Vaccine 42 (2024) 3355–3364

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Safe plant Hsp90 adjuvants elicit an effective immune response against SARS-CoV2-derived RBD antigen

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ARTICLE INFO

Keywords:
Plant Hsp90
Adjuvants
RBD
Immune response
SARS-CoV2

ABSTRACT

To better understand the role of pHsp90 adjuvant in immune response modulation, we proposed the use of the Receptor Binding Domain (RBD) of the Spike protein of SARS-CoV2, the principal candidate in the design of subunit vaccines. We evaluated the humoral and cellular immune responses against RBD through the strategy "protein mixture" (Adjuvant + Antigen). The rRBD adjuvanted with rAtHsp81.2 group showed a higher increase of the anti-rRBD IgG1, while the rRBD adjuvanted with rNbHsp90.3 group showed a significant increase in anti-rRBD IgG2b/2a. These results were consistent with the cellular immune response analysis. Spleen cell cultures from rRBD + rNbHsp90.3-immunized mice showed significantly increased IFN-γ production. In contrast, spleen cell cultures from rRBD + rAtHsp81.2-immunized mice showed significantly increased IL-4 levels. Finally, vaccines adjuvanted with rNbHsp90.3 induced higher neutralizing antibody responses compared to those adjuvanted with rAtHsp81.2. To know whether both chaperones must form complexes to generate an effective immune response, we performed co-immunoprecipitation (co-IP) assays. The results indicated that the greater neutralizing capacity observed in the rRBD adjuvanted with rNbHsp90.3 group would be given by the rRBD-rNbHsp90.3 interaction rather than by the quality of the immune response triggered by the adjuvants. These results, together with our previous results, provide a comparative benchmark of these two novel and safe vaccine adjuvants for their capacity to stimulate immunity to a subunit vaccine, demonstrating the capacity of adjuvanted SARS-CoV2 subunit vaccines. Furthermore, these results revealed differences in the ability to modulate the immune response between these two pHsp90s, highlighting the importance of adjuvant selection for future rational vaccine and adjuvant design.

1. Introduction

During the pandemic of COVID-19, scientists showed that vaccination is one of the most promising actions to prevent infectious diseases that affect human health [1]. At record-breaking speed, the scientific community has worked jointly to develop different vaccine formulations that contribute to the protection of humanity [2]. However, some needs persist to improve existing vaccines or produce new ones that guarantee the vaccination of the entire world population with democratic and broad access, especially in emerging countries. Likewise, the appearance of new variants continues today, and the rational design of vaccines that adapt to these new COVID-19 variants or those that may arise in the

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https://doi.org/10.1016/j.vaccine.2024.04.036
Received 8 January 2024; Received in revised form 10 April 2024; Accepted 10 April 2024
Available online 17 April 2024

future will continue to be essential to achieve greater efficacy and a long-lasting period of protection [3]. Therefore, it is clear that the future challenge is to design a wide variety of alternatives to anti-COVID-19 vaccines or vaccination strategies that guarantee high tolerability and low side effects while avoiding reactogenicity. In this regard, subunit vaccines based on recombinant proteins or peptides are much safer due to they do not contain any pathogenic components and do not cause undesirable side effects [4]. However, they tend to be less immunogenic. For this reason, the immunogenic capacity of the subunit vaccines should be improved by using appropriate adjuvants in the vaccine [1].

Adjuvants not only enhance the immunogenic capacity of the vaccine formulation but also contribute to decreasing the amount of antigen needed for each vaccine dose or reducing the number of vaccine doses [5]. In addition, adjuvants stimulate a Th2-biased immune response, enhancing antibody production [6]. In this sense, the Hsp90s have been extensively studied, and their immunomodulatory properties have contributed significantly to improving vaccine design against infectious diseases caused by intracellular pathogens [7]. The HSP90s from bacteria, parasites, and mammals even from plants have been used as adjuvants in vaccine formulations, such as fusion proteins, complexes peptides/HSP90, or as mixtures of peptides + HSP90 [8]. Although the most used strategies in the design of vaccines based on HSP90 as adjuvants consist of the covalent union or formation of a union complex between the antigenic peptides and these chaperones, the mix of the HSP90 with the antigens of interest is a valuable strategy [9–14]. Several works showed that antigenic peptides + HSP90 administration as a mixture can modulate the humoral and cellular immune responses produced against the antigens, improving protection against intracellular pathogens such as parasites and viruses [8,13,15–17]. Recently, results obtained by our group showed that different Hsp90 isoforms derived from plants differentially modulate immune response profiles. We observed that the Hsp90 fused to the peptide would elicit a Th1 immune response, while the mixture of Hsp90 + peptide induces a Th1/Th2 immune response against the antigens [7,14,17]. Hsp90 from plants has the advantage of being a safe source with which humans have permanent contact. However, this advantage over other adjuvant systems still requires a greater understanding of the role and capabilities of plant Hsp90 (pHsp90) in immune response modulation. This result implies an increase in the number of vaccine models. Taking advantage of the fact that SARS-CoV2 is still a permanent infection in the world and a model of how the generation of alternative vaccines allowed us to control the effects of this pandemic, here, we propose the use of a short version of the Receptor Binding Domain (RBD) of the Spike protein of SARS-CoV2, the main candidate in the development of subunit vaccines. We evaluated humoral and cellular immune responses against RBD through the strategy "protein mixture" (adjuvants + antigens). Furthermore, in this work, we analyzed the potential of the different formulations studied to neutralize viral infections.

2. Materials and methods

2.1. Plasmid construction Receptor Binding Domain

The Receptor Binding Domain (RBD), residues 401 to 541, from Spike protein was reported of SARS-CoV2 Wuham to isolate Wuhan-Hu-1, complete genome, NCBI, No. Accession 6XR8_A [18]. The *RBD* gene was synthesized and codon-optimized by GenScript Company (USA) and cloned into a 6xHis-pRSET-A expression plasmid to obtain the construct pRSET-A-RBD.

2.2. Expression and purification of recombinant RBD

The expression of recombinant RBD was induced with 1 mM isopropyl-β-d-thiogalactoside (IPTG) for 6 h and soluble RBD was purified using a nitrilotriacetic acid-Ni2 + column (Qiagen) [19]. In previous works, we observed that the treatment of recombinant antigens with endotoxin removal polymyxin B resin does not modify the humoral and cellular immune response profiles in comparison with untreated recombinant antigens [14,17,19]. The concentration of LPS detected in the recombinant proteins was lower than 5 ng/ml [14,17,19]. Therefore, the purification of the recombinant AtHsp81.2 and NbHsp90.3 was performed under native conditions as mentioned in Bengoa-Luoni et al. [17].

2.3. Mice and vaccination

Male and female C57BL/6 (H-2b) 8-week-old mice were purchased from the FCEyN-UBA bioterium (Facultad de Exactas y Naturales of Universidad Nacional de Buenos Aires). For immunization, mice were randomly grouped into seven groups: Group rNbHsp90.3: 6 μg of rNbHsp90.3; Group rRBD + rNbHsp81.2: 4 μg of rRBD mixed with 6 μg of rAtHsp81.2, Group rRBD + rNbHsp90.3: 4 μg of rRBD mixed with 6 μg of rNbHsp90.3, Group PBS (negative control) and Group rRBD + Alum (positive control): 4 μg of rRBD mixed with 0.5 mg of Aluminum hydroxide. We followed the guide for the care and use of laboratory animals of Universidad Nacional de General San Martín (CICUAE, IIBIO-UNSAM). The mice had access to food and water ad libitum and were kept in breeding rooms at 22 °C, with a photoperiod of 12 light hours.

2.4. Antibody response and isotype determination

rRBD-specific antibodies were analyzed by indirect ELISA as previously described [13,19]. Sera were obtained from the immunized mice at 0-, 21-, 42-, 63-, 84-, and 105- days post-first immunization. Briefly, 96-well ELISA plates (ExtraGENE) were coated with 5 μg/ml rRBD at 4 °C overnight. For the IgGt determination, rat anti-IgGt-horseradish peroxidase conjugate (Cell Signaling Technology Inc) was used as a secondary antibody. For the isotype determination, rat anti-mouse IgG1-, IgG2b, or IgG2a-horseradish peroxidase conjugates (Sigma-Aldrich) were used. Immune complexes were revealed using tetramethylbenzidine substrate (TMB; Invitrogen). Plates were read at 655 nm with an ELISA reader (Synergy H1; Bio-Tek). All samples were measured by duplicate.

2.5. Cytokine analysis

Supernatants from splenocyte cultures obtained from immunized mice (2–4 mice per group) at 126 days post-first immunization were analyzed to determine the cytokine production as described in Corigliano et al. [20]. Briefly, 2 x 10^6 cells/well were stimulated with 10 μg/ml of rRBD. As a non-stimulation control, cells were cultured in only a medium. The production of IL-4 and IL-10 was measured in the supernatants at 48 h post-stimulation, while the production of IFN-γ was measured at 72 h post-stimulation by capture ELISA kits (Becton Dickinson).

2.6. Co-Immunoprecipitation (Co-IP)

Co-IP assays were performed as described in Vanagas et al. [21]. Briefly, an equimolar mixture of rRBD + rAtHsp81.2, rRBD + rNbHsp90.3, or rRBD alone was incubated with protein A/G Plusagarose (sc-2003, Santa Cruz). Immunocomplexes were first washed with washing buffer I (50 mM Tris, pH 8, 200 mM NaCl and 0.05 % Igepal100), then were washed with buffer II (50 mM Tris, pH 8, 300 mMNaCl and 0.05 % Igepal100), and finally were washed with buffer TE (10 mM Tris, pH 8, 1 mM EDTA. The pellet was resuspended in the SDS-PAGE loading buffer. Samples were loaded in a 12 % SDS-PAGE gel for immunoblotting. A protease inhibitor cocktail (Sigma) was included in every step.

2.7. Pseudovirus neutralization assay

Neutralization assays were performed using pseudotyped lentiviruses carrying the Spike protein of SARS-CoV-2 [22,23]. These viruses were generated by co-transfection of HEK293T cells with the plasmids pCMV14-3X-Flag-SARS-CoV-2 S (gifted by Zhaohui Qian, Addgene plasmid #145780), psPAX2 (gifted by Didier Trono, Addgene plasmid #12260), and pLentipuro3/TO/V5-GW/EGFP-Firefly Luciferase (gifted by Ethan Abel, Addgene plasmid #119816). Transfected cells were cultured for 48 h in DMEM supplemented with 10 % FBS and 1 % PenStrep at 37 °C and 5 % CO2. The supernatant containing viral particles was collected at 24 and 48 h and subsequently filtered through a 0.45-µm filter (Millipore). The viral particles were concentrated by centrifugation at 3000 x g overnight at 4 °C and the pellet was resuspended in DMEM. HEK293T cells constitutively ex-pressing human angiotensin-converting enzyme 2 (HEK293-hACE2) were used for the neutralization assay. One day before the assay, HEK293-hACE2 cells were seeded in 50 µl of medium in 96-well plates. Serial dilutions of sera (from 1/80 to 1/1280) were prepared in separate 96-well plates and incubated with the SARS-CoV-2 pseudotype virus for 1 h at 37 °C. Serum dilutions of the virus were added to each well of a 96-well plate and inoculated for 30 min at 400 × g. After 48 h, transduction efficiency was determined via luciferase activity. Transduced cells were lysed by adding 50 µl of 2× lysis buffer (25 mM Tris hydrochloride pH 8, 2 mM EDTA, 2 mM DTT, 1 % Triton X-100, and 10 % glycerol) and incubated for 10 min with agitation. Twenty µl of the cell lysate was transferred to a black 96-well FluoroNunc plate. One hundred µl of the reaction buffer containing luciferase substrate (25 mM Tricine hydrochloride pH 7.8, 0.05 mM coenzyme A, 3.3 mM DTT, 1 mg/mL BSA, 5 mM magnesium sulfate, 0.5 mM ATP pH ~7–8 (Sigma-Aldrich), 0.5 mM EDTA pH 8.0, 0.05 mg/mL D-luciferin (Gold Biotechnology)) was added to initiate the reaction. Luminescence was quantified using a microplate reader (DTX 880; Beckman Coulter). The luciferase units were graphed and standardized within Prism (GraphPad) by setting a baseline value using cells alone as zero and a maximum value of 100 % using a 1:2 virus-alone ratio. IC50 values were determined from curve fitting through nonlinear regression of log(inhibitor) against the normalized response.

2.8. Statistical analysis

Statistical analysis was generated using the Prism 5.0 Software (GraphPad). Two-way analysis of variance (ANOVA) was used to compare experimental groups with control groups. The values of significance were $p < 0.5$.

3. Results

3.1. The novel immune potent recombinant plant Hsp90 adjuvanted anti-COVID-19 vaccine, inducing RBD-specific antibodies in mice

Previously, Jangra et al. [24] showed that the monomeric SARS-CoV2 spike protein receptor binding domain (RBD) has lower immunogenicity than the full-length spike (S) protein. Therefore, we selected RBD as an antigen for a better understanding of differentiated immune responses triggered by novel plant HSP90 (pHSP90) adjuvants. The RBD contains the region of the S protein, which binds to the human ACE2 receptor (hACE2) and allows viral entry. Furthermore, RBD has most epitopes targeted by neutralizing antibodies (nAbs) and multiple T-cell

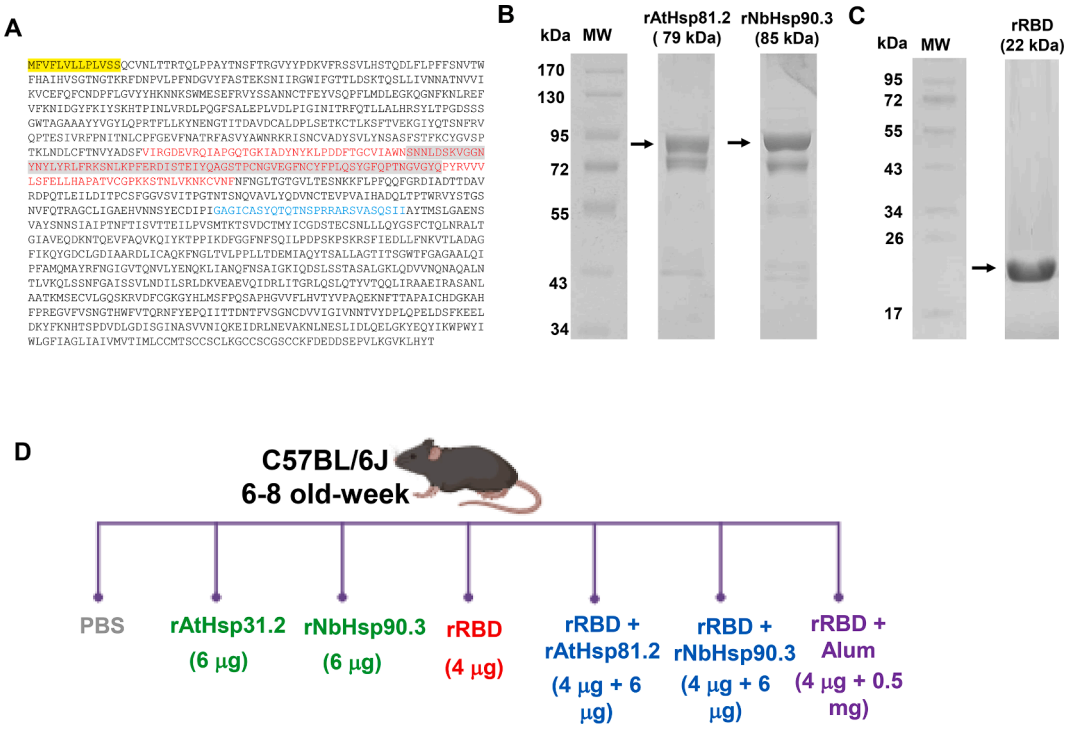

Fig. 1. The solubilized and purified *Arabidopsis thaliana* Hsp81.2 (AtHsp81.2), *Nicotiana benthamiana* Hsp90.3 (NbHsp90.3), and RBD recombinant proteins. A. The complete amino acid sequence of the Spike glycoprotein according to ACCESSION 6XR8_A (National Institutes of Health, National Center for Biotechnology Information database)[46]. The red letter indicates the region cloned to generate rRBD. Highlighted in gray is the ACE2 receptor binding RBD region[52]. Highlighted in yellow is the signal peptide. Highlighted in blue is the cleavage recognition sequence. B. SDS-PAGE analysis under reducing conditions of rAtHsp81.2 and rNbHsp90.3 expressed and purified from *E. coli* Rosetta (DE3) pLys S. C. SDS-PAGE analysis under reducing conditions of rRBD motif expressed and purified from *E. coli* BL21 pLys S. D. C57BL/6 females and males mice were injected intramuscularly with four µg of rRBD alone (rRBD group) or formulated with six µg of rAtHsp81.2 (rRBD + rAtHsp81.2 group) or rNbHsp90.3 (rRBD + rNbHsp90.3 group) or with 0.5 mg of alum (rRBD + alum group) or PBS 1X (PBS group). MW: prestained molecular weight protein marker. Fig. 1A was created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

response epitopes [25–27].

The advantage of the recombinant protein strategy is that the region expressed can be chosen, minimizing undesirable regions. At the beginning of the project, it was still unknown whether undesirable epitopes of the spike protein could induce adverse immune responses. Therefore, we selected a minimal region containing the Receptor Binding Motif (RBM) that can efficiently generate neutralizing antibodies. In this way, we could reduce undesirable effects if they exist. Fig. 1a shows the expressed RBD region (V401 to F541), which includes the ACE2 binding site (RBM) flanked by 32 and 30 residues at N- and C-terminal regions, respectively (Fig. 1A). The solubilized and purified *Arabidopsis thaliana* Hsp81.2 (AtHsp81.2), *Nicotiana benthamiana* Hsp90.3 (NbHsp90.3), and RBD recombinant proteins were observed in the purified fraction of SDS-PAGE (Fig. 1B and 1C). To evaluate whether rAtHsp81.2 and rNbHsp90.3 increase rRBD immunogenicity and modulate humoral response profile, we immunized C57BL/6J mice intramuscularly (i.m.) with 4 μg per mouse of rRBD formulated with 6 μg per mouse of rAtHsp81.2 or rNbHsp90.3 at Days 0 and 21 (Fig. 1D). Mice in the control groups received only rRBD (4 μg), only adjuvant (6 μg) or PBS. We immunized a seventh group with rRBD (6 μg) + alum (0.5 mg) as a positive control group. The doses used are in the range of those used by us in previous studies to evaluate the adjuvant capacity of pHsp90 [14,20] as well as those used for immunizations with Spike antigen as described [22,28,29].

After each injection and during 105 days at 21 interval-days, blood was collected and analyzed by an enzyme-linked immunosorbent assay (ELISA) using rRBD (Fig. 2A). Mice immunized with rRBD formulated with rAtHsp81.2 or rNbHsp90.3 adjuvants induced serum rRBD-specific IgG 42 days after the second immunization (Fig. 2B), with significantly higher IgG in groups that received adjuvanted versus unadjuvanted antigen. Notably, adjuvanted rRBD with alum induced lower antigen-specific IgG than adjuvanted antigen with pHsp90 (Fig. 2B). Interestingly, female and male mice immunized with rRBD + rpHsp90s did not show differences in the levels of anti-rRBD IgG. However, rRBD + alum-immunized female mice showed lower anti-rRBD IgG levels than male mice immunized with this formulation (Fig. 2C and 2D). In addition, control groups showed minimal antigen-specific IgG.

To analyze the type of profile of the induced humoral response, we measured the subtypes IgG2b, IgG2a, and IgG1 at day 84 post-first immunization as a representation of Th1 and Th2-type responses, respectively (Fig. 3A). Similar to other experiences, alum promotes a higher IgG1 response compared to IgG2 [30]. On the other hand, although we did not observe differences in IgGt levels between both rpHsp90s, we noted a different profile regarding the levels of IgG2a/IgG2b and IgG1 induced by these adjuvants (Fig. 3B–D). Interestingly, whereas rAtHsp81.2 induced high levels of IgG1 compared to rNbHsp90.3 and non-adjuvanted RBD (Fig. 3B), rNbHsp90.3 induced high levels of IgG2a/b, suggesting a Th1-biased immune response (Fig. 3C and D). Likewise, analyzing the ratio of IgG1 vs IgG2 levels, alum induces a Th2-type response, while AtHsp81.2 induces a mixed Th1/Th2-type

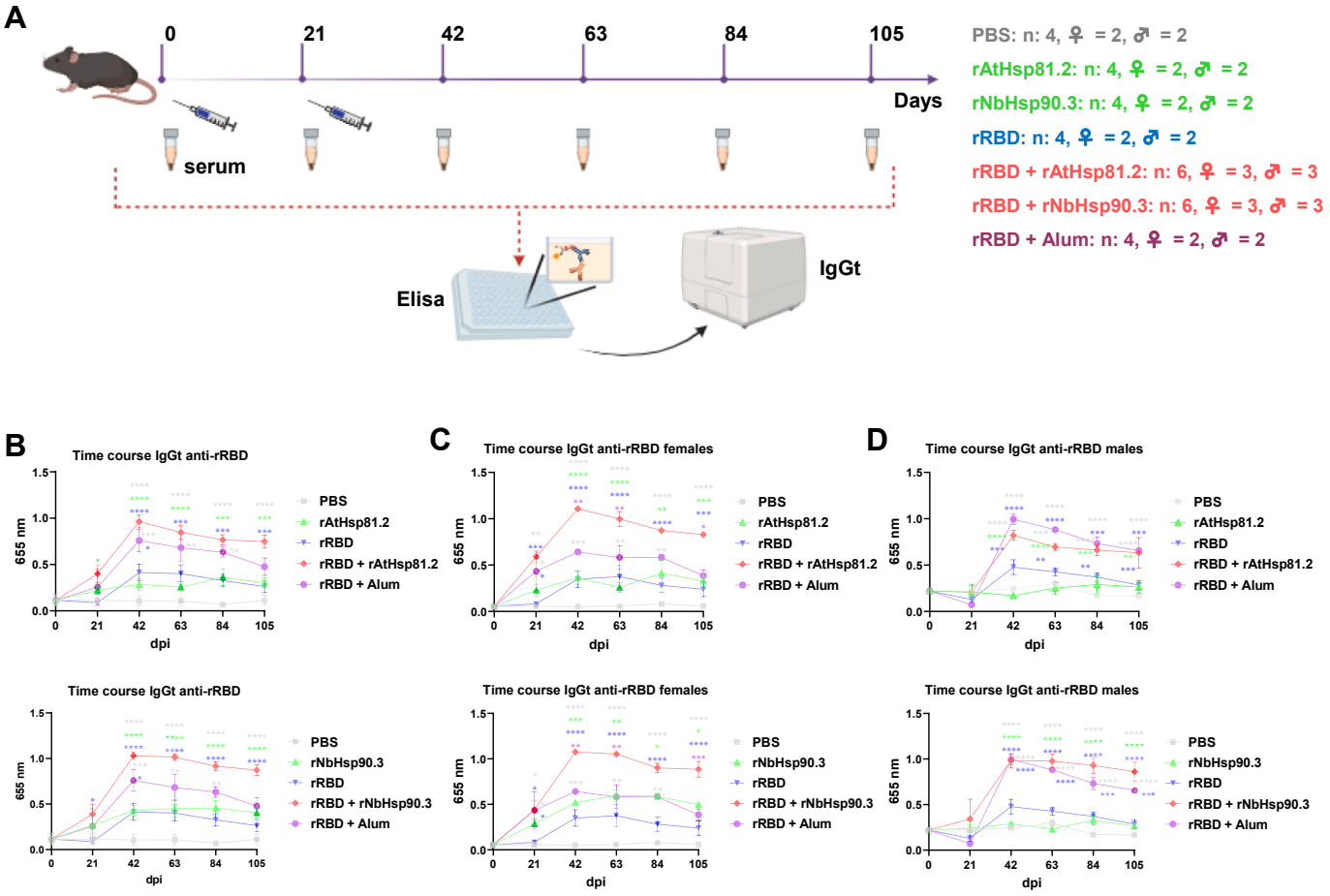

Fig. 2. Two immunizations with rRBD adjuvanted with rAtHsp81.2 or rNbHsp90.3 are sufficient to induce a robust IgG total response in both female and male mice. A. Study design including vaccination, sampling time points, and ELISA. The vaccination was performed twice at 21-day intervals via intramuscular route. Blood samples were collected at 0-, 21-, 42-, 63-, 84-, and 105-days post-vaccination in mice for serological assays. B-D. rRBD-specific IgG levels in both female and male mice (B), in female mice (C) and male mice (D). Data are presented as mean ± SEM. Statistical analyses were performed using two-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, and ****$p < 0.0001$ shown only for rAtHsp81.2/rNbHsp90.3/Alum + rRBD compared to other goups. Fig. 2A was created with BioRender.com. A representative experiment of 3 independent replicates with similar results is shown.

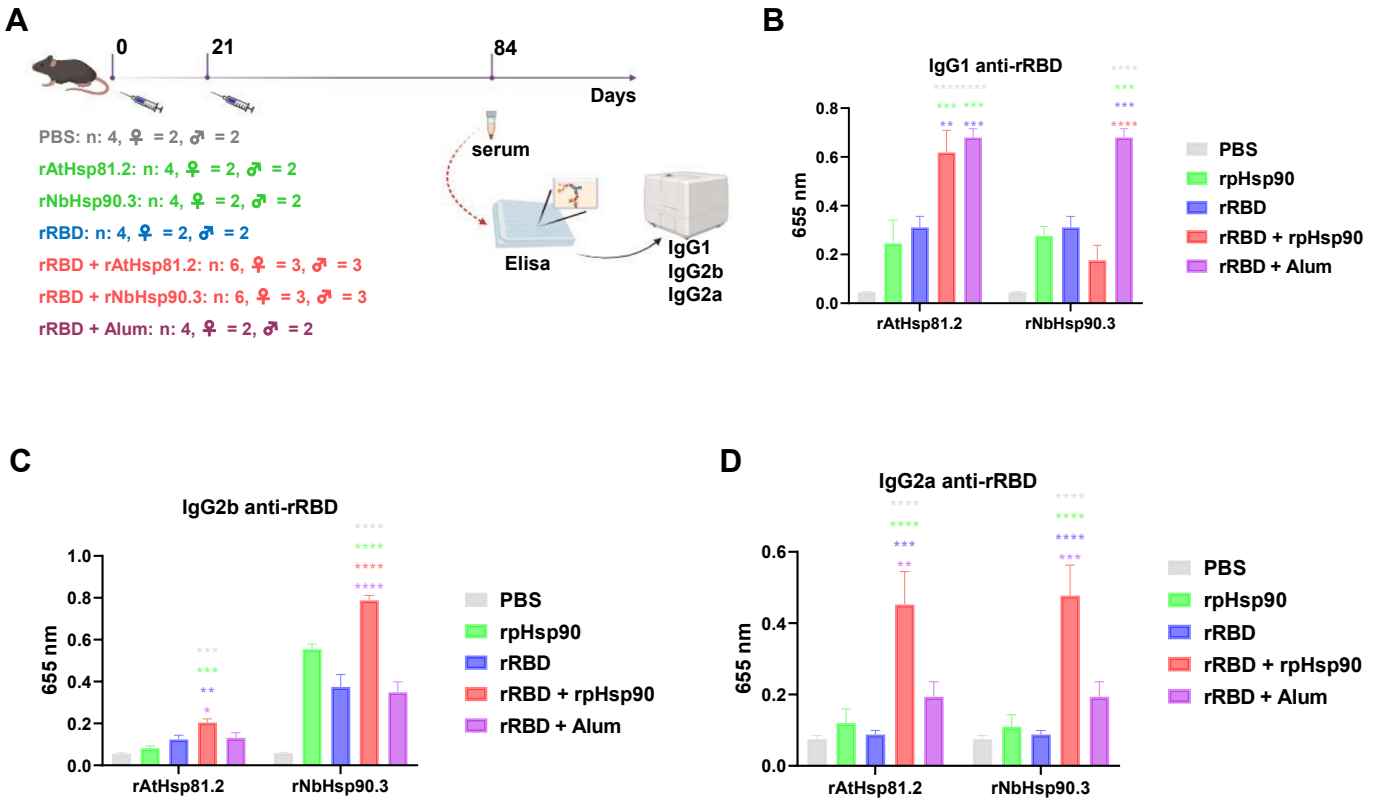

Fig. 3. Different profile of humoral responses induced by rRBD adjuvanted with rAtHsp81.2 versus rNbHsp90.3. A. Study design including vaccination, sampling time points, and ELISA. The vaccination was performed twice at 21-day intervals via intramuscular route. B-D. rRBD-specific IgG subclass antibodies for IgG1 (B), IgG2b (C), and IgG2a (D), were measured at 84 days post-vaccination. Data are presented as mean ± SEM. Statistical analyses were performed using two-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, and ****$p < 0.0001$ shown only for rAtHsp81.2/rNbHsp90.3/Alum + rRBD compared to other groups. Fig. 3A was created with BioRender.com. A representative experiment of 3 independent replicates with similar results is shown.

response, and NbHsp90.3 induces a Th1-type immune response (Fig. 3B–D).

3.2. RBD immunization with different recombinant plant Hsp90 adjuvants induces a distinctive profile of cytokines

To evaluate the cellular immune response induced by vaccination, we immunized five groups as follows: two groups of four mice each received rRBD adjuvanted with AtHsp81.2 or rRBD adjuvanted with NbHsp90.3, and the remaining three groups of two mice each received non-adjuvanted rRBD, rRBD adjuvanted with alum or PBS (Fig. 4A). At 126 days post-first immunizations, splenocytes were isolated, which were stimulated with rRBD to measure the levels of IFN-γ, IL-10, and IL-4 in the supernatants by indirect ELISA (Fig. 4B–D).

We observed that splenocytes from mice vaccinated with rRBD adjuvanted with AtHsp81.2 secreted increased levels of IL-4 upon stimulation, a Th2 cytokine, compared to non-adjuvanted rRBD group (Fig. 4B). Interestingly, cells collected from the spleens of animals immunized with rRBD adjuvanted with NbHsp90.3 produced significantly higher, IFN-γ levels, a Th1 cytokine, compared to PBS, non-adjuvanted rRBD and rRBD adjuvanted with AtHsp81.2 groups (Fig. 4D). At the same time, NbHsp90.3 and alum adjuvanted groups showed a significant increase in IL-10 secretion compared to non-adjuvanted rRBD and rRBD adjuvanted with AtHsp81.2 groups (Fig. 4D). In addition, stimulated splenocyte from mice from all the included groups induced significant IL-10 secretion compared to non-stimulated splenocytes (Fig. 4D). In summary, *in vivo* studies in the mouse model demonstrated that adjuvating rRBD (in low dose) with rNbHsp90.3 or rAtHsp81.2 induced potent humoral and cellular immune responses, but with differences in the cytokine profile elicited, that were higher than those elicited by the formulation adjuvanted with alum.

3.3. Vaccination with rRBD adjuvanted with NbHsp90.3 but not rRBD adjuvanted with AtHsp81.2 elicits SARS-CoV2 neutralizing antibody responses

To investigate the adjuvant capacity of each pHsp90 to enhance protection against infection with a SARS-CoV2 variant, we analyzed the virus neutralization capability of sera from mice immunized with rRBD adjuvanted with AtHsp81.2 or with NbHsp90.3 at 42 days post-first immunization. For the neutralization assay, we use a lentivirus-based pseudovirus (PSV) (Fig. 5A). Sera were incubated with entry into hACE2 expressing HEK293T cells and quantified as a function of the luciferase reporter gene transduction. We found that while both adjuvants enhanced the immunogenicity of rRBD, each adjuvant formulation showed differences in neutralizing antibody capacity. and less so in the rRBD + rAtHsp81.2 group (Fig. 5B and C). In the alum group, we did not detect neutralizing-antibody responses. Although a positive correlation is expected between the presence of antibodies and their neutralizing power, some works showed that the mere presence of antibodies does not guarantee neutralization [31], partly because of the quality of the antibodies due to conformational changes of the neutralizing epitopes [32–34]. In this sense, it cannot be ruled out that immunization schedules and/or the dose used in the vaccine formulations may have affected the neutralizing efficiency of the antibodies. Therefore, it is possible that the quantity or quality of the antibodies from the RBD + alum group was not optimal for demonstrating neutralization. The rRBD adjuvanted with rNbHsp90.3 group had high nAb titers (1:365) and also significantly higher titers than the alum, non-adjuvanted antigen, and PBS groups (Fig. 5B). In addition, nAbs (<1:160) against the PSV were detected in at least some animals in rRBD adjuvanted with AtHsp81.2

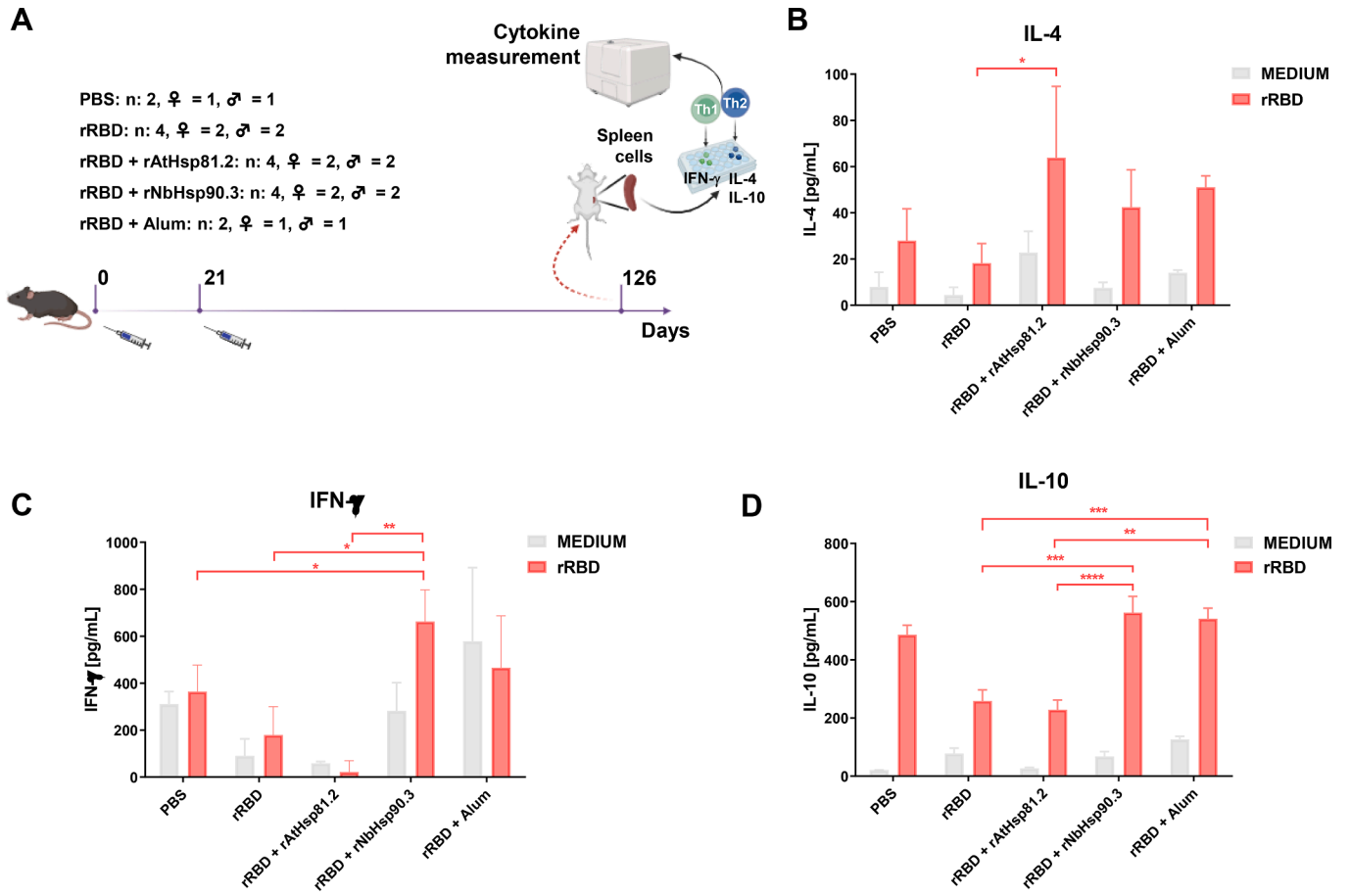

Fig. 4. rRBD adjuvanted with rNbHsp90.3 or rAtHsp81.2 shows differences in cytokine profile. A. Study design including vaccination, sampling time points, and cytokine measurement. At 126 days post-vaccination, levels of secreted cytokines were measured in the cell supernatant by sandwich ELISA for IL-4 (B), IFN-γ (C), and IL-10 (D). Data are presented as mean ± SEM. Statistical analyses were performed using two-way ANOVA with Tukey's multiple comparisons test. *p < 0.05; **p < 0.01; ***p < 0.001, and ****p < 0.0001 shown only for rAtHsp81.2/rNbHsp90.3/Alum + rRBD compared to other groups. Fig. 4A was created with BioRender.com. A representative experiment of 2 independent replicates with similar results is shown.

group (Fig. 5C). While rRBD + rNbHsp90.3 and rRBD + rAtHsp81.2 formulations elicited similar rRBD-specific IgG levels, the higher nAb titers observed with rNbHsp90.3 suggest improved antibody quality.

3.4. Complex assembly of rRBD with rNbHsp90.3, but not with rAtHsp81.2

An intriguing question of our model is whether both chaperones must form complexes to generate an effective immune response that affects the accompanying antigens [8]. With this idea in mind, we performed co-immunoprecipitation (co-IP) assays. rNbHsp90.3 or rAtHsp81.2 were mixed separately with the rRBD antigen and, after incubation, were co-IP with their respective antibodies (Fig. 6A). Fig. 6B shows that rRBD interacted with rNbHsp90.3, but not with rAtHsp81.2 confirming the assembly of the rRBD/rNbHsp90.3 complex. Co-IP performed by using rRBD alone as a control shows no reactivity with any of the antibodies assayed (Fig. 6C and Supplementary Fig. 1S). These data indicate that the greater neutralizing capacity observed in the rRBD adjuvanted with rNbHsp90.3 group would be given by the rRBD-rNbHsp90.3 interaction rather than by the quality of the immune response triggered by the adjuvants.

4. Discussion

The pandemic produced by SARS-CoV2 showed the importance of having a massive battery of safe immunization systems to respond quickly and stop its spread, saving a large proportion of the population from death, alleviating the health-system collapse, and breaking off the economic debacle. It is worth mentioning that in a record time, a large set of vaccines were approved (less than a year) throughout 2020, and around the world, nearly 11 billion vaccines were administered.

Although previous studies carried out on vaccines against Ebola or Mers-CoV have contributed to laying the foundations for vaccine development in pandemic situations [35], the pandemic experience with SARS-CoV2 consolidated the concept of rapid approval of vaccines through the intervention of international organizations such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for health emergencies [36]. A vaccine against SARS-CoV2 named ArVac-CG, based on a recombinant RBD antigen, was recently approved in Argentina [37]. Although this establishes solid foundations for future pandemic experiences that could take place in Argentina, emerging and socially and economically vulnerable countries remain at a disadvantage if new pandemic situations happen again. For this reason, the production of vaccines and their equitable distribution worldwide will require more significant deployment than those observed during this pandemic. In this sense, we consider it essential to continue incorporating safe vaccine models that can be quickly approved and that can also be produced in large quantities at acceptable costs.

Hsp90s have mainly contributed to improving vaccine development against infectious diseases, especially against intracellular pathogens. Several reports have demonstrated that Hsp90s from different sources are potent adjuvants, generating an appropriate immune response against infectious diseases [8]. Here, we showed that plant Hsp90 can be

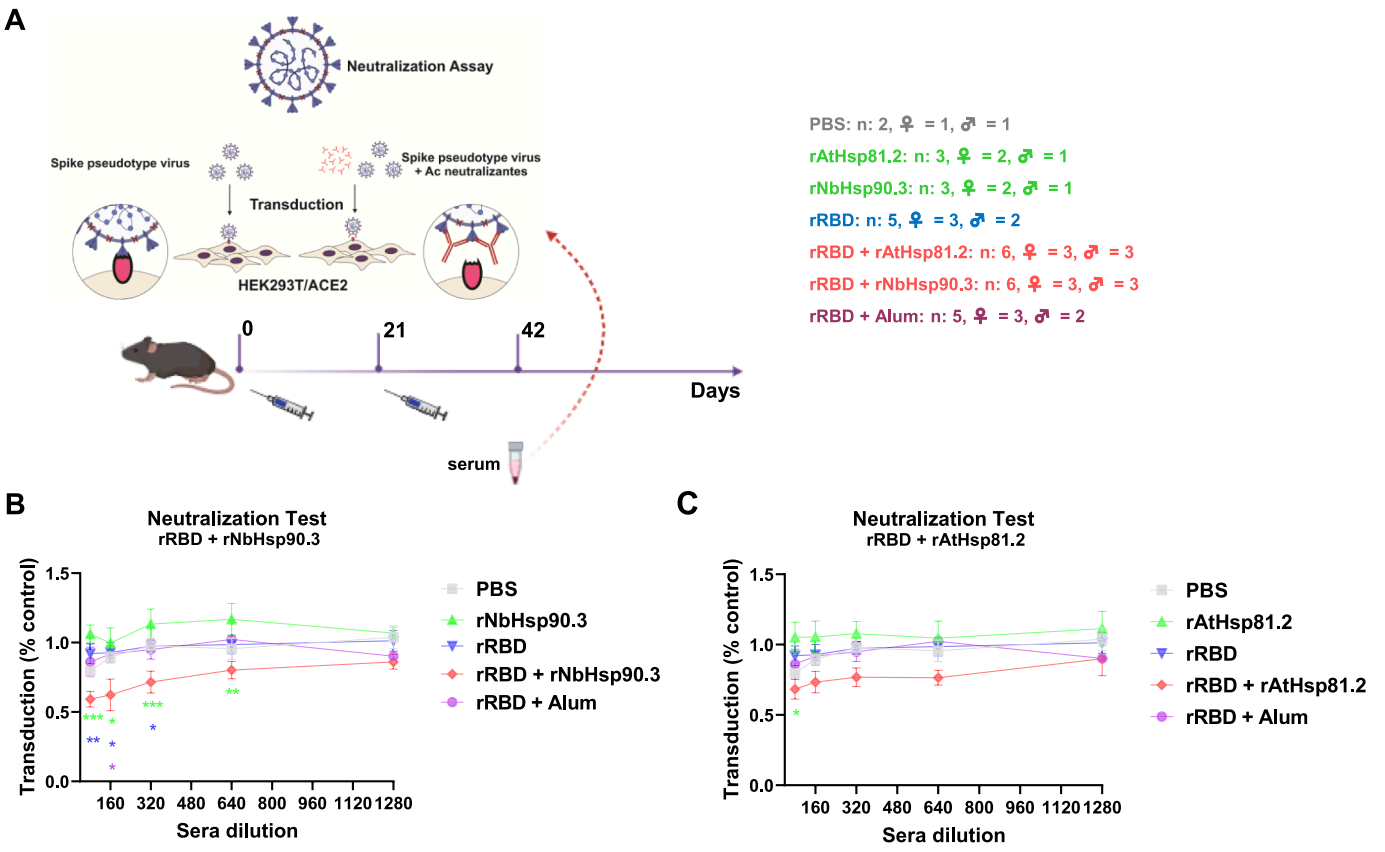

Fig. 5. rRBD adjuvanted with recombinant plant Hsp90 elicits a neutralizing antibody response. A. Schematic diagram of the vaccination and neutralization study. B-C. Anti-RBD neutralizing activity by virus neutralization tests in rRBD + rNbHsp90.3 group (B), and rRBD + rAtHsp81.2 group (C) 42 days post-immunization. Data are presented as the mean ± SEM. Statistical analyses were performed using a two-way ANOVA with Tukey's multiple comparison test. *p < 0.05; **p < 0.01; and ***p < 0.001 shown only for rAtHsp81.2/rNbHsp90.3 + rRBD compared to other groups. Fig. 5A was created using BioRender.com. A representative experiment with independent replicates and similar results is shown.

used as an efficient adjuvant to stimulate an effective immune response against a recombinant form of the SARS-COV2 RBD antigen. We observed that NbHsp90.3 generated the production of neutralizing antibodies and a Th1-type cellular immune response with the production of IFN-γ. On the contrary, AtHp81.2 induced a mixed Th2 humoral immune response with IL-4 production. In general, Hsp90s derived from different organisms, including those from plants, had already been shown to have immunogenic capacities and immunomodulatory properties[7] but had not yet been studied as adjuvants for SARS-COV2. The fact that both pHsp90s generated different types of immune responses could be an advantage when choosing one or the other adjuvant. One of the most commonly used adjuvants for vaccine development in emergencies, which is allowed for use in humans, is aluminum salts, which are also widely used in vaccination for COVID-19 [3]. Aluminum salts act to stimulate immune responses, especially of the Th2 type [38,39], related to the B lymphocyte stimulation for antibody production independent of TLR and CD4+ T helper cell responses [40]. In general, this kind of activation is poor [41]. Therefore, alum adjuvants are used with other salts or adjuvants to enhance the response. For emergencies, a combination of imidazoquinoline class molecules (TLR7 and TLR8 agonist) adsorbed onto alum has also been approved for use in humans [42]. This combination facilitates the generation of cell-mediated immunity [43,44]. In addition, other adjuvants used with recombinant proteins for anti-COVID-19 vaccines are CpG, SQBA, AS03, and MF59, among others [3,22]. The advantage of using a plant version of Hsp90 as an adjuvant is that this protein is a natural plant compound with which humans can maintain permanent contact without showing toxicity. pHsp90s have been shown to stimulate the humoral and cellular immune response through cross-presentation by the internalization of exogenous Hsp90s complexed to or fused to a peptide in early endosomes and the induction of inflammatory cytokines via TLR4 and TLR2 [8]. Similarly, pHsp90 has been shown to stimulate humoral and cellular responses in other models [14,17] in addition to interacting with TLR4 to mediate MHC I activation [8,20]. This would provide a new type of adjuvant to expand the vaccine production possibilities in general and in future pandemics.

As far as we know, the RBD protein (V401; F541) analyzed in this work is a shorter version than others previously studied. Mainly, it is smaller in the N-terminal region than several of those already used, such as NARUVAX-C19 (Q321, S521) [45], ArVac-CG (319R, 537 K)[37] or (R328, T531) [22]. All of these were effective in eliciting a protective immune response. Once again, it shows the versatility of recombinant techniques to design responsive antigens limited to the region of interest. Our strategy was to present the RBD to the immune system to obtain neutralizing antibodies. However, we also detected a cellular response in mice immunized with rRBD adjuvanted with NbHsp90.3, even though no T epitopes have been identified in this fragment during natural infection [46]. This fact may suggest that Hsp90 could help antigen presentation exogenously [47,48] or through the internalization of the peptides from the endosome to the cytosol by the proteasome to the representation of Hsp90-associated peptides [8,49], at the same time that Hsp90s *per se* could trigger the secretion of cytokines.

Hsp90s are specialized chaperones that can bind to a group of client proteins that are not necessarily unfolded [50]. Therefore, it is unknown whether its role as an adjuvant is due to its intrinsic capacity to stimulate the immune response in the formulation or is due to its ability to bind the immunogen as occurs in cancer models [51–55]. Here, we observed that of the two chaperones, only rNbHsp90.3 would form a complex with

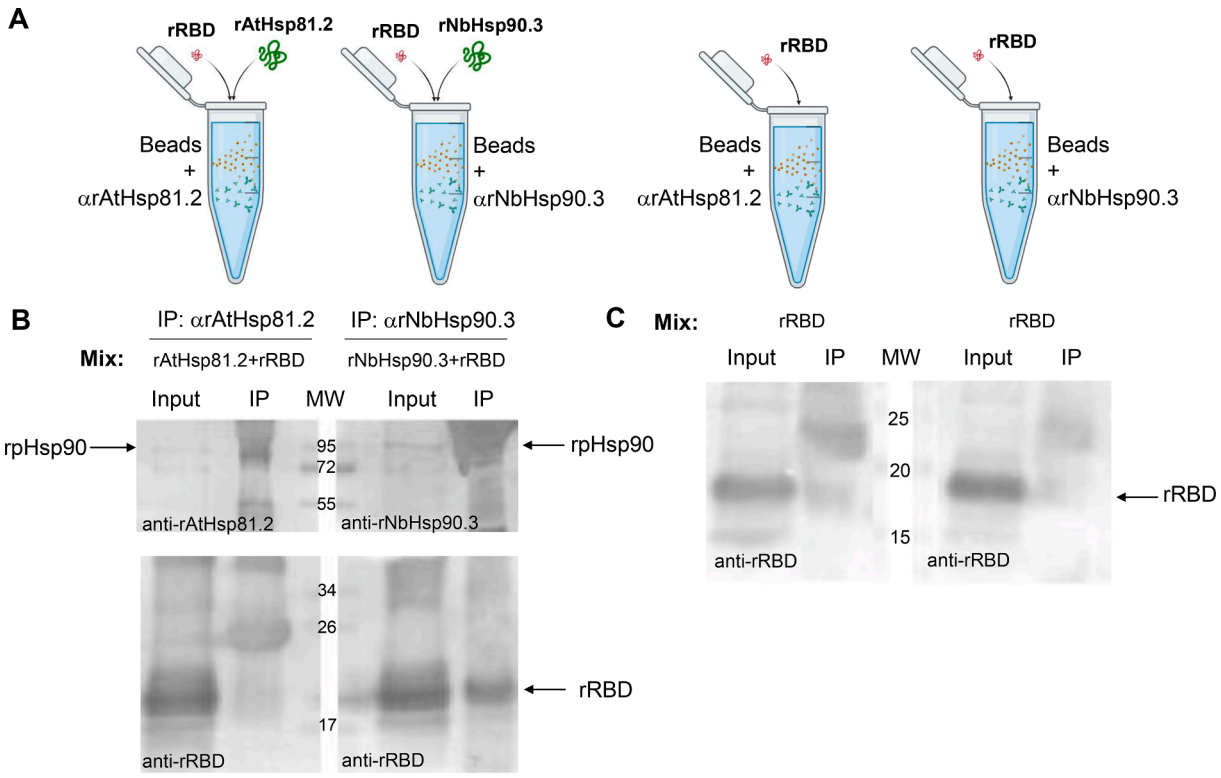

Fig. 6. rRBD interacted with rNbHsp90.3, but not with rAtHsp81.2. A. Schematic diagram of co-immunoprecipitation (co-IP) assays. B-C. Co-immunoprecipitation-WB analysis from rRBD + rNbHsp90.3, rRBD + rAtHsp81.2 (B), or rRBD alone (C). A representative Western blot is shown for anti-rNbHsp90.3, anti-rAtHsp81.2, and anti-rRBD. IP: immunoprecipitation. MW: prestained molecular weight protein marker. Fig. 6a was created with BioRender.com. A representative experiment of 3 independent replicates with similar results is shown.

rRBD, while the rAtHsp81.2 + rRBD formulation would only be a mixture. Interestingly, the rRBD/rNbHsp90.3 complex induced an immune response towards a Th1 profile, which correlates with the immunomodulatory properties commonly described for Hsp90s [8], while the formulation with AtHsp81.2 would elicit an immune response towards a Th2 profile, which is likely for a recombinant antigen alone. This implies that although this mixing strategy has the advantage of being quickly developed, it will be necessary to guarantee that the antigen and the adjuvant form complex in future vaccine formulations if a Th1 profile is required. In this sense, we hypothesize that differences in the immune activation mechanisms of both chaperones may explain the profile of the triggered response. While rNbHsp903 would be presenting the complexed antigen to the immune system through MHC I on DCs for T cell activation, rAtHsp81.2 could help to stimulate the immune response generated by rRBD as a conventional adjuvant. However, we cannot rule out that these differences in the profile of the immune response observed are related to differences in the intrinsic properties of each chaperone or to the structure and characteristics of each antigen. Likewise, in cases where the antigen and the adjuvant do not generate a complex or the antigen/adjuvant mixture does not trigger a potent protective response, another alternative is the "fusion protein" strategy. However, it is more laborious, and the expression levels of the recombinant proteins vary from case to case. In this case, it was also shown to be highly efficient in generating an adequate immune response [7,8,14].

5. Conclusion

The emergence of the SARS-CoV-2 pandemic was a major global challenge for the entire health, research, and vaccine production systems in record time. Initially, numerous studies briefly addressed the lack of knowledge about the characteristics of the infection. Soon, it was clear that the vaccine would be a fundamental tool to control the infection. Fast-track approval has promoted the use of different vaccines. In that sense, there is some uncertainty about the efficiency and feasibility of a safe vaccine. Fortunately, all the vaccines generated and approved have shown to be protective and with few adverse effects. However, having several vaccine systems is advantageous to enable a better response for this type of situation. In addition to the immunogenic properties of pHsp90s being similar to those observed in other Hsp90s, they would be a safe system for humans since it is a chaperone with which, as already mentioned, there is permanent contact through food. We were able to show that pHsp90 can indeed be an adjuvant to take into account for the development of anti-COVID-19 vaccines. Interestingly, we also show that these properties may be related to their ability or not to form complexes with the antigen of interest, which should be analyzed on a case-by-case basis.

Funding

The work was supported by ANPCyT (PICT 2020-639 and PICT cat. I 56) and CONICET (PIP 2021-1168).

Institutional review board statement

All mice were maintained under specific-pathogen-free conditions and handled according to the approved institutional animal care and use committee protocols of the Universidad de General San Martín (C.I.C.U.A.E., IIB-UNSAM, 09/2016).

Informed consent statement

Not applicable.

Author contributions

M.C. and S.O.A. designed and coordinated the research and analyzed data. V.A.R.D. performed all experiments and analyzed data. A.A., L.F. M.M, and M.A.S. helped to perform the recombinant protein purifications. C.J.G. and A.O. performed the neutralizing-antibody response assays and helped to interpret experiments and analyze data. M.G.C. and V.A.S. helped to perform the cytokine analysis. A.L. performed the mice immunizations. All authors wrote the initial draft, and all authors contributed to the final manuscript.

CRediT authorship contribution statement

Victor A. Ramos-Duarte: Methodology, Investigation, Formal analysis. **Alejandro Orlowski:** Writing – review & editing, Supervision, Formal analysis. **Carolina Jaquenod de Giusti:** Writing – review & editing, Methodology. **Mariana G. Corigliano:** Supervision, Methodology. **Ariel Legarralde:** Methodology. **Luisa F. Mendoza-Morales:** Methodology. **Agustín Atela:** Methodology. **Manuel A. Sánchez:** Methodology. **Valeria A. Sander:** Writing – review & editing, Supervision. **Sergio O. Angel:** Writing – original draft, Investigation, Conceptualization. **Marina Clemente:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

S.O.A. Angel, M. Clemente, M.G. Corigliano, and V.A. Sander are members of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Professor of Universidad Nacional General San Martin (UNSAM). C. Jaquenod de Giusti and A. Orlowski are members of CONICET and professors of Universidad Nacional de La Plata (UNLP). V. A. Ramos-Duarte and L.F. Mendoza-Morales are fellows of CONICET. M. A. Sáchez is a fellow of Agencia Nacional de Promoción de Ciencia y Técnica (ANPCyT). A. Legarralde is a technical staff of CONICET. A. Atela is a student of UNSAM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2024.04.036.

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