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Review Article

Versatile effects of bacterium-released membrane vesicles on mammalian cells and infectious/inflammatory diseases

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Abstract

Gram-negative bacterium-released outer-membrane vesicles (OMVs) and Gram-positive bacterium-released membrane vesicles (MVs) share significant similarities with mammalian cell-derived MVs (*eg*, microvesicles and exosomes) in terms of structure and their biological activities. Recent studies have revealed that bacterial OMVs/MVs could (1) interact with immune cells to regulate inflammatory responses, (2) transport virulence factors (*eg*, enzymes, DNA and small RNAs) to host cells and result in cell injury, (3) enhance barrier function by stimulating the expression of tight junction proteins in intestinal epithelial cells, (4) upregulate the expression of endothelial cell adhesion molecules, and (5) serve as natural nanocarriers for immunogenic antigens, enzyme support and drug delivery. In addition, OMVs/MVs can enter the systemic circulation and induce a variety of immunological and metabolic responses. This review highlights the recent advances in the understanding of OMV/MV biogenesis and their compositional remodeling. In addition, interactions between OMVs/MVs and various types of mammalian cells (*ie*, immune cells, epithelial cells, and endothelial cells) and their pathological/preventive effects on infectious/inflammatory diseases are summarized. Finally, methods for engineering OMVs/MVs and their therapeutic potential are discussed.

Keywords: bacterium-released membrane vesicles; microvesicles; inflammation; vaccine; infectious diseases

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Introduction

Shedding of membrane vesicles (MVs) has been recognized as a universal mechanism for intercellular communication and is evolutionarily conserved across eukaryotes, bacteria and archaea[1-4]. These extracellular MVs are nanosized, spherical, bilayered proteolipids harboring specific subsets of bioactive proteins, lipids, nucleic acids, and metabolites. Notably, diverse names have been employed to indicate different species-derived extracellular vesicles[1-4]. For example, MVs for those of archaea and Gram-positive bacterial origin[1-3]; outer-membrane vesicles (OMVs) for those of Gram-negative bacterial origin[1-3]; and exosomes/microvesicles for those of mammalian cell origin[4]. Over the past several decades, tremendous efforts have been aimed at elucidating the versatile roles of mammalian cell-released MVs (*ie*, microvesicles and exosomes) in processes of human disease and injury repai[4-11]. However, bacterium-released MVs, especially their interactions with mammalian cells and their eventual impact on human health and disease, have received less attention. The first observation of bacterial MVs was reported by Chatterjee and Das in 1967 and revealed that the cell wall of *Vibrio cholerae* was bulged out and appeared to be pinched off the bacterial surface[12]. Since then, Gram-negative bacterial OMV-related studies have made steady progress, and the number of publications has increased rapidly in the last 5 years. Furthermore, direct evidence of MV formation in Gram-positive bacteria (*eg*, *Staphylococcus aureus, Bacillus subtilis, Bacillus anthracis,* and *Streptomyces coelicolor)* has also recently been obtained using transmission electron microscopic and proteomic analyses[13-16]. Most studies have focused on the analysis of OMVs produced by Gram-negative bacteria, including both pathogenic and non-pathogenic bacteria, whereas the knowledge of MVs secreted from Gram-positive bacteria is limited. Nonetheless, current evidence suggests that both OMVs and MVs can transport their cargo to recipient cells of the same species, other bacterial species, or mammalian cells[17-21]. Accordingly, bacterial OMVs/MVs have been shown to play diverse and critical roles in nutrient uptake, antimicrobial

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defense, horizontal gene transfer, biofilm nucleation, and the trafficking of microbial products such as virulence factors and toxins during infection[19-25]. The similarities and differences between MVs and OMVs are briefly listed in Table 1. In this review, we highlight recent findings on 1) the biogenesis and characterization of Gram-negative bacterium-released OMVs and Gram-positive bacterium-released MVs; 2) interactions between OMVs/MVs and mammalian cells; and 3) the pathological/beneficial effects of OMVs/MVs on infectious diseases. Finally, we discuss how to engineer these bacterial MVs as therapeutic agents for the treatment of human diseases.

Biogenesis of bacterial MVs

It has been recognized that all Gram-negative bacteria can naturally produce OMVs, which are typically ~10–300 nm in diameter (Figure 1A)[25]. Gram-positive bacteria lack an outer membrane, but some strains (eg, *S. aureus*, *B. anthracis*, *Listeria monocytogenes*, *Bacillus subtilis*, and *Clostridium perfringens)* also generate MVs (~20–150 nm in diameter) that are derived from the cytoplasmic membrane (Figure 1B)[13]. Nonetheless, how Gram-positive bacteria generate MVs remains unclear. Most of the knowledge presented here regarding the biogenesis of bacterial MVs has been collected from studies of Gram-negative bacteria, as summarized below.

The envelope structure of Gram-negative bacterium

It is becoming clear that multiple mechanisms are involved in the production of OMVs in Gram-negative bacteria[26]. To easily understand the process of OMV budding and detachment, it is necessary to comprehend the unique architecture of the Gram-negative envelope (Figure 2). The cell wall of Gram-negative bacteria consists of the outer membrane, the cytoplasmic (inner) membrane, and the periplasmic space in between, which contains a layer of peptidoglycan (PG)[27]. The outer membrane is composed of an exterior leaflet of lipopolysaccharide (LPS: O-antigen, core and lipid A) and an interior leaflet of phospholipids (Figure 2). The inner cytoplasmic membrane consists of a typical phospholipid bilayer that serves as an electrochemical barrier. The periplasm is an oxidative environment that promotes protein folding but does not contain nucleotide sources of energy, such as ATP or GTP[28]. The net-like PG layer within the periplasm maintains the bacterial shape and provides protection from osmotic changes and shear stress. Envelope proteins are either soluble (periplasmic proteins), membrane associated, integral or anchored into the leaflet of either membrane via covalently attached lipid appendages (lipoproteins). There are multiple crosslinks within the envelope that are important for maintaining the stability of the bacterial wall[29-33]. These crosslinks include (Figure 2): 1) the covalent crosslinking of Braun's lipoprotein (Lpp) in the outer membrane with the PG sacculus[29-31]; 2) the non-covalent interactions between the PG and outer-membrane protein A (OmpA), which is an outer-membrane porin[32, 33]; and 3) the non-covalent interactions between the PG and

Table 1. The similarities and differences between MVs and OMVs.

	Membrane vesicles (MVs)	Outer membrane vesicles (OMVs)
Origin	Cytoplasmic membrane of Gram positive bacteria	Outer membrane of Gram negative bacteria
Size	~20–150 nm in diameter	~10–300 nm in diameter
Components	• Various fatty acids and phospholipids; • Cytoplasmic proteins; • Membrane-associated virulence proteins/enzymes; • lipoteichoic acid (LTA); • Peptidoglycan • DNAs • sRNAs	• Outer membrane proteins; • Periplasmic proteins and virulence factors; • Less cytoplasmic and inner-membrane proteins; • LPSs and phospholipids; • Peptidoglycan • DNAs • sRNAs

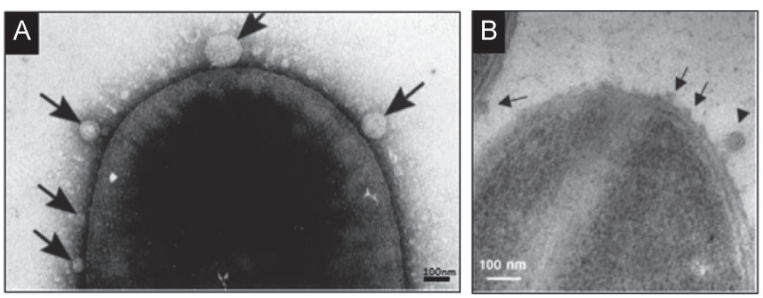

Figure 1. Transmission electron micrograph of bacteria reveals: (A) OMVs released by Gram-negative bacteria, *Serratia marcescens* UG-159 (arrows) (This image is adapted from Ref22, Li Z, *et al.* J Bacteriol 1998); (B) MVs released by Gram-positive bacteria *S. aureus* (arrows) (This image is adapted from Ref79, Lee EY, *et al.* Proteomics, 2009).

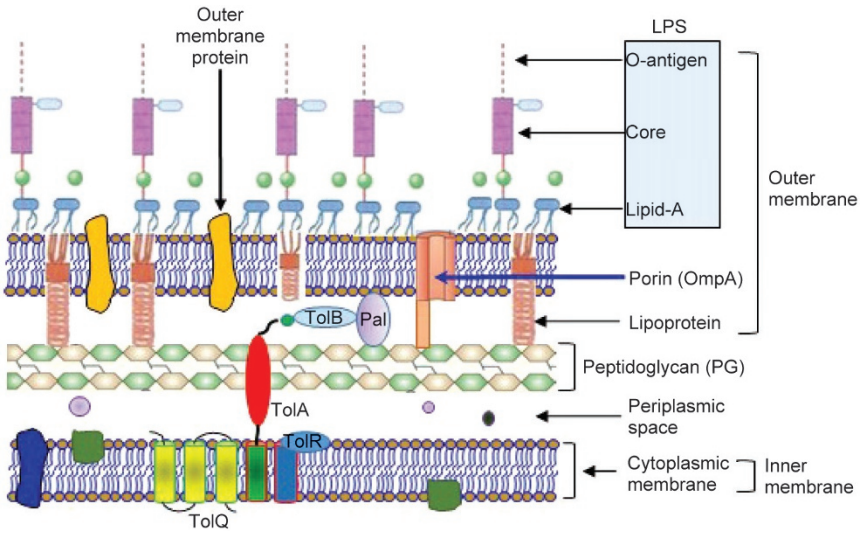

Figure 2. The architecture of the Gram-negative cell envelope. The envelope consists of an outer membrane (OM), an inner membrane (IM), and a periplasm between the two membranes, which contains a thin layer of peptidoglycan (PG).

the Tol–Pal (peptidoglycan-associated lipoprotein) complex, which spans the envelope from the outer membrane across the periplasm to the cytoplasmic membrane[32, 33]. In general, OMV biogenesis relies on the dissociation of the outer membrane from the underlying PG in areas devoid or depleted of attachments and subsequent fission without compromising envelope integrity[34].

The models proposed for OMV biogenesis

Depletion/reduction of envelope crosslinks

It is well known that crosslinks bridge the outer membrane to the PG in most Gram-negative bacteria (*eg*, *E. coli*). For example, OmpA is an outer-membrane porin that contains a periplasmic binding site for diaminopimelic acid (DAP), a PG component[35]. The Tol–Pal complex is a cell-division component that aids in invagination of the outer membrane and in membrane stability and interacts with PG[32, 36, 37]. Lpp is an extremely abundant outer-membrane lipoprotein, one-third of which is covalently crosslinked to PG. Lpp is evenly distributed throughout the entire cell wall, whereas Pal is preferentially located at the cell poles[36, 37]. Accordingly, bacteria lacking OmpA (*eg*, *E. coli*, *Salmonella* spp and *Acinetobacter baumannii* mutants) display increased OMV production, which is plausibly ascribed to decreased crosslinking between PG and the outer membrane[38-41]. Similarly, disruption of the Tol–Pal links causes membrane instability and increased OM shedding[32, 36, 37]. Mutations have further shown that vesiculation levels are dependent upon the proteins that crosslink the OM to the cell wall[32, 36, 37]. Nonetheless, while either a temporary decrease in overall crosslink abundance or a localized displacement of crosslinks is thought to increase OMV biogenesis, a complete lack of Lpp-PG crosslinks can cause membrane instability, leading to cellular leakage[32, 39, 41]. However, in some cases, a partial reduction in the number of Lpp-PG crosslinks could increase OMV production[42]. For instance, the amount of Lpp crosslinked to PG in the hyper-vesiculating *nlpI E. coli* mutant was ~40% lower than that in wild-type *E. coli*[42]. *Nlp*I is an outer-membrane lipoprotein that was recently discovered to control the activity of Spr, a PG endopeptidase that cleaves peptide crosslinks in PG[42-44]. Therefore, mutation of nlpI in *E. coli* prevents the formation of proper crosslinks between PG and Lpp and eventually leads to increased OMV production. Recent studies have also shown that the generation of OMVs in *Neisseria meningitidis* was affected by PG architecture, as OMVs from this bacterium were found to contain lower levels of the three lytic transglycosylases MltA, MltB and Slt[45]. Together, these observations support a model in which OMVs bud off at sites with locally decreased levels of crosslinks between the outer membrane and PG and with locally reduced PG hydrolase activity (Figure 3A).

Accumulation of misfolded proteins or envelope components in the periplasmic space

Schwechheimer *et al*[46] recently investigated several bacterial mutants and discovered that increased OMV production is not solely dependent on Lpp crosslinking; rather, it can also occur via a mechanism that is independent of the total levels of PG-bound Lpp. These hypervesiculating bacteria exhibited accumulation of misfolded proteins or envelope proteins, PG fragments and/or aberrant LPS in the periplasmic space[46]. Such accumulations can be triggered by, for example, defects in cell wall remodeling or temperature stress[47-49]. Following the accumulation of these contents in some regions of the periplasmic space, the outer membrane then bulges outward and buds off, effectively removing the undesirable envelope components from the cell[47-49] (Figure 3B).

Enrichment of the outer membrane with lipids and lipid-binding molecules

Recent studies have shown that *Pseudomonas syringae* OMV lip-

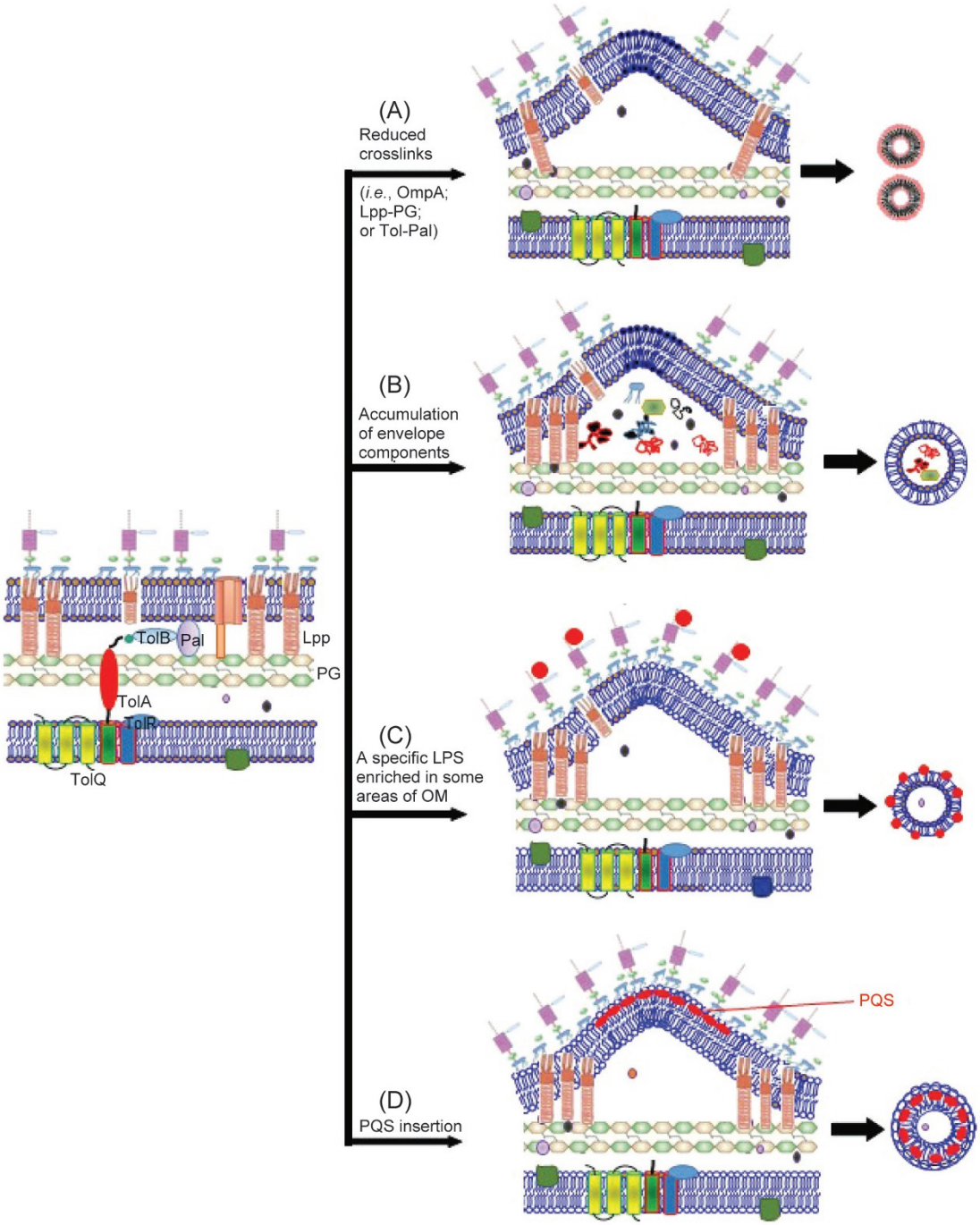

Figure 3. Proposed models for the biogenesis of outer membrane vesicles (OMVs). (A) The linkage between the outer membrane and the underneath peptidoglycan layer is disrupted. (B) A physical force induced by accumulation of misfiled or overexpressed envelope proteins pushes out outer membrane vesicles. (C) The accumulation of LPS molecules with atypical structures or charges leads to the curvature of outer membrane. (D) Local curvature of bacterial outer membrane is stimulated by extracellular signals (*ie*, PQS).

ids differ from the lipids of the OM of the bacterium[50]. These findings have led to a model in which membrane curvature is induced by the accumulation of LPS molecules with atypical structures or charges (Figure 3C). LPS is the major constituent of the outer leaflet of the OM of most Gram-negative bacteria. The LPS molecules themselves are not homogeneous, as the length and content of the polysaccharide chain varies among the different molecules. It has been proposed that subsets of these molecules may gather in patches along the OM, inducing higher degrees of membrane curvature at particular locations, either due to charge repulsion[51] or their molecular shape[52]. In addition, the Pseudomonas quinolone signal (PQS) of *Pseudomonas aeruginosa* can enhance anionic repulsions between lipopolysaccharide molecules, resulting in membrane blebbing

by sequestering divalent cations, which are important in forming stabilizing salt bridges between the negatively charged B-band lipopolysaccharide molecules[53-55]. Recently, it was proposed that the PQS induces OMV formation through a mechanism of asymmetric expansion of the outer leaflet of the OM[53-55] (Figure 3D). Although the PQS-based model is one of the best studied so far, whether it is utilized by other strains of Gram-negative bacteria to produce OMVs remains unclear.

Phospholipid accumulation in the outer leaflet of the OM causes OMV biogenesis

Recently, Roier *et al*[56] reported a new model for OMV biogenesis involving the VacJ/Yrb ABC (ATP-binding cassette) transport system, a proposed phospholipid (PL) transporter. They observed that disruptions within the VacJ/Yrb ABC (ATP-binding cassette) transport system increased OMV production in the human pathogens *H. influenzae* and *V. cholerae* without compromising OM integrity. Similarly, mutations in homologues of *E. coli* also increased vesiculation. Using lipidome analyses, they further found that OMVs from VacJ/Yrb-defective mutants in *H. influenzae* were enriched in phospholipids and certain fatty acids. Given that PL transporters are essential for maintaining the lipid asymmetry in the OM, the asymmetric expansion of phospholipids in the outer leaflet would initiate an outward bulging of the OM, leading to the generation of OMVs. These findings suggest a new general mechanism of OMV biogenesis based on phospholipid accumulation in the outer leaflet of the outer membrane. Importantly, this mechanism is highly conserved among Gram-negative bacteria and can account for OMV formation under all growth conditions[56]. Thus, this model of OMV biogenesis may be applicable to a broad range of Gram-negative bacteria and might have important pathophysiological roles *in vivo*.

Other models for OMV biogenesis

Recently, two additional novel models of OMV biogenesis have been reported. The first model, proposed by Elhenawy *et al*[57], shows that lipid A deacylation could impose shape modifications that result in the curvature of the outer membrane and subsequent OMV formation. They utilized *Salmonella enterica* serovar Typhimurium as a model organism and tested the effect of lipid A remodeling on OMV biogenesis. They observed that expression of the lipid A deacylase PagL resulted in increased vesiculation without inducing an envelope stress response. Mass spectrometry analysis further revealed profound differences in the patterns of lipid A expression in the OM and OMVs, with deacylated lipid A forms accumulating exclusively in OMVs. These findings suggest a novel mechanism for OMV biogenesis that involves outer membrane remodeling through lipid A modification.

The second model of OMV biogenesis was proposed by Turnbull *et al*[58], who showed that the explosive cell lysis of *Pseudomonas aeruginosa* produces shattered membrane fragments that rapidly form MVs. They identified that a prophage endolysin encoded within the R- and F-pyocin gene cluster is essential for explosive cell lysis. Endolysin-deficient mutants are defective in MV production. These findings reveal that explosive cell lysis, mediated through the activity of a cryptic prophage endolysin, acts as a mechanism for the production of bacterial OMVs.

Growth conditions and genetic factors that affect OMV biogenesis

The formation of OMVs was initially thought to be a physical process associated with the routine wear and tear of the OM of the cell[52]. However, recent discoveries show that OMV generation is an active resource-depleting process, which suggests that OMVs are not by-products of simple material shearing of the membrane but that energy expenditure is likely required for the sorting of biomolecules into OMVs[25]. While the exact signaling pathway of OMV biogenesis is not currently known, many factors have been identified to affect the generation of OMVs, as described below.

Growth conditions and environmental stress

Numerous studies have indicated that the production of OMVs changes with different growth conditions. For example, high growth temperatures can increase the number of OMVs produced. McBroom & Kuehn[47] found that temperature stress in *E. coli* leads to accumulation of misfolded proteins that ultimately are removed by being packaged in OMVs. Although they did not discuss it, the increased fluidity of the OM at higher temperatures may also have partially contributed to the increased generation of OMVs. In addition, it has been reported that oxygen stress can increase vesiculation in *P. aeruginosa* and *Neisseria meningitidis*[59, 60]; DNA-damaging antibiotics can stimulate OMV production in *P. aeruginosa* via the SOS response[61]; and medium composition can influence vesiculation in *Lysobacter* sp. XL1[62]. In the case of OMVs of *Pseudomonas putida* DOT-T1E, chemical stresses such as toxic concentrations of long-chain alcohols and EDTA (which chelates ions needed for the normal growth of bacterial cells), as well as physical stresses such as osmotic pressure and heat shock, caused the bacterium to release OMVs[63, 64]. Notably, while OMV production is increased in bacteria in response to stress conditions, OMVs are also released even when cells grow without external stress[65]. Indeed, exponentially growing bacteria produce more OMVs, and similar findings were observed in growing bacteria at division septa, possibly because of the increased peptidoglycan turnover that occurs during cell division[65, 66]. In addition, pathogenic species of bacteria generally release more OMVs than their non-pathogenic counterparts, and it is likely that OMV secretion has been adapted by pathogens to enhance their virulence[67, 68]. For example, a *P. aeruginosa* strain isolated from a cystic fibrosis patient was found to release 3- to 4-fold more OMVs than the laboratory-adapted strain PAO1[68]. Finally, nutrient availability can also affect OMV biogenesis, and the specific effects are dependent on the species of bacteria[69, 70]. OMV biogenesis is upregulated in *Lysobacter* in response to low nutrient conditions, whereas for *Pseudomonas fragi*, upregulation of OMV biogenesis occurs in response to nutrient-rich meat media[69, 70].

Considering these observations, it can be concluded that OMV production occurs in normal growth conditions but is also affected by multiple stress conditions.

Genetic factors

It is well recognized that both OMV production and OMV cargo loading are gene-regulated and do not result from random occurrences or cell death[26, 51, 71]. For example, the lipids of OMVs from *P. aeruginosa* are mainly composed of a highly charged form of LPS known as B-band LPS, whereas this molecule is only a minor component of the bacterial OM[51]. The enrichment of B-band LPS in OMVs rules out random membrane shedding or lysis, which would be expected to produce vesicles that are identical to the cell envelope in composition. Indeed, using an *E. coli* transposon mutant screen, McBroom *et al*[34] identified a number of gene mutations that could upregulate OMV production. Two of the genes they identified encode putative cell-envelope-localized proteins (*ypj*A and *nlp*A). Recently, Kulp *et al*[72] also assessed the genetic basis of OMV production by measuring vesiculation levels in the gene-deletion strains of the *E. coli* Keio library. They observed that nearly 150 new genes affected the process of vesiculation, suggesting that OMV production can be stimulated or inhibited through a gene-modulated pathway.

Furthermore, it has been observed that the OMVs of *S. enterica* contain higher levels of PagC and OmpX proteins[73]. Accordingly, overexpression of PagC and OmpX genes in bacteria could accelerate the generation of OMVs[73]. Similarly, the *E. coli* inner-membrane protein NlpA was found to regulate the biogenesis of OMVs[74]. By contrast, it was previously shown that lipoproteins are specifically depleted or decreased in OMVs of *E. coli* [32]. Accordingly, disruptions in the expression of lipoprotein genes (*eg*, OmpA and the Tol–PAL complex) in *E. coli* have been shown to increase OMV production[32]. It is important to note here that *Pseudomonas aeruginosa* possesses fewer lipoproteins than *E. coli* [75], which may explain why *P. aeruginosa* usually produces significantly higher levels of OMVs than *E. coli*[76]. Along this line, some OM proteins such as Omps (proteins related to Tol–Pal systems and YbgF) are suspected to have a role in the bulging of the bacterial OM, which marks the onset of OMV production[77]. Together, these observations provide multiple lines of compelling evidence showing that OMV biogenesis could be regulated at the genetic level.

Composition of bacterial MVs

Structural studies of OMVs/MVs have invariably shown that they are spherical and enclosed by a membrane bilayer and have a strain-specific characteristic size distribution[13-15]. The contents of OMVs/MVs from various species of bacteria have been characterized using protein mass spectrometry (MS), lipid and SDS-PAGE profiles, and RNA sequencing[25-27, 78-84]. It has been shown that OMVs contain outer-membrane proteins, lipopolysaccharide, periplasmic components including peptidoglycan and virulence factors, and inner-membrane and cytoplasmic proteins as well as DNA and RNA[78-84] (Figure 4), whereas MVs contain various fatty acids and phospholipids, cytoplasmic proteins, membrane-associated virulence proteins/enzymes, lipoteichoic acid (LTA), peptidoglycan, DNA and sRNAs. These OMV/MV contents can be found in the EVpedia public database (http://evpedia.info).

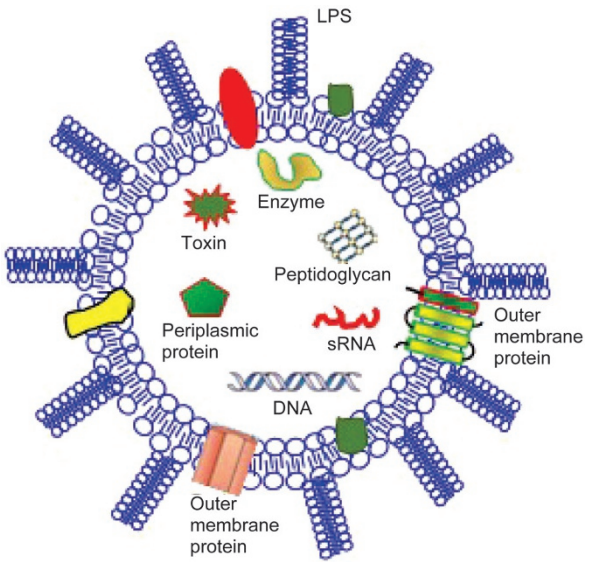

Figure 4. Composition of outer membrane vesicles (OMVs). Generally, OMVs contain outer membrane components (*ie*, OM-anchored lipoproteins, OM phospholipids and LPS), periplasmic proteins (*ie*, autolytic enzymes), peptidoglycan, and virulence factors (*ie*, toxins in pathogenic bacterium-derived OMVs), DNAs and small non-coding RNAs (sRNAs).

Proteins

The protein content of OMVs/MVs has been studied thoroughly by many groups using different bacterial strains[78-84]. The proteins identified in these Gram-negative OMVs include OM structural proteins, porins, ion channels, transporters for different molecules, periplasmic and cytoplasmic enzymes, and proteins related to stress responses[84]. Over the last decade, mass spectrometry-based proteomic studies have identified more than 3500 proteins that are encased in OMVs (http://evpedia.info). It is important to note that 1) not all outer-membrane proteins from a given strain are sorted into OMVs[85], although membrane proteins make up a significant portion of the OMV proteome; 2) cytoplasmic and inner-membrane proteins identified in OMVs are not contaminants from lysed cells in the cultures but might have resulted from an active sorting mechanism[84]; 3) enzymes found in OMVs include proteases, peptidases, nucleases and β-lactamases[85-88]; and 4) protein profiles are different in the OMVs harvested from different strains of bacteria and from the same strain at different growth phases or in response to different stresses[89]. OMVs may also contain toxins and other virulence factors[25]. The presence of virulence factors and toxins is usually observed in the OMVs produced by different pathogenic bacteria[78].

More recently, researchers have characterized the protein

compositions of MVs from several Gram-positive bacteria, such as *S. aureus*, *B. anthracis*, *Listeria monocytogenes*, *Bacillus subtilis*, and *Clostridium perfringens*[13-15, 54, 79, 90]. The first high-throughput proteomic analysis was performed by Lee *et al* on *S. aureus* ATCC 14458 extracellular vesicles[79]. They identified a total of 90 proteins in *S. aureus*-derived MVs. Importantly, *S. aureus*-derived MVs are especially enriched with transporter proteins (*eg*, the protein translocase SecD/SecF and hydroxamate siderophores), antibiotic resistance-related proteins [β-lactamase, membrane-associated global regulator (MsrR), and penicillin-binding proteins (PBP1, PBP2, and PBP3)], diverse virulence proteins (toxins, adhesins, proteolysin, coagulase, and other related enzymes), and many cytoplasmic proteins, such as ribosomal proteins, DNA polymerases, tRNA synthetases, and several metabolic enzymes. Furthermore, Jiang *et al*[90] recently identified a total of 431 proteins in the MVs released from Gram-positive *C. perfringens*. They found the most abundant proteins in *C. perfringens*-derived MVs were membrane (24%) and cytoplasm (18%) proteins, but they found no alpha-toxin or NetB toxin in these MVs. Additional MV proteomic studies on other Gram-positive bacteria have revealed that there are some differences in the specific protein compositions of these vesicles. Together, these results indicate that the makeup of the OMV/MV proteome may help to shed light on the pathophysiological functions of OMVs/MVs.

Phospholipids

In an early study, the OMVs of *E. coli* were identified to have a similar phospholipid profile to the OM[91]. Various studies have subsequently been carried out to compare the phospholipids from the OM and OMVs and have confirmed that OM phospholipids are present in the membranes of OMVs[50, 78, 80, 92]. However, there is evidence that OMVs contain some lipids that are not detected in the OM[93]. Tashiro *et al*[92] recently observed that the phospholipid composition of the OM and OMVs from *P. aeruginosa* was different. In the OM, phosphatidylethanolamine was abundant, while phosphatidylglycerol was found in OMVs[92]. Furthermore, they found that the relative amount of saturated fatty acyl chains was higher in OMVs than in the OM[92], making the OMVs more rigid than the OM. In a later study, Tashiro *et al*[94] proposed that the release of OMVs may make the bacterial OM more fluid and that *P. aeruginosa* may shed OMVs to maintain the optimum fluidity for the prevailing growth conditions. Recently, several lipidomic analyses revealed that Gram-positive bacterial MVs contain various fatty acids[21, 95, 96]. For example, both *B. anthracis* and *S. pneumoniae* MVs are enriched in short-chain saturated fatty acids (C12–C16), such as myristic and palmitic acid, compared with bacterial cell membranes[95, 96]. Using a matrix-assisted laser desorption ionization (MALDI)-based mass spectrometry approach, Resch *et al*[21] recently identified that Group A streptococcus (GAS)-derived MVs contain more than 85 individual lipid species corresponding to glycoglycerolipids and both anionic and cationic phospholipids (PLs). They further observed that anionic phosphatidylglycerols (PGs) were significantly enriched in GAS MVs. In contrast, cardiolipin (CL) comprised only a minor fraction of MV lipids relative to the GAS membrane. Differential enrichment of MV lipid species relative to the membrane provides further evidence for the existence of a regulated mechanism contributing to MV biogenesis, as opposed to nonspecific membrane shedding.

LPSs

It is well known that LPSs in the outer membrane are a characteristic feature of the Gram-negative cell envelope. Therefore, it is obvious that bacterium-released OMVs contain LPS molecules; however, not all LPSs of the parent cell appear in OMVs[50-52, 97]. For example, OMVs from *P. aeruginosa* are mainly composed of the negatively charged B-band of LPS instead of the more neutral A-band in the outer membrane[51]. Haurat *et al*[52] further showed that in the case of the dental pathogen *P. gingivalis*, deacetylated lipid A was accumulated in OMVs. By contrast, no differences were observed in the lipid A profiles of the outer membrane and OMVs in *Bacteroides fragilis*[97]. Collectively, these observations suggest that LPSs are selectively sorted into OMVs.

Nucleic acids (DNA and RNA)

It has been demonstrated that MVs/OMVs contain DNA components, including both plasmid DNA and chromosomal DNA[98-100]. Although DNA fragments from lysed cells could potentially bind to the positively charged surface of OMVs, treatment of OMVs with DNase proved that some DNA fragments were trapped in the lumen of the OMVs[100]. A detailed study of the presence of nuclease-resistant DNA in MVs and OMVs from Gram-positive and Gram-negative bacteria, respectively, was carried out by Dorward & Garon (1990)[101]. They observed that DNA in the form of circular plasmids, linear plasmids and chromosomal fragments is likely to be present in Gram-negative OMVs but not in Gram-positive MVs. However, Jiang *et al*[90] recently showed that Gram-positive *C. perfringens*-derived MVs did contain partial chromosomal DNA fragments encoding the 16S rRNA, plc and pfoA genes. At present, the mechanisms by which DNA is sorted into MVs/OMVs are not clear. It has been suggested that MVs/OMVs could pick up free DNA fragments present in their environment and that DNA can enter through the periplasm during MV/OMV biogenesis[100]. Notably, DNA has not been found in the OMVs of many other bacteria strains[102]. For example, three different strains of *Porphyromonas gingivalis* were found to produce OMVs that did not contain any DNAs[102].

Recently, using an RNA deep sequencing (RNA-Seq) approach, several groups have identified that both MVs and OMVs contain a significant amount of RNAs[21, 83, 103]. For example, Sjöström *et al*[103] detected more than 200 short RNA (sRNA) sequences in the OMVs released by *V. cholerae* strain A1552. Interestingly, the majority of the most abundant RNA sequences identified in *V. cholerae* OMVs correspond to non-coding regions. Similarly, Koeppen *et al*[83] identified 481,480 unique sRNA sequences in OMVs purified from the superna-

tants of three planktonic cultures of *P. aeruginosa* strain PA14. They observed that the median sequence length of the sRNAs was 24 nucleotides, with a minimum of 15 and a maximum of 45 nucleotides. The 1733 most abundant sRNA sequences in OMVs (*ie*, at least 64 sequence reads) mapped to 68 loci in the *P. aeruginosa* genome. These authors further used bioinformatic approaches and found that the ten most abundant sRNAs in OMVs were likely to form stable secondary structures and that these sRNAs could potentially interact with human mRNAs.

In the case of Gram-positive MVs, Resch *et al*[21] recently characterized RNA species present in MVs released by *Streptococcus pyogenes*, also known as group A streptococcus (GAS), using RNA deep-sequencing. They found that a total of 207 RNA species were differentially abundant in MVs relative to bacteria. Of these, 120 RNAs were more abundant in MVs, including the zinc-responsive adhesive competence repressor adcR, anaerobic ribonucleoside triphosphate reductase nrdG, exonuclease-helicase subunit A rexA, and type 1 restriction endonuclease hsdR genes; and 87 RNAs were less abundant in the MV fraction, including the streptolysin S gene *sagA* and the glucosamine-6-phosphate isomerase gene *nagB*. These findings indicate that Gram-positive bacterial MVs are enriched with intragenic RNAs of the originating bacteria. Nonetheless, whether non-coding shorter RNAs are included in these MVs remains unclear.

Other molecules

MVs/OMVs also contain a variety of ions, quorum sensing signaling molecules and metabolites[104, 105]. However, the appearance of these molecules in MVs/OMVs has not been studied extensively to date. Certainly, many other types of bioactive molecules will be identified in MVs/OMVs in the future.

Roles of bacterial MVs in bacterial survival

Many different functions have been demonstrated for MVs/OMVs, making them important players in the physiology of Gram-positive and -negative bacteria[13, 15-26]. The prevalent idea is that MVs/OMVs are indispensable for the survival of these bacteria[22]. The contributions of MVs, mostly OMVs, to bacterial survival have been extensively investigated over the past several decades[13, 15-26]. Some of the prominent functions are discussed below.

Defense and resistance

The outer membrane of Gram-negative bacterial cells is the first line of defense against external stress conditions. In response to unfavorable conditions, bacteria have developed mechanisms of generating MVs to quickly remove surface-attacking agents and internal misfolded or toxic materials[22]. For example, upon treatment of bacteria with lytic bacteriophages, vesiculation of the bacterial outer membrane increases, generating an abundance of decoy membranes in the environment, which could help the bacteria avoid infection by phages[106, 107]. OMVs can also bind or absorb some antibacterial molecules (eg, complement and antibiotics) and, consequently, inactivate these molecules or transport them away from bacteria[80, 107-110]. For example, in *E. coli*, the addition of OMVs or the use of a hypervesiculating mutant increased immediate resistance to the antimicrobial peptides polymyxin B and colistin[107]. Similarly, OMVs from *P. syringae* reduced the levels of colistin and melittin (another antimicrobial peptide) in solution by sequestering these compounds[80]. Moreover, MVs/OMVs can also carry enzymes that mediate antibiotic protection. For instance, β-lactamase can be packaged into MVs released from Gram-positive bacteria, and co-incubation of such MVs with β-lactam-sensitive species improves resistance to these antibiotics[108, 110]. Recently, OMVs from amoxicillin-resistant *M. catarrhalis* (Gram-negative) were found to carry active β-lactamase and to protect amoxicillin-sensitive *M. catarrhalis* from antibiotic-induced killing[86]. Furthermore, it has been demonstrated that the OMVs produced by one bacterial species are able to protect other bacterial species from antibiotic stress, emphasizing the ecological role of OMVs in microbial niches[53, 86, 88]. For example, OMVs from amoxicillin-resistant *M. catarrhalis* have also been shown to improve the amoxicillin resistance of non-typeable *H. influenzae* and *S. pneumoniae*, two bacteria that typically co-infect the respiratory tract[86].

Nutrient acquisition

Numerous studies have shown that OMVs are able to promote nutrient acquisition in bacterial communities and thereby enhance bacterial survival[22, 45, 53, 105, 111]. This is mainly ascribed to the preferential packaging of diverse enzymes (eg, glycosidases, proteases, alkaline phosphatase and enolase), metal ion-binding proteins/molecules, and surface receptors in OMVs[45, 53, 105, 111]. For example, the signal molecule 2-heptyl-3-hydroxy-4-quinolone (also known as Pseudomonas quinolone signal, PQS) is highly enriched in *P. aeruginosa* OMVs and binds iron, which is essential for bacterial viability and often limiting in biological environments[53, 111]. PQS-Fe-OMVs could then be absorbed into the OM by fusion, or OMVs could release PQS-bound iron in the vicinity of the cell and thereby enable the bacteria to scavenge iron, leading to enhanced survival. Notably, the OMVs from *N. meningitidis* are also enriched in the zinc acquisition proteins ZnuA and ZnuD[45], suggesting that the role of OMVs in metal acquisition is not limited to iron.

In addition, OMVs themselves could serve as nutrient sources for bacteria. For example, OMVs from the marine cyanobacterium *Prochlorococcus* have been shown to support growth of the heterotrophs *Alteromonas* and *Halomonas* as the sole carbon source[105]. Similarly, OMV-associated DNA and proteins may serve as a source of nitrogen and phosphorous for bacterial growth.

Facilitation of biofilm formation

Biofilms are surface-adhering structures made from a matrix consisting of exopolysaccharides, DNA, proteins and many other molecules, in which bacterial cells remain embedded[112].

In general, bacteria form biofilms as a response to stress[112]. The number of OMVs released by bacteria increases during stress, suggesting a relationship between OMVs and biofilm formation[63, 112, 113]. Indeed, it has been reported that the addition of OMVs to an *H. pylori* culture stimulated biofilm production[114]. Interestingly, exogenous addition of DNA to bacteria can also stimulate biofilm production[115]. Considering that OMVs carry DNA, it is therefore possible that OMVs trigger biofilm formation through surface-associated DNA. However, the detailed mechanism underlying the role of OMVs in biofilm formation remains to be investigated.

Killing of competing microbial cells

As discussed above, OMVs can help protect bacteria from antibiotics and other stress conditions. In addition to this function, OMVs produced from one bacterium can also kill other competing microbes in the vicinity. For example, Li *et al* previously showed that the OMVs of various Gram-negative strains could kill many Gram-positive and Gram-negative bacteria[22]. Their study investigated the effectiveness of OMVs in killing target bacteria with different peptidoglycan chemotypes. They found that killing was most effective when the target possessed a peptidoglycan that was similar to the OMV donor. If the peptidoglycan hydrolases present in the OMVs are the same as those of the target strain, then they cannot cleave the peptidoglycan layer. Along this line, Vasilyeva *et al*[62] recently found that *Lysobacter* sp. XL1 secreted bacteriolytic enzymes in OMVs. This activity of OMVs shows that they might be capable of distinguishing between self and non-self cells in a mixed community. At present, while the concept of using OMVs as a new antimicrobial agent has been explored, whether they can be a commercially feasible option remains to be tested in the future.

In summary, bacteria tend to produce more MVs/OMVs as a survival mechanism under stress conditions. In fact, hypervesiculating mutants have been shown to better adapt to stress conditions compared with wild-type strains[25, 107]. During temperature stress, OMVs can quickly remove misfolded proteins[64]. Upon antibiotic exposure, OMVs can sequester[107] or degrade[108] the antibiotics. When stress is induced by antibodies or bacteriophages, OMVs can act as decoy targets, protecting cells by titration of the antibodies or phages[107]. The production of OMVs is also found to increase with nutrient stress, which shows that OMVs have a role in bacterial nutrition, as discussed above. Finally, OMVs are able to facilitate biofilm formation and kill competing microbial cells.

Versatile effects of bacterial MVs on mammalian cells

Beneficial effects of OMVs on epithelial cells

The integrity of the epithelial barrier is critical in maintaining homeostasis in the body, and its dysfunction is linked to inflammatory, allergic and metabolic diseases[116, 117]. Interactions between the gut microbiota and the intestinal epithelium are crucial for the integrity of this barrier. Therefore, alterations in the microbiota composition or aberrant responses to luminal bacteria or dietary components can result in increased intestinal permeability, which may lead to the development of the aforementioned pathologies. In this context, many studies have been conducted to investigate the therapeutic potential of certain commensal and probiotic strains to ameliorate inflammatory bowel diseases in clinical trials (see reviews elsewhere[118, 119]) or in animal models of colitis[120-125]. In mouse colitis models, beneficial bacteria reduce inflammatory cytokines, normalize gut permeability, and reinforce the epithelial barrier[124]. Recently, several studies have indicated that these effects may be mediated, at least in part, by bacterial-secreted OMVs[124-126]. For example, Alvarez *et al*[126] reported that OMVs and soluble factors released by probiotic *E. coli* Nissle 1917 and commensal ECOR63 were able to enhance barrier function by upregulating ZO-1 and claudin-14 and downregulating claudin-2 in intestinal epithelial cells.

Pathogenic effects of OMVs on epithelial cells

By contrast, MVs/OMVs released from pathogenic bacteria in the gut could interact with epithelial cells and ultimately cause inflammation[127-129]. *H. pylori* is an example of a pathogen that resides in the mucosa and colonizes the gut of approximately 50% of the human population[130]. In most patients, this infection is asymptomatic, but in approximately 20% of patients, the infection leads to chronic gastritis as well as an increased risk of developing peptic ulcer disease, gastric lymphoma, or gastric carcinoma[130]. Surprisingly, *H. pylori* has been found to be a non-invasive pathogen. However, LPS-enriched OMVs secreted from *H. pylori* bind to and invade gastric epithelial cells partly through clathrin-mediated endocytosis[131]. By adhering to cells via the adhesins BabA and SabA and delivering the vacuolating cytotoxin VacA, *H. pylori*-derived OMVs could trigger inflammation through the persistent delivery of antigens such as proteases and urease to the gastric mucosa[132, 133].

Enterotoxigenic *E. coli*, a major cause of diarrhea and infant mortality in third world countries, has also been analyzed for OMV binding to epithelial cells[134-136]. The heat-labile enterotoxin LT is secreted by the outer membrane of the bacteria as well as OMVs[136]. LT-containing vesicles and OMVs lacking the toxin were compared for their capacity to invade adrenal and intestinal epithelial cells. It was clearly shown that only LT-carrying vesicles could invade cells in a time-, temperature-, and receptor-dependent manner[136]. The vesicles attached to the cells at 4°C, and the LT was internalized at 37 °C[136]. This was mediated through cholesterol-rich lipid rafts, and OMVs sometimes co-localized with the endocytosis protein caveolin. Kesty *et al*[136] concluded that the OMVs are targeted transport vesicles of the enterotoxin.

Furthermore, *Cronobacter sakazakii*, a causative agent of infant meningitis and enterocolitis, produces OMVs that are internalized by intestinal epithelial cells, inducing cell proliferation and IL-8 secretion and facilitating bacterial persistence[137]. Moreover, bacterial RNA contained within OMVs produced by *E. coli*, *P. aeruginosa*, *P. gingivalis*, and *V. cholerae* has immuno-stimulatory capabilities in epithelial cells[81, 83, 138, 139].

Additionally, OMVs have the potential to bind to epithelial cells in the respiratory tract as well as in the mucosa,

anatomical sites where bacteria interact with their host[140, 141]. For instance, *Legionella pneumophila* could secrete OMVs that attach to lung alveolar epithelial cells, stimulating the secretion of inflammatory cytokines (*ie*, IL-6, IL-7, IL-8, and IL-13 as well as GM-CSF, IFN-γ, and MCP-1)[140]. The OMVs of *M. catarrhalis*, a respiratory pathogen that commonly causes otitis media in children, also bind to epithelial cells[141]. *M. catarrhalis* OMVs bind to TLR2 in epithelial cell lipid rafts, which are subdomains of the membrane with distinct protein and lipid compositions and enriched in cholesterol and sphingolipids[141]. The OMVs are subsequently internalized, causing a pro-inflammatory response and resulting in increased IL-8 secretion and ICAM-1 expression. Moreover, OMVs can be observed as bacterial tools that interact with and regulate the inflammatory response of epithelial cells at sites that are distant from the parent bacteria[141].

Versatile effects of OMVs on immune cells

In addition to interacting with the host's epithelial cells, bacterium-released OMVs can directly affect various immune cell populations, including neutrophils, macrophages and dendritic cells (DCs). Some effects of OMVs on immune cells are discussed below.

Effects of OMVs on neutrophils.

OMVs regulate neutrophil function in different ways that are dependent on their composition and bacterial origin. Lapinet *et al*[142] showed that stimulation of human neutrophils with *N. meningitides*-derived OMVs caused secretion of TNF and IL-1β and upregulation of CXCL8, CCL3 and CCL4 expression. These responses were further enhanced by IFNγ, suggesting that *N. meningitidis* OMV-triggered immune responses can be potentiated during a chronic inflammatory state in which IFNγ is present[142]. By contrast, Davis *et al*[143] observed that OMVs from non-pathogenic *E. coli* encase cytotoxic necrotizing factor type 1 (CNF1), a bacterial toxin that impairs the phagocytic and chemotactic abilities of neutrophils. Interestingly, OMVs from *N. meningitidis* and *Histophilus somni* can induce the formation of neutrophil extracellular traps (NETs), which contain DNA, antimicrobial peptides and histones that form extracellular fibers to trap and kill extracellular pathogens[144-146].

Effects of OMVs on macrophages.

Recent studies have elucidated the mechanisms by which OMVs can induce macrophage-mediated diseases. *Legionella pneumophila* (*L. pneumophila*) is an intracellular, Gram-negative pathogen that causes a severe form of pneumonia[147]. In humans, it infects alveolar macrophages, where it blocks lysosomal degradation and forms a specialized replication vacuole[147]. Recently, Jung *et al*[148] demonstrated that incubating differentiated THP-1 macrophages with *L. pneumophila* OMVs led to a TLR2-dependent classical activation of the macrophages and the release of pro-inflammatory cytokines in a dose-dependent manner. Furthermore, treatment of THP-1 cells with OMVs prior to infection reduced *L. pneumophila* replication in the THP-1 cells. However, with prolonged infection times, the OMV pre-treated macrophages became more permissive for bacterial replication than untreated cells. They further identified that *L. pneumophila* OMVs were initially potent pro-inflammatory stimulators of macrophages, acting via TLR2, IRAK-1, and NF-κB, while at later time points, OMVs facilitated *L. pneumophila* replication by miR-146a-dependent IRAK-1 suppression. Therefore, OMVs might promote the spread of *L. pneumophila* in the host.

Furthermore, when examining lung tissue from patients infected with *L. pneumophila,* Jager *et al*[149] observed that OMVs were distributed within the cytoplasm of infected macrophages, suggesting that OMVs directly interact with macrophages *in vivo*. Indeed, fluorophore labeling was used to confirm the direct binding and internalization of *L. pneumophila* OMVs into human macrophages, where they consequently stimulated TNF-α production[148]. Similarly, incubation of monocytes and macrophages with *N. meningitidis* OMVs has been shown to induce the production of CCL2, CCL3, CCL5 (also known as RANTES), CXCL8, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70 and TNF[150]. Notably, OMVs activate macrophages to induce adaptive immune responses, as *N. meningitidis* OMVs upregulate the expression of HLA-DR, the co-stimulatory molecules CD80 and CD86, and intercellular adhesion molecule 1 (ICAM1) by macrophages[150].

In addition to having pro-inflammatory effects, OMVs are able to elicit anti-inflammatory effects that benefit the pathogen of origin or that promote secondary bacterial infections. For example, Winter *et al*[151] recently reported that *H. pylori* OMVs could induce the production of the immunosuppressive cytokine IL-10 by human peripheral blood mononuclear cells to suppress inflammation and facilitate bacterial survival. Likewise, *P. gingivalis* OMVs could inhibit CD14 expression in macrophages, rendering these cells unresponsive to TLR4 signaling triggered by secondary *E. coli* LPS stimulation[152]. Pollak *et al*[153] also showed that *B. abortus* OMVs attenuated TLR2, TLR4 and TLR5 responses, dampened IFNγ-induced MHC class II expression and promoted the internalization of *B. abortus* bacteria by THP-1 cells. Collectively, these studies indicate that OMVs play an important role by interacting with macrophages/monocytes and act both as pro- and anti-inflammatory mediators, possibly depending on the specific strain and on environmental conditions.

Effects of OMVs on dendritic cells (DCs)

OMVs have been shown to be internalized into dendritic cells (DCs) and to program DCs to induce the differentiation of IL-10-producing $CD4^+Foxp3^+$ Treg cells in a PSA-dependent manner[125]. Alaniz *et al*[154] reported that OMVs from *Salmonella* spp. induced the expression of CD86 and MHC class II molecules on DCs and the production of TNF and IL-12 and promoted the development of protective B cell and T cell responses. Similarly, Durand *et al*[155] showed that *N. meningitidis* OMVs induced DC maturation, characterized by the upregulation of MHC class II and co-stimulatory molecules and the production of pro-inflammatory cytokines and chemokines. Mechanistically, *N. meningitidis* OMVs are able to facili-

tate their delivery to and internalization by DCs by binding to the host factor bactericidal permeability-increasing protein (BPI)[156]. BPI is a host immune factor that binds and neutralizes endotoxin in addition to promoting bacterial clearance[157]. Taken together, these observations reveal that OMVs affect DCs by inducing their maturation and facilitating antigen presentation and by limiting their cytokine responses to either OMVs or secondary bacterial antigens.

Effects of OMVs on other types of mammalian cells

Endothelial cells and OMVs

In an early study, Shoberg and Thomas[158] showed that *Borrelia burgdorferi* produces OMVs that contain some of the outer-surface proteins of the bacterium (eg, OspA and OspB). They further observed that such OMVs can bind and enter into cultured human umbilical vein endothelial cells (HUVECs) in a dose-dependent, OMV component-dependent and passage number-dependent manner. Consistent with this, several studies later reported that *P. gingivalis*-derived OMVs were capable of stimulating the expression of E-selectin and ICAM-1 and suppressing capillary tube formation by cultured HUVECs[39, 159-161]. Recently, Kim *et al*[162] found that OMVs derived from intestinal *E. coli* upregulate functional ICAM-1, E-selectin, or VCAM-1 expression on the surface of human microvascular endothelial cells via the activation of NF-κB. When administered intraperitoneally to mice, these OMVs induce neutrophil aggregation in the lung endothelium in an ICAM1- and TLR4-dependent manner, suggesting that OMVs are used by pathogens to sequester leukocytes in the endothelium[162]. Likewise, Dr. Sullivan's group[163, 164] demonstrated that *E. coli* OMVs induce the production of IL-6, tissue factor, thrombomodulin, and the adhesion molecules P-selectin and E-selectin by human endothelial cells, which leads to the recruitment of pro-inflammatory leukocytes, platelet aggregation and coagulation. A recent study by Jia *et al*[165] further showed that *P. gingivalis* OMVs suppressed endothelial nitric oxide synthase (eNOS) in both cultured HUVECs and the mouse aortic endothelium.

By contrast, OMVs have also been observed to limit inflammatory responses at the endothelium; for example, the addition of *P. gingivalis* OMVs to IFNγ-stimulated endothelial cells inhibited the upregulation of MHC class II molecules on the cell surface, thus modulating antigen presentation and favoring survival of the pathogen[166]. Hence, similar to the dual effects that are discussed above in epithelial cells and immune cells, OMVs have both immunostimulatory and immunomodulatory effects on endothelial cells.

Platelets and OMVs

It has been reported that *P. gingivalis* OMVs are potent inducers of mouse platelet aggregation *in vitro*[167]. Similarly, *N. meningitidis* OMVs contribute to thrombosis via increased platelet-platelet and platelet-leukocyte aggregation[168].

Fibroblasts, osteoblasts and synovial cells

Friedrich *et al*[169] recently showed that OMVs released by *T. forsythia* not only interact with human macrophages but also stimulate human periodontal ligament fibroblasts to release pro-inflammatory mediators that are potentially relevant to the development of periodontitis (i.e., TNF-α, IL-6, IL-8, and MCP-1). Consistent with this, multiple groups observed that *P. gingivalis* OMVs could be internalized into human oral keratinocytes and gingival fibroblasts[139, 160, 170]. In addition, osteoblasts and synovial cells can internalize *K. kingae* OMVs, leading to the production of GM-CSF and IL-6, both of which are implicated in pathogen-induced bone and tissue inflammation and destruction[171].

Pathological roles of bacterial MVs in infectious/inflammatory diseases

As discussed above, MVs/OMVs can be internalized into various types of host cells and subsequently induce the activation of inflammatory pathways. Furthermore, MVs/OMVs can be detected in the blood and cerebrospinal fluid during clinically severe bacterial infections. It has been proposed that MVs/OMVs in the blood and body fluids may significantly contribute to inflammation and mortality in human diseases[172-174].

Gram-negative bacterium-derived OMVs in infectious/inflammatory diseases

Currently, it is recognized that OMVs released by Gram-negative bacteria can evoke pathological conditions *in vivo*. For example, OMVs from pathogenic bacteria contribute to the hypercoagulable response in sepsis by influencing the inflammatory and coagulation cascades[163, 164]. In a mouse model, intraperitoneal injection of OMVs derived from intestinal *E. coli* induced a sepsis-like syndrome associated with systemic induction of TNF-α and IL-6 and with significant lethality[173]. Similarly, continuous infusion of *E. coli* OMVs into rats elicited physiological, biochemical, and histological changes that resembled clinical sepsis symptoms[174]. Indeed, LPS-carrying OMVs were found to contribute to fatal endotoxin levels observed in the serum of a patient [175].

In addition, some Gram-negative bacterial OMVs can mediate pulmonary inflammation. For instance, OMVs derived from *M. catarrhalis* and *Klebsiella pneumoniae* have been shown to induce neutrophil and lymphocyte infiltration into the lungs of mice[141, 176]. Moreover, Park *et al*[173] recently showed that intranasal administration of *P. aeruginosa* OMVs into mice caused pulmonary inflammation without any live bacteria *in vivo*. Thus, it is speculated that bacterial OMVs may be causative agents in the pathogenesis of specific infectious diseases. However, little is known about the dissemination and distribution of these vesicles *in vivo*, although this information could provide critical clues into their functions in living organisms. Recently, for the first time, Jang *et al*[177] examined the *in vivo* distribution of *E. coli* OMVs after intraperitoneal injection in mice. They observed that the OMVs spread throughout the body and accumulated in the liver, lungs, spleen, and kidney within 3 h after administration. They also performed functional analyses on the consequences of OMV administration by investigating systemic and lung inflammation[177] and

observed (1) increased levels of TNF-α and IL-6 in serum and bronchoalveolar lavage fluid as an early systemic inflammatory response, (2) fewer leukocytes and platelets in the blood, (3) decreased body temperature, and (4) increased ICAM-1 levels in the lung. These observations suggested that bacterial OMVs could act as effective mediators of long distance communication *in vivo*.

Gram-positive bacterium-derived MVs in infectious/inflammatory diseases

While direct evidence of MV formation in Gram-positive bacteria has just emerged, several recent studies have also suggested that these Gram-positive bacterial MVs may significantly contribute to pathophysiological conditions during bacterial infection[178, 179]. For example, Hong *et al*[178] observed that the *in vitro* application of *S. aureus* EVs increased the production of pro-inflammatory mediators (IL-6, thymic stromal lymphopoietin, macrophage inflammatory protein-1α, and eotaxin) in dermal fibroblasts. The *in vivo* application of *S. aureus* EVs into tape-stripped mouse skin caused severe atopic dermatitis-like skin inflammation and epidermal thickening by increasing the infiltration of eosinophils and mast cells. *S. aureus* MVs were also observed to induce both Th1 and Th17 neutrophilic pulmonary inflammation and facilitated airway hypersensitivity to inhaled allergens. More interestingly, Kim *et al*[179] observed the presence of *S. aureus* MVs in house dust and demonstrated their role as important causative agents of pulmonary inflammatory diseases.

Other Gram-positive bacteria, including *Bacillus* spp., *B. anthracis, S. coelicolor, L. monocytogenes*, *S. pneumoniae*, *C. perfringens*, and *S. mutans*, also produce MVs[13]. However, their possible pathological roles *in vivo* have not been investigated. It is clear that these MVs could also contribute significantly to disease processes during bacterial infection.

Preventive and therapeutic effects of bacterial MVs in human diseases

OMVs are naturally released exocytic vesicles of Gram-negative bacteria that function in a number of processes, including biofilm formation, virulence protein secretion, and cell-to-cell communication[26]. Importantly, because of their distinct immunological and structural features, including nanometer-scale vesicle structure, self-adjuvant effectiveness, potential for genetic modification, and the ability to present exogenous proteins and carry immune stimulators, OMVs have received increased attention as emerging and feasible vaccine carriers[180]. Numerous studies have shown that OMV-derived vaccines could induce protective immunity against infections with pathogenic bacteria, such as *N. meningitides, A. baumannii, P. gingivalis, S. Typhimurium, H. pylori,* and *V. cholerae,* in animal models (see reviews elsewhere[180-182]). The following sections highlight recent findings on OMV-derived vaccines and therapeutic agents for human diseases.

Neisseria meningitides-derived OMV vaccine

For several serogroups of *N. meningitidis* (A, C, W, X), effective conjugate vaccines consisting of a capsular polysaccharide coupled to a carrier protein are on the market. However, for serogroup B, this approach is not feasible because this polysaccharide exhibits homology to molecular structures in the human brain, generating the risk of autoimmunity[183]. Therefore, the development of an *N. meningitidis* serogroup B (MenB) vaccine has focused on other approaches, and MenB-derived OMVs, as an effective vaccine, have been extensively studied over the last several decades[183]. Thus far, OMV vaccines have been used successfully to combat outbreaks of meningitis in Cuba, Norway and New Zealand, where monovalent detergent-extracted OMVs were prepared from a manufacturing strain matching the circulating strains causing the epidemic[182]. In the Netherlands, a multivalent immunodominant OM protein porin A (PorA)-based OMV vaccine has been developed based on genetically engineered strains in which multiple *porA* genes were inserted[184]. Recently, trivalent native OMVs derived from three genetically modified *N. meningitidis* serogroup B strains have been evaluated immunologically in mice, rabbits and human infants[185]. This study suggests that the cross-reactive antibodies generated by one or more OMV antigens may provide further protection against serogroup B meningococcal disease caused by heterologous strains.

Acinetobacter baumannii-derived OMV vaccine

Acinetobacter baumannii, a non-fermenting, Gram-negative, aerobic coccobacillus, is currently one of the major causes of hospital-acquired infections and is usually associated with pneumonia and sepsis in intensive care unit patients[186]. Notably, *A. baumannii* has been shown to secrete OMVs into the blood circulation, through which it sheds virulence factors (i.e., AbOmpA protein) and delivers them to mammalian cells, inducing cell death[187]. In recent years, epidemics caused by multi- or pan-drug-resistant strains of *A. baumannii* have been widely investigated and reported[186]. Thus, there is an urgent need to develop innovative approaches to prevent and treat such multi- or pan-drug-resistant infections. To this end, several groups recently identified various vaccine candidates [*eg*, inactivated whole cell, outer-membrane complexes , single outer-membrane proteins (AbOmpA, Bap, and Ata)] that have an effect on controlling *A. baumannii* infections (see reviews elsewhere[188]). McConnell *et al*[189] also indicated that vaccination using OMVs could be considered as a viable strategy for preventing *A. baumannii* infection. However, OMVs contain a wide range of LPSs that may induce uncontrolled inflammatory responses. Therefore, reducing LPS endotoxicity is an important goal for achieving an optimized immune response. Recently, it was identified that heterologous expression of the 3-*O*-deacylase PagL could remove the 3-*O*-linked acyl chain from the disaccharide backbone of lipid A[190]. This suggests that OMVs purified from a bacterium that expresses PagL could be used as an optimized vaccine candidate against *A. baumannii* infection. Indeed, Badmasti *et al*[191] evaluated the immunological effects of OMVs containing the signal peptides $Bap_{(1\text{-}487aa)}$ and $AbOmpA_{(8\text{–}346aa)}$ and PagL as well as their

combinations [PagL-OMVs/$Bap_{(1\text{-}487aa)}$ and $AbOmpA_{(8\text{-}346aa)}$/$Bap_{(1\text{-}487aa)}$] in a C57BL/6 murine model of disseminated sepsis. They observed that although the AbOmpA and Bap peptides were excellent vaccine targets, they did not provide full protection by themselves against *A. baumannii* infection. By contrast, PagL-OMVs/$Bap_{(1\text{-}487aa)}$-immunized mice displayed robust humoral/cellular immune responses, resulting in full protection against *A. baumannii* ATCC 19606 and MDR AB-44 strains. Together, these findings may provide a new strategy for developing an *A. baumannii* vaccine.

Other bacterium-derived OMV vaccines

Vibrio cholerae-derived OMV vaccine

The Camilli laboratory[192-194] has recently developed a *V. cholerae* OMV vaccine delivered intranasally, which represents an alternative vaccine approach to the currently licensed whole-cell-killed (WCK) and live attenuated vaccines, both delivered orally. Schild's group[195] also reported that immunization with genetically detoxified *V. cholerae* OMVs could elicit a protective immune response. They tested the potential of LPS-modified and toxin-negative OMVs isolated from *V. cholerae* and enterotoxigenic *E. coli* (ETEC) as a combined OMV vaccine candidate. They found that immunization with *V. cholerae* or ETEC OMVs induced a species-specific immune response, whereas the combination of both OMV species resulted in a high-titer, protective immune response against both pathogens[195].

Porphyromonas gingivalis-derived OMV vaccine

Periodontitis is the most prevalent infectious disease related to oral and systemic health, and a novel prophylaxis to prevent the disease is therefore highly desirable[196]. Recently, the Senpuku laboratory reported that intranasal immunization using a combination of *P. gingivalis* OMVs and a TLR3 agonist, Poly (I:C), efficiently elicited a *P. gingivalis*-specific antibody response[196]. They observed that intranasal vaccination of OMVs in mice elicited the production of *P. gingivalis*-specific antibodies in blood and saliva in a dose-dependent manner, which was dramatically enhanced by the addition of the TLR3 agonist Poly (I:C). Serum samples from these immunized mice revealed significant inhibition of the gingipain proteolytic activity of not only the vaccine strain but also heterologous strains.

Burkholderia pseudomallei-derived OMV vaccine

Burkholderia pseudomallei (*Bps*) is the causative agent of melioidosis and is endemic in regions of northern Australia and Southeast Asia[197]. *Bps* is naturally resistant to multiple antibiotics, and there is currently no approved vaccine against the organism[197]. Recently, the Morici laboratory has shown that immunization with natural Bps-derived OMVs provides significant protection against lethal aerosol and systemic infections in BALB/c mice[197]. They further evaluated the safety and immunogenicity of escalating doses of the OMV vaccine in rhesus macaques and observed the generation of humoral immune responses with no associated toxicity or reactogenicity in *Bps* OMV-immunized animals[198]. Nonetheless, future studies are warranted to evaluate the protective efficacy of the OMV vaccine in a nonhuman primate model of melioidosis.

Engineered OMVs as a universal vaccine

The following points are well appreciated: 1) heterologous proteins expressed in *E. coli* cells can be distributed in the periplasmic space and packed into shedding OMVs[25]; 2) expressed heterologous proteins can anchor to the surface of *E. coli* cells and are also presented on the shedding OMVs[199]; 3) technologically, fusion with an intrinsic outer-membrane protein (*eg*, a pore-forming hemolytic protein, ClyA) can bring exogenous proteins to the surface of OMVs[200]; and 4) exogenous proteins can also be presented in the inner lumen of OMVs using an appropriate leader protein[25]. Thus, OMVs could be engineered by genetic manipulation to present exogenous proteins of interest. Such engineered OMVs can serve as an antigen-delivery platform for the efficient induction of specific antibody responses. Huang *et al*[201] recently utilized recombinant gene technology to overexpress a previously identified immunogenic outer-membrane protein of *A. baumannii*, Omp22, in the non-pathogenic *E. coli* DH5*a* strain. They observed that *E. coli* DH5*a*-derived OMVs (Omp22-OMVs) could display a complete Omp22 protein on their surfaces without any alterations in their OMV morphology. Immunization with Omp22-OMVs induced high titers of Omp22-specific antibodies. In a murine sepsis model, Omp22-OMV immunization significantly protected mice from lethal challenge with a clinically isolated *A. baumannii* strain, as evidenced by 1) the increased survival rate of the mice; 2) the reduced bacterial burdens in the lungs, spleen, liver, kidney, and blood; and 3) the suppressed serum levels of inflammatory cytokines. These results clearly indicate that engineered OMVs could display a whole heterologous protein on the surface and effectively trigger specific antibody responses and that OMVs may therefore be utilized as a new and feasible antigen delivery tool for a vaccine to induce protective immunity.

However, there are several concerns associated with the commercialization of OMVs as vaccines in humans: 1) although most of the OMVs considered safe for use in vaccine or drug delivery are LPS-free, increasing evidence suggests that other components of the OMVs besides LPS can also provoke systemic inflammatory response syndrome, leading to lethality in mice; and 2) bacteria release relatively low quantities of OMVs, indicating that their cost-effective mass production may become inconvenient. Regarding these concerns, Kim *et al*[202] recently developed bacterial protoplast-derived nanosized vesicles (PDNVs), which are depleted of outer-membrane components, as a safe and effective adjuvant-free vaccine delivery system. By fabricating protoplasts of genetically engineered antigen-expressing *E. coli* strains, antigen-loaded PDNVs can be easily produced with a significantly higher yield, and, importantly, these PDNVs are less toxic than *E. coli* OMVs. Moreover, they observed that immunization with antigen-loaded PDNVs induced potent antigen-specific humoral and cellular immune responses, leading to effective protection against both Gram-negative and Gram-

positive bacterial sepsis in murine models. Given that the preventive effect of a vaccine is specific to the specific antigen, the use of PDNVs loaded with a single or multiple bacterial antigens may enhance future vaccination efficacy against most virulent bacterial infections. Hence, these nanosized bacterial protoplast-derived vesicles may represent a versatile system that could accommodate either viral or bacterial antigens for future applications as a novel universal vaccine to prevent various infectious diseases. Nonetheless, further studies are required to assess the overall safety of PDNV-based vaccination and to elucidate the key components and detailed mechanism underlying the induction of antigen-specific protective immune responses by PDNVs.

Conclusions

Although bacteria-released OMVs have been studied for almost 4 decades, only in recent years have we witnessed the great advances in our understanding of the mechanisms by which OMVs contribute to bacterial-triggered inflammation and pathology and of their ability to modulate the immune system by suppressing inflammation and facilitating the survival of their parent bacterium in the host[1-3]. The recent identification of MVs produced by some Gram-positive bacteria, which have immuno-pathological and potentially immunomodulatory roles similar to those of OMVs, indicates that there is still much to learn about the involvement of bacterial MVs in human diseases[133]. Notably, the observation of OMVs present in indoor dust may suggest their possible contributions to airway disease[179], which should be further explored. In addition, there is increased interest in exploring the possible contributions of the host microbiota to the development of immunity to gastrointestinal diseases[181]. Given the beneficial contribution of commensal OMVs to gastrointestinal integrity, future studies are needed to better understand the potential protective mechanisms of commensal OMVs against pathogens and how to genetically engineer probiotic *E. coli* OMVs.

Moreover, there has been limited use of OMV-based technologies to deliver antigens and protective proteins to specific organs/tissues. Recently, a mutant *E. coli* strain that exhibits reduced endotoxicity toward human cells was engineered to generate OMVs displaying a human epidermal growth factor receptor 2 (HER2)-specific affibody in the membrane as a targeting ligand. These OMVs delivered anti-tumorigenic cargo that resulted in cell death and tumor regression in mice[203]. This study has expanded our potential use of OMVs beyond vaccine carriers and implicates bioengineered OMVs as new cell-specific drug-delivery vehicles for treating various cancers. Finally, while several OMV vaccines have been clinically applied to combat some infectious diseases, concerns such as serum resistance still remain[180-182]. With the technological advances in our ability to modify the composition of OMVs, it is expected that OMVs will have broad therapeutic and preventive applications for human health.

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