into cationic liposomes. Because the development of new approaches to treat TB is mandatory due to the appearance of multidrug resistant strains of *M. tuberculosis* and HIV/TB co-infected individuals [50], we aimed to evaluate the therapeutic efficacy of this new formulation.

The binary NLS/DNAhsp65 complex was initially analyzed by gel agarose electrophoresis (gel retardation assay). We observed that a peptide/DNA molar charge ratio ($R_{NLS+/DNA-}$) equal or higher than 0.5 promotes complete DNA incorporation into the peptide structure (Fig. 2). However, the gel retardation assay does not

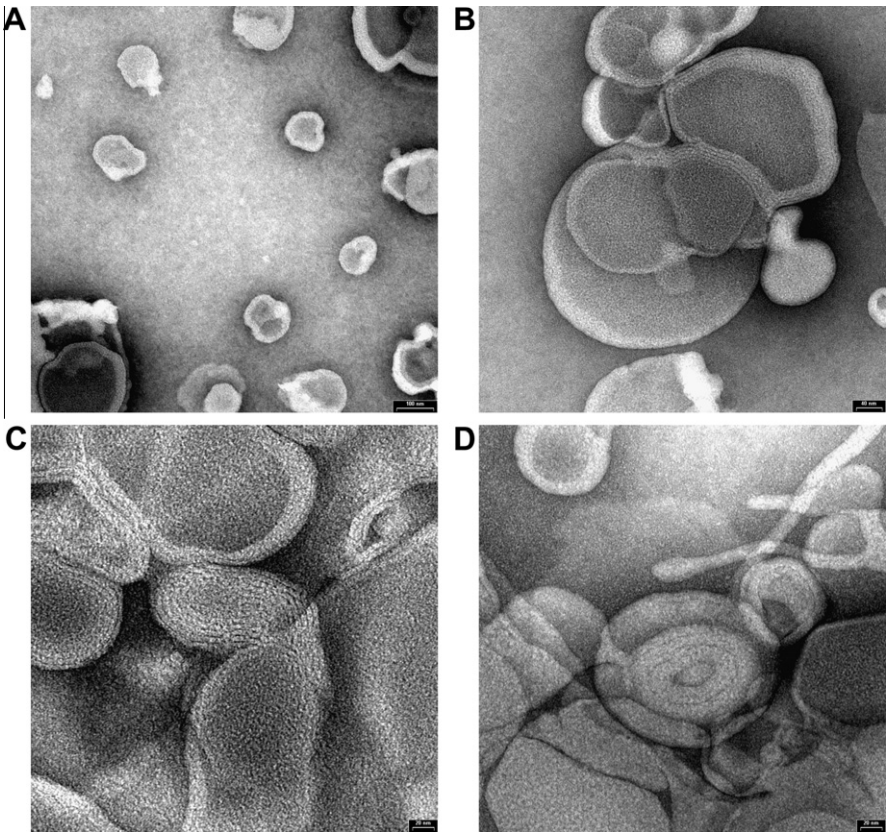

Fig. 5. Negative-staining electron micrographs of the pseudo-ternary complex (liposome/DNA/peptide). Liposomes are composed of EPC/DOPE/DOTAP (50:25:25% molar), the cationic lipid/DNA charge ratio ($R_{LIP+/DNA-}$) was 10, and the peptide/DNA molar charge ratio ($R_{PEP+/DNA-}$) was 1. Scale bars indicate: 100 nm in (A); 40 nm in (B); and 20 nm in (C and D).

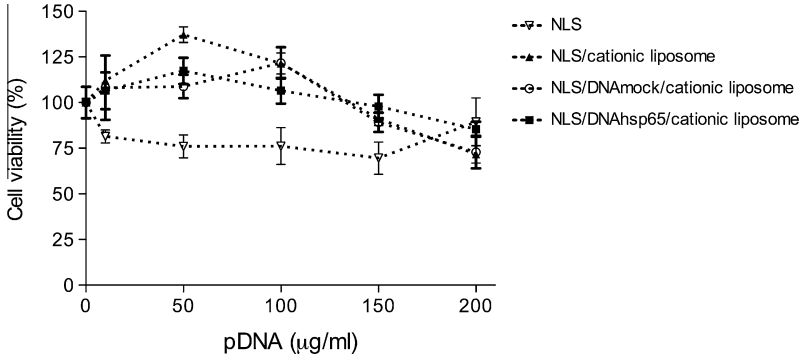

Fig. 6. *In vitro* evaluation of cationic liposome cytotoxicity on the J774 macrophage cell line. Confluent cell populations were incubated with NLS, NLS/cationic liposome, NLS/DNAmock/cationic liposome, or NLS/DNAhsp65/cationic liposome. Macrophages were cultured only in RPMI medium as a control. The formulations were added to the culture in concentrations ranging from 10–200 μg/ml of DNA, equivalent to 20–400 μl/ml of liposome, for 24 h. After treatment, the MTT reagent was added to the culture medium. After 4 h of incubation, the medium was removed and 100 μl of isopropanol containing 0.1 mol/l HCl was added to the wells to dissolve the formazan crystals. Values are the mean ± SD percentage of viable cells compared to the control. The data represent three separate experiments performed in quadruplicate.

supply enough information. The fluorescent probe accessibility assay (specific intercalating dsDNA, Fig. 3) presents a sharp reduction of the fluorescent profile for $R_{NLS+/DNA-}$ higher than 0.5. This behavior suggests that higher $R_{NLS+/DNA-}$ correlates with decreased intercalation, probably due to better DNA condensation by the peptide.

Considering the decreased fluorescence (Fig. 3) and previous reports in the literature that mention the beneficial use of peptide in excess [8,17,36], we selected the $R_{NLS+/DNA-}$ of 1 to continue the evaluation of the average diameter and incorporation into cationic liposomes.

Electrostatic complexation between the peptide and DNA at a $R_{NLS+/DNA-}$ of 1 generated large particles with an average diameter of 31 μm (Table 1). Population heterogeneity was reflected by the large standard deviation (17 μm). Even the temperature control at approximately 4 °C (to decrease the velocity of the electrostatic complexation) and intense vortex was not enough to control the formation of large particles. The zeta potential near neutrality (Table 1) allowed uncontrolled complexation [51].

Despite the large size and near neutral zeta potential, we continued the studies to obtain the pseudo-ternary complex. Our hypothesis was that the differences in zeta potential between the peptide/DNA complex (1.5 mV) and "empty" cationic liposome (26.9 mV) [32] could induce anchorage of the binary complex in the cationic liposome, generating the pseudo-ternary complex.

This hypothesis was supported by previous studies reporting the formation of large particles between plasmid DNA and synthetic peptides (oligopeptides with nine amino acids) (13 μm) and subsequent mixing with cationic liposomes (DMB-Chol and DOPE/120 nm), generating uniform nanoparticles [52]. Unfortunately, these authors did not investigate the zeta potential of the peptide/DNA complex. McKenzie and colleagues also complexed peptides (at different molecular weights and containing different numbers of cysteines) with DNA [37]. In this case, the formation of sulfide bridges can be controlled by adjusting the pH during DNA complexation. The authors demonstrated that different peptides can generate particles that range from 50 to 1600 nm, depending on the peptide type. The mixture of our binary complex (NLS/DNAhsp65) with the cationic liposomes generated nanoparticles of a controlled size and mean diameter of approximately 250 nm (95% of the population in terms of number distribution). The large particles disappeared, suggesting the dissociation of these peptide/DNA micro-structures.

In order to verify whether the peptide was incorporated with the DNA into the liposomes, we evaluated DNA accessibility by a fluorescent probe (Fig. 4). We obtained different peptide/DNA complexes (increasing the peptide amount, $R_{NLS+/DNA-}$ ranging from 0.1 to 1) and mixed them with cationic liposomes. In all cases, we kept the proportion of cationic lipid to DNA constant. We observed a smooth decay in the fluorescent profile, confirming the peptide influence on DNA condensation. The smooth decay was due to excess cationic liposome. We used 10 times more cationic molar charge from the lipids than the cationic molar charge from the peptide (formulation containing $R_{lipid+/DNA-} = 10$ and $R_{NLS+/DNA-} = 1$).

The pseudo-ternary complex at $R_{NLS+/DNA-} = 1$ presented with controlled size, spherical-like shape, and the presence of lamellar liposomes. Indeed, this pseudo-ternary complex presented a zeta potential of 11.3 mV. This value is lower than that of the corresponding cationic liposomes/DNAhsp65 vaccine (27.3 mV ± 2.3) [32], confirming the influence of NLS on the final structure. The pseudo-ternary complex is still cationic, which is fundamental for DNA transfection. This formulation was stable in terms of mean diameter for 4 months (sterile and at 8 °C; data not shown).

The behavior related to the homogeneous formation of the pseudo-ternary complex confirms our hypothesis that the difference in zeta potential between the binary complex and cationic liposomes is the driving force for the formation of the homogeneous pseudo-ternary complex. The peptide nature can also be an important parameter controlling the binary complex (DNA condensation). In our case, the peptide was cyclized (sulfate bridges), which probably contributes to the formation of large NLS/DNA particles. Additional studies will be conducted by our group to investigate the complexation of binary complexes (NLS/DNA) at a higher zeta potential with cationic liposomes.

Because many formulations with cationic charges often exhibit cell toxicity, which is not desirable for *in vivo* purposes, we evaluated it *in vitro* on a macrophage-derived cell line. The addition of NLS to these formulations did not confer cytotoxicity, showing similar results as previously designed formulations [32]. Some components of this complex could maintain the low cytotoxicity, as the addition of EPC was previously shown to contribute to cytotoxicity [33].

We moved forward to evaluate the therapeutic efficacy of these formulations in mice with TB disease. Surprisingly, the DNAhsp65/cationic liposome treatment was not effective for killing the mycobacteria (Fig. 7). On the other hand, the presence of NLS on this formulation completely changes this scenario, reaching comparable results in mice treated with naked DNA. Gene therapy with naked DNAhsp65 consumed 400 μg of plasmid (administration of 100 μg on four occasions), whereas the pseudo-ternary complex consumed 4-fold less DNAhsp65. Considering that several studies have shown that the expression of some antigens could be augmented up to 63 times in the presence of NLS [53], one hypothesis appeared feasible to us. Some cell populations (mainly non-dividing cells) could be refractory to plasmid DNA transfection, which could be circumvented with NLS addition, leading to the priming of cells that were not producing or presenting hsp65 to other immune cell populations. Curiously, NLS coupling to gene vaccine seems to confer a striking improvement, mainly on the treatment of TB, because very preliminary results indicate that its presence did not boost the immune response in a prophylactic model (data not shown). Further studies will focus on elucidating the mechanism underlying NLS participation in different immunization schemes.

Taken together, these results suggest the potentiality of the NLS/DNAhsp65/cationic liposome pseudo-ternary complex on improvements in gene therapy against a relevant infectious disease. The optimization of DNA delivery to the cell nucleus through NLS activity could boost gene transcription, leading to an effective immune response against a related pathogen.

5. Conclusion

In this work, we demonstrated the use of a new synthetic peptide containing a NLS (based on the classical Simian Virus SV 40T sequence) and its incorporation into the cationic liposome/DNAhsp65 gene vaccine. This pseudo-ternary complex presented therapeutic effects against TB, even greater than the cationic liposome/DNAhsp65 gene vaccine and similar to naked DNA, effectively

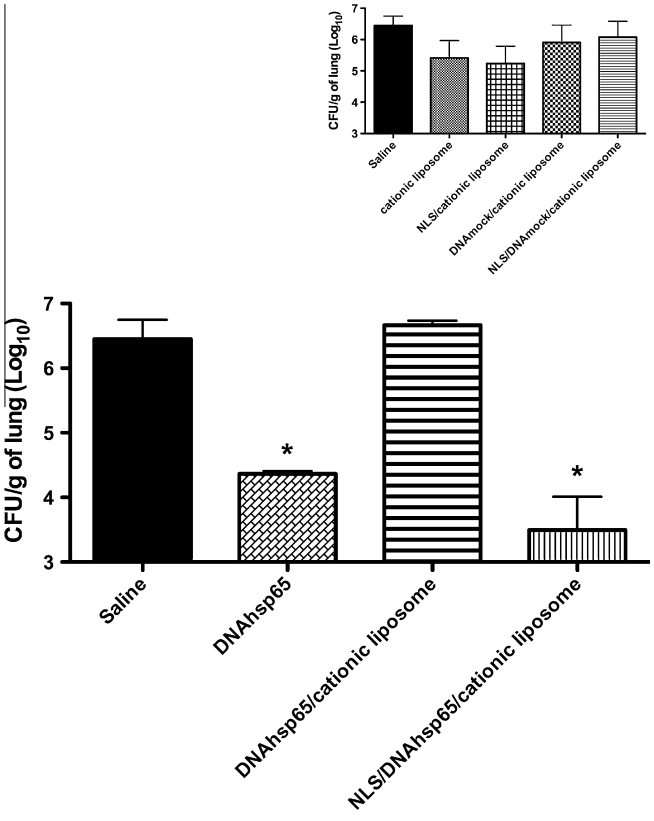

Fig. 7. Determination of *M. tuberculosis* growth in the lungs of infected mice treated with different formulations. BALB/c mice were intratracheally infected with 10^5 Mtb bacilli. Thirty days later, treatment was started, using four doses of naked DNAhsp65 (totaling 400 μg of DNA) or different liposome formulations (totaling 100 μg of DNA carried by 200 μl of liposome) in 10-day intervals. All control groups are shown in the inset. Ten days after the last dose, mice were euthanized and CFU analysis performed. Data represent the mean $\log_{10}$ CFU counts ± SD of six mice per group for one of three independent experiments. $^*P < 0.05$ was considered significant when compared with the saline group.

reducing the total amount of DNAhsp65 administered and via a painless route. The pseudo-ternary NLS/DNAhsp65/cationic liposome is a promising gene vaccine for TB treatment. In addition, we were able to produce multifunctional nanoparticles with different domains (DNA delivery inside cell and nuclear transport), contributing to the development of nanoparticles for *in vivo* applications and gene delivery.

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