

1 MEMBRANE VESICLES DERIVED FROM *BORDETELLA BRONCHISEPTICA*: ACTIVE
2 CONSTITUENT OF A NEW VACCINE AGAINST INFECTIONS CAUSED BY THIS PATHOGEN
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21 **ABSTRACT**

22 *Bordetella bronchiseptica*, a Gram-negative bacterium, causes chronic
23 respiratory-tract infections in a wide variety of mammalian hosts—humans included,
24 albeit rarely. We recently designed *B. pertussis* and *B. parapertussis* experimental
25 vaccines based on outer-membrane vesicles derived from each pathogen and obtained
26 protection against the respective infections in mice. Here, we demonstrate that outer-
27 membrane vesicles derived from virulent-phase *B. bronchiseptica* (OMVBbvir⁺)
28 protect mice against sublethal infection from different *B. bronchiseptica* strains, two
29 isolated from farm animals and one from a human. In all infections, we observed that
30 the *B. bronchiseptica* load was significantly decreased in the lungs of the vaccinated
31 animals: the lung-recovered colony-forming units diminished by at least 4 logs below
32 those detected in the lungs of non-immunized animals (p<0.001). In the OMVBbvir⁺-
33 immunized mice, we detected IgG-antibody titers against *B. bronchiseptica* whole-
34 cell lysates along with an immune serum having bacterial-killing activity that both

35 recognized *B. bronchiseptica* lipopolysaccharides and polypeptides such as GroEL
36 and OMPc and conferred an essential protective capacity against *B. bronchiseptica*
37 infection as detected by passive *in-vivo*–transfer experiments.

38 Stimulation of cultured splenocytes from immunized mice by OMVBbvir⁺
39 resulted in the presence of IL-5, INF- γ , and IL-17 production; indicating that the
40 vesicles induced a mixed Th2, Th1, and Th17 T-cell–immune-response profile. We
41 detected by adoptive transfer assay that spleen cells from OMVBbvir⁺-immunized
42 mice also contributed to the observed protection against *B. bronchiseptica* infection.
43 Outer-membrane vesicles from the avirulent-phase *B. bronchiseptica* and the
44 resulting induced immune sera were also able to protect mice against *B.*
45 *bronchiseptica* infection

47 IMPORTANCE

48 *Bordetella bronchiseptica*, a Gram-negative bacterium, causes chronic
49 respiratory-tract infections in a wide variety of mammalian hosts—humans included,
50 albeit rarely. Several vaccines aimed at preventing *B. bronchiseptica* infection have
51 been developed and used, but a safe and effective vaccine is still needed. The
52 significance and relevance of our research lies in the characterization of the outer-
53 membrane vesicles (OMVs) derived from *B. bronchiseptica* as the source of a new
54 experimental vaccine. We demonstrated here that our formulation based on OMVs
55 derived from virulent-phase *B. bronchiseptica* (OMVBbvir⁺) is effective against the
56 infection caused by *B. bronchiseptica* isolates obtained from different hosts: farm
57 animals and a human. *In-vitro* and *in-vivo* characterization of humoral and cellular
58 immune responses induced by the OMVBbvir⁺ vaccine enabled a better understanding
59 of the mechanism of protection necessary to control *B. bronchiseptica* infection. Here
60 we also demonstrated that OMVs derived from *B. bronchiseptica* in the avirulent
61 phase and the corresponding induced humoral immune response are also able to
62 protect mice from *B. bronchiseptica* infection. This realization provides the basis for
63 novel vaccine developments against not only the acute stages of the disease but also in
64 stages of disease or infectious cycle where avirulence factors could play a role.

66 Key words

67 *Bordetella bronchiseptica*; Outer-membrane vesicles; Vaccine; Phenotypic phases
68

Introduction

Bordetella bronchiseptica is a Gram-negative bacterium that causes respiratory diseases in a variety of mammalian hosts [1]. Though this pathogen rarely infects humans, certain reports have indicated that *B. bronchiseptica* can infect immune-compromised patients or those with underlying respiratory diseases [2-4]. The respiratory infections caused by this zoonotic pathogen could also become chronic, though exhibiting few or no symptoms [5, 6]. The persistence of *B. bronchiseptica* in hosts seems to be facilitated through a modification of the expression of bacterial constituents mainly controlled by a two-component regulatory system encoded by the *bvgAS* locus [7, 8]. This system senses signals from the external environments, regulates the expression of hundreds of genes, and controls different phenotypic phases [9].

The prophylaxis of diseases caused by *B. bronchiseptica* is achieved through vaccination, but to date no satisfactory vaccine to confer protection in animals against the acute or chronic infection caused by *B. bronchiseptica* has been developed. Some of the current vaccines are composed of either killed wild-type bacterial strains (administered parentally) or live attenuated strains (administered intranasally) [10, 11]. Most of the vaccines containing the killed bacteria induce high serum antibody titers but do not always provide an effective protection against infection [10]. The data on the safety and efficacy of live attenuated vaccines are scarce. Moreover, this kind of vaccine is not well accepted because the strains included in the vaccines may revert to full or partial virulence since the basis of the original attenuation is still unknown. As to the acellular *B. bronchiseptica* vaccines, one is composed of the immunogenic *Bordetella* colonization factor-A protein while others contain pertactin (PRN), the outer-membrane protein that is a highly immunogenic virulence factor [12-15]. Although these vaccines appear to resolve mainly the issue related to adverse side reactions, no conclusive evidence has been garnered to support their immunogenicity [13, 16]. Therefore, the identification of appropriate bacterial components for the development of a new vaccine is still needed. In this search, the characteristic constituents of the avirulent phase could be included in such an evaluation since this phase seems to be involved in the infectious process [6, 8, 17].

In the work reported here, we investigated whether a vaccine based on OMVs derived from *B. bronchiseptica* in either the virulent or the avirulent phenotypic phase would be able to generate a protective immunity against an infection caused by *B.*

103 *bronchiseptica*. Vaccines based on OMVs against *B. pertussis* or *B. parapertussis*
104 infections have recently been developed by our group [18-22]. The administration of
105 OMV-based vaccines confers a complete protection against *B. pertussis* or *B.*
106 *parapertussis* in mice. The protection against *B. pertussis* is long lasting and is
107 mediated by both antibodies and CD4⁺ T cells [20]. We have made the interesting
108 observation that the protective capacity of OMVs obtained from a *B. pertussis* strain
109 that expressed the avirulent phenotype was lower than that of the OMVs from
110 virulent-phase *B. pertussis*, but the two were nevertheless protective in the mouse
111 model used [23].

112 These results in combination permit the hypothesis that OMVs derived from
113 *B. bronchiseptica* from either the virulent or the avirulent phase could constitute a
114 suitable candidate for a vaccine against bordetellosis. In fact, the findings described
115 here support this hypothesis since the protection experiments performed in the murine
116 intranasal-challenge model demonstrated that the OMV-vaccine derived from virulent
117 *B. bronchiseptica* was able to effect a significant decrease in the lung colonization of
118 different *B. bronchiseptica* strains obtained from different hosts—*i. e.*, farm animals
119 or a human. Furthermore, by performing *in-vitro* and *in-vivo* experiments, we detected
120 that both a humoral response possessing killing capacity and immune splenic cells
121 contributed to the protection induced by the OMVBbvir⁺-vaccines. Moreover, we
122 have also observed that protective capacity could be induced with a vaccine
123 formulated from OMVs obtained from *B. bronchiseptica* in the avirulent phase.

124

125 MATERIALS AND METHODS

126 Bacterial strains and growth conditions

127 *Bordetella bronchiseptica* strain 9.73 (isolated from a rabbit) [24] and the
128 mutant derivative strain defective in expression of the BvgA protein (blocked in
129 avirulent phase) [25] were used throughout this study. *Bordetella bronchiseptica*
130 strains were grown on Bordet Gengou agar medium (Difco, Houston TX, USA)
131 supplemented with 10% (v/v) defibrinated sheep blood (blood was from Laboratorio
132 Argentino S.A.). For challenge in the animal experiments, the strains from rabbits *B.*
133 *bronchiseptica* 9.73 and *B. bronchiseptica* RB50 (*Bb_{ra}*RB50, kindly provided by Dr.
134 Peggy Cotter of the University of North Carolina) and *B. bronchiseptica* AR705
135 (*Bb_{hu}*AR705, an Argentine clinical isolate obtained from a pediatric patient with cystic
136 fibrosis) were also used.

137

138 Isolation of outer-membrane vesicles (OMVs)

139 To obtain OMVs from the bacterial cells, we used the method previously
140 described by us [18, 22, 26]. The procedure stated in brief: Culture samples from the
141 decelerating growth phase of the bacteria were centrifuged at 10,000 x g for 20 min at
142 4 °C and the pellet obtained resuspended in 20 mM Tris-HCl, 2 mM EDTA, pH 8.5
143 (TE Buffer). Of the resulting pellet, approximately 1 g (wet weight) was resuspended
144 in 5 ml of the TE Buffer. OMV release was promoted by sonication in ice-water; the
145 cells were then removed by centrifugation at 10,000 x g and the OMV supernatant
146 concentrated by ultracentrifugation at 40,000 x g for 3 h. The OMVs thus obtained
147 were stored at 4 °C. Thereafter the OMVs were examined by electron microscopy
148 after negative staining [22].

149

150 Protein assay

151 Protein content was estimated by the Bradford method with BSA as a standard
152 [27].

153

154 One-dimensional electrophoresis and immunoblotting

155 OMV proteins were separated by sodium-dodecyl-sulfate–polyacrylamide-gel
156 electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (Immobilon
157 P, Millipore USA) and probed with either a polyclonal anti(adenylate-cyclase
158 hemolysin) [anti(AC-Hly)] antibody (1:300), an anti-PRN antibody (1:500), an anti-
159 fimbriae (-FIM) antibody (1:500), an anti-flagellin (-FLA; 1:2,000) antibody, or anti-
160 GroEL, an antibody against a protein analogous to the *E. coli* chaperonin GroEL
161 (1:5,000) followed by incubation with anti(mouse IgG) conjugated with alkaline
162 phosphatase at a 1:1,000 dilution. Nitroblue tetrazolium and 5-bromo-4-chloro-3-
163 indolyl-phosphate were used as the phosphatase substrates according to the
164 manufacturer's protocol (Biodynamics SRL Buenos Aires Argentina). Some of the
165 proteins present in the vesicles were identified by mass spectrometry after the initial
166 separation by one-dimensional electrophoresis, as previously described [28, 29].

167 *Bordetella bronchiseptica*–lipopolysaccharide (LPS) electrophoresis was
168 performed at room temperature and constant voltage. The LPS were visualized by the
169 BioRad silver-staining technique.

170

171 **Formulation of vaccines**

172 To use the OMVs as acellular vaccines, the vesicle preparations were
173 detoxified by mixing with aqueous formaldehyde (0.37% [v/v] and incubating at 37
174 °C overnight, and aluminum hydroxide (0.2 mg/ml) was then added as an adjuvant.

175 To prepare whole-cell vaccine (WCVBbvir⁺), a suspension containing 2×10^{10}
176 colony-forming units (CFUs)/ml of heat-killed (56 °C for 20 min) *B. bronchiseptica*
177 9.73 were detoxified in the same manner and were then mixed with aluminum
178 hydroxide (0.2 mg/ml) as an adjuvant.

180 **Expression of inflammatory markers upon systemic delivery of OMVs**

181 In order to assess the proinflammatory capacity of the OMV-based vaccine
182 formulation, mouse-blood samples were collected 4 h after each immunization by
183 submandibular bleeding. Serum interleukin-6 (IL-6) was measured by enzyme-linked
184 immunosorbent assay (ELISA) with the kit BD OptiEIA (BD Biosciences, CA USA)
185 according to the manufacturer's instructions.

187 **Active immunization and intranasal challenge**

188 Four-week-old female BALB/c mice obtained from Biol SAIC, Argentina
189 were used for all assays. As described previously [29], the immunization protocols
190 comprised a two-dose schedule with the formulations described above over a period
191 of 2 weeks. Two weeks after the second immunization, mice were subjected to a nasal
192 challenge with a sublethal dose (10^5 – 10^6 CFUs in 40 µl) of *B. bronchiseptica* strain.
193 The lungs of the challenged mice were excised and collected for bacterial counts at 7
194 days after the challenge. The number of CFUs was determined as previously
195 described [29]. At least three biological replicates were performed.

197 **ELISA**

198 Plates were coated with sonicated *B. bronchiseptica* (whole-cell lysates) in 0.5
199 M carbonate buffer, pH 9.5 in an overnight incubation at 4 °C and then blocked with
200 3% (v/v) skimmed milk in blocking buffer (2 h at 37 °C) before incubation with
201 serially diluted mouse-serum samples (1 h at 37 °C). The bound IgG was detected
202 after a 2-h incubation with horseradish-peroxidase– (HRP)-conjugated goat anti-
203 (mouse IgG) at a titer of 1:20,000 (Thermo Fisher Scientific, Buenos Aires
204 Argentina). For measuring IgG isotypes, detection of bound antibody was determined

205 with HRP-labeled subclass-specific anti-(mouse IgG1) at 1:8,000 or IgG2a (1:1,000;
206 Sigma Aldrich, USA). As substrate 1.0 mg/ ml o-phenyldiamine (OPD, Bio Basic
207 Canada Inc) in 0.1 M citrate-phosphate buffer, pH 5.0 containing 0.1% hydrogen
208 peroxide was used. For measuring IgG isotypes, the detection of bound antibody was
209 determined with the peroxidase bound to subclass-specific anti-mouse-IgG1 (1:8,000)
210 or -IgG2a (1:1,000; Sigma, Aldrich). Optical densities (ODs) were measured with
211 Titertek Multiskan Model 340 microplate reader (ICN, USA) at 492 nm, and the OD
212 was plotted as a function of the log of the (serum dilution)⁻¹. The inflection point of
213 the curve was determined by the GraphPadPrims® software. Titers were defined as
214 the reciprocal of the serum dilution giving an OD corresponding to the inflection
215 point of the curve.

216

217 **Bactericidal Assay**

218 The bactericidal activity of the sera collected from mice two weeks after
219 immunization with the OMVBbvir⁺ vaccine (*i. e.*, the immune sera) and from non-
220 immunized mice (*i. e.*, the naïve sera) were tested *in vitro*. Both, immune and naïve
221 sera inactivated by heat, and phosphate-buffered saline (PBS) were use as controls.
222 Virulent *B. bronchiseptica* 9.73 were grown on Bordet Gengou agar medium and
223 diluted to 1 x 10⁵ CFUs/ml in PBS containing MgCl₂ 0.05 M, CaCl₂ 0.15 mM. Forty-
224 five µl of serum or PBS were mixed with 5 µl of a suspension containing 500 CFUs
225 of the bacteria. After 1 h of incubation at 37 °C, serial dilutions of the samples were
226 spread on Bordet Gengou agar plates and incubated for 48–72 h to determine the
227 CFUs. At least three biological replicates were performed.

228

229 **Analysis of cellular response elicited by vaccination**

230 The cellular response was analyzed as previously described [20]. The
231 procedure stated in brief: Spleen cells from mice immunized with the OMV-based
232 vaccine were harvested 8 weeks after the last immunization and seeded in 48-well
233 culture plates at 10⁶ per well in a volume of 500 µl of RPMI 1640 cell-culture
234 medium supplemented with 10% (v/v) fetal-bovine serum (Invitrogen, Buenos Aires
235 Argentina) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. All the
236 spleen cells were either stimulated with OMVs derived from *B. bronchiseptica* (5
237 µg/ml) or exposed to medium alone. Supernatants were removed after 72 h of

incubation at 37 °C in an atmosphere of 5% CO₂ and the production of interferon- γ (IFN- γ), IL-17, and IL-5 determined by ELISA (BD Biosciences, CA USA), according to the conditions specified by the manufacturer.

Adoptive transfer

Pooled serum (100 μ l) or spleen cells (5×10^6) from non-immunized mice or from mice immunized with the OMV-based vaccine two weeks previously were transferred intraperitoneally to female BALB/c mice. Twenty-four hours thereafter the mice were infected with a sublethal dose (10^5 – 10^6 CFUs 40 in μ l) of *B. bronchiseptica* 9.73 and the subsequent protection assessed by determining the CFU counts in the mouse lungs 7 days after the challenge.

In order to evaluate the contribution of CD4⁺ T cells in the protection, adoptive transfer assays were also performed using spleen cells obtained from animals immunized with OMVBbvir⁺ and depleted in CD4⁺ T cells. Depletion of CD4⁺ T cells in vaccinated mice was performed by intraperitoneal injection with the monoclonal antibody from GK1.5 hybridoma (61.2 mg/ml) specific for CD4. The dosing schedule consisted in the administration to the donor animals of 200 μ l of the antibody the day before spleens were collected for the passive immunization protocol. After spleen cells transfer, receptor animals received 2 doses of anti-CD4 antibody to ensure the depletion. Depletion of CD4⁺ T cells was confirmed by a reduction of at least 95% of the lymphocyte CD4 (+) population in blood and spleen by flow cytometry. Donor mice treated with control isotype (IgG2bk) were included in the assays for comparison purposes.

Statistical analysis

The data were evaluated statistically by one-way analysis of variance (ANOVA) followed by the Tukey test *post-hoc* (via the GraphPad Prism® software). Differences were considered significant at a $p < 0.05$.

RESULTS

Isolation and characterization of the OMVs obtained from *B. bronchiseptica* 9.73 grown in the virulent phase

The OMV samples obtained from *B. bronchiseptica* 9.73 grown in the virulent phase (OMVBbvir⁺) were negatively stained and examined by electron microscopy

272 (Fig. 1, Panel A). The procedure was repeated at least 8 times, and in all the samples
273 the size range (at dimensions of 50–150 nm) was both consistent from batch to batch
274 and similar to that previously described for OMV preparations derived from *B.*
275 *pertussis* [22]. To further characterize these OMVBbvir⁺, one-dimensional
276 electrophoresis (Fig. 1, Panel B) and immunoblotting (Fig. 1, Panel C) were
277 performed. By this assay we could detect that the OMVBbvir⁺ isolates contained AC-
278 Hly, PRN, and FIM2 (Fig. 1, Panel C).

279

280 **IL-6 levels after immunization**

281 Usually after the systemic immunization of an animal, a rise in
282 proinflammatory cytokines can be detected. The levels are related to the
283 proinflammatory capability of the formulation employed. IL-6 is among the
284 proinflammatory cytokines usually chosen as an indicator of this activity [30]. In our
285 experiments, the OMVBbvir⁺-containing formulations induced levels of IL-6 (425.54
286 ± 34.00 pg/ml) that were significantly lower than those detected for the *B.*
287 *bronchiseptica* whole-cell vaccine ($53,673.08 \pm 5,987.44$ pg/ml).

288

289 **Protection against intranasal *B. bronchiseptica* challenge after vaccination with** 290 **OMVBbvir⁺**

291 To evaluate the protective capability of the OMVBbvir⁺ vaccine against *B.*
292 *bronchiseptica* challenge, we used the murine model of intranasal infection. The
293 CFUs recovered from the lungs of the OMVBbvir⁺-immunized mice were compared
294 with those assayed in mice immunized with the *B. bronchiseptica* 9.73 whole-cell
295 vaccine prepared in our laboratory from wild-type bacteria in the virulent phase
296 (WCVBbvir⁺, Fig. 2, Panel A). Significant differences in the lung bacterial counts
297 between the OMVBbvir⁺-immunized animals and non-immunized mice were obtained
298 ($p < 0.001$; Fig. 2, Panel A). The number of colonies recovered from the lungs of the
299 OMVBbvir⁺-immunized mice at day 7 after challenge was at least 4 logs lower than
300 those detected in the non-immunized animals and similar to those from the
301 WCVBbvir⁺-immunized mice (Fig. 2, Panel A).

302 We next sought to investigate if the protective capability induced by the
303 OMVBbvir⁺ vaccine extended to strains other than *B. bronchiseptica* 9.73. To that
304 end, we performed *in-vivo* protection assays using two other strains of *B.*
305 *bronchiseptica* for challenge, one from a farm animal (*Bb_{ra}*RB50) and the other

306 recovered from a pediatric patient with cystic fibrosis (*Bb_{hu}*AR705). Mice were
307 accordingly immunized twice with the OMVBbv⁺ formulation and then challenged 2
308 weeks after the second immunization with a sublethal dose of each *B. bronchiseptica*
309 strain:—*i. e.*, *Bb_{ra}*RB50 and *Bb_{hu}*AR705. As a negative control we used non-
310 immunized mice. For both strains used in the bacterial challenge, significant
311 differences in lung *B. bronchiseptica* colony-formation counts of more than 4 logs
312 were obtained between the immunized animals and the negative control group (p
313 <0.001; Fig. 2, Panel B).

314

315 **Characterization of the humoral immune response induced by OMVBbv⁺**

316 To characterize the immune response induced by the OMVBbv⁺ vaccine
317 derived from *B. bronchiseptica* 9.73, titers of antibody were quantitatively measured
318 (Table 1). In comparison with the antibody levels detected in negative-control
319 animals, higher serum levels of specific IgG were found at 14 days after the OMV
320 priming (Table 1). Further tests were then conducted to determine the antibody
321 subtypes. Mice immunized with OMVBbv⁺ produced high titers of specific IgG1
322 and IgG2a antibodies. The specific IgG1 titer was higher than that of the specific
323 IgG2a (IgG1:IgG2a = 2.2; Table 1). Examination of these IgG subclasses indicated
324 that the mice had responded to the OMVBbv⁺ vaccination with a mixed Th1/Th2
325 profile, but with mainly a skewed Th2-type immune response.

326 To identify the main immunogenic proteins present in the OMVs, we analyzed
327 the antibody profile by immune proteomics. This analysis was achieved by examining
328 the reactivity of sera induced by the OMVBbv⁺ vaccine against proteins from the
329 OMV derived from *B. bronchiseptica*. Fig. 3, Panel A shows protein bands detected in
330 OMVBbv⁺ that cross-reacted with sera induced by vaccination with OMV. The
331 identity of some of those antigens was determined after the selected immunoreactive
332 bands were excised from the one-dimensional electrophoretic gels and then analyzed
333 by mass spectrometry. Bands a, and b in Fig. 3, Panel A, for example, were identified
334 as the GroEL-like protein and the outer-membrane protein OMPc, respectively. The
335 mass-spectrometric identification of the protein indicated as GroEL in particular was
336 subsequently confirmed by immunoreactivity with a GroEL-specific antibody (not
337 shown).

338 To investigate the presence of specific antibodies against the LPS in the
339 OMVBbv⁺-immune serum, we performed a Western-blot analysis to determine the

mobility on SDS PAGE of the purified wild-type *B. bronchiseptica* 9.73 LPS. The 3 expected bands were observed: a diffuse one (the lipid-A-KDO core O antigen) 341 containing the O antigen (a single sugar polymer consisting of 2,3-dideoxy-di-N- 342 acetylgalactosaminuronic acid) and the two other faster migrating bands—*i. e.*, Band 343 A (the lipid-A-KDO core) and Band B (lipid-A-KDO) [31]. As anticipated, these 344 LPS-associated bands were recognized by the immune serum induced by OMVBbvir⁺ 345 vaccination (Fig. 3, Panel B).

347 Since the activated components of complement had been previously reported 348 to mediate—at least in part—the killing of *B. bronchiseptica* bacteria by direct 349 bacterial lysis, we next performed a killing assay with immune serum [32]. For this 350 determination, a suspension of 500 *B. bronchiseptica* 9.73 bacteria was incubated in 351 50 μ l of 90% (v/v) serum in PBS to ensure that the serum components were not 352 limiting. This assay revealed that the *B. bronchiseptica* 9.73 bacteria were sensitive to 353 the immune sera (to a degree of *ca.* 50% survival), but resistant to naïve serum (*i. e.*, 354 with 100% surviving; Fig. 3, Panel C). In control experiments using heat-inactivated 355 sera we found 100% of bacterial survival for both immune and naïve sera.

356 To examine the specific role of antibodies in the control and clearance of *B.* 357 *bronchiseptica*, serum from naïve or immunized animals was adoptively transferred 358 into naïve animals 24 h before challenge with *B. bronchiseptica* (5×10^5 CFUs *B.* 359 *bronchiseptica* 9.73). This OMVBbvir⁺-induced serum—collected from the mice 14 360 days after the second dose—cleared *B. bronchiseptica* from the mouse lungs by day 7 361 after the inoculation (at a reduction of 3.5 logs), whereas naïve serum had no 362 significant effect (Fig. 3, Panel D).

363 All the results presented here indicated that the OMVBbvir⁺ vaccine induced a 364 robust humoral immunity that effected an induced protection against *B.* 365 *bronchiseptica*—at least partially—as a consequence of the killing activity of the 366 serum.

367 368 **Induction of a mixed Th1, Th2, and Th17 immune response by OMVBbvir⁺** 369 **vaccination**

370 To characterize the T-cell profile induced by the OMVBbvir⁺ vaccine, we 371 determined the levels of IFN- γ , IL-17, and IL-5 (markers of the respective Th1, Th17, 372 and Th2 immune responses) produced by stimulated spleen-cell cultures (Fig. 4). 373 Lymphocyte-proliferation assays revealed that OMVBbvir⁺ vaccination effectively

374 stimulated lymphocyte proliferation (not shown). Two months after immunization,
375 higher concentrations of IFN- γ ($5,514.48 \pm 1,198.51$ pg/mL; Fig. 4, Panel A) and IL-
376 17 ($8,645.97 \pm 1,796.29$ pg/mL; Fig. 4, Panel B) were produced by spleen cells from
377 OMVBbvir⁺-immunized mice than those present in non-immunized mice (Fig. 4). IL-
378 5 was also detected (469.14 ± 67.03 pg/mL) in the supernatants of stimulated
379 splenocytes from OMVBbvir⁺-immunized mice (Fig. 4, Panel C). All these findings
380 strongly indicate that OMVBbvir⁺ vaccination induces a mixed Th1-Th17-Th2
381 spleen-cell profile.

382 To investigate the role of OMVBbvir⁺ immune spleen cells in protection, we
383 injected BALB/c mice i.p. with 5×10^6 intact spleen cells from nonimmunized
384 animals or from mice that had been immunized with OMVBbvir⁺ vaccine 2 weeks
385 before. Twenty-four hours later, the mice were infected with 5×10^5 CFUs of *B.*
386 *bronchiseptica* 9.73 and then sacrificed 7 days later to determine the number of CFU
387 counts in the lungs (Fig. 4, Panel D). The transfer of spleen cells from immunized
388 animals, but not from the non-immunized mice, resulted in a reduction in bacterial
389 colonization of approximately 2 logs. To look specifically at the function of CD4⁺ T
390 cells in the protection against *B. bronchiseptica* colonization; spleen cells from mice
391 immunized with OMVBbvir⁺ and treated with anti-CD4 antibody were administered
392 to naive mice 24 h before challenge with a sublethal dose of *B. bronchiseptica*.
393 Depletion of CD4⁺ T cells in the spleen of the donor animals, increased the counts of
394 bacteria recovered from the lungs of the recipient animals. The bacterial counts
395 detected in these animals were similar to those found in the lungs of mice that
396 received the spleen cells of naive animals (Fig. 3 Panel D).

397 All these results indicate that spleen cell-mediated immunity, and in particular
398 the CD4⁺ T cells, plays a role in the protection against *B. bronchiseptica* induced by
399 the OMVBbvir⁺ vaccine.

400

401 **Protection against intranasal *B. bronchiseptica* challenge after vaccination with** 402 **OMV from *B. bronchiseptica* blocked in the avirulent phase**

403 Since the avirulent phase of *B. bronchiseptica* could be involved in some stage
404 of the infectious cycle (i.e during the chronic infection), we sought to evaluate
405 whether an experimental vaccine containing the OMV derived from *B. bronchiseptica*
406 9.73 blocked in the avirulent phase (OMVBbvir⁻) was able to confer protection
407 against *B. bronchiseptica* infection. To this end, OMVs were obtained from a mutant

408 previously constructed in our laboratory that was defective in the two-component
409 signal-transduction regulatory system BvgA of *B. bronchiseptica* [25]. The
410 characterization of OMVBbvir⁻ evidenced no differences from the OMVBbvir⁺ in
411 their size distribution, as evaluated by electron microscopy (Fig. 5, Panel A); but
412 certain changes in the electrophoretic profile were detected, even though the major
413 components were the same as those of the OMVBbvir⁺ (Fig. 5, Panel B, left lane). The
414 lack of expression of the main virulence factors and the expression of the flagellin as
415 an avirulent marker were evidenced upon immunoblotting (Fig. 5, Panel B, right
416 lanes). After this initial characterization of the OMVs, intranasal *B. bronchiseptica*
417 challenges were performed. In those experiments, we analyzed the effect of two
418 previous administrations of the OMVBbvir⁻ preparation on the subsequent
419 colonization of the lungs of the experimental mice by the *B. bronchiseptica* strain 9.73
420 ($\approx 10^6$ CFUs in 40 μ l; Fig. 5, panel C and D). The results were compared with those
421 obtained for mice that had been preimmunized with the OMVBbvir⁺ vaccine (Fig. 5,
422 Panel C). PBS-injected mice served as a negative control. Significant differences in
423 the lung bacterial counts between the immunized animals and the negative control
424 group were observed ($p < 0.001$; Fig. 5). Of major significance was the observation
425 that protection against *B. bronchiseptica* challenge was also achieved at same level by
426 the OMVBbvir⁻ immunization. The number of CFUs recovered from the lungs at day
427 7 after challenge likewise dropped by at least 4 orders of magnitude compared to that
428 of the non-immunized mice (Fig. 5, Panel C).

429 For this experimental vaccine, we also evaluated the specific role of induced
430 antibodies in the control and clearance of *B. bronchiseptica*. For that purpose, serum
431 from naïve or immunized animals was adoptively transferred into naïve animals 24 h
432 before a challenge with *B. bronchiseptica* (5×10^5 CFUs of *B. bronchiseptica* 9.73).
433 As had been detected with the OMVBbvir⁺ vaccine, the OMVBbvir⁻-induced
434 serum—collected from mice 14 days after the second dose—cleared *B. bronchiseptica*
435 (by a reduction of 3.5 logs) from the lungs of the challenged mice by day 7 post-
436 inoculation, whereas the naïve serum had no significant effect (Fig. 5, Panel D).

437 The results presented in this final section would clearly indicate that both the
438 OMVBbvir⁻ vaccine and its induced humoral immunity possessed the capability of
439 protecting mice against an infection caused by *B. bronchiseptica*.

440

441 DISCUSSION

442 In this report we describe the development and evaluation of OMVs obtained
443 from *B. bronchiseptica* as vaccines against lung colonization. As we had previously
444 observed in the OMVs derived from *B. pertussis*, the sizes of the OMVs from *B.*
445 *bronchiseptica* 9.73 (at dimensions of 50–150 nm) were consistent from batch to
446 batch. Furthermore, the well known principal *B. bronchiseptica* surface immunogens
447 such as PRN, AC-Hly, and FIM were also detected in the OMVs through
448 immunoblotting. The OMVBbvir⁺ thus characterized were then used in the murine
449 model to examine their safety and protective capability. In the first experiments
450 described, we performed comparisons with a whole-cell vaccine prepared in our
451 laboratory since a previous report had indicated that the same type of formulation in
452 dogs reduced the disease burden and the lesions in the vaccinated animals relative to
453 those of infected naïve controls [10]. We observed that two doses of our experimental
454 OMVBbvir⁺ vaccine administered 2 weeks before *B. bronchiseptica* challenge fully
455 protected the BALB/c mice against the colonization of different *B. bronchiseptica*
456 strains obtained from diverse hosts—two representative ones being shown: a farm
457 animal and a human). As to the safety of these preparations, in comparison with the
458 whole-cell vaccine, we observed only a minimal rise in the levels of the
459 proinflammatory IL-6 in serum just after OMV vaccination. These results would
460 position the OMV-based vaccines above the classic cellular preparations; as
461 OMVBbvir⁺ induces equal levels of protection, but along with adequate levels of
462 safety. Another significant and relevant result detected with our experimental vaccine
463 was that ability to induce protection against different isolates of *B. bronchiseptica*
464 obtained from diverse hosts. This result becomes especially pertinent upon
465 consideration of the diversity of genotypes already reported for *B. bronchiseptica* [33,
466 34] since this finding would indicate that the formulation of a specific OMV-based
467 vaccine for each specific isolate would be unnecessary.

468 In the present work, we also detected that the antibody titers of OMVBbvir⁺-
469 vaccinated mice were higher on day 14 after the second dose and before *B.*
470 *bronchiseptica* challenge than those of the non-immunized control group. Moreover,
471 this OMVBbvir⁺-vaccine-induced immune serum recognized a group of antigens that
472 included OMPc and the GroEL-like protein along with LPS. That the OMPc and
473 GroEL-like protein had been previously detected in mice immunized with OMV
474 derived from *B. pertussis* [23] was of interest to us. As discussed in a previous
475 publication, both proteins in *Bordetella* and other microorganisms have been

described as promising vaccine targets [35, 36]. By immunoblotting assays we also detected that the OMVBbvir⁺ was able to induce antibodies against these LPS, an immunogenic bacterial component with proven protective capability [37]. Based on the detection of these protection-inducing immunogens, we next sought to investigate if the OMVBbvir⁺ vaccine-induced antibodies alone were sufficient to confer protection against *B. bronchiseptica*. The passive-transfer assays we therefore performed demonstrated that the vaccine-induced serum reduced the number of viable bacteria in the lungs by about 4 logs. This protective role of the antibodies was in accordance with previous results reported by other authors [38]. In fact, protection against *B. bronchiseptica* infection had been evidenced with antibodies induced by either infection or vaccination [38] through mechanisms that appeared to be different: whereas the immunity induced by previous infection offered significant protection even in the absence of complement or the induction of the monocyte IgG receptors FcγRs, the vaccination-induced protection required both complement and the FcγRs [38].

In addition, we found that CD4⁺ T cells also contributed to the protection exhibited by the OMVBbvir⁺-based vaccine. The protection against *B. bronchiseptica* infection induced by the OMV-based vaccine seems to involve a dual mechanism involving both a humoral and a cellular immune response as was evidenced by adoptive transfer experiments.

Based on our previous results with OMVs derived from *B. pertussis* in the avirulent phase, [23] and taking into account the potential role of that phase in the induction of *B. bronchiseptica* the infection process (i.e chronic infection), we decided to evaluate if the OMVs derived from *B. bronchiseptica* blocked in the avirulent phase (OMVBbvir⁻) were also able to induce protection. The OMVBbvir⁻ formulation indeed proved to be protective, as judged by the significant decrease of 4 logs observed in the lung bacterial counts between avirulent OMVBbvir⁻-immunized mice and the non-immunized control group ($p < 0.001$; Fig. 5, panels C and D). The detected flagella of *B. bronchiseptica* in the OMVBbvir⁻ preparation probably contributes to the protective capacity of the OMVBbvir⁻ formulation since it has been described as a potent proinflammatory factor that induces chemokines, cytokines and expression of the host defense gene [39]. In addition, it was demonstrated that *B. bronchiseptica* flagellin is able to effectively signal through both human and mouse the Toll-like receptor 5 [39]. The protective effect is elicited by non-replicative

510 avirulent components that have thus far not been reported as capable of conferring
511 protection against *B. bronchiseptica*. To our knowledge, the only report regarding a
512 vaccine with avirulent *B. bronchiseptica*, was done in dogs with a live, intact avirulent
513 strain [40]. Moreover, the authors demonstrated that the vaccinated dogs were
514 protected against colonization by as early as 48 h after immunization [40].

515 Considered all together, the results presented here provide clear evidence that
516 the OMVs derived from *B. bronchiseptica* exhibit a high level of protection against *B.*
517 *bronchiseptica* that is not dependent on bacterial virulence-factor expression.
518 Moreover, we further demonstrate that the protection induced by these OMVBb-based
519 vaccines is mediated mainly by antibodies but also by CD4+T cells.

520

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527

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530 DFH. DFH, MEG and DB, are members of the Scientific Career of CONICET. MEZ
531 and EB are fellows from CONICET.

532

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673

674

675 **Table 1. Serum IgG and isotypes antibodies titers.**

	Total IgG	IgG2a	IgG1	IgG1:IgG2a
OMVBbvir ⁺ immunized	259.6 ± 32.4	250.2 ± 71.9	558.7 ± 124.3	2.2
Non-immunized	9.3	ND	11.3	

676

677 LEGENDS TO THE FIGURES

678 **Fig. 1.** Panel A: Transmission-electron-microscopy image of the negatively stained
679 preparations obtained from *B. bronchiseptica* 9.73 in the virulent phase (OMVBbvir⁺;
680 scale bar: 200 nm). Panel B: Analysis of OMVBbvir⁺ by 12.5% (w/v) SDS-PAGE.
681 The bands were visualized by staining with Coomassie brilliant blue R-250.
682 Molecular weights in kDa are indicated on the left. Panel C: Immunoblots of
683 OMVBbvir⁺ and purified proteins indicating the bands binding to anti-AC-Hly-, anti-
684 PRN-, and anti-FIM2-specific polyclonal mouse antibodies. In the figure, the sources
685 of the samples are indicated above the panels.

686

687 **Fig. 2.** Effect of (i.p.) immunization with active OMVBbvir⁺ in the mouse-intranasal-
688 challenge model. WCVBbvir⁺ was used as a positive control. Panel A: *B.*
689 *bronchiseptica* 9.73 was used as challenge bacteria (5×10^5 CFUs in 40 μ l). Panel B:
690 Rabbit *B. bronchiseptica* RB50 and the human clinical isolate *B. bronchiseptica*
691 AR705 were also used as challenge bacteria (5×10^5 CFUs in 40 μ l). In all instances
692 three biological replicates were performed, with the results from a representative one
693 being presented. Results depicted are the means of four mice per group at 7 days after
694 challenge. The line indicates the lower limit of detection. In both panels, the number
695 of bacteria recovered from the mouse lungs, expressed as the \log_{10} means \pm SEM
696 (error bars) of the CFUs per lungs, is plotted on the *ordinates* for the samples from the
697 lungs of the experimental groups indicated on the *abscissas*. The *bronchiseptica*
698 strains used for the challenges are depicted above the panels. The asterisks (*)
699 indicate significant differences at a $p < 0.001$.

700
701 **Fig. 3.** Panel A: Analysis of OMVBbvir⁺ antigenic reactivity by 12.5% (w/v) SDS-
702 PAGE visualized by staining with Coomassie Brilliant Blue (Lane 1). Molecular
703 weights in kDa are indicated on the left. Immunoblots (Lane 2) with the OMVBbvir⁺-
704 protein sample were probed against polyclonal antiserum obtained from mice
705 immunized with OMVBbvir⁺. Bands a and b were identified as GroEL and OMPc
706 respectively. Panel B: Analysis of purified *B. bronchiseptica* LPS by 15% (w/v) SDS-
707 PAGE (Lane 1) and by immunoblotting with the polyclonal antiserum obtained from
708 mice immunized with OMVBbvir⁺ (Lane 2). The arrows indicate the locations of the
709 O antigen and the bands A and B that were recognized by the antiserum used in
710 immunoblotting in Lane 2. Panel C: *In-vitro*-killing assay of *B. bronchiseptica* with
711 immune sera derived from OMVBbvir⁺-vaccinated animals and from non-immunized
712 mice. Three biological replicates were performed, with the results from a
713 representative one being presented. The asterisks (*) indicate significant differences at
714 a $p < 0.05$. In the panel, the percent survival of the bacteria is plotted on the *ordinate*
715 for each of the sera indicated on the *abscissa*. Panel D: Effect of passive
716 immunization with sera collected from OMVBbvir⁺ immunized mice. *B.*
717 *bronchiseptica* 9.73 was used as the challenge bacterium (5×10^5 CFUs in 40 μ l).
718 Three biological replicates were performed, with the results from a representative one
719 being presented. The data are the mean values from four mice per group at 7 days
720 after challenge. The line indicates the lower limit of detection. In the panel, the

number of bacteria recovered from the mouse lungs, expressed as the $\log_{10}(\text{means} \pm \text{SEM} [\text{error bars}])$ of the CFUs per lungs, is plotted on the *ordinate* for each of the experimental groups on the *abscissas*. The asterisks (*) indicate significant differences at a $p < 0.001$.

Fig. 4. Panels A–C: Cytokine production by splenocytes from immunized mice. BALB/c mice were immunized with two doses of OMVBbvir⁺ or left non-immunized. Two months after the final immunization, the mice were sacrificed and their cultured spleen cells stimulated with OMVBbvir⁺ or simply incubated in the culture medium (negative control). After 72 h, the concentrations of IFN- γ (Panel A), IL-17 (Panel B), and IL-5 (Panel C) were determined by ELISA in the culture supernatants. The results are expressed as the mean values (\pm the standard error) of three experiments with 4 mice per group. Significant differences were analyzed for each cytokine between non-immunized and immunized mice ($p \leq 0.01$, Panel A) or $p < 0.001$ (panels B and C). In each of the three panels, the concentration of the cytokine indicated on the *ordinate* in -pg.ml^{-1} that was assayed in the culture supernatant is plotted for the two experimental conditions—exposure to medium or vesicles—designated on the *abscissa*. Panel D: Effect of passive immunization with spleen cells collected from OMVBbvir⁺ immunized mice with or without depletion of CD4⁺ T cells. Donor animals were i.p. injected with anti-CD4 antibody or the corresponding isotype control 24 h before the spleens were collected. After the i.p. injection of spleen cells from OMVBbvir⁺-immunized or non-immunized mice, as indicated on the *abscissa*, the recipient mice were challenged with *B. bronchiseptica* 9.73 (5×10^5 CFUs in 40 μl). Three biological replicates were performed, with the results from a representative one being presented. The data represent the means of four mice per group at 7 days after challenge. The line indicates the lower limit of detection. In the panel, the number of bacteria recovered from the mouse lungs, expressed as the $\log_{10}(\text{means} \pm \text{SEM} [\text{error bars}])$ of the CFUs per lungs, is plotted on the *ordinate* for the samples from the lungs of the mice receiving the spleen cells from the source indicated on the *abscissa*. In all four panels, the white bars indicate spleen cells from non-immunized mice and the black bars those from immunized mice. The asterisks (*) indicate significant differences at a $p < 0.001$.

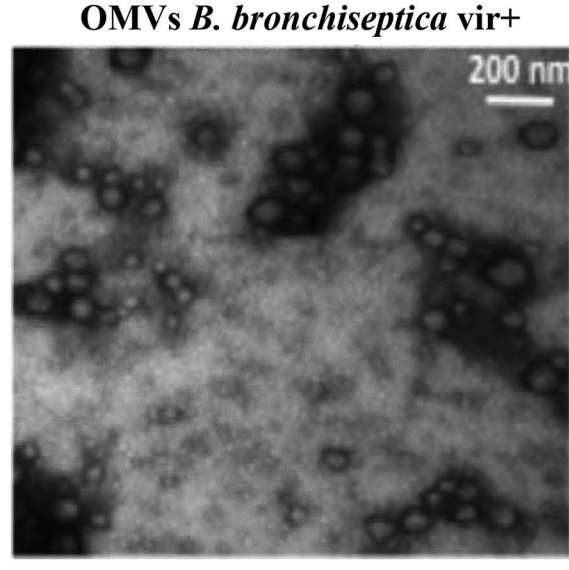
754 **Fig. 5.** Panel A: transmission-electron-microscopy image of negatively stained OMVs
755 obtained from *B. bronchiseptica* 9.73 in the avirulent phase (OMVBbvir⁻; scale bar:
756 200 nm). Panel B: Analysis of OMVBbvir⁻ by 12.5% (w/v) SDS-PAGE with
757 visualization by Coomassie Brilliant Blue. Molecular weights are indicated (on the
758 left side). On the right side, immunoblots of OMVBbvir⁻ and the purified
759 recombinant proteins AC-Hly, PRN, FLA, and FIM2, as detected by the respective
760 specific polyclonal mouse antibodies. Panel C: Effect of active (i.p.) immunization
761 with OMVBbvir⁻ in the mouse intranasal challenge model. Panel D: Effect of passive
762 immunization with sera collected from OMVBbvir⁻-immunized mice. For panels C
763 and D, *B. bronchiseptica* 9.73 was used as the challenge bacterium (1×10^6 CFUs in
764 40 μ l). Three biological replicates were performed, with the results from a
765 representative one being presented. The data represent the means of four mice per
766 group at 7 days after challenge. The line indicates the lower limit of detection. In the
767 panels, the number of bacteria recovered from the mouse lungs, expressed as the
768 \log_{10} means \pm SEM (error bars) of CFUs per lungs, is plotted on the *ordinates* for the
769 lung samples from the mice receiving the immunogen—in the form of vesicles (Panel
770 C) or serum (Panel D)—indicated on the *abscissas*. The asterisks (*) indicate
771 significant differences at a $p < 0.001$.

772

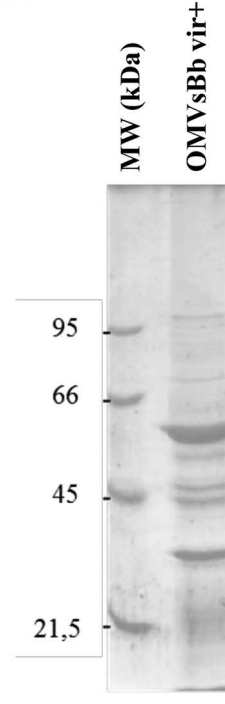
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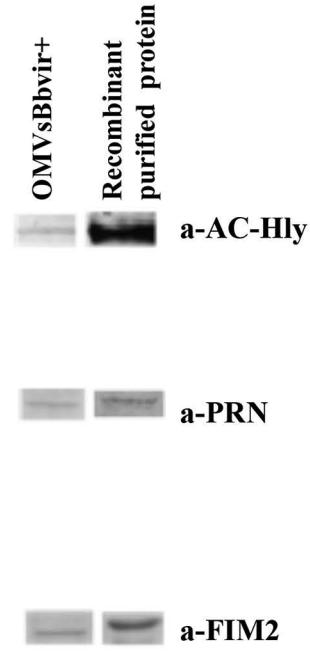
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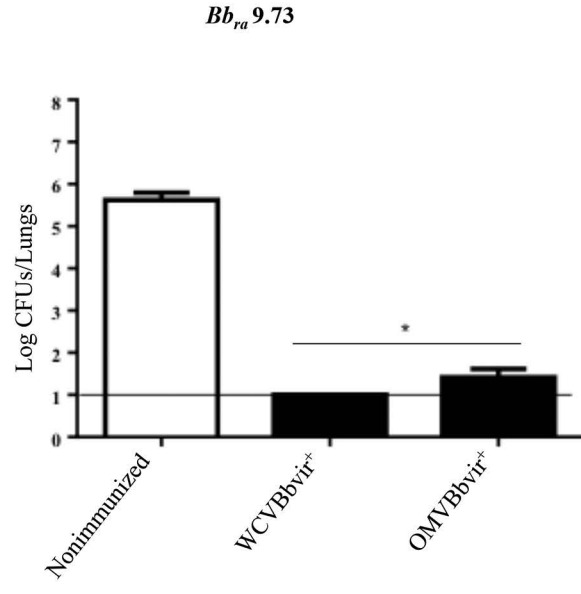
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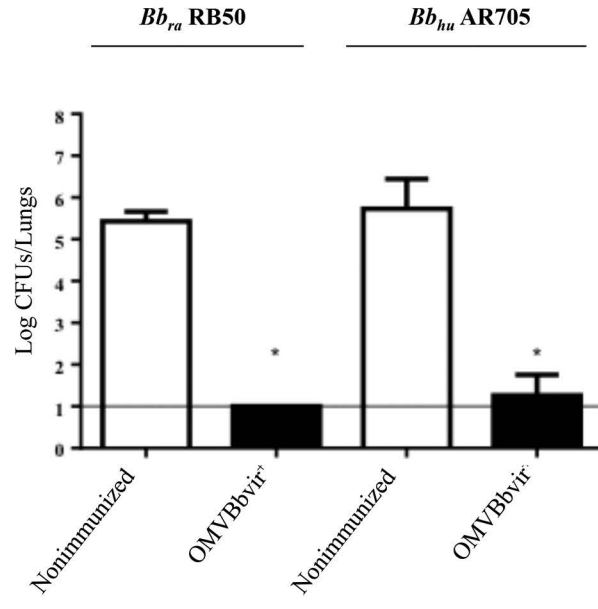
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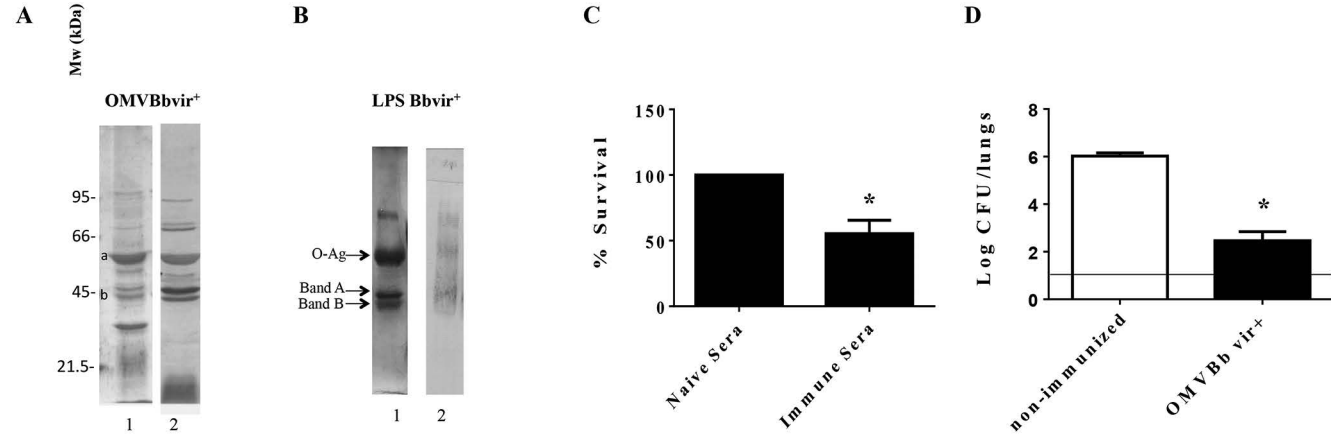


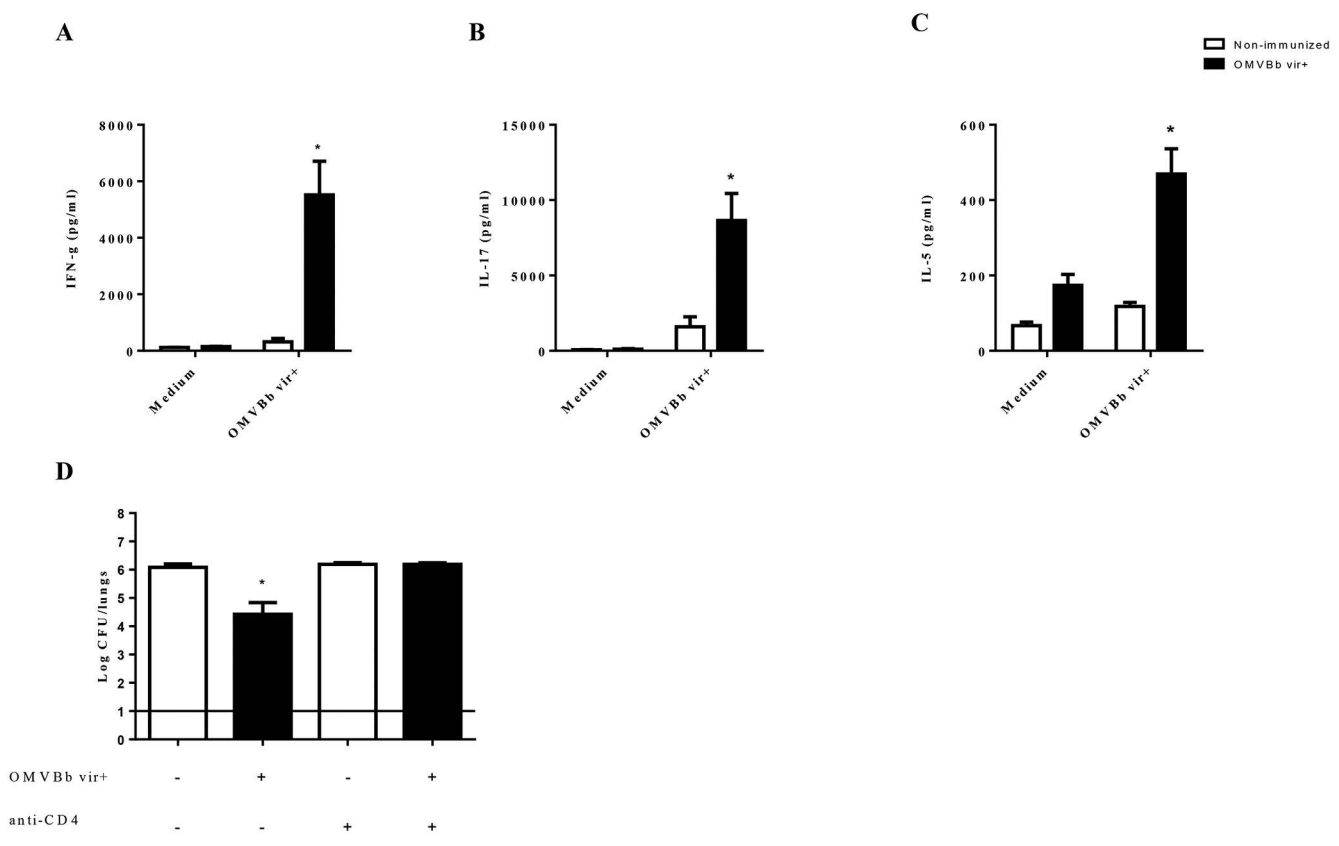
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B

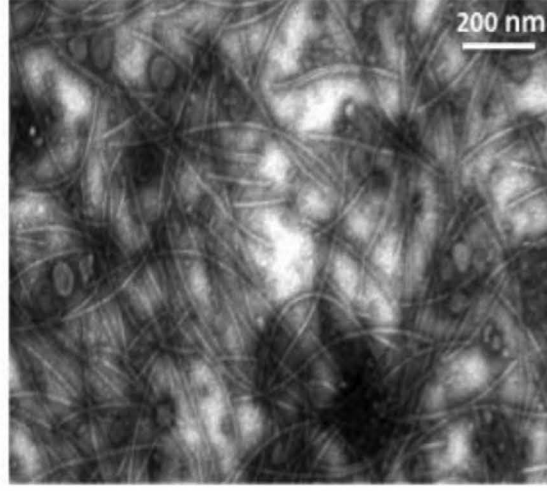




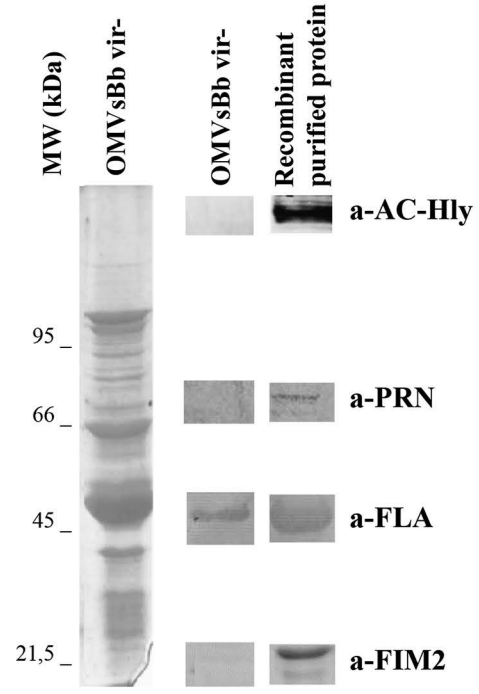


A

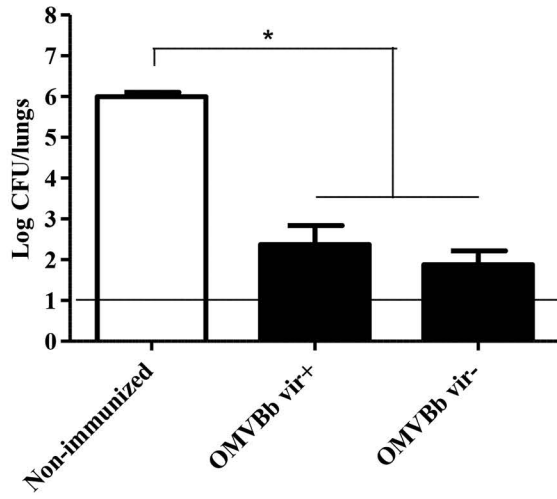
OMVs *B. bronchiseptica* vir-



B



C



D

