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Plant heat shock protein 90 as carrier-adjuvant for immunization against a reporter antigen

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ABSTRACT

Here, we evaluated the modulation of the immune response induced by Hsp90 of *Nicotiana benthamiana* (NbHsp90.3) against the Maltose Binding Protein (MBP) as a reporter antigen. Equimolar quantities of recombinant proteins were administered in mice as follows: MBP alone (MBP group), a mixture of MBP and rNbHsp90.3 (MBP + rNbHsp90.3 group) and the fusion of MBP to rNbHsp90.3 (MBP-rNbHsp90.3 group). The covalent linkage between NbHsp90.3 and MBP to bring a fusion protein was essential to induce the strong specific antibody response with predominance of IgG2a. Eighty-four days after the first immunization, splenocyte proliferation from MBP-rNbHsp90.3-immunized mice was consistently higher than that from MBP and MBP + rNbHsp90.3 groups. In addition, splenocytes from MBP-rNbHsp90.3 immunized mice produced higher levels of IFN-γ than controls. Finally, both formulations with rNbHsp90.3 significantly enhanced the MHC class I expression levels, but only rNbHsp90.3 covalent bound to MBP induced a specific cellular immune response against MBP measured as increased percentage of $CD8^+$ T cells. Taken together, these results suggest that plant HSP90s could be incorporated as adjuvants in vaccines that require the generation of a Th1 response along with a CD8 cytotoxic cell response to confer immunity.

1. Introduction

The main goal of adjuvant research is to find new adjuvants able to stimulate Th1, Th2 or both responses but with reduced toxicity [1–3]. In this context, the study of the immunomodulatory properties of molecules and proteins derived from different organisms can contribute to find new and more efficient adjuvants.

Heat Shock Proteins (HSPs) such as Hsp60, Hsp70 and Hsp90 have been recently reported to play important roles in antigen presentation, activation of lymphocytes and macrophages, and maturation of dendritic cells [4,5]. Different studies have demonstrated that immunization of animals with an HSP-peptide/protein complex, an artificially re-constituted HSP-peptide complex or an HSP-antigen fusion protein, in the absence of adjuvants, elicits strong antigen-specific immune responses and facilitates antigen cross-presentation in antigen-presenting cells [6–16]. Also, evidence indicates that antigens can be presented through HSPs in the context of the MHC class I and elicit a cytotoxic T lymphocyte-response [17–19]. Thus, the modulatory properties present in HSPs support the idea that these proteins could be used as carrier-adjuvant in immunization protocols.

The immunological properties of Hsp70 and Hsp90 from humans and other organisms as bacteria and parasites are also present in their plant orthologs [20–22]. We have previously shown that recombinant plant Hsp90 proteins induce prominent proliferative responses of CD19-bearing populations, suggesting a direct effect of these proteins on B lymphocytes [21]. Therefore, plant Hsp90s are an attractive option to use as novel and interesting carriers-adjuvants for proteins or peptides of immunoprotective value. Following with the characterization of the immunomodulatory properties of plant Hsp90s, in the present study, we evaluated the modulation of the immune response induced by Hsp90 of *Nicotiana benthamiana* (NbHsp90.3) against the Maltose Binding Protein (MBP) as a reporter antigen.

2. Material and methods

2.1. Plasmids and recombinant proteins

Plasmid pRSET-rNbHsp90.3 expressing the 6-histidine tag fused to NbHsp90.3 has been described previously [21]. The plasmid

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pMAL-rNbHsp90.3 was obtained cloning the NbHsp90.3 in frame to the carboxyl terminus of the MBP encoded by the vector pMAL-cRI (New England BioLabs, Ipswich, MA, USA) in the *Pst*I and *Bam*HI restriction sites. The recombinant protein MBP alone was obtained from the pMALcRI plasmid.

The recombinant proteins expressed by clones pMAL-rNbHsp90.3 and pMALcRI were induced and purified as described in the manufacturer's instructions (New England BioLabs). The recombinant protein NbHsp90.3 expressed by clone pRSET-rNbHsp90.3 was purified with a nitrilotriacetic acid-Nicolumn (QIAGEN, Manchester, UK) as described previously [21]. To eliminate endotoxins, the recombinant proteins MBP, rNbHsp90.3 and MBP-rNbHsp90.3 were passed through a polymyxin B-agarose column following the manufacturer's instructions (SIGMA, St. Louis, MO, USA). The concentration of lipopolysaccharide (LPS) in free-LPS recombinant proteins was measured using the HEK-Blue™ LPS Detection Kit (InvivoGen, San Diego, CA, USA) as described previously [21]. LPS contamination in MBP, rNbHsp90.3 and MBP-rNbHsp90.3 was lower than 1 ng/ml.

2.2. Animals

Female BALB/c (H-2^d) mice were bred and housed at the animal facilities of the Biotechnology Research Institute (IIB), National University of General San Martín (UNSAM), Buenos Aires, Argentina, and used at 8–10 weeks of age. All procedures requiring animals were performed in agreement with institutional guidelines and approved by the Independent Ethics Committee for the Care and Use of Experimental Animals of the National University of General San Martin (C.I.C.U.A.E., IIB-UNSAM, 09/2011).

2.3. Immunizations

The immunization schedule was the same as that described by Rico et al. [23] and Echeverria et al. [24]. Mice ($n = 8$ per group) were immunized intraperitoneally (i.p.) with each antigen on days 0 and 21. In order to administrate approximately equimolar quantities of recombinant proteins, the immunization doses for each antigen formulation were: MBP (42 kDa, 1 μg), rNbHsp90.3 (82 kDa, 2 μg), and MBP-rNbHsp90.3 (126 kDa, 3 μg) in 100 μl of phosphate-buffered saline (PBS 1X). The control group was injected with 100 μl of PBS. Mice were periodically bled from the tail vein and sera were stored at −20 °C until analyzed.

2.4. Antibody titers and isotype determination

Sera from immunized mice were analyzed for the presence of specific antibodies. Antigen-specific antibodies were analyzed by ELISA as previously described [25]. Briefly, 96-well microtiter plates (Immuno Plate Maxisorp; Nunc, Rochester, NY, USA) were coated overnight at 4 °C with 5 μg/ml of MBP. Goat anti-mouse IgG-horseradish peroxidase conjugate (1:5000) was used as a secondary antibody (SIGMA), and rat anti-mouse IgG1- or IgG2a-horseradish peroxidase conjugates (1:3000) (BD Biosciences, San Jose, CA, USA) were used for isotype analysis. Immune complexes were revealed with tetramethylbenzidine chromogen (TMB, One-Step; Invitrogen, Carlsbad, CA, USA), and optical density was read at 630 nm with an automatic ELISA reader (Synergy H1, Bio-Tek, Winooski, VT, USA). Serial dilutions of sera were carried out to determine the titer, which was defined as the highest serum dilution that gave a value above the absorbance value for pre-immune sera plus two standard deviations (cut off).

2.5. Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed as previously described [21]. Briefly, 84 days after the first immunization, viable splenocytes were plated in RPMI medium at 2.5×10^5 cells/well in 96-well flat-bottom microculture plates (Immuno Plate Maxisorp; Nunc). Cells were stimulated with 10 μg/ml of MBP. Positive controls were assayed with 5 μg/ml concanavalin A (ConA) (SIGMA). Non-stimulated cells were used as negative controls. Splenocytes were incubated for 72 h at 37 °C in 5% CO_2 and 1 μCi of [^{3}H]-thymidine (20 Ci/mmol, Perkin Elmer, Waltham, MA, USA) was added to the last 24 h of culture period. Incorporated radioactivity was measured in a liquid scintillation Beta counter (Beckman, Brea, CA, USA). Results are expressed as stimulation index (SI), defined as the mean number of counts per minute (cpm) from MBP-stimulated cells divided by the mean number of cpm from non-stimulated cells.

2.6. Cytokine analysis

Spleens from 5 immunized mice from each group were removed 84 days after the first immunization. Splenocytes cultures (1.25×10^6 cells/well) were stimulated with 10 μg/ml of MBP. Supernatants were harvested at 48 h to analyze IL-4 and IL-10 or 72 h for IFN-γ and stored at −70 °C until samples were measured by ELISA. Briefly, 96-well flat-bottom microtiter plates (Immuno Plate Maxisorp, Nunc) were coated overnight at 4 °C with 3 μg/ml of the capturing rat anti-mouse-IFN-γ, IL-10 and IL-4 monoclonal antibodies (BD Biosciences) diluted in 0.1 M Na_2HPO_4 pH 9. The supernatants from the cell cultures were tested in duplicate and serial dilutions of recombinant murine IFN-γ, IL-10 and IL-4 (BD Biosciences) proteins were used at 20–4000 pg/ml for the standard curves. After incubation, the plates were washed and 1 μg/ml of biotinylated rat anti-mouse IFN-γ, IL-4 and IL-10 (BD Biosciences) monoclonal antibodies were added. Streptavidin-peroxidase conjugate (SIGMA) was added to the washed wells. Bound complexes were detected with TMB (One-Step; Invitrogen), and optical density was read at 630 nm with an automatic ELISA reader (Synergy H1, Bio-Tek).

2.7. Flow cytometry

Spleens from five immunized mice from each group were removed 84 days after the first immunization. Splenocytes (2.5×10^5cells/well) were incubated for 72 h with 10 μg/ml of MBP. Then, the cells were incubated with phycoerythrin (PE)-conjugated anti-mouse CD4 (BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 (BD Biosciences) or with PE-conjugated anti-mouse CD8 (BD Biosciences) and FITC-conjugated anti-mouse CD3 (BD Biosciences). To analyze the expression of MHC class I surface molecules, spleens from five immunized mice from each group were removed 17 h after the first immunization. Splenocytes (1×10^6) were incubated with (FITC)-conjugated anti-mouse CD11c (BD Biosciences) and with Alexa Fluor 647 anti-mouse MHCI (Biolegend, San Diego, CA, USA) for 1 h. Cells were acquired using a FACScan flow cytometer (BD, San Jose, CA, USA). Dead cells were excluded on the basis of forward- and side-cell scatter. Results were analyzed using WinMDI 2.9 (De Novo Software, Los Angeles, CA, USA).

2.8. Statistical analysis

Statistical analysis was carried out with Prism 5.0 (GraphPad, San Diego, CA, USA) using one-way analysis of variance (ANOVA). Bonferroni test "*a posteriori*" and Kruskal Wallis with Dunns test "*a posteriori*" were carried out to compare means among groups. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Expression and purification of recombinant proteins

Fig. 1 shows the purified recombinant proteins. The generation of the recombinant NbHsp90.3 (rNbHsp90.3) has been described previously [21]. The MBP-rNbHsp90.3 fusion protein was generated by the fusion of the NbHsp90.3 gene to the MBP gene contained in the plasmid pMALcr1 (Fig. 1A). The fusion protein migrated with an apparent molecular weight of ~130 kDa, which was recognized by a polyclonal antibody against *Arabidopsis thaliana* Hsp81.2 (anti-AtHsp81.2) (Fig. 1B and C). The other bands observed might be degradation products since these secondary bands were also recognized by a polyclonal antibody anti-AtHsp81.2 (Fig. 1C).

3.2. Antibody response against MBP in immunized mice

To analyze the value of NbHsp90.3 as carrier-adjuvant, mice were immunized with equimolar doses of MBP-rNbHsp90.3, MBP + rNbHsp90.3 or MBP alone. The immunization schedule involved only two immunizations 21 days apart. A control group of mice injected with PBS was included. None of the immunizations included an adjuvant. The efficacy of vaccination was followed by serological analysis. The presence of anti-MBP IgG antibodies was evaluated over a period of 84 days (Fig. 2A). On day 42, all mice immunized with MBP formulations elicited a strong humoral response against MBP. However, on day 64, MBP and MBP + rNbHsp90.3 groups presented similar IgG antibody titers (8000), whereas mice immunized with the fusion protein showed titers of 16,000. Moreover, on day 84 the antibody responses in the MBP and MBP + rNbHsp90.3 groups decreased drastically (titers of 4000), while mice immunized with MBP-rNbHsp90.3 still showed a very high antibody response (titers of 16,000) (Fig. 2A). No MBP-specific IgG was detected in the pre-immune sera (data not shown) or in the PBS group (Fig. 2A). Furthermore no development of humoral immune responses against NbHsp90.3 was observed since no rNbHsp90.3-specific IgG was detected in any of the immunized mice (Fig. 2A).

The profile of the antibody response was assessed by analyzing the presence of MBP-specific IgG1 and IgG2a subtypes in sera. Immunization with MBP-rNbHsp90.3 clearly showed a Th1 humoral immune response (Fig. 2B). Similar to the results obtained with total IgG, all the three groups that received MBP presented high titers of specific IgG2a 42 days after the first immunization (Fig. 2B). However, on day 84 the MBP-rNbHsp90.3 group was the only one that showed high MBP-specific IgG2a responses with a titer of 16,000 (Fig. 2B). These results were confirmed by analyzing IgG2a serum levels with a fixed sera dilution (1:500) over the 84 days period (Fig. 1S). From this analysis it can be also clearly seen that, animals immunized with the fusion protein kept the same IgG2a increased level up to day 84, while in the other groups, this subclass dropped significantly from day 42 (Fig. 1S). On the other hand, the levels of anti-MBP IgG1 elicited by immunization with MBP-rNbHsp90.3 were not significantly higher than those observed in the other groups (Fig. 2B and 1S).

We also analyzed, by western blot, whether antibodies elicited after MBP-rNbHsp90 immunization could cross-react with mouse-Hsp90 (Fig. 3). A specific 95-kDa band, which corresponds to the mouse Hsp90 protein, was recognized by a monoclonal antibody specific for human Hsp90 in the extract of *naïve* mouse splenocytes (Fig. 3A). However, this 95-kDa band was not revealed by the sera from MBP-rNbHsp90.3-immunized mice (Fig. 3B). In addition, another lower molecular weight band was revealed in the protein extracts of splenocytes from *naïve* mice and also when the membranes were incubated with the secondary antibody alone, suggesting that this band is recognized by non-specific binding of the secondary antibody (Fig. 3B). In agreement with ELISA analysis, the western blot assay showed that sera from MBP-Hsp90.3-immunized mice revealed MBP and MBP-rNbHsp90.3 proteins, but did not react with the rNbHsp90.3 protein (Fig. 3B).

Table 1
Flow cytometry analysis of the lymphocyte subsets in splenocytes from mice immunized with PBS, MBP, MBP + rNbHsp90.3, and MBP-rNbHsp90.3.

Immunization	$CD3^+CD8^+$ (%)	$CD3^+CD4^+$ (%)	Ratio ($CD3^+CD8^+$/$CD3^+CD4^+$)
PBS	35.30 ± 1.17	65.89 ± 0.64	0.54 ± 0.02
MBP	35.02 ± 1.81	64.40 ± 0.96	0.54 ± 0.02
MBP + rNbHsp90.3	35.47 ± 1.89	66.38 ± 0.73	0.53 ± 0.03
MBP-rNbHsp90.3	39.73 ± 0.60[a]	62.90 ± 1.92	0.63 ± 0.03[a]

[a] $p < 0.01$.

3.3. Cellular response against MBP in immunized mice

In vitro stimulation with MBP was performed to analyze whether splenocytes from immunized mice were able to secrete IFN-γ, IL-4 and/or IL-10. Individual mouse splenocyte suspensions were obtained 84 days after the first immunization. Splenocytes from mice immunized with the fusion protein MBP-rNbHsp90.3 secreted significantly higher levels of IFN-γ than the PBS, MBP and MBP + rNbHsp90.3 groups (Fig. 4A). In contrast, IL-4 and IL-10 concentrations were below detectable levels in all groups (<20 pg/ml) (data not shown).

The ability of the fusion protein to elicit proliferation was also examined. After *in vitro* stimulation with MBP, the proliferation levels of splenocytes from the MBP-rNbHsp90.3 group ($P < 0.01$) were significantly higher than those from MBP, MBP + rNbHsp90.3 and PBS groups (Fig. 4B). No significant differences were detected between mice immunized with MBP or MBP + rNbHsp90.3 and control mice (PBS). Finally, we evaluated the induction of CD8 T cell responses by MBP-rNbHsp90.3. As shown in Table 1, although the percentage of $CD3^+CD4^+$ T lymphocytes in the CD3 compartment was slightly lower in the MBP-rNbHsp90.3 group than in the other groups, they were not statistically different ($P > 0.05$). However, the percentage of $CD3^+CD8^+$ was significantly increased in the MBP-rNBHsp90.3 group (Table 1, Fig. S1). In consequence, the $CD3^+CD8^+$ T cells/$CD3^+CD4^+$ T cells ratio was significantly higher in the MBP-rNbHsp90.3 group than in the rest of the groups (Table 1). In order to determine whether the increase of $CD8^+$ T cell correlates with increased MHC class I expression, we analyzed the cell surface MHC class I levels. Interestingly, both formulations with rNbHsp90.3 significantly enhanced the MHC class I levels (Fig. 5). However, only rNbHsp90.3 covalent bound to MBP induces a specific cellular immune response against MBP.

4. Discussion

Heat Shock Proteins (HSPs) have been proposed as immunostimulatory molecules to enhance antigen-specific immunity [10,13,14]. Several reports showed that HSPs can be carriers for antigens of different pathogens that require a Th1 response to confer immunity [6,14,16]. Here, we evaluated the adjuvant properties of NbHsp90.3 by analyzing its potential to induce an immune response against a reporter antigen in mouse vaccination.

Both humoral and cellular immune responses against MBP were elicited by administering the MBP-rNbHsp90.3 fusion protein. The inoculation of mice with MBP-rNbHsp90.3, but not with the mix or MBP alone, elicited a strong and prolonged specific anti-MBP antibody response. Noteworthy, MBP-rNbHsp90.3 immunization induced the highest amount of IgG2a, while similar levels of IgG1 were observed in all groups. These results suggest that the physical linkage between NbHsp90.3 and MBP was

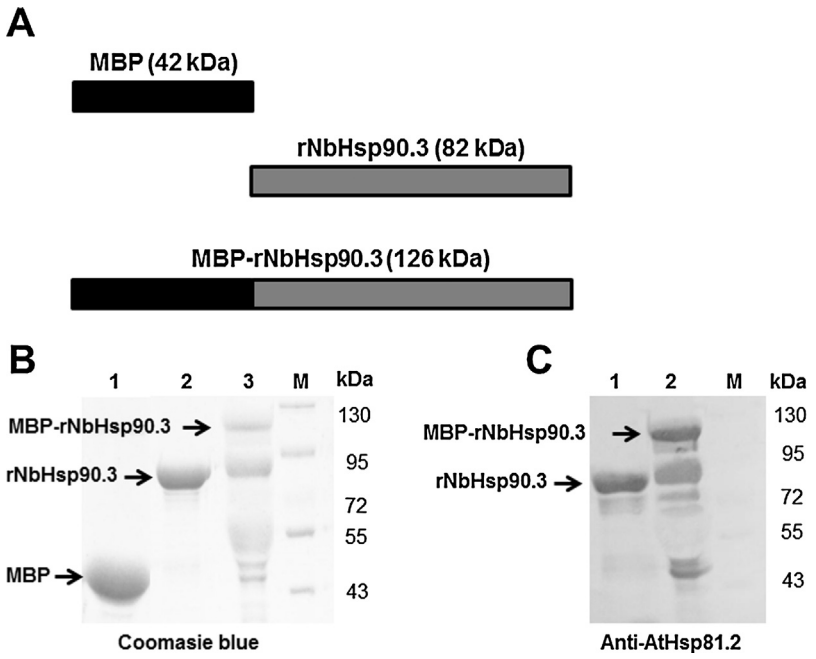

Fig. 1. Analysis of recombinant *N. benthamiana* Hsp90.3 protein (rNbHsp90.3), recombinant maltose binding protein (MBP) and recombinant MBP-rNbHsp90.3 fusion protein (MBP-rNbHsp90.3). (A) Schematic representation of recombinant MBP, rNbHsp90.3, and MBP-rNbHsp90.3 fusion protein and their encoding plasmids. (B) Recombinant MBP (lane 1), rNbHsp90.3 (lane 2), and MBP-rNbHsp90.3 fusion protein (lane 3) after purification, migration (SDS-PAGE), and staining with Coomassie blue. (C) Western blot of rNbHsp90.3 (lane 1) and the MBP-rNbHsp90.3 fusion protein (lane 2) revealed with the polyclonal antibodyanti-*Arabidopsis thaliana* Hsp81.2 (anti-AtHsp81.2, 1:500). The sizes (in kilodaltons) of molecular size markers (lane M) are indicated on the right.

essential to induce the high specific antibody response. In addition, no specific antibodies against rNbHsp90.3 were detected in MBP-rNbHsp90.3-immunized mice suggesting that immunization with MBP-rNbHsp90.3 fusion protein only elicited a specific humoral response against MBP. Furthermore, the antibodies induced by immunization with MBP-rNbHsp90.3 showed no cross-reactivity with the mouse Hsp90, suggesting that there is no concern for the potential to develop autoimmune diseases as a result

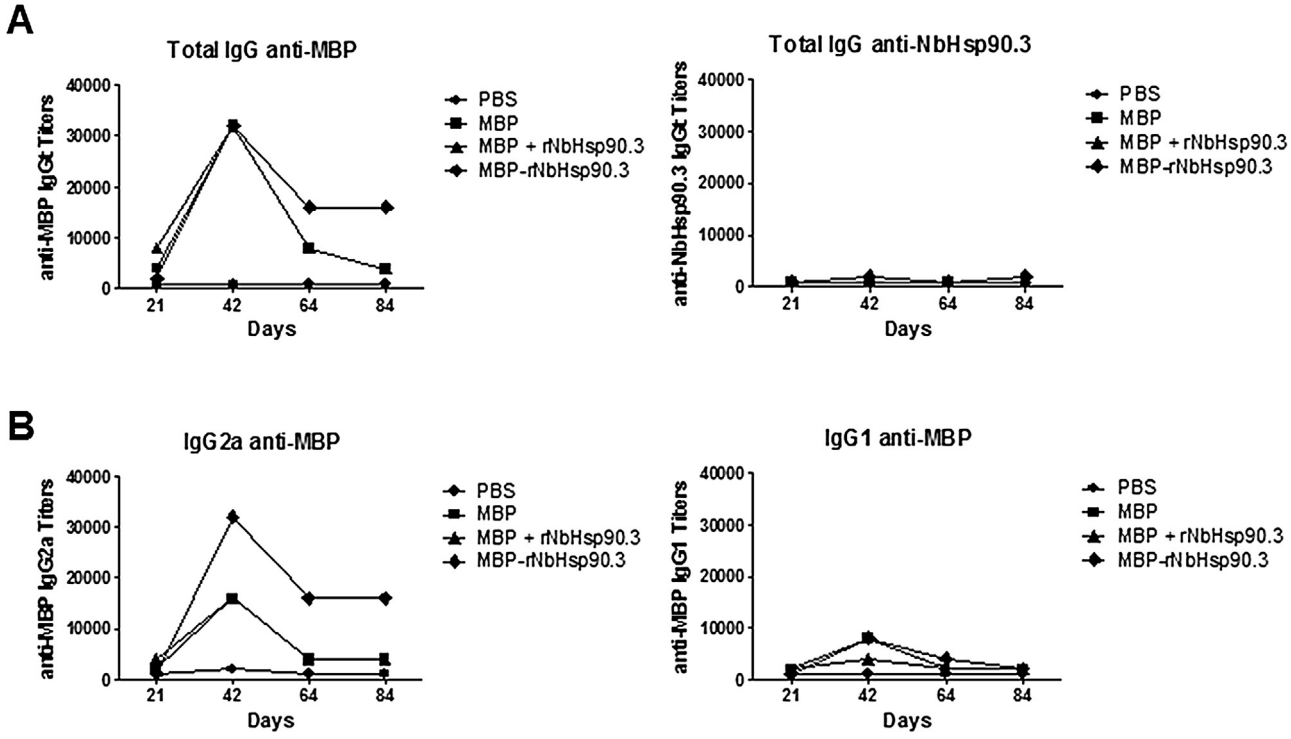

Fig. 2. Humoral immune response. (A) Specific IgG antibody production against MBP and rNbHsp90.3. (B) IgG isotype antibody response generated against MBP. Serum samples were obtained at the times indicated (in days) after the first immunization with MBP, the MBP + rNbHsp90.3 mixture, or the MBP-rNbHsp90.3 fusion protein. Serum samples from each group of mice (8 mice per group) were pooled at each time point, and serial dilutions were analyzed by direct ELISA. Results are expressed as endpoint titers, defined as the highest dilution analyzed that gave a value above the cut-off. The cut-off value was estimated as an absorbance value for pre-immune sera plus two standard deviations in 1:500 dilution (optical density, ~0.04). Two independent ELISA were performed for each pooled serum samples and the analysis was performed over two independent experiments.

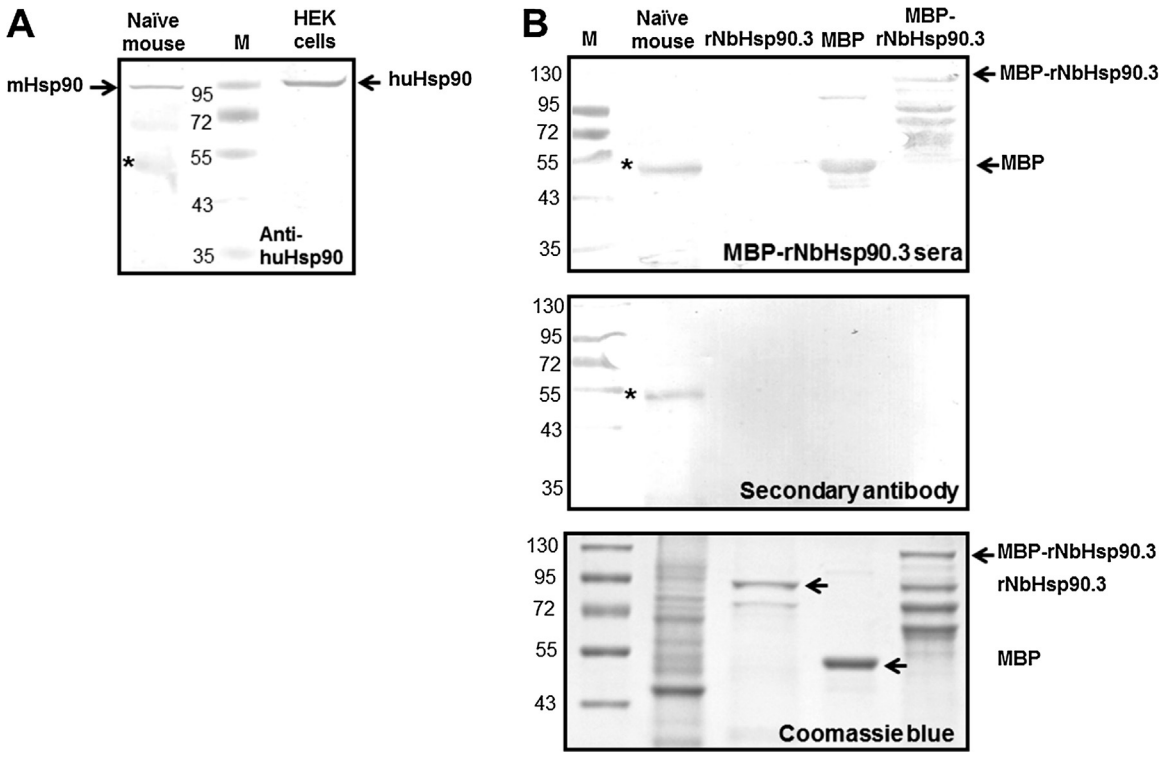

Fig. 3. Western blot analysis of the specificity of the anti-Hsp90 antibodies in immunized mice. (A) Total proteins (60 μg) from naïve mouse spleens and total proteins (60 μg) of HEK cells were separated on SDS–12% PAGE gels, transferred to PVDF membranes (GE Healthcare) and incubated with monoclonal antibody anti-human HSP90 (StressGen, Bruges, Belgium). The sizes (kilodaltons) of the molecular size markers (Thermo scientific, Hudson, NH, USA) (lane M) are indicated. (B) Total proteins (60 μg) from naïve mouse spleens, rNbHsp90.3 protein (5 μg), MBP (5 μg) and MBP-rNbHsp90.3 fusion protein (5 μg) were separated on SDS–12% PAGE gels, transferred to PVDF membranes (GE Healthcare, UK) and incubated with serum from a mouse immunized with the MBP-rNbHsp90.3 fusion protein (upper part of the figure), or incubated with the secondary antibody alkaline phosphatase conjugated goat anti-mouse IgG (SIGMA, St. Louis, MO, USA) (middle of the figure). Equivalent protein gels were separated on SDS–12% PAGE gels and stained with Coomassie blue (bottom of the figure). An unspecific band detected with the monoclonal antibody anti-human Hsp90, the sera and the secondary antibody is indicated with an asterisk. Sera and antibody dilutions were assayed as follows: sera 1:1000; anti-human Hsp90 1:1000 and secondary antibody 1:5000.

of the highly conserved sequences among HSPs from different species. The absence of anti-NbHsp90.3 antibodies and self-HSP cross-reactivity encourages the use of this protein as carrier-adjuvant. Similar results were observed with Hsp70 *Leishmania infantum* [23]. This protein used as adjuvant did not induced cross-reactivity against self-HSP in a mouse model. These results suggest that several HSPs, not just the HSPs from the same species, could be used as adjuvants in vaccine development with the advantage that plant HSPs are not derived from human pathogens.

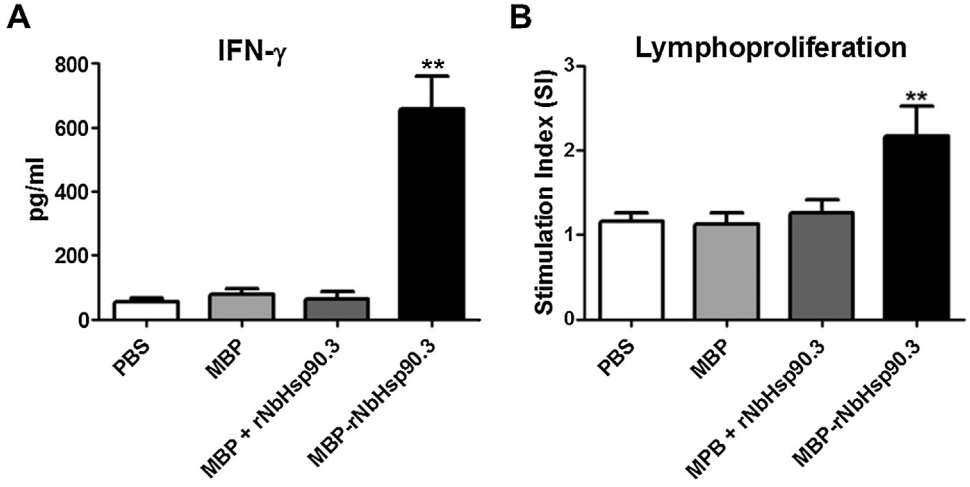

Fig. 4. Cellular response against MBP. Splenocytes from mice immunized with PBS, MBP, MBP + rNbHsp90.3, MBP-rNbHsp90.3 were *in vitro* stimulated with 10 μg/ml of MBP for 72 h to analyze: (A) IFN-γ production and, (B) lymphoproliferation. Four mice per group were analyzed. Results are expressed as stimulation index (SI), defined as the ratio between the mean of counts per minute (cpm) of MBP-stimulated cells over the mean of cpm from non-stimulated cells. To analyze cytokines, cell culture supernatants were assayed by ELISA. Data are shown as pg/ml (mean + SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's Multiple Comparison post-test. The asterisks indicate the statistically significant differences between different groups. $^{**}p < 0.01$.Two independent ELISA were performed for each supernatant and the analysis was performed over two independent experiments.

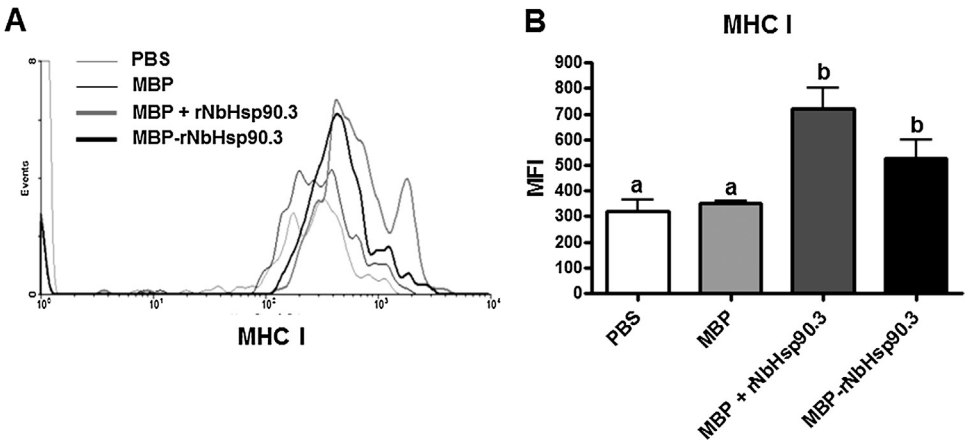

Fig. 5. MHC class I surface molecules expression. Splenocytes from mice immunized with PBS, MBP, MBP + rNbHsp90.3, MBP-rNbHsp90.3 were obtained 17 h after the first immunization. Spleen cells (1×10^6) were incubated with (FITC)-conjugated anti-mouse CD11c and with Alexa Fluor 647 anti-mouse MHCI. (A) Expression of MHC I on $CD11^+$ cells. (B) Mean fluorescence intensity (MFI) for each stimulus. Data are shown as percentages (mean + SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Kruskal Wallis with Dunns post-test. Different letters indicate statistically significant differences ($P < 0.05$; MBP-rNbHsp90.3 vs. PBS, MBP and MBP + rNbHsp90.3).

Previous reports have shown that the fusion of MBP to HSPs from *L. infantum* induce a specific IgG2a antibody response against the reporter antigen [23,24]. Also, previous works have analyzed the adjuvant properties of Hsp90s from mammals and parasites and demonstrated the ability of these Hsp90s to induce a Th1 immune response against cancers and infectious diseases [11,16]. In fact, Hsp90s are able to induce a T cell immune response against low amounts of the carried antigenic peptide [28]. Our results showed that MBP-rNbHsp90.3-immunized mice secreted mainly the IgG2a isotype indicating the involvement of cell-mediated immune responses via Th1 cells.

T cell activation through Hsp90s leads to the production of IFN-γ, supporting the idea that Hsp90s are able to induce a Th1 type response [29]. Echeverria et al. [11] showed that the antigen Rop2 from *Toxoplasma gondii* fused to Hsp90 from *L. infantum* (LiHsp83) generates a Th1 immune response with IFN-γ secretion in different mouse strains. In addition, Mohit et al. [16] demonstrated that vaccination with the human papilloma virus E7 protein fused to the gp96 protein (endoplasmic reticulum form of mammal Hsp90) directs the immune responses towards Th1 immunity with production of high IFN-γ levels. Herein, rNbHsp90.3 also elicited a strong and highly polarized Th1 humoral and cellular response.

Several publications have shown that Hsp70 and Hsp90 bound to peptides can interact with the antigen-presenting cells and mediate efficient peptide cross-presentation to $CD8^+$ cytotoxic T lymphocytes through the MCH class I molecules [30,31]. For this reason, we analyzed the $CD4^+$ and $CD8^+$ populations after *in vitro* MBP re-stimulation of splenocytes from immunized mice. We observed significant differences in the relative proportion of $CD3^+CD8^+$ T cells/$CD3^+CD4^+$ T cells between the MBP-rNbHsp90.3 group and the other vaccinated groups. The percentage of $CD3^+$ $CD8^+$ T cells significantly increased in MBP-rNbHsp90.3-immunized mice. In addition, the results showing that rNbHsp90.3 was able to enhance MHC class I levels suggests that rNbHsp90.3 linked to MBP, may provide an appropriate stimuli for presentation on MHC class I molecules and elicit a memory immune response against the carried antigen. Although we have no evidence that plant Hsp90s are able to interact with receptors in antigen-presenting cells, the mitogen B cell properties via TLR4 interaction described previously [21] and the adjuvant properties described here suggest that these chaperones would share similar signaling pathways of innate and adaptive immune responses to those already described for human Hsp90s [15,32,33]. Thus, plant Hsp90s could be incorporated in vaccines to stimulate CD8 T cell responses which are crucial against tumors and intracellular infections caused by viruses, bacteria and parasites.

Several studies have shown that plant tissue would contribute to the modulation of the immune response induced by antigens expressed and produced in plants [34–36]. Recently, Buriani et al. [22] observed that the complex plant Hsp70-*influenza A virus* nucleoprotein induces a specific cellular and antibody response in different mouse strains. In addition, Streatfield [37] suggested that the fusion of a foreign protein or peptide to a second recombinant protein can stabilize the target protein or peptide. In this sense, HSPs could be directly expressed in plants as recombinant proteins fused to the vaccine antigens and given the Hsp90 chaperone properties, it might be suggested that the fusion to HSPs could contribute to the stabilization of the carried antigenic protein and to the improvement of the expression levels, making the plant expression platform more competitive than conventional expression systems. Therefore, the strong and long-term Th1 antigen specific humoral and cellular response and the increased $CD8^+$ T cells observed when the reporter antigen was administered bound to rNbHsp90-3 encourage the idea that plant HSP90s would be excellent carriers/adjuvants of vaccine proteins and peptides and would thus improve the stability/immunogenicity especially for those antigens expressed in plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.09.047.

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