Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Surface display of a borrelial lipoprotein on meningococcal outer membrane vesicles

Merijn L.M. Salverda[a,*], Sanne M. Meinderts[a,1], Hendrik-Jan Hamstra[b], Alex Wagemakers[c], Joppe W.R. Hovius[c], Arno van der Ark[a], Michiel Stork[a], Peter van der Ley[a]

[a] Institute for Translational Vaccinology (InTraVacc), Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands
[b] Immunology of Infectious Diseases and Vaccines (IIV), National Institute of Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3721 MA Bilthoven, The Netherlands
[c] Department of Internal Medicine, Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 5 August 2015
Received in revised form 9 December 2015
Accepted 11 January 2016
Available online xxx

Keywords:
OMV
Outer Membrane Vesicles
Surface display
Heterologous expression
Lipoprotein
Borrelia burgdorferi
Neisseria meningitidis
OspA
fHbp

ABSTRACT

Outer Membrane Vesicles (OMVs) are gaining attention as vaccine candidates. The successful expression of heterologous antigens in OMVs, with the OMV functioning both as adjuvant and delivery vehicle, has greatly enhanced their vaccine potential. Since there are indications that surface exposed antigens might induce a superior immune response, targeting of heterologous antigens to the OMV surface is of special interest. Several systems for surface display of heterologous antigens on OMVs have been developed. However, these systems have not been used to display lipidated membrane-associated proteins known as lipoproteins, which are emerging as key targets for protective immunity. We were therefore interested to see whether we could express a foreign lipoprotein on the outer surface of OMVs. When outer surface protein A (OspA), a borrelial surface-exposed lipoprotein, was expressed in meningococci, it was found that although OspA was present in OMVs, it was no longer surface-exposed. Therefore, a set of fusions of OspA to different regions of factor H binding protein (fHbp), a meningococcal surface-exposed lipoprotein, were designed and tested for their surface-exposure. An N-terminal part of fHbp was found to be necessary for the successful surface display of OspA on meningococcal OMVs. When mice were immunized with this set of OMVs, an OspA-specific antibody response was only elicited by OMVs with clearly surface-exposed OspA, strengthening the idea that the exact positioning of an antigen in the OMV affects the immune response. This method for the surface display of heterologous lipoproteins on OMVs is a step forward in the development of OMVs as a vaccine platform.

1. Introduction

Outer Membrane Vesicles (OMVs) are spherical buddings of the outer membrane (OM) that are spontaneously produced by Gram-negative bacteria. They are composed of OM proteins, LPS, phospholipids, and entrapped periplasmic components. Because of their excellent immunostimulatory properties [1–3] and ease of production, OMVs are receiving more and more attention as vaccine candidates. Immunization studies in mice have demonstrated that OMVs can protect against challenges with various pathogenic bacteria [4–12]. For *Neisseria meningitidis*, OMV vaccines have been extensively investigated in clinical trials, and three OMV-based vaccines against *Neisseria* (MenBvac, MeNZB, and Bexsero) are already available for human use [13–15].

Because of their intrinsic adjuvant properties, the use of OMVs as a delivery vehicle for heterologous antigens has gained considerable interest [16]. Several studies have demonstrated that the expression of heterologous antigens in the periplasm or OM of Gram-negative bacteria can lead to their inclusion in OMVs [1–3,17–20]. Importantly, such recombinant OMVs can induce an immune response to the heterologous antigen in immunized mice [2,3,12,18,19], and protect them against an otherwise lethal challenge with the pathogen from which the antigen originates [3,18].

To what extent the specific location of a heterologous antigen within the OMV (periplasm/inside of the OM/outside of the

* Corresponding author.
E-mail address: merijn.salverda@intravacc.nl (M.L.M. Salverda).
[1] Present address: Sanquin Research, Department of Blood Cell Research, Plesmanlaan 125, 1066 CX Amsterdam.

http://dx.doi.org/10.1016/j.vaccine.2016.01.019
0264-410X/

OM) affects the immune response remains an open question. Theoretically, the outer surface of the OMV appears to be the best option, as this provides the best accessibility for the binding of B-cell receptors [18]. There is indeed accumulating evidence that surface exposed antigens evoke superior antibody responses [21–25], which makes the precise targeting of heterologous antigens to the OMV surface of special interest.

Various expression systems that specifically target the expression of heterologous proteins to the outer surface of bacterial cells have been developed. However, many of these systems can only display small parts of proteins and suffer from low expression levels [26]. The two most versatile approaches fuse (parts of) heterologous proteins to Ice Nucleation Protein [27,28] or autotransporters [22,29–32] to reach the cell surface.

We were interested to find out whether it is possible to express heterologous lipoproteins on the outer surface of OMVs. Lipoproteins are membrane-bound proteins that are emerging as key targets for protective immunity, because of their excellent immunostimulatory properties (the N-terminal lipidation motif is recognized by the mammalian innate immune system through the Toll-like receptor TLR2 [33,34]) and role as virulence factors. For example, OspA (*Borrelia burgdorferi*) and fHbp (*N. meningitidis*) have both been extensively studied as vaccine components against Lyme disease [35–39] and meningitis [40,41], respectively.

Surface expression of heterologous lipoproteins on OMVs has not been explored so far, because surface lipoproteins are normally removed from the OMV during the detergent-based removal of LPS. However, recent biotechnological developments in *Neisseria* have led to a detergent-free OMV extraction process that allows surface exposed lipoproteins to remain attached to the OMV [42,43], opening up the possibility for surface expression of heterologous lipoproteins on these so-called native OMVs (nOMVs).

In Gram-negative bacteria, most lipoproteins are found on the periplasmic side of the inner or outer membrane. They are transferred from the inner membrane to the outer membrane by the Lol (localization of lipoproteins) machinery [44]. Lipoproteins that are located on the extracellular side of the outer membrane are less common, and systems or signals guiding transfer over the outer membrane have not yet been elucidated.

Because of its surface localization in *Borrelia* and the detailed knowledge regarding its immunogenicity and structure, OspA was chosen as a suitable lipoprotein to test heterologous expression in *Neisseria* nOMVs. OspA has been extensively investigated as a vaccine component against Lyme disease, the most common vector-borne disease in Europe and the United States [37,38,45].

In the current study, OspA was successfully expressed in *N. meningitidis* cells and nOMVs, but it could not be detected on the meningococcal cell surface. This indicates mislocalization to the periplasm or the periplasmic side of the OM. In order to redirect OspA to the cell surface of *Neisseria*, the globular domain of OspA was fused to different parts of fHbp, a well-studied meningococcal surface lipoprotein. It was found that fusion to specific N-terminal parts of fHbp allows surface expression of the fHbp–OspA fusion constructs. Moreover, it was demonstrated that these surface-exposed fHbp–OspA hybrids elicit strong antibody responses in immunized mice.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli strains JM109 (Promega) and TOP10F' (Invitrogen) were used for cloning steps involving vectors pGEM-T Easy and pEN11, respectively. *Neisseria meningitidis* capsule deficient strain HB-1 carrying an inactivated *lpxL1* gene [46] was used as host for shuttle vector pEN11 [47] carrying OspA or fHbp-OspA fusion constructs. Genomic DNA of *Borrelia burgdorferi* strain B31 [48] was used as template for OspA-based PCR reactions.

2.2. Recombinant DNA technology

All primers used in this study are shown in Table 1. Hybrids of fHbp (*N. meningitidis*) and OspA (*B. burgdorferi*) were constructed using Overlap Extension PCR [49]. All PCRs were carried out using the Accuprime *Taq* DNA Polymerase System (Invitrogen) to ensure both high fidelity amplification and the addition of 3′ A-overhangs. See Fig. 1 for a schematic overview of the different constructs and Supplementary data for cloning strategies.

2.3. Expression of constructs in N. meningitidis cells and nOMVs

Protein samples of cells or nOMVs were separated by SDS-PAGE on 12% Precise Protein Gels (Thermo Scientific). Separated proteins were then transferred to 0.45 μm nitrocellulose membranes (Bio-Rad). Membranes were incubated for 1 h on a rolling table in a 1:1000 dilution of anti-OspA (Rockland) in Tris buffer (0.1 M Tris, 1.54 M NaCl, and 5% Tween-80). The membrane was then transferred to a 1:2000 dilution of goat-anti-rabbit IgG AP (Southern BioTech) in Tris buffer supplemented with 0.5% Protifar (Nutricia). Blots were developed using the AP Conjugate Substrate Kit (BioRad).

2.4. Immunostaining

Briefly, *N. meningitidis* cells immobilized on coverslips were inactivated and then sequentially incubated in PBS with 3% BSA (for blocking), primary antibodies (anti-OspA and anti-fHbp), and fluorescent secondary antibodies. A more detailed description of the procedure is given in the supplementary data.

2.5. Purification of nOMV vaccines

The isolation of nOMVs for the immunization of mice was based on the detergent-free process developed in van de Waterbeemd et al. [43]. Note that the process was adjusted slightly for constructs that showed poor expression. See supplementary data for further details.

2.6. Mice and immunization

Animal experiments were approved by the Animal Ethics Committee. Groups of five female, six- to eight-week-old BALB/cOlaHsd mice (Harlan) were immunized subcutaneously with 200 μl of nOMVs, at either low concentration (5 μg/ml) or high concentration (20 μg/ml). Next to the groups that received nOMVs 'loaded' with OspA (two groups) or fHbp–OspA fusions (16 groups), two control groups received 'empty' nOMVS harvested form cells carrying the pEN11 plasmid with the *imp* gene replacing the ospA-constructs [47]. An additional control group was immunized with PBS, resulting in a total of 21 groups. Mice were immunized at days 0 and 28 and sacrificed 14 days after the last immunization. Blood was collected in Vacuette Z Serum Clot Activator tubes (Greiner Bio-One) and centrifuged at 2000 RPM for 15 min. Subsequently, sera were collected and stored at -20 °C for further analysis.

2.7. Analysis of sera

Sera were first pooled by group (five mice) and analyzed for the presence of antibodies by Western blot. Membranes were loaded with proteins from *E. coli* TOP10F' cells carrying either pEN11-Imp or pEN11-OspA. Membranes were incubated for 1 h on a rolling table with pooled sera diluted 1:1000 in Tris buffer, followed by 1 h of incubation in a 1:2000 dilution of secondary antibody (goat-anti-mouse IgG AP, Southern BioTech) in Tris buffer with 0.5% Protifar.

Please cite this article in press as: Salverda MLM, et al. Surface display of a borrelial lipoprotein on meningococcal outer membrane vesicles. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.01.019