Journal of Infection 86 (2023) 563–573

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

# Intranasal immunization with outer membrane vesicles (OMV) protects against airway colonization and systemic infection with *Acinetobacter baumannii*

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ARTICLE INFO

*Article history:*
Accepted 23 February 2023
Available online 27 February 2023

*Keywords:*
ESKAPE
Vaccine
OMV
Bacteria
Mucosal

SUMMARY

*Objectives:* The multidrug-resistant bacteria *Acinetobacter baumannii* is a major cause of hospital-associated infection; a vaccine could significantly reduce this burden. The aim was to develop a clinically relevant model of *A. baumannii* respiratory tract infection and to test the impact of different immunization routes on protective immunity provided by an outer membrane vesicle (OMV) vaccine.
*Methods:* BALB/c mice were intranasally challenged with isolates of *oxa23*-positive global clone GC2 *A. baumannii* from the lungs of patients with ventilator-associated pneumonia. Mice were immunized with OMVs by the intramuscular, subcutaneous or intranasal routes; protection was determined by measuring local and systemic bacterial load.
*Results:* Infection with *A. baumannii* clinical isolates led to a more disseminated infection than the prototype *A. baumannii* strain ATCC17978; with bacteria detectable in upper and lower airways and the spleen. Intramuscular immunization induced an antibody response but did not protect against bacterial infection. However, intranasal immunization significantly reduced airway colonization and prevented systemic bacterial dissemination.
*Conclusions:* Use of clinically relevant isolates of *A. baumannii* provides stringent model for vaccine development. Intranasal immunization with OMVs was an effective route for providing protection, demonstrating that local immunity is important in preventing *A. baumannii* infection.
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## Introduction

Around 58% of Ventilator-associated pneumonia (VAP) cases are caused by aerobic Gram-negative bacteria.[1] The most common Gram-negative bacteria isolated from VAP patients are *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriacae*.[2] *A. baumannii* has emerged as one of the most problematic pathogens in clinical settings, particularly in ICUs.[3] Typical mortality rates of *A. baumannii* VAP range from 55% to 66%, caused by a combination of the underlying critical states of the patients and the difficulty in treating such MDR infections.[2] Many *A. baumannii* strains are extremely drug-resistant or pandrug-resistant, leaving colistin and tigecycline as the only two drugs capable of treating such an infection.[4] Antimicrobial resistance to Colistin is on the rise:[5] recently, there has been a high prevalence of carbapenem-resistant *Oxa23* expressing strains, particularly in the GC2 phylogenetic cluster.[6] With increased antimicrobial resistance and the challenges in the discovery of new antibiotic classes, new therapeutic options are needed to successfully control antimicrobial-resistant bacterial infections.[7] Vaccines represent one of the most promising options to control antimicrobial resistance (AMR). Effective vaccines would prevent infections caused by MDR bacteria. Vaccines have also been shown to significantly reduce antibiotic prescription and thus reduce AMR.[8]

Outer membrane vesicles (OMVs) are an emerging technology for inducing anti-bacterial immunity. OMVs are exocytic vesicles produced

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https://doi.org/10.1016/j.jinf.2023.02.035
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naturally by many Gram-negative bacteria. They are thought to serve multiple functions from cell-to-cell communication, protein secretion, virulence and aiding in biofilm formation. OMVs have a lipid bilayer that contains lipopolysaccharide and cell surface proteins. They are good potential vaccine candidates because they can be taken up by mammalian cells, they are strongly immunogenic[9] and proteins found in OMVs have a native conformation.[10,11] OMVs have been shown to elicit effective immune responses against several bacterial pathogens. Vaccination with a meningococcal OMV gave rise to serum bactericidal antibody responses against gonococci and meningococci.[10] Using a mouse model, OMVs are effective in preventing intestinal colonization of *Vibrio cholerae* through anti-OMV IgG antibodies.[9] Subcutaneous immunization with *Bordetella pertussis* OMVs induced comparable levels of protection to either the whole cell inactivated pertussis or the acellular pertussis vaccines.[11]

An important consideration for vaccine development is the delivery route.[12] This is both for practical reasons such as ease of administration and dose reduction, and also for improving the quality of the response. For pathogens that infect mucosal surfaces, such as respiratory pathogens, it may be more relevant to induce a local immune response. Mucosal surfaces, such as the respiratory tract, have highly specialized innate and adaptive responses to protect against pathogens and control growth of commensal microorganisms.[13] Although locally produced IgG and IgM are important in humoral immunity at mucosal surfaces, secretory IgA has also been shown to be key in developing a protective humoral response against mucosal infection.[13] Mucosal vaccinations elicit good local mucosal and systemic immune responses and would be easier to administer than injectable vaccines, resulting in a decreased risk of infection.[13,14] Mucosal immunization may also lead to qualitatively better immune responses, for example, in humans intranasal immunization induced upper airway IgA responses.[14]

The aim of this study was to test whether OMVs produced by clinically relevant *A. baumannii* strains elicit a protective immune response against challenge with clinically relevant strains. We wanted to determine which route of immunization that would give the best protection. To test this, we developed novel models of respiratory infection using clinical isolates of *A. baumannii*. We observed that whilst intramuscular immunization with OMVs induced a humoral immune response, it was not protective against intranasal infection. However, subcutaneous immunization substantially reduced lower airway bacterial load. Strikingly, nasal immunization with OMVs led to a reduction of bacteria in the upper airway and blocked systemic dissemination.

## Materials and methods

### *Bacterial strains*

*A. baumannii* (ATCC 17978) is a readily available, well-studied laboratory reference strain that has no drug resistance.

Clinical isolates of *A. baumannii* strains BAL_084 and BAL_276 were isolated from ICU patients in Ho Chi Minh.[6] All *A. baumannii* strains were grown at 37 °C, 200 rpm in Lysogeny broth (LB) or statically at 37 °C on HiCrome Acinetobacter Agar Base (HIMEDIA M1938: Trafalgar).

### *OMV production, quantification, and characterization*

Swabs were taken from areas of dense growth of each strain and grown planktonically in LB overnight. The culture was centrifuged at 7000g for 15 min at 4 °C and the supernatant containing the OMVs was vacuum filtered using a 0.45 μm filter. The filtered supernatant was then ultracentrifuged at 100,000 g for 2 h at 4 °C to pellet the OMVs. The OMV pellet was resuspended in phosphate-buffered saline (PBS). A sample of the subsequent OMV and PBS solutions was taken and grown overnight at 37 °C on LB agar to check for sterility.

OMVs were quantified by incubation with lipophilic dye FM4-64 in a black 96-well polypropylene microtiter plate. Fluorescence was then read using an EnVision Plate Reader (Perkin Elmer) with excitation 520 nm and emission 700 nm. Data were then expressed as fluorescence units (FU) relative to the volume of sample in the well.

LPS levels on the OMVs were measured using a Pierce Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific), the plate was read at 405 nm using a Fluostar (Omega) plate reader. The final LPS concentration of the neat OMVs was 0.6 ng/μl, which would equate to approximately 0.3 ng or 1.2 ng LPS per immunization dose of 100 Fus or 500 Fus, respectively.

OMVs were visualized via scanning electron microscopy (Fig. S1), which showed vesicles ranging between 20 and 200 nm in diameter.

### *Animal studies*

All animal studies were performed in accordance with the United Kingdom's Home Office guidelines under animal study protocol number (P4EE85DED). All work was approved by the Animal Welfare and Ethical Review board at Imperial College London. Studies also followed NC3Rs guidelines, and all work was carried out in biosafety level-two facilities.

Specific pathogen-free 6–8 week old female BALB/c mice were bought from Charles River Laboratories (Ware, UK). Mice were maintained in individually ventilated cages in groups of five animals per cage and received food and water ad libitum. Cages were all kept in the same specific pathogen-free room, which was maintained on a 12 h light/dark cycle at 20–24 °C with 55 ± 10% humidity. For challenge studies, mice were anaesthetized through inhalation of isoflurane and dosed intranasally with 100 μl of $5 \times 10^6$ CFU/ml or $5 \times 10^7$ CFU/ml *A. baumannii* (ATCC 17978), 100 μl of $10^8$ CFU/ml *A. baumannii* (BAL_276), 100 μl of $10^8$ CFU/ml *A. baumannii* (BAL_084) or 100 μl of LB broth. For vaccination studies, mice were intramuscularly or subcutaneously injected with 100 FU of OMVs from *A. baumannii* (from strains ATCC 17978, BAL_276 or BAL_084) in a 50 μl volume, responses were compared against an equivalent volume of PBS. For intranasal immunization mice were anaesthetized through inhalation of isoflurane and dosed with 500 FU *A. baumannii* (BAL_276) OMVs in 100 μl. Mice were initially weighed 4 h after infection and then daily thereafter.

### *Bacterial load quantification*

Bronchoalveolar lavage (BAL) and nasal fluid were collected during culling by flushing the lungs and nasal cavity each with 1 ml of PBS as described.[15] Lungs and spleen were dissected out, into 1 ml Dulbecco's modified eagle media (DMEM) before homogenizing through cell strainers. Resultant BAL fluid, nasal fluid, lung mash and spleen homogenates were serially diluted 1:10 in PBS and plated onto HiCrome Acinetobacter Agar Base plates. Plates were incubated overnight at 37 °C and individual colonies were counted at appropriate dilutions and averaged to determine CFU per ml.

### *Characterization of bronchoalveolar lavage immune cells*

After plating a sample of BAL for bacterial load quantification, the residual BAL was centrifuged, and the cell pellet was treated with ammonium-chloride-potassium (ACK) lysing buffer. The total number of live cells was estimated using trypan blue and manually counting cells in a Neubauer chamber. The ACK treated BAL fluid was cytocentrifuged onto glass microscope slides and left to dry before haematoxylin and eosin staining for differential white blood cell counting, which was also done manually.

### *Cytokine detection*

When the BAL fluid was first centrifuged, the supernatant was kept and frozen at −80 °C. IL-6 levels in the BAL fluid were assessed

using mouse IL-6 DuoSet kits (R&D Systems). In the BAL_276 infection model development, cytokines at 24 h after infection were measured in BAL using a custom mouse 10-spot U-PLEX kit (K15069L-2; Meso Scale Diagnostics), including the following analytes (lower limit of detection in pg/ml in bracket): GM-CSF (0.16), IFN-γ (0.16), IL-5 (0.63), IL-6 (4.8), IP-10 (0.5), KC (0.43), MCP-1 (1.4), MIP-1α (0.21), MIP-1β (13), and TNF (1.3).

### *IgG, IgM and IgA ELISA*

Blood was collected through tail bleeding mice before boosting, before challenge and from a terminal bleed during the cull. Blood was left to clot and then centrifuged to collect the serum, which was stored at −80 °C. Plates were incubated with OMVs and mouse kappa and lambda capture antigens and incubated at room temperature for 2 h. Plates were blocked with 1% BSA PBS. Mouse serum samples and total IgG, IgG1, IgG2a, IgM or IgA standards were added to the plate and the plate was incubated overnight at 4 °C. Total IgG (BioRad STAR120p), IgG1 (STAR132P), IgG2a (STAR133P), IgM (Jackson Immuno Research 115-035-020) and IgA (SouthernBiotech 1040-08) were detected. ELISAs were developed using TMB and stopped using $2NH_2SO_4$. Plates were read at 450 nm using a Fluostar (Omega). Standard curves were produced and concentrations of OMV-specific antibody were determined by interpolation.

### *Statistical analysis*

Calculations as described in figure legends were performed using Prism 9 (GraphPad Software Inc., La Jolla, CA, USA).

## Results

### *Intranasal infection with A.baumannii (ATCC 17978) results in low levels of colonization and mild disease in BALB/c mice*

To establish whether the laboratory reference strain *A. baumannii* (ATCC 17978) caused respiratory disease in BALB/c mice we infected mice intranasally. Mice intranasally challenged with ATCC 17978 lost a small amount of weight 24 h after infection, significantly more than the naïve group ($p \leq 0.05$, Fig. 1A). Infected animals had significantly more IL-6 in the BAL at 4 and 24 h after infection ($p \leq 0.01$, Fig. 1B), this was concomitant with a significant increase in neutrophil recovery from the airways at 24, and 48 h after infection ($p < 0.01$, Fig. 1C). By 72 h after infection, both IL-6 and neutrophils into the lungs had returned to baseline levels.

We measured the amount of *A. baumannii* in the airways (Fig. 1D), lungs (Fig. 1E) and spleen (Fig. 1F) after infection. No bacteria were recovered from the airway, lungs or spleen of the naïve group. The number of bacteria recovered from airways (BAL) of the infected groups decreased very quickly after 4 h and no bacteria were recovered from 24 h after infection. There were significantly more bacteria recovered from the challenge group's lungs ($p \leq 0.001$) at 4 h than the naïve group. After this, the numbers of bacteria recovered slowly fell, bacteria were still recovered from 3 out of 5 mice of the challenge group at 72 h. Significantly more bacteria were recovered from the spleens of the challenge groups at 4 ($p \leq 0.05$) and 24 h ($p \leq 0.01$) compared to the naïve group. This cleared by 72 h after infection. This demonstrates that *A. baumannii* strain ATCC 17978 can be used to cause a limited respiratory infection, from which the mice recover 72 h after challenge.

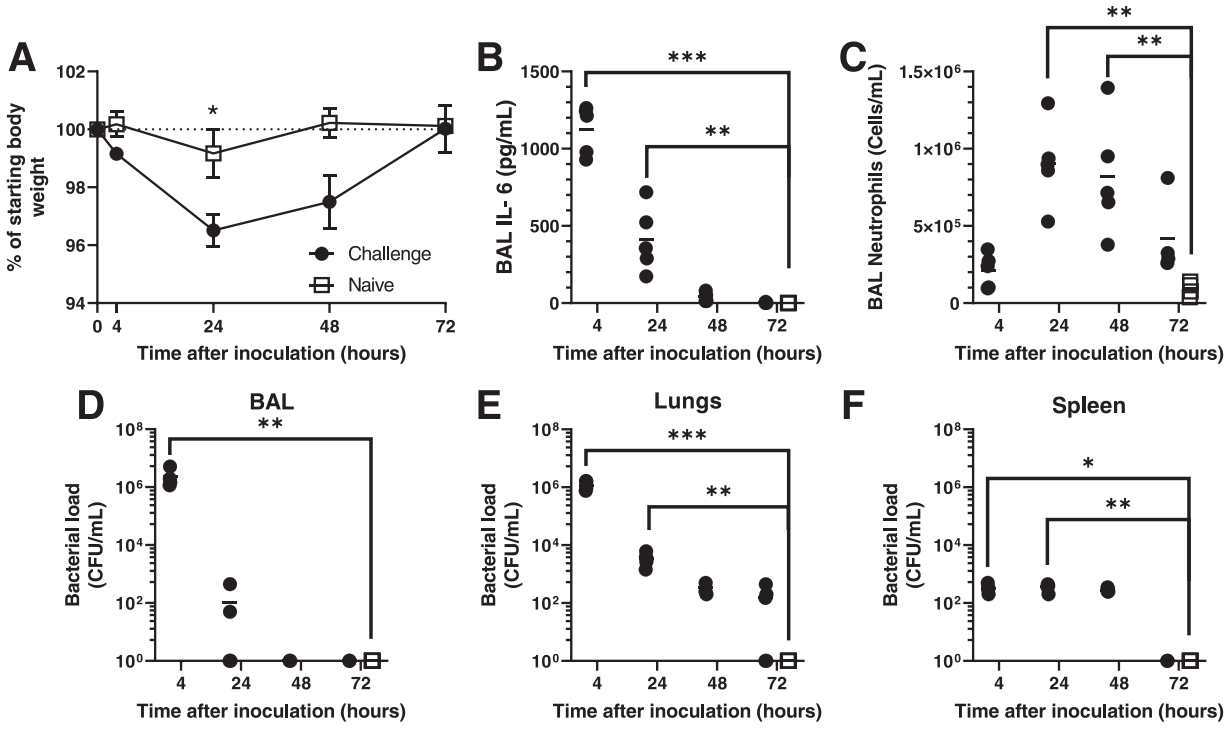



**Fig. 1.** Infection with ATCC *A. baumannii* strain causes limited colonization and disease. Adult BALB/c mice were intranasally dosed with 100 µl containing $5 \times 10^6$ CFU per mouse *A. baumannii* (strain ATCC 17978) (Challenge) or LB (Naïve) and culled 4, 24, 48 or 72 h later. (A) Weight after infection. (B) IL-6 concentration in the BAL fluid, measured using ELISA. **(C)** Concentration of neutrophils in the BAL fluid, estimated by haematoxylin and eosin staining followed by manual counting. Bacterial load in BAL (D). Lungs (E) and Spleen (F) were estimated by serially diluting samples and plating onto HiCrome Acinetobacter agar base plates. Kruskal-Wallis tests were used to test for significant differences in bacterial load, concentration of neutrophils and IL-6 concentration. Multiple t tests were used to test for significant differences in change in bodyweight. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Points represent means of mice (A) or individual animals (B-F).

*Intramuscular injection with OMVs from A.baumannii (ATCC 17978) reduces bacterial load and protects against challenge in BALB/c mice*

Having developed an in vivo respiratory tract infection model using ATCC 17978, we tested whether OMVs could protect against intranasal infection. Mice were immunized intramuscularly with OMVs derived from *A. baumannii* (ATCC 17978) in a prime, boost regime (30 days apart). There was detectable OMV-specific IgG on day 30 after infection (Fig. 2A), which increased on day 56 and was significantly higher than the naïve group ($p \leq 0.05$).

Both OMV-immunized and unimmunized mice were intranasally challenged with ATCC 17978. Immunized and naïve mice lost weight 24 h after challenge (Fig. 2B). The OMV immunized mice had a significantly less IL-6 in their BAL fluid (Fig. 2C) compared to the naïve group ($p \leq 0.05$). Both groups had very similar levels of neutrophil infiltration into their lungs (Fig. 2D). There was significantly less bacteria recovered from the BAL fluid (Fig. 2E) of the OMV immunized mice compared to the naïve group ($p \leq 0.05$). There was no difference in the bacteria recovered from the lungs of the OMV immunized group (Fig. 2F) compared to the naïve group. Taken together, ATCC 17978 OMVs induced an antibody response in the mice that was partially protective, reducing inflammation and bacterial load in the lungs.

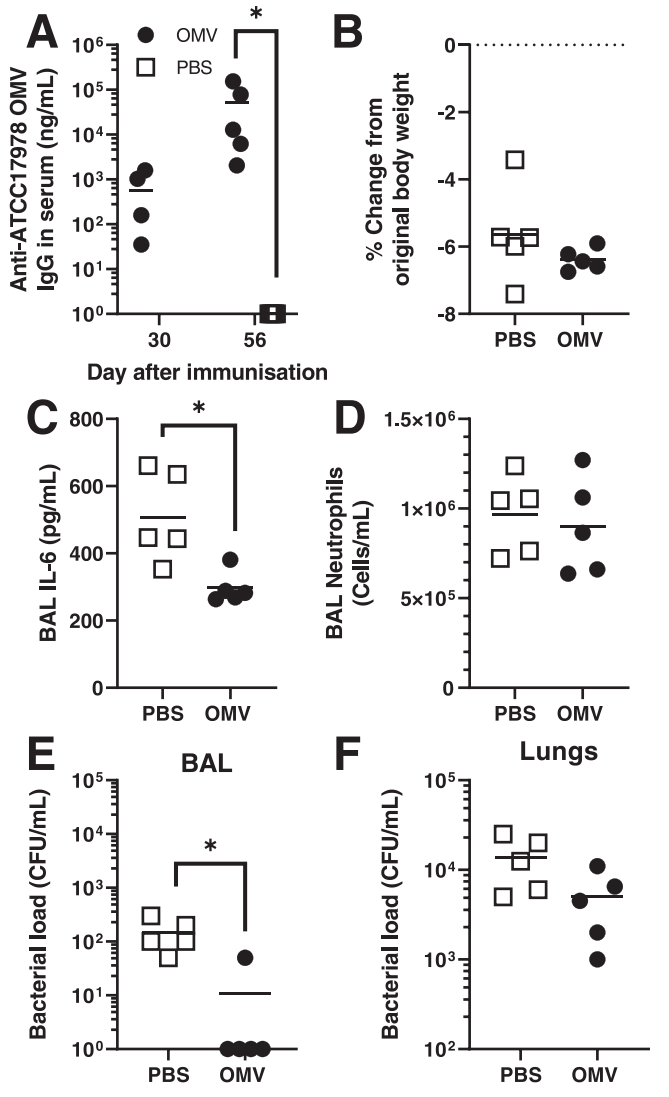



**Fig. 2.** OMVs from *A. baumannii* (strain ATCC 17978) protect against challenge. Adult BALB/c mice were intramuscularly injected with 100 fluorescence units of ATCC 17978 OMV (OMV) or PBS (Naïve) at day 0 and boosted at day 30. Levels of ATCC 17978 OMV specific IgG (A) in serum measured by ELISA. At day 56 all mice were intranasally infected with $5 \times 10^6$ CFU *A. baumannii* (strain ATCC 17978) and culled 24 h later. (B) Weight 24 h after infection. IL-6 concentration (C) and neutrophils (D) in the BAL fluid. Bacterial load in BAL (E) and lungs (F) after challenge. Mann-Whitney tests were used to test for significant differences in IgG and IgM concentrations in serum, bacterial load, change in bodyweight, IL-6 concentration and neutrophil concentration. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Points represent individual animals, lines mean.

*Intranasal infection with clinical isolates of A.baumannii causes increased levels of colonization and severe disease in BALB/c mice*

Having seen that OMVs provide partial protection against the ATCC 17978 strain in our model, we explored whether OMV immunization could provide protection against challenge with more relevant clinical isolates. We tested four relatively recently isolated strains of *A. baumannii*; BAL_191, BAL_215 BAL_084 and BAL_276, from bronchoalveolar lavage of ICU patients with VAP.[6] All strains are from the GC2 clonal group, BAL_191 is in subclade E with a KL49 capsule, BAL_215 is in subclade C with a KL58 capsule, BAL_084 is in subclade B with a KL58 capsule and BAL_276 is in subclade D with a KL2 capsule. All strains are carbapenem resistant via the *Oxa23* gene, encoding a class D carbapenemase enzyme.

BALB/c mice were intranasally dosed with *A. baumannii* (BAL_276). Bacterially challenged mice lost significantly more weight at 24 ($p \leq 0.0001$), 48 ($p \leq 0.0001$) and 72 ($p \leq 0.01$) hours after challenge compared to the naïve control group (Fig. 3A). Airway IL-6 concentration was highest at 4 h after infection (Fig. 3B) and significantly higher at 4 ($p \leq 0.0001$) and 24 ($p \leq 0.01$) hours after challenge than control mice. Neutrophil infiltration into the airways increased over time in the challenge group until it peaked at 48 h (Fig. 3C). There were significantly more neutrophils recovered from the airways of the challenge group at 24 ($p \leq 0.01$) and 48 ($p \leq 0.0001$) hours than the naïve group. A panel of cytokines was measured in the airways 24 h after infection (Fig. 3D): all the cytokines assessed were significantly greater in the infected animals.

Significantly more *A. baumannii* were recovered from the lower airways of infected mice than control mice (Fig. 3E; $p \leq 0.0001$); 4 h was the peak, and levels decreased after this point. A similar pattern was seen in the lungs (Fig. 3F), Nasal wash (Fig. 3G) and spleen (Fig. 3H) of infected animals, with the maximum recovered 4 h after infection and a decrease over time. Comparing recovery from the different compartments, the airways and the spleen had cleared the bacteria by 72 h after infection, but there were still recoverable *A. baumannii* in both the lungs and the nasal wash (Fig. 3I). We then performed a longer time course study over 7 days to investigate the course of disease and how long bacteria were recoverable from infected animals. Infected mice lost weight from day 1 of infection and had not fully returned to the same weight as control mice by day 7 (Fig. 3J). There were no detectable bacteria in lungs, airways or spleen 7 days after infection; however, there were detectable bacteria in the nasal lavage (Fig. 3K).

Having seen that one of the clinical *A. baumannii* isolates tested (BAL_276) could cause disseminated respiratory infection in mice, we tested three other recent representative clinical isolates. Mice were intranasally inoculated with BAL_191, BAL_215 or BAL_084. Mice infected with BAL_084 or BAL_215 lost significantly more weight (Fig. 4A) than the control group 24 h after infection ($p \leq 0.05$). There were increased levels of IL-6 in the airways of all challenge mice (Fig. 4B), but there was a significant difference between the mice challenged with BAL_215 compared to the naïve mice ($p \leq 0.05$). Bacteria were recovered from the airways (Fig. 4C), lungs (Fig. 4D) and nose (Fig. 4E). Both BAL_215 and BAL_084 showed significantly more bacteria in the BAL fluid (BAL_215: $p \leq 0.01$, BAL_084: $p \leq 0.05$) lungs ($p \leq 0.05$) and nasal wash ($p \leq 0.05$) than the naïve controls, reflecting the weight loss. We, therefore, show that multiple clinical isolates of *A. baumannii* can infect BALB/c mice

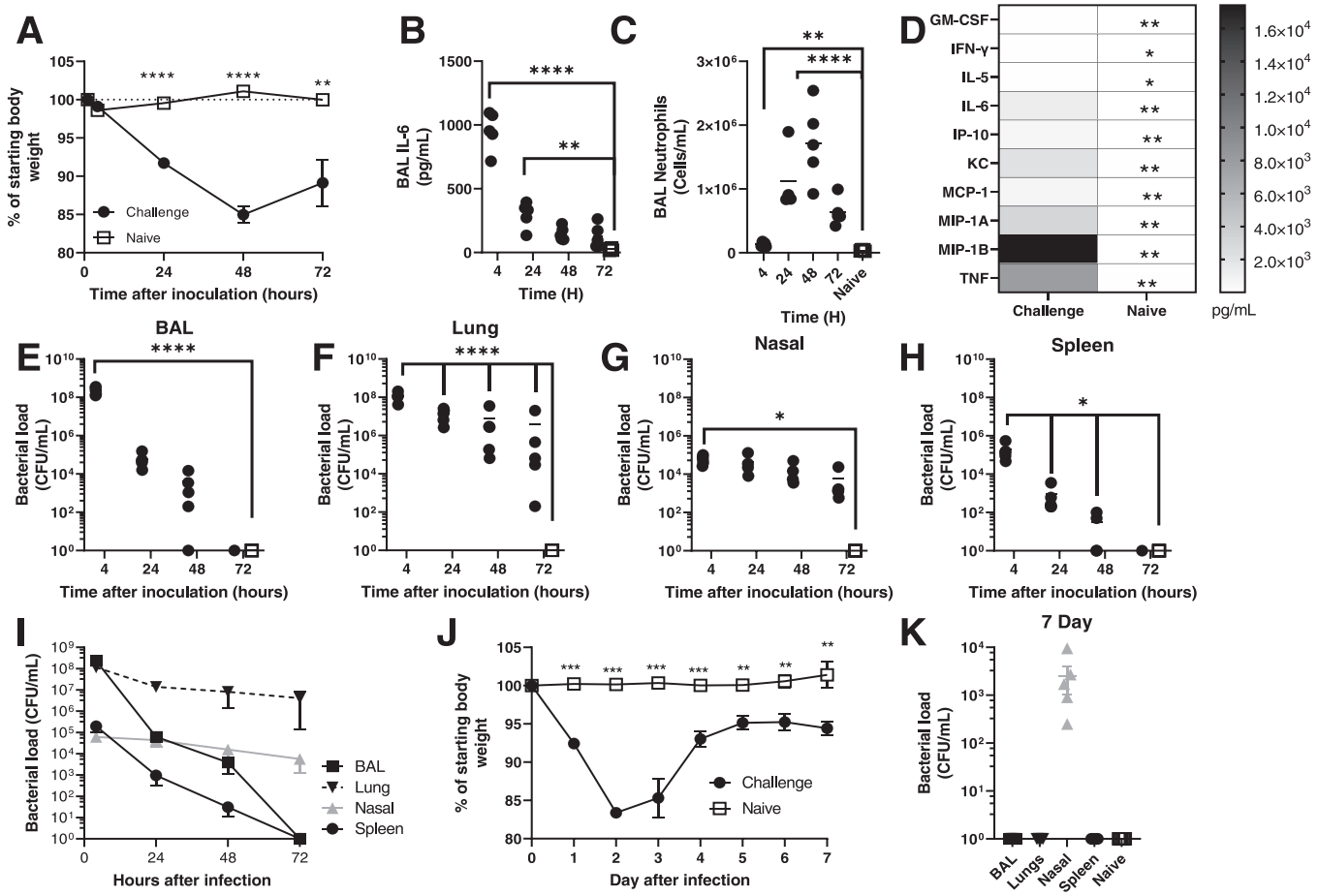



**Fig. 3.** Infection with a GC2 clade clinical isolate of *A. baumannii* (strain BAL_276) causes severe disease and systemic colonization. Adult BALB/c mice were intranasally dosed with $10^7$ CFU per mouse *A. baumannii* (strain BAL_276) (Challenge) or LB (Naïve) and culled 4, 24, 48 or 72 h later. Weight after infection (A). IL-6 concentration (B) and neutrophils (C) in the BAL fluid. BAL cytokines at 24 h measured by MSD (D). Bacterial load in BAL (E) lungs (F), Nasal lavage (G) and spleen (H) after challenge. Comparison of bacterial clearance from different compartments (I). In an extended timecourse, mice were infected intranasally and weight change tracked for seven days (J), bacterial load on day 7 after infection (K). One-way ANOVA was used to test for significant differences in bacterial load, concentration of neutrophils and IL-6 concentration. Multiple t tests were used to test for significant differences in change in bodyweight and cytokine concentrations. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Points represent mean of mice (A, D, I and J) or individual animals (B, C, E–H and K).

causing more severe disease and a greater bacterial burden than ATCC17978, with long-term colonization of the nasal cavity.

### *Intramuscular-delivered OMVs induce an antibody response, but only partial protection against a clinical isolate of A.baumannii*

Having established a respiratory tract challenge model with clinical isolates, we investigated whether OMVs derived from these isolates could protect against infection. Mice were immunized intramuscularly (IM) with OMVs derived from BAL_276 in a prime-boost regime (30 days apart). IgM (Fig. 5A) and IgG (Fig. 5B) specific to OMVs from BAL_276 and the related BAL_084 were measured at day 57, just prior to challenge. There was significantly greater IgM specific to OMVs from either bacterial strain in the terminal bleed sera of the immunized compared to the control group (BAL_276: $p \leq 0.001$, BAL_084: $p \leq 0.0001$). A similar pattern was seen with IgG, immunized animals had significantly greater antibody responses than control to both BAL_276 ($p \leq 0.01$) and BAL_084 ($p \leq 0.05$) OMVs. Antibody responses to the heterologous OMVs from BAL_084 were lower than the homologous BAL_276 OMVs.

Both immunized and naïve mice lost weight 24 h after intranasal challenge with BAL_276 (Fig. 5C) and there were similar levels of IL-6 in the BAL fluid (Fig. 5D). The immunized mice had slightly raised levels of neutrophil infiltration into their lungs than the non-immunized mice (Fig. 5E), though this was not significant. There was a small but significant reduction in bacteria recovered from the BAL (Fig. 5F; $p \leq 0.05$), lungs (Fig. 5G; $p \leq 0.01$), Spleen (Fig. 5H; $p \leq 0.05$) and nasal wash (Fig. 5I; $p \leq 0.05$) of the OMV immunized mice compared to the naïve group. This shows that IM injected OMVs provide partial protection against infection with a clinical isolate of *A. baumannii*.

### *Subcutaneously delivered OMVs improve protection against infection with a clinical isolate of A.baumannii*

Having observed partial bacterial clearance following intramuscular vaccination with OMVs derived from BAL_276 we wanted to compare routes of delivery to see if this affected the protection. Mice were immunized IM or subcutaneously (SC) with BAL_276 OMVs in a prime, boost regime (30 days apart). There was significantly greater specific IgM (Fig. 6A) 30 days after the first immunization in the IM ($p \leq 0.001$) and SC ($p \leq 0.001$) immunized group than control groups, but not at day 60. SC immunization led to significantly greater IgG responses 30 days (Fig. 6B; $p \leq 0.0001$) and 60 days ($p \leq 0.01$) after the first immunization. To investigate whether there was a qualitative difference between the responses, we compared IgG1 and IgG2a subtypes. SC immunized mice had significantly greater IgG1 than the PBS control mice (Fig. 6C; $p \leq 0.01$); both immunized groups had significantly increased levels of IgG2a compared to naïve mice (Fig. 6D; $p \leq 0.05$).

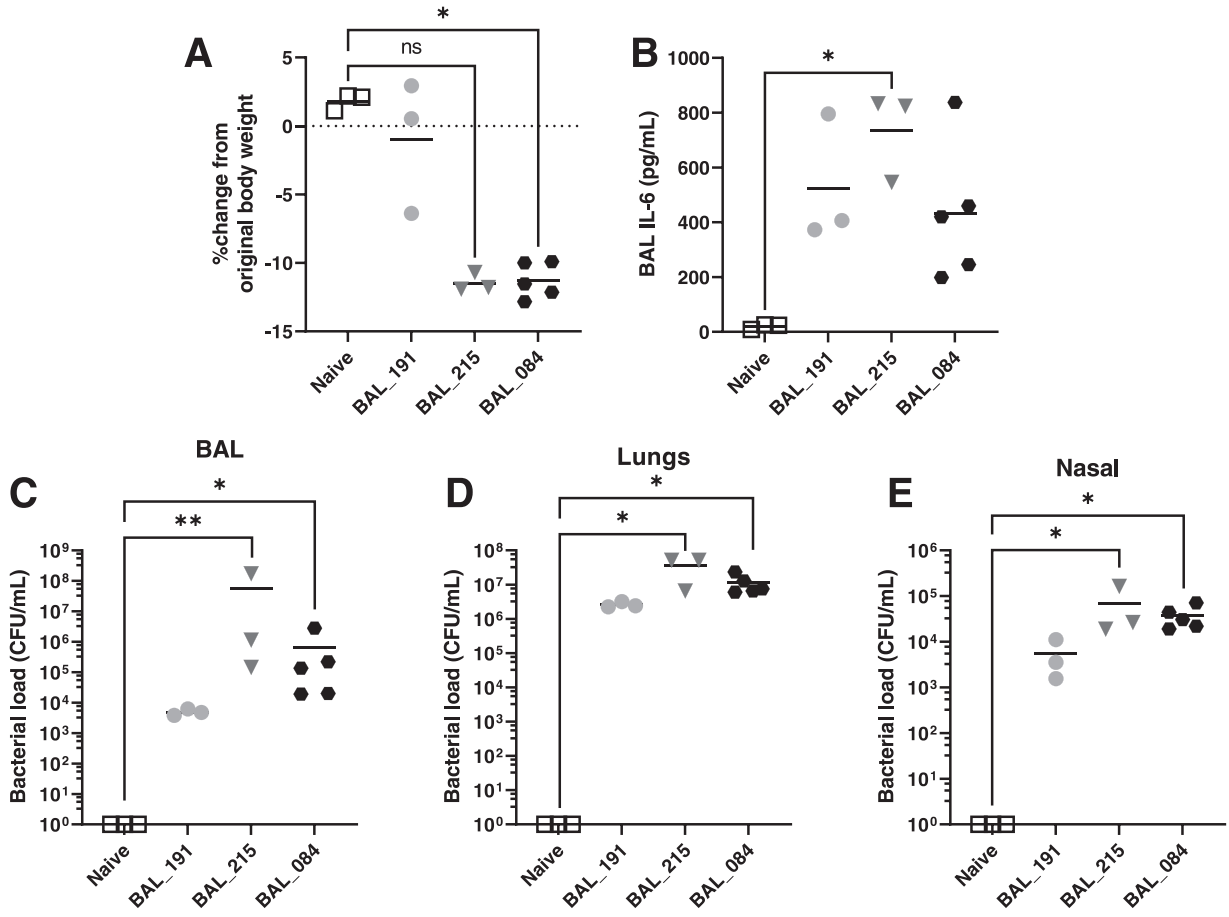



**Fig. 4.** Infection with clinical isolates of *A. baumannii* (strain BAL_084, BAL_191 and BAL_215). Adult BALB/c mice were intranasally dosed with *A. baumannii* strains BAL_191, BAL_215 or BAL_084 or LB (Naïve) and culled 4, 24, 48 or 72 h later. (A) Weight change 24 h after infection. IL-6 concentration (B) in the BAL fluid. Bacterial load in BAL (C) lungs (D) and nasal lavage (E) after challenge. Kruskal Wallis was used to test for significant differences in weight loss, bacterial loads and IL-6 concentrations. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. Points represent individual animals, lines represent means.

Mice were intranasally challenged with BAL_276 and responses compared between the three groups. Both immunized and naïve mice lost weight 24 h after challenge (Fig. 6E). The SC immunized mice had a significantly lower concentration of IL-6 in their BAL fluid compared to the naïve group (Fig. 6F; $p \leq 0.05$). There was significantly less bacteria recovered from the BAL of SC immunized mice compared to the naïve mice (Fig. 6G; $p \leq 0.05$), with a 4 log reduction, compared to a 1 log reduction in IM immunized mice. Likewise, SC-immunized mice had a large and significant reduction in bacterial load compared to the naïve groups in lungs (Fig. 6H; $p \leq 0.05$) and spleens (Fig. 6I; $p \leq 0.01$); bacteria were only recovered from 1 out of 5 spleens of SC immunized animals. There was no difference in bacteria recovered from the nose (Fig. 6J). Taken together, SC immunization here was more protective than IM, leading to greater antibody responses, less inflammation in respiratory airways and decreased bacterial burden.

*Intranasally delivered OMVs induced a strong OMV-specific IgA response in the respiratory tract and improved nasal protection after challenge with a clinical isolate of A.baumannii*

To determine whether mucosal immunization with OMVs would enhance protection against respiratory pathogen *A. baumannii* and induce a mucosal immune response, mice were immunized intranasally (IN), SC and IM with OMVs. OMVs derived from BAL_276 and were used in a prime boost regime (21 days apart). There were significantly increased levels of BAL_276 OMV-specific IgA in the BAL fluid (Fig. 7A; $p \leq 0.001$) and nasal wash (Fig. 7B; $p \leq 0.0001$) compared to all other groups 24 h after challenge. All mice lost weight after challenge, but the SC-immunized mice lost significantly less weight than the control mice 24 h after challenge (Fig. 7C; $p \leq 0.05$). Mice continued to lose weight by 48 h after challenge but there were no significant differences between the groups. Total cells in the BAL fluid were measured after challenge and increased between 24 and 48 h after challenge. At 48 h after challenge the IM immunized mice had significantly increased levels of WBC infiltration into their lungs compared to IN immunized mice (Fig. 7D; $p \leq 0.01$).

There were no significant differences in the bacteria recovered from the BAL fluid between the groups at either 24 or 48 h after challenge (Fig. 7E). The bacteria recovered from the noses of the immunized mice was significantly reduced compared to the control mice 24 h after challenge (Fig. 7F; $p \leq 0.05$). By 48 h after challenge, only the 3 of the IN-immunized mice had managed to completely clear bacteria from their noses. There were no significant differences in the bacterial loads in the lungs of any of the groups at either time point (Fig. 7G). The amount of bacteria recovered from the spleens of the mice was significantly higher in the IN-immunized mice compared to all other groups 24 h after challenge (Fig. 7H; $p \leq 0.01$). However, 48 h after challenge bacteria had been completely cleared from the spleens of all mice in the control, IM and IN groups. IN instillation of OMVs induced a strong and significant IgA response in the respiratory tract of mice, with no IgA responses detected from the IM or SC-immunized mice. Additionally, IN-immunized mice had the best protection against nasal infection, with 3 mice completely clearing all bacteria from the nose within 48 h of challenge.

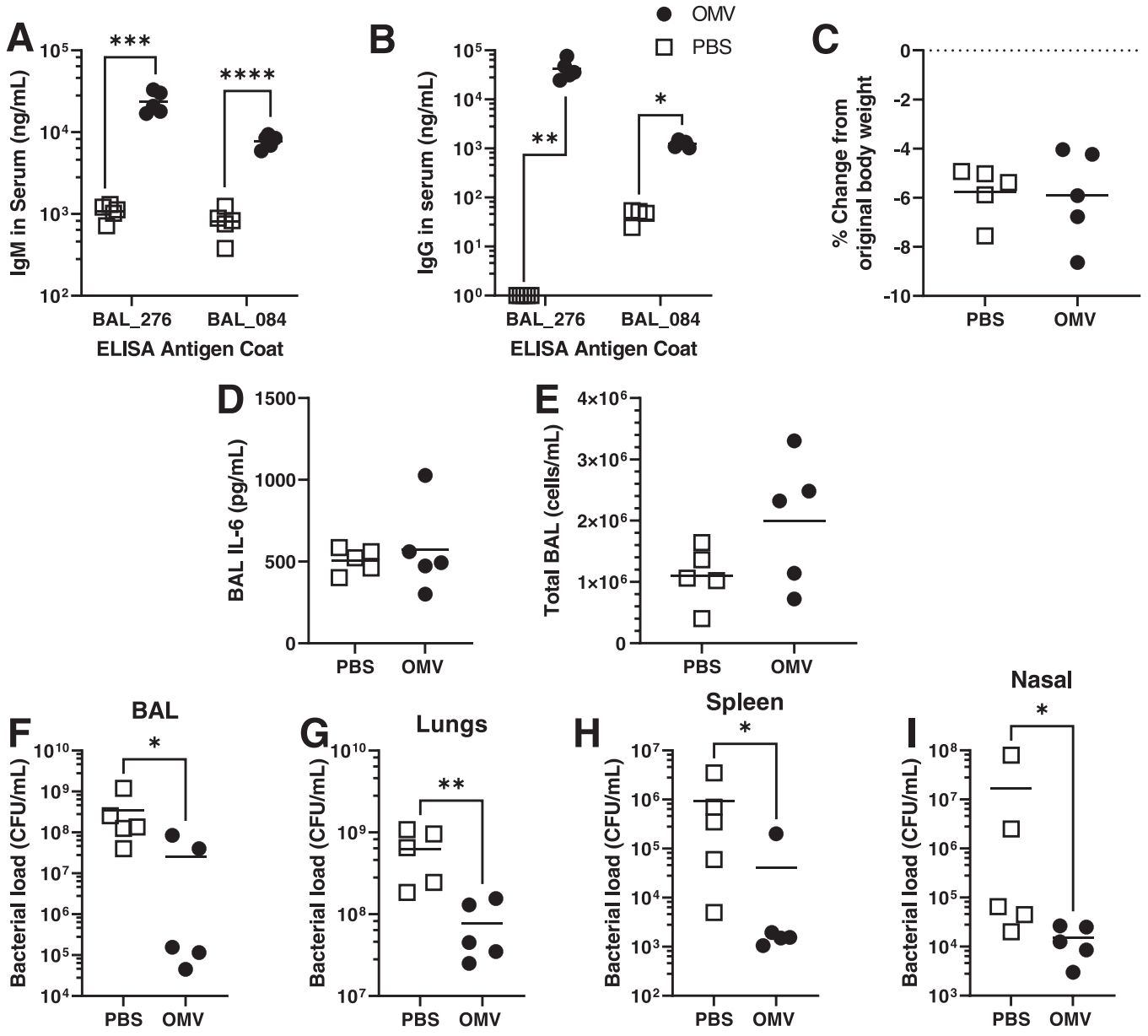



**Fig. 5.** OMVs delivered IM give partial protection against homologous bacterial challenge. Adult BALB/c mice were intramuscularly injected with 100 fluorescence units of BAL_276 OMV (BAL_276 OMV) or PBS at day 0 and boosted at day 30. Anti-BAL_276 and BAL_084 OMV IgM (A) or IgG (B) in sera from the terminal bleed. At day 56 mice were intranasally infected with $10^7$ CFU *A. baumannii* (strain BAL_276) and culled 24 h later. Weight (C), IL-6 (D) BAL cell count (E) was measured 24 h infection. Bacterial load at 24 h in BAL (F), Lungs (G), Spleen (H), Nasal wash (I). Mann-Whitney tests were used to test for significant differences in IL-6, bacterial load, change in weight and total cells. Multiple t tests were used to test for significant differences in IgG and IgM concentrations in serum. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, ****$p \le 0.0001$. Points represent individual animals.

*Intranasally delivered OMVs further improve protection against infection with a clinical isolate of A.baumannii*

Because *A. baumannii* infects the respiratory tract, we were interested in whether mucosal immunization would enhance protection. Mice were immunized intranasally (IN) with OMVs derived from BAL_276 in a prime boost regime (21 days apart). IgM responses (Fig. 8A were slightly increased 21 days after immunization in the OMV immunized group, though this was not significant. There were significantly increased levels of BAL_276 OMV-specific IgG at days 21 (Fig. 8B; $p \le 0.01$) and 43 ($p \le 0.05$) in the immunized group compared to the control group. BAL_276 OMV-specific IgA was significantly greater in the nasal wash of immunized mice 24 h after challenge (Fig. 8C; $p \le 0.05$). Whilst both the immunized and naïve mice lost weight 24 and 48 h after challenge, the immunized group lost significantly less weight than the naïve group at the 24 h time point (Fig. 8D; $p \le 0.01$). Both groups had similar levels of IL-6 in their BAL fluid (Fig. 8E). There was significantly less bacteria recovered from the BAL fluid of the immunized mice compared to the naïve mice (Fig. 8F; $p \le 0.05$), with a 3.5 log reduction. There was also a large and significant reduction of bacterial loads in the immunized mice in the nose (Fig. 8G; $p \le 0.01$), lungs (Fig. 8H; $p \le 0.01$) and spleen (Fig. 8I; $p \le 0.01$). Therefore, immunization with OMVs via the IN route induced local IgA antibody responses and also significantly reduced weight loss and bacterial burden, while completely preventing systemic dissemination of bacteria from the lungs to the spleen.

## Discussion

The high levels of antibiotic resistance along with the likelihood of new resistance developing rapidly to newly-developed antibiotics

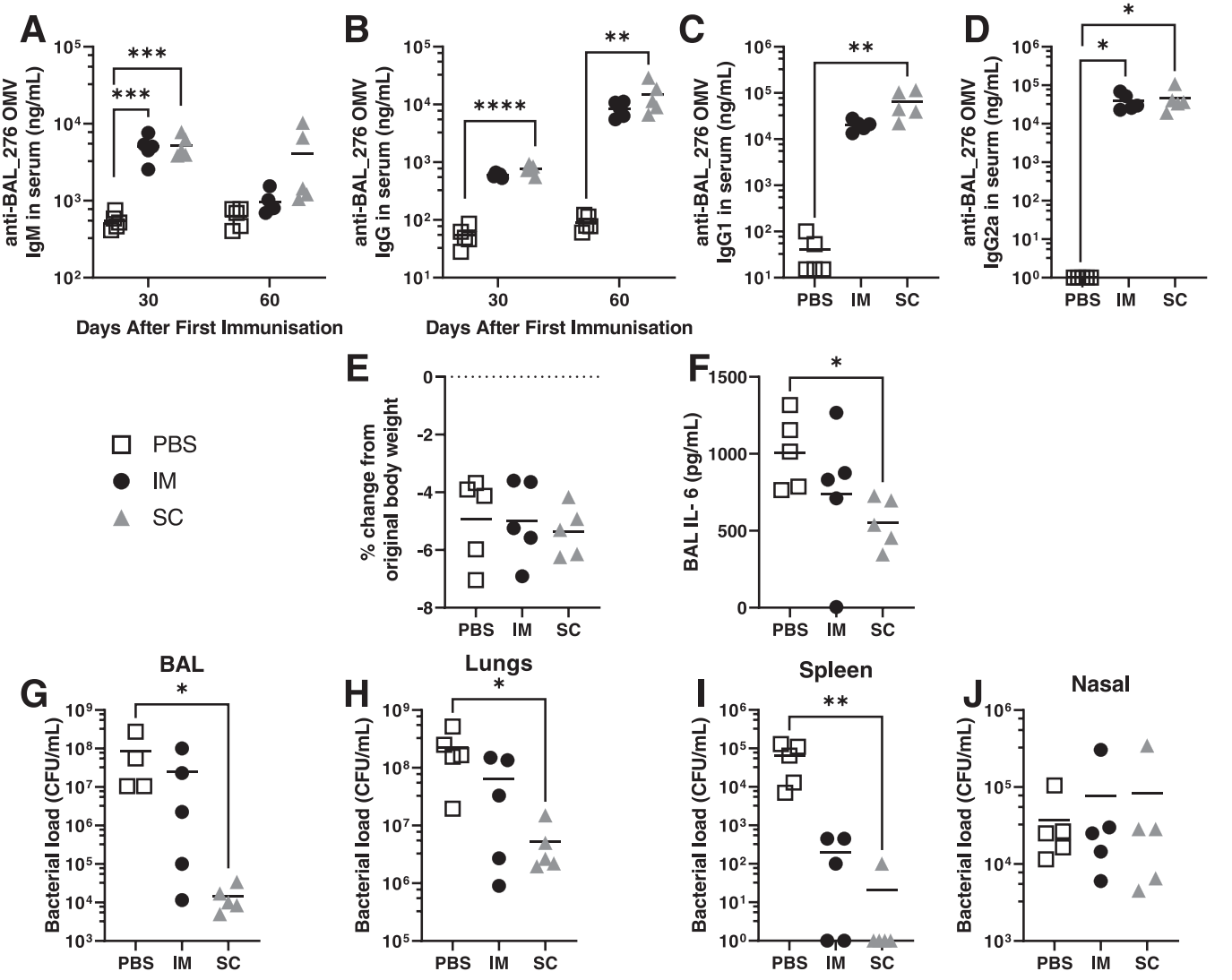



**Fig. 6.** OMVs from *A. baumannii* (strain BAL_276) reduce bacteria load better when injected subcutaneously. Adult BALB/c mice were intramuscularly or subcutaneously injected with 100 fluorescence units of BAL_276 OMV (OMV) or intramuscularly with PBS (Naïve) at day 0 and boosted at day 27. Anti-BAL_276 OMV IgM (A) or IgA (B) IgG1 (C) or IgG2 (D) in sera. At day 91 mice were intranasally dosed with $1.4 \times 10^7$ CFU *A. baumannii* and culled 24 h later. Weight (E) BAL cell count (F) was measured 24 h infection. Bacterial load at 24 h in BAL (G), Lungs (G), Spleen (H), Nasal wash (I). Kruskal Wallis was used to test for significant differences in concentrations of antibodies, bacterial load and change in weight. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Points represent individual animals.

suggest that vaccines should play a key role in the prevention of *A. baumannii* infections and outbreaks. Preclinical models play an important part in the development of vaccines and in providing immunological understanding into how they provide protection. Challenge with clinical isolates in the models presented here lead to a persistent colonization of the upper respiratory tract alongside systemic bacterial dissemination. Using these models, we tested an OMV-based vaccine, comparing different routes of administration. We showed that OMVs are immunogenic in all routes tested and that intranasal immunization provides the most robust protection against challenge.

OMVs derived from *A. baumannii* induced a systemic humoral response with significantly increased levels of specific IgG and IgM in the sera. This reduced inflammation in the lungs by reducing bacterial burden. This is in concurrence with a previous study that IM delivered OMVs led to protection against models of *A. baumannii* sepsis and pneumonia.[16] It has been proposed that protection is via bacterial opsonisation,[17] and opsonising antibodies were induced by intramuscular OMV delivery.[16] OMVs are a potent vaccine for inducing antibacterial protection.[18] As well as being used directly for the delivery of antigens, they can also act as adjuvants – for example, as seen in Bexsero or as a delivery vehicle. Genetic engineering of the bacterial strain, in particular removing the *tolR* gene can lead to much greater OMV production, and this has been utilized in the generation of a *Shigella sonnei* vaccine (sometimes called the Generalized Modules for Membrane Antigens (GMMA) approach).[19] It is of note that in *A. baumannii* it was possible to generate OMVs without needing *tolR* mutation.

The route of vaccination alters the immune response inducing different mechanisms of protection.[20] In our study, subcutaneously immunized mice were better protected against challenge with reduced inflammation in the lungs and reduced bacterial burdens compared to intramuscularly immunized mice. The only differences that we observed in antibody responses of the two routes of immunization were that subcutaneously immunized mice had increased levels of IgG1 compared to the intramuscularly immunized mice. This suggests that there could be a qualitative difference responsible for improved level of protection. Increased levels of IgG1 suggest subcutaneous immunization could be skewing the immune response more towards a Th2 response[21] than intramuscular vaccination. Similar results have been seen upon vaccination with recombinant OmpA, where mice develop a Th2-skewed response.[22]

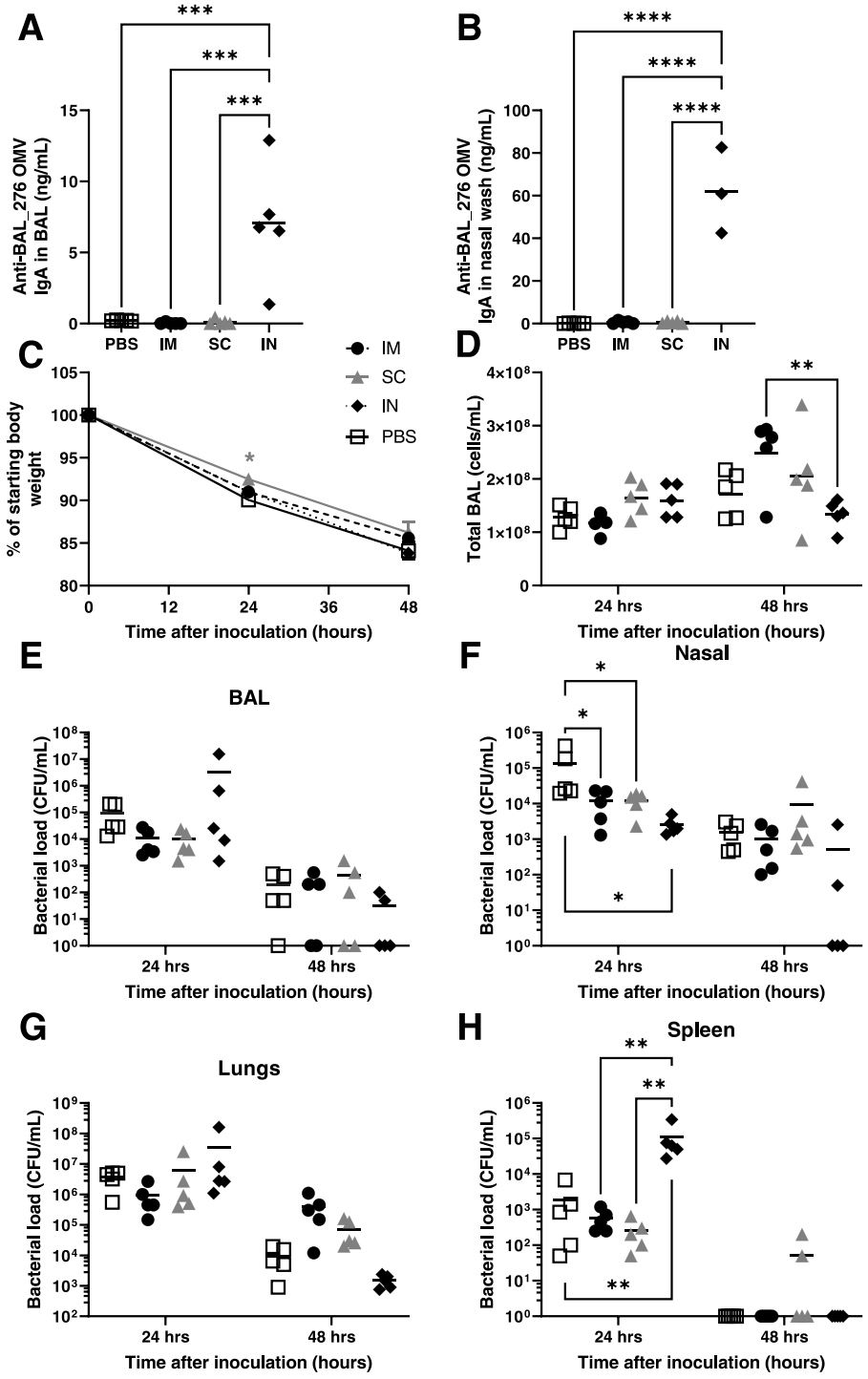



**Fig. 7.** Intranasally instilled OMVs from *A. baumannii* (strain BAL_276) induce a significant IgA response. Adult BALB/c mice were intramuscularly, subcutaneously or intranasally immunized with 100 fluorescence units of BAL_276 OMV (OMV) in 50 µl or intramuscularly with PBS (Naïve) at day 0 and boosted at day 21. Anti-BAL_276 OMV IgA in BAL fluid (A) and nasal wash (B) 24 h after challenge. At day 42 mice were intranasally dosed with $1 \times 10^7$ CFU *A. baumannii* and culled 24 or 48 h later. Weight (C) and BAL cell count (D) were measured after infection. Bacterial load at 24 and 48 h in BAL (E), Nasal wash (F), Lungs (G) and Spleen (H). One-way ANOVAs were used to test for significant differences in concentrations of antibodies. Two-way ANOVAs were used to test for significant differences in weight loss, cell count and bacterial load. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Points represent individual animals.

Additionally, as well as the IgG1 response a strong IgG2a response was also seen after subcutaneous and intramuscular immunization, with similar results being reported in other *A. baumannii* OMV vaccination models.[16] Due to its important role in neutrophil recruitment, it is suspected, but not confirmed, that IL-17 could be playing a role during *A. baumannii* infection.[17]

Intranasal vaccination with OMVs proved to be the most effective at protecting against homologous infection with *A. baumannii*. Of particular note were the increased levels of IgA and the reduction of the bacterial load seen in the nasal wash. Mucosal IgA plays a key part in protecting against pathogens at mucosal surfaces by preventing attachment of pathogens to epithelial cells.[23] Systemic

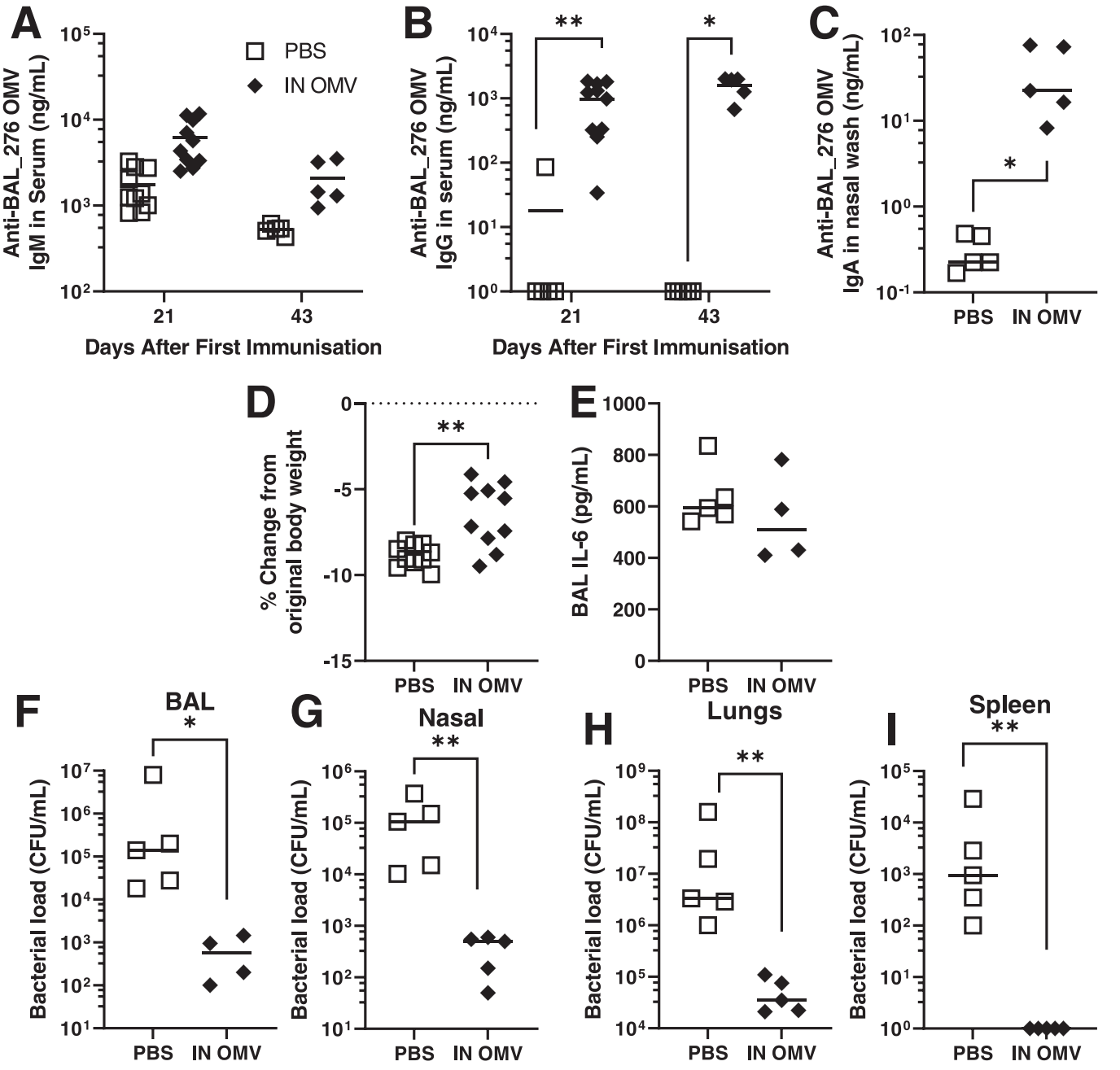



**Fig. 8.** OMVs from *A. baumannii* (strain BAL_276) protect when administered intranasally. Adult BALB/c mice were intranasally vaccinated with 500 fluorescence units of BAL_276 OMV in 100 µl or with 100 µl PBS at day 0 and boosted at day 21 and challenged on day 42. Mice were culled 24 h after infection. Levels of BAL_276 OMV specific IgM (A) or IgG (B) in serum and IgA (C) in nasal wash were measured using ELISA. Weight (D) and IL-6 (E) were measured 24 h after infection. Bacterial load at 24 h in BAL (F), Lungs (G), Spleen (H), Nasal wash (I). Kruskal Wallis was used to test for significant differences in IgG, IgM, IgA and IL-6. Nonparametric t tests were used to test for significant differences weight loss and bacterial loads. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Points represent individual animals, lines means.

vaccination methods do not induce the production of mucosal IgA.[23] Prevention of nasopharyngeal carriage of many respiratory pathogens is an important part of both individual and herd immunity.[24] A similar reduction in airway colonization following intranasal OMV vaccination has been seen for transgenic OMVs expressing proteins from *Streptococcus pneumoniae.*[25] In addition to IgA, it is likely that the mucosal IN vaccination has induced a protective tissue-resident memory (Trm) cell response,[26] particularly a protective Th17 response. Intratracheal immunization against *Klebsiella pneumoniae* led to Th17 CD4+ TRM cells providing serotype-independent protection from infection in the lungs;[27] with the shared capsule types between *A. baumannii* and *K. pneumoniae,*[28] it is possible Th17 Trm could also be an important part of the immune response against *A. baumannii*.

Since *A. baumannii* infections are most commonly seen in Ventilator-Associated Pneumonia, understanding the best way to deploy potential vaccines is important. One consideration is prevention of upper respiratory tract carriage because it can act as a reservoir of infection in ventilator-associated pneumonia[29–31]. An effective vaccine could prevent bacterial colonization of the airways and therefore also help reduce cases of pneumonia that occur as a result of aspiration. Similarly to the Bexsero vaccine, an effective *A. baumannii* vaccine will likely need to be a multivalent, multi-component vaccine in order to provide protection against multiple

strains. We showed that intranasal immunization was the most effective, reducing upper airway colonization. These findings support further development of OMVs as a vaccine platform against *A. baumannii* and warrant further exploration of intranasal delivery as a route of immunization.

## CRediT authorship contribution statement

**SH:** Conceptualization, Investigation, Writing – original draft. **SB:** Resources, Writing – review & editing. **KF:** Investigation. **AK:** Investigation, Methodology. **PK:** Conceptualization. **SR:** Conceptualization, Investigation, Methodology, Resources, Funding acquisition, Writing – review & editing. **JT:** Conceptualization, Investigation, Funding acquisition, Writing – original draft.

## Funding

This project was supported by a BBSRC DTP iCASE Award, BactiVac Catalyst funding (BVNCP3-07) and an Imperial College Confidence in Concept Award. PK and SR acknowledge support of the Bill and Melinda Gates Foundation Grant OPP1159947.

## Conflict of interest

SR is an employee of Kymab.

## Acknowledgments

The authors thank Kasia S for assistance in the in vivo studies and Lesley Rawlinson for lab management. For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) license to any Author Accepted Manuscript version arising.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2023.02.035.

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