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Assessment of the adjuvant activity of mesoporous silica nanoparticles in recombinant *Mycoplasma hyopneumoniae* antigen vaccines

Veridiana Gomes Virginio [a,b], **Natalia Costantin Bandeira** [a],
Fernanda Munhoz dos Anjos Leal [a,b], **Marcelo Lancellotti** [d,e], **Arnaldo Zaha** [a,b,c],
Henrique Bunselmeyer Ferreira [a,b,c,*]

[a] *Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil*

[b] *Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil*

[c] *Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociências, UFRGS, Porto Alegre, RS, Brazil*

[d] *Laboratório de Biotecnologia, Instituto de Biologia, Departamento de Bioquímica, UNICAMP, Campinas, SP, Brazil*

[e] *Faculdade de Ciências Farmacêuticas, UNICAMP, Campinas, SP, Brazil*

* Corresponding author at: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, 91501-970 Porto Alegre, RS, Brazil.

E-mail address: henrique@cbiot.ufrgs.br (H.B. Ferreira).

Abstract

The adjuvant potential of two mesoporous silica nanoparticles (MSNs), SBa-15 and SBa-16, was assessed in combination with a recombinant HSP70 surface polypeptide domain from *Mycoplasma hyopneumoniae*, the etiological agent of porcine enzootic pneumonia (PEP). The recombinant antigen ($HSP70_{212-600}$), previously shown as immunogenic in formulation with classic adjuvants, was used to immunize BALB/c mice in combination with SBa-15 or SBa-16 MSNs, and the effects obtained with these formulations were compared to those obtained with alum, the adjuvant traditionally used in anti-PEP bacterins. The $HSP70_{212-600}$ + SBa-15 vaccine elicited a strong humoral immune response, with high serum total IgG levels, comparable to those obtained using $HSP70_{212-600}$ + alum. The

HSP70$_{212-600}$ + SBa-16 vaccine elicited a moderate humoral immune response, with lower levels of total IgG. The cellular immune response was assessed by the detection of IFN-γ, IL-4 and IL-10 in splenocyte culture supernatants. The HSP70$_{212-600}$ + SBa-15 vaccine increased IFN-γ, IL-4 and IL-10 levels, while no stimulation was detected with the HSP70$_{212-600}$ + SBa-16 vaccine. The HSP70$_{212-600}$ + SBa-15 vaccine induced a mixed Th1/Th2-type response, with an additional IL-10 mediated anti-inflammatory effect, both of relevance for an anti-PEP vaccine. Alum adjuvant controls stimulated an unspecific cellular immune response, with similar levels of cytokines detected in mice immunized either with HSP70$_{212-600}$ + alum or with the adjuvant alone. The better humoral and cellular immune responses elicited in mice indicated that SBa-15 has adjuvant potential, and can be considered as an alternative to the use of alum in veterinary vaccines. The use of SBa-15 with HSP70$_{212-600}$ is also promising as a potential anti-PEP subunit vaccine formulation.

Keywords: Microbiology, Biotechnology, Immunology

1. Introduction

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (PEP), a respiratory disease that causes significant economic losses to the pig industry [1]. Disease spread is partially controlled and symptoms are minimized by the use of commercially available vaccines, which consist of inactivated whole cells (mostly bacterins). Anti-PEP bacterins, however, have been unable to prevent transmission or the establishment of the bacteria in the swine lungs [2, 3].

Experimental formulations using *M. hyopneumoniae* recombinant antigens potentially protective for pigs have emerged as attractive alternatives for anti-PEP vaccine development PEP [4, 5, 6], and a subunit vaccine based on a recombinant *M. hyopneumoniae* P102 adhesin has already been patented (http://www.google.com/patents/WO1999026664A1?cl=en). The efficacies of anti-PEP subunit vaccine formulations, however, are so far restricted, and limitations in the levels of induced immune response and conferred protection may be, at least in part, due to the type of used adjuvant. These components are critical in the vaccine formulations for the augmentation of the proper immunogenicity of the biological antigen [7]. The repertoire of safe and effective adjuvants for commercial use is still very limited and needs urgent expansion, in order to provide options for the development of more efficient vaccine formulations [8]. Adjuvants like Freund's adjuvant, ISCOM-matrix and B subunit of *Escherichia coli* heat-labile enterotoxin LTB have been used in experimental protocols of vaccination against PEP in animal models (see, for example, [9, 10, 11]), but their use in veterinary vaccines is not allowed, due to toxicity and undesirable side effects.

The anti-PEP bacterins commercially available are formulated with aluminium salts (alum) and/or oil-based emulsion (Serkel Pneumo – Vencofarma, and Respisure® 1 One – Pfizer, respectively), the adjuvants most commonly used in veterinary and human vaccines [12]. However, these adjuvants, especially alum, although licensed, can induce swelling, skin nodules and allergic reactions in the injection sites [13, 14].

Nanotechnology has emerged as a promising alternative for the development of novel adjuvants for formulation of improved vaccines [15]. Mesoporous silica nanoparticles (MSNs) are attractive adjuvant candidates, due to their good biocompatibility, low cytotoxicity and excellent ability for surface functionalization and molecule anchoring [16, 17]. The SBa-15 and SBa-16 MSNs have been proposed as candidate vaccine adjuvants, based on preliminary results obtained using them in combination with bovine serum albumin, and a few bacterial or viral antigens [18, 19, 20, 21]. Further studies, however, are still needed in order to better evaluate their abilities to carry and deliver different recombinant polypeptides to vaccinated animals.

In the search for antigens suitable for use in anti-PEP subunit vaccines, the *M. hyopneumoniae* heat shock protein of 70 kDa (HSP70) has been shown to be antigenic and recognized by sera from *M. hyopneumoniae*-infected swine [22]. Moreover, a recombinant version of an extracellular antigenic domain of HSP70 combined with Freund's adjuvant demonstrated promising results in eliciting both humoral and cellular specific responses [9]. Here the vaccinal potential of this recombinant HSP70 domain is being assessed in combination with the SBa-15 and SBa-16 MSNs as adjuvants, to develop an efficient anti-PEP vaccine formulation, suitable for use in pigs. Our results demonstrated that SBa-15 MSN was a better adjuvant than SBa-16 and alum, being extremely effective to elicit both a strong humoral immune response and a mixed Th1/Th2-type cellular immune response. Moreover, SBa-15, and also SBa-16, did not induce local, undesirable side effects in the animals upon immunization. The potential of the HSP70/SBa-15 formulation as an anti-PEP vaccine for pigs is discussed.

2. Materials and methods

2.1. Expression and purification of the antigenic *M. hyopneumoniae* HSP70 recombinant polypeptide

An extracellular recombinant polypeptide comprising the C-terminal amino acid residues 212-600 of the *M. hyopneumoniae* HSP70 protein (MHP7448_0067) [23] was used as antigen. Its coding DNA sequence (CDS) was previously cloned and expressed in *Escherichia coli* essentially as described by Virginio et al. [9]. The purified HSP70$_{212-600}$ was quantified by spectrophotometry in a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.2. Immunization of mice with HSP70$_{212-600}$ combined with SBa-15 or SBa-16 mesoporous silica nanoparticles

Twelve-week-old female BALB/c mice (5 mice/group) were immunized intraperitoneally with three doses (at days 0, 15 and 30) of 25 μg of the HSP70$_{212-600}$ purified recombinant polypeptide combined with each MSN individually (SBa-15 or SBa-16) at 250 μg of final concentration [20], diluted in PBS (200 μL/dose). In the immunization positive control group, mice were immunized with 100 μL doses of the HSP70$_{212-600}$ polypeptide combined with the Alhydrogel 2% VacciGrade (Invivogen) alum adjuvant in a 1:1 ratio, according the manufacturer's instructions. Mice immunized only with each of the MSNs or with Alhydrogel 2% VacciGrade in the same conditions described above were used as immunization negative control groups. Blood samples were collected from the facial vein [24] at 0 day (preimmune negative control) and 15, 30 and 45 days after the first immunization (DAI). Euthanasia was carried out 60 DAI, prior to spleen removal. All procedures involving animals were previously approved by the Ethics Committee on Animal Use of the Universidade Federal do Rio Grande do Sul (protocol CEUA/UFRGS no. 28847/2016), and were performed according to international standards for the welfare of laboratory animals.

2.3. Assessment of the humoral immune response

Serum total IgG responses were determined by indirect ELISA, as described by Virginio et al. [25] with modifications in the dilution of sera and secondary antibodies. Serum samples from mice immunized with the HSP70$_{212-600}$ polypeptide and different adjuvants were diluted in Blotto 5% at a 1:50,000 ratio for HSP70$_{212-600}$ + SBa-15 and HSP70$_{212-600}$ + Alhydrogel 2% VacciGrade, and at a 1:1000 ratio for HSP70$_{212-600}$ + SBa-16. Goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma) was used as a secondary antibody, diluted in Blotto 5% at a 1:4000 ratio for HSP70$_{212-600}$ + SBa-15 and HSP70$_{212-600}$ + Alhydrogel 2% VacciGrade, and at a 1:1000 ratio for HSP70$_{212-600}$ + SBa-16.

2.4. Assessment of the cellular immune response

Cellular immune responses were assessed as described by Bargieri et al. [26] with some modifications as follows. Spleens of mice immunized with the HSP70$_{212-600}$ recombinant polypeptide combined with SBa-15 or SBa-16 MSNs were removed aseptically at 60 DAI. Splenocytes were suspended in RPMI 1640 medium (Thermo Scientific) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U penicillin-streptomycin/mL and 50 μM 2-mercaptoethanol, and then plated in duplicates onto 24-well plates at a concentration of 5×10^6 cells/well. Cell suspensions were incubated at 37 °C in 5% CO_2 for 72 h with the HSP70$_{212-600}$ purified recombinant polypeptide (10 μg/mL). Concanavalin A (Sigma), in the

same concentration of the $HSP70_{212-600}$ polypeptide (10 μg/mL), was used as a positive control of T cell stimulation. The detection of IFN-γ, IL-4 and IL-10 cytokines in splenocyte culture supernatants was performed using commercial mouse cytokine ELISA kits (BD Biosciences) according the manufacturer's instructions.

2.5. Statistical analysis

Levels and concentrations of antibodies and cytokines between different groups were compared using One-way ANOVA (GraphPad Prism 6). The Bartlett's test was used for the analysis of homogeneity of variances and the Sidak's post-test was used for multiple comparisons. Differences were considered to be significant if P-values ≤ 0.05.

3. Results

3.1. Expression and purification of the $HSP70_{212-600}$ recombinant antigen

The $HSP70_{212-600}$ recombinant antigen was successfully expressed in BL21 Star and purified with a yield of 10 mg/L of culture. Purity and integrity of the recombinant antigen were confirmed by SDS-PAGE 12%, in which a single band with the expected molecular mass of approximately 50 kDa was observed (data not shown).

3.2. Humoral immune response elicited by the $HSP70_{212-600}$ recombinant antigen combined with SBa-15 or SBa-16 silica nanoparticles
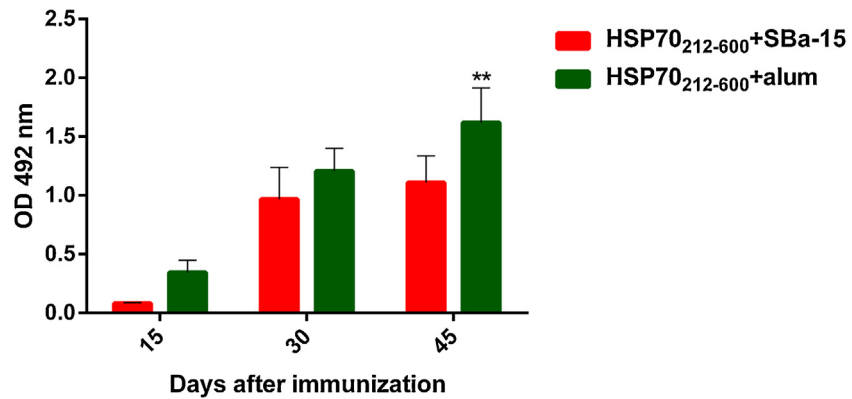
Mice immunized with the $HSP70_{212-600}$ recombinant antigen elicited humoral immune responses using either SBa-15 or SBa-16 MSNs as adjuvants (Fig. 1). At 30 and 45 DAI, animal immunized with the $HSP70_{212-600}$ + SBa-15 vaccine produced significantly higher levels of serum total IgG against $HSP70_{212-600}$, in comparison to preimmune sera and to sera from mice immunized with SBa-15 alone ($P \leq 0.05$ and $P \leq 0.01$, respectively) (Fig. 1A).

Mice immunized with the $HSP70_{212-600}$ + SBa-16 vaccine also presented significantly higher levels of serum total IgG at 15 and 45 DAI in comparison to both preimmune sera and sera from mice immunized only with SBa-16 ($P \leq 0.05$ for both) (Fig. 1B). At 30 DAI, however, averaged differences between $HSP70_{212-600}$ + SBa-16 vaccinated animals and negative controls were noticeable but not statistically significant ($P = 0.24$, for preimmune sera, and $P = 0.22$, for mice immunized only with SBa-16), due to the higher standard deviation (SD).
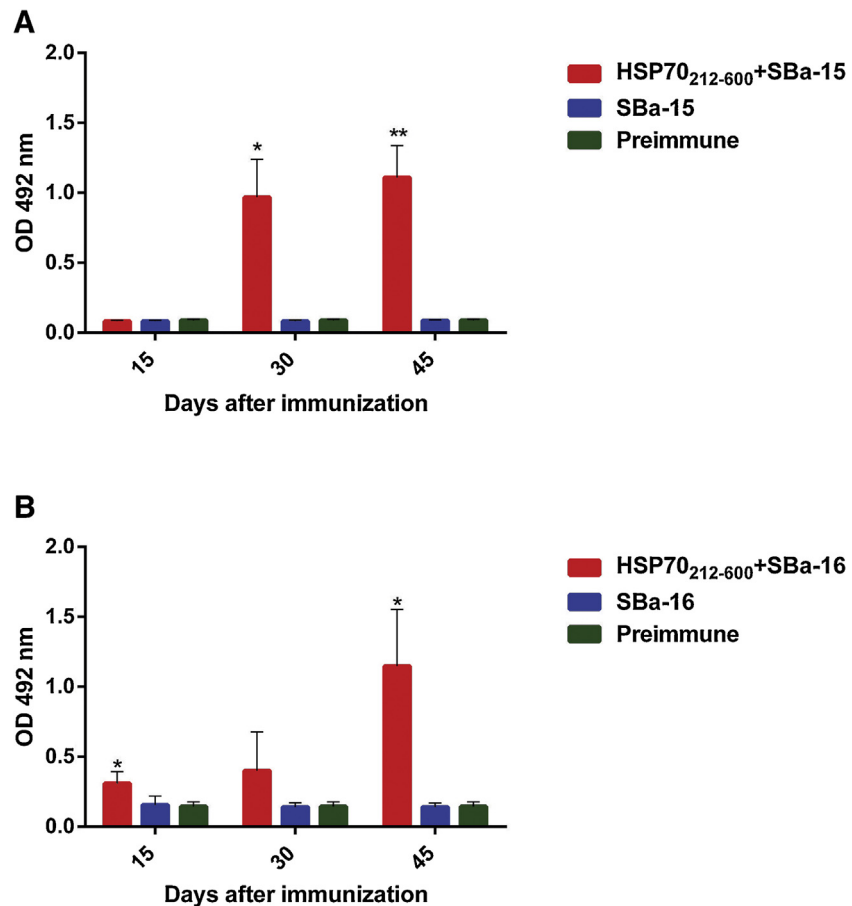
Fig. 1. Serum total IgG responses of mice immunized with the $HSP70_{212-600}$ recombinant antigen in combination with SBa-15 or SBa-16 MSNs as determined by indirect ELISA. Mice were immunized with 25 μg/dose of $HSP70_{212-600}$ + 250 μg/dose of SBa-15 (A) or SBa-16 (B). Microtiter plates were coated with 0.4 μg/well of $HSP70_{212-600}$ recombinant antigen. Sera were collected at 15, 30 and 45 DAI, and diluted 1:50,000 for $HSP70_{212-600}$ + SBa-15 and 1:1000 for $HSP70_{212-600}$ + SBa-16. Presented data are the mean optical densities at 492 nm (OD_{492}) ± SD from five mice in each group. The asterisks indicate significant differences of antibody responses (*$P \leq 0.05$, **$P \leq 0.01$) in comparison to negative controls (preimmune sera or sera from mice immunized with the SBa-15 adjuvant alone).

At 15 and 30 DAI, the $HSP70_{212-600}$ + SBa-15 vaccine elicited similar, not statistically significant different ($P = 0.06$ and $P = 0.063$, respectively) levels of serum total IgG in comparison to those obtained with the $HSP70_{212-600}$ + Alhydrogel 2% VacciGrade alum formulation (Fig. 2). At 45 DAI, however the formulation with alum elicited significantly higher levels of serum total IgG ($P \leq 0.01$) than the $HSP70_{212-600}$ + SBa-15 vaccine. Therefore, the $HSP70_{212-600}$ + SBa-15 vaccine successfully induced a humoral response in immunized mice, with similar results to those obtained upon immunization with the same recombinant antigen in a formulation with the alum adjuvant. The $HSP70_{212-600}$ + SBa-16 vaccine, however, was much less effective, as could be observed in Fig. 1. Detection of specific antibodies against $HSP70_{212-600}$ upon immunization with

Fig. 2. Total IgG responses in the sera of mice immunized with the $HSP70_{212-600}$ recombinant antigen in combination with SBa-15 MSNs or Alhydrogel 2% VacciGrade alum as determined by indirect ELISA. Mice were immunized with 25 μg/dose of $HSP70_{212-600}$ + 250 μg/dose of SBa-15 or 100 μL/dose (1:1 v/v) of Alhydrogel 2% VacciGrade alum. Microtiter plates were coated with 0.4 μg/well of $HSP70_{212-600}$ recombinant antigen. Sera were collected at 15, 30 and 45 DAI, and diluted 1:50,000. Presented data are the mean optical densities at 492 nm (OD_{492}) ± SD from five mice in each group. The asterisks indicate significant differences of antibody responses (**$P \leq 0.01$) between the groups of mice immunized with SBa-15 or alum as adjuvant.

SBa-16 were detectable only using serum and secondary antibody dilutions much lower than those used in the assays with sera from animals immunized with SBa-15 or alum formulations (1:1000 for both primary and secondary antibodies in the case of $HSP70_{212-600}$ + SBa-16 versus 1:50,000 for serum and 1:4000 for secondary antibody in the cases of $HSP70_{212-600}$ + SBa-15 and $HSP70_{212-600}$ + alum).

3.3. Cellular immune response elicited by $HSP70_{212-600}$ recombinant antigen combined with SBa-15 or SBa-16 silica nanoparticles

In assays performed with splenocyte cultures, the $HSP70_{212-600}$ + SBa-15 vaccine elicited significantly higher levels of the IFN-γ (92.4 pg/mL), IL-10 (523.9 pg/mL) and IL-4 (85.5 pg/mL) cytokines in the culture supernatants than those observed in the control culture supernatants of splenocytes of mice immunized with $HSP70_{212-600}$ + SBa-15 without *in vitro* stimulation with recombinant antigen, and of splenocytes of mice immunized with SBa-15 alone but with *in vitro* stimulation with $HSP70_{212-600}$ ($P \leq 0.01$ for all comparisons) (Table 1). For both negative control groups cytokine production was below the detection threshold of the assay.

The $HSP70_{212-600}$ + SBa-16 vaccine, on the other hand, failed to elicit detectable levels of IFN-γ, IL-10 and IL-4 cytokines (data not shown), while the $HSP70_{212-600}$ + Alhydrogel 2% VacciGrade control vaccine elicited an unspecific cellular immune response. This unspecific cellular response was characterized by detection of

Table 1. Cytokine production by splenocytes from BALB/c mice immunized with the $HSP70_{212-600}$ combined with the SBa-15 MSNs.

Groups	Cytokine production in pg/mL (mean $\pm$ SD)		
	IFN-γ	IL-4	IL-10
$HSP70_{212-600}$ + SBa-15	92.4 ± 46.1[**]	85.5 ± 63.6[**]	523.9 ± 359.1[**]
$HSP70_{212-600}$ + SBa-15 without *in vitro* stimulation control	nd	nd	nd
SBa-15 control	nd	nd	nd

The data represent the mean $\pm$ SD (n = 5).

nd (not detected) indicates cytokine production below the detection limit for IFN-γ (3.1 pg/mL), IL-4 (7.8 pg/mL) or IL-10 (31.3 pg/mL) in the assay.

[**] The asterisks indicate significant differences in the cytokines production ($P \leq 0.01$), compared to the negative controls. Negative controls were samples from mice immunized with $HSP70_{212-600}$ + SBa-15 but without *in vitro* stimulus of the spleen cells with the $HSP70_{212-600}$ antigen (no *in vitro* stimulation control), and from mice immunized with SBa-15 adjuvant only but with *in vitro* stimulus of the spleen cells with the $HSP70_{212-600}$ antigen (SBa-15 control).

similar levels of cytokines in mice immunized with the $HSP70_{212-600}$ recombinant antigen combined with alum and mice immunized only with alum (data not shown).

4. Discussion

Genetically engineered vaccines based on a single recombinant antigen and/or domain are often poorly immunogenic, and, therefore, it is necessary to increase their immunogenicity with the use of appropriate adjuvants [27]. Moreover, different adjuvants need to be tested for any given recombinant antigen in order to find the best antigen-adjuvant combination for vaccinal use.

The screening of novel adjuvants for use in vaccination of large animals, as pigs, may be based on the results of experiments in mice [11]. The mice model allows obtain valid immunological insights on candidate vaccine formulations at a relatively low cost. However, further research will be necessary to assess the immune response and efficacy of the selected antigen-adjuvant formulation in the target species (pig). In assays with swine it will be possible to correlate the immune response induced upon immunization with candidate vaccine formulations with protection against *M. hyopneumoniae* infection.

Nanoparticles, including MSNs, can function as adjuvants, as they enhance antigen processing, by functioning as a delivery system, and/or activate or enhance immunity, acting as immunostimulants [20, 28]. Since MSNs are biologically compatible, exhibit low cytotoxicity, and have a relatively low cost of production, they are ideal candidates for the development of a new generation of adjuvants [16, 17]. In addition, MSNs are tolerated in the mammalian system at relatively high concentrations [29].

SBa-15 MSNs have shown good potential as adjuvant in combination with some recombinant antigens [21, 30]. However, their potential to induce a cellular immune response has never been previously assessed in a splenocyte culture supernatant system for evaluation of their effect on cytokine production. Here, we assessed the SBa-15 and SBa-16 MSNs potential as adjuvants in combination with an extracellular $HSP70_{212-600}$ recombinant antigen, previously characterized as a potential vaccinal antigen for PEP [9]. Both humoral (total IgG production) and cellular (cytokine production) responses were assessed.

SBa-15 MSNs demonstrated better adjuvant potential in the vaccinated mice, eliciting higher levels of total IgG and a specific cellular immune response, with high IFN-γ, IL-10 and IL-4 production, upon immunization with the $HSP70_{212-600}$ antigen. In contrast, SBa-16, also in combination with the $HSP70_{212-600}$ antigen, elicited lower levels of total IgG and failed to induce cytokine production.

The formulation of the $HSP70_{212-600}$ antigen with alum, traditionally used as adjuvant in veterinary vaccines, was efficient as the $HSP70_{212-600}$ + SBa-15 vaccine regarding its ability to induce a total IgG response, but induced a totally unspecific cellular immune response. Alum based adjuvants are not effective for all antigens and have been reported to have poor or inefficient induction of cell-mediated immunity [15, 31, 32]. Considering that cellular immunity is regarded as important in conferring protection against *M. hyopneumoniae* [33, 34], recombinant formulations with SBa-15 as adjuvant, potentially more efficient to induce specific cytokine production, may be a good alternative to the use of alum as in some anti-PEP vaccines.

The $HSP70_{212-600}$ + SBa-15 vaccine successfully induced the production of IFN-γ, IL-4 and IL-10 cytokines, corroborating the results obtained with the $HSP70_{212-600}$ recombinant antigen in formulation with Freund's adjuvant [9]. The elicited production and secretion of IFN-γ and IL-4 cytokines characterizes a mixed Th1/Th2-type cellular immune response, as IFN-γ induces strongly opsonizing IgG2a responses [35, 36], enhancing phagocytosis by alveolar macrophages (a Th2 response), and IL-4 activates B cells and induces class switching to the IgG1 isotype (a Th1 response) [35, 36].

The mixed Th1/Th2-type of cellular immune response elicited by the $HSP70_{212-600}$ + SBa-15 vaccine is adequate for an anti-PEP vaccine, as, according to Lee et al. [37], an ideal anti-PEP vaccine should enhance Th1 responses while concomitantly maintaining Th2 responses. A Th1 antibody response is involved in protection, while a Th2 response is helpful for clearing the infection. Moreover, the elicited IL-10 production may have an additional and interesting effect for an efficient anti-PEP vaccine. IL-10 secretion reduces the severity of in the bronchoalveolar lymphoid tissue by preventing activation of macrophages, the expression of proinflammatory cytokines, and the recruitment of neutrophils [38], an important

factor that regulates the inflammatory process [39, 40] that may minimize the disease severity by reducing the development of lesions in the lungs.

The better performance of the SBa-15 in a $HSP70_{212\text{-}600}$ + MSNs vaccine formulation than that of SBa-16 may be related with the different structural properties of these two MSNs. SBa-15 and SBa-16 are morphologically different [41]. While SBa-15 MSNs have an elongated vermicular shape, with an average size of 590 nm and a hexagonal array of 5.7 nm pores, SBa-16 MSNs are rounded in shape, with a 15–20 μm diameter, and have a cubic arrangement of uniform 3.7 nm pores. The presence of larger mesopores in the SBa-15 MSNs provides easy passage for efficient transport of reactants and products without interparticles aggregation and pore blockage [42]. The SBa-15 structure in terms of particle size, pore size and distribution, and specific external surface area, allows a better adsorption of antigens, either on the on the surface or within the mesopores of the nanoparticles [17]. SBa-15 structure and properties likely enhance the co-transport and co-delivery of antigens from the peripheral injection site to the main lymphoid organs via the lymphatic system, which also helps to overcome antigen degradation [17, 43]. In addition, the nanoparticle sizes can affect uptake by antigen-presenting cells (APCs), and smaller nanoparticles, like SBa-15, may enter lymph node more easily than larger ones, like SBa-16 [44].

In combination with the $HSP70_{212\text{-}600}$ antigen, SBa-15 MSNs presented an adjuvant effect comparable to that of the alum, tested in this work, and with the Freund's adjuvant, tested by Virginio et al. [9]. However, SBa-15 MSNs may present some advantages in relation to these traditional adjuvants, which may have unacceptable side effects and lack of biocompatibility [45]. Freund's adjuvant is known to induce high toxicity and severe reactions, limiting its use in vaccines [15], while the application of the alum as adjuvant induces local reactions which increase with each injection [46], and may destabilize the adsorbed proteins, decreasing their performance as vaccinal antigens [47]. Swelling and other apparent local reactions, however, were not observed in the mice vaccinated with the $HSP70_{212\text{-}600}$ domain combined either with SBa-15 or SBa-16, demonstrating the biocompatibility.

The use of MSNs in vaccination has, to date, focused on attaching the antigens within or directly to MSN structures, a procedure called surface functionalization, to enhance antigen uptake by immune cells [15]. Surface functionalization usually requires multiple steps of chemical reaction or physical stress, which can add significant complexity and cost to the entire process, and may structurally alter the antigen or increase formulation toxicity [48, 49]. In our work, the simple mixing of the $HSP70_{212\text{-}600}$ antigen with the non-modified MSNs, without any surface functionalization, provided an effective adjuvanting action, at least in the case of SBa-15, avoiding the complexity of conjugation or incorporation and simplifying

the application of these MSNs as adjuvants. In the case of SBa-16 MSNs, its efficient use as adjuvant, at least for immunizations with the $HSP70_{212\text{-}600}$ antigen, may require some kind of surface functionalization, which remains to be investigated.

The results presented here suggested that the SBa-15 MSNs act as an adjuvant even when the antigen is not specifically conjugated to or incorporated within the nanoparticles. At least in combination with the $HSP70_{212\text{-}600}$ antigen, the SBa-15 presented an adjuvant effect comparable to that of alum, with the advantage of being less harmful to the inject animals, and, therefore, more biocompatible. Moreover, the $HSP70_{212\text{-}600}$ + SBa-15 formulation was able to induce a mixed Th1/Th2-type response with an additional IL-10-mediated anti-inflammatory effect in mice. Such responses would be of high interest for the design of efficient anti-PEP subunit vaccine formulations, which may include not only $HSP70_{212\text{-}600}$, but also other recombinant antigens.

Declarations

Author contribution statement

Veridiana G. Virginio: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Natalia C. Bandeira: Performed the experiments.

Fernanda M. A. Leal: Performed the experiments; Analyzed and interpreted the data.

Marcelo Lancellotti: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Arnaldo Zaha: Analyzed and interpreted the data.

Henrique Ferreira: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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