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Immunization with a recombinant BibA surface protein confers immunity and protects mice against group B *Streptococcus* (GBS) vaginal colonization

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ABSTRACT

Streptococcus agalactiae or group B *Streptococcus* (GBS) is a Gram-positive bacterium divided into ten distinct serotypes that colonizes the vaginal and rectal tracts of approximately 30% of women worldwide. GBS is the leading cause of invasive infection in newborns, causing sepsis, pneumoniae and meningitis. The main strategy to prevent GSB infection in newborns includes the use of intrapartum antibiotic therapy, which does not prevent late-onset diseases and may select resistant bacterial strains. We still do not have a vaccine formulation specific for this pathogen approved for human use. Conserved surface proteins are potential antigens that could be targets for recognition by antibodies and activation of cell opsonization. We used a serotype V GBS (GBS-V)-derived recombinant surface protein, rBibA, and evaluated the potential protective role of the induced antigen-specific antibodies after parenteral or mucosal immunizations in C57BL/6 mice. *In vitro* and *in vivo* assays demonstrated that vaccine formulations containing BibA combined with different adjuvants induced serum IgG and/or secreted IgA antibodies, leading to enhanced opsonophagocytosis of GBS-V cells and reduced invasion of epithelial cells. One BibA-based vaccine formulation adjuvanted with a nontoxic derivative of the heat-labile toxin produced by enterotoxigenic *Escherichia coli* (ETEC) strains was capable of inducing protection against vaginal colonization and lethal parenteral challenge with GBS-V. Serum collected from vaccinated mice conferred passive protection against vaginal colonization in naïve mice challenged with GBS-V. Taken together, the present data demonstrate that the BibA protein is a promising antigen for development of a vaccine to protect against GBS infection.

1. Introduction

Streptococcus agalactiae or Group B *Streptococcus* (GBS) is a Gram-positive coccus, β-hemolytic, facultative anaerobe organized in pares or small chains [1]. GBS is one of the most important risk factors for the development of neonatal disease and is the main cause of sepsis and meningitis in newborns [2,3]. The GBS serological classification is based on a specific capsular polysaccharide, comprising ten distinct capsular serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) [4,5]. All GBS serotypes are capable of causing invasive disease, but serotypes Ia, Ib, II, III and V are responsible for the majority of invasive diseases in newborns and adults [1,6].

GBS asymptomatically colonizes the urogenital and gastrointestinal tracts of 30% to 50% of healthy adults [7–9]. During pregnancy, the vaginal tract provides ideal conditions for GBS multiplication due to its acidity [10], and GBS transmission to a fetus can occur by ascension of bacteria from the vagina to the amniotic fluid and transmission to a newborns can occur through mucosal exposition during labor (vertical transmission) [11]. In fact, vaginal colonization of the maternal genital tract characterizes the primary

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risk factor for early-onset disease, which occurs within the first week of life [12,13].

In the 1980s, clinical assays demonstrated the efficacy of intrapartum antibiotic therapy in preventing GBS transmission to newborns and early-onset disease [14,15]. However, this strategy is associated with increased rates of Gram-negative bacterial infection and is not effective against late-onset disease [16]. For these reasons, there is a need for the development of vaccine formulations against GBS to prevent infection [17].

With the advent of reverse vaccinology, hypothetical immunogenic proteins can be identified by *in silico* analysis [18]. The use of GBS surface protein antigens in vaccine formulations is supported by the correlation between relatively high susceptibility to severe neonatal infection and low levels of antibodies specific for surface proteins [19]. In addition, the use of proteins as vaccine antigens would be a low-cost alternative to protein-conjugated polysaccharides and would favor broader coverage with regard to different GBS serotypes [20].

One example of a GBS surface protein used in mouse immunization is the C5a peptidase protein, that led to immune responses capable of enhancing GBS killing by macrophages [21,22]. Another example is the combination of the Rib and Alpha proteins, and combination immunization was capable of promoting *in vitro* opsonophagocytosis and protection in an animal model [23,24]. This vaccine formulation is presently being evaluated in a phase 1 clinical trial, but further information is not currently available [25]. Another surface protein used as an antigen for mouse immunization is the immunogenic bacterial adhesin BibA. This protein was shown to promote mouse protection from lethal challenge, but the study did not evaluate the impact on GBS vaginal colonization or epithelial cell invasion [26].

Mucosal vaccination can promote relatively strong local responses against mucosal pathogens, inhibiting bacterial adhesion [27]. In addition, incorporation of adjuvants is usually required to enhance the immune responses, particularly at mucosal sites, induced by subunit vaccines. One option is α-galactosylceramide (αGalCer), an invariant natural killer T cell activator that potentiated mucosal immune responses in an oral vaccine against ETEC [28]. Previous studies have also shown that administration of αGalCer in intranasal or oral vaccine formulations induces antigen-specific T cell responses in mucosal tissues, resulting in protection against mucosal pathogens [29]. Another option is the use of nontoxic derivates of the heat-labile toxin (LT) produced by enterotoxigenic *Escherichia coli* strains, one of the most effective mucosal immunomodulators [30,31]. The LTK63 derivative has been described to enhance adaptive immune responses, inducing both antibody and T cell responses and thus resulting in protective immunity against different pathogens [31,32].

A vaccine given to pregnant women could be a relatively powerful tool to prevent GBS disease by protecting mothers and children [33]. In this context, BibA is an adhesin involved in immune evasion and promotes adhesion to human epithelial cells [34]. In addition, BibA represents a virulence factor that contributes to phagocytosis resistance and has been indicated to be a vaccine antigen of interest [35]. Thus, our propose was to further extend the evaluation of the immunogenic properties of the group B *Streptococcus* bacterial adhesin BibA, testing vaccine formulations delivered via a parenteral or mucosal administration route, to assess epithelial cell invasion and phagocytosis enhancement. In the present study, we confirmed that intranasal immunization of mice with BibA conferred protection against both vaginal colonization and lethal challenges with a GBS serotype V (GBS-V) strain, reinforcing the relevance of this antigen in the development of anti-GBS vaccines.

2. Material and methods

2.1. Mice and ethics statement

This study was performed according to the guidelines of the Brazilian National Council for Control of Animal Experimentation (CONCEA). Experimental protocols were approved by the Ethics Committee on Animal Use of the University of São Paulo (CEUA-ICB/USP) under protocol number 41/2017. Mice were obtained from the Medicine Faculty at the University of São Paulo (USP). Five animals per cage were housed and bred in the Microbiology Department Animal Facility. Food and water were provided *ad libitum*, and all handling and experimental procedures followed the Institutional Animal Care and Use Committee guidelines.

2.2. Bacterial strain and growth conditions

The GBS strain V 2603 V/R (ATCC BAA-611) was used in colonization and lethal challenges. Bacteria were grown at 37 °C without shaking in Todd-Hewitt broth (Difco, Michigan, USA), containing 0.3% yeast extract, 15 μg/mL nalidixic acid and 8 μg/mL gentamicin. Aliquots of bacteria were stored at −80 °C. The identity of *S. agalactiae* was confirmed by assessing colony morphology on blood agar plates. Standard strains for different serotypes of GBS were obtained from the University Hospital of São Paulo, as previously described [36].

2.3. Recombinant BibA expression and purification

A plasmid containing the *bibA* gene was synthetized by GenScript (Piscataway, NJ, USA). The recombinant pET28a-BibA plasmid was introduced into the *Escherichia coli* BL21(DE3) and BL21 (DE3) pLysS strains for expression tests in Luria broth (LB) containing 50 μg/ml kanamycin. Cells were grown at 37 °C and 200 rpm until an optical density of 0.7 ($OD_{600\ nm}$) was reached and induced with 0.5 mM IPTG for 4 h at 37 °C and 200 rpm. For purification of the recombinant protein, cultures (3 L) of *E. coli* BL21(DE3) transformed with pET28a-BibA were induced under the conditions described above. After induction, the cells were harvested, resuspended in buffer (10 mM Tris-HCl, 300 mM NaCl, and 20% glycerol, pH 8.6) and lysed in a cell homogenizer APLAB-10 (ARTEPEÇAS, São Paulo, BR). Soluble fractions were purified by nickel affinity chromatography using a HisTrap™ HP column (GE Healthcare Life Sciences, Buckinghamshire, UK), followed by gel exclusion chromatography using HiPrep™ 26/60 Sephacryl® S-200 HR. Purified fractions were analysed by SDS-PAGE, quantified and stored at −80 °C.

2.4. Circular dichroism characterization

The circular dichroism (CD) spectrum of the recombinant protein was collected on a Jasco J-810 spectropolarimeter (Jasco Inc., Easton, MD). Samples were analysed in 20 mM phosphate buffer in a quartz cuvette (path length of 0.2 cm) at room temperature. Data were reported as the average molar ellipticity of four accumulated scans ($[\theta]^\circ\ M^{-1}\ cm^{-1}$) at wavelengths between 190 and 240 nm. A plot was generated after K2D3 analysis.

2.5. Immunization procedures

To generate polyclonal serum, 5-week-old specific pathogen-free female BALB/c mice (5 animals *per* group) underwent a three-dose immunization regimen (days 0, 14, and 28) via the subcutaneous (s.c.) route, with 1×10^8 CFU (colony forming units) of

heat-inactivated GBS-V (10 min at 100 °C) and 1 μg of LT-1 adjuvant administered. One day before each immunization, serum samples were harvested from the submandibular plexus. Vaginal colonization and lethal challenge experiments were performed with 10-week-old specific pathogen-free female C57BL/6 mice (5 animals *per* group). Vaccines were delivered via different routes as follows: i) the subcutaneous (s.c.) route, with 20 μg of rBibA and 50 μg of $Al(OH)_3$ (Rehydragel, Reheis, NJ, USA) in a final volume of 50 μL; and ii) the intranasal (i.n.) route, with 20 μg of rBibA, 20 μg of rBibA and 8.3 μg of LTK63, 20 μg of rBibA and 5 μg of αGalCer (KRN7000) or a mock treatment (saline and 8.3 μg of LTK63, used as control). Immunizations via the i.n. route were carried out with a micropipette and a total volume of 10 μL. One day before each immunization, serum samples were collected for subsequent analyses.

2.6. Detection of antibody responses by ELISA

Antigen-specific antibodies raised in vaccinated mice were assayed in the serum, saliva, feces and vaginal fluids by enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates (Nunc International, Rochester, NY) were coated overnight with 2 μg of rBibA in carbonate buffer, pH 9.6. The plates were blocked for 2 h at 37 °C with PBS containing 3% milk. Serial dilutions of tested serum samples were added, and the plates were incubated for 1 h at room temperature. The plates were subsequently washed, and serum IgG and IgA were detected with HRP-conjugated anti-mouse IgG (1:3000), IgG1 (1:10,000), IgG2c (1:3000) or IgA antibodies (1:2500) (Sigma-Aldrich, St. Louis, MO, USA). After incubating for 1 h, the plates were washed, and an OPD peroxidase substrate was added for color development. The wells were read at 492 nm with an ELISA microplate reader (Biotek Synergy H1 Hybrid Reader, Bio-Tek Instruments, Winooski, VT), and the titers were defined as the highest sample dilution able to generate 0.2 absorbance above the absorbance of the preimmune serum.

2.7. Whole-cell ELISA

This assay was conducted as described previously [37], with minor modifications. GBS cells of serotype Ia, Ib, II, III, IV or V were grown in THB medium overnight. The bacteria were inoculated in fresh medium and grown to an optical density of 1 ($OD_{600\ nm}$). The cells were washed twice with PBS and adjusted to 1.5×10^8 CFU/mL, and 96-well microplates were coated with 100 μL of the bacterial suspension (1.5×10^7 CFU) and incubated overnight in the presence of PBS at 37 °C in a 5% CO_2 atmosphere. Following coating, the assay was conducted as described above for the standard ELISA.

2.8. Antibody-antigen affinity assay

The antibody-antigen affinity index was measured using an ELISA protocol with the incorporation of one additional dissociation step with ammonium thiocyanate. Plates were coated with rBibA as described above. Serum samples were tested at dilutions corresponding to an optical density of 1 ($OD_{492\ nm}$). After incubation, different concentrations of ammonium thiocyanate (0–5 M) were added to the microplate wells, and the plates were incubated at room temperature for 15 min. The plates were then washed and incubated with an HRP-conjugated anti-mouse IgG antibody. The percentage of antibodies bound to rBibA was determined by the following formula: $OD_{492\ nm}$ recorded for samples treated with ammonium thiocyanate × 100/$OD_{492\ nm}$ of the same sample in the absence of ammonium thiocyanate.

2.9. Binding of anti-BibA antibodies to native and heat-denatured proteins

rBibA was heat-denatured by incubation at 100 °C for 15 min, followed by a 15-min incubation on ice. Native and heat-denatured proteins were spotted on nitrocellulose membranes over the range of 0.5–5 μg. The membranes were blocked with a 5% milk PBS solution for 2 h, washed with PBS-Tween 0.05% and incubated with anti-GBS-V polyclonal serum (1:500) for 1 h. The membranes were then washed with PBS-Tween, incubated with an HRP-conjugated anti-mouse IgG antibody (1:3000) for 1 h, washed again with PBS-Tween, and developed by incubation with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, Waltham, USA).

2.10. Opsonophagocytic assay

Opsonophagocytic assays (OPAs) were conducted with serum samples collected from immunized mice as described previously [38], with modifications. Aliquots containing 1×10^5 cells of the macrophage J774 cell line (ATCC, CCL-240) were plated in 96-well microplates (Nunc). The plates were incubated overnight at 37 °C in a 5% CO_2 atmosphere. The GBS-V strain, grown overnight in Todd-Hewitt broth, was inoculated in fresh medium and incubated at 37 °C in a 5% CO_2 atmosphere until an optical density of 1 ($OD_{600\ nm}$) was reached. The bacterial pellet was washed twice with PBS, resuspended in DMEM and used in the assays. Reactions were performed by incubating 2×10^3 CFU of GSB-V with heat-inactivated (56 °C for 30 min) test serum diluted 1:16, with 12.5% of fresh mouse complement source and 1% gelatine in Hank's Balanced Salt Solution, for 30 min at 37 °C and then centrifuged at 500*g*. Control reactions were performed with non-immune serum or without GBS-V cells. Opsonized GBS-V cells were incubated with J774 cells at a ratio of 50:1 at 37 °C for 1 h with no agitation. The supernatants were collected, serially diluted and plated on selective blood agar plates. The experiment was performed in triplicate. Colonies were counted after a 24-h incubation at 37 °C in a 5% CO_2 atmosphere. The percentage of killed bacteria was determined by comparing the colony counts in tests carried out without effector cells (100% surveillance) to those of the tested samples, subtracting the percent that survived from 100%.

2.11. Invasion assay

A549 human lung epithelial cells (ATCC® CCL-185™) were seeded in 96-well flat-bottom microplates (Nunc) at a density of 1×10^5 cells. Aliquots containing 5×10^6 CFU of GBS-V (MOI of 50) were incubated with polyclonal serum raised in vaccinated mice at a final dilution of 1:100 for 45 min at 37 °C. Opsonized bacterial cells were incubated with A549 cells for 3 h at 37 °C with no agitation. Wells were washed with PBS and treated with 100 μg/mL gentamicin for 2 h to kill membrane-attached bacterial cells. The A549 cells were washed once again to remove the antibiotic and subsequently lysed with 0.025% Triton X-100. The lysates were serially diluted (1:2) and plated on blood agar plates with 8 μg/mL gentamicin and 15 μg/mL nalidixic acid. Colonies were counted after a 24-h incubation at 37 °C in a 5% CO_2 atmosphere. Qualitative evaluation of A549 cell invasion was conducted after gentamicin treatment. Cells were washed with PBS and incubated with 100 μL of a 50 μg/mL solution of acridine orange and ethidium bromide for 5 min in the dark. Cells were visualized under an AMG EVOS fluorescence microscope.

2.12. Immunofluorescence

Overnight-cultured GBS-V cells were diluted 1:10 in PBS, and 30 μL of bacterial suspension was added to a 96-well immunofluorescence plate (Corning). The cells were fixed by incubation with 200 μL of a 4% formaldehyde solution for 15 min on ice. After washing, the wells were blocked for 30 min at 37 °C with a 5% milk solution. The wells were incubated with the following primary antibodies: i) anti-*S. agalactiae* monoclonal antibody (224/46) (1:100) (Novus Biologicals), ii) polyclonal serum raised against GBS-V (1:50), iii) polyclonal serum raised against BibA-alum (1:50), and iv) control serum raised against saline (1:50). After washing, a goat anti-mouse IgG secondary antibody conjugated to Alexa 488 (Thermo Fisher) was added to the wells (1:100) and incubated for 1 h in the dark. The cells were washed and evaluated with an AMG EVOS fl LED fluorescence microscope.

2.13. Vaginal colonization and lethal challenge

First, GBS-V cells underwent virulence activation through three intraperitoneal passages in 10-week-old C57BL/6 mice. The spleens were collected from euthanized mice, treated with 100 μg/mL gentamicin, macerated and plated on selective blood agar plates. Vaginal colonization challenges were carried out two weeks after administration of the third dose, with female C57BL/6 mice treated via i.p. injection of 0.5 mg of medroxyprogesterone acetate (Depo-Provera®) for estrous cycle synchronization. One day later, each mouse was colonized with 5×10^7 CFU of GBS mixed with 10% gelatine at a ratio of 1:1 in a 10-μL final volume. Vaginal washes, saliva (induced by i.p. injection of 0.5 mL of 0.2% pilocarpine), feces and blood were collected 3 days after vaginal colonization. CFU numbers were determined by serial dilutions and plating on blood agar. For the lethal challenge, 11-week-old pathogen-free C57BL/6 mice (10 animals *per* group) were i.p. challenged with 1×10^8 CFU of GBS-V. Mouse deaths were recorded over the next 10 days, with daily evaluation of disease signs.

2.14. Protection conferred by passive immunity

Aliquots containing 5×10^7 CFU of GBS-V were opsonized after incubation for 30 min at 37 °C with polyclonal serum raised in mice immunized with BibA-alum or BibA-LTK63 or mock treated (n = 10 mice *per* group). Serum samples were used at a dilution corresponding to an absorbance of 1.0 ($OD_{492\,nm}$) or at 1:100 for the control serum samples collected from the mock-treated mice. After opsonization, bacteria were centrifuged at 4000*g* for 5 min, suspended in a final volume of 5 μL and mixed with 10% gelatine at a ratio of 1:1. Each sample was inoculated vaginally into non-immunized mice. Vaginal washes were collected 3 days after the challenge.

2.15. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad). The data are represented as the arithmetic mean ± SD, and differences were compared using one-way or two-way analysis of variance (ANOVA) with Tukey's, Dunnett's, and Bonferroni's multicomparison *post hoc* tests or using the log-rank (Mantel-Cox) test for survival analysis.

3. Results

3.1. Expression and purification of GBS-V rBibA

E. coli BL21 strains carrying pET28a-BibA expressed a recombinant protein with an apparent molecular weight of 84.8 kDa after induction (Fig. S1A). The recombinant protein accumulated in the soluble fraction of cellular extracts of both BL21(DE3) and BL21 (DE3) pLysS, but the *E. coli* BL21(DE3) strain expressed higher amounts of the protein (Fig. S1). The protein was purified by nickel affinity chromatography followed by size-exclusion chromatography with a yield of approximately 40 mg/L of induced culture and a purity above 90% (Fig. S1).

Once purified, rBibA was analysed by circular dichroism (CD) polarimetry. The CD profile generated with the recombinant protein showed a local minimum at 207.7 nm and a local maximum at 189.8 nm (Fig. S2). The results generated after K2D3 analysis indicated a predicted alpha-helix content of 64% for rBibA and confirmed that the recombinant protein produced in *E. coli* remained highly folded. Both native rBibA and heat-denatured rBibA were recognized by serum antibodies raised in mice immunized with whole GBS cells or with the recombinant protein, as determined by immunoblotting and ELISA (Fig. S2). These results indicate that the conformational and linear epitopes present in the GBS-V BibA protein are also present in the recombinant protein produced in *E. coli*.

Serum collected from mice immunized with BibA and alum was tested for the ability to recognize different GBS serotypes by whole-cell ELISA. All serotypes tested (Ia, Ib, II, III, IV, and V) were recognized by the serum samples collected from mice immunized with rBibA (Fig. 1A). Notably, the reaction with bacteria of serotype Ia, II or III was stronger than that with serotype V cells, the serotype used to obtain the *bibA* gene sequence for expression of the recombinant protein. These results could be explained by the variable levels of BibA expression observed among different GBS strains and serotypes. In addition, some GBS strains are capable of secreting most of the expressed protein [34,35].

Immunofluorescence assays performed with polyclonal serum samples raised against whole inactivated GBS-V or BibA-alum detected GBS-V cells (Fig. 1B-ii and 1B-iii). A stronger signal was recorded with a commercial anti-*S. agalactiae* monoclonal antibody (mAb 224/46) (Novus Biologicals) (Fig. 1B-i). Altogether, these data demonstrate that despite the natural polymorphism in the protein, antibodies raised in mice immunized with GBS-V BibA cross-reacted with proteins expressed by different GBS strains of diverse serotypes on the cell surface.

3.2. Immunization of mice with GBS-V rBibA

To test the immunogenicity of rBibA, female C57BL/6 mice were immunized with different formulations containing purified rBibA delivered via a parenteral (s.c.) or mucosal (i.n.) route. Parenterally (s.c.) immunized mice received 20 μg of rBibA admixed with 50 μg of $Al(OH)_3$. Mice immunized via the i.n. route received 20 μg of rBibA admixed with 8.3 μg of LTK63 or 5 μg of αGalCer. After a three-dose immunization regimen, serum and vaginal wash samples were collected to evaluate the presence of antigen-specific IgG antibodies. Significant increases in BibA-specific IgG antibody levels were detected in serum samples collected from mice immunized with rBibA, with higher titers raised in the parenterally immunized mice, followed by mice intranasally immunized with rBibA admixed with LTK63 or αGalCer (Fig. 2A).

BibA-specific IgG antibodies were detected in the vaginal washes of the mice immunized with BibA adjuvanted with alum or LT-K63R but not in those immunized with αGalCer (Fig. 2B). Analysis of the serum IgG subclass responses indicated that the antigen-specific IgG1/IgG2c ratio was significantly higher in the mice immunized via the s.c. route than in those immunized via the i.n. route regardless of whether LTK63 or αGalCer was used as the adjuvant (Fig. 2C). Individual serum BibA-specific IgG titers show more variability among the intranasally immunized mice

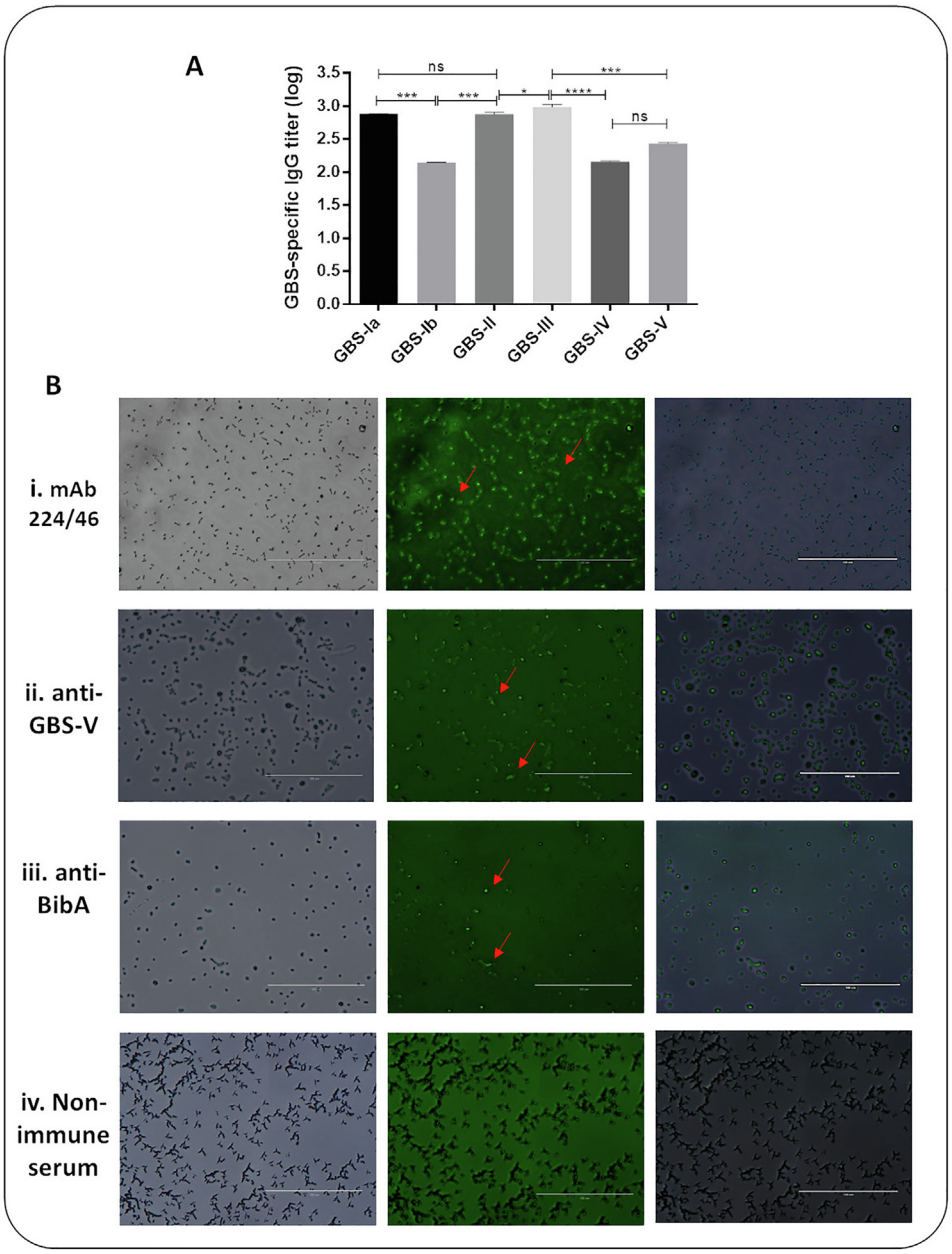

Fig. 1. Antibodies raised in mice immunized with rBibA bind to the native protein exposed on the surface of GBS cells of different serotypes. (A) Whole-cell ELISA performed with GBS cells belonging to different serotypes and serum samples collected 2 weeks after the third immunization with rBibA admixed with alum. Values represent the mean ± SD. Significant differences between groups were determined using two-way ANOVA with Tukey's *post hoc* test. ****p < 0.0001, ***p < 0.001, and *p < 0.05; ns: nonsignificant. (B) Immunofluorescence detection of GBS-V using antibodies raised in BALB/c mice immunized with rBibA. Tested antibodies: **i)** anti-*S. agalactiae* monoclonal antibody (mAb 224/46) (Novus Biologicals), ii) polyclonal serum raised against whole inactivated GBS-V (anti-GBS-V), iii) serum samples from mice immunized with rBibA-alum (anti-BibA), and iv) serum samples collected from saline-treated mice (non-immune serum). Cells were evaluated on an AMG EVOS fl LED fluorescence microscope at a magnification of 40×. Scale bar: 100 μm. The columns show transmitted light (left), GFP fluorescence (center) and merged images (right). Red arrows point GBS-V cells detected by the indicated antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than among the mice immunized via the s.c. route. Nonetheless, no statistically significant differences were observed in the serum antibody responses of the mice immunized with one of the three tested adjuvants (Fig. 2D).

Higher antigen affinity was detected in the serum raised in the mice immunized with rBibA-alum (Fig. 2E). The amount of ammonium thiocyanate required to dissociate 50% of the binding of the polyclonal antibodies raised in the different immunization groups was 1.3 M for rBibA-alum-immunized mice, 0.9 M for BibA-LTK63-immunized animals and 0.2 M for rBibA-αGalCer-immunized animals (Fig. 2F). Immunization with rBibA alone did not induce a sufficient antibody response to test the subclass responses or antigen affinity.

Secreted IgA responses were also followed in the different immunization groups. Mice were immunized with rBibA and challenged intravaginally with GBS-V cells, and three days later, vaginal wash, saliva, feces and serum samples were collected for determination of the BibA-specific secreted IgA responses. BibA-specific IgA responses were detected only in the mice immunized with LTK63 (Fig. 3). The same result was obtained for the detection of serum IgA responses (Fig. 3C). None of the groups showed detectable IgA antibodies in the feces (data not shown).

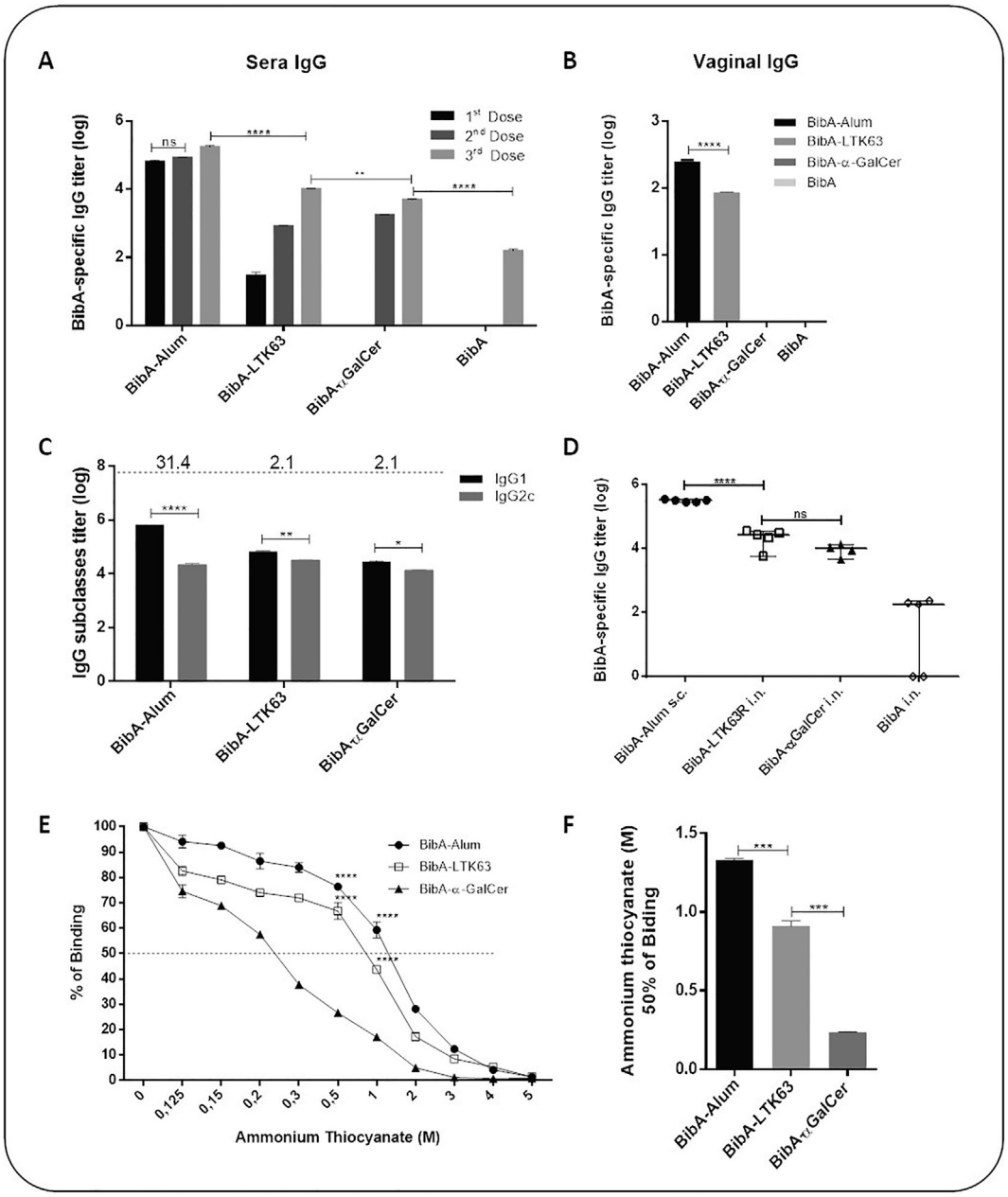

Fig. 2. Characterization of antibody responses elicited in mice immunized with rBibA. (A) Female C57BL/6 mice were immunized with three doses of rBibA alone (20 μg) or rBibA coadministered with one of the tested adjuvants: LTK63 (7.8 μg, i.n.), αGalCer (5 μg, i.n.), or alum (50 μg, s.c.). Analyses of serum antigen-specific IgG responses were carried out with pooled blood samples collected 2 weeks after each vaccine dose. (B) Analyses of secreted vaginal antigen-specific IgG responses were performed with pooled vaginal samples collected 2 weeks after the third immunization. (C) The anti-BibA IgG subclass responses in serum samples collected 2 weeks after the third immunization were evaluated. The ratio of IgG1:IgG2c is presented on top of the respective bars. (D) Individual serum BibA-specific IgG titers were determined after the third immunization dose. (E, F) The affinities of anti-BibA antibodies for purified rBibA were determined. (E) Values are expressed as the percentage of antibodies that remained bound to the solid phase-adsorbed antigen in the presence of ammonium thiocyanate relative to amount of antibody bound in the reaction performed without ammonium thiocyanate. (F) The concentration (in M) of ammonium thiocyanate required to dissociate 50% of the antibody bound to the antigen was determined. Anti-BibA titers were determined in triplicate (n = 5 mice per group). Values represent the mean ± SD of the IgG titers. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ represent significant differences among groups determined using two-way ANOVA with Tukey's *post hoc* test for (A) and with Bonferroni's *post hoc* test for (C,E), or one-way ANOVA with Bonferroni's *post hoc* test for the remaining assays.

3.3. Protective immunity raised in mice immunized with rBibA

The protective roles of antibodies raised in mice immunized with the rBibA-containing vaccine formulations were assessed with two different *in vitro* assays. The opsonophagocytic assay was performed with J774 macrophages in the presence of GBS-V previously treated with anti-BibA antibodies raised in vaccinated mice. The results demonstrated that compared with those raised in mice in the other test groups, the anti-rBibA serum antibodies raised in mice vaccinated with rBibA admixed with LTK63 promoted statistically significant increases in the killing of GBS-V by macrophages (Fig. 4A). The opsonophagocytic effect, although at lower values, was also detected with serum samples collected from mice immunized with rBibA admixed with alum or αGalCer.

The second *in vitro* assay evaluated the functionality of anti-BibA antibodies based on the invasion and killing of epithelial cells (A549 cell line) by GBS previously treated with serum samples collected from vaccinated mice. As demonstrated in Fig. 4B and 4C,

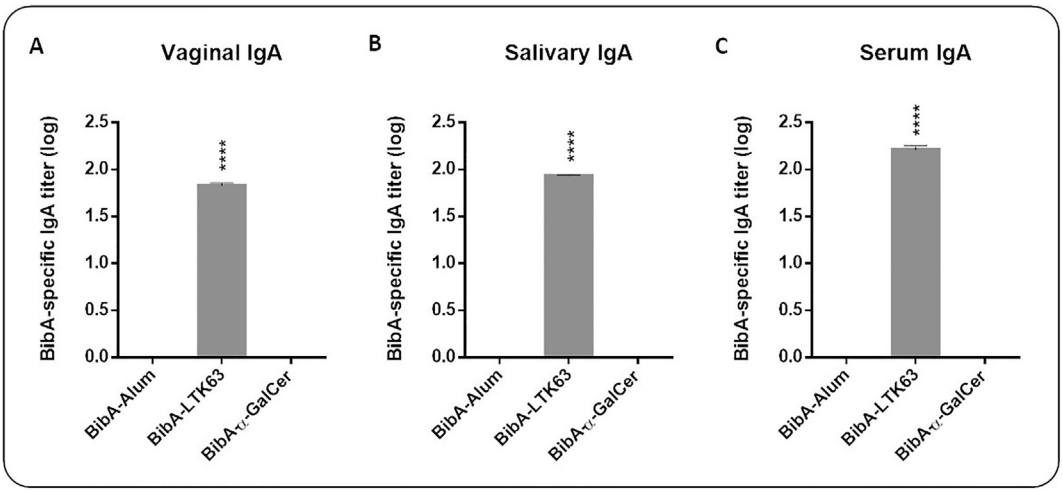

Fig. 3. Secreted BibA-specific IgA responses in mice immunized with rBibA. Secreted IgA responses were measured in vaginal washes, saliva and serum from three-dose vaccinated mice three days after vaginal challenge with GBS-V. (A) Vaginal BibA-specific IgA responses, (B) salivary BibA-specific IgA responses, and (C) serum BibA-specific IgA responses. Samples were collected 3 days after vaginal colonization, pooled and assayed by IgA-ELISA with plates coated with 2 μg of rBibA as the solid-phase bound antigen. Data represent two independent experiments, and the results are presented as the mean ± SD. ****$p < 0.0001$ represents significant differences among groups determined using one-way ANOVA with Bonferroni's *post hoc* test.

serum samples collected from mice immunized with rBibA admixed with LTK63 or αGalCer were more efficient than serum samples collected from mice immunized with rBibA and alum, but treatment with all tested sera collected from vaccinated mice conferred statistically significant protection against the killing of A549 cells by GBS compared with cells exposed to untreated GBS-V or GBS-V treated with control serum samples (Fig. 4B).

Similar responses were measured *in vitro* using dual fluorescence staining of infected cells: acridine orange stains viable cells (green), while ethidium bromide stains dead or dying cells (red). It was possible to verify the presence of ethidium bromide-stained cells in samples incubated with GBS-V treated with non-immune serum samples or untreated GBS-V (Fig. 4C and 4D). Cells exposed to GBS-V previously incubated with serum samples collected from mice immunized with rBibA-LTK63 or BibA-αGalCer showed survival values similar to those of cells not exposed to GBS-V. Altogether, these results indicate that anti-rBibA antibodies are capable of neutralizing GBS to prevent killing of eukaryotic cells under *in vitro* conditions.

Finally, the protective immunity induced by immunization with rBibA with regard to lethal i.p. challenge with GBS-V was evaluated. As shown in Fig. 5A, mice immunized with BibA admixed with alum or LTK63 were protected from a lethal parenteral challenge with GBS-V. On the other hand, mock-treated mice showed 90% mortality after GBS-V i.p. challenge, and the surviving animals showed motor sequelae, probably due to neurological disturbances caused by infection with GBS-V. We also measured the impact of immunization on vaginal colonization of C57BL/6 mice by GBS-V. For that purpose, female mice underwent estrus cycle synchronization before inoculation with GBS-V (Fig. S3). Notably, only mice immunized with rBibA admixed with LTK63 showed reduced vaginal colonization with GBS-V, which probably correlated with the local production of specific sIgA antibodies (Fig. 5B). We also performed a passive immunization protocol using serum samples raised in mice immunized with BibA-alum, BibA-LTK63 or saline (mock group) to test the impact on vaginal colonization by GBS-V. For that purpose, serum-treated bacteria were used in vaginal colonization tests performed with naïve mice. The quantification of bacterial cells in vaginal washes of mice showed that the mice inoculated with GBS-V treated with antibodies raised in mice immunized with BibA and LTK63 showed a significant reduction in colonization (80% protection) compared with the mice challenged with GBS-V treated with non-immune serum (Fig. 5C). The mice challenged with GBS-V treated with serum samples collected from mice immunized with rBibA and alum also showed partial protection (40% protection) against vaginal colonization by GBS-V (Fig. 5C). These data confirm that antibodies raised in mice immunized with purified rBibA confer passive protection against vaginal colonization by GBS-V.

4. Discussion

Previous studies have attempted to identify surface proteins expressed in all GBS serotypes [21-23,39] to be used as vaccine antigens for protection against GBS infection due to the lower production costs and broader coverage of protein-based vaccine formulations compared with those of polysaccharide-based vaccine formulations [20]. On this basis, the BibA protein, which characterizes a virulence factor involved in phagocytosis resistance, represents a promising vaccine candidate of great interest [35]. In this work, we successfully expressed and purified BibA from *E. coli.* We obtained high yields of a rather pure recombinant protein, and structural characterization of rBibA revealed a prevailing alpha-helix secondary structure, which is compatible with a previous description of the native protein [35].

Different antigen delivery routes have been tested in the development of GBS vaccines [40,41]. Parenteral routes, such as subcutaneous or intramuscular injection, are potent inducers of systemic responses, but they usually fail to induce mucosal immunity or promote weak local immune responses compared to delivery of antigens to mucosal sites [42]. In addition, the administration of vaccines via parenteral routes is usually more complex and requires trained personnel, which has relatively high costs and causes pain [43]. In contrast, mucosal vaccination may induce antigen-specific humoral and cellular responses in both the systemic and mucosal compartments [42], indicating mucosal vaccination is a good alternative for a GBS vaccine approach.

In this study, we tested immunizations with rBibA by comparing a parenteral administration route (s.c.) with alum as the adjuvant and a mucosal route (i.n.) with two different adjuvants, αGalCer and LTK63, to attempt to maximize the induced immune

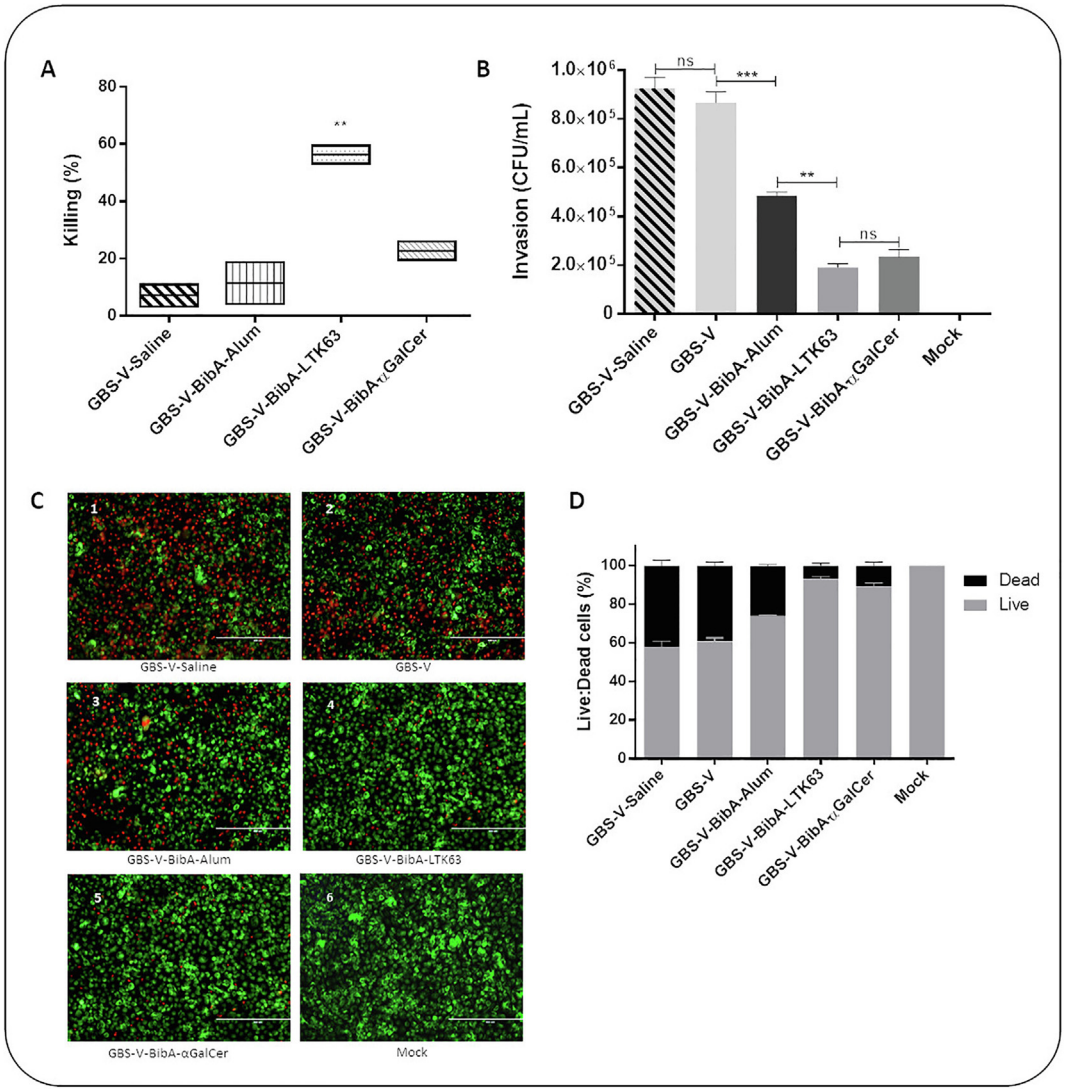

Fig. 4. Protective immunity raised in mice immunized with the tested vaccine formulations containing rBibA. (A) Opsonophagocytic assay using J774 cells and GBS-V cells previously treated with pools of polyclonal serum from three-dose vaccinated mice (n = 5 mice per group). Opsonized GBS-V cells were incubated with J774 cells for 1 h at 37 °C in triplicate. Boxes represent the percentage of killed GBS cells, centralized at the mean value. (B–D) Invasion of A549 cells by GBS-V pre-incubated with a pool of polyclonal serum raised in mice immunized with the tested vaccine formulations (n = 5 mice per group). (B) Quantification of intracellular GBS-V cells after opsonization and incubation for 3 h at 37 °C with A549 cells. Cell lysate samples were plated on blood agar. (C) Survival of A549 cells after exposure to GBS-V previously treated with the different tested serum samples. Cells were labelled by dual fluorescence staining with acridine orange (viable cells shown in green) and ethidium bromide (dead/dying cells shown in red) and visualized under an AMG EVOS fl LED fluorescence microscope. Scale bar: 400 μm. (D) Counting of live and dead A549 cells. Data represent two independent experiments performed in triplicate, and the results are presented as the mean ± SD. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ represent significant differences between groups compared with the GBS-V-Saline group using two-way ANOVA with (A) Dunnett's or (B) Tukey's *post hoc* test. ns: nonsignificant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

responses [44]. Intranasally administered BibA-LTK63 led to the induction of secreted IgA in both the upper and lower respiratory tracts and genital tract, and the same response pattern has been described in previous studies using the i.n. immunization route [45]. In Baker *et al.* [41], i.n. immunization with GBS induced local and systemic immune responses related to GBS clearance. Taken together, these data suggest that local IgA induction is an important factor that can confer protection against GBS colonization, the first step in invasive GBS disease.

We have now demonstrated that immunization with BibA admixed with each of the three tested adjuvants was able to induce systemic IgG responses. Additionally, in comparison with that of the remaining groups, the serum raised with BibA-alum had a higher affinity for the target antigen. However, previous reports evaluating immune responses to *Streptococcus pneumoniae* suggest that antibody avidity does not necessarily correlate with opsonophagocytosis and host protection [46]. Our data show that even with a lower antigen avidity, the serum raised in mice immunized with BibA-LTK63 was capable of inducing GBS phagocytosis, a key parameter involved in GBS killing, corroborating the lack of direct links between the antigen-avidity of antibodies and their functionality *in vitro* and *in vivo* [38].

In recent decades, several vaccine formulations based on the use of a capsular polysaccharide have been tested. However, these formulations were able to protect against only the capsular serotype used in the vaccine formulation [47]. The BibA protein has been detected in most, if not all, GBS strains previously analysed, and it was found both on the cell surface and in the culture supernatant [34,35]. Although four different alleles have been identified among GBS of different serotypes, the overall sequence is rather well conserved among them [35], and, more importantly, as corroborated by our results, antibodies raised

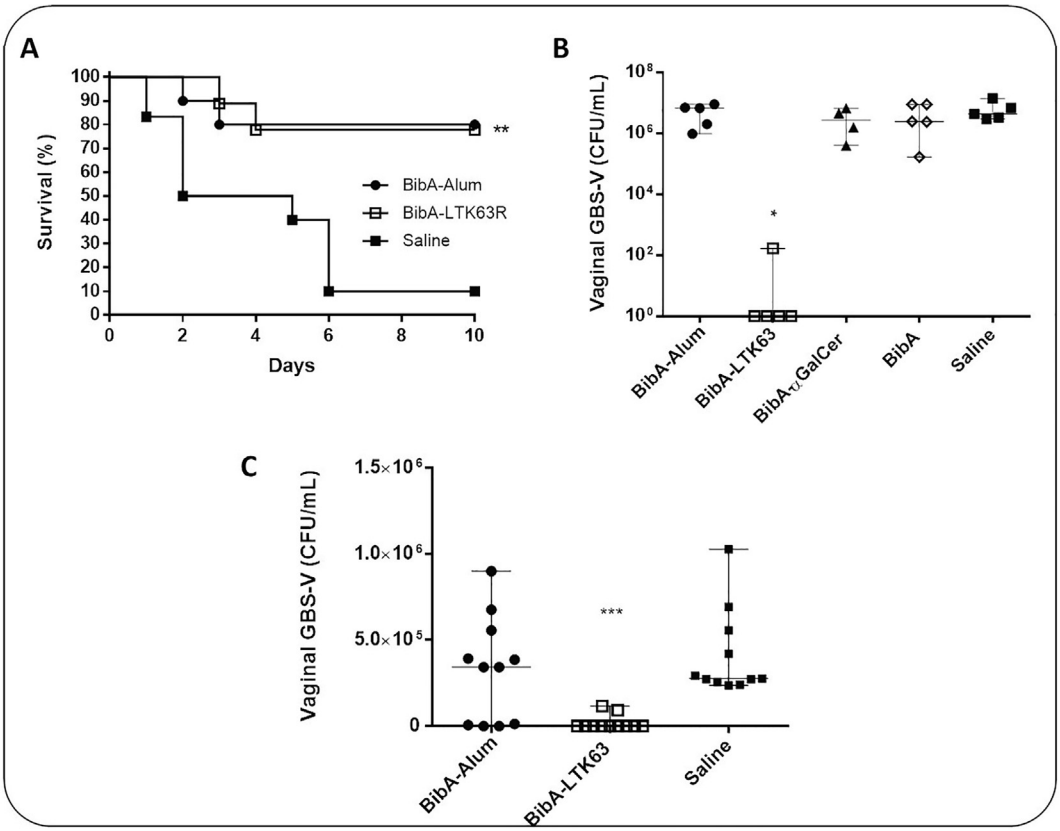

Fig. 5. Active and passive protective immunity against GBS vaginal colonization and lethal i.p. challenge in mice vaccinated with rBibA. (A) Immunized mice underwent a lethal i.p. challenge with 1×10^8 CFU of GBS-V (n = 10 per group). Survival was followed for 10 days. Differences in survival were determined by the log-rank (Mantel-Cox) test. (B) Immunized mice underwent vaginal colonization with 5×10^7 CFU of GBS-V (n = 5 mice per group). Vaginal washes were collected 3 days after vaginal colonization. Colonies were recovered on blood agar plates. (C) Passive protection was evaluated in naïve female mice that underwent vaginal colonization with 5×10^7 CFU of GBS-V previously treated with serum raised against rBibA-alum, rBibA-LTK63 or saline (n = 10 mice per group). Vaginal washes were collected 3 days after vaginal colonization. Colonies were counted after plating on blood agar. These experiments were repeated twice with similar results obtained in the two experiments. The results are presented as the median. ***p < 0.001, **p < 0.01 and *p < 0.05 represent significant differences between groups compared with the saline group determined using one-way ANOVA with Dunnett's *post hoc* test.

against one variant recognize the variants found in other GBS serotypes [34]. This cross-reactivity is extremely important in the GBS vaccine context since it supports the use of a protein antigen capable of inducing protective cross-reactive antibody responses in vaccinated subjects.

Different factors contribute to cell adhesion and mucosal colonization by different GBS strains [48]. BibA contributes to the binding of GBS to epithelial cells and confers anti-phagocytic activity to bacterial cells by binding to the C4bp complement factor [35]. Our results confirm that anti-BibA serum samples decreased the invasion of epithelial cells under *in vitro* conditions, thus confirming the role of these antibodies raised by vaccination in protective immunity, particularly at mucosal sites. In accordance with previous observations [26,35], our present results demonstrated that anti-BibA antibodies, particularly the antibodies raised in mice immunized with BibA-LTK63, neutralized the anti-phagocytic activity of the target protein and enhanced the killing of opsonized GBS, which suggests a relevant role for LTK63 adjuvant in anti-GBS vaccine formulations. As adhesion to host epithelial cells characterizes a central step in the pathogenesis of GBS [49], antibodies directed against BibA can impair the adherence process, reduce the GBS invasive capability and contribute to the prevention of invasive infections.

Previous studies with BibA did not explore the role of immune responses in protection against vaginal colonization or the invasion process [26], critical steps in GBS pathogenesis. Our data demonstrated that the BibA-LTK63 vaccine formulation was capable of preventing vaginal colonization in 80% of immunized mice challenged with GBS-V. Baker *et al.* [41] suggested that mucosal vaccination could provide superior protection against genital pathogens, leading to GBS clearance at mucosal sites. In contrast, the i.p. lethal challenge model helped us demonstrate that antibodies raised in mice immunized via a parenteral or mucosal route were capable of neutralizing the lethal effects mediated by GBS, mimicking an invasive form of the infection. These data emphasize the contribution of serum anti-BibA responses to bacteremia prevention in both newborns and the elderly. In addition, the evaluation of passive protection against vaginal colonization confirmed the protective role of antibodies raised against the purified rBibA protein, where the LTK63-adjuvanted vaccine produced protection in 80% of mice. All these data indicate that BibA is a potent antigen capable of inducing protective immune responses against GBS-V colonization and infection.

Our data strongly support that immunization with BibA, particularly after delivery to mucosal sites and in combination with strong mucosal adjuvants, can induce antibody responses capable of protecting mice against vaginal colonization and lethal parenteral challenge with GBS-V. Additionally, as demonstrated by our results, anti-BibA responses have the potential to confer cross-reactive protective immunity against different GBS serotypes. Thus, our data further support previous findings [26,34,35] that BibA is a promising antigen target for the development of GBS vaccines capable of preventing GBS colonization in mothers and, consequently, preventing disease in newborns.

5. Conclusion

Our results demonstrated that immunization of mice with the recombinant group B *Streptococcus* immunogenic bacterial adhesin BibA enhanced the magnitude of the antibody responses elicited against GBS. The rBibA-LTK63 vaccine formulation was capable of inducing serum IgG responses capable of increasing opsonophagocytic killing and reducing cell invasion, important steps in impairing disease development. More relevantly, mice immunized with rBibA and LTK63 or alum were protected from a lethal i.p. challenge with GBS-V. On the other hand, only female mice immunized with rBibA admixed with LTK63 were protected from vaginal colonization by GBS-V. In addition, serum raised against rBibA with a vaccine adjuvanted with either LTK63 or alum was capable of conferring passive protection against vaginal colonization with GBS-V in naïve mice. Taken together, these data show that BibA is a strong antigen candidate for inclusion in vaccine formulations against different GBS serotypes, contributing to the prevention of vaginal colonization and systemic invasion, which are necessary steps in the development of invasive and potentially deadly diseases.

CRediT authorship contribution statement

Nayara Fernanda Barros dos Santos: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing - original draft, Writing - review & editing. **Lukas Raposo da Silva:** Investigation, Methodology, Writing - review & editing. **Fagner James Martins Dantas Costa:** Investigation, Methodology. **Daniely Maranhão de Mattos:** Investigation, Methodology. **Enéas de Carvalho:** Investigation, Methodology. **Luís Carlos de Souza Ferreira:** Conceptualization, Data curation, Formal analysis, Project administration, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing. **Rita de Cássia Café Ferreira:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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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Appendix A. Supplementary data

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