Outer Membrane Vesicle-Coated Nanoparticle Vaccine Protects against Acinetobacter baumannii Pneumonia and Sepsis

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1. Introduction

Antimicrobial resistance (AMR) is an exigent threat to global public health, with recent statistical estimates of 4.95 million deaths associated with, and 1.27 million directly attributable to, multidrug-resistant (MDR) bacterial infections worldwide in 2019.[1] As successful development of new antibiotics has dwindled in recent decades, vaccine technologies have continued to advance.[2,3] Thus, prevention of infections by emerging MDR pathogens through novel vaccine development has gained support as a critical element of the battle against AMR.[4–6]

The application of innovative nanotechnologies in vaccine formulation has increased sharply in recent years, offering advantages of improved stability, protection from premature degradation, and precise delivery to desired cellular targets.[7–9] The tunability of nanoparticles to precise sizes for maximal immunogenicity and uptake by antigen presenting cells is a particularly important facet of these lines of translational research.[7]

Outer membrane vesicles (OMVs) are naturally occurring, nonreplicating, highly immunogenic, spherical nanostructures that are shed from the surface of Gram-negative bacteria.[10–12] The diverse biological functions of OMVs include horizontal gene transfer,[13] delivering microRNAs[14] and virulence factors[15,16] into host cells, immune evasion by inactivating host defense factors,[17,18] resistance to antibiotics,[19] and nutrient acquisition.[20] quorum sensing,[21] and stress responses.[22] Because they display an array of bacterial surface proteins, lipids, and carbohydrates, as well as cell wall components with adjuvant properties, several strategies have explored OMVs as an alternative to whole cell bacterial vaccines.[10–12,23,24] Indeed, OMVs from Neisseria meningitidis serogroup B are included along with bacterial proteins from the pathogen in a multicomponent vaccine (Bexsero).
that is approved for human use. Nevertheless, the development of OMVs as a vaccine or drug product poses certain complexities, in particular related to heterogeneity in size, yield, potential differences in the internal cargo they carry, and their structural stability in face of various physical or chemical stresses.

In this study, we explore whether a nanotechnology based on citrate-stabilized gold nanoparticles (AuNPs) could be used to enhance the consistency and stability of an OMV-based bacterial vaccine while maintaining optimal immunogenicity. For these proof-of-principle studies, we selected the frequently MDR opportunistic Gram-negative bacterial pathogen Acinetobacter baumannii, which is named an urgent threat by the U.S. Centers for Disease Control and Prevention and sits atop the World Health Organization global priority pathogens list of antibiotic-resistant bacteria. Nosocomial A. baumannii infections such as hospital- or ventilator-acquired pneumonia (HAP/VAP), sepsis, urinary tract infections, and wound infections have associated mortality rates between 25% and 80%, depending on infection type and location. MDR prevalence among A. baumannii strains is extremely high at 80%, with >50% of isolates on most continents resistant to carbapenems, an antibiotic class of last resort. A. baumannii infects medically vulnerable populations—risk factors include mechanical ventilation, prolonged antimicrobial or immunosuppressive therapy, comorbidities such as diabetes and chronic lung disease, severe trauma or burns, and most recently, COVID-19 pneumonia.

A vaccine against endemic A. baumannii could provide essential protection to vulnerable populations without further exacerbating antibiotic resistance. Although such vaccine candidates have been tested in rodents, none have progressed past preclinical investigations. With respect to A. baumannii OMVs (Ab-OMVs), their secretion is dependent on outer membrane protein A (OmpA), which is subsequently highly enriched on the Ab-OMV surface. OmpA facilitates delivery of A. baumannii virulence factors into the host cell cytoplasm. As Ab-OMVs are coated with OmpA, virulence factors, and membrane lipids, they are strongly immunogenic; however, their heterogeneity and variable cargo pose a challenge for large-scale clinical testing and manufacturing.

Here, we apply cell membrane coating technology to functionalize AuNPs with Ab-OMVs and engineered a tunable A. baumannii nanoparticle vaccine (Ab-NP). Use of a nanoparticle core enabled us to fine-tune nanovaccine size for efficient lymphatic transport and immune processing, otherwise hard to achieve with pure OMV-based formulations. We found that Ab-NP vaccination induced robust IgG antibody responses in both rabbits and mice, and that the antisera promoted opsonophagocytic killing (OPK) of A. baumannii by human neutrophils. Ab-NP vaccination induced superior B cell recruitment to the draining lymph nodes and increased dendritic cell activation compared to Ab-OMV only and PBS controls. Most importantly, passive immunization with immune serum and active Ab-NP vaccination strongly protected mice against lethal sepsis and pneumonia in challenge studies with a hypervirulent A. baumannii strain. Combining immunogenic OMVs with nanotechnology thus merits further exploration as a platform for vaccine development against A. baumannii and other MDR Gram-negative bacterial pathogens.

2. Results

To engineer Ab-OMV bound nanoparticles (Ab-NPs), we coated 30 nm citrate-stabilized gold particles (AuNPs) with OMVs isolated and purified from the hypervirulent, clinical outbreak-associated A. baumannii strain Lac-4 by an established protocol (Figure 1; see Experimental Section for details). AuNPs have been utilized in nanotechnology for decades and were chosen as the core for this novel application due to their reliability, ease of synthesis, and superior uptake by immune cells. The small size of AuNPs also allows for efficient lymphatic drainage into secondary lymphoid organs for antigen presentation and processing.

An inherent property of uncoated AuNPs is their tendency to rapidly aggregate in PBS, which can be visualized by a change in color (Figure 2A). Optimal membrane coating was determined by evaluating the stability of Ab-NPs fabricated at various Ab-OMV to AuNP ratios (Figure S1). At a 1:1 weight ratio, the resulting Ab-NPs were stable upon transfer to PBS, and this ratio was utilized in subsequent experiments. A sharp drop in membrane protein loading efficiency beyond the 1:1 ratio indicated a saturation in the membrane coating (Figure 2B). For the optimized Ab-NP formulation, protein loading was 88.6 ± 0.45 μg Ab-OMVs per mg of AuNPs with a loading yield of 8.14 ± 0.04%; considering the high density of gold, excellent coverage of the AuNPs by the bacterial membrane was achieved. Consistent with OMVs from other Gram-negative bacteria, dynamic light scattering (DLS) measurements revealed high heterogeneity in size distribution of Ab-OMVs, with diameters ranging from 30 to 5000 nm (Figure 2C and 2D). In contrast, AuNPs were uniformly distributed with a hydrodynamic size of 34.18 ± 1.63 nm and a slight shift in size to 45.54 ± 0.27 nm after coating, consistent with the addition of a several nm thick membrane layer. Successful coating was further verified by zeta potential measurements where the value of uncoated AuNPs increased after coating to a value statistically similar to Ab-OMVs (Figure 2E). Coated Ab-NPs maintained remarkably consistent size distribution even after 7 days due to the stability provided by the OMV layer (Figure 2F). Ab-OMV association with AuNPs and the completeness of the membrane coating were visually confirmed by transmission electron microscopy (TEM), where spherical AuNP cores were found wrapped with an additional thin layer of membrane (Figure 2G). In contrast, visualization of Ab-OMVs showed heterogeneous vesicles of varying sizes (Figure 2H). Coomassie blue dye staining of A. baumannii Lac-4 lysate, Ab-OMVs, uncoated AuNPs, and Ab-NPs confirmed protein banding patterns in the coated Ab-NPs comparable to OMVs (Figure 2I). Importantly, Ab-NPs were not cytotoxic to murine bone-marrow derived dendritic cells (BMDCs) at concentrations up to 10 μg mL−1 (Figure 2J). Together these biophysical studies document the uniformity and preliminary safety profile of the Ab-NP vaccine candidate.

To test whether Ab-NPs could generate a protective antibody response, we obtained immune rabbit serum by vaccinating two New Zealand white rabbits with 1 mg of Ab-NPs without additional adjuvants. Compared to prevaccination (prevax) serum controls, postvax rabbit serum had significantly increased immunoglobulin G (IgG) titers (Δ2.5 log10-fold, Figure 3A) and enhanced binding intensity and percent IgG binding...
To assess the protective capacity of the postvax serum in vivo, we established an intraperitoneal (IP) sepsis model with the hypervirulent A. baumannii Lac-4 strain. Mice rapidly succumbed to infection in a dose-dependent fashion with an LD$_{50}$ of $10^8$ colony-forming units (CFUs) of the bacterium (Figure 3F). Hypothermia is a hallmark of experimental Gram-negative sepsis, and A. baumannii induced an acute dose-dependent temperature drop in the mice within 6 h of bacterial challenge (Figure 3G). For passive immunization studies, mice received either postvax or naive rabbit serum intravenously at days –2 and 0 (Figure 3H), with successful antibody transfer confirmed by submandibular cheek bleeding and ELISA—namely, an $\approx 2.5$ log$_{10}$-fold increase in anti-A. baumannii IgG titer in animals receiving postvax serum compared to naive serum (Figure 3I). Two days following passive immunization, mice were challenged IP with A. baumannii Lac-4. All animals that received two doses of postvax serum survived the infection compared to 20% and 100% mortality among the single dose postvax and naive serum cohorts, respectively (Figure 3J). Passive antibody transfer also protected mice from sepsis-induced hypothermia, and controlled bacterial burdens.

We next examined the antigenicity and murine safety profile of Ab-NP vaccination. Mice were vaccinated with three successive doses of Ab-NPs, red blood cell-coated AuNPs (RBC-NPs), or mock as vehicle controls (Figure 2B) and similar or enhanced APC responses of Ab-NP vaccination in murine models. Vaccination induced neutrophil recruitment, reduced systemic inflammation, protected mice from sepsis-induced hypothermia, and controlled bacterial burdens.

Figure 1. Acinetobacter baumannii OMV-derived nanoparticle vaccine. OMVs were isolated from the hypervirulent A. baumannii clinical isolate Lac-4 and associated with inert gold nanoparticle cores (AuNPs) to generate an A. baumannii nanoparticle vaccine (Ab-NP). Vaccination with Ab-NPs evoked A. baumannii specific immunity in mice and rabbits. Passive vaccination with immune rabbit serum protected mice against bacterial challenge in a lethal sepsis model. Active vaccination with Ab-NPs protected mice against bacterial challenge in lethal pneumonia and sepsis models. Vaccination induced neutrophil recruitment, reduced systemic inflammation, protected mice from sepsis-induced hypothermia, and controlled bacterial burdens.

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versus RBC-NP or mock control (Figure S3A and S3B). Likewise, toxicity of Ab-NP vaccination to the hematopoietic compartment was not observed, as white blood cell (WBC) count increased only 1.5-fold on day 3 compared to day 0 prior to immunization; less than a 10-fold change is considered safe\(^\text{[54,55]}\) (Figure S3C and S3D). Moreover, no leukopenic toxicity was seen for Ab-NP relative to RBC-NP (Figure S3E, cyclophosphamide used as positive control). To exclude potential impacts on kidney function, we analyzed serum creatinine 24 h after the first vaccination dose for an acute measurement and 24 h post the third dose for delayed toxicity (Figure S3F). No increases in serum creatinine levels were seen in mice after Ab-NP vaccination, indicating kidney function was not impaired. We further examined two markers for liver function, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and found no changes from baseline for either enzyme after Ab-NP vaccination (Figure S3G and S3H). These initial analyses are encouraging that Ab-NP vaccination is safe in the murine preclinical model.

Knowing our vaccine can generate a specific antibody response and is tolerated by mice, we next investigated the efficacy of protection. Mice were vaccinated with three doses of RBC-NPs, Ab-OMVs, or Ab-NPs. Two weeks after the third dose, mice were challenged intraperitoneally with Ab Lac-4 in a disseminated sepsis model. Immunized mice were completely protected against lethal septic challenge with the hypervirulent \(A.\) \(baumannii\) strain (Figure 5C). To establish the duration of protection generated by Ab-NP vaccination, we vaccinated mice with RBC-NPs or Ab-NPs and monitored titers for 6 months. Although anti-Ab Lac-4 titers declined modestly between 12 and 26 weeks, Ab-NP vaccination still protected 90% of mice from a lethal sepsis infection without additional booster doses (Figure S3I and S3J). Thus, we find our three-dose regimen is highly efficacious at protecting mice from lethal...
A. baumannii infection for a period of at least 6 months. To determine whether protection in immunized mice correlated with lower bacterial burdens, we first established that A. baumannii Lac-4 rapidly disseminates to systemic organs 4 h after IP infection in a dose-dependent manner (Figure 5D). In separate cohorts of mice, we found that Ab-NP immunization markedly...
accelerated bacterial clearance, with a 3 to 5 log10-fold reduction in *A. baumannii* CFUs in organs (spleen, liver, and kidney) and undetectable CFU in the blood (Figure 5E).

Gram-negative sepsis is characteristically accompanied by an overwhelming cytokine storm—thus we investigated whether Ab-NP vaccination could contain uncontrolled release of inflammatory mediators following infection. Notably, Ab-NP vaccination dampened systemic inflammation as measured by significant decreases in serum IL-6, IL-1β, and TNF-α levels (Figure 5F–H). Neutrophils are an essential immune responder during *A. baumannii* infections,[56,57] and neutropenia is a significant risk factor for bacteremia and associated mortality.[58,59] To assess Ab-NP vaccine effects on neutrophil responses in the mouse model, we harvested and processed spleens 4 h post-infection for single-cell isolation, staining, and analysis by flow cytometry. Ab-NP-vaccinated mice had significantly more splenic neutrophils, both in percentage and total numbers, relative to RBC-NP and mock-treated mice (Figure 5I–K). These data suggest Ab-NP vaccination boosts the recruitment of neutrophils to the spleen upon Ab-NP trapping and/or increases neutrophil survival during the direct host–pathogen encounter.

*A. baumannii* is a leading cause of hospital- and ventilator-acquired pneumonia.[30] In a murine intratracheal challenge model of pneumonia (Figure 6A), we found that the hypervirulent *A. baumannii* Lac-4 strain produced similar mortality rates at a 10-fold lower challenge dose than the commonly studied AB5075 strain[60] (Figure 6B). To assess the protective effect of Ab-NP vaccination in this model, mice were immunized with mock, RBC-NPs, or Ab-NP and bled weekly for IgG antibody titers. Four weeks post initial vaccination, mice were challenged intratracheally with *A. baumannii* Lac-4. Consistent with our findings in the sepsis model, Ab-NP immunized mice, which had significantly higher titers than the control mice, were completely protected from pneumonia-induced mortality (Figure 6C and D).

Figure 4. Ab-NPs induce antigen presenting cells in draining lymph nodes. A) Mice were vaccinated with PBS (mock), 100 ng Ab-OMVs, or 100 ng Ab-NPs. 24 h later, inguinal lymph nodes (ILNs) were harvested, processed for single-cell isolation, and stained for analysis by flow cytometry. Gates were drawn using single-stained controls and FMOs. Dendritic cells were gated on cells, singlets, live, F4/80+CD11c+ and then further individually gated for activation markers CD40, CD80, CD86, and MHC-II. B cells were gated cells, singlets, live, CD19+. Macrophages were gated cells, singlets, live, F4/80+CD11c+. B) Quantification of percent of B cells, dendritic cells (DCs), and F4/80+ macrophages of total live cells in the ILN. C) quantification and D) representative flow plots of the activation markers CD40, CD80, CD86, and MHC-II expressed by CD11c+ dendritic cells in inguinal lymph nodes 24 h postvaccination. A) Representative of two independent experiments (n = 10), B) and C) two independent experiments were pooled with each dot representing a single mouse (n = 10).
Lastly, we assessed the long-term protective capacity of Ab-NP vaccination in a pneumonia model by vaccinating mice with RBC-NPs or Ab-NPs. Mice were challenged intratracheally 30 weeks postvaccination with Ab Lac-4 and monitored daily. We saw Ab-NP vaccination provided 100% protection even after 7 months with no additional boosters (Figure 6E), consistent with our findings that vaccination also protects long-term against disseminated sepsis (Figure 5E).

3. Discussion

A. baumannii is an increasingly important cause of nosocomial pneumonia and sepsis for which treatment and environmental eradication are exceptionally difficult.[61–63] We have developed an A. baumannii OMV-based, nanoparticle-stabilized vaccine prototype that protects mice from lethal sepsis and pneumonia. Ab-NP vaccination induces a robust IgG antibody response.
specific to A. baumannii that promotes human neutrophil opsonophagocytic killing and confers protection against sepsis on passive transfer. Active immunization with Ab-NP protected against lethal A. baumannii sepsis with rapid reduction of bacterial counts in blood and organs and likewise provided protection against the pathogen in a pneumonia model. Ab-NP vaccination stimulated superior B cell recruitment to inguinal lymph nodes and increased DC activation and maturation relative to PBS and Ab-OMV controls, and further promoted the survival and recruitment to the spleen of neutrophils that are essential in innate defense against A. baumannii.

The extreme prevalence of MDR⁶⁴ and remarkable ability to acquire novel resistance among circulating A. baumannii strains prompts an urgent need for nonantibiotic solutions. While multiple vaccine candidates have been tested against A. baumannii, none have progressed to clinical studies.³² Successful vaccine candidates against other Gram-negative bacteria have utilized OMVs, the non-replicative, cargo-containing vesicles released by A. baumannii Gram-negative species.²⁶,⁶⁵ Ab-OMVs potential as vaccine antigens lies in the immunogenicity of their membrane antigens, ability to potentiate uptake by immune cells, and stimulatory contents.²⁶,⁶⁵ Despite these advantages, OMVs exhibit high heterogeneity in size distribution, which could influence their uptake by antigen presenting cells. Macrophages prefer larger particles (0.5–5 μm) while dendritic cells preferentially ingest smaller debris (0.04–0.1 μm).⁶⁶ Furthermore, antigen size significantly instructs the immune response, with particles between 40 and 50 nm inducing increased CD8 and CD4 Th1 responses relative to particles >0.5 μm.⁶⁷

When designing our vaccine, we considered manufacturing concerns as well as efficacy and safety. Paramount to Good Manufacturing Practices (GMP) regulations promulgated by the U.S. FDA are quality control practices that ensure safe, efficacious, and highly reliable products. While OMVs are strongly immunostimulatory, their heterogeneity results in batch variability that could render large scale manufacturing impractical or cost prohibitive, whereas the defined nature of the Ab-NP formulation could help to overcome these challenges. AuNPs have been utilized for decades in biological research and have a proven record of reliable and tunable fabrication.⁶⁸ We investigated whether combining OMVs with nanotechnology could serve as a safe and consistent platform for vaccine delivery.

We engineered a unique nanoparticle vaccine against A. baumannii by coating AuNPs with OMVs released by a hypervirulent clinical isolate of the pathogen (Lac-4). The Ab-NP vaccine is highly immunostimulatory and protective in both sepsis and pneumonia models in mice even up to 6 months after vaccination. Consistent with our targeted antigen size, Ab-NP vaccination induced significant B cell recruitment and DC reaction to pathogens. Additionally, Ab-NP vaccinated mice showed increased CD8 and CD4 Th1 responses relative to particles >0.5 μm.⁶⁷

Figure 6. Ab-NP vaccination protects against lethal pneumonia. A) Mortality curves in mice infected with 10⁸ CFUs AB5075, 10⁷ or 10⁶ CFUs Ab Lac-4 intratracheally (IT) in sterile PBS. Mice were anesthetized with 100 mg kg⁻¹ ketamine and 10 mg kg⁻¹ xylazine prior to infection. Two independent experiments were pooled for each infectious dose (n = 10 per group). B) Active immunization scheme with PBS (mock), red blood cell NPs (RBC-NPs, nanoparticle control), or Ab-NPs. Mice were immunized IP with 1 μg antigen in 100 μL sterile PBS on days 0, 7, and 14. Ab Lac-4 IgG titers were assessed weekly prior to immunization by submandibular blood collection and ELISA. Mice were challenged on day 28 and monitored for survival for seven days. C) IgG antibody titers specific for Ab Lac-4 from mice prevaccination and postvaccination prior to challenge. Each dot represents one mouse. Representative of two independent experiments (n = 10 per group). ****p < 0.0001. D) Mortality curves in immunized mice infected with 10⁸ CFUs Ab Lac-4 IP. Mice were monitored for survival for seven days. Pooled survival from two independent experiments per group (n = 10). E) Mortality curves in immunized mice infected with 10⁷ CFUs Ab Lac-4 IP 30 weeks postvaccination. Mice were monitored for survival for 7 days. Pooled survival from two independent experiments per group (n = 10).
maturation in draining lymph nodes that exceeded immunization with Ab-OMVs alone. Robust IgG antibody responses specific to A. baumannii after vaccination protected in active and passive immunization and potentiated the killing capacity of healthy human neutrophils, supporting our hypothesis that the combination of nanoparticle technology and OMV immunogenicity is an effective approach for vaccine design. While we elected to employ AuNPs in our study due to proven consistency and utility in fabrication, other nanomaterials such as polymeric or iron oxide nanoparticles can similarly act as the stabilizing core.[41] The choice of core material can bestow additional functionalities, such as inclusion of additional adjuvants or magnetic guidance, although these additions were not necessary in our current Ab-NP formulation.

One potential hurdle that has previously arisen with OMV vaccines is the presence of lipopolysaccharide (LPS), a major component of Gram-negative cell membranes.[69] Humans are approximately 1000 times more sensitive to LPS than mice, which can lead to serious side effects such as endotoxic shock.[70] Interestingly, A. baumannii is one of three Gram-negative bacteria that is capable of surviving loss of the immunostimulatory lipid A component of LPS.[30] Unlike LPS-deficient Neisseria meningitidis and Moraxella catarrhalis that were both constructed in vitro,[71,72] recent studies have found that naturally occurring A. baumannii clinical isolates modify LPS synthesis genes to escape host immunity and antibiotic pressure.[73] While these strains are less immunostimulatory in vitro,[74] their presence in the clinical disease suggests that LPS deficiency does not entail the same fitness or virulence deficit observed with LPS-deficient N. meningitidis and M. catarrhalis.[71,72] A previous study found that adventured Ab-OMVs derived from an LPS-deficient strain of A. baumannii provided 75% protection in a lethal sepsis model.[75] If the presence of LPS in Ab-NP proved concerning for toxicity or safety issues, substitution of LPS-minus Ab-OMVs into the nanoparticle platform could be achieved.

Until recently, murine sepsis models had been limited to extremely high inoculums and immunocompromised mice,[50,76] but our studies employed the clinical isolate Lac-4 that is acutely lethal in healthy mice at much lower doses than other commonly utilized A. baumannii strains. However, A. baumannii frequently infects individuals whose immune systems are impaired through injury, trauma, disease, or immunosuppression. We thus plan future studies to examine the efficacy of Ab-NP in specialized experimental mouse models of chemotherapy-induced neutropenia, diabetes, pregnancy, or major surgery. Future directions of this work also include expanding the repertoire of A. baumannii strains used to prepare the OMVs, since although OmpA has >90% conservation across strains,[77] outer membrane and capsule heterogeneity are likely to result in variable cross-strain protection from a single OMV-derived vaccine.[78]

In summary, we have found Ab-NP to be an effective and novel nanoengineered vaccine candidate that is highly protective against A. baumannii sepsis and pneumonia. Our work provides proof-of-principle that OMV-coated NPs can serve as a consistent and tunable platform for rapid vaccine production and testing for high priority MDR Gram-negative pathogens.

4. Experimental Section

Experimental Design: The objective of this study was to design and validate an OMV-coated nanoparticle vaccine that protects against lethal sepsis and pneumonia caused by a hypervirulent clinical isolate of A. baumannii.

Bacterial Strains and Culture: A. baumannii Lac-4 is an outbreak strain isolated from an LA county hospital in 1997[44] and obtained from Wangelx Chen (National Research Council, Canada).[76] The strain was grown in tryptic soy broth (TSB) with aeration at 37 °C. Stationary phase overnight cultures were sub-cultured in TSB and grown to mid-logarithmic phase with aeration at 37 °C and washed with PBS prior to use in experiments. Strain AB5075 was isolated from a patient with tuberculosis and was handled as above substituting the similar growth medium cation-adjusted Mueller Hinton Broth (Ca-MHB).

Ab-OMV Derivation: Single colonies of A. baumannii Lac-4 were inoculated in 1.5 L flasks of TSB and grown for 16 h at 37 °C with aeration. Stationary-phase cultures were spun down in a Sorvall RC-6 refrigerated floor centrifuge for 10 min at 10,000 rpm. The supernatant was filtered through a 0.45 μm PES vacuum filter (Thermo Fisher, 167-0045) and concentrated nearly 100 times by tangential flow filtration. Ab-OMVs were spun down by ultracentrifugation at 150,000 × g for 2 h at 4 °C, resuspended with water, and stored in −80 °C.

Ab-NP Production: Citrate stabilized 30 nm AuNPs (NanoComposix, AUCN30) were mixed with Ab-OMVs at different weight to protein ratios and sonicated in a bath sonicator for 2 min for coating. The mixture was then washed once with water by centrifugation at 5000 × g for 15 min to remove any free Ab-OMVs and used immediately. RBC-NP controls were fabricated in a similar fashion with murine RBC membranes.

Ab-NP Characterization: Protein loading was determined using a bicinchoninic acid kit (Thermo Fisher, 23227), and size, polydispersity index, and zeta potential were measured by dynamic light scattering. OMV loading efficiency was defined by the percent of OMVs bound to AuNPs compared to the input OMVs. Stability was tested by adjusting Ab-NP to 1 × PBS and storing the sample at 4 °C for a week. To image the nanoparticles under TEM, samples were concentrated down to 0.5 mg mL⁻¹ and 5 μL was deposited onto a TEM grid. The sample was dried and then negatively stained with 1% uranyl acetate for visualization. To confirm proper protein transfer, all samples were normalized to a protein concentration of 0.25 mg mL⁻¹ diluted with lithium dodecyl sulfate loading buffer (Thermo Fisher, NP0007), and run at 165 V for 45 min in MOPS running buffer (Thermo Fisher, B0001) for separation. The separated proteins were visualized by staining the gel with InstantBlue (Abcam, ISBL1) for 1 h followed by overnight washing with water. BDNC viability was evaluated by incubating varying concentrations of Ab-NP with 25 000 BMDC in 96-well plates for 3 days at 37 °C, 5% CO₂. Viability was evaluated using PrestoBlue according to the manufacturer’s instructions.

Immunized Rabbit Sera: Two New Zealand White rabbits were immunized with four successive doses of 0.25 mg Ab-NP subcutaneously with each dose 2 weeks apart at AbCore (Ramona, CA). Rabbits were bled before (prevax) and after vaccination (postvax) and serum was isolated.

Murine and Rabbit IgG Neutralization Assays: For ELISA assay, low-binding Immulon 4 HBX 96-well plates (Thermo Fisher, 3555) were coated with 1 × 10⁴ CFU/well of heat-killed A. baumannii Lac-4 in sodium bicarbonate buffer (Sigma, C3041). Coated plates were incubated overnight at 4 °C, washed 3 times with PBST and blocked with 1% Reagent Diluent #2 (R&D Systems, DY995) for 1 h at room temperature or overnight at 4 °C. Isolated rabbit or mouse serum was serially diluted in 1% Reagent Diluent #2 and incubated overnight at 4 °C. Plates were washed 3 times with PBST and detected with goat anti-rabbit (1:4000) or anti-rabbit (1:4000) IgG (H&L, abcam, ab100105) in 1% Reagent Diluent #2 for 90 min at room temperature. Plates were washed 3 x with PBST and detected with streptavidin (R&D Systems, DY998) in 1% Reagent Diluent #2 for 30 min. Plates were washed 3 times with PBST and detected with TMB (BD, 555214) according to the manufacturer’s instructions. Detection was halted with 2N H₂SO₄, and plates read on a spectrometer.
at 450 nm. Titers were determined using four-parameter logistics curve fit (Prism). For flow cytometry analysis, single-cell isolation and gating were performed on the submandibular vein, and serum was isolated on days 0 (before vaccination), 1, and 15. Serum creatinine was assessed using a creatinine assay kit according to the manufacturer’s instructions (Cayman Chemical, 700460). Serum AST and ALT were determined using ALT and AST colorimetric activity assay kits, respectively (Cayman Chemical, 701640, 700260). Serum AST and ALT were determined using a submandibular cheek bleed on Days –2 and 0 and by ELISA. Mice were challenged with $10^7$ CFUs Ab Lac-4 IP on Day 0 and monitored every 6 h for body temperature for 24 h and twice daily for survival for 7 days.

**Passive Immunization:** Eight to 12 week-old female C57BL/6 mice were immunized intravenously through retro-orbital injection with 200 μl of naive rabbit serum (Sigma, R4505) or 200 μl immune rabbit serum from immunized AbCore rabbits. One group of mice received a second dose of immune rabbit serum 24 h after the initial dose. Mice were briefly anesthetized with isoflurane prior to injection. Prevac and postvac IgG titers were determined by submandibular cheek bleeding on Days –2 and 0 and by ELISA. Mice were challenged with $10^7$ CFUs Ab Lac-4 IP on Day 0 and monitored every 6 h for body temperature for 24 h and twice daily for survival for 7 days.

**Nanoparticle Vaccination:** Eight to 5-week-old female C57BL/6 mice were immunized intraperitoneally with 1 μg Ab-NP, Ab-OMVs, or RBC-NPs in 100 μl PBS on days 0, 7, and 14. Mock mice were immunized intraperitoneally with 100 μl PBS. Mice were cheek-bled on days 0, 7, 14, 21, and 28, and serum was isolated for antibody titers. For safety studies, mice were cheek-bled on days –1, 3, and 21 using EDTA vacutainers (BD, 365974). White blood counts (WBCs) were assessed by automated hemogram by the Animal Care Program Diagnostic Lab. Weights were monitored on days –1, 3, 7, 10, 14, 17, 21, and 28. Cyclophosphamide mice received 50 mg kg$^{-1}$ cyclophosphamide (Sigma, 239785) IP on days 0, 1, and 2 with WBC assessment on day 7 via automated hemogram analysis. For the sepsis model, mice were injected intraperitoneally with $10^7$ CFUs A. baumannii Lac-4 and monitored twice daily for 7 days for survival. For the pneumonia model, mice were anesthetized with 100 μg kg$^{-1}$ ketamine (Koetis) and 10 mg kg$^{-1}$ xylazine (VetOne), injected intratracheally with $10^7$ CFUs A. baumannii in 40 μl PBS using an operating otoscope (Welch Allen) and monitored twice daily for 7 days for survival. For bacterial load determination, mice were injected intraperitoneally with $10^7$ CFUs A. baumannii Lac-4, humanely euthanized with CO₂ according to IACUC approved protocols, and the spleen, top left lobe of the liver, and kidneys were harvested for enumeration 4 h postinfection as described above. Blood was isolated by cardiac puncture and mixed with sodium heparin for enumeration, or into serum separator tubes for cytokines and antibody titers. The peritoneum was lavaged with 10-ml sterile, ice-cold PBS. Lavage fluid was spun down for 15 min at 4000 RPM. The pellet was reconstituted in sterile PBS, serially diluted, and plated on TSA for enumeration.

**Dendritic Cell Activation and Vaccination:** Six to eight-week-old female C57BL/6 mice were briefly anesthetized with isoflurane and immunized subcutaneously in the flank with 100 ng Ab-OMVs or Ab-NP. Mock mice were immunized with PBS. Mice were humanely euthanized 24 h postvaccination and the inguinal lymph nodes were processed for single-cell isolation and stained for the antigen presenting markers CD19 (BD, 557398), CD11c (Invitrogen, 45-0114 82), and F4/80 (eBioscience, 25-4801-82) as well as the macrophage marker CD206 (eBioscience, 01-9741 82). The gene expression of TNF-α, IL-6, IL-10, IL-1β, and IL-12p40 was evaluated by qPCR.

**Flow cytometry analysis:** Flow cytometry analysis was performed using FlowJo v10.8.1. Single color compensation was performed for every experiment with more than one fluorophore. Gates were drawn using unstained, single-stained, and FMO controls.

**Supporting Information** is available from the Wiley Online Library or from the author.
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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

V.N., L.Z., E.B., J.Z., and R.H.F. conceived the idea and designed the experiments. E.B., J.Z., T.Q., N.K., R.H.Z., N.D.M., and A.H. performed all experiments. All authors analyzed and discussed the data. E.B., J.Z., R.H.F., L.Z., and V.N. wrote the article.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

Acinetobacter baumannii, multidrug-resistant bacterial infection, nanoparticle vaccine, outer membrane vesicle, pneumonia, sepsis

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