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Outer Membrane Vesicle—Host Cell Interactions

Jessica D Cecil^{#1}, Natalie Sirisaengtaksin^{#2}, Neil M O'Brien-Simpson^{1,*}, and Anne Marie Krachler^{2,*}

¹Oral Health Cooperative Research Centre, Melbourne Dental School, Bio21 Institute, The University of Melbourne, Melbourne, Victoria, Australia.

²Department of Microbiology and Molecular Genetics, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, Texas, USA.

[#] These authors contributed equally to this work.

Abstract

Outer membrane vesicles (OMVs) are nanosized proteoliposomes derived from the outer membrane of Gram-negative bacteria. They are ubiquitously produced both in culture and during infection, and are now recognized to play crucial roles during host-microbe interactions. OMVs can transport a broad range of chemically diverse cargoes, including lipids and lipopolysaccharides, membrane embedded and associated proteins and small molecules, peptidoglycan and nucleic acids. Particularly virulence factors such as adhesins and toxins are often enriched in OMVs. Here, we discuss a variety of ways in which OMVs facilitate host-microbe interactions, including their contributions to biofilm formation, nutrient scavenging, and modulation of host cell function. We particularly examine recent findings regarding outer membrane vesicle – host cell interactions in the oral cavity and the gastrointestinal tract.

Introduction to outer membrane vesicles

Outer membrane vesicles (OMVs) are nanosized, spherical proteoliposomes. They are secreted via vesiculation of the outer membrane by Gram-negative bacteria as part of the normal growth process (1). OMVs play diverse roles in intracellular communication, microbial virulence, and in modulating the host immune response (2).

The surface of OMVs is composed of a phospholipid bilayer with an outer layer of lipopolysaccharide, outer membrane proteins, and receptors. Internally, OMVs possess a thin layer of peptidoglycan and contain periplasmic proteins as well as nucleic acids (2-6), (Figure 1). Specific components may selectively be enriched or depleted from OMVs, suggesting that vesiculation is a deliberate and regulated process (6, 7). Many theories exist on the mechanism of vesiculation, including the accumulation of envelope components, increased membrane curvature, or reduction in lipoprotein to peptidoglycan crosslinks (2, 8-11). In all cases, vesiculation requires the outer membrane to separate from the underlying peptidoglycan layer and outwards budding until a vesicle can detach from the bacterial

* Address correspondence to: Anne Marie Krachler, anne.marie.krachler@uth.tmc.edu, +1-713-500-5456 and Neil M O'Brien-Simpson, neil.obs@unimelb.edu.au, +61-3-9341-1550.

surface. The exception is the recently described mechanism of vesicle formation by “explosive” cell lysis, which is initiated by a prophage endolysin (12).

Variations in temperature, growth medium, growth phase, and many other factors can quantitatively and qualitatively influence vesiculation and OMV composition. For example, OMVs secreted by biofilms, as opposed to planktonic bacteria, are smaller, more gelatinous, and produced in larger quantities (13). Vesiculation in *Helicobacter pylori* biofilms is enhanced by the addition of serum to the growth medium (14). OMVs produced during stationary growth have different physiochemical properties than those produced during exponential phase, including differences in protein and lipid composition, a higher buoyant density, and higher negative charge (15, 16). Vesiculation also increases in response to nutrient restriction, exposure to chemical stressors, and during infection (10, 17-20). This implies that vesiculation is an envelope stress response promoting bacterial survival (21-23). Mutations leading to increased vesiculation enhance the pathogenic potential of bacteria, despite the associated increase in metabolic burden (24). Stress-inducing conditions are often used to stimulate vesiculation, although the composition of such preparations is altered by the stress (25). Alterations in amounts of lipoprotein, LPS, and other pathogen-associated molecular patterns (PAMPs) contained in such OMVs likely trigger different immunological responses, and this should be considered when using them to study host-microbe interactions.

The sensitivity of OMVs to altered growth conditions as well as the inherent variability between OMVs of different species and strains demands precise and standardised methods of growth, and OMV isolation. Additionally, biochemical and immunological characterization of OMVs is greatly complicated by their nanosize, which precludes many established methods of quantification and analysis. These obstacles complicate OMV research and make comparisons and conclusions regarding OMV-host cell interactions difficult. We and others have reported methods to separate immunogenic cell debris from OMVs, and combined this with enumeration of the OMVs via flow cytometry and nanoparticle tracking techniques to allow quantitative comparison of OMV-host cell interactions (26-28).

Techniques used to characterize and visualize OMV-host cell interactions were recently reviewed elsewhere (29). Here, we discuss the role of OMVs in microbe-host interactions, with particular emphasis on two areas that have seen major recent advances: OMVs in microbe host-interactions in the oral cavity, and in the gastrointestinal tract.

OMV-host cell interactions in the oral cavity

Chronic periodontitis is an inflammatory, polymicrobial disease promoting the progressive destruction of bone and ligament tissue supporting the teeth (30). Destruction of these support structures lead to tooth mobility and loss (30, 31). Chronic periodontitis is associated with an increased risk of cardiovascular disease, adverse pregnancy outcomes, respiratory infections, and rheumatoid arthritis (32, 33).

The subgingival plaque is home to multispecies biofilms and its development is strongly associated with the onset of chronic periodontitis. These biofilms are protected within the periodontal pocket, the space around a tooth left behind by degraded bone and tissue. Although more than 700 bacterial species make up this structured biofilm (34), only a handful of them are associated with disease progression (35). Increased concentrations of the Gram-negative, anaerobic, proteolytic bacteria *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* are strongly associated with symptoms of chronic periodontitis (36-38), and all three species secrete OMVs (Figure 1).

Role of OMVs in plaque formation

P. gingivalis is the most widely studied bacterial species in the periodontal disease field and a major contributor to chronic periodontitis. Implantation of *P. gingivalis* was sufficient to induce periodontitis in non-human primates, supporting its role as a keystone pathogen in the disease (39). Its success as an oral pathogen is largely attributed to its arsenal of pathogenicity factors, many of which are secreted from the cell in OMVs (40, 41). The most prominent of these are gingipains, secreted cysteine proteases (42). *P. gingivalis* OMVs are enriched in gingipains, compared to the bacterial surface (41, 43). Gingipains promote *P. gingivalis* cell spread throughout periodontal tissue, by promoting the destruction of supportive bone and tissue within the oral cavity, and host cell invasion (44-46).

P. gingivalis-derived OMVs exhibit a functional flexibility that allows for the elimination of competitors and promotion of bacteria advantageous to *P. gingivalis* (47). They are also capable of inhibiting and dispersing competing biofilms, such as those composed of *Streptococcus gordonii*, in a gingipain-dependent manner (47). *P. gingivalis* OMVs have a strong tendency to form aggregates, both between themselves and other microbes, which facilitates the co-aggregation of non-aggregating species, such as *T. denticola*, *Eubacterium saburreum* and *Capnocytophaga ochracea* (4, 9). *T. forsythia* also benefits from the secretion of gingipain-containing OMVs, which enhance the attachment of whole cell *T. forsythia* to epithelial cells (48). These studies suggest that *P. gingivalis* OMVs influence the bacterial composition of the periodontal plaque. *T. forsythia* secrete OMVs that may also encourage and strengthen subgingival biofilms. They contain both the sialidase SiaHI and the β -N-acetylglucosaminidase HexA, which are thought to be involved in biofilm formation (7, 49, 50).

OMV interactions with gingival tissues

Nanoparticles of 10-100 nm diameter penetrate the extracellular matrix protecting host cells (51). Therefore, it has been proposed that periodontal OMVs act as a novel secretion system that delivers virulence factors deep into host tissues, eliminating the need for direct bacterial contact (52). The OMVs' ability to adhere to and fuse with host cells provides a mechanism of cellular entry for pathogenicity factors (53, 54).

P. gingivalis secretes OMVs that are internalized by both gingival epithelial and endothelial cells (55-57), (Figure 2). Multiple internalization pathways for these OMVs have been proposed. These include caveolin-dependent endocytosis and a fimbriae-dependent pathway which relies on lipid raft mediated endocytosis (58). The exact manner of endocytosis depends

upon vesicle size (58). Once internalized, *P. gingivalis* OMVs survive briefly within endocytic organelles before being sorted into lysosomal compartments and being degraded (55, 59). Despite the fast turnover of *P. gingivalis* OMVs within host cells, their entry still causes functional impairment of epithelial cells by degrading signaling molecules required for cellular migration (60). *P. gingivalis* OMVs also disrupt oral squamous epithelial cell monolayers, by inducing gingipain-dependent cell detachment (61), (Figure 2). Likewise, *T. denticola* secretes dentilisin-containing OMVs that disrupt tight junctions in epithelial monolayers. The protease activity of dentilisin results in the degradation of intercellular adhesion proteins, which facilitates bacterial penetration of underlying tissues (52, 62).

A contributing factor to advanced periodontal tissue destruction is host cell death, reported to affect both epithelial cells (63) and fibroblasts (64) in gingival biopsies of periodontitis patients. *T. denticola* outer membranes and purified outer membrane proteins Msp and chymotrypsin-like proteinase are highly cytotoxic to periodontal ligament epithelial cells due to their pore-forming activity (65), (Figure 2). Lipooligosaccharide on *T. denticola* OMVs is highly toxic to gingival epithelial cells (66). Additionally, *P. gingivalis* OMVs hinder the proliferation of fibroblasts and endothelial cells and suppress angiogenesis *in vitro*, contributing to inhibited wound repair in periodontal tissues (67). Interestingly, low concentrations of *T. forsythia* OMVs promote cell survival in human gingival fibroblasts over short time periods (7). *P. gingivalis* OMVs protect endothelial cells by reducing eNOS expression, an indicator of oxidative damage and metabolic dysfunction (68). In some cases, cell death is preceded by autophagy, a highly regulated process leading to degradation of damaged organelles and cytosolic products (69, 70). Autophagy is an important mechanism during periodontal inflammation (71), and is stimulated by peptidoglycan contained within bacterial OMVs (72).

Manipulation of the host immune response by OMVs

OMVs from *P. gingivalis*, *T. denticola*, and *T. forsythia* interact intimately with mucosal epithelial cells, connective tissue fibroblasts, endothelial cells, and innate immune cells to facilitate and dysregulate inflammation within gingival tissue (26). These OMVs activate pattern recognition receptors (PRRs) in gingival epithelial cells, resulting in cell activation, cytokine secretion, or apoptotic cell death (Figure 2). OMVs interact with macrophages through PRRs to induce the secretion of both pro-inflammatory and anti-inflammatory cytokines that dysregulate chronic inflammation (73). The effects of pro-inflammatory cytokines in periodontitis include the activation of neutrophils, T and B lymphocytes, macrophages, natural killer cells, and osteoclasts. This promotes connective tissue destruction and alveolar bone resorption, all clinical hallmarks of chronic periodontitis (74-76), (Figure 2).

Human periodontal ligament fibroblasts express significantly higher levels of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) when exposed to *T. forsythia* OMVs than in response to *T. forsythia* bacteria (7). IL-8 and MCP-1 are chemoattractants that induce the migration of neutrophils and monocytes, respectively, to the site of inflammation (75, 77), (Figure 2). Once recruited to tissues, monocytes are stimulated by *P. gingivalis* OMVs to

induce foam cell formation (78), release inducible nitric oxide synthase (iNOS) and nitric oxide (NO), (79) and secrete significant amounts of cytokines (Figure 2).

P. gingivalis OMVs induce IL-8 secretion from human gingival fibroblasts (80) and LOS on *T. denticola* OMVs induces strong pro-inflammatory responses from gingival fibroblasts, including secretion of IL-6, IL-8, MCP-1, prostaglandin E, matrix metalloproteinase-3, and NO (66). The continuous secretion of cytokines from host tissues promotes periodontal tissue destruction, and leads to the recruitment of innate and adaptive immune cells to the site of infection.

P. gingivalis OMVs also inhibit the surface expression of Human Leukocyte Antigen – antigen D related (HLA-DR) molecules on human umbilical cord vascular endothelial cells, limiting Major histocompatibility complex class II-induced active immunity (56).

Gingipains contained within the OMVs compromise the protective action of human serum through the degradation of IgG, IgM, and complement factor C3 (81, 82). Further, *P. gingivalis* OMVs mediate LPS tolerance in monocyte/macrophage cell lines, limiting pro-inflammatory responses. Prior exposure to *P. gingivalis* OMVs greatly inhibits TNF α and IL-1 β secretion in response to either *E. coli* LPS or whole-cell, live *P. gingivalis* (77, 83). Likewise, *T. denticola* lipooligosaccharide and the outer membrane protein Msp can induce macrophage tolerance to further stimulation with intact bacteria (84), (Figure 2).

Tolerance to LPS assists the host by minimizing inflammatory damage induced by high OMV/bacterial concentrations and prolonged or repeated exposure; however, it also benefits bacterial survival by inhibiting bacterial clearance. *P. gingivalis* OMVs also manipulate adaptive immunity and elicit humoral immune responses. Intranasal immunization with *P. gingivalis* OMVs induces *P. gingivalis*-specific IgG and IgA antibodies in blood as well as mucosal IgA in saliva (3, 85, 86). The structural and functional stability of *P. gingivalis* OMVs, combined with their ability to induce mucosal immunity make them a promising candidate for future immunization studies (87).

OMV-host cell interactions in the gastrointestinal tract

The human intestinal mucosa is one of the largest interfaces mediating host-microbe interactions. The human gut alone plays host to more than 500 species of bacteria, which fall largely into four major phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria (88, 89). These microbial populations play diverse and critical roles in intestinal homeostasis and overall human health. Certain types of bacteria may benefit the host, as they produce metabolites that allow energy recovery and aid in nutrient absorption (90). Commensal bacteria may also positively promote differentiation and proliferation of the intestinal epithelial cell layer (91). However, disruption in the composition of the gut microflora may contribute to many different pathologies, including inflammatory bowel diseases, obesity, cancer, diabetes, and neurological disorders, among others (92-96). Although the intestinal epithelium communicates with the gut microbiota, the two entities are physically separated by a mucosal barrier (97). As in other environments, gut bacteria generate OMVs, which they use as a means of bacteria-host communication at the mucosal interface (Figure 3). The intestinal milieu and mucus layer have been reported to stimulate vesiculation (20).

Role of OMVs in intercellular trafficking

Many toxins once classically viewed as secreted proteins, have recently been shown to be associated with and trafficked by OMVs. These include, most prominently, Shiga toxins of enterohemorrhagic *E. coli* (EHEC) (98, 99). EHEC is a foodborne pathogen causing hemorrhagic colitis and hemolytic uremic syndrome, a life-threatening complication of EHEC infection that may result in kidney failure (100). OMV-associated Shiga toxin is sufficient to cause hemolytic uremic syndrome in a mouse model (101). EHEC vesicles traffic accessory toxins, including hemolysin and cytolethal distending toxin V, into host cells (102, 103). Exposure to the gastrointestinal milieu, including conditions of low pH and the presence of mucin and antimicrobial peptides, stimulates the release of OMVs from EHEC and other enteric pathogens (20, 104). A majority of cholera toxin (CTx) produced by *V. cholerae* is also secreted in OMV-associated form, rather than as soluble protein as previously thought (105). Since CTx is contained within the vesicle lumen, it is taken up in a manner independent of host glycoproteins such as Lewis X glycan, which acts as a receptor for soluble CTx (106, 107). The finding that many toxins are vesicle-associated significantly impacts our understanding of their entry kinetics, intracellular trafficking and intoxication of host cells (103). Vesicle-associated lipopolysaccharide modulates OMV entry kinetics (28), and OMV-associated LPS is the main trigger for cytosolic caspase-11 which contributes to innate immune responses during EHEC infection (108).

Subversion of gastrointestinal immunity by OMVs

Helicobacter pylori is a pathogen that colonizes the upper gastrointestinal tract of around half the human population, although carriage remains asymptomatic in 80% of patients. However, persistent infection induces chronic inflammation of the gastric mucosa, which can lead to gastric ulceration and cancer (109). *H. pylori* releases cytotoxic proteins, including the vacuolating cytotoxin VacA, as OMV cargo (110, 111). These OMVs contribute to the carcinogenic potential of *H. pylori* infections (112), although the exact mechanisms remain elusive. OMVs are small enough to penetrate the mucosal barrier and deliver immunomodulatory molecules to the gastric epithelium (Figure 3). This interaction between host cells and bacterial proteins dysregulates both pro- and anti-inflammatory processes, contributing to overall pathogenesis. For example, *H. pylori*-derived OMVs induce T cell apoptosis, partially due to carriage of VacA (113). At lower concentrations, they inhibit T cell activation in a cyclo-oxygenase-2 dependent manner, by increasing the expression of the anti-inflammatory cytokine IL-10 (114). *H. pylori* OMVs also subvert the activation of dendritic cells, by modulating both the Akt-Nrf2 and mTOR-IKK-NF- κ B signaling axes (Figure 3). This induces the expression of heme oxygenase-1, which regulates dendritic cell maturation and function (115).

OMVs from commensals play a potent role in mediating anti-inflammatory responses and microbial immune tolerance (Figure 3). Peptidoglycan from commensal *E. coli* is delivered to cytosolic nucleotide-binding oligomerization domain – containing protein 1 (NOD1) through OMVs, and this process is involved in intestinal homeostasis (116). In the absence of these mechanisms, the host is more susceptible to inflammatory disease, such as colitis (117). On the other hand, overgrowth of certain members of the microbiota, for example

Bacteroides thetaiotaomicron, can lead to colitis, due to their production of sulfatase which permits access of OMVs to immune cells and pro-inflammatory responses (118).

OMVs as a platform for vaccine development

Acute diarrheal diseases remain a major cause of mortality worldwide, and particularly affect infants and the elderly. Despite intense efforts, vaccines that both efficiently target enteric pathogens and are effective in populations in endemic regions have remained elusive. However, the finding that OMVs play a crucial role in the pathogenesis of enteric infections has brought about a renewed interest and directed a new wave of research in this area. OMVs have been tested as antigens for vaccines that target pathogenic *E. coli* (119), *Salmonella* (120), *Vibrio cholerae* (121, 122), and combinations of pathogens (123). OMVs carry a complex mixture of biologically active molecules, including lipopolysaccharide, proteins, and phospholipids. This means they stimulate more protective immune responses than purified proteins, and elicit more robust protection than recombinant protein vaccines (124, 125). They may also offer prolonged protection compared to protein-based vaccines (126).

OMVs derived from *Salmonella enteritidis* provided intranasally or intraperitoneally elicited both robust humoral and mucosal immune responses, and were protective against *S. enteritidis* infection (127). Engineered strains overexpressing the small RNA MicA displayed hypervesiculation, with OMVs enriched in outer membrane porins. These provoked robust Th1- and Th17-type immune responses, and were protective against lethal challenge with *Salmonella* (128). In another study, a modified strain expressing penta-acetylated lipid A was used, which produced OMVs that retained their immunogenicity against enterotoxigenic *E. coli*, but with reduced reactogenicity (124).

Outlook and future research

Many advancements have been made in the field of OMV research over the past few years, and we have significantly furthered our understanding of OMV-host cell interactions, including in previously understudied niches such as the oral cavity. An emerging field is the investigation of OMVs secreted by the microbiota, and the contributions of these to gut homeostasis. Recent studies indicate an extensive metabolic link between the intestinal microbiota and host, and OMVs serve as a shuttle of small molecules between bacteria and host (129). Another exciting area is the investigation of immune responses triggered by OMVs. A detailed understanding of these interactions will allow us to tailor OMV-based vaccines, and improve our ability to target pathogens which have so far evaded vaccination efforts. These developments ensure the investigation of OMV-host interactions will stay a busy and rewarding field in years to come.

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Figure 1: Structure and composition of bacterial outer membrane vesicles.

Examples of purified outer membrane vesicles isolated from (A) *Porphyromonas gingivalis*, (B) *Treponema denticola* and (C) *Tannerella forsythia*. Outer membrane vesicles were purified using an optiprep gradient and visualized using Cryo-TEM as previously described (73). Scale bars, 200 nm. (D) Typical composition of bacterial outer membrane vesicles.

Figure 2: A summary of outer membrane vesicle interactions within gingival tissues.

Bacteria accrete on the tooth's surface and form a bacterial (plaque) biofilm that is adjacent to the epithelial cells in the gingival (gum) tissue. Outer membranes vesicles secreted from bacteria in this plaque biofilm bind to and penetrate into the mucosal tissue and generate host cell interactions and responses. These response culminate into a chronic inflammatory response resulting osteoclast activation which in turn promotes bone resorption and eventual tooth loss.

Figure 3. A summary of outer membrane vesicle interactions with the gastrointestinal tract. Environmental pressures, such as low pH, mucin or peptides or overgrowth of certain bacteria result in the release of outer membrane vesicles in the gastric lumen. These OMVs are able to penetrate the mucosal barrier and via different mechanisms adhere and interact with the underlying epithelial cell and immune cells inducing homeostasis or pathology.