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IMXQB-80: A *Quillaja brasiliensis* saponin-based nanoadjuvant enhances Zika virus specific immune responses in mice

Samuel Cibulski [a,b,1], Thais Fumaco Teixeira [b,1], Ana Paula Muterle Varela [b,1], Matheus Fabião de Lima [a], Gabriela Casanova [c], Yuri Mangueira Nascimento [d], Josean Fechine Tavares [d], Marcelo Sobral da Silva [d], Patrícia Sesterheim [e], Diogo Onofre Souza [f], Paulo Michel Roehe [b], Fernando Silveira [g,*]

[a] Centro de Biotecnologia – CBiotec, Laboratório de Biotecnologia Celular e Molecular, Universidade Federal da Paraíba, João Pessoa, Paraíba, Brazil
[b] Departamento de Microbiologia Imunologia e Parasitologia, Laboratório de Virologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Porto Alegre, Rio Grande do Sul, Brazil
[c] Unidad de Microscopía Electrónica de Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
[d] Institute for Research in Pharmaceutical and Medications, Universidade Federal da Paraíba, João Pessoa, Paraíba, Brazil
[e] Centro de Cardiologia Experimental, Instituto de Cardiologia/Fundação Universitária de Cardiologia, Porto Alegre, RS, Brazil
[f] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[g] Departamento de Desarrollo Biotecnológico, Instituto de Higiene, Facultad de Medicina, Universidad de la República (UdelaR), Montevideo, Uruguay

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ABSTRACT

Vaccine adjuvants are compounds that enhance/prolong the immune response to a co-administered antigen. Saponins have been widely used as adjuvants for many years in several vaccines – especially for intracellular pathogens – including the recent and somewhat revolutionary malaria and shingles vaccines. In view of the immunoadjuvant potential of *Q. brasiliensis* saponins, the present study aimed to characterize the QB-80 saponin-rich fraction and a nanoadjuvant prepared with QB-80 and lipids (IMXQB-80). In addition, the performance of such adjuvants was examined in experimental inactivated vaccines against Zika virus (ZIKV). Analysis of QB-80 by DI-ESI-ToF by negative ion electrospray revealed over 29 saponins that could be assigned to known structures existing in their congener *Q. saponaria*, including the well-studied QS-21 and QS-7. The QB-80 saponins were a micrOTOF able to self-assembly with lipids in ISCOM-like nanoparticles with diameters of approximately 43 nm, here named IMXQB-80. Toxicity assays revealed that QB-80 saponins did present some haemolytical and cytotoxic potentials; however, these were abrogated in IMXQB-80 nanoparticles. Regarding the adjuvant activity, QB-80 and IMXQB-80 significantly enhanced serum levels of anti-Zika virus IgG and subtypes (IgG1, IgG2b, IgG2c) as well as neutralized antibodies when compared to an unadjuvanted vaccine. Furthermore, the nanoadjuvant IMXQB-80 was as effective as QB-80 in stimulating immune responses, yet requiring fourfold less saponins to induce the equivalent stimuli, and with less toxicity. These findings reveal that the saponin fraction QB-80, and particularly the IMXQB-80 nanoadjuvant, are safe and capable of potentializing immune responses when used as adjuvants in experimental ZIKV vaccines.

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1. Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus that, during recent epidemics, has been implicated in the devastating congenital zika syndrome (CZS), a condition associated to microcephaly, congenital malformations, fetal demise, Guillain-Barré syndrome (GBS) and other severe neurological disorders [1,2]. Due to the explosive character of the epidemics, ZIKV infection was declared a public health emergency of international concern by the World Health Organization (WHO) in 2016 [3]. Since then, intensive global efforts have been made to understand ZIKV biology and to develop prophylactic measures, including the development of vaccines. In consonance with this trend, a vaccine portfolio has been developed which includes several ZIKV vaccine candidates based on inactivated or attenuated virus, nucleic acids and subunit-based preparations [4].

Effective vaccines against intracellular pathogens (such malaria, tuberculosis, or viral infections) requires both humoral immunity and cellular immunity for clearance of infected cells [5,6]. Thereby, adjuvants have been added to vaccine formulations aiming to enhance the speed and magnitude of immune stimulus, reduce of

* Corresponding author.
E-mail address: fernandosilveiragonzalez@gmail.com (F. Silveira).
[1] These authors contributed equally to the work.

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antigen dose, increase cross-protection and improve response in specific populations (elderly), targeting of adaptive immune responses to antigens.

In this sense, limitations of aluminium salts (the most widely used adjuvant in human and veterinary vaccines) that are unable to elicit cellular responses against intracellular pathogens [7], have driven the development of new alternative compounds. As such, triterpenoid saponins, such as Quil A®, extracted from *Quillaja saponaria* Molina, have been often employed as adjuvants for many years in several vaccines of veterinary use [5]. However, saponins have several drawbacks associated to the natural origin of the product, such as its chemical instability, scarcity, heterogeneity, dose-limiting toxicity and poorly understood mechanism of action; these have limited the wider usage of such compounds as adjuvants [5,6].

In this context, saponins isolated from *Quillaja brasiliensis* (A. St.-Hil. et Tul.) Mart, a native tree from southern Brazil and Uruguay, have been thoroughly studied by our team as a promising alternative to the development of new vaccine adjuvants. The saponin fractions, named QB-90 and QB-80, have confirmed its potential as immune adjuvants when added to a number of distinct viral antigens in murine models [8–13]. Additionally, these fractions show substantial chemical similarity to Quil A® saponins [8,14–17], with the advantage of inducing fewer toxic effects than Quil A® [8,11,13].

The main drawback of the use of saponins as adjuvants is its toxicity. However, this can be reduced by using lipid-based delivery systems [18]. One of such preparations, called immunostimulating complexes (ISCOM) consists of 40 nm cage-like self-assembled nanostructures combining Quil A® with cholesterol, phospholipids and antigen [19]. A similar formulation, named ISCOMATRIX™, is another nanoparticulate adjuvant preparation which does not contain the target antigen [20,21].

Recently, we reported an alternative ISCOM or ISCOM-matrix-like nanoadjuvants replacing Quil A® by QB-90 (IQB-90 or IMXQB-90). These nanoadjuvants were shown to trigger early immune responses with long-lasting induction of antibodies, while stimulating the generation of cytotoxic T lymphocytes and a balanced Th1/Th2 response in mice [22,23]. Here, in view of immunoadjuvant potential of *Q. brasiliensis* saponins, we aimed to characterize QB-80 and a nanoadjuvant prepared with it (IMXQB-80). These were evaluated in its performances as immune adjuvants in experimental inactivated vaccines against ZIKV.

2. Material and methods

2.1. Virus and cells

ZIKV strain 17 SM, isolated from a patient in São Paulo city, Brazil [24], was propagated in Vero cells (CRL-1586, originally obtained from ATCC), and quantified by standard plaque assays. Viral stocks were stored at −80 °C until use. The cells were expanded in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (penicillin 100 U/mL; streptomycin 100 μg/mL) (Gibco, USA). Cells were grown at 37 °C in an atmosphere of 5% CO_2 and were used for virus multiplication and neutralization tests.

2.2. Q. brasiliensis QB-80: Isolation and partial characterization

Q. brasiliensis (A. St.-Hil. et Tul) Mart. leaves were collected in Canguçu, RS, Brazil (31°23′42″ S-52°40′32″ W) (voucher ICN 142953, deposited at the Herbarium of the Federal University of Rio Grande do Sul). Extraction and purification of saponins were carried out as previously described [25].

For saponin identification, a direct injection (DI) followed by electrospray ionization (ESI) and Time-of-Flight detection (DI-ESI-ToF) were applied on a micrOTOF II high-resolution mass spectrometer (Bruker, USA). Spectrometer analysis parameters were: 4.0 kV capillary, ESI in negative ion mode, a 500 V end plate offset, a 40.6 psi nebulizer, a dry gas (N_2) flow rate of 8.0 L/h and a temperature of 300 °C. Spectra (m/z 50–3,000) were recorded every 2.0 s. The ESI Tuning mix® (Sigma Aldrich, USA) was used as the internal calibrator.

2.3. IMXQB-80 nanoadjuvant: preparation and characterization

IMXQB-80 nanoadjuvant were prepared by the modified ethanol injection technique [22,26]. Briefly, an ethanol-dissolved cholesterol (Sigma-Aldrich, USA) and di-palmitoylphosphatidyl choline (Avanti Polar Lipids, USA) were rapidly injected into a phosphate-buffered saline containing QB-80 (1 mg/mL), which were finally stirred during 48 h at 4 °C. The nanoadjuvant so obtained were named IMXQB-80 and characterized as follows.

The hydrodynamic diameter of the nanoadjuvant was evaluated using dynamic light scattering (DLS) performed using a Malvern Zetasizer System (Malvern, USA), vertically polarized coherent He-Ne laser as light source (λ = 632.8 nm). Zeta potential was measured by laser-Doppler electrophoresis (LDE) in the above described instrument. Zeta potential and the size distribution were stated as an average of ten measurements. All measurements were carried out at 25 ± 0.1 °C.

Furthermore, the preparation was visualized by transmission electron microscopy (TEM). For this, 10 μL of the nanoadjuvant was placed on a copper grid covered with a formvar and carbon film for 2 min, after which the remaining liquid was drained, and the sample was negatively stained with uranyl acetate (2% w/v) for 2 min. The analysis was performed in a Jeol JEM 1010 transmission electron microscope at an accelerating voltage of 80 kV and at magnifications between 30,000 and 150,000X.

2.4. Toxicity assays

The haemolytic activity of saponins is a major indicative of cytotoxicity. QB-80 and the nanoadjuvant derived from QB-80 (IMXQB-80) were tested over a range of concentrations for induction of haemolysis as previously described [11,22]. Physiological saline solution and *Q. saponaria* saponins (Sigma Aldrich, USA) at 250 μg/mL were used as indicators of 0% and 100% haemolysis, respectively. Samples were tested in triplicate in V-bottom microtitre plates. The haemolytic activity was expressed as the endpoint concentration capable of inducing haemolysis in 50% of the RBCs (HD_{50}).

Cytotoxicity was determined using the MTT assay. Briefly, L-929 cells (originally ATCC CCL-1) seeded at a concentration of $3.0x10^4$ per well on 96-well microplates and maintained at 37 °C under a humid atmosphere with 5% CO_2. After 24 h, different concentrations of QB-80 or IMXQB-80 were added to each well in triplicate. The plates were incubated as above; after 24 h, 10 μL of 5 mg/mL MTT (Sigma Aldrich, USA) were added to each well and the cells were incubated for a further 4 h and revealed and analyzed as described [22].

The toxicity of QB-80 and IMXQB-80 was further evaluated in the *Artemia salina* (brine shrimp) lethality test as previously described [27]. *A.* salina encysted eggs (10 mg) (Maramar, Brazil) were incubated in 500 mL of sterile seawater (pH 7,4) under artificial light at 28 °C. After incubation for 48 h, nauplii were collected with a Pasteur pipet. QB-80 and IMXQB-80 samples were dissolved in seawater separately as follows. Aliquots of seawater (100 μL)

were placed in each well of a 96-well microtiterplate. Subsequently, 100 μL aliquots of each of the QB-80 or IMXQB-80 were added and serially (two-fold) diluted. The last row was left with seawater only as a control. One hundred microliters of a suspension containing on average 10 nauplii was added to each well. Plates were then incubated at room temperature and examined after 24 h. Dead larvae in each well were counted by stereomicroscopic examination. Lethality was expressed as LD_{50}. HD_{50}, IC_{50} (for cytotoxicity assay) and LD_{50} values were generated using a variable-slope sigmoidal dose–response computer model in a GraphPad Software (version 8, USA).

2.5. Production of ZIKV antigen

For antigen production, ZIKV was inoculated in Vero cells with a multiplicity of infection (m.o.i.) of 0.001 and collected after 5–7 days. The suspension obtained was clarified using low seed centrifugation and filtered through a 0.45 μm filter. Viral particles were subsequently purified by ultracentrifugation at 100.000 × g on a 20% sucrose cushion. The pellet containing ZIKV virions was inactivated with 0.05% formalin at 22 °C for 7 days. The formalin was then removed by extensive dialysis. Antigen concentration was then carried out using Amicon Ultra Centrifuge Filters (molecular weight cut-off of 30 K, Sigma-Aldrich, USA). Antigen inactivation was confirmed by three passages in Vero cells. The amount of protein antigen in purified inactivated ZIKV-vaccine (PIV ZIKV) was quantified by fluorimetry in a Qubit apparatus (ThermoFisher Scientific, USA) using the Qubit protein assay kit.

2.6. Vaccine formulation and mice immunization

Five to six weeks old female C57BL/6J mice were purchased from Centro de Modelos Biológicos Experimentais (CeMBE/PUCRS) and acclimatized for 72 h prior to use. Mice were maintained under controlled temperature. Food and water were provided *ad libitum*. All procedures were carried out in strict accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health (USA) and National Council for Animal Experimentation Control (Brazil). Project was approved by institutional Animal Care and Use Committee (CEUA) under protocol UP 5402/17.

The experimental vaccines were prepared under aseptic conditions, filtered through 0.22 μm and kept at 4 °C until administration. Mice were divided into five groups (each with nine animals) and were inoculated subcutaneously (in the hind neck) twice, on days 1 and 14, with 5 μg of PIV ZIKV antigen plus 100 μL saline (no adjuvant group), or with either alum (Alhydrogel®, InvivoGen, USA, 100 μg), QB-80 (10 μg) or IMXQB-80 (2.5 μg). Saline-treated animals were included as controls. The concentration of IMXQB-80 (2.5 μg) used in this study was defined as saponin concentration in nanoadjuvant. Mice were bled immediately before immunizations on days 0, 14 and 28.

2.7. Determination of ZIKV-specific antibodies by enzyme linked immunosorbent assay (ELISA)

ZIKV antigen of ELISA was produced by inoculating Vero cells (150 cm^2 bottles) with ZIKV (m.o.i. 0.001). When cytophatic effect reached ~70% of the monolayer, the supernatant was removed, and the monolayer washed twice with PBS. This was then treated with 5 mL of 0.1% of octyl β-D-glucopyranoside (OGP, Sigma-Aldrich, USA) at 4 °C for 2 h with slight agitation. The soluble fraction was then recovered by low-speed centrifugation. The protein content was quantified by fluorimetry (Qubit, Fisher-Scientific) and the preparation stocked at −80 °C. For the assays, the antigen was diluted in PBS (pH 7.2) and 96 well high-binding plates (Greiner Bio-One, Germany) were sensitized with 100 μL volumes of antigen (4 μg/mL) at 4 °C, overnight. Plates were then washed three times with PBS containing 0.05% Tween®-20 (PBS-T20, Sigma-Aldrich, USA) and blocked with BSA fraction V (1% in PBS-T20) (Sigma-Aldrich, USA) at 37 °C for 1 h.

Appropriately diluted samples in PBS-T20 were added to wells in duplicate (100 μL/well) and incubated for 1 h at 37 °C. After washing three times with PBS-T20, anti-mouse HRP-conjugates to IgG (Sigma-Aldrich, USA), IgG1, IgG2b and IgG2c (both from BioLegend, USA) diluted in PBS-T20 (1:10,000) were added to each well (100 μL/well); plates were incubated for 1 h at 37 °C. After three washings, 100 μL of OPD (*ortho*-phenylenediamine, Amresco, USA) with 0.003% H_2O_2 were added to wells, and plates further incubated for 15 min at 37 °C. Reactions were stopped with 50 μL/well of 1 N HCl. Optical densities (OD) were measured in an ELISA plate reader (SpectraMax, Molecular Devices, USA) at 492 nm. A pool of positive sera was used to draw a standard curve. Samples were tested in duplicate, at two dilutions. Antibody titres were expressed in arbitrary units per mL (AU/mL).

2.8. Determination of neutralizing antibodies by plaque reduction neutralization (PRNT) assay

Neutralizing antibody titers were determined by plaque reduction neutralization test (PRNT) in Vero cells cultured in 6-well plates. Sera were heat inactivated at 56 °C for 30 min and pooled by group. Sera pools were tested at 1:10 dilution (in DMEM), mixed with an equal volume of 50 PFU/well of ZIKV17 SM and incubated at 37 °C for 1 h. Serum-virus mixture was then added to Vero cells in duplicate and incubated at 37 °C for 1 h. After, serum-virus mixture was removed, and cells were overlaid with DMEM containing 1% agarose and incubated for 72 h. The plates were stained with violet crystal and counted. Neutralizing antibody titres were expressed as percentages of plaque reduction. The experiments were performed three times.

2.9. Statistical analyses

Data were analyzed using Kruskal-Wallis test with Dunn's post-test. Analyses and plots were performed using the GraphPad Prism version 8 (GraphPad Software, USA). Results are expressed as the median value from individuals in each group ± SEM. A P value equal or less than 0.05 was considered statistically significant.

3. Results

3.1. Characterization of QB-80 saponins by DI-ESI-ToF

Initially, the saponin rich fraction QB-80 was investigated by DI-ESI-TOF in the negative ion mode. Presumptive identification of saponins detected in QB-80 fraction is carried out based on its homologous, well characterized, saponins from *Q. brasiliensis* and *Q. saponaria*. The full scan of DI-ESI-MS is shown in Fig. 1. Using this approach, it was possible to identify 29 saponins in the QB-80 preparation (based in the m/z value) (Table 1).

3.2. QB-80, cholesterol and di-palmitoylphosphatidyl choline self-assemblies in ISCOM-like nanoadjuvant

In this work we used another fraction of active saponins from *Q. brasiliensis* (QB-80) combined with cholesterol and phospholipid under controlled conditions to obtain IMXQB-80, which also

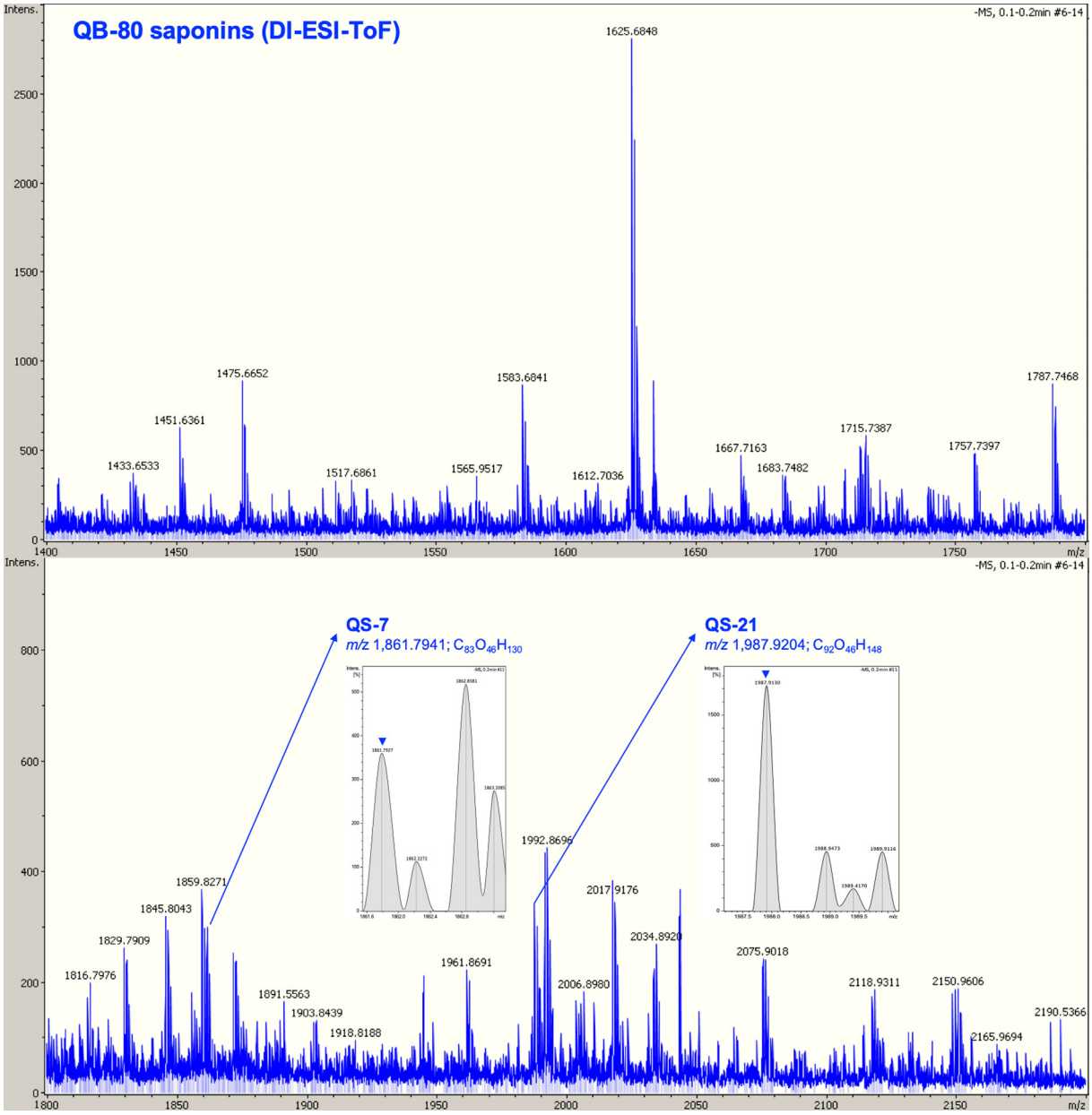

Fig. 1. QB-80 saponins profile obtained using DI-ESI-ToFusing a high-resolution mass spectrometer. For presumptive saponin identification a DI-ESI-ToF strategy were applied using a micrOTOF II high-resolution mass spectrometer. Spectra (*m/z*1, 400–1,800, figure top and 1,800–2,200, figure bottom), recorded every 2.0 s was shown. In detail, two remarkable *Q. saponaria* saponins that could be identified in QB-80 fraction (QS-21 and QS-7). The experiments were performed three times.

formed cage-like structures (ISCOM-matrices). IMXQB-80 structures were examined by dynamic light scattering (DLS) (Fig. 2A) and transmission electron microscopy (TEM) (Fig. 2B). The average hydrostatic diameter of the cage-like structures was 104.5 nm (±0.82 nm) (Fig. 2A), that is, larger than ISCOM-like structures previously documented [28]. The polydispersity was low (0.220), indicating a homogeneous size distribution in IMXQB-80. Measurements of zeta potential by LDE indicated that IMXQB-80 nanoadjuvant have an overall negative surface charge (zeta potential = −13.65 ± 0.212 mV).

The ISCOM-like structure of IMXQB-90 nanoadjuvant was confirmed by TEM (Fig. 2B). The average diameter observed was 43.6 nm (± 3.77 nm). In most fields, the ~ 40 nm nanoadjuvant were evident; however, different populations of micellar formulations of varying sizes and shapes could also be observed (mainly aggregates).

3.3. The haemolytic and cytotoxic activity of QB-80 is abrogated in IMXQB-80 nanoadjuvant

The haemolytic activity of QB-80 saponins (HD_{50} of 88.7 μg/mL) was confirmed here (Fig. 3A). However, the haemolytic activity with the IMXQB-80 nanoparticulate adjuvant, was abrogated (Fig. 3A).

The cytotoxic potentials of QB-80 and IMXQB-80 were evaluated in L-929 cells with the MTT assay. The QB-80 saponins showed moderate cytotoxic potential (IC_{50} of 69.7 μg/mL), where as IMXQB-80 showed no measurable toxicity (Fig. 3B).

The brine shrimp lethality assay was also used as a preliminary assessment of toxicity [29]. In this assay, QB-80 saponins showed a moderate toxicity (LD_{50} of 726.7 μg/mL), whereas IMXQB-80 revealed no toxicity, as no decrease in nauplii survival rate was observed (Fig. 3C).

Table 1
QB-80 saponins tentatively identified by DI-ESI-ToF.

$[M-H]^-$ (m/z)	*Q. saponaria* or *Q. brasiliensis* homologous saponin
1433,6527	S13, 24
1435,6631	4
1475,6632	S13, 24
1517,6860	S13, 24
1567,6853	11b, 18a, 23
1583,6823	11b, 23
1597,6879	13a, 23
1625,6903	15b
1627,6983	13a, 23
1655,6961	20a
1667,7144	15b
1699,7419	8, 10
1713,7596	B4/B6, 27–29, 13b, 9/7, 30
1715,7353	13b, 23
1745,7459	14b, 23
1757,7283	13b
1787,7390	16b, 23, 21a
1829,7683	16b, 23, B4/B6, 27–29
1855,9005	S4/S6, S2
1861,7941	18b,23, QS7
1871,8772	S4/S6, 20, 21
1875,8379	B4/B6, 27–29, 18a, 23
1901,8711	B4/B6, 27–29
1903,8538	18b, 23
1917,8012	18a, 23
1961,8582	18a,23
1987,9204	S4/S6, QS21
2017,9311	B2, B8
2149,9707	B4, B6, QS18

Presumptive identification of QB-80 saponins was carried out based on m/z value of homologous *Q. saponaria* and *Q. brasiliensis* saponins [15,16,31].

3.4. Immunization to ZIKV with adjuvanted preparations (QB-80 or IMXQB-80) induce high titres of anti-ZIKV IgG, including neutralizing antibodies

The immunization schemes designed for the experimental vaccines used in the present study are shown in Fig. 4A. The vaccines were administered subcutaneously with one of following formulations: containing 5 μg/dose antigen (PIV ZIKV) adjuvanted with either QB-80, IMXQB-80 or alum. These were compared to the immune stimulating effect of inactivated ZIKV vaccine with no adjuvant (PIV ZIKV).

The levels of anti-ZIKV IgG antibodies fourteen days after the priming dose of the preparations containing saponins (QB-80 or IMXQB-80) were significantly enhanced compared to control group (no adjuvanted PIVZIKV) ($P < 0.001$, $P < 0.01$). However, this effect was not observed in the group of mice that received the alum-adjuvanted vaccine (Fig. 4B, light colors). After boostering (28 days post-priming), levels of anti-ZIKV IgG were significantly enhanced by QB-80 and IMXQB-80 (Fig. 4B, dark colors). In particular, IgG titres in groups that received either QB-80 or IMXQB-80-adjuvanted vaccines were significantly higher in relation to control group ($P < 0.0001$, $P < 0.001$, respectively), yet higher than those that received the alum-adjuvanted formulation group ($P < 0.05$) (Fig. 4B, dark colors). Animals which were inoculated with saline only had no detectable levels of antibodies to ZIKV (data not shown).

Regarding IgG1 (Th2-related isotype), groups that received QB-80 and IMXQB-80 adjuvanted preparations showed significant differences when compared to the control group ($P < 0.001$, $P < 0.0001$) (Fig. 4C). IgG1 titres in the alum-based vaccine showed the same behavior as the control group that received no adjuvant ($P = 0.2636$). Mice immunized with QB-80 or IMXQB-80 showed a similar increase of serum titres of IgG2b and IgG2c (Th1-related isotypes) which were significantly higher than those obtained for the control group and alum-group (Fig. 4DE).

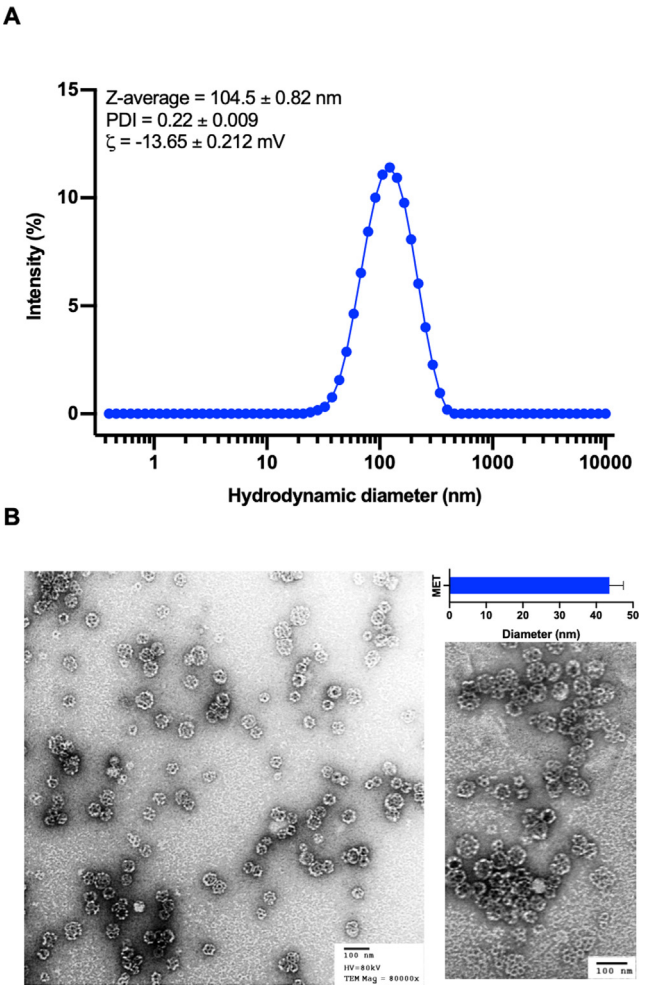

Fig. 2. IMXQB-80nanoadjuvant characterization. (A) DLS measurements of average hydrodynamic diameter, polydispersity index, and zeta potential of IMXQB-80 nanoadjuvant. (B) TEM images of IMXQB-80. Images were obtained after negative staining with uranyl acetate. In detail, a plot indicates the median size and SD of IMXQB-80 nanoadjuvant.

The neutralizing antibody responses were examined by PRNT. Potent neutralizing activity (>80%) was induced by the two vaccines with saponin-based adjuvants (QB-80 and IMXQB-80; Fig. 5). These were of the same magnitude as those induced by the alum adjuvanted vaccine (Fig. 5).

4. Discussion

The development of vaccines capable of triggering powerful immune responses with minimal side effects, yet effective for all age groups (e.g. elderly population) represents a major challenge. The addition of adjuvants to vaccines is one of the strategies which have been employed to enhance immunogenicity of vaccines [30]. However, mostly due to residual toxicity of adjuvant preparations, just a few adjuvants have been licensed to date for use in human vaccines. Saponin-based adjuvants (SBA) have been proposed as an alternative to classical (alum-based) adjuvants for the design of new vaccines [5,6].

Here, we show that QB-80, a *Quillaja brasiliensis* saponin fraction present saponins structures analogous to those detected in

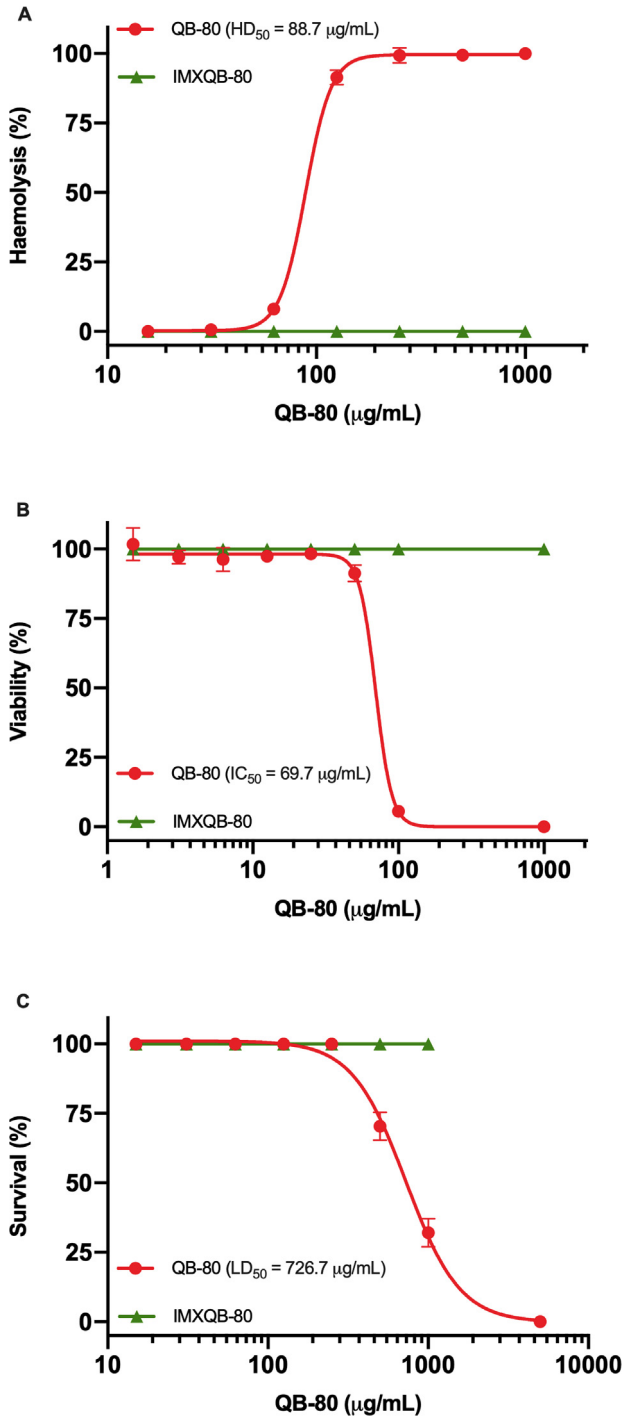

Fig. 3. Toxicity assays. (A) Haemolytic activity of QB-80 and IMXQB-80. Haemolysis was expressed as percentual in relation to saline (0%) and *Q. saponaria saponins* (250 μg/mL; 100%). (B) Cytotoxicity of QB-80 and IMXQB-80 were evaluated on L-929 cells. Cell viability was measured by MTT assay 24 h after treatment. (C) Brine shrimp lethality assay. Nauplii were exposed to different concentrations of QB-80 and IMXQB-80. After 24 h, dead nauplii were counted and expressed as % survival. All results are presented as the mean value ± SD. HD_{50}, IC_{50} and LD_{50} were calculated and plotted in GraphPad Prism 8.

Q. saponaria fractions [15,16]. In addition, the results presented here are in line with previous results reported by our group, regarding chemical similarities between QuilA® and *Q. brasiliensis* fractions [13,14]. In QB-80 fraction, two remarkable *Q. saponaria* saponins could be identified: QS-21 (*m/z* 1,987.9204; molecular formulae $C_{92}O_{46}H_{148}$) and QS-7 (*m/z* 1,861.7941; molecular formulae $C_{83}O_{46}H_{130}$) (Fig. 1). These saponins are well characterized compounds known to possess potent adjuvant features, inducing strong antibodies and $CD8^+$ CTL responses to many antigens. Additionally, it has a lower lytic activity over red blood cells and the two saponins produce a synergistic response when combined at suboptimal doses [31]. Recently, two vaccines with SBAs were licensed for human use (Shingrix™ and Mosquirix™), and many are enrolled in clinical trials. Both contain in its formulations QS-21 [6], a saponin found in *Q. saponaria* and *Q. brasiliensis*.

Despite the fact that the full structure characterization of these myriad of plant metabolites is yet to be elucidated, the results obtained in this work by DI-EDI-ToF and previously by MALDI-ToF MS (matrix-assisted laser desorption/ionization) analyses [13], support and reinforces a sharing of saponins between two species of Quillajaceae. Indeed, DI-ESI-ToF, as well as, MALDI-ToF MS analysis are a rapid and cost-effective tools, which should be further explored as it may contribute to obtain standards and achieve reproducible protocols for extraction, purification and characterization of *Q. brasiliensis* saponins. Taken together, these results are particularly relevant since they contribute to elucidate the constituents of QB-80 bioactive saponin fraction.

The effectiveness of saponins from *Quillaja* genus as an adjuvant is impressive good [5]. However, as mentioned above, one critical issue regarding its use as vaccine adjuvants is potential to induce toxicity. It has been reported that saponins displaying high affinity to cholesterol are able to create pores in mammalian cell membranes, thus contributing to cells damage [31]. In attempting to mitigate such effects different micellar formulations have been developed. ISCOM and ISCOM-matrices, as well as other micellar liposome-based formulations (AS01) were shown to be capable of inhibiting interactions between saponins and membranes, to abrogate haemolytic activity, to reduce toxicity and adverse reactions and, at the same time, to enhance the immune responses [18,21,30,32]. Previously, we reported a formulation of ISCOM and ISCOM-matrices using the QB-90 [33,34] and now, we shown similar ISCOM-like nanoparticles assembled with the saponin-rich QB-80 fraction (IMXQB-80). Regarding the toxicity of IMXQB-80 nanoformulation, the abrogation of haemolytic activity of QB-80 was achieved when nanoformulated (Fig. 3A). The same effect was observed with respect to the viability of the L-929 cells (Fig. 3B) and the brine shrimp lethality assay (Fig. 3C). Thus, it seems that IMXQB-80 nanoadjuvant, as formulated here, were able to somehow prevent possible *in vivo* toxicity.

Using dynamic light scattering analysis, the average hydrostatic diameter of cage-like structures was 104.5 nm (Fig. 2A), superior to expected ISCOM-like structures [28]. However, the expected ISCOM-like structure of IMXQB-80 nanoadjuvant was confirmed by TEM. In most fields, the ~40 nm nanoparticles were evident (Fig. 2B); however, different populations of micellar formulations varying sizes and shapes could also be observed (mainly aggregates). It is known that information from TEM images often do not corroborate with data obtained from DLS as the latter is an intensity-based technique, whereas TEM is a number-based one, making them fundamentally different [35,36]. While samples for DLS are solvated, TEM works on dry samples under ultrahigh vacuum conditions. Furthermore, DLS measures hydrodynamic radius of dispersed particles whereas TEM provides projected surface area based on the amount of incident electrons transmitted through the sample. Therefore, the size obtained by DLS is usually bigger than TEM [37].

Effective vaccines for intracellular pathogens, as ZIKV, must elicit a diverse repertoire of antibodies, including a broad, persistent induction of neutralizing antibodies [38]. Our results show that QB-80 and IMXQB-80 are also able to elicit high levels of anti-ZIKV antibodies, including antibodies with neutralizing activities. The levels of IgG, IgG1, IgG2b and IgG2c detected after vaccination

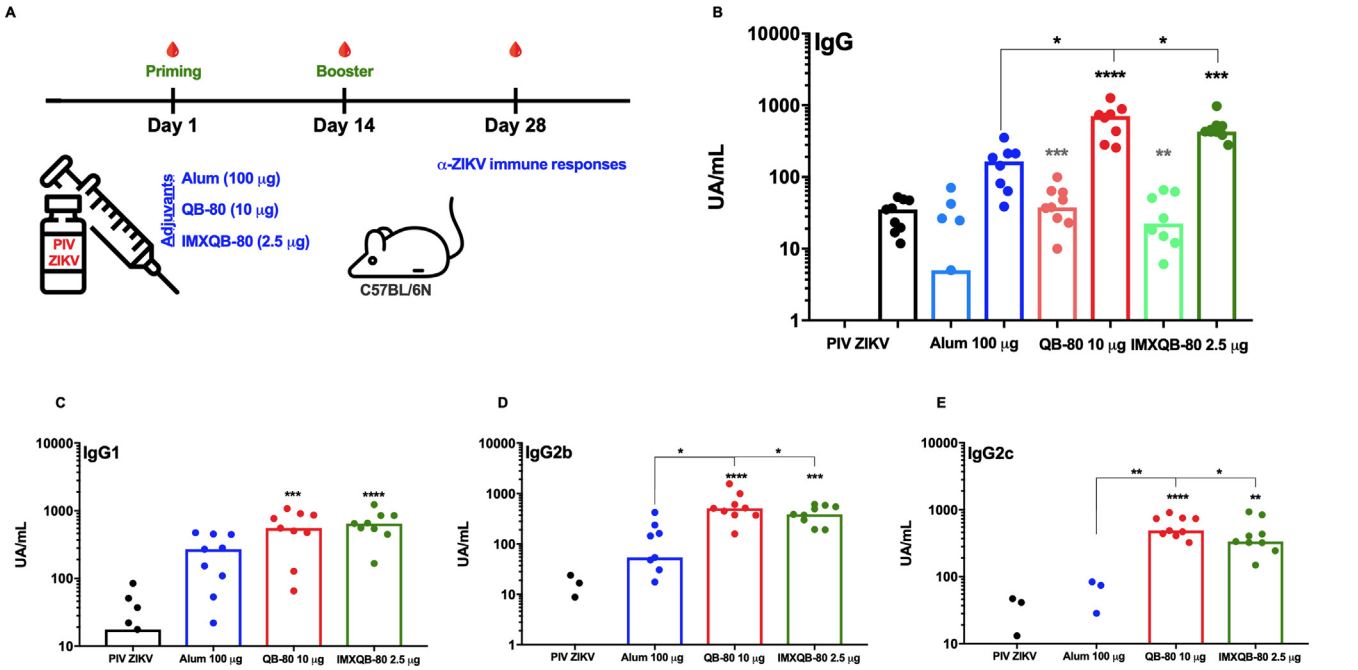

Fig. 4. Antibody responses in immunized mice. Scheme of experimental of immunization (A). Total anti-ZIKV IgG serum titres of on days 14 (light colors) and 28 (dark colors) (B). Anti-ZIKV IgG1 (C), IgG2b (D) and IgG2c (F) titres on day 28 post priming. The values are expressed as the mean value ± S.E.M (n = 9) in arbitrary units. Significant differences are indicated: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) and ****($P < 0.0001$), in relation to the group immunized with unadjuvanted vaccine (PIV ZIKV) or the alum-adjuvanted vaccine group. Significant differences on day 14 are indicated in grey.

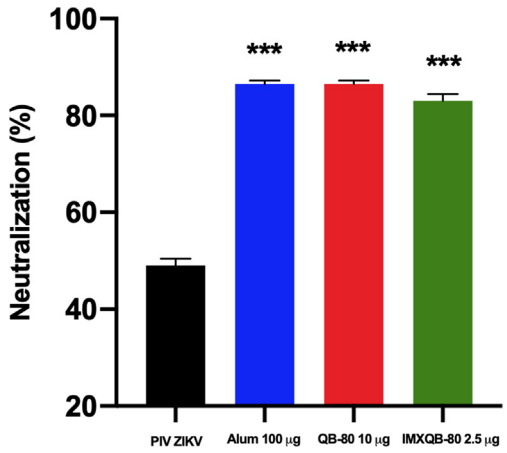

Fig. 5. ZIKV neutralizing antibodies in serum of immunized mice. ZIKV-neutralizing antibodies were evaluated in PRNT assays, two weeks after the administration of the second dose of vaccine immunization. The values are expressed as % PFU neutralization (mean value ± S.D., n = 3). Significant differences are indicated: *** ($P < 0.001$), in relation to the to the group immunized with non-adjuvanted vaccine (PIV ZIKV).

with QB-80 and IMXQB-80 were consistent with those previously obtained with other viral antigens adjuvanted with *Q. brasiliensis* saponins [10–12]. In addition, QB-80 and IMXQB-80 increased anti-ZIKV by increasing IgG1, a Th2 antibody isotype, as well as, IgG2a, IgG2b and IgG2c, with a Th1 phenotype. This Th1-isotype with the aid of $CD4^+$ T cells are regarded as effective against intracellular pathogens [39]. Moreover, for many viral infections, including ZIKV and influenza, IgG2 is the predominant antibody subclass responsible for neutralizing activity [40,41]. Quillajaceae saponins have on numerous occasions been used in veterinary vaccines, experimental and now, approved human vaccines, and are known to rapid and strongly stimulate not only adaptative but also innate immune responses [5,6,21]. Previously, we reported that the Th1 bias profile of *Q. brasiliensis* SBAs induced high levels of IFN-γ and IL-2 mRNA were detected in spleen cells [11], TNF enhanced production of IFN-γ by $CD4^+$ and $CD8^+$ T lymphocytes [12,13]. Thus, vaccines with *Q. brasiliensis* SBAs, particularly in nanoparticulate adjuvant preparations, may be an interesting alternative to currently more widely used vaccine adjuvants, especially for purified or inactivated antigens.

It is worth mentioning that alum-based adjuvants are the most often used adjuvants licensed for human vaccines [7]. In this study, using the PRNT assay, we shown that the neutralizing antibodies elicit by IMXQB-80 nanoadjuvant were the same magnitude as those induced by the alum adjuvanted vaccine. The control group which received a non-adjuvanted vaccine (PIV ZIKV) did not display any significant enhancement in neutralizing antibody titres (Fig. 5). As expected, sera from animals mock-immunized with saline had no detectable ZIKV neutralizing antibodies (data not shown). In summary, immunization with either QB-80 or IMXQB-90 adjuvanted vaccines were able to induce significant levels of ZIKV neutralizing antibodies, compared to those induced by the alum adjuvanted vaccine.

At least, but not least, another question addressed in this work is the assembly of QB-80 saponins as ISCOM-like nanoparticles and their use as adjuvant. Until now, studies with the adjuvant potential of *Q. brasiliensis* saponins have been performed with the crude extract (aqueous extract, AE) and the high purified fractions QB-90 and QB-80, as well as ISCOM particles derived from QB-90 (when the formation nanoparticles occur in the presence of the antigen). In this study, for the first time, we show the great adjuvant potential of QB-80 nanoparticles, formulated without the presence of antigen. This finding brings great possibilities, since the adjuvant potential of saponins is maintained, while toxicity is reduced. Moreover, IMXQB-80, was prepared to contain at 2.5 μg of QB-80/dose; this was sufficient to stimulate immune responses similar to those elicited by a fourfold higher concentration of the QB-80 (10 μg/dose).

5. Conclusions

The QB-80, an immunoadjuvant obtained from the aqueous extract of *Q. brasiliensis* leaves, has at least 29 triterpenic saponins that have been tentatively identified by DI-ESI-ToF. This is the first report on the saponin content of QB-80. These saponins are capable of self-assembling with cholesterol and di-palmitoylphosphatidyl choline to form ISCOM-like nanoparticles with approximately 43 nm, named IMXQB-80. In this work, we have demonstrated that QB-80 SBAs significantly enhances immune responses to an experimental ZIKV vaccine. The nanoadjuvant formulation IMXQB-80, which contains fourfold less saponins, has nevertheless proved to be as effective as immune enhancer as QB-80, with the advantage of mitigating toxic side effects usually associated to SBAs. The immune adjuvant activity was revealed by a significant enhance in serum levels of anti-ZIKA virus IgG and subtypes (IgG1, IgG2b and IgG2c) as well as neutralizing antibodies, when compared to an unadjuvanted vaccine. Overall, our findings reveal that either QB-80 or IMXQB-80 are strong immunoadjuvants and shown to be more potent immune enhancers than alum, a widely used vaccine adjuvant.

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.

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