Display of Recombinant Proteins on Bacterial Outer Membrane Vesicles by Using Protein Ligation

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ABSTRACT The *Escherichia coli* virulence factor hemoglobin protease (Hbp) has been engineered into a surface display system that can be expressed to high density on live *E. coli* and *Salmonella enterica* serovar Typhimurium cells or derived outer membrane vesicles (OMVs). Multiple antigenic sequences can be genetically fused into the Hbp core structure for optimal exposure to the immune system. Although the Hbp display platform is relatively tolerant, increasing the number, size, and complexity of integrated sequences generally lowers the expression of the fused constructs and limits the density of display. This is due to the intricate mechanism of Hbp secretion across the outer membrane and the efficient quality control of translocation-incompetent chimeric Hbp molecules in the periplasm. To address this shortcoming, we explored the coupling of purified proteins to the Hbp carrier after its translocation across the outer membrane using the recently developed SpyTag/SpyCatcher protein ligation system. As expected, fusion of the small SpyTag to Hbp did not hamper display on OMVs. Subsequent addition of purified proteins fused to the SpyCatcher domain resulted in efficient covalent coupling to Hbp-SpyTag. Using in addition the orthogonal SnoopTag/SnoopCatcher system, multiple antigen modules could be coupled to Hbp in a sequential ligation strategy. Not only antigens proved suitable for Spy-mediated ligation but also nanobodies. Addition of this functionality to the platform might allow the targeting of live bacterial or OMV vaccines to certain tissues or immune cells to tailor immune responses.

IMPORTANCE Outer membrane vesicles (OMVs) derived from Gram-negative bacteria attract increasing interest in the development of vaccines and therapeutic agents. We aim to construct a semisynthetic OMV platform for recombinant antigen presentation on OMVs derived from attenuated *Salmonella enterica* serovar Typhimurium cells displaying an adapted *Escherichia coli* autotransporter, Hbp, at the surface. Although this autotransporter accepts substantial modifications, its capacity with respect to the number, size, and structural complexity of the antigens genetically fused to the Hbp carrier is restricted. Here we describe the application of SpyCatcher/SpyTag protein ligation technology to enzymatically link antigens to Hbp present at high density in OMVs. Protein ligation was apparently unobstructed by the membrane environment and allowed a high surface density of coupled antigens, a property we have shown to be important for vaccine efficacy. The OMV coupling procedure appears versatile and robust, allowing fast production of experimental vaccines and therapeutic agents through a modular plug-and-display procedure.

KEYWORDS autodisplay, antigen display, outer membrane vesicle, vaccine, protein ligation, nanobody, SpyTag

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In Gram-negative bacteria, secretion and surface display of heterologous proteins are difficult to achieve due to the presence of a complex, multilayered cell envelope. Several secretion systems have evolved to overcome this barrier and deliver proteins in the extracellular environment. Of these, the type V secretion system, also known as the autotransporter (AT) pathway, appears best suited for piggyback transport of cargo proteins because it combines simplicity with a high transport capacity (1, 2). Based on this secretion system, we have developed an innovative vaccine delivery platform by which attenuated bacterial cells or derived outer membrane vesicles (OMVs) can be decorated with multiple heterologous antigens of choice for optimal stimulation of the immune system (3, 4).

As an AT carrier, we use the *Escherichia coli* hemoglobin protease (Hbp). Like other classical ATs, Hbp is organized in three domains: (i) the signal peptide at the N terminus that triggers targeting to and translocation across the inner membrane via the generic protein-conducting Sec-translocon, (ii) the secreted passenger domain that carries the actual effector function of the AT, and (iii) the β -domain at the C terminus that adopts a β -barrel conformation in the outer membrane (OM) and plays a crucial role in transfer of the passenger domain across the OM (Fig. 1A and D). The latter step is supported by the generic insertase for outer membrane proteins (OMPs), the Bam complex, in a concerted, poorly understood mechanism (5). At the cell surface, the passenger domain starts to fold, which provides a pulling force to energize translocation across the outer membrane (6). Finally, upon completion of folding of the passenger- β -domain tandem and release from the Bam complex, the passenger is cleaved from its β -domain through an autocatalytic mechanism that takes place in the barrel interior (7). The cleaved and released passenger domain is a long β -helical stem structure from which small loops and larger functional domains protrude (8). We have identified loops and domains that can be replaced by heterologous antigens without affecting expression and secretion (9). Furthermore, the autocatalytic cleavage site was mutated to create an Hbp carrier that is displayed at the cell surface rather than being released, providing permanent exposure of fused antigens at some distance from the cell surface.

As a host for the Hbp antigen display carrier, we have used an attenuated *Salmonella enterica* serovar Typhimurium strain that provokes strong mucosal and systemic responses of both cellular and humoral nature (3, 10, 11). Furthermore, as an alternative nonliving platform, we have explored OMVs derived from the same strain (4). OMVs are an attractive vaccine formulation because, though nonreplicating and safe to use, they have intrinsic immunostimulatory properties that are a facsimile of their live parental cells (12, 13). All Gram-negative bacteria produce OMVs that are shed from the outer membrane during growth. OMVs are spherical nanostructures (30 to 200 nm in diameter) composed of phospholipids, lipopolysaccharide (LPS), and proteins derived from the periplasm and outer membrane (14). To increase the production of OMVs, we have created a $\Delta tolIRA$ derivative of the attenuated *S. Typhimurium* strain and have shown that Hbp-antigen fusions are efficiently included and surface exposed in these OMVs (4). Importantly, *in vitro* and *in vivo* studies in mice have confirmed the immunogenicity and protective efficacy of OMV-based prototype vaccines decorated with antigens inserted in the Hbp carrier, providing strong proof-of-concept evidence for the utility of the platform (15, 16).

Despite being an efficient and versatile system for bacterial-based display of heterologous proteins, the capacity of Hbp to translocate complex and/or bulky fusion partners across the cell envelop is somewhat limited (3, 4, 15, 17). This problem can partly be solved by splitting up the antigen and dividing the resulting fragments over the various permissive integration sites in Hbp (3, 9, 15). However, this strategy may result in the loss of conformational epitopes. Since the density of antigens at the OMV surface has been shown to be important for protective efficacy, we have therefore investigated modifications that increase the display capacity of the Hbp system while maintaining a high expression level.

As a strategy to circumvent the membrane translocation steps and their inherent limitations, antigens could be conjugated to already surface-exposed structures. The

FIG 1 Schematic representations of the proteins used in this study. (A) Hbp fusions. Wild-type Hbp is synthesized with an N-terminal signal sequence (ss) that is cleaved off after translocation across the inner membrane. The C-terminal β -domain (black) integrates into the outer membrane, facilitating translocation of the passenger domain. After translocation, autocatalytic cleavage separates the passenger and the β -domain (after Asn1100). The passenger domain contains five subdomains (white, numbered 1 to 5) protruding from a β -helical stem structure (blue). The derived HbpD(Δ d1) display platform lacks subdomain 1 and the autocatalytic cleavage site (9). Ligation tags and catchers were integrated at the site of subdomain 1. (B) Catcher-fused model proteins. The SpyCatcher-SnoopCatcher fusion protein (SpC-SnC) was constructed by Veggiani et al. and contains a 34-amino-acid α -helical linker (19). GFPnanobody-SpyCatcher (GFPnb-SpC) contains an N-terminal PelB signal sequence (ss) for translocation into the periplasm and a hexahistidine tag (H6) for metal affinity purification. (C) SpyCatcher-fused antigens. The fusion proteins shown contain pneumococcal antigens PspA α and SP1690, SpyCatcher (SpC), and SnoopTag (SnT) for protein ligation, an HA tag for detection, and a hexahistidine tag (H6) for metal affinity purification. (D) Cartoon of Hbp-mediated Spy ligation to the surface of outer membrane vesicles. HbpD(Δ d1)-SpT (coloring and numbering as in panel A) is embedded in the membrane of an outer membrane vesicle. The SpyTag (red) has covalently bound to a chimera of the SpyCatcher (green) and a cargo protein (purple, model structure of SP1690) through the formation of an isopeptide bond between the SpyTag and the SpyCatcher. The protein structures were generated using PyMOL.

long stem structure of the Hbp display platform is in concept ideally suited to provide exposed ligation sites. To couple purified recombinant antigens to Hbp on OMVs, we explored the SpyCatcher (SpC)/SpyTag (SpT) technology (18). This protein ligation system is based on a domain of fibronectin binding protein FbaB of *Streptococcus pyogenes* in which an intramolecular isopeptide bond is formed between an adjacent lysine and aspartic acid residue through an autocatalytic mechanism (18). By splitting this domain into a 13-amino-acid (aa) peptide, termed SpyTag, and its remaining 138-aa fragment, termed SpyCatcher, a SpyCatcher/SpyTag pair was created for intermolecular coupling. Fused to different proteins, the SpyTag and SpyCatcher reconstitute the domain upon mixing and form a stable intermolecular covalent bond under a wide range of conditions (pH, temperature, and buffer). Following the same strategy, another protein ligation system has been developed based on the *Streptococcus pneumoniae* adhesin RrgA termed SnoopTag (SnT)/SnoopCatcher (SnC) (19). The SpyCatcher/SpyTag conjugation system was further adapted into a tripartite system by splitting up the SpyCatcher into the SpyLigase domain and the 10-aa peptide KTag (KT) (20). The SpyTag and the KTag each contain one of the cognate amino acid residues (Asp and Lys, respectively) that form the isopeptide bond, while the catalytic activity is included in the SpyLigase domain that can be added separately.

While the Spy ligation system works very efficiently for soluble proteins, reconstitution of the adhesin domain might be inhibited in the microenvironment of the bacterial outer membrane surface that displays a high content of long and bulky lipopolysaccharide molecules. Here we analyzed the potential of Spy- or SnoopCatcher and Spy- or SnoopTag protein ligation technology for the decoration of bacterial cells and OMVs with heterologous recombinant protein partners. The data presented show that a SpyTag genetically fused to the distal end of the Hbp display carrier is well accessible for Spy ligation and allows efficient directional loading of *Salmonella* OMVs and cells with pure, complex antigens, but also with nanobodies, enabling immune and tissue targeting. The OMV and display technology combined with protein ligation thus constitutes a robust plug-and-(dis)play system for tailored OMV vaccine design.

RESULTS

Spy ligation to Hbp on the surface of OMVs. To investigate the suitability of the SpyCatcher (SpC)/SpyTag (SpT) system for ligation of proteins to Hbp fixed on the surface of OMVs, we fused the SpyTag to the N terminus of the Hbp display construct HbpD(Δ d1) (Fig. 1A). In our experience, this position in Hbp is the most tolerant toward fusion of heterologous sequences (17). To test display in the context of OMVs, the fusion construct was expressed in the hypervesiculating *S. Typhimurium* Δ tolRA strain, and OMVs were collected by ultracentrifugation. As expected, the small tag (13 amino acids [aa] long) did not impede expression and surface display of Hbp (Fig. 2A, compare lanes 1 and 8, and Fig. 3A, lanes 7 to 9), providing a density of the SpyTag at the OMV surface that is similar to that of the major outer membrane protein OmpA. Importantly, the SpyTag is located at the tip of the β -helical stem structure of Hbp, providing considerable distance to the membrane surface and consequent accessibility to cognate coupling partners.

As proof of concept for Spy-mediated ligation, OMVs decorated with HbpD(Δ d1)-SpT were incubated with purified SpyCatcher-SnoopCatcher hybrid protein (SpC-SnC) (Fig. 1B) in incremental molar ratios, and subsequently the samples were analyzed by SDS-PAGE and total protein staining. An adduct with an electrophoretic mobility corresponding to that expected of the HbpD(Δ d1)-SpT-SpyCatcher-SnoopCatcher ligation product, 145.7 kDa, appeared at the expense of HbpD(Δ d1)-SpT (Fig. 2A, lanes 2 to 7). In a control reaction mixture containing OMVs displaying the carrier HbpD(Δ d1) without a SpyTag, this ligation product was not detected (Fig. 2A, lanes 8 and 9). The amounts of unligated and ligated Hbp protein were determined by densitometric scanning. Saturation of coupling, at a maximum of $\sim$ 85% of the Hbp molecules ligated, was reached at an approximate 4-fold molar excess of cargo protein relative to the Hbp carrier (Fig. 2B). While the reaction reached completion after approximately 16 h, the

FIG 2 Ligation of OMVs displaying SpyTag using increasing amounts of SpyCatcher-SnoopCatcher (SpC-SnC). (A) SDS-PAGE and Coomassie staining analysis of reaction mixtures of OMVs harboring HbpD(Δd1)-SpT and SpC-SnC incubated for 21 h at 4°C. Reactant proteins, the adduct, and the major outer membrane protein OmpA are indicated on the right-hand side of the panel. A proteolytic product of the adduct is indicated with an asterisk. The sizes of the molecular mass markers are indicated on the left side of the panel. (B) Quantification of ligation efficiency to HbpD(Δd1)-SpT. The intensities of the adduct and HbpD(Δd1)-SpT in the gel shown in panel A were determined by densitometry. The percentage of HbpD(Δd1)-SpT ligated with SpyCatcher-SnoopCatcher is plotted as a function of the molar ratio SpyCatcher-SnoopCatcher:HbpD(Δd1)-SpT. (C) SDS-PAGE and Coomassie staining of reaction mixtures containing a 4:1 molar ratio of SpyCatcher-SnoopCatcher to HbpD(Δd1)-SpT incubated for 1, 2, 4, 8, 16, or 24 h at 4°C. HbpD(Δd1)-SpT and the adduct are indicated on the right-hand side of the panel. A proteolytic product of the adduct is indicated with an asterisk. The sizes of the molecular mass markers are indicated on the left side of the panel. (D) Quantification of ligation efficiency to HbpD(Δd1)-SpT as in panel B. The percentage of HbpD(Δd1)-SpT ligated with SpyCatcher-SnoopCatcher is plotted as a function of time.

majority of the product was formed within the first hour of incubation (Fig. 2C and D). These results indicate that the SpyTag fused to displayed Hbp is well accessible for coupling at the surface of OMVs.

To investigate if the Spy and Snoop ligation systems are more generally compatible with the Hbp display platform, the SpyCatcher (SpC), KTag (KT), SnoopCatcher (SnC) and SnoopTag (SnT) were also individually introduced at the N terminus of the Hbp

FIG 3 Surface exposure and ligation analysis of HbpD(Δ 1) fusion proteins carrying Spy- or SnoopTag and Spy- or SnoopCatcher moieties. (A) The exposure of the HbpD(Δ 1) fusion proteins containing SpyCatcher (SpC), SnoopCatcher (SnC), SpyTag (SpT), SnoopTag (SnT), or KTag (KT) on the exterior surface of the OMVs was analyzed by proteinase K treatment. OMVs, in the absence or presence of 1% Triton X-100, were incubating with proteinase K. The samples were analyzed by SDS-PAGE and Coomassie staining. Successful exposure of HbpD(Δ 1) fusion proteins follows from their degradation upon treatment of OMVs with proteinase K. A proteinase K-sensitive intracellular loop of OmpA is not accessible under these conditions, unless the OMVs were dissolved with Triton X-100 first, indicating that OMVs remained intact throughout the procedure. Full-length HbpD(Δ 1) fusion proteins, OmpA, and proteinase K, as well as degradation products of the HbpD(Δ 1) fusion proteins (Δ 1) and of OmpA (Δ 2), are indicated on the right side of the panel. The sizes of the molecular mass markers are indicated on the left. (B) Ligation to OMVs displaying various Spy- or SnoopTag and Spy or SnoopCatcher moieties. Protein ligation of soluble model proteins to OMVs harboring HbpD(Δ 1) fusions containing SpyCatcher, SnoopCatcher, SpyTag, SnoopTag, or KTag was tested (lanes 1 to 6). The soluble model proteins used are indicated on the left side of the $\pm$ matrix. Reaction mixtures were incubated for 24 h at 4°C and analyzed by SDS-PAGE with Coomassie staining. Unreacted HbpD(Δ 1) fusion proteins are indicated with dots in the image. The ligation efficiencies [percentage of HbpD(Δ 1) or SpT/SnT-MBP converted] are shown below the lanes. The sizes of the molecular mass markers are indicated on the left side of the panel.

display construct HbpD(Δ 1) and expressed on OMVs. All fusion proteins were present in the OMV fraction in a density similar to that of the major OMPs (Fig. 3A). For all fusion proteins, protease accessibility experiments confirmed their localization at the cell surface, indicating that the Spy- or SnoopTags and Spy- or SnoopCatchers did not impede translocation of Hbp across the cell envelope (Fig. 3A). To further substantiate surface localization and suitability for ligation of cognate partner proteins, ligation tests were carried out. OMVs decorated with the Hbp fusion proteins were incubated with purified recombinant proteins that were used by Howarth and coworkers as tools to demonstrate bipartite and tripartite ligation: SpyCatcher-SnoopCatcher, SpyTag-maltose-binding protein (MBP), SnoopTag-MBP, SnoopTag-mEGFP-SpyTag, SUMO-KTag, and SpyLigase (18–20). Summarizing, in the context of OMVs, the highest efficiency of display of ligated product was obtained with the

HbpD(Δ d1)-SpT construct (Fig. 3B, lane 3). In comparing the display efficiencies of (bipartite) ligated adducts, the constructs rank HbpD(Δ d1)-SpT > HbpD(Δ d1)-SpC > HbpD(Δ d1)-SnT > HbpD(Δ d1)-SnC (Fig. 3B, lanes 1 to 4). Apparently, the 10.0-kDa SpyCatcher and the 12.6-kDa SnoopCatcher had no major impact on the display of the Hbp carrier and folded into their catalytic structure at the OMV surface, enabling binding to Spy- or Snoop-tagged partner proteins.

In tripartite ligation, the SpyCatcher domain is further split up into the so-called KTag harboring the lysine residue that is part of the isopeptide bond and the larger catalytic "SpyLigase" domain. The isolated SpyLigase is able to couple a KTag-tagged protein and a SpyTag-tagged protein in *trans*, expanding the potential applications of this technology (20). In contrast to the bipartite system, the tripartite system did not yield any appreciable ligation product in the OMV context (Fig. 3B, lanes 5 and 6). To verify the activity of the SpyLigase used, purified SUMO-KTag and SpT-MBP were shown to be coupled upon coincubation, although not very efficiently (7% ligation efficiency [data not shown]).

Spy ligation of antigens to Hbp. Due to inherent limitations in the secretion of Hbp across the outer membrane, translocation of large or structurally complex antigens genetically fused to Hbp generally results in relatively low-level surface exposure (3, 4, 15, 17). Encouraged by the efficient enzymatic coupling of cargo proteins to surface-exposed Hbp using Spy/Snoop technology, we now tested this approach for the decoration of OMVs with complex antigens. As proof of concept, we explored coupling of the large N-terminal α 1 α 2-domain (213 aa, 24.4 kDa, further referred to here as the PspA α' -domain) of the surface-exposed pneumococcal PspA antigen (from strain TIGR4) to OMVs predecorated with Hbp using Spy technology. Previously, efficient display required splitting up the PspA α' -domain and incorporating the two resulting fragments at different sites in Hbp (HbpD-PspA[α 1- α 2]) (15). Yet, the highest achievable concentration of HbpD-PspA[α 1- α 2] in OMVs was still only one-third of that of antigen-free HbpD. For coupling, we fused the SpyCatcher to the complete PspA α' -domain. For purposes described in the next section, we further included a hemagglutinin (HA) tag and a SnoopTag at the C terminus of the fusion construct (SpC-PspA α' -SnT) (Fig. 1C).

Incubation of *S. Typhimurium* OMVs decorated with HbpD(Δ d1)-SpT with a 10-fold molar excess of SpC-PspA α' -SnT resulted in efficient ($\sim$ 85%) ligation, as judged by SDS-PAGE analysis (Fig. 4, lane 2). For further proof of principle, we followed the same strategy using the conserved pneumococcal antigen SP1690 (21). SP1690 is a 47.3-kDa component of an ABC-type oligosaccharide transport system belonging to the type 2 periplasmic-binding fold superfamily (22). Initial attempts to display intact SP1690 as a fusion to Hbp were unsuccessful, necessitating the splitting of SP1690 into four fragments that were inserted into two Hbp carriers (two fragments per carrier). However, even upon splitting, only moderate amounts of SP1690 were displayed at the surface (data not shown). For Spy ligation, we created a fusion protein of SpyCatcher and full-length SP1690 (only omitting the N-terminal Cys residue that is most likely acylated in the native protein as predicted from its lipoprotein-like signal peptide) (23). A FLAG tag and a SnoopTag were included at the C terminus of the fusion construct (SpC-SP1690-SnT). Similar to SpC-PspA α' -SnT, efficient coupling ($\sim$ 85%) was observed upon incubation with OMVs decorated with HbpD(Δ d1)-SpT (Fig. 4, lane 3). A further increase in complexity of the fusion partner was achieved by fusing the complete PspA α' -domain and full-length SP1690 in one construct. The resultant $\sim$ 87-kDa bivalent antigen was coupled through Spy technology to Hbp-decorated OMVs at equally high efficiency (Fig. 4, lane 4). Besides the full-length adducts of HbpD(Δ d1)-SpT and the different antigen constructs, proteins of intermediate size were detected. These products are most likely formed by proteolytic events within the ligated antigen segment but constitute only a minor fraction of the ligated material. Together the data indicate that large complex antigens and even chimeras of complex antigens can be displayed at high density using the combined Hbp and Spy technologies.

Ligation of multiple antigens to Hbp using iterative Spy and Snoop coupling. Vaccine efficacy may be enhanced by incorporating multiple antigens into a single

FIG 4 Spy ligation of pneumococcal antigens to OMVs. OMVs harboring HbpD(Δ d1)-SpT were mixed with a 5- to 10-fold molar excess of SpC-PspA α' -SnT (α'), SpC-SP1690-SnT (1690), or SpC-PspA α' -SP1690-SnT (α' -1690). The reaction mixtures were incubated for 24 h at 4°C and analyzed by SDS-PAGE with Coomassie staining. Reactant proteins are indicated on the right-hand side of the panel. Adducts are indicated with arrowheads, and the proteolytic products of the adducts are indicated with asterisks. The sizes of the molecular mass markers are indicated on the left side of the figure.

vaccine. Multiple stages of infection can be addressed, and evasion of the immune system will be more difficult when multiple targets of the pathogen are targeted. Alternatively, a multivalent vaccine can protect against multiple pathogens or multiple serotypes of a particular pathogen. To create a multivalent vaccine, multiple different batches of OMVs decorated with singular antigens fused to the Hbp carrier can be combined in a prototype vaccine. However, the concentration of each antigen in the combined formulation will be reduced relative to the total OMV content, which harbors the adjuvant activity but is also reactogenic at high concentrations. As an alternative strategy, we have recently shown the display of three mycobacterial antigens inserted in a single Hbp molecule (3). This approach, however, requires a laborious trial-and-error procedure to establish the optimal use of insertion sites. “Posttranslocational” coupling of purified chimeric antigens through Spy technology can be considered yet another method for multivalent display as detailed in the previous section.

In concept, the combined use of the Spy and Snoop ligation systems also allows a more attractive iterative approach to couple multiple antigens to the Hbp platform while maintaining high antigen concentrations per OMV. First, we tested whether HbpD-PspA α' formed by the ligation of SpC-PspA α' -SnT to HbpD(Δ d1)-SpT could be further extended by adding SP1690 fused to SnoopCatcher. To allow subsequent coupling of purified SpC-PspA α' -SnT, the SP1690 fusion protein also contained a SpyTag at its C terminus. A similar iterative extension approach was shown by Veggiani et al. to efficiently polymerize affibodies into multimeric chains (19). Importantly, the process was started with an affibody fused to a SpyCatcher immobilized on a solid support. Subsequently the material was incubated with affibody containing both a SpyTag and a SnoopTag at the N terminus and C terminus, respectively. After the unreacted affibody was washed away, the material was incubated with a SpyCatcher-SnoopCatcher chimera to extend the chain. By iterating this process, chains of affibodies were formed. Since immobilizing and washing the OMVs is technically more challenging, we explored the principle of the strategy in one reaction mixture. Our

FIG 5 Adduct extension by alternating Spy ligation and Snoop ligation. OMVs harboring HbpD(Δ d1)-SpT were mixed with a 1.5-fold molar excess of SpC-PspA α' -SnT and incubated for 2 h at 4°C. Subsequently a similar amount of SnC-SP1690-SpT was added. After 2 h at 4°C again, the same amount of SpC-PspA α' -SnT was added, and after another 2 h at 4°C, the same amount of SnC-SP1690-SpT was added. Finally, the mixture was incubated for a further 15 h at 4°C to allow completion of the reaction. After centrifugation, the pellet fraction (ligated) was analyzed by SDS-PAGE with Coomassie staining (A, lane 4). (B) Immunoblotting analysis of OMV pellet fraction after ligation and reference OMVs as described under panel A. Monoclonal antibodies were used for immunodetection of the HA tag in SpC-PspA α' -SnT and of the FLAG tag in SnC-SP1690-SpT. The adducts are indicated with arrowheads: HbpD-P, HbpD(Δ d1)-SpT-SpC-PspA α' -SnT; HbpD-P-S, HbpD(Δ d1)-SpT-SpC-PspA α' -SnT-SnC-SP1690-SpT; HbpD-P-S-P, HbpD(Δ d1)-SpT-SpC-PspA α' -SnT-SnC-SP1690-SpT-SpC-PspA α' -SnT. The sizes of the molecular mass markers are indicated on the left side of the panels.

approach therefore has the disadvantage that SpC-PspA α' -SnT and SnC-SP1690-SpT may link to each other, but not to HbpD(Δ d1)-SpT. Moreover, polymers of SpC-PspA α' -SnT and SnC-SP1690-SpT might cyclize and form unreactive end products that are not linked to the Hbp platform. To limit undesirable product formation, OMVs containing HbpD(Δ d1)-SpT were incubated sequentially with SpC-PspA α' -SnT and SnC-SP1690-SpT in two rounds, followed by centrifugation and analysis of the OMVs by SDS-PAGE. A complex pattern of adducts was observed, which likely resulted from polymer formation combined with limited proteolysis (Fig. 5A, lane 4).

Importantly, three major adducts that probably correspond to single, double, and triple ligation events dominate. Omitting the Spy/Snoop ligation terms from the nomenclature for clarity, these probably represent HbpD-PspA α' with an expected molecular mass of 157 kDa, HbpD-PspA α' -SP1690 with an expected mass of 222 kDa, and HbpD-PspA α' -SP1690-PspA α' with an expected mass of 262 kDa, respectively. The identities of these three adducts were confirmed by immunodetection using anti-HA and anti-FLAG antibodies that recognize the HA and FLAG epitopes integrated in

SpC-PspA α' -SnT and SnC-SP1690-SpT, respectively (Fig. 5B). Quantification of HbpD(Δ d1)-SpT and the three major adducts (Fig. 5A, lane 4) indicated a total ligation efficiency of $\sim$ 85% and a ratio of $\sim$ 5:1:1 (157, 222, and 262 kDa, respectively) for the adducts. The data thus show that the principle of polymerization worked, although not very efficiently. Only a minor part of the Hbp molecules is coupled to two or more antigen molecules, and long polymeric structures could not be detected.

Spy ligation of nanobodies to Hbp. Decoration of bacteria or OMVs with lectins, adhesins, or affinity handles, such as antibodies or nanobodies, could be used as a tool for OMV targeting to certain tissues and immune cells, thereby increasing the efficacy of antigen delivery. Nanobodies are particularly attractive for this purpose because of their versatility and ease of recombinant production. They are much smaller (15 kDa) than regular antibodies since they consist only of a single variable domain fragment (V_{HH}), as found in heavy-chain-only antibodies that occur naturally in camelids and sharks. Previous attempts to incorporate nanobodies into Hbp by gene fusion have met with variable and generally low display efficiencies (data not shown).

To provide proof of concept for coupling of nanobodies to Hbp on OMVs through Spy technology, we produced a fusion construct in which SpyCatcher and a green fluorescent protein (GFP)-specific nanobody (GFPnb) (24) are connected through a flexible linker to enable independent folding. Sequential incubation of HbpD(Δ d1)-SpT-decorated bacteria with purified GFPnb-SpC and GFP should lead to covalent coupling of GFPnb-SpC and binding of GFP, provided that GFPnb is correctly folded in the context of the fusion construct. Consequently, the bacteria should get a green coat, which can be visualized by whole-cell immunofluorescence. *S. Typhimurium* cells expressing HbpD(Δ d1)-SpT were first incubated with GFPnb-SpC to allow coupling, which was confirmed by SDS-PAGE. As expected, an adduct of $\sim$ 140 kDa was detected, consistent with the combined mass of Hbp-SpT (117.1 kDa) and GFPnb-SpC (24.2 kDa) (Fig. 6A, lane 1). The cells were harvested and briefly incubated in phosphate-buffered saline (PBS) containing purified GFP. Whole-cell immunofluorescence revealed a clear circumferential staining, as expected for cells decorated with GFP (Fig. 6B). As a control for nonspecific interaction of GFP with the bacterial surface in this procedure, the same sequential labeling was carried out using a catalytically inactive SpyCatcher fused to GFPnb. The catalytic Glu₇₇ in SpyCatcher was substituted for by a Gln (SpC EQ), which has been shown to abolish covalent bond formation (18). Using purified GFPnb-SpC EQ, the $\sim$ 140-kDa adduct was not detected (Fig. 6A, lane 2), and surface labeling with GFP did not occur (Fig. 6B), confirming the specificity of the labeling procedure. Although GFP labeling is better suited for visualization using whole cells, the GFPnb-SpC coupling step was also shown to occur on the surface of OMVs decorated with Hbp-SpT, again depending on the catalytic integrity of the SpyCatcher part of the GFPnb-SpC hybrid (Fig. 6C).

DISCUSSION

OMVs attract increasing interest in the development of vaccines and therapeutic agents (13, 25). Compared to live attenuated vaccines, OMVs raise fewer safety concerns because of their nonreplicative nature. Yet, the intrinsic constituents of OMVs such as OMPs, LPS, and other pathogen-associated molecular patterns (PAMPs) activate innate as well as adaptive immune responses, leading to the induction of adaptive immunity. This makes the addition of adjuvant unnecessary, which is an important advantage for vaccine approval.

We aim to construct a semisynthetic OMV platform for recombinant antigen presentation in nanoparticles. The OMVs are derived from an attenuated auxotrophic (*aroA* mutant) *Salmonella Typhimurium* strain that has been engineered in three consecutive steps. First, to increase OMV production, we have genetically inactivated TolRA, which contributes to connecting protein structures between the inner and outer membranes (4). Second, we have adapted the *E. coli* AT Hbp into a multivalent antigen display carrier in which heterologous sequences can be grafted through genetic fusion (3). The fusion constructs are expressed in the hypervesiculating Δ *tolRA* strain, resulting in

FIG 6 Binding of GFP to bacterial cells upon ligation and display of a GFP nanobody. (A) *S. Typhimurium* cells expressing HbpD(Δd1)-SpT were incubated with GFPnb-SpC or with the unreactive GFPnb-SpC EQ. The cells were subsequently incubated in the presence of GFP and analyzed by SDS-PAGE and Coomassie staining (A). HbpD(Δd1)-SpT (H), the adduct (a), and a proteolytic product of the adduct (*) are indicated. The sizes of the molecular mass markers are indicated on the left side of the panel. (B) Fluorescence microscopy analysis of cells produced under panel A. Phase-contrast images (upper panels) and the corresponding fluorescence images (lower panels) are shown. The lower right panel, showing the fluorescence of the cells incubated with GFPnb-SpC EQ, is split into two parts. In the upper left half, the contrast is enhanced to visualize the weak fluorescence signal (autofluorescence of the cells), while the lower right half shows the absence of signal at the same settings as the left panel. (C) *S. Typhimurium* SL3261 ΔtolRA-derived OMVs harboring HbpD(Δd1)-SpT were incubated with GFPnb-SpC or with the unreactive GFPnb-SpC EQ and analyzed by SDS-PAGE followed by Coomassie staining. HbpD(Δd1)-SpT (H), the adduct (a; arrowhead), and a proteolytic product of the adduct (*) are indicated. The sizes of the molecular mass markers are indicated on the left.

high-density display of multiple antigens on the OMV surface for optimal exposure to the immune system (4). The immunogenicity and protective efficacy of the antigens presented in this way on OMVs have been confirmed in a mouse colonization model for *Streptococcus pneumoniae* (15). Third, to achieve a good balance between immunogenicity and reactogenicity, we genetically modified the hexa-acylated lipid A that has strong proinflammatory properties and may lead to systemic toxicity (16). The *msbB* gene, which encodes one of the lipid A acylases, was inactivated, resulting in a penta-acylated lipid A base of the LPS, thus reducing the Toll-like receptor 4 (TLR4)-dependent stimulation of the host immune system (26, 27). Importantly, immunogenicity and protective efficacy appeared unaffected, confirming the persistent adjuvanticity of the detoxified platform (16).

In this study, a fourth modification of the OMV-Hbp platform is described that addresses the main limitation of the current version: the restricted capacity with respect to the number, size, and structural complexity of the antigens fused to the Hbp carrier. Instead of using genetic fusion, antigens were linked enzymatically to Hbp present at high density at the surface of OMVs using Spy- and Snoop-coupling technology. We

have focused on SpyTag-tagged Hbp and cognate SpyCatcher-antigen fusions, but other options are certainly feasible (Fig. 3). The high ligation efficiency of around 85% suggests that the SpyTag that we fused to the N terminus of surface-displayed Hbp is readily available for SpyCatcher-antigen ligation and is apparently unobstructed by the membrane environment. This in turn allowed a high surface density of coupled antigens, which we have shown to be important for vaccine efficacy (15). Using Spy technology, Alves et al. showed the ligation of phosphotriesterase to bacterial outer membrane protein OmpA, but in that study the reaction took place at the intracellular side of the outer membrane and at a relatively low efficiency (28). In other studies, the Spy technology has been applied to decorate virus-like particles to high densities with antigens for vaccine purposes (29–32).

In principle, any antigen that can be purified as a SpyTag or SpyCatcher chimera can be coupled to OMVs using isopeptide bonding technology, including proteins that are modified (glycoproteins and lipoproteins), oligomeric proteins, and oligosaccharide structures. Multiple antigens can be coupled by simply using purified fusion constructs consisting of multiple antigens and an added Spy- or SnoopCatcher domain. Insertion of both a SpyTag and SnoopTag in the same Hbp carrier or on a second coexpressed carrier is expected to further increase the flexibility and valency of the antigen display.

Another strategy to ligate multiple different antigens to the Hbp carrier is to use recombinant antigens that are flanked by either a SpyTag and SnoopCatcher or a SnoopTag and SpyCatcher. Essentially, by iterative extension on Hbp-SpT, a string of compatible antigen modules can be stacked on the Hbp carrier, although we reached no more than three antigen ligation events in substantial amounts. Formation of the antigen string before coupling or using an alternating incubation regime (20) will likely improve the efficiency of this approach and produce repetitive antigen structures to boost the immune response (33).

Although we focus on OMV nanoparticles as vectors for antigen delivery, they may also be used as drug delivery vehicles. Both applications would benefit from directed targeting to certain tissues or immune cells. Coupling of adhesins or affinity molecules appears an attractive strategy to add this extra functionality to OMVs. Previously we have inserted a nanobody into Hbp to achieve display, but with limited success (data not shown), indicating that the nanobody is largely translocation incompetent. In contrast, the efficient coupling of Spy- or SnoopCatcher-nanobody chimeras to vesicles, shown in this study, will permit for instance targeted drug delivery in tumor tissue (34, 35). For OMV vaccine development, it will be interesting to include surface-exposed nanobodies that recognize receptors specific for certain dendritic cell types. In this way, specific T-cell responses can be induced toward the presented antigens (36).

In conclusion, the OMV coupling procedure appears versatile and robust, allowing fast production of experimental vaccines and therapeutic agents through a modular plug-and-display procedure. Importantly, the optimized platform can be combined with other initiatives that expand the OMV toolbox, such as glycoengineering to display surface glycans from pathogenic bacteria and modifying the endogenous LPS to tailor immune responses (37, 38).

MATERIALS AND METHODS

Strains. *E. coli* BL21(DE3), *S. Typhimurium* SL3261 (10), and the isogenic SL3261 Δ tolRA mutant strain (4) were grown in lysogeny broth (LB; 10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl).

S. Typhimurium SL3261 Δ tolRA Δ msbB (16) was grown in TYMC (10 g/liter tryptone, 5 g/liter yeast extract, 2 mM MgSO₄, 2 mM CaCl₂). The growth medium was supplemented with 0.2% glucose. Where appropriate, antibiotics were added to the following concentrations: ampicillin, 100 μ g/ml; kanamycin, 50 μ g/ml; and chloramphenicol 30, μ g/ml. Unless stated otherwise, cultures were incubated at 37°C with shaking.

Expression plasmids. pET28a SpyCatcher-SnoopCatcher, pET28a-SpyTagMBP, pET28a SnoopTag-MBP, pET28a SnoopTag-mEGFP-SpyTag, pET28a SUMO-KTag, and pDEST14 SpyLigase were obtained from Addgene. The DNA primers used are listed in Table 1.

To construct pHbpD(Δ d1)-SpC, a SpyCatcher-encoding fragment was amplified by PCR using pET28a-SpyCatcher-SnoopCatcher as a template and the primers SpyCat fw and SpyCat rv. Using the Clontech In-Fusion method, the resulting fragment was cloned into SacI/BamHI-digested pHbpD(Δ d1)-Ag85B, a derivative of pHbpD(Δ d1)-ESAT6 carrying the mycobacterial Ag85B gene between the SacI/BamHI

TABLE 1 DNA primers used in this study

Primer name	5'→3' sequence ^a
ACYC Duet UP1	GGATCTCGACGCTCTCCCT
GFPnb rv	CGGTACC ACCGCTGCCTTTGCTGCTAACGGTAACC
SpyC fw	GCAGCGGT GGTACC GCGATAGTGCTACCCATATTAATTCTC
SpyC rv	GCGGCCGC AAGCTTT TACTAATGATGGTGATGATGACCGCTGCCAATATGAGCGTCACC
SpyC EQ fw	GAAATATACATTTGTCCAAACCGCAGC
SpyC EQ rv	GCTGCGGTTTgACAAATGTATATTTTC
SpT-H6 us	CGGCGCCACATCGTGATGGTGGACGCTACAAGCCGACGAAGGGTAGTGGTGAAAGTGGTCATCATCATCACCATCATTAGTAA
SpT-H6 ls	AGCTTTTACTAATGATGGTGATGATGATGACCACTTTACCACTACCCCTTCGTCGGCTTGAGGCGTCCACCATCAGATGTGGGCGCGGTAC
SnT-H6 us	CGGCAAACTGGGCGATATTGAATTTATTAAGTGAACAAAGGTAGTGGTGAAAGTGGTCATCATCATCACCATCATTAGTAA
SnT-H6 ls	AGCTTTTACTAATGATGGTGATGATGATGACCACTTTACCACTACCCCTTGTTCACCTTAATAAAATCAATATCGCCAGTTTGCCGGTAC
NcoI SpyC fw	ATATAT CCATGGG CGATAGTGCTACCCATATTAATTCTC
EcoRI HA SpyC rv	ATATAT GAATT CGCCGACCCCGCATAGTCAGGAACATCGTATGGGTATCCCGAACCAATATGAGCGTCACCTTAG
Sall SpT fw	ATATAT GTCGAC GCGCAGCGGTGGTACC
T7 Terminator	GCTAGTTATTGCTCAGCGG
NcoI SnC fw	ATATAT CCATGGG CAAGCCGCTGCGTGG
EcoRI FLAG SnC rv	ATATAT GAATT CCCCGCTACCGCTTTATCGTCATCATCTTATAGTCACCGCCGCTACCGCCTTTCGGCGGTATCGG
Sall SpT fw	ATATAT GTCGAC GCGCAGCGGTGGTACC
EcoRI αTIGR4 fw	ATATAT GAATT CGAAGAAAGTCCGCGAGTTG
Sall αTIGR4 rv	ATATAT GTCGAC GGTGCCATCATCCGG
EcoRI SP1690 fw	ATATAT GAATT CTCAGGAAAAAAGAAGTACAACTAGTAC
Sall SP1690 rv	ATATAT GTCGAC CTGAACAGCCTCAAATAAATCATTTAATTG
SpyCat fw	GGAAGTCTTGGCGGGAGCTCCGATAGTGCTACCCATATTAATTCTC
SpyCat rv	TACCGCTGCCGGATCCAATATGAGCGTCACCTTTAGTTG
SnoopCat fw	GGAAGTCTTGGCGGGAGCTCCAAGCCGCTGCGTGGTGCC
SnoopCat rv	TACCGCTGCCGGATCCTTTCGGCGGTATCGGTTTATTG
pEH_Xba_InFu fw	TTGCTAACTTTCTAGATTACAAAAC
Hbp-SpyTag rv	TACCGCTGCCGGATCCCTTCGTCGGCTTGATAGGCGTCCACCATCAGATGTGGGCGGAGCTCCCCGCAAGACTTC
Hbp-KTag rv	TACCGCTGCCGGATCCATCAGCTTTTGAGAAATTAATATGGGTAGCGGAGCTCCCCGCAAGACTTC
SnoopTag S/B fw	CCAAACTGGCGATATTGAATTTATTAAGTGAACAAAG
SnoopTag S/B rv	GATCCTTTGTTCACCTTAATAAATCAATATCGCCAGTTTGAGCT

^aOverlapping regions for overlap PCR are underlined, restriction enzyme recognition sites used for cloning are in bold, overhangs used for In-Fusion cloning are in italic, and mutagenic nucleotides are in lowercase.

restriction sites (9). This yielded pHbpD(Δd1)-SpC. The same strategy was followed for construction of pEH3-HbpD(Δd1)-SnC, but now the primers used were SnoopCat fw and SnoopCat rv.

For construction of pHbpD(Δd1)-SpT, a PCR fragment was generated encoding a fusion product between an N-terminal segment of Hbp and a downstream SpyTag. Plasmid pEH3-Hbp was the template, and the primers used were pEH_Xba_InFu fw and Hbp-SpyTag rv (17). The product was cloned into the XbaI/BamHI sites of pHbpD(Δd1)-Ag85B using the In-Fusion methodology to yield pHbpD(Δd1)-SpT. The same strategy was used to create pHbpD(Δd1)-KT but now using the primer pair pEH_Xba_InFu fw and Hbp-KTag rv. To construct pHbpD(Δd1)-SnT, a SnoopTag-encoding fragment was generated by annealing the long oligonucleotides SnoopTag S/B fw and SnoopTag S/B rv. The resulting product was ligated into the SacI/BamH sites of pHbpD(Δd1)-Ag85B, yielding pHbpD(Δd1)-SnT.

pET22b GFPnb was a kind gift from Hansjörg Götzke, Center for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden.

To construct pET22b GFPnb-SpC, the gene encoding a GFP-nanobody (GFPnb) was PCR amplified from pET22b GFPnb using primers ACYC Duet UP1 and GFPnb rv. The SpyCatcher-encoding sequence was PCR amplified from pET28a SpyCatcher-SnoopCatcher using primers SpyC fw and SpyC rv, introducing a carboxy-terminal His₆-tag. After overlap PCR, the GFPnb-SpC sequence was cloned with XbaI-HindIII into pET22b (Novagen), yielding pET22b GFPnb-SpC.

To construct pET22b GFPnb-SpC EQ, the E77Q codon mutation was introduced by PCR amplification from pET28a SpyCatcher-SnoopCatcher using either primers SpyC fw and SpyC EQ rv or SpyC EQ fw and SpyC rv followed by overlap PCR using both PCR products. The final product was cloned with KpnI-HindIII into pET22b GFPnb-SpC, replacing SpC, yielding pET22b GFPnb-SpC EQ.

To construct pET22b GFPnb-SpT and pET22b GFPnb-SnT, oligonucleotides SpT-H6 us and SpT-H6 ls or SnT-H6 us and SnT-H6 ls were mixed at a 0.25 mM concentration in 100 mM potassium acetate plus 30 mM HEPES (pH 7.5), heated to 100°C, and allowed to gradually cool down to ambient temperature. The annealed oligonucleotides were cloned into KpnI-HindIII-opened pET22b GFPnb-SpC, yielding pET22b GFPnb-SpT and pET22b GFPnb-SnT, respectively.

To construct pET28 SpC-PspAα'-SnT, pET28 SnC-PspAα'-SpT, pET28 SpC-SP1690-SnT, and pET28 SnC-SP1690-SpT, the SpyCatcher-encoding sequence was PCR amplified from pET28a SpyCatcher-SnoopCatcher using primers NcoI SpyC fw and EcoRI HA SpyC rv, introducing an HA tag. The product was cloned with NcoI-EcoRI into pET28a, yielding pET28 SpC-HA. Next, a SnoopTag-His₆-encoding sequence was PCR amplified from pET22b GFPnb-SnT using primers Sall SpT fw and T7 Terminator. The product was cloned with Sall-XhoI into pET28 SpC-HA, yielding pET28 SpC-HA-SnT-His₆. The SnoopCatcher-encoding sequence was PCR amplified from pET28a SpyCatcher-SnoopCatcher using primers NcoI SnC fw and EcoRI FLAG SnC rv, introducing a FLAG tag. The product was cloned with NcoI-EcoRI into pET28a, yielding pET28 SnC-FLAG. Next, a SnoopTag-His₆-encoding sequence and a SpyTag-His₆-encoding

TABLE 2 Overview of the plasmids used in this study

Plasmid name	Protein expressed	His ₆ tag ^a	Source or reference
pET28a SpyCatcher-SnoopCatcher	SpyCatcher-SnoopCatcher fusion linked via an L9 α -helix linker	N	Addgene 72324
pET28a-SpyTagMBP	SpyTag-MBP	N	Addgene 35050
pET28a SnoopTag-MBP	SnoopTag-MBP	N	Addgene 72323
pET28a SnoopTag-mEGFP-SpyTag	SnoopTag-mEGFP-SpyTag	N	Addgene 72325
pET28a SUMO-KTag	SUMO-KTag	N	Addgene 51723
pDEST14 SpyLigase	SpyLigase	N	Addgene 51722
pHbpD(Δ d1)-SpT	Hbp display platform with SpyTag at site of domain 1 (amino terminus)		This work
pHbpD(Δ d1)-SnT	Hbp display platform with SnoopTag at site of domain 1		This work
pHbpD(Δ d1)-SpC	Hbp display platform with SpyCatcher at site of domain 1		This work
pHbpD(Δ d1)-SnC	Hbp display platform with SnoopCatcher at site of domain 1		This work
pHbpD(Δ d1)-KT	Hbp display platform with KTag at site of domain 1		This work
pET22b GFPnb	PelBss-GFPnb ^b	C	24
pET22b GFPnb-SpC	PelBss-GFPnb-SpyCatcher fusion	C	This work
pET22b GFPnb-SpC EQ	PelBss-GFPnb fused to SpyCatcher E77Q mutant ^c	C	This work
pET22b GFPnb-SpT	PelBss-GFPnb-SpyTag fusion	C	This work
pET22b GFPnb-SnT	PelBss-GFPnb-SnoopTag fusion	C	This work
pET28 SpC-PspA α' -SnT	SpyCatcher-HA-PspA ₃₂₋₂₄₄ -SnoopTag	C	This work
pET28 SnC-PspA α' -SpT	SnoopCatcher-FLAG-PspA ₃₂₋₂₄₄ -SpyTag	C	This work
pET28 SpC-SP1690-SnT	SpyCatcher-HA-SP1690-SnoopTag	C	This work
pET28 SnC-SP1690-SpT	SnoopCatcher-FLAG-SP1690-SpyTag	C	This work
pET20b GFP-His ₆	GFP-His ₆	C	Kind gift from J. W. de Gier

^aN, His₆ tag on the amino terminus; C, His₆ tag on the carboxy terminus.

^bPelBss-GFPnb, PelB signal sequence-GFP-binding nanobody.

^cE77 is a key residue for isopeptide bond formation.

sequence were PCR amplified from pET22b GFPnb-SnT and pET22b GFPnb-SpT, respectively, using primers Sall SpT fw and T7 Terminator. The products were cloned with Sall-XhoI into pET28 SpC-HA and pET28 SnC-FLAG, yielding pET28 SpC-HA-SnT-His₆ and pET28 SnC-FLAG-SpT-His₆, respectively. Finally, DNAs encoding amino acids 32 to 244 of PspA or 24 to 445 of SP1690 (both *Streptococcus pneumoniae* serotype 4 [TIGR4] sequences) were PCR amplified using primers EcoRI α TIGR4 fw and Sall α TIGR4 rv or EcoRI SP1690 fw and Sall SP1690 rv, respectively. The products were cloned with EcoRI-Sall into pET28 SpC-HA-SnT-His₆ and pET28 SnC-FLAG-SpT-His₆, yielding pET28 SpC-alpha-SnT, pET28 SnC-alpha-SpT, pET28 SpC-SP1690-SnT, and pET28 SnC-SP1690-SpT.

Table 2 gives an overview of the expression plasmids used in this study.

Protein purification. (i) Cytoplasmic proteins. *E. coli* BL21(DE3) cells harboring a pET28 or pDEST14 expression plasmid were grown in LB containing 0.2% glucose and kanamycin to an optical density at 600 nm (OD₆₀₀) of 0.4 to 0.5. Protein expression was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and the cells were incubated for a further 2 h. Subsequently the cells were washed with PBS (pH 7.4) and stored at -20°C . The cells were resuspended in buffer A (50 mM NaPO₄, 300 mM NaCl [pH 7.4]), and phenylmethylsulfonyl fluoride (PMSF) was added to a concentration of 125 μM . The cells were disrupted by two passages through a One Shot cell disruptor (Constant Systems, Ltd.) at 1.2×10^8 Pa. Cell debris and membranes were removed by centrifugation at $10,000 \times g$ and $293,000 \times g$, respectively, at 4°C . His₆-tagged proteins were isolated from the cleared lysate using Talon Superflow medium (GE Healthcare Life Sciences) according to the manufacturer's instructions. Eluates were dialyzed overnight at 4°C against 500 to 1,000 volumes of PBS (pH 7.4). After dialysis, glycerol was added to 10%, and aliquots were stored at -80°C .

E. coli BL21(DE3) cells harboring a pET20b GFP-His₆ expression plasmid were grown at 30°C in LB containing 0.2% glucose and ampicillin to an OD₆₀₀ of 0.2 to 0.3. Protein expression was induced by the addition of IPTG to a final concentration of 0.4 mM, and the cells were incubated for a further 4 h. GFP-His₆ was purified from the cells as described above.

(ii) Periplasmic proteins. A procedure based on that described by Pardon et al. in *Nature Protocols* was used (39). *E. coli* BL21(DE3) cells harboring a pET22b GFPnb-SpC or pET22b GFPnb-SpC EQ expression plasmid were grown in LB containing 0.2% glucose and ampicillin to an OD₆₀₀ of 0.8 to 0.9. Both fusion proteins were equipped with a cleavable PelB signal sequence for translocation into the periplasmic space. Isolation of the GFPnb-SpC fusions from the oxidizing periplasmic environment allowed formation of an intramolecular disulfide bond ensuring nanobody functionality. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM, and the cells were incubated for a further 20 h at 12°C with shaking. The cells were harvested by centrifugation and stored at -20°C . The cells were resuspended in PBS (pH 7.4) and incubated for 30 min at 21°C in a Thermomixer (Eppendorf) at 1,400 rpm. Next, the cells were removed by centrifugation ($10,000 \times g$) and the His₆-tagged proteins were isolated as described above in the section "Protein purification. (i) Cytoplasmic proteins."

OMV isolation. *S. Typhimurium* SL3261 Δ tolRA cells harboring the pHbpD(Δ d1)-SpyTag expression plasmid were grown in LB containing glucose and kanamycin to an OD₆₀₀ of 0.2 to 0.3. Protein expression was induced by the addition of IPTG to a final concentration of 0.1 mM, and the cells were incubated for a further 2 h. Cells were removed by two successive centrifugation steps at $5,000 \times g$. OMVs were

isolated from the second supernatant by centrifugation at $235,000 \times g$, resuspended in PBS (pH 7.4) containing 15% glycerol, and stored at -80°C .

Alternatively, in view of future therapeutic purposes, OMVs were isolated from *S. Typhimurium* SL3261 $\Delta tolRA \Delta msbB$ cells harboring the pHbpD(Δ d1)-SpyTag expression plasmid. These cells were grown at 30°C in TYMC medium containing glucose and kanamycin. Fresh medium containing 1 mM IPTG was inoculated to an OD_{600} of 0.02 and incubated at 30°C with shaking for 17 h. Cells were removed by two successive centrifugation steps at $5,000 \times g$. The supernatant was passed through 0.45- μm -pore-size filters (Millipore) and centrifuged at $235,000 \times g$ for 75 min to sediment the OMVs. The vesicles were resuspended in PBS (pH 7.4) containing 15% glycerol.

Spy ligation with OMVs containing Hbp(Δ d1)-SpT and increasing amounts of SpC-SnC. To OMVs (isolated from *S. Typhimurium* SL3261 $\Delta tolRA$ cells) containing approximately 62 pmol of Hbp(Δ d1)-SpT, purified SpC-SnC was added in the ratios indicated in Fig. 2A. The mixtures were supplemented with PBS (pH 7.4) to reach a final volume of 20 μl . After 21 h of incubation at 4°C , the reaction mixtures were analyzed by SDS-PAGE with Coomassie staining.

Proteinase K treatment of OMVs containing HbpD(Δ d1)-SpyCatcher (SpC), -SnoopCatcher (SnC), -SpyTag (SpT), -SnoopTag (SnT), or -KTag (KT) fusion proteins. OMVs were diluted in 50 mM Tris-Cl (pH 7.5)–1 mM CaCl_2 to obtain a concentration of approximately 2 pmol of HbpD(Δ d1) fusion protein per μl . The suspension was split into three aliquots. To one aliquot, Triton X-100 was added to a concentration of 1% to dissolve the OMVs. After incubation of all aliquots on ice for 15 min, proteinase K was added (0.1-mg/ml final concentration) to the dissolved membranes and to one of the other two aliquots. After 30 min of incubation at 37°C , PMSF was added to 0.2 mM. The reaction was stopped by the addition of PMSF to 0.2 mM and incubation on ice for 10 min. The samples were analyzed by SDS-PAGE with Coomassie staining.

Protein ligation with Hbp(Δ d1)-SpC, -SnC, -SpT, -SnT, or -KT in OMVs. To OMVs (isolated from *S. Typhimurium* SL3261 $\Delta tolRA$ cells) containing approximately 200 pmol of Hbp(Δ d1)-SpC, -SnC, -SpT, -SnT, or -KT, a 2-fold molar excess of purified SnT-mEGFP-SpT, SpC-SnC, or SUMO-KT was added. (For the specific combinations, see Fig. 3B.) Where appropriate, 800 pmol of purified SpyLigase was added and the mixtures were supplemented with PBS (pH 7.4) to reach a final volume of 50 μl . After 24 h of incubation at 4°C , the reaction mixtures were analyzed by SDS-PAGE with Coomassie staining.

Spy ligation of antigens to Hbp in OMVs. To 500 μl of OMVs (isolated from *S. Typhimurium* SL3261 $\Delta tolRA \Delta msbB$ cells) containing HbpD(Δ d1)-SpyTag, obtained from a 1,000- OD_{600} -unit equivalent ($\text{OD}_{600} \times \text{ml}$) of cell culture, 900 μl of purified SpC-PspA α' -SnT (140 μM), SpC-SP1690-SnT (176 μM), or SpC-PspA α' -SP1690-SnT (72 μM) was added, and the mixture was incubated for 24 h at 4°C . The reaction mixtures were analyzed by SDS-PAGE with Coomassie staining and with immunodetection after Western blotting. For immunodetection, antipolyhistidine antibody (H1029; Sigma) was used.

Adduct extension by alternating Spy ligation and Snoop ligation. To 100 μl of OMVs (isolated from *S. Typhimurium* SL3261 $\Delta tolRA$ cells) containing HbpD(Δ d1)-SpyTag, obtained from a 100- OD_{600} -unit equivalent ($\text{OD}_{600} \times \text{ml}$) of cell culture, 80 μl of purified SpC-PspA α' -SnT (86 μM) was added. After 2 h of incubation at 4°C , 120 μl of purified SnC-SP1690-SpT (57 μM) was added. After 2 h of incubation at 4°C , another 80 μl of purified SpC-PspA α' -SnT was added. After a further 2 h of incubation at 4°C , another 120 μl of purified SnC-SP1690-SpT was added, and the mixture was incubated for 15 h at 4°C . The OMVs were isolated by centrifugation at $293,000 \times g$ for 45 min at 4°C . The OMVs were resuspended in PBS (pH 7.4) containing 15% glycerol and analyzed by SDS-PAGE with Coomassie staining and with immunodetection after Western blotting. For immunodetection, monoclonal anti-FLAG M2 antibody (F3165; Sigma), HA tag monoclonal antibody (2-2.2.14) (ThermoFisher Scientific), and antipolyhistidine antibody (H1029; Sigma) were used.

Spy ligation of GFPnb-SpC to Hbp in cells. *S. Typhimurium* SL3261 cells harboring pHbpD(Δ d1)-SpyTag were grown in LB containing 0.4% glucose and chloramphenicol to an OD_{600} of 0.3 to 0.4. Protein expression was induced by the addition of IPTG to a final concentration of 1 mM, and the cells were incubated for a further 2 h. Subsequently the cells were harvested, resuspended to an OD_{600} of 10 in PBS (pH 7.4) containing 3 μM GFPnb-SpC or GFPnb-SpC EQ, and incubated for 60 min at 25°C in a Thermomixer (Eppendorf) at 1,000 rpm. After incubation with the nanobody-SpyCatcher protein, the cells were resuspended to an OD_{600} of 10 in PBS (pH 7.4) containing 3 μM GFP and incubated at 4°C for 5 min. Finally, the cells were resuspended to an OD_{600} of 10 in PBS (pH 7.4) and analyzed by SDS-PAGE with Coomassie staining and microscopy.

Spy ligation of GFPnb-SpC to Hbp in OMVs. One microliter of OMVs containing HbpD(Δ d1)-SpyTag, obtained from a 1- OD_{600} -unit equivalent ($\text{OD}_{600} \times \text{ml}$) of cell culture, was diluted in 100 μl of PBS (pH 7.4) containing 3 μM GFPnb-SpC or GFPnb-SpC EQ. The mixtures were incubated for 60 min at 25°C in a Thermomixer (Eppendorf) at 1,000 rpm and analyzed by SDS-PAGE with Coomassie staining and with immunodetection after Western blotting. For immunodetection, antipolyhistidine antibody (H1029; Sigma) was used.

Quantitation of ligation efficiency. After SDS-PAGE of proteins and staining, Coomassie brilliant blue G250 gels were scanned on a GS-800 calibrated densitometer (Bio-Rad). The intensities of protein bands were determined using ImageJ (<http://imagej.nih.gov/ij/>). After correction for the difference in molecular masses, the fractions of Hbp (unmodified and ligated) were calculated.

Microscopy. Cells were photographed using an F-View II charge-coupled device (CCD) camera (Olympus) mounted on a BH2 microscope with an RFCA fluorescence attachment using a DAP0100UV PL 1.30 oil 160/0.17 objective and a GFP fluorescence filter cube (BP495/DM505) (Olympus). Image files were processed using ImageJ (<http://imagej.nih.gov/ij/>).

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