






Review Article

The complex, bidirectional role of extracellular vesicles in infection

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Cells from all domains of life release extracellular vesicles (EVs), packages that carry a cargo of molecules that participate in communication, co-ordination of population behaviours, virulence and immune response mechanisms. Mammalian EVs play an increasingly recognised role to fight infection, yet may also be commandeered to disseminate pathogens and enhance infection. EVs released by bacterial pathogens may deliver toxins to host cells, signalling molecules and new DNA to other bacteria, and act as decoys, protecting infecting bacteria from immune killing. In this review, we explore the role of EVs in infection from the perspective of both the pathogen and host, and highlight their importance in the host/pathogen relationship. We highlight proposed strategies for EVs in therapeutics, and call attention to areas where existing knowledge and evidence is lacking.

Introduction

Infectious diseases are diverse and can elicit varied clinical outcomes, from asymptomatic infection to serious disease and death. In an infection, a microbe colonises a host and multiplies at a site it is not normally found, with clinical manifestations resulting from damage from the host/microbe interactions [1]. Understanding the interactions at play during infection is especially important to identify molecular targets that can be utilised for the detection, prevention and treatment of infectious threats. We see the importance of this knowledge to ensure global public health security as we enter an era of widespread antimicrobial resistance [2] and as the COVID-19 pandemic unfolds [3].

Extracellular vesicles (EVs) are membrane-bound packages released by cells that contain an array of molecules carried as cargo. We will use the term ‘EVs’ as recommended by the international society for EVs (ISEV) as a ‘generic term for particles naturally released from any cell that are delimited by a lipid bilayer and cannot replicate’ [4]. Once thought to be unremarkable cellular ‘garbage bags’ [5], EVs are now well established as fundamental cell-to-cell communicators [6]. In this review, we explore the complex bidirectional role of EVs in the host/pathogen relationship, with a primary focus on bacterial infections in the human host. We will investigate how pathogen EVs escalate the process of infection; while by detecting bacterial cells or their EVs, the host immune response may produce EVs to combat infection (Figure 1). After consideration of the roles of EVs in infection, we will examine the evidence for the future of EVs in diagnostics and therapeutics and identify areas with potential for new research in the future.

Bacterial EVs in infection

It was originally thought that only Gram-negative bacteria produced EVs. Gram-negative bacteria have inner and outer membranes that are separated by a thin peptidoglycan cell wall, and historically their EVs were called outer membrane vesicles (OMVs), a term that recognises their origin [7].

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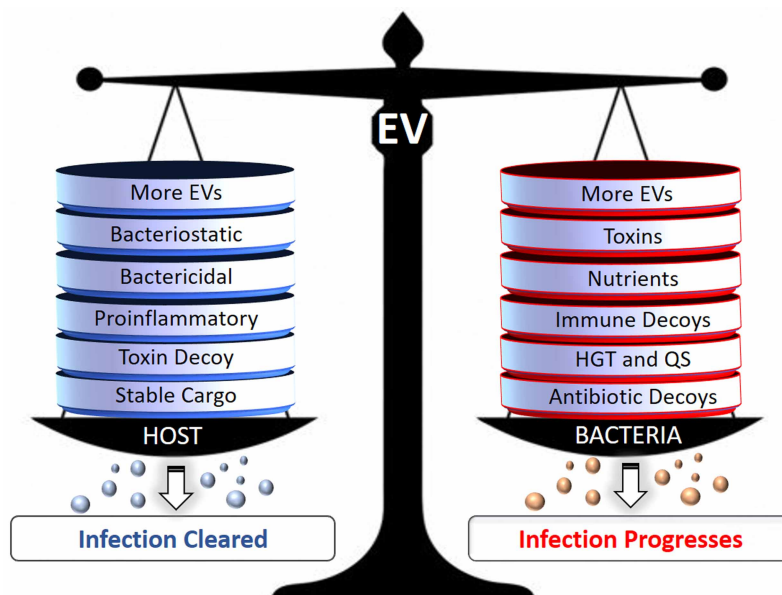


Figure 1. EVs in infection.

EV production is often increased during infection, from both the host and pathogen. EVs from the host clear infections by promoting inflammatory responses and carrying bacteriostatic and bactericidal factors. In contrast, pathogenic bacterial EVs carry toxins and provide nutrients, as well as acting as decoys to the host immune system and contributing to antibiotic resistance.

Gram-positive bacteria have a single cell membrane surrounded by a thick peptidoglycan layer [8], and were only shown to produce EVs 30 years after their Gram-negative counterparts [7]. It is now considered that all prokaryotes can produce EVs mainly through either bacterial cell lysis or membrane blebbing mechanisms [8].

The composition and biogenesis of bacterial EVs (bEV) has been recently reviewed in Nagakubo et al. [9]. Overall, bEVs normally range between 20 and 400 nm in diameter and may carry diverse molecular cargo, including proteins, DNA, RNA, lipids, lipopolysaccharides and other organic small molecules that can enable communication with host cells and other bacteria, and provide functionalities that enhance survival.

Bacterial EVs in the pathogenesis of infection

An indication that bEVs contributed materially in the infection process came from the demonstration that for the cytotoxin of *Escherichia coli* [10] and the VacA toxin of gastric pathogen *Helicobacter pylori* [11] that delivery of activated toxin could occur as part of a bEV cargo. Subsequently, many recognised toxins have been added to the list that may be delivered by bEVs (see Table 1), including those of different pathogenic *E. coli* (reviewed in [12]), other gastrointestinal pathogens like *Vibrio cholerae* [13] and *Listeria monocytogenes* [14], pathogens of the oral cavity like *Aggregatibacter actinomycetemcomitans* [15] and *Porphyromonas gingivalis* [16], as well as opportunistic pathogens like *Pseudomonas aeruginosa* [17] and *Staphylococcus aureus* [18]. In general, uptake of the toxins as part of the bEV cargo occurs via endocytosis of vesicles via clathrin, caveolin or lipid raft mediated mechanisms, or via cholesterol-dependent membrane fusion (reviewed in [19]).

Although secreted bEVs may often be perceived as one population, the heterogeneity of bEVs is now becoming better understood; subtypes are now known to have different compositions [8] and specific roles in host pathogenesis and immunogenicity. For example, Turner et al. [20] showed that *H. pylori* bEVs differentiated by size have a different protein composition and will enter host cells by different mechanisms; O'Donoghue et al. [21] demonstrated that the structural modifications of O antigen in LPS carried on the surface of *E. coli* bEVs can influence the route of entry and enhance bEV uptake into host cells.

The cargo manifest of bEVs delivered to mammalian cells now goes beyond classical toxins and has identified the possibility of new virulence mechanisms involving small RNAs [22]. Moreover, the delivery of a cargo highlights that pathogenesis will likely result from the combined effects of these virulence factors [12,14,17], and the challenge may be to distinguish the roles of each when other factors are present [23].

Table 1. The contribution of bEVs to pathogenesis during bacterial infections.

Bacterial species	bEV effect on pathogenesis	Reference
<i>Aeromonas</i> spp.	Promote biofilm formation	[113]
<i>Aggregatibacter actinomycetemcomitans</i>	Induce a cytolethal distending effect on HeLa and HGF cells by delivering the genotoxin CDT	[15]
<i>Campylobacter jejuni</i>	Exerts cell-distending effects typical of CDT on human intestinal cells	[114]
<i>Escherichia coli</i>	Export ClyA cytotoxin and elicit dose-dependent haemolytic response on blood cells	[10]
Enterohemorrhagic <i>E. coli</i> (EHEC)	Induces pro-inflammatory cytokine response in intestinal cells Delivers cocktail of toxins (Stx2a, CdtV, EHEC hemolysin) to cells, which are then trafficked to their disparate sites of action	[115] [120]
Enterotoxigenic <i>E. coli</i> (ETEC)	Specifically, the target transport of active enterotoxin and other bacterial envelope components into intestinal epithelial cells.	[121]
Uropathogenic <i>E. coli</i> (UPEC)	bEVs carry RNA and LPS that inhibit pro-inflammatory cytokine response in human uroepithelial cells	[23]
<i>Francisella tularensis</i>	FtIA lipase carried by bEVs enhances internalisation in mouse macrophages	[116]
<i>Helicobacter pylori</i>	bEVs contain VacA (vacuolating cytotoxin) is internalised by gastric epithelial cells, and may induce pathogenic effects different from that of soluble VacA Protect cells from extracellular ROS-mediated killing	[11,117] [118]
<i>Listeria monocytogenes</i>	Protect from autophagy and cell death induced by its own pore-forming toxin LLO, for survival in kidney cells bEV-carried RNA induces expression of IFN- β in BMDMs	[119] [63]
<i>Moraxella catarrhalis</i>	Carry serum resistance proteins that bind and inactivate C3 of the complement system, thus acting as decoys	[24]
<i>Mycobacterium tuberculosis</i>	Carry iron-binding factors that scavenge this nutrient and promote bacterial survival	[27]
<i>Vibrio cholerae</i>	bEV-carried bioactive cholera toxin (CT) increases cAMP levels in intestinal epithelial cells	[13]
<i>Porphyromonas gingivalis</i>	bEVs are targeted to host lysosomal compartments in HeLa and immortalised human gingival epithelial cells; <i>P. gingivalis</i> bEVs degrade host cellular functional proteins	[16]
<i>Pseudomonas aeruginosa</i>	bEV-carried host colonisation factors (alkaline phosphatase, β -lactamase, haemolytic phospholipase C and Cif) are transported to airway cells where they cause cytotoxicity Short RNAs associated with bEVs may inhibit the host immune response by attenuating IL-8 secretion induced by bEVs themselves Carry iron-binding factors that scavenge this nutrient and promote bacterial survival Carry quorum sensing signals that promote many genes involved in virulence	[17] [22] [28] [29,30]
<i>Salmonella enterica</i>	Deliver genotoxic CDT secreted by intracellular bacteria to bystander cells and induce DNA damage	[46]
<i>Staphylococcus aureus</i>	Carry α -haemolysin which contributes to HeLa cytotoxicity, and induces lysis in erythrocytes	[18]

Abbreviations: bEVs, bacterial extracellular vesicles; BMDMs, bone marrow-derived macrophages; cAMP, cyclic adenosine monophosphate; CDT, cytolethal distending toxin; CdtV, cytolethal distending toxin V; CT, cholera toxin; IFN- β , interferon β ; IL, interleukin; LLO, listeriolysin O; LPS, lipopolysaccharide; ROS, reactive oxygen species; Stx2a, Shiga toxin 2a

In concert with damaging the host with toxins, bEVs have been implicated in the preparation of an environment in the host that favours an infection. An attractive concept is the immune decoy, as exemplified by the *Moraxella catarrhalis* bEV which carries serum resistance proteins that bind important immune mediators,

such as C3 of the complement system, and inactivate the complement cascade [24], leaving the bacteria the opportunity to colonise the host and proliferate. BEVs can further aid in colonisation by contributing to the formation of the biofilm matrix [25,26], and in the acquisition of key nutrients, with iron-acquisition offering the best example to date: *Mycobacterium tuberculosis* and *P. aeruginosa* bEVs carry iron-binding factors that scavenge this nutrient for bacterial cells, promoting bacterial survival within the iron-restricted host environment [27,28]. Finally, bEVs can influence the activity of other bacteria, such as through the delivery of quorum sensing (QS) signals that promote the dispersal of biofilms [29,30].

In terms of placing bEVs at the sites of infection or inflammation in humans, there are still technical and ethical challenges to overcome. Experimental animal models have, however, been exploited to show bEVs travelling to remote sites after injection into the animal, and to demonstrate bEV presence and inflammatory effects, in target organs [31–33]. For example, the intraperitoneal injection of Cy7-labelled *E. coli* bEVs followed by near-infrared imaging in SKH-1 E hairless mice demonstrated travel to liver, lung, spleen, and kidney within 3 h [31]. Moreover, ELISAs using polyclonal anti-OMV antibodies detected bEV cargo in infected tissues of similarly challenged C57BL/6 mice, along with evidence of a systemic inflammatory response that had dissipated with the clearance of bEVs, although elevated leucocyte levels were observed in bronchoalveolar lavage fluid as a late reaction [31]. More recently, a similar approach was used to follow fluorescently labelled bEV of the oral pathogen *A. actinomycetemcomitans* where following cardiac injection into monocyte-specific CX3CR1-GFP mice they have been detected in the brain colocalized with meningeal macrophages and microglial cells by intravital imaging techniques. This work suggests that bEVs can cross the blood-brain barrier (BBB) and links bEVs in the brain to elevated production of pro-inflammatory cytokines to support a connection between periodontitis and neuroinflammatory disease [32,33]. The observations made investigating bEV transport in mice add support to a role for bEVs in the aetiology of some instances of sterile inflammation. The bEVs of *E. coli* can elicit severe immune responses and signs of septic shock in rodents in the absence of living bacteria [34–36], and EVs from murine faeces induce strong local and systemic inflammatory responses when injected intraperitoneally, whereas the faecal EVs from germ-free mice provoke a much weaker response across a range of inflammatory markers [36].

Antibiotics introduce a new challenge for bacteria in an infection, and we now see bEVs acting as ‘decoys’ for antibiotics directed at membrane-bound targets [37], carrying enzymes that degrade antibiotics in the surrounding milieu [38,39], or enabling horizontal gene transfer (HGT) of antimicrobial resistance genes to susceptible bacterial cells [40]. A summary of the pathogenic diversity of bEVs from various bacteria is presented in Table 1.

Host conditions influencing bacterial EVs

Although EV production is a continuous process throughout bacterial growth stages, there are specific environmental conditions favouring bEV biogenesis, and that influence bEV composition [8]. The human body is a dynamic environment and changing conditions may alter pathogen bEV production both qualitatively and quantitatively. Most obvious is iron-limitation, which as a single stimulus (i) increases *M. tuberculosis* bEV production to increase the capacity for iron acquisition [27]; (ii) significantly changes the biophysical properties and proteome of *E. coli* bEVs [41,42]; and (iii) modifies the LPS structure in *H. pylori* bEVs [43], suggesting there is an iron-responsive selection of bEV cargo or a favoured EV biogenesis route responsive to iron availability.

Less well-defined host conditions can influence bEV production too. For *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) bEV-associated RNAs, including RNAs involved in virulence, are specifically enriched under conditions that mimic the macrophage’s intracellular environment or the lumen of the intestine [44]. In particular, it has been shown that intracellular pathogens release bEVs at their intracellular location to effectively deliver virulence factors [45,46]. For intracellular *M. tuberculosis*, bEVs are the primary medium for the export of lipoglycans and lipoproteins that impair antibacterial functions of the infected macrophages, and when released from the macrophage, circulate bacterial components beyond the site of infection to influence the responses of uninfected cells [45]. For *Salmonella*, the intracellular expression of a DNA-damaging cytolethal distending toxin (CDT) occurs in the *Salmonella*-containing vacuole (SCV), with secretion from the bacterium in bEVs. CDT-bEV trafficking from the SCV then leads to their release from the infected cell from where they are endocytosed by bystander cells, leading to DNA damage [46]. Additionally, while antibiotic treatment offers a challenge to bacterial survival in an infection, it can also increase bEV production, especially

where the antibiotic causes envelope stress [47–52]. Understanding how diverse factors come together to impact bEV production and content may help us to understand their biological contribution to infection.

Bacterial EVs contribute to immune response

bEVs can stimulate effective antibacterial host responses [53–58]. The effects are thought to result from receptor interactions at host cell surfaces and at intracellular locations. bEVs carry many of the surface determinants found on the parent cell [59,60], and these may be recognised as invaders by host cells. For example, upon intranasal administration in mice *S. aureus* bEVs interact with cell surface TLR2 receptors and induce Th1 and Th17 cell responses and bring about neutrophilic pulmonary inflammation [55]. The LPS of Gram-negative bacteria offers well-documented pathways of extracellular sensing by TLR4 surface receptors that can be employed by immune cells to generate potent innate immune responses when exposed to bEVs [57].

bEVs and cargo trafficked into the cell can interact with a range of intracellular receptors and, for LPS entering the cell as bEV cargo, elicit entirely different responses when compared with TLR4 sensing of LPS. The response is characterised by activation of caspase-4/5 and NLRP3 inflammasome, leading to a signature secretion of cytokines IL-1 β and IL-18, and a strong antibacterial inflammatory response [58]. It is proposed that the immunostimulatory detection of intracellular bEV components may represent a cardinal sign of infection by multiplying bacteria. Research now reports bEVs to be internalised by many types of host cells, including macrophages, neutrophils, antigen-presenting cells (APCs), dendritic cells (DCs), and epithelial cells [19–22,29]. With each cell type having different response capabilities according to their roles in the human body, there is a substantial research gap determining the effects of bEVs in each cell type they encounter and the role in the progression of the infection those responses may have, which is important in inflammatory diseases such as sepsis [62].

Knowledge of the fate and effect of the delivered bEV cargo is incomplete. In particular, there is a need to better understand the effect of bEV-RNA as, while some studies show the induction of specific cytokines [32,63], others present evidence for the suppression of cytokine secretion by epithelial cells or lymphocytes [22,23,64].

Host EV-mediated response to infection

Eukaryotic cells produce a heterogeneous group of EV subtypes, categorised by size and biogenesis mechanism as small EVs (sEVs; <200 nm in diameter) that include most exosomes; microvesicles (microEVs; 200–1000 nm); apoptotic bodies (>500 nm) [4,65]. An intracellular endosomal biogenetic route defines exosomes, and they are enriched in markers useful for identification [66,67]. MicroEVs are predominantly formed by blebbing and pinching from the plasma membrane [65]. In humans, EVs have been reported in all body fluids [68–72] and are produced by many different cell types [71,73–76]. EVs play an important role in extending the functional range of the bioactive molecules released by cells, increasing their stability, and targeting their delivery to achieve higher localised concentrations [77,78]. It is now clear that EVs have a role in both the co-ordination and the delivery of antimicrobial immune responses.

In infections, the cells having the first contact with pathogens (e.g. epithelial cells, macrophages) release EVs containing bioactive molecules that stimulate pro-inflammatory responses [79–81]. Immune cells stimulated during infections may release EVs that carry antimicrobial factors [82–84] or act as decoys that protect cells by binding to bacterial toxins [85].

EV production by some host cell types increases in infectious disease [86,87], and viruses, fungi, bacteria, and parasites have all been shown to directly stimulate host EV production [50,54,55,88]. A quantitative increase in host EV release has been demonstrated following an extracellular challenge of alveolar epithelial cells with heat-killed bacteria, finding that the elicitor of such response was bacterial CpG DNA binding to endosomal TLR9 receptors [85]. Macrophages and endothelial cells infected by viable bacteria are similarly stimulated to release EVs [79–81]. In addition to increasing the production of EVs, infections change the composition of the EVs released by host cells [79].

Change in host EV production due to infection

Epithelial [77,89] and immune [79–81,90] cells and platelets [91,92] that come into contact with bacterial pathogens [77,79,80,89,90] or the toxins pathogens secrete [91,92], release EVs that contain signals, including regulatory RNAs [77,93] cytokines [91,92], pathogen-associated molecular patterns (PAMPs) [79,80,89,90] and even toxins [46,94] that may activate endothelial cells [81,92], amplify the release of proinflammatory signals

from immune cells [79,90], recruit macrophages/neutrophils [91,95] or promote B-cell/T-cell interactions that lead to antibody production [71,96,97].

The molecular processes involved in these responses are well characterised, but the recognition that EVs can improve their efficiency can offer new insights into the complex system that targets our immune system to the task of eliminating pathogens.

Host EVs as antimicrobial responses

The EVs released by immune cells as part of an effective immune response have been shown to exhibit antimicrobial effects *in vitro* [82] and are suggested to be associated with strong antimicrobial responses *in vivo* [98]. The antimicrobial responses described are attributed to the presence of bacteriostatic [72,82] and bactericidal [72] components of the EV cargo. For example, Hiemstra et al. [72] showed that human urinary EVs are enriched in proteins with immune functions, such as bacteriostatic proteins mucin-1, fibronectin, CD14, and the bactericidal proteins lysozyme C, calprotectin, and dermcidin [72]. In addition, the authors found that human urinary EVs inhibited the growth of laboratory-adapted, uropathogenic and probiotics strains of *E. coli*, and effected bacterial killing by a lytic mechanism [72]. The molecular mechanisms of these factors are often well characterised, but it is their delivery as part of an EV that can provide new understanding of the processes involved.

The broader study of host EVs and their interactions with bacteria suggest novel antimicrobial mechanisms await discovery and characterisation. For example, where EVs stabilise bioactive molecules, such as RNAs, they may enable more efficient delivery to bacteria, and effect non-lethal control of microbial behaviour [99]. To this end, we have previously reviewed host molecules that may contribute to changes in the resident and pathogenic microbiota [100], and here we highlight the role that EVs may play in connecting these molecules with bacteria.

Finally, EVs produced by the host in response to the presence of a pathogen, in this case, CpG DNA by TLR9, a process we have already described as a response involving bEVs [85], may protect cells from microbial attack by effectively mimicking the targets of bacterial toxins and acting as decoys [85]. Here, changes in the trafficking of staphylococcal alpha haemolysin receptor ADAM10 occur, leading to the release of exosomes enriched for ADAM10 on their surface that neutralise the toxin and protect from the damage normally caused during infections [85].

EVs as infection biomarkers, in vaccines and therapeutics

EVs are found in all body fluids, and are now exploited as non-infectious disease biomarkers, such as for cancer [101,102]. As we begin to understand the composition and distribution characteristics of EVs during infectious disease, novel biomarkers may be identified offering the potential to develop EV-based diagnostics, especially if we are able to overcome the difficulties that limit the detection of bEVs in body fluids [103]. In particular, there is potential for serum EVs to report on the presence of biofilm infections to support a rapid diagnosis [104].

As well as detecting pathogens, an understanding of EV biology offers possible ways to protect from infection. Pathogen EVs often carry PAMPs which allow them to stimulate the immune system effectively [54,61,105,106], giving them the potential to be used as vaccines. An excellent example of this are bEVs from *Neisseria meningitidis*, which have safely and successfully been used as the basis for a vaccine against meningococcal disease, as they induce potent antibacterial immune responses [106]. The success of bEV-based vaccines gives encouragement to those seeking new strategies to immunise against pathogens that have thus far proven difficult [106], perhaps through the use of pathogen EVs as in the *N. meningitidis* vaccine [106] or designer EVs from genetically modified bacteria [107,108].

Rather than using pathogen EVs, another approach has been suggested that uses the EVs produced by beneficial bacteria. In one example, the commensal gastrointestinal bacterium *Bacteroides fragilis* selectively packages polysaccharide A (PSA) in bEVs that have been demonstrated to induce immunomodulatory effects and prevent experimental colitis in mice [109], a finding that suggests some commensal bEVs may be the basis of therapeutic formulations, or even be a rationale for new probiotic approaches. The evidence now emerging to suggest that bEVs may travel between organ systems in the body [110,111] and even cross the BBB [33] may identify new probiotic approaches and mechanisms by which, e.g. the gut microbiota can influence the health at other sites in the body.

Lastly, EVs may offer a new approach to the formulation and targeted delivery of antimicrobials to manage infections. Many strategies have been proposed, including the artificial loading of EVs with antimicrobials and the decoration of antimicrobial-loaded nanoparticles to enhance selective uptake by the pathogen [59,105,112]. It may be that these designer EVs have a role in the future to meet the challenges of antimicrobial resistance and new infectious diseases.

Perspectives

- *Importance:* EVs provide the opportunity for multicomponent pathogen/host interactions, facilitating the transport and delivery of bioactive cargoes and offering the potential to mimic cells to intercept molecules that would be harmful.
- *Current thinking:* Current thinking states that EVs are produced by all cells. The pathogen uses EVs to deliver toxins to damage the host, to prepare a microenvironment favouring proliferation, and to communicate with other bacteria to facilitate QS and horizontal gene transfer. The infected host is stimulated to produce EVs that amplify and co-ordinate proinflammatory responses and deliver antimicrobial effector molecules. A challenge now is to investigate how well this adversarial model describes what really happens in an infection.
- *Future directions:* The future of EV research may elucidate the role of novel virulence factors (e.g. small RNAs) and establish how the components of the EV and bEV cargoes, often well characterised in isolation, act in combination. The knowledge gained will offer the potential for new approaches for rapid diagnoses, vaccine developments and effective therapeutics. As EV research in infectious disease gains impetus it will be important to follow the ISEV guidelines for isolation and analysis of EVs so we can be confident that any effects attributed to EVs and bEVs are correctly assigned.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviation

BBB, blood-brain barrier; bEV, bacterial EVs; CDT, cytolethal distending toxin; EVs, extracellular vesicles; ISEV, society for EVs; OMVs, outer membrane vesicles; PAMPs, pathogen-associated molecular patterns; PSA, polysaccharide A; QS, quorum sensing; SCV, *Salmonella*-containing vacuole

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