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Short Communication

A Lipopolysaccharide-free Outer Membrane Vesicle Vaccine Protects against *Acinetobacter baumannii* Infection

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Short title: LPS-free OMVs protect against *A. baumannii*

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Key Words: *Acinetobacter baumannii*, outer membrane vesicles, vaccine, lipopolysaccharide

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Abstract

Outer membrane vesicles (OMVs) were isolated from an *Acinetobacter strain* deficient in lipopolysaccharide (LPS) due to a mutation in *lpxD* (IB010). Two immunizations with 10 µg of IB010 OMVs elicited total IgG, IgM, IgG1 and IgG2c titers similar to those observed after immunization with OMVs derived from the parental strain (ATCC 19606) and IB010 OMVs plus purified LPS. Immunization with IB010 OMVs resulted in significantly reduced post-infection spleen bacterial loads and serum IL-1β and IL-6 levels compared to control mice in a disseminated sepsis model. Mice immunized with 10 µg IB010 demonstrated significant, but partial, protection (75%) against infection, whereas mice immunized with ATCC 19606 OMVs or IB010 OMVs plus purified LPS were completely protected. Immunization of mice with 100 µg of IB010 OMVs completely protected mice from infection. This study demonstrates that LPS deficient *A. baumannii* produces OMVs, and that immunization with these OMVs elicits protective immunity against infection.

Introduction

Bacterial outer membrane vesicles (OMVs) contain multiple antigenic proteins from the bacterial outer membrane, making them attractive antigens for the development of vaccines. Immunization with OMVs has been demonstrated to induce protective immunity to multiple Gram negative bacterial pathogens, including *Neisseria meningitidis [1], Escherichia coli* [2], and *Bordetella pertussis* [3], among others. Notably, a licensed vaccine for the prevention of meningitis caused by serogroup B *N. meningitidis* (Bexsero™) contains OMVs as one of four antigens [1].

Infections caused by multidrug resistant *Acinetobacter baumannii* are increasingly difficult to treat given the lack of antibiotics that retain activity against these strains. In this context, the development of alternative prevention and treatment strategies, such as vaccines, is warranted. *A. baumannii* OMVs have been shown to induce a robust antibody response against multiple outer membrane proteins, significantly reduced post-infection bacterial loads, and protected mice against infection in experimental models of infection [4-6]. Recently, we isolated strains of *A. baumannii* with mutations in genes involved in lipid A biosynthesis, *lpxA*, *lpxC* and *lpxD* [7, 8]. These strains are deficient in lipopolysaccharide (LPS) and demonstrate very low levels of endotoxin activity. The goal of the present study was to determine if *A. baumannii* strains deficient in LPS were able to produce OMVs, and characterize the immune response to vaccination with LPS-free OMVs.

Materials and Methods

Bacterial strains and OMV preparation

A. baumannii ATCC 19606 is an antibiotic susceptible reference strain. IB010 is an LPS-deficient derivative of ATCC 19606 that is deficient in LPS biosynthesis due to a 462 base pair deletion in the *lpxD* gene [8]. OMVs from ATCC 19606 and IB010 were prepared and purified by centrifugation as described previously [6], with the only modification being that ATCC 19606 was grown in Mueller Hinton broth, and IB010 was grown in Mueller Hinton broth supplemented with 10 µg/ml of colistin in order to maintain selective pressure on the *lpxD* mutation. Endotoxin levels were determined in three independent assays using the QCL-1000 Limulus Amebocyte Assay (Lonza).

LPS extraction and SDS-PAGE

LPS was extracted from approximately $3.5x10^9$ ATCC 19606 cells with the LPS Extraction Kit (iNtRON Biotechnology, Inc), and eluted in 40 µl of 10 mM Tris-HCl (pH 8.0). The absence of protein in LPS extracts was confirmed by separating 10 µl of purified LPS samples on 12.5 % polyacrylamide gels followed by Coomassie blue staining (Simply Blue Safestain, Thermo Scientific).

For SDS-PAGE, purified OMVs were resuspended in 50 µl of 50 mM Tris-HCl pH 7.2, 8 M urea and 2 M thiourea, and 10 µg of total protein were resolved on a 12 % polyacrylamide gel. Proteins were visualized by Coomassie staining (Simply Blue Safestain, Thermo Scientific).

Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

For TEM, OMV suspensions were fixed with 1.6% cold glutaraldehyde in 0.1 M sodium cacodylate (pH: 7.4) for 1h at 4ºC and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH: 7.4) for 1 h at 4ºC. OMVs were dehydrated in increasing concentrations of acetone and embedded in Spurr's resin. OMVs were then visualized and photographed with a Zeiss Libra 120 transmission electron microscopy (80 kV). For scanning electron microscopy, cell suspensions were fixed as described for OMVs. Cells were coated with a layer of gold and visualized using a Zeiss Auriga microscopy (20 kV).

Immunization, ELISAs, and infection

Purified OMVs were resuspended in PBS to a concentration of 0.1 µg/µl or 1.0 µg/µl and combined 1:1 (v/v) with the aluminum-based adjuvant, Alhydrogel 2% (InvivoGen). Six to 8-week-old, female C57BL/6 mice (University of Seville; n = 8/group) were immunized with 100 µl of the mixture in each quadriceps muscle (total volume 200 µl; vaccine dose 10 µg or 100 µg OMV protein) on days 0 and 14. One group of mice was immunized with a mixture of IB010 OMVs (10 µg protein) plus purified LPS (30 µg). Control mice were immunized with a mixture of PBS and adjuvant.

On days 7 and 21 sera were collected from the retro-orbital sinus. ELISAs were performed as described previously using 96- well plates coated with $5x10^7$ bacteria cells/well of the ATCC 19606 strain [8, 9].

Seven days after the second immunization (day 21), mice were infected with a lethal dose of the ATCC 19606 strain using a previously characterized mouse intraperitoneal sepsis model [10], and monitored for 7 days. Post-infection spleen

bacterial loads and serum levels of the pro-inflammatory cytokines IL-1β and IL-6 were determined (n = 8/group) at 12 hours post-infection as described previously [10].

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego California, USA). Antibody titers, bacterial loads and cytokine levels were compared using the Student's *t* test or the Mann-Whitney U test. Survival was analyzed using the log-rank test. *p* values of < 0.05 were considered significant.

Results

Purification and characterization of LPS-free OMVs

It has been reported that LPS is a major component of OMVs from Gram negative bacteria [11, 12], raising the possibility that *A. baumannii* stains deficient in LPS biosynthesis are unable to produce OMVs. To address this, cultures of IB010, an *A. baumannii* strain deficient in LPS biosynthesis due to a mutation in the *lpxD* gene, and the parental strain, ATCC 19606, were subjected to scanning electron microscopy. OMVs could clearly be visualized budding from bacterial cells in both ATCC 19606 and IB010 cultures (Figures 1A and 1B, respectively), indicating that OMVs are produced in LPS-deficient *A. baumannii*. OMVs from ATCC 19606 and IB010 were purified using centrifugation from culture supernatants as described previously [6], although it should be noted that contamination with non-OMV associated bacterial proteins cannot be completely ruled out. As shown in Figures 1C (ATCC 19606) and 1D (IB010), transmission electron microscopy of OMV preparations from both strains yielded structures similar to what has previously been reported for OMVs from the

ATCC 19606 strain [6]. SDS-PAGE using OMVs from the ATCC 19606 and IB010 was carried out to qualitatively compare the protein content from the strains, demonstrating a similar banding pattern upon Coomassie staining (Figure 1E). OMVs from IB010 demonstrated very low levels of endotoxin activity by Lymphocyte Amebocyte Assay ($6.3 \times 10^{-3} \pm 8.2 \times 10^{-4}$ endotoxin units/μg protein).

Antibody response after immunization with OMVs

To assess the antigenicity of LPS-free OMVs obtained from IB010 compared to OMVs from the parental 19606 strain, mice were immunized with 10 μg of the indicated OMVs based on our previous study demonstrating that this dose induces protective immunity upon vaccination with ATCC 19606 OMVs [6]. Total IgG titers (against ATCC 19606 whole cells) in serum were determined on days 7 and 21 after immunization on days 0 ad 14 (Figure 2A). Mice immunized with all preparations (ATCC 19606 OMVs, IB010 OMVs and IB010 OMVs + purified LPS) demonstrated significant levels of antigen-specific IgG on day 7 compared to control mice receiving adjuvant alone. All groups of mice demonstrated a tendency towards increased total IgG titers at day 21 compared to day 7 after boosting on day 14. Antigen-specific IgG1, IgG2a and IgM titers on day 21 were significantly higher in all groups compared to the control group (Figures 3B, 3C and 3D, respectively). Notably, IgG2a titers in mice vaccinated with IB010 OMVs were significantly lower than in mice immunized with ATCC 19606 OMVs or with IB010 OMVs + purified LPS (Figure 3C).

Effect of vaccination on post-infection bacterial loads, cytokine levels, and survival

Seven days after the second immunization (day 21), groups of mice were infected with 2×10^6 cfu of the ATCC 19606 strain ($302.8 \times LD_{50}$). All groups had

significantly lower bacterial loads in spleens compared to control mice (Figure 3A). There were no differences in bacterial loads between mice immunized with ATCC 19606 OMVs, IB010 OMVs, and IB010 OMVs + purified LPS. Survival was assessed after infection with 2 x 10^6 cfu of the ATCC 19606 strain (302.8 x LD_{50}), demonstrating that all groups showed significant survival compared to the control group (Figure 3B). Importantly, mice immunized with ATCC 19606 OMVs, and IB010 OMVs + LPS demonstrated 100% protection, whereas mice immunized with the IB010 OMVs demonstrated only partial (75%) protection.

As shown in Figures 3C and 3D, serum levels of IL-1β and IL-6 were significantly lower in all groups of vaccinated mice compared to control mice, suggesting that vaccinated mice did not experience the pro-inflammatory cytokine release associated with the development of septic shock.

Immunization with 100 µg of LPS-free OMVs

We next wanted to determine if increasing the dose of IB010 OMVs from 10 µg to 100 µg increased post-infection survival of vaccinated mice. As shown in Figures 4A, 4B, 4C and 4D, two immunizations with 100 µg of IB010 OMVs induced significant levels of total IgG, IgG1, IgG2c and IgM, respectively, compared to control mice. Spleen bacterial loads were approximately 10,000-fold lower in vaccinated compared to control mice 12 hours after infection with 1.8 x 10^6 cfu of the ATCC 19606 strain (272.5 x LD_{50}), similar to the result obtained after vaccination with 10 µg of IB010 OMVs (Figure 3A). Notably, as shown in Figure 4F, mice vaccinated with 100 µg of IB010 OMVs were completely protected from infection with 1.8 x 10^6 cfu of the ATCC 19606 strain (272.5 x LD_{50}).

Discussion

OMVs have multiple characteristics that make them attractive for the development of antibacterial vaccines. First, OMVs contain multiple antigens from the bacterial outer membrane, which may be ideal for eliciting antibodies that can opsonize bacterial cells and facilitate their clearance via phagocytosis. In addition, the inclusion of multiple antigens in antibacterial vaccines may be desirable due to sequence variation of bacterial surface antigens and the ability of bacteria to decrease expression of some surface proteins. Vaccines that generate antibodies against multiple antigens may have increased coverage compared to vaccines based on single recombinant proteins against strains with variation in surface antigen amino acid sequence or expression. Previously-reported vaccines against *A. baumannii* that employ multiple surface antigens, such as inactivated whole cells [8, 13], outer membrane extracts[10, 14], OMVs [4-6], live attenuated cells [15], and bacterial ghosts [16] have been shown to provide protective immunity against multiple strains in animal models. Similar to the present study, these approaches likely elicited an antibody response against multiple surface antigens. A second characteristic of OMVs that makes them attractive for vaccine development is that they are non-living particles that are easily separated from bacterial cells. Thus, they may not be subject to the safety issues associated with vaccines based on whole bacterial cells, such as inactivated cells, which may present concerns due to incomplete inactivation, or attenuated strains, which may have residual virulence or be capable of producing infection in highly immune compromised hosts. A final characteristic of OMVs is their ability to interact with host immune effector cells. OMVs have been shown to stimulate multiple cells of the immune system including neutrophils, macrophages, dendritic cells, and B and T lymphocytes (reviewed in [11]), thus possibly potentiating the immune response to OMV-associated antigens.

LPS is a major component of OMVs derived from Gram negative species [11, 12]. For this reason, we first wanted to determine if *A. baumannii* strains deficient in LPS were able to produce OMVs. Electron microscopy of IB010 cultures and OMV preparations (Figures 1B and 1D) clearly indicated the production of OMVs from this strain, indicating the LPS is not essential for OMV production in *A. baumannii*. OMVs containing truncated forms of LPS have previously been isolated from *N. meningitidis* [17], however to our knowledge this is the first description of OMV production from bacterial cells deficient in LPS. Decreased LPS content in OMV vaccines is desirable given the reactogenicity of LPS from Gram negative species in humans and the limits regarding endotoxin activity required for medication used in humans. Notably, the results presented here indicate that OMVs from LPS-free *A. baumannii* may be somewhat less immunogenic than wildtype OMVs, as indicated by lower IgG2c levels at 21 days (Figure 2C), and afford only partial protection from infection when a dose of 10 µg is used for immunization (Figure 3B). This may be due to adjuvant-like activity of *A. baumannii* LPS, which is known to be a ligand for TLR4 [18]. Alternatively, antibodies directed against LPS may contribute to protective immunity. Importantly, increasing the dose of LPS-free OMVs to 100 µg resulted in complete protection from infection (Figure 4F).

In conclusion, this study demonstrates that OMVs are produced from an *A. baumannii* strain deficient in LPS synthesis, and that immunization with these OMVs elicits protective immunity against *A. baumannii* in an experimental model of infection.

Acknowledgments

This work was supported by Plan Nacional de I+D+i 2013- 2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0009) - cofinanced by European Development Regional Fund "A way to achieve Europe", Operative program Intelligent Growth 2014- 2020, a grant from the la Consejería de Economía, Innovación, Ciencia y Empleo (PIE-CTS-2027), and a grant from the Fundación Progreso y Salud/Consejería de Igualdad, Salud y Politicas Sociales of Junta de Andalucía (PI-0098-2014).M.R.P. was supported by the Subprograma Sara Borrell from the Ministerio de Economía y Competitividad of Spain (CD14/00014).

Conflict of Interest Statement

MRP, MGC, JP and MJM are listed as inventors on a patent application describing the use of LPS-free *A. baumannii* and its derivatives as vaccines. MJM is a founder and shareholder of Vaxdyn, S.L., a biotechnology company developing vaccines for bacterial infections.

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Figure Legends

Figure 1. Visualization and characterization of OMVs from ATCC 19606 and IB010. Scanning electron microscopy of ATCC 19606 (A) and IB010 cultures (B) demonstrating OMV production. Arrows indicate sites of OMV vesiculation from bacterial cells. The bar indicates 5 µm. Transmission electron microscopy of purified OMVs from ATCC 19606 (C) and IB010 (D) cultures. SDS-PAGE and Coomassie staining of 10 µg of total protein of ATCC 19606 and IB010 OMVs.

Figure 2. Antibody response after immunization with ATCC 19606 and IB010 OMVs. Groups of 8 mice were immunized on days 0 and 14 with 10 µg of ATCC 19606 OMVs, 10 µg of IB010 OMVs, 10 µg IB010 OMVs + purified LPS, or with adjuvant alone as a control. Total IgG titers in serum against ATCC 19606 whole cells were determined in serum samples collected on days 7 and 21 (A). IgG1 (B), IgG2c (C), and IgM (D) titers were determined in serum samples collected on day 21. *** $p < 0.001$ and * $p < 0.05$ compared to control mice.

Figure 3. Post-infection bacterial loads, survival and serum pro-inflammatory cytokine levels. Groups of 8 mice were immunized on days 0 and 14 with 10 µg of ATCC 19606 OMVs, 10 µg of IB010 OMVs, 10 µg IB010 OMVs + purified LPS, or with adjuvant alone as a control, and infected with 2×10^6 cfu of ATCC 19606 on day 21. Spleen bacterial loads 12 hours post-infection (A) and post infection survival (B) were determined for groups of mice. Serum levels of IL-1β (C) and IL-6 (D) were determined samples taken 12 hours post-infection. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared to control mice.

Figure 4. Immunization with 100 µg IB010 OMVs. Groups of 8 mice were immunized on days 0 and 14 with 100 µg of IB010 OMVs or with adjuvant alone as a control, and infected with 1.8×10^6 cfu of ATCC 19606 on day 21. Total IgG titers were determined on serum samples taken on days 7 and 21 (A). IgG1 (B), IgG2c (C), and IgM (D) titers were determined in serum samples taken on day 21. Spleen bacterial loads were determined 12 hours post-infection in vaccinated and control mice (E). Post-infection survival was determined in groups of mice over 7 days. *** $p < 0.001$ and ** $p < 0.01$ compared to control mice.

Abstract

Outer membrane vesicles (OMVs) were isolated from an *Acinetobacter strain* deficient in lipopolysaccharide (LPS) due to a mutation in *lpxD* (IB010). Two immunizations with 10 µg of IB010 OMVs elicited total IgG, IgM, IgG1 and IgG2c titers similar to those observed after immunization with OMVs derived from the parental strain (ATCC 19606) and IB010 OMVs plus purified LPS. Immunization with IB010 OMVs resulted in significantly reduced post-infection spleen bacterial loads and serum IL-1β and IL-6 levels compared to control mice in a disseminated sepsis model. Mice immunized with 10 µg IB010 demonstrated significant, but partial, protection (75%) against infection, whereas mice immunized with ATCC 19606 OMVs or IB010 OMVs plus purified LPS were completely protected. Immunization of mice with 100 µg of IB010 OMVs completely protected mice from infection. This study demonstrates that LPS deficient *A. baumannii* produces OMVs, and that immunization with these OMVs elicits protective immunity against infection.

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Statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego California, USA). Antibody titers, bacterial loads and cytokine levels were compared using the Student's *t* test or the Mann-Whitney U test. Survival was analyzed using the log-rank test. *p* values of < 0.05 were considered significant.

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As shown in Figures 3C and 3D, serum levels of IL-1β and IL-6 were significantly lower in all groups of vaccinated mice compared to control mice, suggesting that vaccinated mice did not experience the pro-inflammatory cytokine release associated with the development of septic shock.

Immunization with 100 µg of LPS-free OMVs

We next wanted to determine if increasing the dose of IB010 OMVs from 10 µg to 100 µg increased post-infection survival of vaccinated mice. As shown in Figures 4A, 4B, 4C and 4D, two immunizations with 100 µg of IB010 OMVs induced significant levels of total IgG, IgG1, IgG2c and IgM, respectively, compared to control mice. Spleen bacterial loads were approximately 10,000-fold lower in vaccinated compared to control mice 12 hours after infection with 1.8 x 10^6 cfu of the ATCC 19606 strain (272.5 x LD_{50}), similar to the result obtained after vaccination with 10 µg of IB010 OMVs (Figure 3A). Notably, as shown in Figure 4F, mice vaccinated with 100 µg of IB010 OMVs were completely protected from infection with 1.8 x 10^6 cfu of the ATCC 19606 strain (272.5 x LD_{50}).

Discussion

OMVs have multiple characteristics that make them attractive for the development of antibacterial vaccines. First, OMVs contain multiple antigens from the bacterial outer membrane, which may be ideal for eliciting antibodies that can opsonize bacterial cells and facilitate their clearance via phagocytosis. In addition, the inclusion of multiple antigens in antibacterial vaccines may be desirable due to sequence variation of bacterial surface antigens and the ability of bacteria to decrease expression of some surface proteins. Vaccines that generate antibodies against multiple antigens may have increased coverage compared to vaccines based on single recombinant proteins against strains with variation in surface antigen amino acid sequence or expression. Previously-reported vaccines against *A. baumannii* that employ multiple surface antigens, such as inactivated whole cells [8, 13], outer membrane extracts[10, 14], OMVs [4-6], live attenuated cells [15], and bacterial ghosts [16] have been shown to provide protective immunity against multiple strains in animal models. Similar to the present study, these approaches likely elicited an antibody response against multiple surface antigens. A second characteristic of OMVs that makes them attractive for vaccine development is that they are non-living particles that are easily separated from bacterial cells. Thus, they may not be subject to the safety issues associated with vaccines based on whole bacterial cells, such as inactivated cells, which may present concerns due to incomplete inactivation, or attenuated strains, which may have residual virulence or be capable of producing infection in highly immune compromised hosts. A final characteristic of OMVs is their ability to interact with host immune effector cells. OMVs have been shown to stimulate multiple cells of the immune system including neutrophils, macrophages, dendritic cells, and B and T lymphocytes (reviewed in [11]), thus possibly potentiating the immune response to OMV-associated antigens.

LPS is a major component of OMVs derived from Gram negative species [11, 12]. For this reason, we first wanted to determine if *A. baumannii* strains deficient in LPS were able to produce OMVs. Electron microscopy of IB010 cultures and OMV preparations (Figures 1B and 1D) clearly indicated the production of OMVs from this strain, indicating the LPS is not essential for OMV production in *A. baumannii*. OMVs containing truncated forms of LPS have previously been isolated from *N. meningitidis* [17], however to our knowledge this is the first description of OMV production from bacterial cells deficient in LPS. Decreased LPS content in OMV vaccines is desirable given the reactogenicity of LPS from Gram negative species in humans and the limits regarding endotoxin activity required for medication used in humans. Notably, the results presented here indicate that OMVs from LPS-free *A. baumannii* may be somewhat less immunogenic than wildtype OMVs, as indicated by lower IgG2c levels at 21 days (Figure 2C), and afford only partial protection from infection when a dose of 10 µg is used for immunization (Figure 3B). This may be due to adjuvant-like activity of *A. baumannii* LPS, which is known to be a ligand for TLR4 [18]. Alternatively, antibodies directed against LPS may contribute to protective immunity. Importantly, increasing the dose of LPS-free OMVs to 100 µg resulted in complete protection from infection (Figure 4F).

In conclusion, this study demonstrates that OMVs are produced from an *A. baumannii* strain deficient in LPS synthesis, and that immunization with these OMVs elicits protective immunity against *A. baumannii* in an experimental model of infection.

Acknowledgments

This work was supported by Plan Nacional de I+D+i 2013- 2016 and Instituto de Salud Carlos III, Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía, Industria y Competitividad, Spanish Network for Research in Infectious Diseases (REIPI RD16/0016/0009) - cofinanced by European Development Regional Fund "A way to achieve Europe", Operative program Intelligent Growth 2014- 2020, a grant from the la Consejería de Economía, Innovación, Ciencia y Empleo (PIE-CTS-2027), and a grant from the Fundación Progreso y Salud/Consejería de Igualdad, Salud y Politicas Sociales of Junta de Andalucía (PI-0098-2014).M.R.P. was supported by the Subprograma Sara Borrell from the Ministerio de Economía y Competitividad of Spain (CD14/00014).

Conflict of Interest Statement

MRP, MGC, JP and MJM are listed as inventors on a patent application describing the use of LPS-free *A. baumannii* and its derivatives as vaccines. MJM is a founder and shareholder of Vaxdyn, S.L., a biotechnology company developing vaccines for bacterial infections.

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287 **Figure Legends**

288 **Figure 1.** Visualization and characterization of OMVs from ATCC 19606 and IB010.
289 Scanning electron microscopy of ATCC 19606 (A) and IB010 cultures (B)
290 demonstrating OMV production. Arrows indicate sites of OMV vesiculation from
291 bacterial cells. The bar indicates 5 µm. Transmission electron microscopy of purified
292 OMVs from ATCC 19606 (C) and IB010 (D) cultures. SDS-PAGE and Coomassie
293 staining of 10 µg of total protein of ATCC 19606 and IB010 OMVs.

294

295 **Figure 2.** Antibody response after immunization with ATCC 19606 and IB010 OMVs.
296 Groups of 8 mice were immunized on days 0 and 14 with 10 µg of ATCC 19606
297 OMVs, 10 µg of IB010 OMVs, 10 µg IB010 OMVs + purified LPS, or with adjuvant
298 alone as a control. Total IgG titers in serum against ATCC 19606 whole cells were
299 determined in serum samples collected on days 7 and 21 (A). IgG1 (B), IgG2c (C), and
300 IgM (D) titers were determined in serum samples collected on day 21. *** $p < 0.001$
301 and * $p < 0.05$ compared to control mice.

302

303 **Figure 3**. Post-infection bacterial loads, survival and serum pro-inflammatory cytokine
304 levels. Groups of 8 mice were immunized on days 0 and 14 with 10 µg of ATCC 19606
305 OMVs, 10 µg of IB010 OMVs, 10 µg IB010 OMVs + purified LPS, or with adjuvant
306 alone as a control, and infected with 2×10^6 cfu of ATCC 19606 on day 21. Spleen
307 bacterial loads 12 hours post-infection (A) and post infection survival (B) were
308 determined for groups of mice. Serum levels of IL-1β (C) and IL-6 (D) were determined
309 samples taken 12 hours post-infection. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$
310 compared to control mice.

311

Figure 4. Immunization with 100 µg IB010 OMVs. Groups of 8 mice were immunized on days 0 and 14 with 100 µg of IB010 OMVs or with adjuvant alone as a control, and infected with 1.8×10^6 cfu of ATCC 19606 on day 21. Total IgG titers were determined on serum samples taken on days 7 and 21 (A). IgG1 (B), IgG2c (C), and IgM (D) titers were determined in serum samples taken on day 21. Spleen bacterial loads were determined 12 hours post-infection in vaccinated and control mice (E). Post-infection survival was determined in groups of mice over 7 days. *** $p < 0.001$ and ** $p < 0.01$ compared to control mice.

Figure 1

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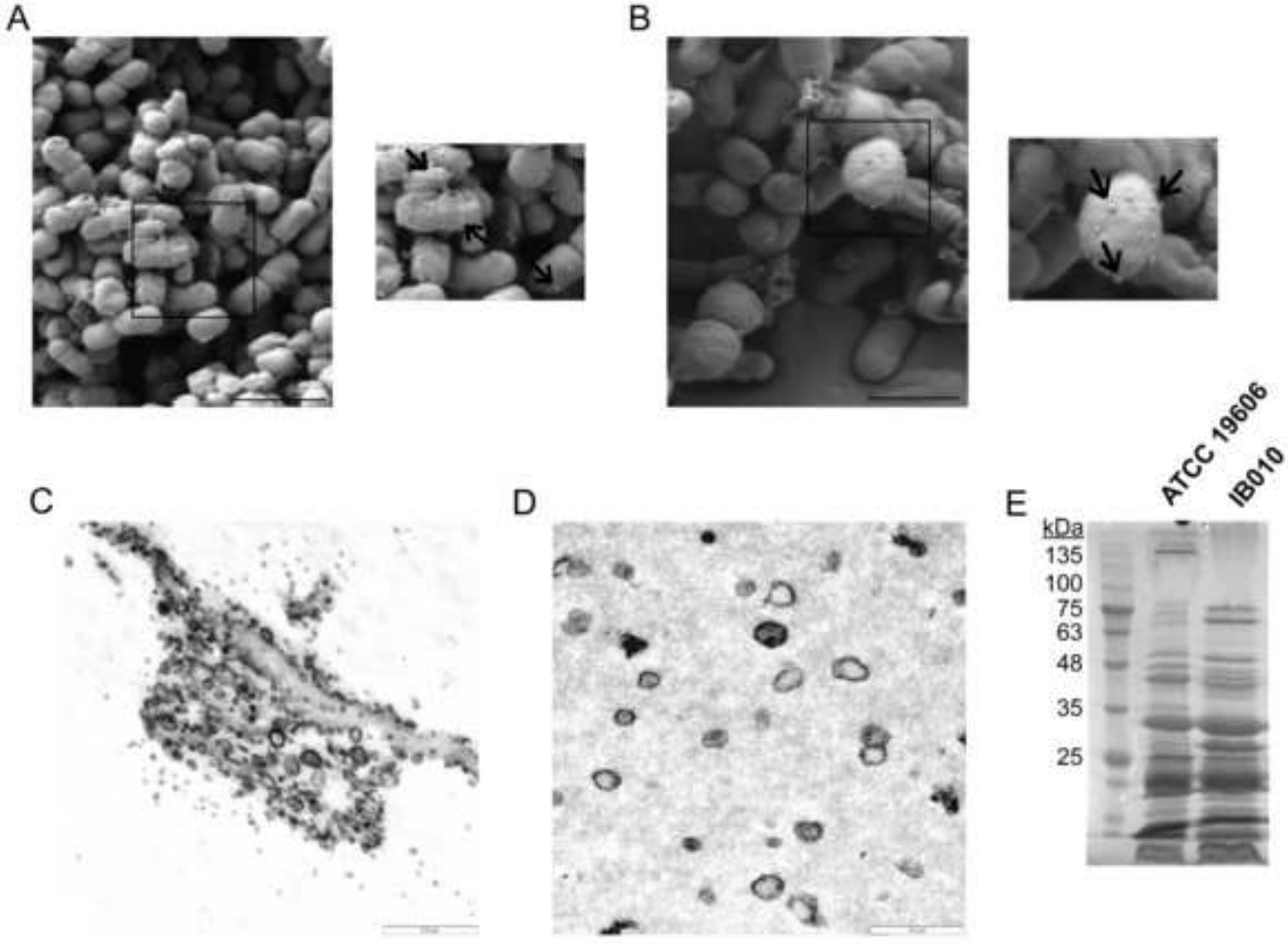

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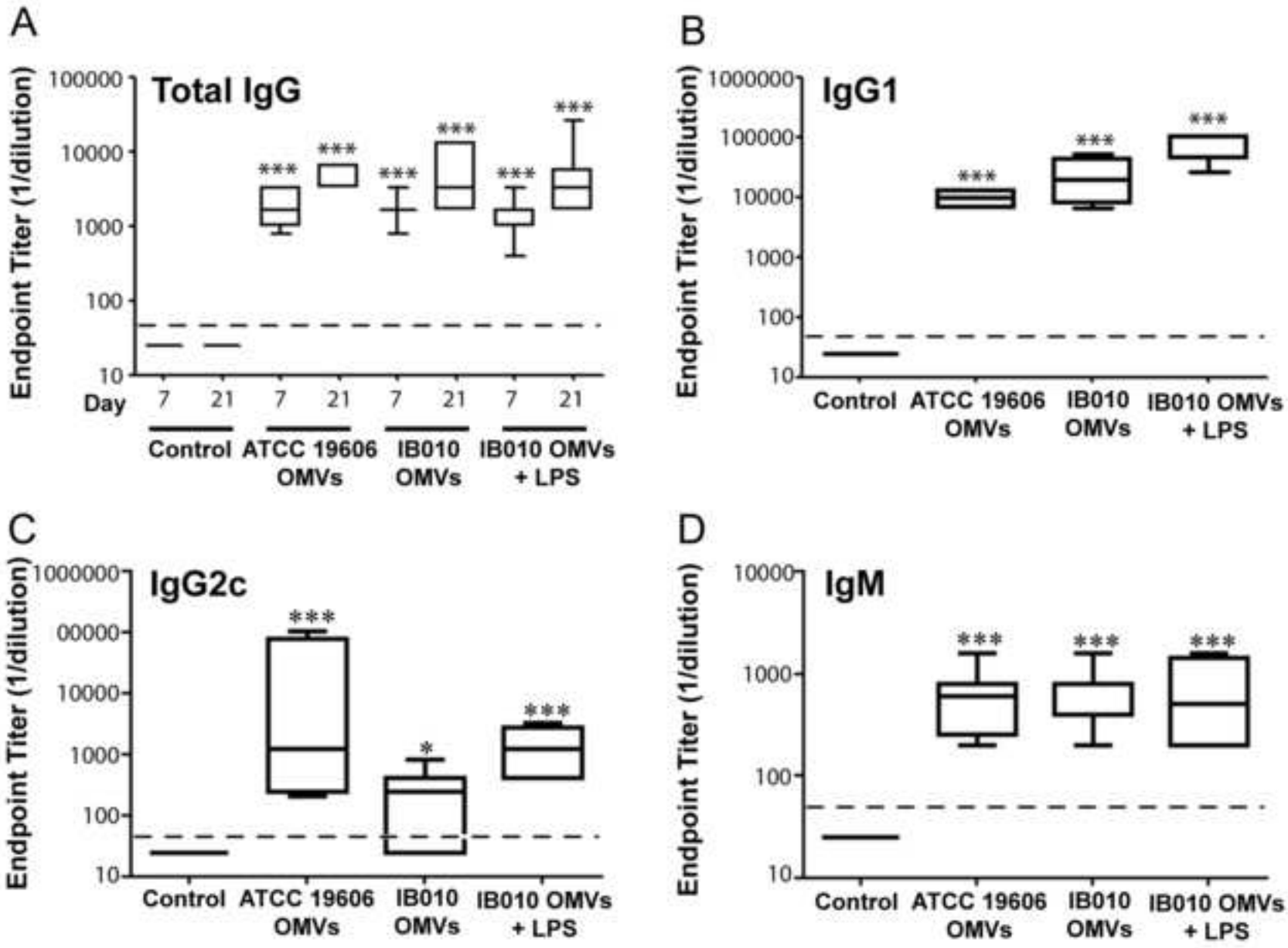

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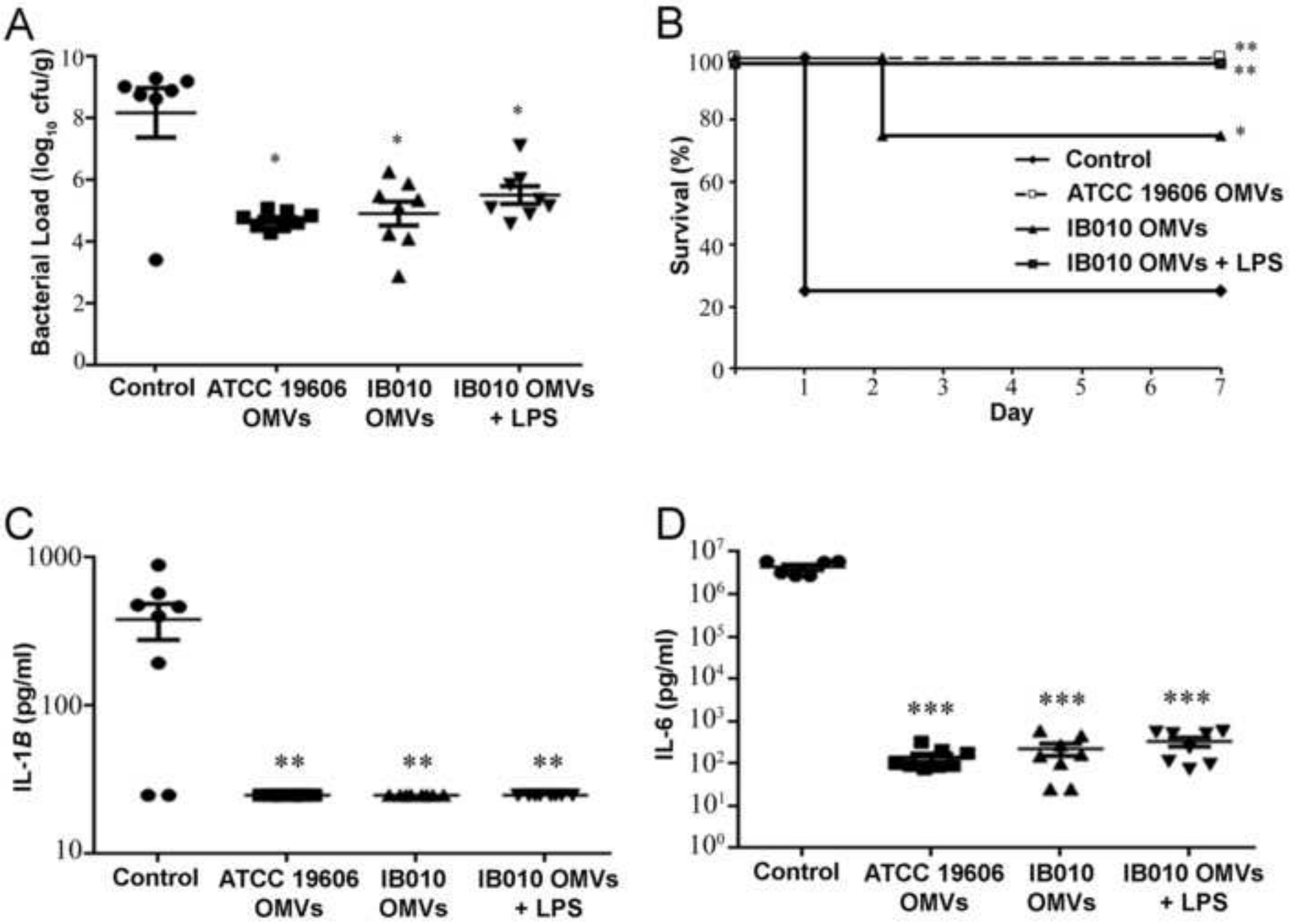

Figure 4

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