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Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonization

Kirsten Kuipers[a,1], Maria H. Daleke-Schermerhorn[b,c,1], Wouter S.P. Jong[b,c], Corinne M. ten Hagen-Jongman[b,c], Fred van Opzeeland[a], Elles Simonetti[a], Joen Luirink[b,c,*], Marien I. de Jonge[a,**]

[a] Laboratory of Pediatric Infectious Diseases, Department of Pediatrics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands
[b] Section Molecular Microbiology, Department of Molecular Cell Biology, Faculty of Earth and Life Sciences, VU University, Amsterdam, The Netherlands
[c] Abera Bioscience AB, Stockholm, Sweden

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ABSTRACT

Bacterial outer membrane vesicles (OMVs) are attractive vaccine formulations because they have intrinsic immunostimulatory properties. In principle, heterologous antigens incorporated into OMVs will elicit specific immune responses, especially if presented at the vesicle surface and thus optimally exposed to the immune system. In this study, we explored the feasibility of our recently developed autotransporter Hbp platform, designed to efficiently and simultaneously display multiple antigens at the surface of bacterial OMVs, for vaccine development. Using two *Streptococcus pneumoniae* proteins as model antigens, we showed that intranasally administered *Salmonella* OMVs displaying high levels of antigens at the surface induced strong protection in a murine model of pneumococcal colonization, without the need for a mucosal adjuvant. Importantly, reduction in bacterial recovery from the nasal cavity was correlated with local production of antigen-specific IL-17A. Furthermore, the protective efficacy and the production of antigen-specific IL-17A, and local and systemic IgGs, were all improved at increased concentrations of the displayed antigen. This discovery highlights the importance of an adequate antigen expression system for development of recombinant OMV vaccines. In conclusion, our findings demonstrate the suitability of the Hbp platform for development of a new generation of OMV vaccines, and illustrate the potential of using this approach to develop a broadly protective mucosal pneumococcal vaccine.

1. Introduction

Outer membrane vesicles (OMVs), ubiquitously released from the outer membrane (OM) of Gram-negative bacteria, are promising as vaccines because they combine antigen and adjuvant in a single formulation. The intrinsic adjuvant activity provided by the presence of various pathogen recognition receptor ligands, such as lipopolysaccharide and immunogenic surface proteins, forms an attractive combination with the non-living, particulate nature of the OMVs [1–3]. OMVs have protected animals against various pathogens [4,5], and a licensed OMV vaccine against *Neisseria meningitidis* has been proven safe and protective in humans [6]. The interest in OMVs expanded as heterologous proteins were successfully incorporated into vesicles [7–11], and engineered OMVs were shown to elicit antigen-specific immune responses [9–11] and protection in mice [9].

Accumulating evidence indicates that both the magnitude and the breadth of the immune response can be improved by secreting or displaying antigens at the surface of bacterial and viral vaccine vectors [12–17]. Instigated by these observations, we recently engineered the *Escherichia coli* autotransporter (AT) Hemoglobin

Abbreviations: aa, amino acid; AT, autotransporter; CFU, colony forming units; Hbp, Hemoglobin protease; i.n., intranasal; OM, outer membrane; OMV, Outer membrane vesicle; PBS, phosphate buffered saline; PCV, pneumococcal conjugate vaccine; Ply, pneumolysin; PspA, pneumococcal surface protein A.

* Corresponding author at: Department of Molecular Cell Biology, Faculty of Earth and Life Sciences, VU University, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands. Tel.: +31 205987175.
** Corresponding author at: The Deparment of Pediatrics, Radboud University Medical Center, Philips van Leydenlaan 15, 6525 GA, Nijmegen, The Netherlands. Tel.: +31 243666406.
E-mail addresses: s.luirink@vu.nl (J. Luirink), marien.dejonge@radboudumc.nl (M.I. de Jonge).
[1] K.K and M.D.H.S contributed equally to this work.

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protease (Hbp) into a platform for efficient display of heterologous polypeptides at the surface of live bacteria [18,19], bacterial ghosts [20] and OMVs [21]. The AT pathway is the most widespread system for transport of proteins across the Gram-negative cell envelope, employing a relatively simple two-step mechanism. The AT is first transported across the inner membrane by the Sec machinery, after which its C-terminal domain inserts into the OM and forms a β-barrel that together with a central linker domain mediates transport of the functional N-terminal passenger domain to the cell surface or medium [22]. Based on the available crystal structure of the Hbp passenger [23], a side-domain replacement strategy was developed that allows fusion of multiple heterologous sequences to a single, stable Hbp scaffold [18]. Using various mycobacterial, chlamydial and influenza antigens, the Hbp platform was demonstrated to be a versatile tool for the simultaneous display of multiple sizeable antigens at the surface of live bacteria [19] and OMVs [21].

Here, we set out to test the applicability of the Hbp platform for development of recombinant OMV vaccines, using as model antigens the well-known protective proteins pneumococcal surface protein A (PspA) and pneumolysin (Ply) of the nasopharyngeal colonizer *Streptococcus pneumoniae* [24–26]. Current pneumococcal conjugate vaccines (PCVs), consisting of capsular polysaccharides, have strongly reduced the incidence of severe disease caused by vaccine-specific *S. pneumoniae* serotypes [27]. However, the efficacy of PCVs is reduced by serotype replacement [28–30] and capsular switching events [31], and their accessibility in developing countries is restricted by complex, costly manufacturing and the requirement for needle-based administration. These limitations can potentially be overcome by development of a novel vaccine based on conserved, non-capsular, proteinaceous antigens that is suitable for intranasal administration. We describe here the first *in vivo* challenge study designed to test a vaccine formulation consisting of OMVs that display heterologous proteins at the surface. We investigated whether intranasal immunization with *Salmonella enterica* serovar Typhimurium OMVs efficiently displaying sizeable fragments of PspA and Ply at the surface could protect mice against pneumococcal colonization. By assessing immune responses and bacterial load following intranasal *S. pneumoniae* challenge in mice immunized with various vaccine formulations and regimes, we demonstrate the potential of the Hbp platform for recombinant OMV vaccine development and we identified parameters that are critical for obtaining protective efficacy.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. Typhimurium SL3261 Δ*tolRA* [21], and *E. coli* TOP10F' and BL21(DE3) were grown at 37 °C in LB medium containing 0.2% glucose. When appropriate, kanamycin was used at a concentration of 25 μg/ml and chloramphenicol at 30 μg/ml. *S. pneumoniae* TIGR4 [32] was grown and vaccination stocks containing 10^6 colony forming units (CFU)/10 μL in phosphate buffered saline (PBS) were prepared as described [33].

2.2. Plasmid construction

Construction of pEH3-based [34] plasmids for expression of Hbp fused to pneumococcal antigen fragments and pET16b(+) vectors (Novagen) for expression of PspA and the PdT derivative of Ply [35] is described in the Supplementary Materials and Methods.

2.3. Hbp expression, OMV isolation and protein analysis

S. Typhimurium SL3261 Δ*tolRA* harboring pEH3 vectors was grown until an OD_{660} of ~0.6, at which expression of Hbp derivatives was induced from the *lacUV5* promoter in the presence of 1 or 100 μM IPTG for 1 h.

To isolate OMVs, culture supernatants obtained by low-speed centrifugation were passed through 0.45-μm-pore-size filters (Millipore) and centrifuged at 208,000 × *g* for 60 min, separating OMVs from soluble proteins. Pelleted OMVs were washed by resuspension in PBS containing 500 mM NaCl (1 OD unit of OMVs per μl) and centrifugation at 440,000 × *g* for 2 h, after which they were taken up in PBS containing 15% glycerol (1 OD unit of OMVs per μl). An amount of 1 OD unit of OMVs is derived from 1 OD_{660} unit of cells.

Proteinase K accessibility of OMV proteins was analysed as described [21], and proteins were analysed by SDS-PAGE and Coomassie G-250 (BioRad) staining or immunoblotting using antisera recognizing the β-domain of Hbp (SN477) [36], PspA or pneumolysoid, the detoxified derivative of Ply (own lab collection). Densitometric analysis on Coomassie-stained gels was carried out using a Molecular Imager GS-800 Calibrated Densitometer (Biorad) and Quantity One software (Biorad).

2.4. Mouse immunizations and challenge

Seven week-old female C57BL/6 mice (Charles River Laboratories) were intranasally (i.n.) immunized three, two or one time(s) with 8 OD units of OMVs (corresponding to ~4 μg total protein) in a volume of 10 μL, at two-week intervals, under anaesthetics (2.5% v/v isoflurane, AU Veterinary Services). Three weeks after the final immunization mice were challenged i.n. with 10^6 CFU of *S. pneumoniae* TIGR4 [33]. Three days after infection, mice were euthanized, and blood and mucosal nasal tissue were harvested. Nasal tissue was homogenized using an IKA T10 basic blender, and serially diluted samples were plated on Gentamicin Blood Agar (Mediaproducts BV) to determine bacterial recovery (log CFU/organ). All animal work was performed with approval of the Radboud University Medical Center Committee for Animal Ethics. Consequently, three mice were euthanized and excluded from further experimental analysis after reaching a humane endpoint, i.e., over 20% weight loss, potentially caused by a reaction to lipopolysaccharide present in the OMVs.

2.5. Detection of antibody responses by enzyme-linked immunosorbent assay analysis

Antigen and OMV-specific IgG in nasal samples and sera were detected as described in the Supplementary Materials and Methods.

2.6. Measurement of local IFNγ and IL-17A

Cytokine production in mouse nasal samples were determined with Cytometric Bead Array (Becton Dickinson) according to manufacturer's instructions, using the Mouse Enhanced Sensitivity buffer kit in combination with the Enhanced Sensitivity Flex set for IFNγ and IL-17A (Becton Dickinson). Concentrations were calculated using Soft Flow FCAP Array v1.0 (Becton Dickinson).

2.7. Statistical analyses

All statistical analyses were performed using GraphPad Prism version 5.0 (Graphpad Software). For bacterial recovery data, the Grubbs outlier test was used to test for significant outliers. The One-Way Anova Kruskall Wallis with Bonferroni post-test for multiple groups or Mann–Whitney *t*-test for two groups were used for comparisons of protection and immune responses. To determine the relation between IL-17A and protection, a Spearman Correlation test was applied.