

# Chapter 8

## Immunodetection and Pathogenesis Mediated by Bacterial Membrane Vesicles



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**Abstract** Animals have evolved sophisticated means to detect and defend themselves against colonization by microorganisms. Mammals differentiate self from nonself by the use of germline encoded pattern recognition receptors (PRRs) of the innate immune system in addition to pathogen-specific recognition by the adaptive immune system. Conserved microbial structures, termed microbe-associated molecular patterns (MAMPs) are recognized by the innate immune system. Pathogens succeed in subverting these immune mechanisms by the production of effector proteins and modification of MAMPs. While many PRRs and their cognate ligands have been extensively examined, most studies addressed the immune response toward the whole microorganism, isolated MAMPs, or effector proteins. Both Gram-negative and Gram-positive bacteria produce membrane vesicles that contain several MAMPs and bacterial effector proteins, and the study of the interplay of these with immune PRRs is a recently emerging field. Here we summarize key components of the innate and adaptive immune systems and discuss current knowledge regarding the immune recognition and immunomodulatory functions of bacterial membrane vesicles in mammalian hosts.

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## 8.1 Bacterial Membrane Vesicles

Both Gram-negative and Gram-positive bacteria produce vesicles known as outer membrane vesicles (OMVs) or membrane vesicles (MVs), respectively. They contain components derived from the parent bacterium, including proteins, lipids, and nucleic acids (reviewed in Schwechheimer and Kuehn 2015). Originally thought to be an artifact of bacterial growth, recent studies have demonstrated that OMVs and MVs may contribute to disease pathogenesis and progression due to their immunostimulatory cargo. OMVs are naturally released by bacteria as part of their normal growth. There are a number of mechanisms by which OMVs are produced and bacteria can actively increase their production (Kulp and Kuehn 2010). In contrast to OMVs, the mechanisms of MV biogenesis are not well characterized. This is because Gram-negative OMVs were discovered long before Gram-positive MVs, and consequently we have a greater understanding of OMV production, composition, and their effects on the host. Although research has been focused on examining the immunostimulatory effects of OMVs derived from pathogens, recent studies have begun investigating the role of OMVs from commensal bacteria in modulating the hosts immune response. In contrast, due to their more recent discovery, limited studies have examined the immunostimulatory effects of Gram-positive MVs on the host.

A number of studies have suggested that OMV production may be increased during the course of infection; however, the mechanisms behind OMV biogenesis during infection have not been completely elucidated. It has been identified that the disruption of genes encoding for outer membrane proteins and peptidoglycan synthesis can lead to changes in OMV production (McBroom et al. 2006). OMV production is also thought to be a response to stress stimuli such as antibiotic treatment, envelope stress, and increase in temperature (Baumgarten et al. 2012; Kadurugamuwa and Beveridge 1995; Macdonald and Kuehn 2013; McBroom and Kuehn 2007; Yun et al. 2018). Moreover, the immunogenicity of OMV cargo can be altered by certain bacteria, such as *Vibrio cholerae*, in response to changes in the expression of virulence factors upon quorum sensing (Bielig et al. 2011). Therefore, it is thought that during infection, factors such as environmental stress, bacterial regulators, and host immune molecules contribute to increased production of bacterial OMVs. Collectively, this increased production of OMVs and their composition of immunogenic cargo is thought to contribute to pathogenesis.

### 8.1.1 Bacterial Membrane Vesicles Contain Immunogenic Cargo

Numerous studies have demonstrated the pro-inflammatory nature of Gram-negative OMVs, their ability to stimulate the immune system, and their use as vaccines for humans (Holst et al. 2009; Kaparakis-Liaskos and Ferrero 2015; Manning and

Kuehn 2011; Ellis and Kuehn 2010; Pathirana and Kaparakis-Liaskos 2016). OMVs have been observed in a number of samples from human patients and tissue biopsies. For example, OMVs from *Neisseria meningitidis* were observed using electron microscopy in the cerebrospinal fluid of an infected infant, and in samples from a patient with fatal meningococcal disease (Stephens et al. 1982; Namork and Brandtzaeg 2002). Additionally, OMVs from *Helicobacter pylori* have been found in gastric biopsies from infected individuals and in cultures from several clinical patient samples (Fiocca et al. 1999; Keenan et al. 2000). OMVs have also been observed in samples from the sinus of a child infected with *Moraxella catarrhalis* (Vidakovics et al. 2010), and in the lungs of patients with nontypeable *Haemophilus influenzae* infections (Ren et al. 2012). In addition to their presence during infection, a number of studies have revealed that OMVs contain specific microbe-associated molecular patterns (MAMPs), known to stimulate the immune system, such as lipopolysaccharide (LPS), peptidoglycan (PG), nucleic acids, as well as other bacterial cell wall components (Vanaja et al. 2016; Chatterjee and Das 1967; Renelli et al. 2004; Choi et al. 2011).

In addition to their ability to carry immunostimulatory cargo, OMVs have been shown to be important in the structure and function of biofilms, in the environment as well as during the course of infection (Schooling and Beveridge 2006; Yonezawa et al. 2009). Additionally, they are able to act as bacteriophage and antibiotic decoys and can “trap” complement proteins produced by the host immune system (reviewed in Manning and Kuehn 2011; Tan et al. 2007). Because of this, it is thought that OMVs may be harnessed by bacteria to further establish colonization and pathogenesis within the host.

Conversely, the biogenesis, contents and immunostimulatory ability of Gram-positive MVs is not well characterized. Some studies have reported that Gram-positive MVs contain immunogenic cargo (Lee et al. 2009; Gurung et al. 2011; Peschel et al. 2001; Rivera et al. 2010); however, further studies are required to elucidate their immunogenic profiles and interactions with the host immune system. Chapter 3 contains a more detailed review of the biogenesis and functions of Gram-positive MVs.

The contribution of bacterial OMVs and MVs to pathogenesis and immunity is quickly becoming apparent as more studies uncover their roles as a novel transport system for bacterial derived molecules. Here we discuss the various mechanisms by which OMVs are able to interact with the host’s innate and adaptive immune system, their contributions to pathogenesis, as well as the recent discovery of immunogenic Gram-positive MVs.

## 8.2 Bacterial Membrane Vesicles Interact with and Modulate the Hosts Innate Immune System

In order to survive within a human host, microorganisms must circumvent host immune defenses. All multicellular organisms have developed innate defense mechanisms which have the capacity to be activated by foreign microorganisms and that

function to defend the host by destroying pathogens and neutralizing their virulence factors (Medzhitov and Janeway 1997). One of the major mechanisms by which bacteria are able to interact with host cells is by the secretion of immunostimulatory products (Young et al. 1999; Bruno et al. 2009). Bacterial membrane vesicles are released from both Gram-negative and Gram-positive bacteria and act as secretory transporters for proteins, lipids, and other bacterial products to host cells (reviewed in Ellis and Kuehn 2010). The innate immune system is the first line of defense against invading and resident bacteria, and it is able to rapidly detect and initiate an immune response to bacteria membrane vesicles. In humans, the immune system is comprised of innate and adaptive immunity. The innate immune system is the frontline defense against bacteria, whereas adaptive immunity functions to develop pathogenic-specific immune responses while generating long-lived immunity.

### **8.2.1 *OMVs Protect Bacteria Against Host Innate Immune Molecules***

The hosts innate immune system is multifaceted in its ability to respond to pathogens and is composed of a range of defense mechanisms which include innate immune receptors, innate immune cells, and the production of antimicrobials and inflammatory molecules. For example, pathogens can trigger the activation of the complement system, a group of small proteins made by hepatocytes which circulate in the bloodstream. The complement system enhances the ability of antibodies and phagocytic cells to clear microbes, in addition to damaging bacteria by attacking their cell membrane (Rus et al. 2005). Although part of the innate immune system, complement can also regulate aspects of the adaptive immune system by recruiting and activating immune cells using anaphylatoxins, small peptide mediators (Barrington et al. 2001).

Pathogens are often able to inhibit activation of the complement system; however, the mechanisms by which this can occur have not been entirely elucidated (Joiner 1988). It has been shown that *Porphyromonas gingivalis* OMVs can degrade complement components such as the complement 5a (C5a) cluster of differentiation 88 receptor (CD88) (Jagels et al. 1996) and CD14 from the surface of human macrophage cells. CD88 and CD14 are both involved in the recognition of bacteria (Duncan et al. 2004) and their targeting leads to reduced efficacy of immune responses and allows bacteria to evade the immune system. Similarly, *Haemophilus influenzae* can survive complement-mediated attacks when supplemented with *M. catarrhalis* OMVs, further potentiating a mutually beneficial role for invading pathogens and their OMVs (Tan et al. 2007).

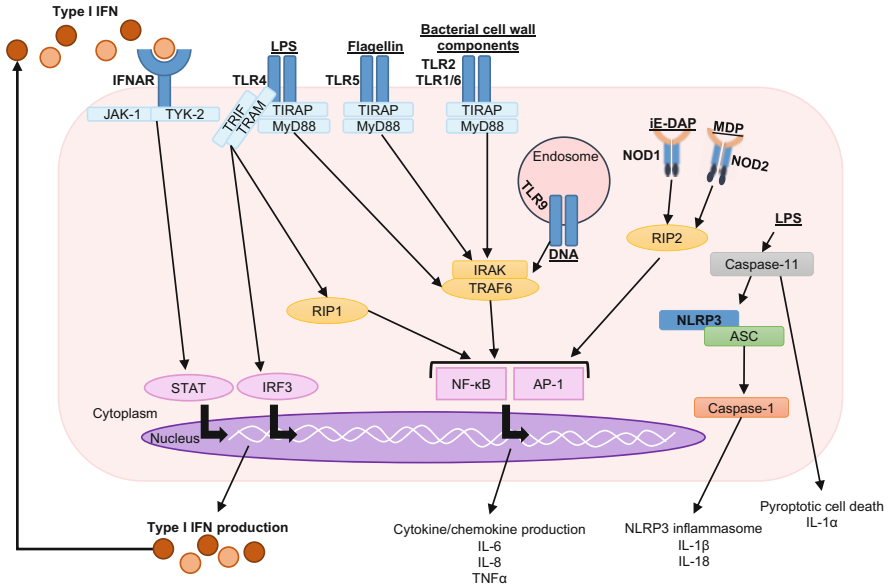
Moreover, the innate immune system also employs antimicrobial peptides (AMPs), such as defensins, as another defense mechanism against invading pathogens. AMPs can directly attack invading microbes to limit pathogenesis; however, resistance to AMPs has been observed for a number of pathogens, including *Staphylococcus aureus* (Peschel et al. 2001), *Salmonella enterica* serovar

Typhimurium (*S. Typhimurium*) (Gunn et al. 2000), *Listeria monocytogenes* (Abachin et al. 2002), and *Pseudomonas aeruginosa* (Moskowitz et al. 2004). OMVs have been demonstrated to contribute to AMP resistance, as *V. cholerae* OMVs were shown to protect other bacteria from membrane attack by AMPs (Duperthuy et al. 2013). This was proposed to be a more general mechanism of immune evasion, where OMVs can function to sequester host immune molecules (Band and Weiss 2015). Moreover, the host limits the availability of transition metals by siderophores, such as calprotectin, to restrict pathogen growth, referred to as “nutritional immunity” (Hood and Skaar 2012).

### ***8.2.2 Recognition of Bacterial Membrane Vesicles by Host Pattern Recognition Receptors Results in a Pro-Inflammatory Response***

An integral feature of the innate immune system involves a number of specific pattern recognition receptors (PRRs), which recognize MAMPs and subsequently results in the generation of a pro-inflammatory innate immune response (Janeway and Medzhitov 2002). MAMPs are conserved microbial structures such as bacterial nucleic acids and bacterial cell wall components including LPS and PG, which are distinguishable from host molecules (Boller and Felix 2009). PRRs are protein receptors expressed by a number of innate immune cells such as neutrophils, macrophages, and dendritic cells (DCs) (Thomas and Schroder 2013; Takeuchi and Akira 2010) as well as non-immune cells, such as epithelial cells (Kawai and Akira 2010). Once PRRs are activated by detection of their respective MAMP ligand, a signaling cascade initiates the release of inflammatory mediators such as cytokines and chemokines which are responsible for the recruitment and the activation of immune cells and for mediating an inflammatory response (Kawai and Akira 2010). PRRs are vital for the recognition of, and downstream effects initiated by invading bacteria.

PRRs include Toll-like receptors (TLRs) which are membrane bound (Fig. 8.1), intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Fig. 8.1), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and C-type lectins, which can be either soluble or function as membrane associated receptors (Kawai and Akira 2008; Kanneganti et al. 2007). TLRs and NLRs are activated upon recognition of MAMPs, such as components of the bacterial cell wall or microbial DNA (Fig. 8.1). RIG-I-like receptors are responsible for the recognition of virally derived dsRNA within the cytoplasm (Wells et al. 2011), and C-type lectins contribute to innate immune responses to many pathogens (reviewed in Robinson et al. 2006). Activation of PRRs leads to a number of outcomes, namely the induction of signaling pathways resulting in the transcription of pro-inflammatory cytokines that drive inflammation, in an effort to combat the invading pathogen (Fig. 8.1). Bacterial membrane vesicles can activate these signaling pathways in a number of ways, which will be discussed below.



**Fig. 8.1** Bacterial products are detected by TLRs and NLRs by the host cell. On the surface of the host cell, TLR5 detects flagellin, TLR2 and TLR1/6 detect bacterial cell wall components and TLR4 detects LPS. Within the cytoplasm, caspase-11 detects LPS, and NOD1 and NOD2 detect peptidoglycan subunits iE-DAP and MDP, respectively. Endosome-bound TLR9 detects bacterial DNA. Detection of bacterial MAMPs leads to the recruitment of adaptor proteins, downstream activation of transcription factors and subsequent production of pro-inflammatory chemokines and cytokines or pyroptotic cell death in the case of caspase-11 activation. Type I IFN production can also occur in response to the detection of bacteria or bacterial products, via the JAK/STAT pathway

### 8.2.2.1 Recognition of Bacteria by Toll-like Receptors

Toll-like receptors (TLRs) are a family of transmembrane proteins expressed by both leukocytes and non-immune cells, such as epithelial and endothelial cells. They contain an extracellular leucine-rich repeat (LRR) domain that is necessary for the detection of MAMPs, a transmembrane domain and a cytoplasmic tail (Toll/interleukin-1 receptor (TIR) domain). TLRs can be found on the cell surface or on intracellular endosomal membranes. To date, 10 members of the TLR family have been identified in humans (Akira and Takeda 2004). TLRs are capable of detecting a range of molecules derived from bacteria, viruses, and fungi. Importantly, TLRs detect molecules such as LPS, PG, and nucleic acids from both Gram-positive and Gram-negative bacteria (Fig. 8.1).

Upon detection of MAMPs, most TLRs form either homodimers or heterodimers (Fig. 8.1). Once activated, adaptor molecules are recruited to TLRs to initiate downstream signaling. TLR2, TLR4, TLR5, and TLR1/6 signal via the adaptor proteins Myeloid Differentiation Primary Response Gene 88 (MyD88) and TIR-Domain-Containing Adaptor Protein (TIRAP). This leads to the activation of IL-1

Receptor-Associated Kinases (IRAKs) and the adaptor molecules TNF Receptor-Associated Factor 6 (TRAF6) and eventually the nuclear translocation of transcription factors Nuclear Factor Kappa B (NF- $\kappa$ B) and Activator Protein 1 (AP-1). TLR9 also uses this pathway; however, it is located on the endosomal membrane (Fig. 8.1). Both NF- $\kappa$ B and AP-1 have functions in the expression of pro-inflammatory cytokines (Fig. 8.1). As a result of NF- $\kappa$ B and AP-1 activation, cytokines and chemokines are produced, for example interleukin-6 (IL-6), IL-8 (CXCL8) and Tumor Necrosis Factor Alpha (TNF/TNF $\alpha$ ).

Additionally, TLR4 can signal independently of MyD88, via TIR-Domain-Containing Adaptor Inducing Interferon- $\beta$  (TRIF) and TRIF-related adaptor molecule (TRAM) (Fig. 8.1). This leads to the activation of adaptor molecules, such as Receptor-Interacting Protein 1 (RIP1), and the translocation of NF- $\kappa$ B and AP-1 or Interferon Regulatory Factor-3 (IRF3) to the nucleus. IRF3 is a transcription factor which induces the production of type 1 interferons (Fig. 8.1, Kumar et al. 2009). Type I IFNs can signal in a paracrine or autocrine manner by interacting with Interferon Alpha/Beta Receptor (IFNAR) on the cell surface, and signal using the Janus Kinases and Signal Transducer and Activation of Transcription Proteins (JAK/STAT) pathway (Fig. 8.1, Akira and Takeda 2004). Type 1 interferons have been demonstrated to limit bacterial infections by upregulating the production of host defense molecules (reviewed in Boxx and Cheng 2016). Therefore, membrane-bound TLRs are collectively important receptors of bacteria and bacterial products. In addition, intracellular receptors from other protein families are also vital in the host response to internalized MAMPs, as discussed below.

### 8.2.2.2 Recognition of Bacteria by Cytoplasmic Innate Immune Receptors

In the cytoplasm of the host cell, a large family of PRRs known as NOD-like receptors (NLRs) are expressed. They are characterized by the presence of a central conserved NTPase (NACHT) domain, required for nucleotide binding and self-oligomerization (Inohara et al. 2000). These proteins generally contain a variable N-terminal effector-binding domain, which is responsible for protein-protein interactions, as well as C-terminal LRRs to detect MAMPs (Inohara and Nunez 2001). NOD1 and NOD2 are cytoplasmic receptors that recognize bacterial peptidoglycan (PG). NOD1 recognizes  $\gamma$ -D-Glu-mDAP (iE-DAP), a dipeptide present in the PG of almost all Gram-negative and some Gram-positive bacteria. NOD2 recognizes muramyl dipeptide (MDP) which is common to PG of all bacteria (Girardin et al. 2003a, b).

Activation of either NOD1 or NOD2 leads to the recruitment of Receptor-Interacting Serine/Threonine-Protein Kinase 2 (RIP2, RIPK2) by their CARD domain, the activation of NF- $\kappa$ B (Inohara et al. 2000) and the production of pro-inflammatory cytokines and chemokines (Fig. 8.1). Other NLRs, such as those from the NLR-pyrin domain containing (NLRP) subfamily are namely responsible for the formation of “platforms,” known as inflammasomes, in response to host

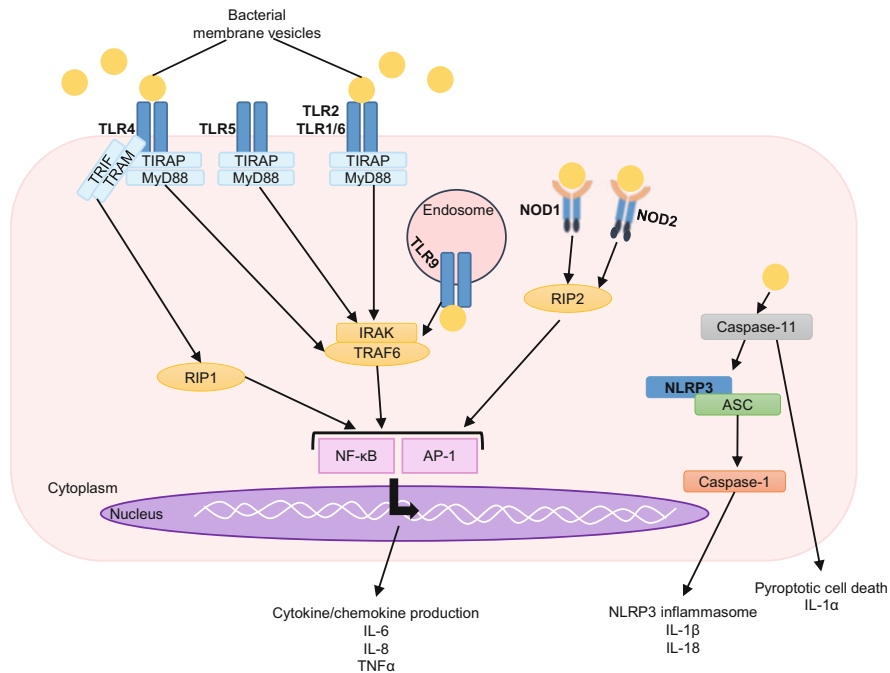
signals, which contribute to inflammation (Elliott and Sutterwala 2015). NLRP3, for example, can be triggered by caspase-11 activation by cytoplasmic LPS (Shi et al. 2014), cell membrane damage, and extracellular ATP released by damaged cells (Fig. 8.1, Martinon et al. 2002). Activation of these inflammasome forming NLRs leads to the recruitment of the adaptor Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC) that links NLRP3 to caspase-1 (Fig. 8.1). This complex, termed inflammasome, triggers caspase-1 activation, resulting in the production and release of the key inflammatory mediators IL-1 $\beta$  and IL-18, and leads to pyroptotic cell death (Schroder et al. 2010).

The activation of PRRs is a pivotal prerequisite to prime the adaptive immune system and to induce antigen-specific responses to bacterial pathogens (Fritz et al. 2007). As a result, TLRs and NLRs are key regulators of the innate immune response (Milward et al. 2007; Faure et al. 2000). Collectively, there are a range of mechanisms by which bacteria can interact with host PRRs to trigger TLR and NLR signaling to mediate a pro-inflammatory response.

### **8.2.2.3 Bacterial Membrane Vesicles Are Detected by Host Pattern Recognition Receptors Resulting in the Induction of an Innate Immune Response**

The mucosal epithelial cell surface is the first line of defense in the host, and often the location where bacterial vesicles first come into contact with the host. The gut epithelium and the underlying lamina propria contains a diverse subset of innate and adaptive immune cells within the gut-associated lymphoid tissue (GALT). Long seen as a classical barrier, we now know that the intestinal epithelium is actively involved in immune regulation (Rhee et al. 2004). Epithelial cells express PRRs and can detect pathogens and respond to cytokines, such as IL-22, to produce antimicrobial peptides to control the microbiota composition and to secrete cytokines that recruit immune cells (Peterson and Artis 2014). The mucosal epithelial cell surface has evolved to cope with the constant presence of microbial products and foreign antigens without inducing inflammation. Indeed, the presence of microbiota and their derived MAMPs are needed for the development of the GALT and to maintain tissue homeostasis (Stappenbeck et al. 2002). A wealth of current work aims to delineate how the host can mediate tolerance of commensals at the mucosal surface, while still being able to detect and respond toward bacterial pathogens. We still know little about the microbial substances and molecular details that mediate these responses, and the role of OMVs and MVs in these processes remains largely elusive. It has been demonstrated that the interaction of OMVs with host epithelial cells results in the production of cytokines and chemokines. Therefore, OMVs and MVs are thought to be a mechanism whereby bacteria can transport their products to non-phagocytic cells at the mucosal surface in order to modulate innate and adaptive immune responses which will be discussed below.





**Fig. 8.2.** Bacterial membrane vesicles interact with PRRs on the surface and cytoplasm of host cells to initiate an innate immune response. OMVs produced by *E. coli*, *V. cholerae*, and *H. pylori*, for example, can interact with TLR4, TLR2, and TLR1/6 on the surface of the cell. OMVs produced by *H. pylori* and *A. actinomycetemcomitans* can interact with NOD1 and NOD2, respectively. The activation of these PRRs results in the induction of an intracellular signaling cascade that ultimately results in the production of pro-inflammatory cytokines and chemokines

### 8.2.2.4 Gram-Negative OMVs Are Detected by PRRs Expressed by Epithelial Cells

A number of *in vitro* studies using human epithelial cells have reported the production of chemokines and cytokines in response to stimulation by bacterial OMVs. *H. pylori* is a gastric pathogen, which produces OMVs demonstrated to modulate the proliferation of, and IL-8 production by gastric adenocarcinoma cells (Ismail et al. 2003). This was the first study to indicate that OMVs could induce pro-inflammatory cytokine production by human epithelial cells. Further investigation revealed that the production of IL-8, stimulated by *H. pylori* OMVs was dependent on detection of their peptidoglycan cargo by the intracellular NOD1 receptor (Fig. 8.2, Kaparakis et al. 2010). Similarly, OMVs from the pathogen *V. cholerae* were found to induce the production of IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in a NOD1-dependent manner (Fig. 8.2, Chatterjee and Chaudhuri 2013; Bielig et al. 2011). NOD2 is able to detect PG from both Gram-positive and Gram-negative membrane vesicles, and has been shown to induce a pro-inflammatory immune

response upon the detection of OMVs, such is the case for the pathogen *Aggregatibacter actinomycetemcomitans* (Fig. 8.2, Thay et al. 2014). In addition, OMVs from the Gram-negative pathogen *Legionella pneumophila* were found to induce the production of a number of chemokines and cytokines by human alveolar epithelial cells (Galka et al. 2008). OMVs from periodontal pathogens have also been implicated in the progression of disease by interaction with epithelial cells, similarly to the studies discussed above (Cecil et al. 2019).

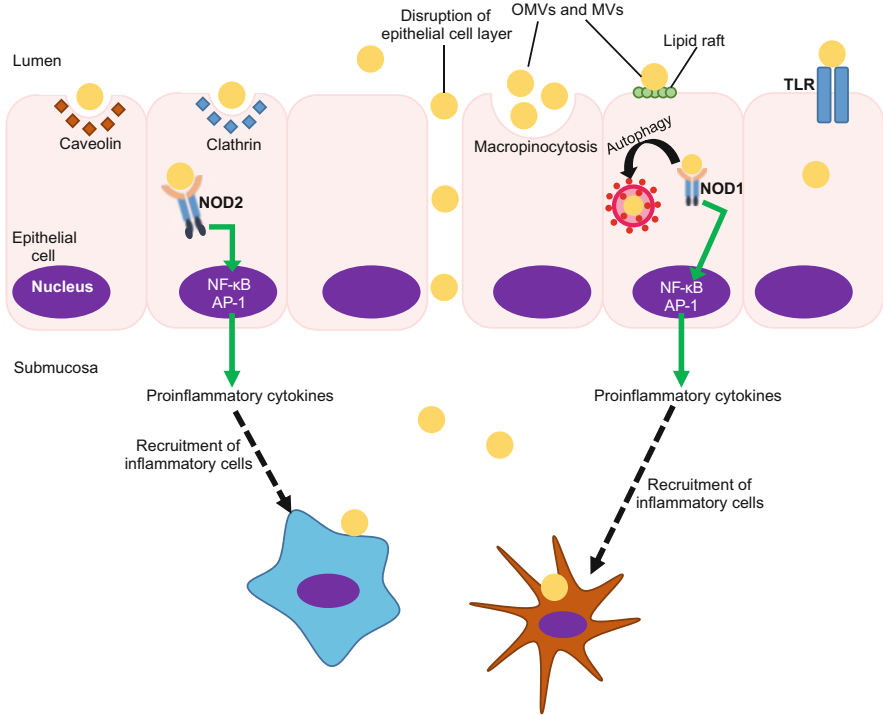
Opportunistic pathogens can utilize OMVs as a virulence factor when establishing disease. *P. aeruginosa* produces OMVs which can stimulate IL-8 production by primary human bronchial epithelial cells (Bauman and Kuehn 2006). OMVs from the opportunistic pathogen, *A. baumannii* were also found to upregulate the production of a number of cytokines and chemokines by human epithelial cells (Jun et al. 2013).

Lastly, OMVs derived from the commensal Gram-negative bacterium *Bacteroides fragilis* have been shown to modify the expression of TLR2 and TLR4 in epithelial cells, in addition to increasing the expression of anti-inflammatory cytokines (Ahmadi Badi et al. 2019). OMVs from commensal and probiotic *E. coli* can interact with epithelial cells and activate NOD1, leading to the release of the pro-inflammatory cytokines IL-6 and IL-8 (Fig. 8.2, Canas et al. 2016). Collectively, these studies demonstrate the complex role Gram-negative OMVs play at the epithelium in immune tolerance, and conversely, in the initiation of an innate immune response by pathogens.

#### 8.2.2.5 Gram-Positive MVs Interact with Host Epithelial Cells

Conversely, a limited number of studies have demonstrated the immunogenic ability of Gram-positive MVs. A proteomic study examining MVs produced by *S. aureus* found that they are enriched in penicillin-binding proteins which contribute to antibiotic resistance as well as proteins which have pathological functions during infection (Lee et al. 2009). Later, it was demonstrated that *S. aureus* MVs can induce inflammation in dermal fibroblasts, but not human epithelial cells, suggesting a potential role for MVs in mediating inflammation during atopic dermatitis (Jun et al. 2017). Another study found that MVs from *S. aureus* were able to enter and induce host cell death in the human epithelial cell line (HEp-2) (Gurung et al. 2011).

In addition to epithelial cells, MVs are thought to interact with various other cell types throughout the host, both immune and non-immune. Overall, bacterial membrane vesicles are known to interact with epithelial cells and are thought to disseminate throughout the host to interact with cells, such as macrophages, neutrophils, and DCs, distal to the site of infection.



**Fig. 8.3** Bacterial membrane vesicles interact with epithelial cells via a range of different mechanisms to mediate pathogenesis in the host. OMVs and MVs enter host cells via lipid rafts. OMVs can enter cells in a lipid-raft independent manner, via caveolin- or clathrin-mediated endocytosis, macropinocytosis, or by TLR-mediated endocytosis. Adherence and entry into host cells results in the production of pro-inflammatory cytokines and the recruitment of inflammatory innate immune cells. Within epithelial cells, OMVs can be degraded by autophagy. These processes, as well as the delivery of cytotoxic molecules by OMVs to the host cell can initiate the disruption of the epithelial cell layer, allowing OMVs to enter the submucosa

### 8.3 Bacterial Membrane Vesicles Adhere to, Fuse and Enter Host Cells to Mediate a Pro-Inflammatory Response

OMVs have been identified in a diverse range of host tissues, signifying their ability to travel and exist in a range of environments in the host. In addition to interacting with host surface PRRs, OMVs have been shown to adhere, fuse with, and enter host cells in order to contribute to inflammation, pro-inflammatory immune response, and mediate pathogenesis (Fig. 8.3, Bomberger et al. 2009; Furuta et al. 2009; Kaparakis et al. 2010). Early studies demonstrated the ability of OMVs to fuse with host cells and subsequently release their contents into the cytoplasm (Kadurugamuwa and Beveridge 1998). More recent studies confirmed these findings, and additional examples of OMV uptake into host cells were identified.

### **8.3.1 Lipid Raft-Dependent Entry of OMVs and MVs into Epithelial Cells**

The most commonly observed mechanism of entry for bacterial OMVs into non-phagocytic host cells involves cholesterol-rich lipid rafts (Fig. 8.3). This has been demonstrated for entry of OMVs produced by Gram-negative pathogens such as enterotoxigenic *E. coli*, *Campylobacter jejuni*, *H. pylori*, *H. influenzae*, *P. aeruginosa*, and *P. gingivalis* (Bauman and Kuehn 2009; Sharpe et al. 2011; Kaparakis et al. 2010; Kesty et al. 2004; Elmi et al. 2012; Furuta et al. 2009; Bomberger et al. 2009). Although not well-characterized, MVs are also thought to enter host cells via cholesterol-rich lipid rafts (Gurung et al. 2011; Thay et al. 2013).

### **8.3.2 Lipid Raft-Independent Mechanisms of OMV Entry into Epithelial Cells**

OMVs have also been shown to enter host cells by mechanisms which are independent of lipid rafts (Parker et al. 2010). Clathrin-mediated endocytosis of bacterial OMVs into host cells can be triggered by receptor-mediated binding of ligands at the cell surface and subsequent internalization (Fig. 8.3) (reviewed in O'Donoghue and Krachler 2016). The internalization of clathrin-coated pits leads to degradation of the material contained within these endocytic vacuoles (Rewatkar et al. 2015). Several studies have identified OMV entry into host epithelial cells via clathrin-mediated endocytosis, including OMVs produced by *H. pylori* (Olofsson et al. 2014). However, other studies have demonstrated that inhibition of clathrin-mediated endocytosis did not abolish, but only reduced the entry of *H. pylori* OMVs into host epithelial cells implying there are several mechanisms of entry into host cells (Parker et al. 2010). Caveolin-mediated endocytosis has also been implicated in the uptake of bacterial OMVs into host cells (Fig. 8.3, reviewed in O'Donoghue and Krachler 2016). Another suggested mechanism of OMV entry into host cells is by macropinocytosis; however, this is not thought to be a deliberate process (Fig. 8.3, Lim and Gleeson 2011). OMVs produced by *P. aeruginosa* have been shown to enter airway epithelial cells by macropinocytosis (Bomberger et al. 2009). Furthermore, it has recently been shown that OMV size can regulate their mechanism of entry into host cells, as *H. pylori* OMVs utilized distinct mechanisms of endocytosis or micropinocytosis to enter host cells based on their size (Turner et al. 2018). Collectively, these studies show that OMVs can utilize a range of mechanisms to enter host cells, and their mechanism of entry may be regulated based on receptor-mediated entry, OMV size or their content.

### **8.3.3 Detection and Degradation of Intracellular OMVs by Epithelial Cells**

Until recently, the intracellular fate of OMVs following their entry into host cells remained unknown. It was identified that upon entry into host epithelial cells, *H. pylori* and *P. aeruginosa* OMVs were detected by the innate immune receptor NOD1, resulting in the induction of an inflammatory response (Kaparakis et al. 2010). This finding was further supported by subsequent studies showing the ability of *V. cholerae* OMVs to be detected by NOD1 and NOD2 which resulted in NF- $\kappa$ B activation and the production of pro-inflammatory cytokines (Chatterjee and Chaudhuri 2013; Bielig et al. 2011). It was later shown that NOD1 was essential for the degradation of intracellular OMVs in host epithelial cells, as internalized OMVs were degraded via the host cellular degradation pathway of autophagy in a NOD1-dependent manner (Fig. 8.3, Irving et al. 2014). Specifically, the detection of PG contained within *H. pylori* and *P. aeruginosa* OMVs by the immune receptor NOD1, resulted in their degradation via autophagy (Fig. 8.3, Irving et al. 2014). Therefore, despite OMVs having a range of mechanisms to enter host cells, they can rapidly be degraded once intracellular via the host degradation pathway of autophagy.

Alternatively, bacterial OMVs are not only restricted to interacting with the mucosal cell surface or being internalized by host epithelial cells, as they are capable of disrupting and passing the epithelial cell layer entirely (Fig. 8.3). This has been demonstrated in studies using OMVs from *Treponema denticola* which can disrupt intact epithelium as a means to further mediate pathogenesis in the host (Chi et al. 2003), discussed further below.

## **8.4 Bacterial Membrane Vesicles Are Cytotoxic and Disrupt the Epithelial Cell Barrier**

A significant aspect of pathogenesis is the invasion of bacteria or their products into the host tissue. Bacterial membrane vesicles have a range of mechanisms whereby they can function to destroy the epithelial cell barrier integrity and promote cell death to ultimately create damage and promote disease in the host. These mechanisms involve disruption of the epithelial cell barrier and having cytotoxic activities.

### **8.4.1 OMVs Are Cytotoxic to Host Cells**

OMVs have been shown to disrupt the epithelial cell layer, enabling further penetration of bacteria and their products (Fig. 8.3). This has been observed for OMVs including those derived from enterobacteria such as *E. coli* (Wai et al. 2003; Kouokam et al. 2006), and the dental pathogens *T. denticola* and *P. gingivalis* (Chi et al. 2003;

Nakao et al. 2014). Once OMVs disrupt the integrity of the epithelial cell layer and gain access to the submucosa, they can interact directly with immune cells and stimulate an inflammatory response (Fig. 8.3, Nakao et al. 2014).

In addition to the disruption of the epithelium, OMVs can interrupt division of epithelial cells and induce death of innate immune cells, such as macrophages, neutrophils, and DCs. For example, *Acinetobacter baumannii* was shown to deliver cytotoxic OMVs to macrophages (Jin et al. 2011), and OMVs derived from *Actinobacillus actinomycetemcomitans* contained leukotoxins which could kill neutrophil-like cells in vitro (Kato et al. 2002). Moreover, *C. jejuni* OMVs contained cytolethal distending toxin, which disrupted the epithelial cell layer by interrupting cell division (Fig. 8.3, Lindmark et al. 2009).

Antibiotic treatment and stress of bacterial cells has widely been demonstrated to affect the production of OMVs; however, only a few studies have addressed the potential downstream immunostimulatory functions of these OMVs. One study demonstrated that *A. baumannii* produced twofold more OMVs when treated with antibiotics, and these OMVs had an altered proteome compared to control OMVs. When stimulated with OMVs obtained from cells treated with antibiotic, human lung carcinoma cells underwent severe apoptotic cell death, which is suggested to be attributable to the differential expression of OMV proteins in the presence of antibiotic stress (Yun et al. 2018). Collectively, these studies highlight the cytotoxic potential of OMVs on host cells, and the ability of bacteria to modify the cytotoxic abilities of OMVs during conditions of antibiotic stress, to ultimately mediate pathogenesis in the host.

#### 8.4.2 MVs Are Cytotoxic to Host Cells

Gram-positive MVs have been shown to also have cytotoxic effects on host cells. MVs produced by *S. aureus* have been shown to deliver the  $\alpha$ -haemolysin toxin to host cells, inducing cell death (Thay et al. 2013). However, not all MVs of the same genus are cytotoxic or pathogenic, highlighting that these MVs may have different functions in vivo. For example, analysis of MVs from the Gram-positive resident skin bacterium, *Staphylococcus haemolyticus*, revealed an enrichment of proteins involved in the acquisition of iron by MVs from both commensal and pathogenic *S. haemolyticus* (Cavanagh et al. 2018). However, MVs from a clinical isolate of a coagulase-negative staphylococci strain were enriched in proteins involved in antimicrobial resistance, such as  $\beta$ -lactamase and other virulence factors when compared to MVs isolated from a commensal *S. haemolyticus* strain (Cavanagh et al. 2018). This suggests that bacteria from the same genus can produce MVs which differ in functions within the host. Therefore, it should be noted that MVs produced by a number of commensal and probiotic strains are not cytotoxic to host epithelial cells (Canas et al. 2016; Shen et al. 2012). This further demonstrates the dichotomy between MVs produced by commensal and pathogenic microbes and their role in host immunity and pathogenesis, and suggests that bacterial vesicles have a range of functions in the host.

## 8.5 Bacterial Membrane Vesicles Interact with Host Innate Immune Cells

In addition to their interactions with epithelial cells and cytotoxic abilities, bacterial OMVs have been shown to induce inflammatory responses in innate immune cells such as macrophages, DCs, and neutrophils, which are phagocytic cells responsible for detecting and responding to pathogens. These cells express PRRs, including TLRs and NLRs at high levels (Kawai and Akira 2010). Due to their immunostimulatory abilities, OMVs are thought to interact directly with immune cells within the host and the mechanisms by which they interact with host immune cells are discussed below.

### 8.5.1 OMVs Interact with Macrophages

A number of studies have examined the effects of OMVs on innate immune cells such as macrophages. Analysis of macrophages from the lung tissue of patients infected with *L. pneumophila* revealed OMVs within the cytoplasm, suggesting that OMVs directly interact with macrophages in vivo (Jager et al. 2014). Another intracellular pathogen, *S. Typhimurium*, is able to penetrate and divide within innate immune cells. One study investigated the release of OMVs from *S. Typhimurium* within macrophages, and showed that OMVs containing virulence proteins were transported to the macrophage cytoplasm from within the *Salmonella*-containing vacuole (Yoon et al. 2011). Additionally, OMVs from *S. Typhimurium* could activate DCs and macrophages and stimulate their expression of a number of inflammatory molecules, including TNF $\alpha$  and IL-12 (Alaniz et al. 2007).

OMVs produced by the oral pathogen *P. gingivalis* are thought to contribute to atherosclerosis by inducing lipid droplet accumulation in macrophages (Qi et al. 2003). *P. gingivalis* OMVs have also been shown to induce the production of nitric oxide and the expression of inducible nitric oxide synthase by mouse macrophages to exacerbate disease (Imayoshi et al. 2011). Additionally, following treatment with *P. gingivalis* OMVs, macrophages were shown to have reduced CD14 on their surface (Duncan et al. 2004). CD14 is a co-receptor involved in TLR4-mediated recognition of LPS, and therefore loss of CD14 results in a reduced immune response to secondary *E. coli* LPS stimulation. These OMVs also suppressed TNF $\alpha$  production, further illustrating the effects of OMVs on immune cells (Duncan et al. 2004). Although detected by membrane-bound TLR4, cytoplasmic LPS can also activate the intracellular sensor caspase-11 in mice and caspase-4/5 in human, resulting in the release of IL-1 $\beta$  and pyroptotic cell death. A recent study reported the ability of *E. coli* OMV-associated LPS to enter the cytosol of host macrophages resulting in caspase-11 activation (Fig. 8.2, Gu et al. 2019). The transport of OMV-associated LPS into the cytosol of macrophages required TLR4 activation and occurred in a TIR domain-containing adaptor-inducing interferon- $\beta$  (TRIF)-dependent manner (Fig. 8.2, Gu et al. 2019).

Similarly, *P. aeruginosa* OMVs have been shown to upregulate the production of inflammatory cytokines such as macrophage inflammatory protein-2 (MIP-2), TNF $\alpha$  and IL-6 in mouse macrophages (Ellis et al. 2010). *H. pylori* OMVs have also been shown to promote IL-6 production by human peripheral blood mononuclear cells (Winter et al. 2014) and *Salmonella* spp. OMVs induced production of TNF and nitric oxide by mouse macrophages (Alaniz et al. 2007).

*N. meningitidis* OMVs have also been shown to induce cytokines and chemokines such as CC-chemokine ligand 2 (CCL2), CCL3, CCL5, CXCL8 (IL-8), IL-1 $\beta$ , IL-6, IL-10, IL-12p40, IL-12p70, and TNF production in monocytes and macrophages (Tavano et al. 2009). These OMVs also induced the production of monocyte-derived thrombotic factors such as tissue factor and plasminogen activator inhibitor 2. These molecules facilitate intravascular coagulation, microthrombosis, and organ dysfunction which is observed during septicemia caused by this bacterium (Tavano et al. 2009). Additionally, this study revealed that *N. meningitidis* OMVs can activate macrophages to induce adaptive immune responses by the upregulation of co-stimulatory molecules HLA-DR, CD80, and CD86, and intracellular adhesion molecule 1 (ICAM1) (Tavano et al. 2009).

#### 8.5.1.1 Anti-Inflammatory Effects of OMVs on Host Macrophages

Despite a number of pro-inflammatory properties, OMVs can also have anti-inflammatory effects on macrophage precursor cells, known as monocytes. *Neisseria gonorrhoeae*, the causative agent of gonorrhea in humans, has been shown to release OMVs within the mucosa that contain PorB. PorB is a protein which targets the macrophage mitochondrial membrane resulting in host cell death, thus down-regulating the innate immune response to the pathogen (Deo et al. 2018). Moreover, *H. pylori* OMVs induce the production of anti-inflammatory IL-10 by human peripheral blood mononuclear cells to limit inflammation and bacterial clearance, facilitating persistence of the pathogen (Winter et al. 2014). Similarly, *Brucella abortus* OMVs inhibit TLR2, TLR4, and TLR5 responses, limit IFN $\gamma$ -induced major histocompatibility complex (MHC) class II expression, and promote the internalization of *B. abortus* bacteria by monocytes in an effort to enhance pathogenesis (Pollak et al. 2012). Overall, OMVs have been shown to interact with different cell types, such as macrophages and monocytes to result in pro- or anti-inflammatory responses; however, this has not been demonstrated as widely for Gram-positive MVs.

#### 8.5.2 OMVs and MVs Interact with Neutrophils

Similar to macrophages, neutrophil responses can be modulated by bacterial OMVs in different ways. When stimulated with OMVs derived from *N. meningitidis*, human neutrophils produced TNF $\alpha$  and IL-1 $\beta$  and exhibited upregulation of IL-8,



CCL3, and CCL4 expression (Lapinet et al. 2000). Likewise, OMVs from uropathogenic *E. coli* which contained a toxin, cytotoxic necrotizing factor type 1, impaired the phagocytic and chemotactic abilities of neutrophils (Davis et al. 2006). Additionally, *E. coli* OMVs were recently demonstrated to effectively recruit neutrophils into the lung by stimulating the release of IL-8 in TLR4-dependent and NF- $\kappa$ B manners (Fig. 8.3, Lee et al. 2018).

During cell death, neutrophils produce neutrophil extracellular traps (NETs) which contain antimicrobial peptides, DNA, and histones. NETs are fibrous in structure to assist in trapping and killing pathogens (Brinkmann et al. 2004). OMVs produced by *N. meningitidis* and *Histophilus somni* induced neutrophil cell death and NET formation resulting in the killing of these pathogens. Although *N. meningitidis* OMVs induced NET formation, they also facilitated pathogen colonization and may be a contributing factor to the substantial amount of OMVs in patients with meningococcal disease (Lappann et al. 2013).

More recently, Gram-positive *S. aureus* MVs were also shown to activate neutrophils and induce an immune response. These MVs induced NET formation and neutrophil cell death. Moreover, mice immunized with MVs had increased production of antibodies such as immunoglobulin M (IgM) and total IgG, and were protected against subsequent *S. aureus* challenge (Askarian et al. 2018). Collectively, these studies reveal the multiple mechanisms whereby bacterial membrane vesicles interact with neutrophils to ultimately facilitate the onset of pathogenesis in the host.

### 8.5.3 OMVs Induce DC Maturation

In addition to their interactions with other immune cells, OMVs from pathogens induce the maturation of DCs, which is important for antigen presentation and adaptive immunity. DC maturation is characterized by their upregulation of MHC class II and production of pro-inflammatory cytokines and chemokines (Inaba et al. 1990). *N. meningitidis* OMVs facilitate their own delivery and internalization into DCs (Schultz et al. 2007), and are known to induce DC maturation, as characterized by the upregulation of MHC class II and co-stimulatory molecules, and their production of pro-inflammatory cytokines and chemokines (Durand et al. 2009). Similarly, *Salmonella* spp. OMVs induced the expression of CD86 and MHC class II on DCs, and their production of TNF and IL-12, as well as promoting protective T and B cell responses (Alaniz et al. 2007). These studies demonstrate the abilities of OMVs to induce DC maturation, leading to antigen presentation to further facilitate an adaptive immune response.

### 8.5.4 *The Effects of Commensal OMVs on Innate Immune Cells*

The effects of commensal OMVs on various innate immune cells is of interest due to their ability to traverse the epithelial cell layer (Chi et al. 2003). Commensal bacteria have been long thought to shape the immune system, and maintain homeostasis between tolerance and immunity (Macpherson and Harris 2004). The contributions of bacterial OMVs and MVs to the host-commensal homeostatic balance is of interest due to the versatility of bacterial vesicles. The Gram-negative commensal *B. fragilis* was found to produce OMVs containing LPS, a well-characterized MAMP. These OMVs were shown to have a protective effect against experimental colitis in mice, by stimulating DCs through TLR2 to adopt an anti-inflammatory profile (Shen et al. 2012). Additionally, OMVs from the commensal *Bacteroides thetaiotaomicron* have been demonstrated to package and deliver BtMinpp (the bacterial homolog of eukaryotic multiple inositol polyphosphate phosphatase 1) into host cells. This is thought to have beneficial effects for both the host and the bacterial community by providing the anticarcinogenic properties of BtMinpp and nutrients, respectively (Stentz et al. 2014).

Conversely, *B. thetaiotaomicron* OMVs were demonstrated to enter and activate mouse macrophages inducing an innate immune response, which may contribute to pathogenesis involved in inflammatory bowel disease (Hickey et al. 2015). Furthermore, OMVs from *Bacteroides vulgatus* were found to induce semi-maturation of bone marrow-derived cells (BMDCs). OMV-treated BMDCs had increased expression of MHC class II, CD40, CD80, and CD86 on their surface. After stimulation with colitogenic *E. coli*, BMDCs treated with OMVs from *B. vulgatus* had unchanged expression of MHC class II and secretion of TNF in the supernatant, indicating the induction of tolerance to bacterial challenge (Maerz et al. 2018). This study highlighted the role of commensal OMVs in immune cell priming. Collectively, these studies highlight the broad and opposing roles of commensal OMVs in host immunity, and their abilities to interact with innate immune cells to ultimately shape the host immune response.

## 8.6 Interactions of OMVs with Endothelial Cells

In addition to interacting with epithelial cells and innate immune cells, OMVs have been shown to interact with endothelial cells in a number of ways to initiate an immune response. OMVs produced by *E. coli* induce the production of IL-6 (Soult et al. 2013), tissue factor, thrombomodulin and adhesion molecules P- and E-selectin (Soult et al. 2014) by human endothelial cells. This leads to the recruitment of pro-inflammatory immune cells, platelet aggregation, and coagulation (Soult et al. 2013). *E. coli*-derived OMVs also upregulate the expression of ICAM1 and vascular cell adhesion molecule 1 on the surface of human microvascular endothelial cells

(Kim et al. 2013a). Mice injected intraperitoneally with these OMVs showed neutrophil aggregation in the lung endothelium in an ICAM1- and TLR4-dependent manner (Kim et al. 2013a).

As well as pro-inflammatory responses in the endothelium, OMVs from *P. gingivalis* were demonstrated to downregulate inflammation in IFN $\gamma$ -stimulated endothelial cells. OMVs did this by inhibiting the upregulation of MHC class II molecules on the host cell surface, thus limiting antigen presentation (Srisatjaluk et al. 2002). Recently, another study revealed that *P. gingivalis* OMVs suppress endothelial nitric oxide synthase (eNOS) expression in human umbilical vein endothelial cells in vitro, which may contribute to cardiovascular disease (Jia et al. 2015).

The interaction of bacterial vesicles with host cells, including innate immune cells, can lead to activation of the adaptive immune system. Bacterial vesicles have also been described to interact with the adaptive immune system directly, which is discussed below.

## 8.7 Adaptive Immune Responses to Bacterial Membrane Vesicles

The adaptive immune system is responsible for generating a pathogen-specific immune response that is capable of clearing the pathogen, in addition to generating immune memory that can be reactivated when the host reencounters the pathogen. Specifically, the adaptive immune system is responsible for the production of antigen-specific T and B cells that can target the pathogen directly, or produce pathogen-specific antibodies (Iwasaki and Medzhitov 2015). The hallmarks of this system are the random generation of receptor specificities (T cell receptor and immunoglobulins) by somatic recombination. This results in the possibility of generating a nearly unlimited spectrum of antigen specificity. Populations of cells with cognate receptors for the pathogen are selected and enriched by clonal selection, which means that specific activation of the cell bearing the receptor with the highest affinity leads to its proliferation.

Phagocytic cells such as macrophages, DCs and B cells act as antigen-presenting cells (APCs) in this process. These cells can present peptide antigens via surface receptors (MHC class I and II), which can be recognized by naïve T lymphocytes, leading to their activation, proliferation, and differentiation to effector cell subsets (reviewed in Unanue 1984; Guernonprez et al. 2002; Rodriguez-Pinto 2005). Helper T lymphocytes (CD4 positive cells) are also essential for the activation of B cells and their differentiation into antigen producing plasma cells. Collectively, the activation of the adaptive immune response results in the generation of memory T and B cells which can confer long lived immunity, and is the basis for the idea of bacterial membrane vesicles functioning as vaccines.

### 8.7.1 *Bacterial Membrane Vesicles Shape Adaptive Immunity*

Due to their immune stimulating properties and vast evidence of their interactions with the innate immune system, OMVs and MVs are thought to influence the hosts adaptive immune response. OMVs from *H. pylori* were able to adsorb antibodies in sera from infected patients, thus providing protection for the bacteria (Hynes et al. 2005). Following this, a number of in vivo studies have further revealed the complexity of OMVs as vehicles for antigens and their initiation of adaptive immune responses, which will be discussed here briefly.

### 8.7.2 *Generation of OMV-Specific Protective Immune Responses*

Many in vivo studies have demonstrated the generation of specific antibody responses to bacterial OMVs. For example, OMVs produced by *N. meningitidis* induce the upregulation of MHC class II receptors as well as costimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) on macrophages (Tavano et al. 2009). Furthermore, *S. Typhimurium* and *V. cholerae* OMVs were also described as an abundant source of antigens, which could activate CD4<sup>+</sup> T cells in order to generate antibody-specific B cell responses (Alaniz et al. 2007; Chatterjee and Chaudhuri 2013). Specifically, in a mouse model of infection and sepsis, *Burkholderia pseudomallei* OMVs administered subcutaneously were shown to induce serum IgG and T helper 1 (Th1) cell responses (Nieves et al. 2011). A similar finding was observed in mice immunized intraperitoneally with *E. coli* OMVs. Exposure to OMVs from *E. coli* resulted in stimulation of T cell immunity primarily driven by Th1 and Th17 cells (Kim et al. 2013b).

Mice vaccinated intramuscularly with OMVs produced by *A. baumannii* produced high levels of OMV-specific IgG antibodies, and subsequent challenge with *A. baumannii* intranasally resulted in mucosal IgA and IgG responses in a pneumonia model (Huang et al. 2014). These mice were also challenged with *A. baumannii* intraperitoneally as a model for bacterial sepsis, and were found to have increased survival when compared to the control group. In this study, mice passively immunized intravenously with antiserum from vaccinated mice also had protection from *A. baumannii* intranasal challenge. The antisera from immunized mice was also shown to have opsonophagocytic properties and resulted in killing of numerous clinical isolates of *A. baumannii* by macrophages in vitro (Huang et al. 2014).

Furthermore, OMVs produced by streptomycin resistant clinical strains of nontypeable *H. influenzae* used to immunize mice both intranasally and intraperitoneally resulted in robust IgG1 antibody production, and IgA production following intranasal immunization. However, when intraperitoneally immunized mice were challenged with *H. influenzae* strains, they were found to have a higher rate of bacterial colonization when compared to intranasally immunized mice (Roier et al.

2012). These reports demonstrate the shared capacity of OMVs from different bacteria to induce OMV-specific immune responses *in vivo*.

### 8.7.3 *OMVs Can Modulate B Cell Responses*

OMVs have also been demonstrated to modulate B cell responses directly. OMVs produced by *M. catarrhalis* were shown to contain an outer membrane protein known as *Moraxella* immunoglobulin D binding protein (MID). MID is an antigen which potently activates human tonsillar B cells via clustering of B cell receptors which mediates endocytosis. TLR9 was also involved in detection of DNA associated with these OMVs, which induced full B cell activation (Vidakovics et al. 2010). *H. influenzae* also uses a similar approach to modulate B cell responses, by producing OMVs which sensitize B cells resulting in an increase in IgG and IgM production; however, these antibodies do not recognize *H. influenzae* (Deknuydt et al. 2014). In a manner independent of T cells, *Neisseria lactamica* OMVs induce B cell proliferation (Vaughan et al. 2010) and polyclonal antibody production (Vaughan et al. 2009) highlighting the versatile interactions of OMVs with adaptive immune cells.

### 8.7.4 *Gram-Positive MVs Mediate Adaptive Immune Responses*

Only a handful of studies have analyzed the effects of Gram-positive MVs on mediating adaptive immunity. MVs produced by the pathogen *Clostridium perfringens* have been shown to induce the production of high titer IgG1 in mice (Jiang et al. 2014). Additionally, *B. anthracis* MVs have been demonstrated to induce a robust IgM response in mice (Rivera et al. 2010). Mice immunized intramuscularly with *Streptococcus pneumoniae* MVs were protected from intranasal *S. pneumoniae* challenge. These mice had increased IgG levels, and increased survival (Olaya-Abril et al. 2014).

*S. aureus* MVs administered intramuscularly or intravenously have been demonstrated to induce protective immune responses in mice using various models of infection including oropharyngeal or intravenous challenge, respectively, the latter inducing a Th1-mediated response (Choi et al. 2015; Wang et al. 2018). It is because of the antigenic properties of OMVs and MVs that bacterial membrane vesicles are currently being considered as future vesicle vaccines.

### **8.7.5 *Development of OMVs and MVs for Use as Vaccines***

Due to their ability to mediate innate and adaptive immune responses *in vivo*, OMVs have been examined for their ability to confer protection against bacterial challenge and refine their use as vaccine candidates. There are numerous studies that have demonstrated the effect of OMVs on the adaptive immune system, with studies demonstrating the ability of OMVs and MVs to mediate protection from infection, sepsis, and death (Fantappie et al. 2014; Huang et al. 2014; Camacho et al. 2013; Kim et al. 2013b). OMVs have also been proven to be effective at generating a robust immune response and have been found to be more protective against live challenge than vaccination using bacterial membrane fractions (Tavano et al. 2009). In addition, vaccination with OMVs is more effective at generating a bactericidal serum response in mice when compared to serum responses generated as a result of infection alone (Zhu et al. 2005). There are many examples of the effective use of OMVs and MVs as vaccine candidates, in addition to their future therapeutic applications, and these are discussed in further detail in Chap. 10.

## **8.8 Conclusions and Perspectives**

The innate and adaptive immune systems are complex, as are their interactions with invading and commensal organisms. Bacterial membrane vesicles and their immunostimulatory functions have not been fully characterized; however, studies have examined their effects on host epithelial, innate and adaptive immune cells (reviewed in Kaparakis-Liaskos and Ferrero 2015; Manning and Kuehn 2011). OMVs from various bacterial species have been shown to induce a constant interplay between pro-inflammatory and anti-inflammatory signals, and the overall response can vary depending on the amount of OMVs, as well as their content (reviewed in Kaparakis-Liaskos and Ferrero 2015). Bacterial OMVs are able to stimulate TLRs and NLRs of host cells. Furthermore, OMVs and MVs have a wide variety of inherent properties which can be beneficial to the pathogenic capabilities of their parent bacteria, including their contributions to biofilm formation, and antibiotic and host immune molecule defense (Tan et al. 2007; Jagels et al. 1996; Baumgarten et al. 2012; Schooling and Beveridge 2006; Yonezawa et al. 2009). Overall, the vast mechanisms by which OMVs and MVs can interact with and contribute to immunity and pathogenesis are only emerging at present, and further studies should aim to understand the complex nature of membrane vesicle-immune interactions. This knowledge may contribute to forthcoming nanoparticle technologies, whereby OMVs and MVs can be utilized for therapeutics in addition to novel targets to limit bacterial vesicle-mediated pathology.

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