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Review Article

Outer membrane vesicles of *Porphyromonas gingivalis*: Novel communication tool and strategy

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ABSTRACT

Extracellular vesicles (EVs) have been recognized as a universal method of cellular communications and are reportedly produced in bacteria, archaea, and eukaryotes. Bacterial EVs are often called "Outer Membrane Vesicles" (OMVs) as they were the result of a controlled blebbing of the outer membrane of gram-negative bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*). Bacterial EVs are natural messengers, implicated in intra- and inter-species cell-to-cell communication among microorganism populations present in microbiota. Bacteria can incorporate their pathogens into OMVs; the content of OMVs differs, depending on the type of bacteria. The production of distinct types of OMVs can be mediated by different factors and routes. A recent study highlighted OMVs ability to carry crucial molecules implicated in immune modulation, and, nowadays, they are considered as a way to communicate and transfer messages from the bacteria to the host and vice versa. This review article focuses on the current understanding of OMVs produced from major oral bacteria, *P. gingivalis*: generation, characteristics, and contents as well as the involvement in signal transduction of host cells and systemic diseases. Our recent study regarding the action of *P. gingivalis* OMVs in the living body is also summarized.

1. Introduction

Signal transduction between cells, tissues, and organs is meticulously regulated and is involved in cellular development, growth, and diseases. These communications are initiated by direct or indirect interactions. In the case of indirect interaction, "cytokines" or "hormones," released from immune cells and endocrine cells, are involved respectively in cell survival, proliferation, differentiation, and activity.

More recently, a novel intercellular indirect communication system is gathering the attention of scientists. In this system, cells enclose various substances (information) in small particles and then release them to be transferred to other cells. The cargo in which cells contain packaged substances is called extracellular vesicles (EVs); they encompass cell-specific molecules such as nucleic acids, proteins, sugars, and lipids. Over the last decade, research in this area has exponentially expanded — its role has uncovered various insights into the importance of areas such as cancer and immunity. Under physiological or pathological conditions, eukaryotic cells secrete various sizes of vesicles wrapped in plasma or intracellular membrane. These extracellular vesicles are disseminated in fluids – blood, saliva, urine, breast milk, amniotic fluid, etc. – and play an important role in various stages such as development, differentiation, immune response, and aging. Since EVs are presumably released from all cells, they become critical to every evolutionary process in both unicellular and multicellular organisms.

In contrast to the well-established various roles of EVs in eukaryotic cell biology, outer membrane vesicles (OMVs), produced via blebbing of prokaryotic membranes, have frequently been regarded as cell debris or microscopy artificial products. Over the past decades, the role of OMVs produced gram-

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negative bacteria have been analyzed. OMVs nowadays have been considered a very sophisticated mechanism for transporting many molecular effectors, cell-cell interactions, nutrients, interaction, host cell immune dysregulation, modulation, bacterial aggregation, biofilm formation, etc. Some of OMVs-releasing species reported include *Porphyromonas gingivalis* (*P. gingivalis*), *Actinobacillus actinomycetemcomitans, Borrelia burgdorferi*, and *Pseudomonas aeruginosa*. [1–4].

Periodontitis is among the most common human infection, initiated by specific species of bacteria: *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *P. gingivalis* [5]. The pathogenesis of periodontitis is mediated through interactions between these microbial factors and the host cells. In particular, *P. gingivalis* has been frequently associated with specific types of periodontitis and possesses various mechanisms that favor the pathogenic process. *P. gingivalis* has been observed within gingival tissues *in vivo*, suggesting that *P. gingivalis* may invade deeper structures of the connective tissue [6]. On the other hand, *P. gingivalis* has been reported to be heavily involved in a wide variety of systemic diseases including cardiology, rheumatology, diabetology, oncology, immunology, and neurology [7]. Several mechanisms have been reported to associate *P. gingivalis* with these systemic diseases. *P. gingivalis* affects endothelial cells, platelets, leucocytes (mainly monocytes and macrophages), cardiomyocytes, and smooth muscle cells to aggravate cardiovascular diseases such as atherosclerotic cardiovascular diseases and myocardial infarction [8–10]. *P. gingivalis* enhances oxidative stress, inflammatory and prothrombotic responses in endothelial cells [11–13]. *P. gingivalis* fimbria and lipopolysaccharide (LPS) increase the expression of adhesion molecules in endothelial cells, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and monocyte chemoattractant protein (P-seletin and E-selectin) [14,15]. *P. gingivalis* fimbria and LPS also support monocyte to migrate and infiltrate into endothelial cells, which differentiate monocyte into pro-inflammatory macrophages [16,17]. More recently, *P. gingivalis* W83 strain was reported to invade into dermal microvascular cells via Intercellular Adhesion Molecule 1 (ICAM-1) and inhibit the formation of vascular networks [18]. Autoantibody against citrullinated proteins is considered an important pathological base of rheumatoid arthritis (RA). Regarding rheumatology, circulating bacterial antibodies and the anti-cyclic citrullinated peptide induced by periodontal pathogens such as *P. gingivalis* seems to be involved in the progression of RA [19]. *P. gingivalis* is the only microorganism to express the enzyme mediating protein citrullination called peptidylarginine deiminases (PAD) [20]. *P. gingivalis* PAD is proposed to break immunotolerance to citrullinated proteins, leading to the occurrence of RA [20]. Among the reported *P. gingivalis*-elicited mechanisms involved in diabetes, insulin resistance seems to be the most significant. *P. gingivalis* infection stimulates inflammation and elevates inflammatory markers, such as CRP and IL-6, leading to insulin resistance [21]. More recently, it was reported that *P. gingivalis* causes insulin resistance by the phosphorylation of insulin receptor through activation of mammalian target of rapamycin and related genes in the mice fed with high-fat diet [22]. *P. gingivalis* LPS also is involved in obesity-associated insulin resistance by inducing pro-inflammatory adipokine production and oxidative stress in adipocytes [23]. *P. gingivalis,* reportedly, raises the development of oral squamous cell carcinoma, esophageal and pancreatic cancers through epithelial-mesenchymal transition (EMT) of oral epithelial cells, the prevention of epithelial cell apoptosis, and the promotion of immune evasion, etc [8,24–26]. *P. gingivalis* modulates EMT by regulating zinc-finger homeobox proteins (ZEB1 and Zeb2) and GSK-3β/β-catenin/forkhead box-O1 (FOXO1) [27]. Furthermore, *P. gingivalis* controls epithelial apoptosis many apoptotic/anti-apoptotic pathways including PI3K/Akt and JAK/Stat pathways as well as mitochondrial apoptosis-related factors (Bad, Bcl-2, and Bax) [8,26,28]. In the field of neurology, *P. gingivalis* infection has been suggested to be the risk of Alzheimaer's disease (AD) and depression by several epidemiological studies [29,30]. Animal experiments also support the possible relevance of *P. gingivalis* in AD pathogenesis characterized as microglia-mediated neuroinflammation and β-amyloid (Aβ) accumulation in neurons, which impaires cognitive function and reduces in learning and memory [8,31,32]. *P. gingivalis* and its LPS are thought to be involved in the progression of AD via cathepsin B (CatB), a crucial mediator for Aβ deposition and neuroinflammation [31,33]. Inflammatory mediators - TNF-α, IL-1, IL-6, and IL-8 - released from host cells infected with *P. gingivalis* are also reported to associate with the progression of AD by reaching central nervous system via hematoencephalic barieer-free area [34,35].

Interestingly, in addition to these mechanisms described above, several reports demonstrated the involvement of *P. gingivalis* OMVs in the etiology of systemic diseases including diabetes and vascular calcification. *P. gingivalis* OMVs translocates to the liver and attenuates insulin sensitivity and hepatic glycogen synthesis by downregulating the Akt, -GSK3β-FOXO1 pathway [36,37]. *P. gingivalis* OMV induces vascular smooth muscle cell calcification through activation of ERK1/2-Runx2 [38]. These observations lead us to the advanced interpretation that *P. gingivalis*-involved diseases, including periodontitis, are not only caused by direct local infection and transfer of virulence factors into host cells, but also by strategic indirect communication devices of *P. gingivalis*. Among the several factors associated with *P. gingivalis*-involved diseases, increasing evidence suggests that OMVs contribute to the pathogenesis of this bacterium. Therefore, the function of OMVs synthesized from *P. gingivalis* will be reviewed and discussed here.

2. *P. gingivalis* OMVs generation

Based on their membrane properties, bacteria are classified as Gram-negative or Gram-positive. Gram-negative bacteria are characterized by a double plasma membrane layer separated by the periplasm. Most EVs produced by Gram-negative are thought to be OMVs, which are composed of a single bilayer membrane derived from an outer membrane and contain periplasmic contents such as lipids, outer membrane proteins, lipoproteins, and lipids [39,40]. *P. gingivalis*, a gram-negative bacterium, has been observed to produce vesicles on its cell surface and release them to its environments [41,42].

The mechanism of OMVs biogenesis has not been fully elucidated, despite their biological importance. The curvature of the OMVs membrane is approximately 14 times that of the extracellular membrane, suggesting that OMVs generation requires energy expense due to the prominent curvature of the outer membrane [40]. These observations indicate that *P. gingivalis* OMVs generation of could be completed by increasing the production of specific inner or outer leaflet lipids. This is carried out by anionic lipopolysaccharide (A-LPS) on the outer surface and its related C-terminal domain (CTD) family proteins [40].

Various factors have been reported to be involved in the formation of OMVs. Autolysins, endogenous murein hydrolases that cleave covalent bonds in the peptidoglycan of the cell wall, play an important role, physiologically, in bacterial growth, cell division, cell wall remodeling, peptidoglycan turnover, and recycling [43]. Autolysin is involved in the release of *P. gingivalis* OMVs [44]. There are three types of gingipains in *P. gingivalis*: lysine-specific gingipain (Kgp), arginine-specific gingipain A (RgpA), and arginine-specific gingipain B (RgpB) [45,46]. These gingipains are encoded by the *rgpA*, *rgpB*, and *kgp*, genes respectively. In contrast to the mature RgpB, which only possesses a catalytic domain, RgpA and Kgp have

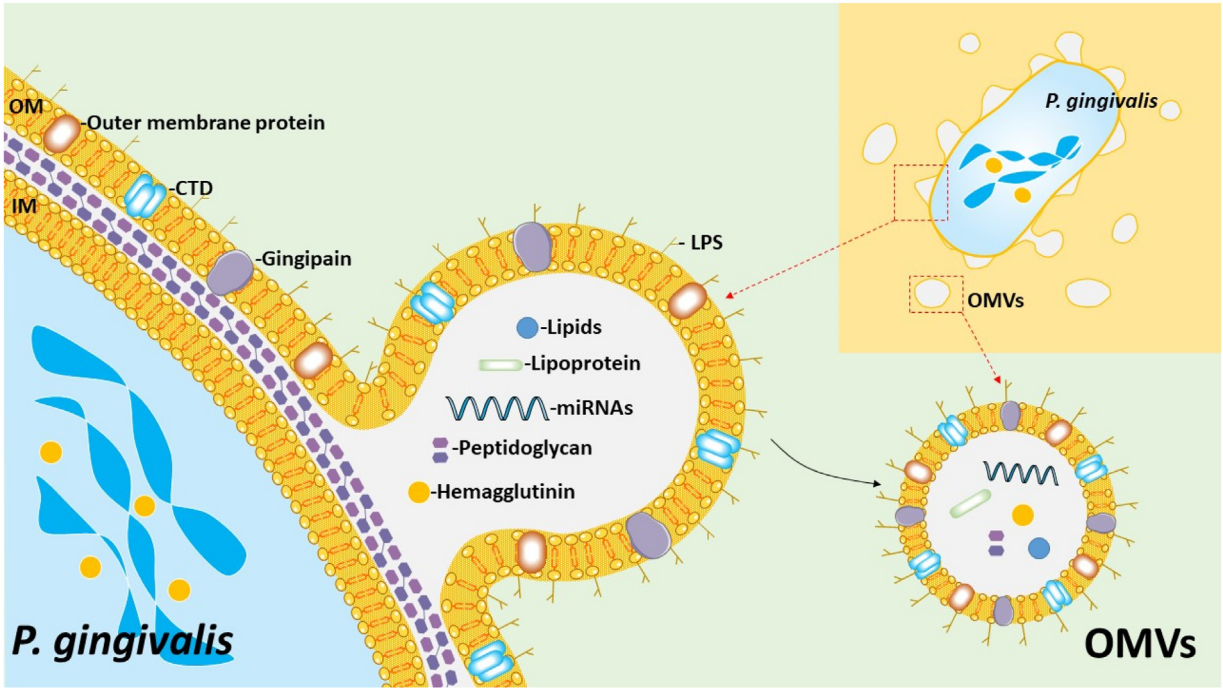

Fig. 1. Schematic summary of the *P. gingivalis* OMVs generation.
OMVs are composed of a single bilayer membrane derived from an outer membrane and contain periplasmic contents such as lipids, outer membrane proteins, lipoproteins, and lipids. The generation of OMVs is considered to be carried out by anionic lipopolysaccharide (A-LPS) on the outer surface and its related C-terminal domain (CTD) family proteins. Dephosphorylation of lipid A of A-LPS by PG0027 induces the destabilization of the outer membrane, resulting in blebbing and the generation of OMVs. LptO is essential for the O-deacylation of LPS and for the coordinated secretion and attachment of A-LPS and CTD proteins. OM: outer membrane; IM: inner membrane.

homologous adhesin/hemagglutinin domains as a part of their C-terminal extension [47]. Among them, the deletion of RgpA results in a reduction of OMVs [48]. *P. gingivalis* LPS is the major component of the outer membrane of the bacteria that stimulates the innate immune system and is a key virulence factor which induce the production of cytokines [49]. *P. gingivalis* has A-LPS, consisting of phosphorylated branched mannan repeating unit attached to the lipid A core [50]. A-LPS and PG0027, outer membrane proteins, are also involved in the formation of OMVs [51]. PG0027 dephosphorylates lipid A of A-LPS; this process may be necessary for optimal OMVs formation. Dephosphorylation of lipid A of A-LPS, controlled by PG0027, induces the destabilization of the outer membrane, resulting in blebbing and the generation of OMVs [51].

The surrounding environment affects the generation of *P. gingivalis* OMVs. *P. gingivalis* can adapt to the surrounding environment, and its gene expression is controlled by extracytoplasmic function (ECF) sigma factors. SigP is one of these factors and mutation of sigP increases the level of OMVs formed on the *P. gingivalis* cell surface [52]. SigP is thought to be involved in activities – gingipain activity, autoaggregation, hemagglutininination, vesicle formation, antimicrobial susceptibility – associated with the bacterial surface [52]. In addition, under hemin-limitation, the gravimetric yield of OMVs increased by 2.5-folds, although cell yield did not changed. Growth in hemin-excess conditions resulted in increased hemin-binding capacities of OMVs [53].

3. *P. gingivalis* OMVs characteristics

There are several cases that *P. gingivalis* bacterial cells are not detected in distant tissues or organs although *P. gingivalis* DNA is present. Compared with *P. gingivalis* bacterial cells, *P. gingivalis* OMVs have various functional advantages. For instance, *P. gingivalis* OMVs possess some well-known bacterial virulence compared to levels found in *P. gingivalis* bacterial cells. There are approximately 3–5 fold increase in gingipain levels in *P. gingivalis* OMVs compared with the levels in surface extracts of the original bacterial cells [54]. *P. gingivalis* OMVs, moreover, show the increased antigenicity, which may result from the more concentrated immune-responsive factors on the vesicles compared with the surface of bacterial cells [55,56]. Additionally, *P. gingivalis* OMVs bring bacterial DNA and signal molecules to distant target organs without degradation. In the mice applied with *P. gingivalis* into gingival sulcus, DNA of *P. gingivalis* was detected in the liver by PCR. However, the bacterial cells was not observed in the liver under strict examination by a transmission electron microscope [57]. It was also reported that in RA patients *P. gingivalis* organisms are not observed in joint fluid although its DNA was detected [58]. *P. gingivalis* OMVs are thought to be an important carrier of *P. gingivalis* DNA to distant organs without the direct translocation of *P. gingivalis* bacteria cells.

Fig. 1 shows a schematic summary of the structure and representative components of *P. gingivalis* OMVs. *P. gingivalis* OMVs were originally reported in the 1980s; however, the biological pathogenic functions were not immediately recognized. Similar to that of other gram-negative bacterial vesicles, the diameter of *P. gingivalis* OMVs is approximately 50–250 nm but is most commonly around 50 nm [59]. OMVs are not only just a part of a bacterial component, but also include a toxic complex of LPS and proteolytic enzymes (proteases). Bacteria discharges vesicles like missiles as a mechanism for survival and also to create an environment for growth and proliferation. LPS and the proteases associated with *P. gingivalis* OMVs are likely to represent the heat-stable and the heat-unstable components, respectively [60].

The major outer membrane proteins Pgm6/7, which are homologous to the OmpA protein in *Escherichia coli,* are important for maintaining the integrity of the outer membrane. The loss of Pgm6/7 induced wavy and irregular OM and increased the number of vesicles and the rate of gingipain activity [61]. PGN_1251 (gtfB) is also involved in the transition of gingipain to the cell surface.

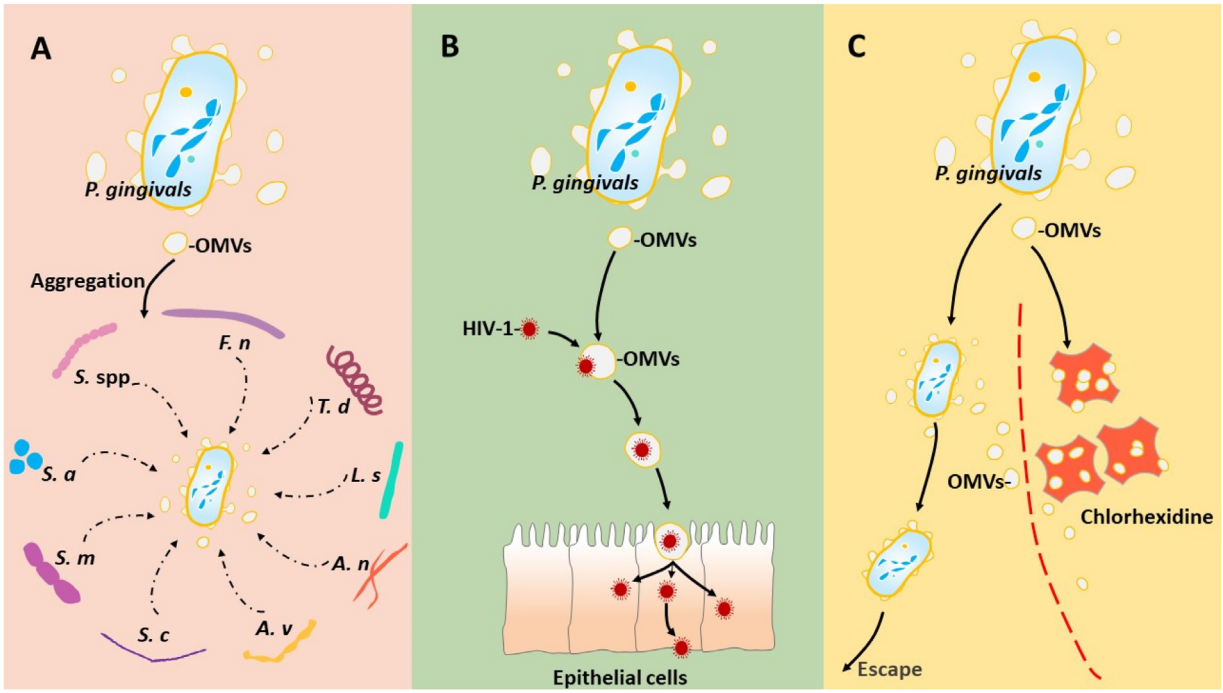

Fig. 2. Involvement of *P. gingivalis* OMVs in aggregation of other microorganisms.
(A), *P. gingivalis* OMVs aggregate *Streptococcus* spp (*S.* spp), *Fusobacterium nucleatum* (*F. n*), *T. denticola* (*T. d*), *L. saburreum* (*L. s*), *Actinomyces naeslundii* (*A. n*), *and Actinomyces viscosus* (*A. v*), *S. cricetus* (*S. c*), and *S. mutans* (*S. m*). Gingipains of *P. gingivalis* OMVs are involved in autoaggregation of *S. aureus* (*S. a*). *P. gingivalis* OMVs supports viral entry into oral epitherial cells. (B), *P. gingivalis* OMVs supports viral entry into oral epitherial cells. (C), *P. gingivalis* OMVs protect themselves and other species of oral bacteria by binding to the antimicrobial chlorhexidine.

PGN_1251 (gtfB) shares homology with genes encoding for glycosyltransferase 1 with several bacteria. Both LPSs containing O side chain polysaccharide (O-LPS) and anionic polysaccharide (A-LPS) were not synthesized in gftB mutant bacteria, resulting in a complete loss of surface-associated proteins including gingipain [62].

Furthermore, *P. gingivalis* possesses a unique CTD system essential for secretion and attachment to the cell surface. The attachment of PgpB proteinases to the outer membrane is associated with the presence of a conserved CTD of approximately 70-amino-acid residues in the encoded sequences [63]. The outer membrane protein LptO is essential for the O-deacylation of LPS and for the coordinated secretion and attachment of A-LPS and CTD proteins [64]. It was also reported that OMVs are enriched in high molecular weight A-LPS molecules. Sorting factors within *P. gingivalis* OMVs is selective, and LPS plays an important role [65].

Lo A, et al. demonstrate that both FimR and FimS are involved in *P. gingivalis* biofilm formation, including the regulation of genes associated with fimbriation (Lo et al., 2010). Mantri et al. compared the protein components of *P. gingivalis* OMVs using a fimbriated strain (33277) and *P. gingivalis* of an afimbriated strain (W83) [54]. Gingipain and hemagglutinin were contained in the OMVs produced from both bacterial strains. FimC, FimD and FimE were found in the OMVs of 33277 strain, but not in W83 vesicles, indicating that different species contain different factors in their own OMVs.

P. gingivalis OMVs have been demonstrated to enter cells such as human oral keratinocytes and gingival fibroblasts more effectively than originating bacterial cells [55]. FimA, a well-known adhesion responsible factor, is considered important for the adhesion and invasion of *P. gingivalis* into the host cells. However, the OMVs derived from the mutant strain of FimA were able to penetrate the cells [54]. On the other hand, in the case of OMVs produced FimR-deleted *P. gingivalis*, the penetration rate was significantly reduced. Thus, FimR is most likely involved in the invasion of *P. gingivalis* OMVs into host cells, not FimA.

4. Supporting other microorganisms

Fig. 2 shows various functions of *P. gingivalis* OMVs. *P. gingivalis* OMVs may protect other bacterial species from complement action; thus, favoring the pathogenic process of periodontitis. *P. gingivalis* OMVs can aggregate a wide range of *Streptococcus* spp., *Fusobacterium nucleatum, T. denticola, L. saburreum, Actinomyces naeslundii, and Actinomyces viscosus* [66,67]. Also, *P. gingivalis* OMVs are capable of attaching to various molecules [68], which supports the aggregation of *S. cricetus* and *S. mutans* as well as the attachment of bacteria such as *A. viscosus* to the tooth surface [69]. Autoaggregation of *S. aureus* in the presence of *P. gingivalis* OMVs is inhibited by L-arginine, L-lysine, and L-cysteine, suggesting that gingipains are involved in these effects.

It has been found that the receptor-independent invasion of human immunodeficiency virus-1 (HIV-1) into epithelial cells is mediated by *P. gingivalis*. *P. gingivalis* OMVs promote mucosal transmission of HIV-1. Dynabeads technology showed that a specific interaction between HIV-1 and *P. gingivalis* OMVs supports viral entry into oral epithelial cells [70]. Subsequently, HIV-1 was reverse-transcribed and viral DNA was integrated into the genome of these cells. OMVs released from *P. gingivalis* are thought to be vehicles for HIV-1 and promote infection to the mucous membranes.

P. gingivalis OMVs bind to the antimicrobial chlorhexidine via LPS and protect themselves and other species of oral bacteria. In such a way, the interaction between bacteria in the oral cavity affects the sensitivity of microbes to drugs [71].

5. Systemic diseases

P. gingivalis exerts its pathogen to different organs (sometimes to distant organs), manipulates the invasion of other cells, disrupts phagocytosis, affects complement-related factors, and induces pro-inflammatory signaling cascades. Other review arti-

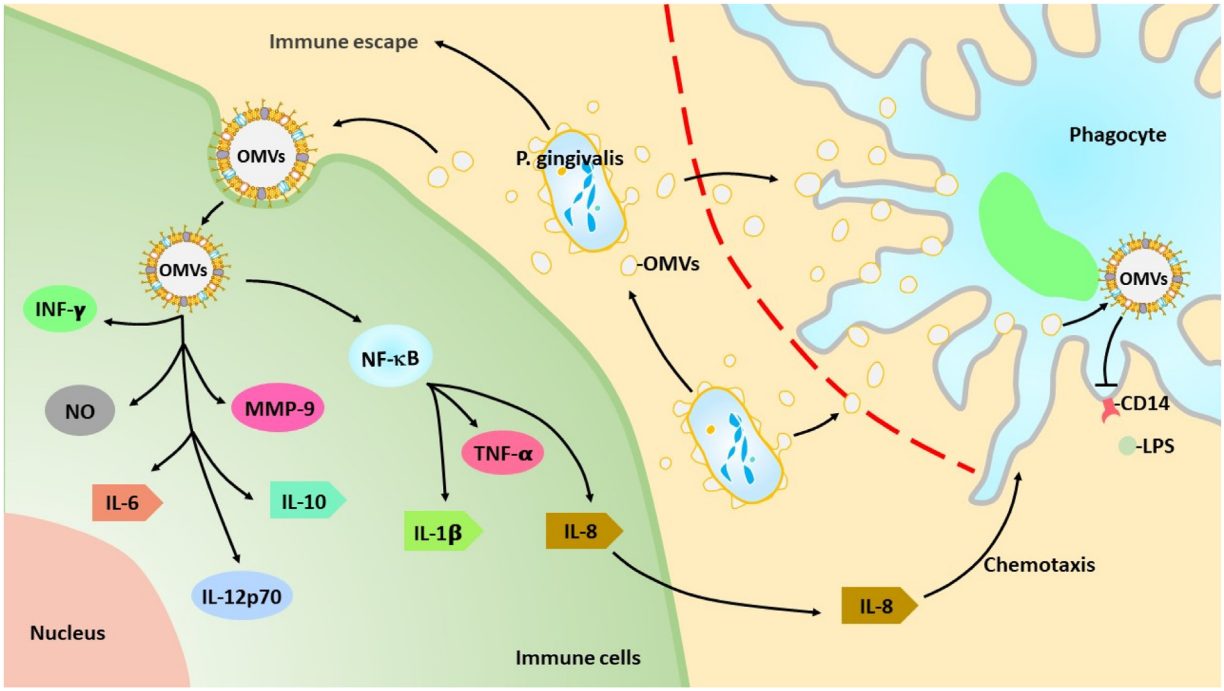

Fig. 3. Schematic summary of the effects of *P. gingivalis* OMVs on host cells.
P. gingivalis OMVs can effectively enter the host cells and destroy the tissues by disrupting the immune system and regulating cellular responses involved in inflammation and acquired immunity.

cles have already described the effects of *P. gingivalis* bacteria itself on systemic diseases such as atherosclerosis and AD as well as its involvement in signal transduction in disease-related cells [72]. Therefore, in this review, the role of *P. gingivalis* OMVs in these systemic diseases is highlighted.

5.1. Arteriosclerosis

Atherosclerosis, a chronic inflammatory disease of the blood vessels, is one of the most common causes of morbidity and mortality worldwide. The association between atherosclerosis and periodontitis has been epidemiologically suggested. In addition, there is research to elucidate this by molecular biological strategy. Bacteria and their products in dental plaque enter the bloodstream from the infected site, and become involved in arteriosclerosis and thromboembolic events [73–76]. Platelet aggregation is considered important for atherosclerotic plaque formation. *P. gingivalis* OMVs possess powerful platelet aggregation activity [77], implying that *P. gingivalis* is most causative agent for arterial infarction.

Foam cells, fat-storage macrophages indicating signs of plaque formation or atherosclerosis, are generally associated with an increased risk of heart attack and stroke. It was also demonstrated that *P. gingivalis* OMVs stimulates foam cell formation in murine macrophage cells [78]. In addition, several studies reported that *P. gingivalis* OMVs induced LDL aggregation, which then induced murine macrophage to form foam cells [79,80]. Thus, *P. gingivalis* OMVs released from periodontitis into the circulation may deliver virulence factors to the arterial wall to initiate or promote foam cell formation in macrophages and contribute to atheroma development. *P. gingivalis* OMVs are more potent inducers of the inflammatory response associated with the development of atherosclerosis [81].

5.2. Alzheimer's disease

Research concerning AD is also being conducted with the focus on the oral cavity and the brain. According to various key evaluations of prospective and retrospective population-based data, the presence of chronic inflammation for over 10 years increases the risk of developing sporadic AD by more than two times [82]. In *in vivo* experiments, *P. gingivalis* infection induces pathological phenomena such as inclusive of extracellular amyloid plaques and enhances phosphorylation of tau, characteristics of AD-like phenotype. Other studies have also investigated the decline in cognitive functions in AD patients with untreated periodontitis [82]. It seems that *P. gingivalis* OMVs are being used effectively as a trigger for inflammation and inflammatory mediators as a result of OMVs attack, which may lead to organic and functional changes that define inflammation and impaired cognitive abilities typical of AD.

5.3. Rheumatoid arthritis

P. gingivalis has been recently hailed as a potential etiology of autoimmune disease RA. In particular, PAD of *P. gingivalis* is involved in the citrullination of bacterial or host cell proteins. PAD then induces the production of anticitrullinated protein antibodies, leading to the loss of tolerance to citrullinated proteins in RA patients. PAD exists in the form of two variants: the outer-membrane-bound state and the soluble secreted state [83,84]. This different feature of PAD is closely related to the transport system for PAD excretion, and PAD binds to the outer membrane via A-LPS anchoring [85]. As a result, in many cases, PAD is present in OMVs secretion (less common in the soluble state). Interestingly, some *P. gingivalis* strains showed a reduced PAD binding to OMVs, which was closely related to the substitution of 373 Glutamine for lysine residues [86]. It is strongly suggested that the glutamine residue in this part is involved in the association between PAD and OMVs. Recently, a comparative analysis of wild type *P. gingivalis* and PAD deficient strains was performed [87]. In this report, 51 citrullinated proteins (51 of 78) of wild type OMVs were identified, of which most were assumed to have been translocated to the bacterial surface. PAD transferred to *P. gingivalis* OMVs seems to be strongly involved in the pathogenesis of RA.

6. The effects of OMVs on host cells

6.1. Immune cells

Fig. 3 shows a schematic summary of the effects of *P. gingivalis* OMVs on host cells. *P. gingivalis* OMVs can be used offensively as delivery systems for virulence factors and defensively to aid in the survival of the bacterium in hostile environments. *P. gingivalis* OMVs can enter cells more effectively than originating bacterial cells [55]. *P. gingivalis* OMVs seem to contribute to tissue destruction by disrupting the immune system and regulating cellular responses involved in inflammation and acquired immunity.

OMVs play a role in local immune evasion strategies that promote monocyte unresponsiveness and make microbial detection difficult [72]. *P. gingivalis* OMVs have been reported to contribute to the loss of LPS receptor, a membrane-bound CD14 receptor. Such a phenomenon results in a hyporesponsiveness of macrophages to LPS stimulation, which may contribute to an increased virulence capacity of *P. gingivalis*. Furthermore, some strains of *P. gingivalis* can raise matrix metalloproteinase 9 (MMP9) activity in macrophages and may be involved in plaque disruption. In the destructive periodontitis, *P. gingivalis* stimulates monocytes primed with IFNγ to release IL-12, thereby enhancing IFNγ accumulation in T-cell populations [88]. Gingipain on the surface of *P. gingivalis* OMVs membrane induces IL-8 cleavage and activation, leading to the recruitment of neutrophils [89]. *P. gingivalis* OMVs induce nitric oxide (NO) production in RAW264.7 cells [90]. Nakao et al. investigated the effect of *P. gingivalis* OMVs on host immune response and tissue destruction during *P. gingivalis* infection. *P. gingivalis* OMVs had high antigenic, and absorption of patient sera with OMVs greatly reduced reactivity with whole cells of *P. gingivalis* [91]. Interestingly, *P. gingivalis* OMVs suppress monocyte unresponsiveness to living *P. gingivalis* but maintain bacterial responsiveness to DNA. TLR2 plays a central role in the innate immunity of *P. gingivalis*, but TLR4 appears to have a selective role in the response to *P. gingivalis* OMVs [92].

Some reports reveal the immense ability of *P. gingivalis* OMVs to induce inflammation compared with that of *P. gingivalis* bacteria itself. Treatment of *P. gingivalis* OMVs immensely stimulates the production of TNFa, IL-12p70, IL-6, IL-10, IFNb, and NO in macrophages, whereas direct infection of *P. gingivalis* shows a significantly lower effect [93]. *P. gingivalis* OMVs stimulation causes a metabolic shift in macrophage and increases the expression of genes important for glycolysis and decreases the expression of genes related to the TCA cycle. *P. gingivalis* OMVs, without the direct infection of *P. gingivalis*, induces inflammasome activation (activation of Caspase-1 and production of IL-ib and IL-18). These findings indicate that *P. gingivalis* and *P. gingivalis* OMVs have different effects on macrophage inflammatory phenotype, mitochondria function, inflammasome activation, etc.

Cecil et al. investigated immuno-modulatory effects of *P. gingivalis* OMVs on monocytes and differentiated macrophages [94]. *P. gingivalis* OMVs were phagocytosed into monocytes and M (naïve) and M (IFNg) macrophages. *P. gingivalis* OMVs induced NF-kB activation and cytokine secretion such as TNFa, IL-8, and IL-1b. *P. gingivalis* OMVs also induced anti-inflammatory IL-10 secretion [94,95]. These observations indicate that *P. gingivalis* OMVs interfere with the reaction of host immune cells and may contribute to local immune evasion.

6.2. Non-immune cells

P. gingivalis OMVs affect non-immune cell function and elicit signal transduction. *P. gingivalis* OMVs significantly inhibit the proliferation of human gingival fibroblasts capillary tube formation in cultured human umbilical vein endothelial cells (HUVEC) [96]. *P. gingivalis* OMVs enter gingival epithelial cells via lipid rafts through the actin filament assembly in phosphatidylinositol 3-kinase and Rac1 dependently. After entry, OMVs-associated gingipains degrade cellular proteins, which are essential for intracellular transferrin and cellular migration, leading to their functional impairment [97]. *P. gingivalis* OMVs induce the expression and activity of eNOS in HUVEC through Rho kinase (ROCK)-stimulated ERK1 / 2 and p38 MAPK [98]. *P. gingivalis* OMVs promote calcification of vascular smooth muscle cells, implying the involvement in arteriosclerosis. *P. gingivalis* OMVs enhance the expression of markers of osteoblast differentiation and calcification in vascular smooth muscle cells and calcification via ERK1 / 2-Runx2 [38]. In addition, *P. gingivalis* OMVs induce oral squamous epithelial cell detachment, which is inhibited by preincubating *P. gingivalis* OMVs with anti-gingipain serum. E-selectin and ICAM-1 are important factors for the adhesion of leukocytes to endothelial cells. *P. gingivalis* OMVs induced the expression of E-selectin and ICAM-1 on the surfaces of vascular endothelial cells. These findings demonstrate that *P. gingivalis* OMVs are capable of inducing acute inflammation, which is characterized by the accumulation of large numbers of neutrophils in connective tissues [99].

7. Vaccination

Periodontitis is the most prevalent infectious disease and is related to oral and systemic health; therefore, novel prophylaxis to prevent the disease is highly desirable. There are several attempts to develop novel vaccines using *P. gingivalis* OMVs and some desirable results have been obtained. Nasally or percutaneously administered 40-kDa outer membrane protein of *P. gingivalis* elicits specific antibodies, which inhibits the co-aggregation activity of *P. gingivalis*, implying that this fragmented outer membrane protein is useful for vaccination against chronic periodontitis [100–102]. A few years later, Nakao et al. assessed the capacity of *P. gingivalis* OMVs as a vaccine antigen by intranasal immunization to BALB/c mice. They proposed that *P. gingivalis* OMVs are an intriguing immunogen for the development of a periodontitis vaccine [56]. Recently, their group performed sub-immunoproteome analysis using *P. gingivalis* OMV-immunized mouse serum samples to identify immunodominant antigens. As a result, it has been reported that LPS and A-LPS-modified proteins in *P. gingivalis* OMVs are immunodominant determinants that lead to the production of *P. gingivalis*-specific antibodies in mice [103].

P. gingivalis OMVs are stable structurally and functionally, resistant to proteinase K, able to withstand long-term storage, and advantageous in delivering components to host immune cells. The challenge to generate antibodies specific to *P. gingivalis* is proceeded by using intranasal vaccination of *P. gingivalis* OMVs [104].

8. Contents of *P. gingivalis* OMVs and movement in the host body

Recent studies using genetic, proteomic, and morphological tools have demonstrated that *P. gingivalis* OMVs include a variety of virulence factors provided by parental cells [70]. As described above, *P. gingivalis* OMVs possess various components of outer membrane constituents, including LPS, muramic acid, a capsule, fimbriae, and gingipains [41,42]. *P. gingivalis* OMVs are potent vehicles for transmitting virulence factors into the host cells and are involved in the etiology of periodontitis.

The proteins in *P. gingivalis* OMVs were examined by LC–MS/MS analysis [105]. Most proteins are derived from outer membrane or periplasm, which are distributed on the vesicle surface, membrane, lumen, etc. Numerous virulence factors including all CTD proteins are shown to be preferentially packaged into *P. gingivalis* OMVs.

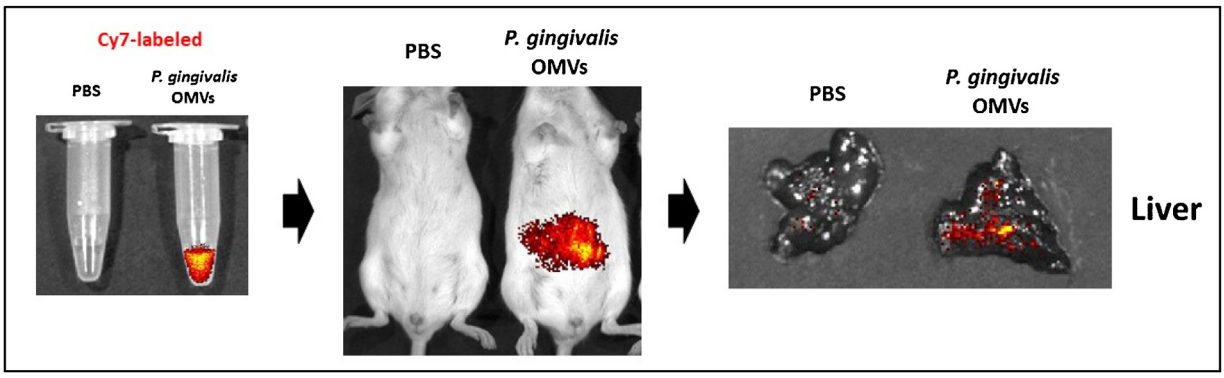

Fig. 4. Experimental strategy for tracking the movement of *P. gingivalis* OMVs *in vivo*.
We established a novel tracking system of *P. gingivalis* OMVs by *in vivo* imaging. *P. gingivalis* OMVs were incubated with Cy7 Mono NHS Ester and Cy7-labeled *P. gingivalis* OMVs were injected intraperitoneally to mice. Cy7 fluorescence was detected in the liver by in vivo imaging system (IVIS) Spectrum.

Small RNAs are also incorporated in *P. gingivalis* OMVs. *P. gingivalis* has also been shown to have small RNAs similar in size to eukaryote microRNAs (miRNAs). Small RNA, similar in size to miRNAs (miRNA-size, small RNAs or msRNA), has a length of 15–25 nucleotides, and its precursor is assumed to be the secondary structure of a hairpin loop. Deep sequencing reveals that msRNA expressed on *P. gingivalis* are secreted via OMVs [106]. *P. gingivalis* OMVs are also found to be capable of transporting RNAs to eukaryotic cells. These exogenous msRNA are reported to suppress cytokines in Jurkat cells [106]. msRNA may be a new bacterial signaling molecule that connects bacteria to humans.

As far as we know, no reports have directly analyzed the existence and pathogenetic properties of *P. gingivalis* OMVs in human clinical isolates. Gabarrini et al. primitively tested *P. gingivalis*'s peptidylarginine deiminase using unfiltered growth medium fractions of 93 clinical isolates, which contain both OMV-associated proteins [86]. In recent years, we showed that *P. gingivalis* OMVs were equipped with *P. gingivalis*-derived proteases gingipains and translocated to the liver in mice (Fig. 4). In these mice, the hepatic glycogen synthesis decreased in response to insulin, and thus, high blood glucose levels were maintained. *P. gingivalis* OMVs also attenuated the insulin-induced Akt/glycogen synthase kinase-3 β (GSK-3β) signaling in a gingipain-dependent fashion in hepatic HepG2 cells [57].

9. Future direction

Small particles secreted from oral bacteria, which was initially thought to be debris or artificial products, are now considered the most sophisticated tools conducted by oral bacteria. Oral bacteria can use these tools to deliver their nucleic acids and proteins to local and distant organs and tissues. In recent years, our preliminary study has shown that *P. gingivalis* invades macrophages and hides pathogenic factors in EVs released by the macrophages. The released EVs in turn can reach distant organs such as the brain and the lungs more efficiently. What tools should we utilize to cope with such a tricky oral bacterial strategy? The communication between oral bacteria and the host cells via small vesicles is still unknown to a large extent, and further clarification is strongly required.

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Conflict of interest

The authors declare that they have no conflict of interest.

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