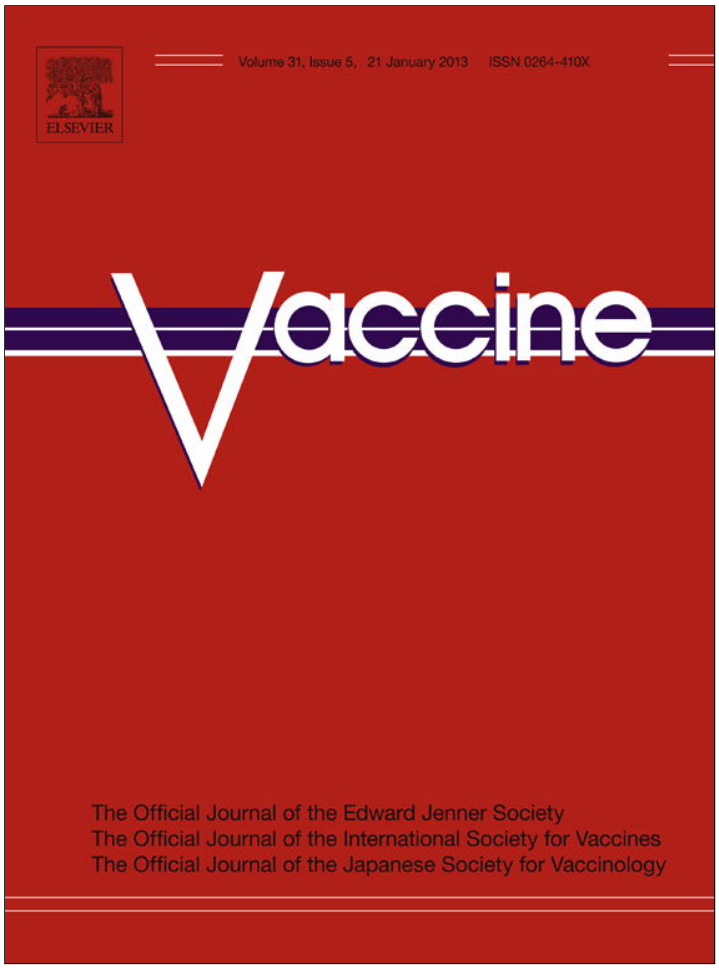

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Tandem repeats of the extracellular domain of Matrix 2 influenza protein exposed in *Brucella* lumazine synthase decameric carrier molecule induce protection in mice

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ABSTRACT

The antigenic variation of influenza virus represents a major prevention problem. However, the ectodomain of the protein Matrix 2 (M2e) is nearly invariant in all human influenza A strains and has been considered as a promising candidate for a broadly protective vaccine because antibodies to M2e are protective in animal models. In this work we evaluated the possible use of *Brucella abortus* lumazine synthase protein (BLS), a highly immunogenic decameric protein, as a carrier of the M2e peptide. Chimeric proteins generated by the fusion of one or four in tandem copies of M2e to BLS were efficiently expressed in *Escherichia coli* and assembled in decameric subunits similarly to the wild type BLS enzyme, as demonstrated by the comparative circular dichroism spectra and size exclusion chromatography and static light scattering analysis. The M2e peptides were stably exposed at the ten N-terminal ends of each BLS molecule. Immunization of mice with purified chimeras carrying only one M2e (BLS-M2e) copy elicited a significant humoral immune response with the addition of different adjuvants. The fusion of four in tandem copies of the M2e peptide (BLS-4M2e) resulted in similar levels of humoral immune response but in the absence of adjuvant. Survival of mice challenged with live influenza virus was 100% after vaccination with BLS-4M2e adjuvanted with Iscomatrix® ($P < 0.001$) and 80% when adjuvanted with alum ($P < 0.01$), while the chimera alone protected 60% of the animals ($P < 0.05$). The approach described in this study is intended as a contribution to the generation of universal influenza immunogens, through a simple production and purification process and using safe carriers that might eventually avoid the use of strong adjuvants.

1. Introduction

Seasonal influenza A outbreaks are caused by viral subtypes already circulating in humans, while pandemics are caused by emerging novel subtypes/strains derived by reassortment with avian or porcine viruses [1,2].

Current vaccines are based primarily on antibody responses against the viral glycoprotein HA. Although antibodies to HA provide potent virus strain-specific protection, due to the frequent antigenic drifts and the occasional antigenic shifts of the circulating virus, a universal influenza vaccine, able to confer cross protection against different influenza variants and subtypes, would represent a dramatic medical advance and may limit or complement the need for annual vaccination. The influenza A nucleoprotein (NP), the Matrix 2 (M2) proteins and more recently, the highly conserved stalk domain of the HA protein are the major target antigens for this kind of cross-reactive vaccines [3–8].

Matrix 2 is an integral membrane protein, which is expressed at the plasma membrane of influenza virus-infected cells, and it is also incorporated in small amounts into budding virions. The mature M2 protein is a homotetramer with proton selective ion channel activity [9–12]. The ectodomain of M2 (M2e) is highly conserved in many influenza virus strains circulating in the human population, and several studies have shown that immunization with this 23-mer peptide can protect mice against homologous and heterologous infection with influenza A virus [5,13,14]. It has also been shown that passive administration of a monoclonal antibody directed to M2e inhibits influenza A virus replication in mice [15].

Previous studies have focused on the M2e peptide fused to a variety of carrier molecules, such as glutathione-S-transferase [16], hepatitis B virus core [13,17], human papilloma virus L protein [18],

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keyhole limpet hemocyanin [19], bacterial outer membrane complex [20], and flagellin [21], among others. To obtain a protective immune response, potent adjuvants such as Freund's adjuvant, bacterial protein conjugates, QS21 or heat labile endotoxins derivatives were used [20–23].

The polymeric enzyme lumazine synthase from *Brucella* spp. (BLS) is an immunodominant *Brucella abortus* derived antigen, able to generate strong humoral as well as cellular immunity [24,25]. This protein folds as stable dimers of pentamers, and has been shown to allow the insertion of foreign peptides or proteins at its N-terminus without disrupting its general folding. The presentation of BLS to the immune system in a highly ordered three-dimensional array enhances the immunogenicity of the heterologous peptides [26,27].

In the present work we evaluated the use of BLS as carrier protein to present the M2e peptide to the immune system. Although immunization of mice with the chimera carrying only one M2e copy elicited a significant humoral immune response in the presence of adjuvants, the addition of four copies of the M2e peptide to the BLS molecule resulted in similar levels of humoral immune response in the absence of any adjuvant. The adjuvanted multicopy molecule was capable to induce 100% protection from viral challenge in BALB/c mice.

2. Materials and methods

2.1. Construction of the BLS-M2e chimeras and expression in Escherichia coli

The selected M2e amino acid sequence was MSLLTEVETPIRNEWGCRCNDSSD [5]. Oligonucleotides were synthesized (GenScript Corporation) and one or four in tandem copies were cloned into the pET11a plasmid containing the open reading frame of BLS [26].

Recombinants BLS, BLS-M2e and BLS-4M2e were expressed in *E. coli* (*DE3*) (Stratagene, La Jolla, CA) competent cells and purified as previously described [26,28] through a Mono-Q column, followed by a Superdex-200 column. The purity of the BLS preparations was determined by 12% SDS-PAGE, and identified by Western blot analysis using a monoclonal antibody anti-BLS [29] or a rabbit serum anti-M2e, obtained by immunization of rabbits with the M2e peptide conjugated to OVA protein. Recombinant proteins were adsorbed with Sepharose-polymyxin B to eliminate LPS contamination. They contained less than 0.05 endotoxin units per 1 mg of protein, as assessed by Limulus Amebocyte Lysate Analysis kit (Sigma, St Louis, MO).

2.2. Stability studies

Circular dichroism (CD) spectra were measured on a spectropolarimeter (JASCO J-810) using a 0.1 path length quartz cells. BLS and BLS chimeras were incubated in 50 mM sodium phosphate, 1 mM DTT, pH 7.0 and monitored by far-UV CD (260–200 nm).

Unfolding of the proteins was monitored by the change in their molar ellipticity at 220 nm as a function of temperature. Thermal denaturation was conducted by slowly increasing the temperature with a Peltier system (JASCO). The range of temperature scanning was 20–100 °C at a speed of 4 °C/min. Molar ellipticity (θ) at 222 nm was measured every 0.2 °C. Fast or slow cooling back to 20 °C (from 100 to 20 °C at a speed of 18 °C/min) did not show a recovery of ellipticity demonstrating the irreversibility of the thermal unfolding. Thus, the temperature midpoint of the thermal transition was considered as an apparent Tm.

The molecular weight of the proteins was determined on a Precision Detectors PD2010 light scattering instrument connected in tandem to a high-performance liquid chromatography system, including a Waters 486 UV detector, and a LKB 2142 differential refractometer. Each protein was loaded on a Superdex 200 column (GE Healthcare Bio-Sciences). BLS-4M2e, BLS-M2e, and BLS were eluted in buffer phosphate containing 0.15 M NaCl. The elution was monitored by the static light scattering (SLS) at 90°, UV absorption at 280 nm, and refractive index (RI) signals and analyzed with the Discovery32 software supplied by Precision Detectors. The molecular weight of each sample was calculated as previously described in detail [30].

2.3. Analysis of humoral immune responses to BLS-M2e chimeras

A peptide with the M2e sequence described above was synthesized at the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy, and the molecular weight was confirmed by mass spectroscopy (courtesy of Dr. S. Tisminetzky).

The adjuvants used in this work were: immune stimulating complex MATRIX Q (IMX®), kindly provided by ISCONOVA AB (0.75 μg per dose); Monophosphoryl Lipid A adjuvant (MPL, Invivogen) (10 μg per dose); α-C-galactosylceramide (αGC), kindly provided by NIH Tetramer Core Facility at Emory University (dissolved in 0.5% Tween 20 in PBS, 1 μg per dose); and aluminum hydroxide (Rehydragel™ HPA, General Chemical) formulated at 15% in PBS.

Six to 8 weeks-old female BALB/c mice (University of La Plata, Argentina) were acclimated and randomly distributed into experimental groups. Mice were maintained in conventional animal facilities in accordance with pertinent Institutional and Federal regulations and policies.

Mice (5 animals per group) were anaesthetized with Isofluorane (Abbot) and immunized by the intramuscular (IM), subcutaneous (SC) or intranasal (IN) route with BLS, BLS-M2e, BLS-4M2e or PBS in the presence or absence of the indicated adjuvant, at days 0, 21 and 42, and bled at 15, 36 and 57 days after the first immunization. The humoral immune responses were evaluated by measuring total specific IgG, IgG1 and IgG2a titers by standard indirect ELISA, in plates coated with 0.3 μg per well M2e synthetic peptide or 0.3 μg per well of recombinant BLS protein. A peroxidase-conjugated polyclonal antibodies to mouse IgG (Dako) plus ABTS was used to measure total specific IgG. For IgG isotypes determination, biotinilated rat anti-mouse IgG2a or anti-mouse IgG1 (BD Pharmingen™) were used followed by addition of avidin-horseradish peroxidase conjugate (BD Pharmingen™) plus ABTS. Each sample was tested in duplicate. Sera obtained from naive animals and assayed in the same plates were used as negative controls. The ELISA endpoint titers were expressed as the reciprocal of the highest serum sample dilution that yielded an OD $\geq$ 3 times the mean value of pre-immune control sera.

2.4. Protection experiments

Mice (5 animals per group) were immunized by SC route with 10 μg of BLS-4M2e, 10 μg of BLS-4M2e formulated with IMX®, 10 μg of BLS-4M2e formulated with aluminum hydroxide or with PBS. Two weeks after the second boost, the mice were anesthetized with a mixture of xylazine (10 mg/kg) and ketamine (100 mg/kg) and challenged by IN inoculation of 15 μl of PBS containing 5 lethal doses (LD_{50}) of A/PR/8/34 influenza A virus (mouse-adapted). Body weight was monitored daily, and mice losing more than 30% of their initial weight were sacrificed and scored as dead as described [8].

2.5. Statistical analysis

Differences between the results obtained in each experimental group were tested for significance by Student's *t*-test.

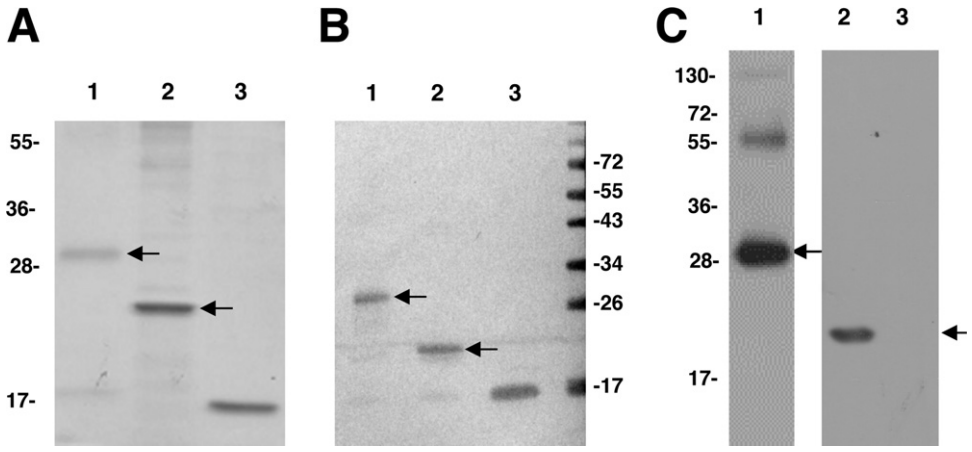

Fig. 1. Expression of the recombinant BLS fusion proteins. (A) SDS-PAGE analysis. The proteins are stained with Coomassie Blue. Lane 1: BLS-4M2e (0.3 μg); lane 2: BLS-M2e (2 μg) and lane 3: BLS (2.5 μg). (B) Western blot of the recombinant chimeras using a monoclonal anti-BLS antibody. Lane 1: BLS-4M2e (0.5 μg); lane 2: BLS-M2e (0.5 μg), and lane 3: BLS (0.7 μg). (C) Western blots analysis of BLS and the chimeras using a polyclonal anti-M2e serum. The arrows indicate the position of the recombinant proteins.

P-values < 0.05 were considered statistically significant. In the Kaplan–Meier curves *P*-values were determined using log-rank test (GraphPad Prism version 4.00 for Windows, GraphPad Software, La Jolla, CA, USA; www.graphpad.com). *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Characterization of the recombinant chimeras

The BLS-M2e and BLS-4M2e chimeras were efficiently expressed in *E. coli*, purified to homogeneity (Fig. 1A), and identified by Western blot analysis using an anti-BLS monoclonal antibody as well as anti-M2e rabbit serum (Fig. 1B and C, respectively). Previous reports have shown that the protection from influenza virus could be significantly increased including more than one copy of the peptide [14,16,31].

The far UV circular dichroism spectra of the BLS-M2e and BLS-4M2e chimeras were almost identical to that of the wild-type protein, indicating that the overall secondary structure did not change following peptide insertion (Fig. 2A).

The agreement in the thermal stability of BLS, BLS-M2e and BLS-4M2e, evaluated by CD spectroscopy, indicates that the domains were properly folded in the structure of the chimeras (Fig. 2B). The

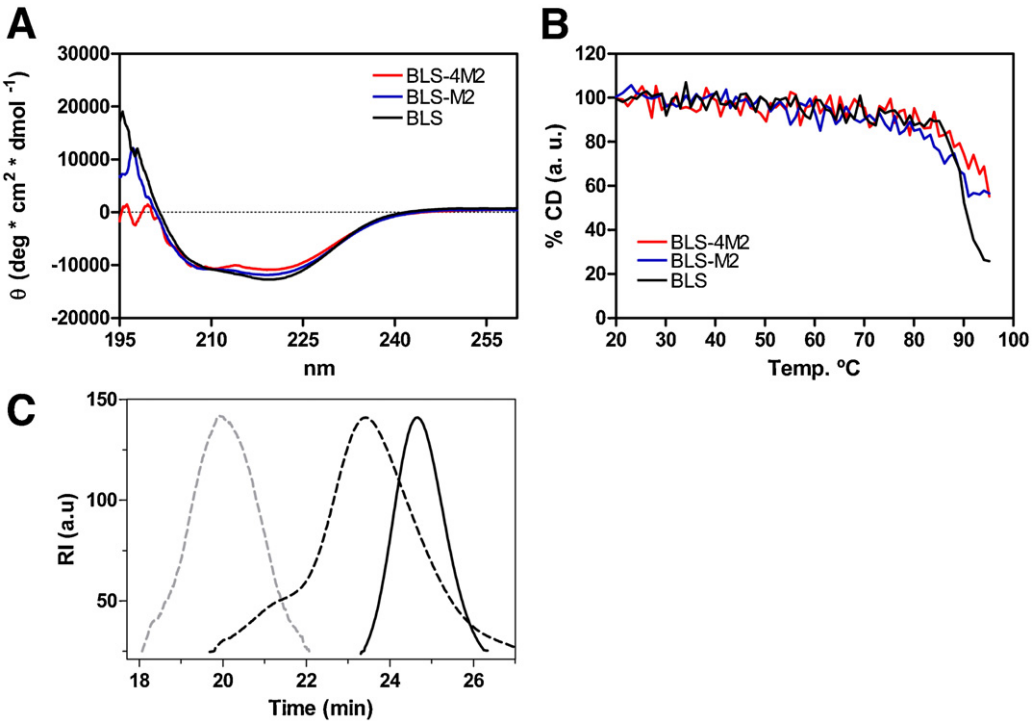

Fig. 2. Stability studies of the carrier BLS and the chimeric BLS plus M2e peptides. (A) The circular dichroism far UV spectra (expressed as molar ellipticity or θ) of BLS (black line), BLS-M2e (blue line) and BLS-4M2e (red line) are superimposable, showing that the chimeras have the same overall secondary structure of BLS. (B) Thermal denaturation of BLS (black line), BLS-M2e (blue line) and BLS-4M2e (red line), monitored by far UV-CD expressed as percent of the signal at 222 nm at 20 °C. (C) Size exclusion chromatography. Each protein was loaded in a Superdex 200 column. The elution was monitored by measuring the light scattering at 90 degree signals, using a flow rate of 0.5 ml/min. The experimental MW of the different proteins was measured as described in Section 2 and the values obtained are: for BLS-4M2e (gray dashed line) 278 kDa (theoretical weight 275 kDa), BLS-M2e (black dashed line) 213 kDa (theoretical 195 kDa) and BLS wt (black continuous line) 198 kDa (theoretical 185 kDa), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

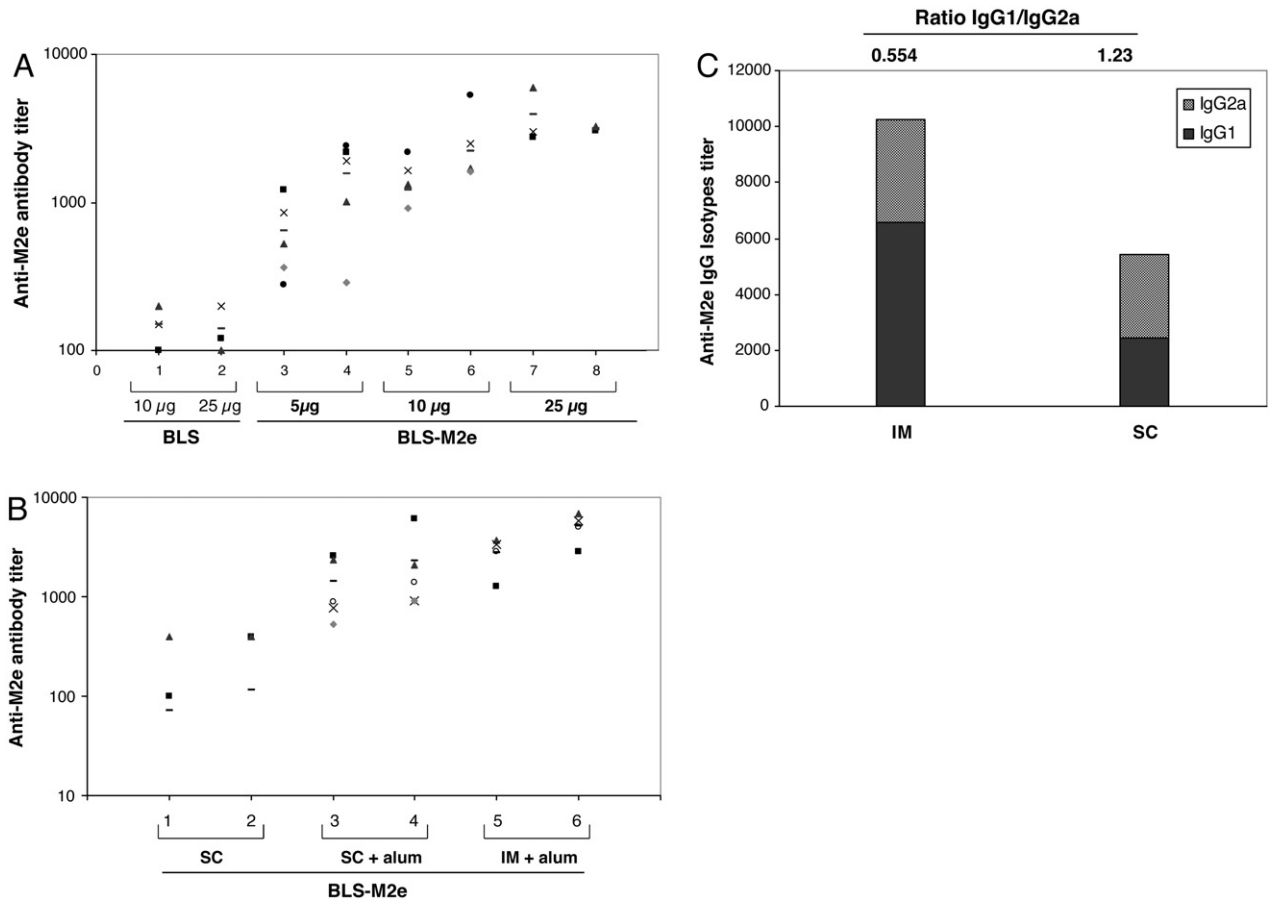

Fig. 3. Detection of M2e specific antibodies in mice immunized with the recombinant BLS-M2e protein. (A) Dose–response curves. BALB/c mice were immunized subcutaneously with 5, 10 or 25 μg of BLS-M2e or BLS protein formulated in aluminum hydroxide and boosted twice at 3-week intervals. Serial dilutions of sera were analyzed by ELISA. Lanes 3, 5, and 7 correspond to the first boost. Lanes 4, 6 and 8 correspond to the second boost. Lanes 1 and 2 correspond to the second boost of mice with 10 or 25 μg of the carrier BLS alone. (B) Different routes of immunization. Groups of mice were immunized by the SC or IM route with 10 μg of BLS-M2e formulated with or without aluminum hydroxide adjuvant, as indicated, and boosted twice at 3-week intervals. The data shown in the panels A and B correspond to individual animals. Lanes 1, 3 and 5, correspond to the first boost. Lanes 2, 4, and 6 correspond to the second boost. The mean values are indicated with a dash (–). (C) IgG1 and IgG2a isotype of the antibodies elicited after inoculation of mice with BLS-M2e by the intramuscular (IM) or subcutaneous (SC) route. Dark gray: IgG1; light gray: IgG2a.

apparent Tm of the thermal transition was approximately 87 °C for the three proteins. The molecular weights determined experimentally by SLS analysis were the expected for 10 molecules of the different chimeras assembled in decameric subunits (Fig. 2C). The values obtained were: for BLS-4M2e, 278 kDa (theoretical weight 275 kDa), BLS-M2e, 213 kDa (theoretical 195 kDa) and BLS wt, 198 kDa (theoretical 185 kDa) respectively.

3.2. Dose–response experiments

Groups of animals were inoculated with 5, 10 or 25 μg of BLS or BLS-M2e formulated in aluminum hydroxide. Fifteen days after the second and third dose, high titers of specific anti-M2e antibodies were elicited in all groups, in a dose-dependent manner, except in the groups vaccinated with the carrier BLS (Fig. 3A). The dose of 10 μg was chosen for subsequent immunization experiments.

3.3. Immune responses elicited in BALB/c mice inoculated by different routes or with different adjuvants

One group of mice received 10 μg of BLS-M2e protein by the SC route, while the second and third groups received 10 μg BLS-M2e formulated with aluminum hydroxide by the SC or IM route, respectively. IM immunization generated higher titers of specific anti-M2e antibodies and more homogeneous responses among individuals (Fig. 3B). The use of alum induced significant higher antibody levels.

IM immunization, resulted in an increased titer of IgG1 antibodies, with a ratio IgG2a/IgG1 of 0.554 suggesting an immune response biased toward Th2 type (Fig. 3C). In contrast, the SC route induced a response with an IgG2a/IgG1 ratio of 1.23, indicative of an antibody response skewed toward a Th1 phenotype. Although, mice infected with influenza virus normally do not generate detectable titers of antibodies recognizing M2, it has been recently reported that in mice vaccinated with M2e chimeric proteins, both kind of M2-specific antibody isotypes were involved in protection against influenza virus [32]. For this reason, the SC route, which in our case induced a more balanced response, was selected for use in the subsequent experiments.

The effect of other recently described adjuvants, such as IMX® and αGC, was tested next. IMX® is an adjuvant capable of inducing significant humoral and cellular immune responses in humans and animals [33] and has been recently tested in human influenza vaccines [34]. αGC is a marine-sponge-derived glycolipid with strong immunomodulatory activities that was successfully tested in other M2e based influenza vaccines [35]. In the case of the BLS-M2e chimera, the inclusion of IMX® had a notable effect in the level of response to immunization, while the addition of αGC was not different from the unadjuvanted chimera (Fig. 4). Control animals immunized with the empty carrier BLS did not show positive titers against M2e (data not shown).

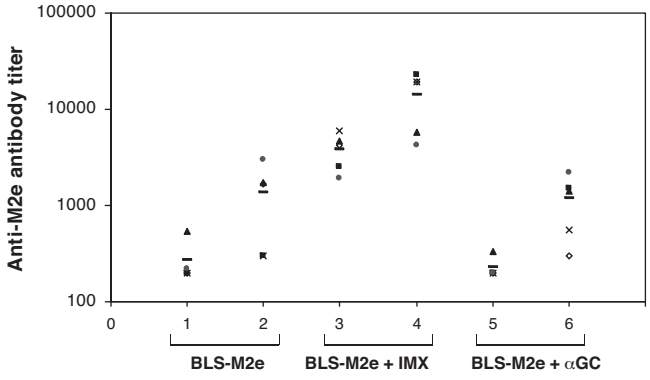

Fig. 4. Influence of the use of different adjuvants in the immune responses to BLS-M2e protein. BALB/c mice were immunized SC with 10 μg BLS-M2e, and boosted twice at 3-week intervals. The chimera was formulated without adjuvant, or adjuvanted as indicated. Levels of specific anti-M2e antibodies (IgG) were analyzed by ELISA in sera collected 2 weeks after the second and third immunization. The data shown correspond to each individual animal. Lanes 1, 3 and 5, correspond to the first boost. Lanes 2, 4, and 6 correspond to the second boost. The mean values are indicated with a dash (–).

Finally, groups of BALB/c mice were inoculated IN with 10 μg of the BLS-M2e subunit, alone or formulated with MPL, IMX® or αGC (described as activators of the mucosal immune system). Sera and saliva collected 2 weeks after the third immunization showed very low ELISA titers in all the conditions tested (data not shown).

Immunization of mice with 10 μg of purified BLS-4M2e or BLS-M2e, without the addition of any adjuvant induced seroconversion in both groups of mice, but the specific anti-M2e antibodies titers were significantly higher in the groups inoculated with the BLS-4M2e chimera (Fig. 5). Antibodies to the carrier BLS in the animals inoculated with BLS-M2e or BLS-4M2e were 1/600,000 and 1/100,000, respectively.

3.4. Protective immune response elicited by the BLS-4M2e chimera

BALB/c mice were vaccinated with 10 μg of BLS-4M2e chimera formulated with alum, IMX® or without any adjuvant. A group vaccinated with PBS and a group of not challenged animals (to provide a control weight curve) were also included. Two weeks after the second boost, the animals were IN challenged with 5 LD_{50} of mouse adapted A/PR/8 influenza A virus. As shown in Fig. 6A, vaccination

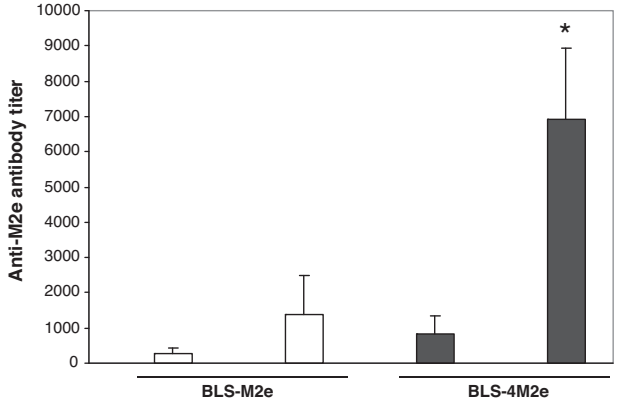

Fig. 5. Antibody responses developed by mice immunized with BLS-M2e or BLS-4M2e chimeras in the absence of adjuvant. Groups of 5 BALB/c mice were immunized with 10 μg of BLS-M2e or BLS-4M2e in PBS and boosted twice at 3-week intervals. Levels of specific anti-M2e antibodies after the first or second boost were analyzed by ELISA. Data are expressed as the mean ± SD. The asterisk denotes significant differences ($P < 0.05$) in the immune response.

of mice with BLS-4M2e chimera alone provided 60% protection. The percentage of protection increased when the chimera was formulated with aluminum hydroxide (80% protection), reaching 100% protection when IMX® was included in the formulation. Average antibody titers before challenge were 1/4260, 1/10,900 and 1/24,931 for groups inoculated with BLS-4M2e, BLS-4M2e + alum and BLS-4M2e + IMX®, respectively. The per cent average body weight loss in each group of mice after challenge is depicted in Fig. 6B. Seven days after challenge, the surviving animals in the 3 groups vaccinated with the chimeras recovered its body weight gradually, approaching the initial weight 16 days post challenge.

4. Discussion

In this work we developed chimeras with the scaffold protein BLS decorated with 10 or 40 copies of a small peptide derived from the M2 protein of influenza, which has been considered a promising candidate for a broadly protective vaccine because antibodies to M2e are protective in animal models and capable of clearing infected cells [15].

Chimeric proteins generated by the fusion of one or four copies of M2e to BLS were assembled as stable subunits, as reported for the wild type BLS enzyme which has been studied either as an adjuvant or as a carrier of antigens derived from several animal pathogens and found to be very efficient to elicit systemic and oral immunity in the absence of other adjuvants [27,36]. These immunological properties of BLS are not related to the enzymatic activity, since a mutant enzymatically inactive retains its carrier capacity (unpublished results).

The humoral immune response elicited by the BLS chimeras in BALB/c mice was dependent on the dose and on the addition of different adjuvants to the formulations. As expected, in similar amounts, the chimera carrying four tandem copies of the M2e peptide elicited higher humoral immune responses in the absence of adjuvant than the chimera carrying only one M2e copy (Fig. 5). However, when the single-copy chimera BLS-M2e was formulated with adjuvants, the humoral immune response increased significantly, ranging from values similar to the unadjuvanted multicopy construct (e.g., 1/8000 with aluminum hydroxide, Fig. 3) to even higher values (1/30,000 with IMX®, Fig. 4). Antibodies to the carrier BLS were always very high, as reported previously for this immunogenic decameric protein [24,26,36].

Vaccination with BLS-4M2e chimera was able to protect BALB/c mice from lethal challenge (Fig. 6). Significant protection was achieved using IMX® (100%) and aluminum hydroxide (80%). The unadjuvanted BLS-4M2e protected 60% of the challenged animals ($P < 0.05$, Fig. 6A). Animal's survival correlated better with antibodies titers. Regarding morbidity, there was a significant difference in terms of loss of body weight between animals inoculated with PBS and animals vaccinated with BLS-4M2e + alum for days 6 and 7 post challenge ($P < 0.05$, Fig. 6B). Of note, recovery of weight was significantly faster in the group adjuvanted with alum than in the group adjuvanted with IMX®, on days 7–10 ($P < 0.05$, Fig. 6B).

In previous studies it was shown that protection induced by the M2e peptide was exclusively mediated by antibodies. In this regard, Jegerlehner et al. [37] reported that vaccination with M2e coupled to hepatitis B core particles induced protective antibodies, whereas the contribution of T cells to protection was negligible. The authors showed that M2-specific antibodies failed to neutralize the virus in vitro and suggested an indirect mechanism of protection such as ADCC. A more recent report using the same antigen [32] showed that antibodies of both IgG1 and IgG2 isotypes were involved in protection of mice from influenza virus. Higher antibody titers of the IgG1 isotype might mediate protection by alveolar macrophages [32]. Other research group reported that the

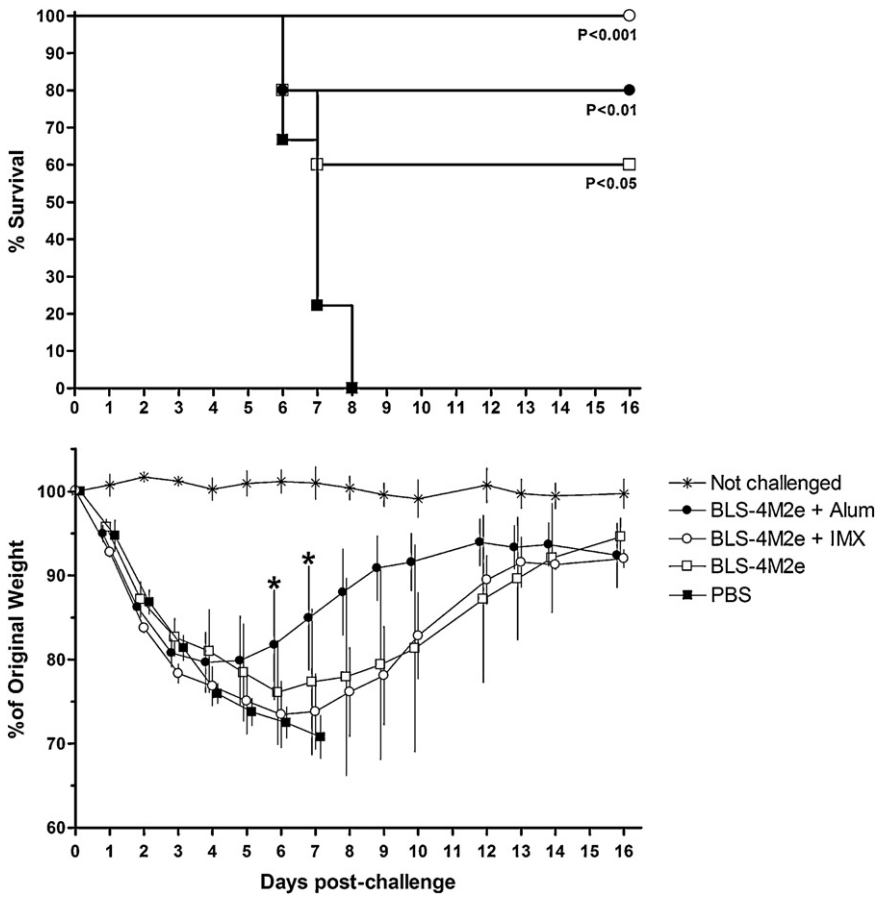

Fig. 6. Protection from challenge with live influenza virus of mice immunized with BLS-4M2e chimera. Groups of five BALB/c mice were vaccinated with the BLS-4M2e chimera, either unadjuvanted, or formulated with IMX® or alum, as well as a group inoculated with PBS. The animals were challenged 2 weeks after the second boost via intranasal inoculation of 5 LD_{50} of mouse adapted A/PR/8/34 influenza A virus. (A) Survival after challenge. Mice vaccinated with BLS-4M2e chimeras were significantly protected compared with mock vaccinated mice. A 100% protection was achieved with BLS-4M2e + IMX® (log-rank test, $P < 0.001$); 80% protection with BLS-4M2e + alum (log-rank test, $P < 0.01$) and 60% with non adjuvanted BLS-4M2e (log-rank test, $P < 0.05$). (B) Weight loss after challenge. Significant differences in morbidity were found between mice vaccinated with PBS or vaccinated with BLS-4M2e + alum in days 6 and 7 after challenge (t-test, $P < 0.05$, indicated by asterisks). Recovery of weight in the group vaccinated with BLS-4M2e + alum was significantly faster at days 7, 8, 9 and 10 pc ($P < 0.05$). A group of not challenged animals was included to provide a control weight curve. SDs are indicated per day and per group.

protection awarded by M2e-based vaccines might be mediated by NK cells via antibody-dependent cytotoxicity (ADCC) or complement-mediated cytotoxicity [37].

In the present study, the SC immunization route induced a response with an IgG2a/IgG1 ratio of 1.23, indicative of a more balanced antibody response (Fig. 3). As antibodies of the IgG2 isotype have been shown to be the most important mediators of ADCC in mice [38], the results obtained in this work after SC immunization and challenge suggest that both kinds of immune mechanisms may be eventually involved [31,37].

Most of the conjugated M2e-based vaccines reported have shown to be effective when applied with adjuvants that might induce serious secondary effects [39]. The approach presented herein is attractive because it employs an antigen generated through a simple production and purification process, exposed by a strong carrier, which might be used with safe adjuvants, such as alum or IMX®, or eventually alone. Correlates of protection for this type of vaccines are still lacking in the Pharmacopeia.

The plasticity of the BLS scaffold for the production of polyvalent chimeras, suggest that M2e peptides from influenza viruses presenting slight differences in amino acid sequences may be coupled to BLS to elicit antibodies with broader specificity, including conserved peptides derived from other influenza proteins.

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