SHORT REVIEW

# Host-derived extracellular vesicles for antimicrobial defense

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**One sentence summary:** Antimicrobial extracellular vesicles produced by organisms ranging from bacteria to humans contribute to host defense against microbes and offer new strategies for the development of therapeutics.
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## ABSTRACT

Extracellular vesicles are of increasing importance in the clinic, as diagnostics for complex diseases and as potential delivery systems for therapeutics. Over the past several decades, extracellular vesicles have emerged as a widespread, conserved mechanism of intercellular and interkingdom communication. The ubiquitous distribution of extracellular vesicles across life offers at least two compelling opportunities: first a path forward in the design of targeted antimicrobial delivery systems; and second, a new way to view host pathogenesis during infection. Both avenues of research are well underway. In particular, preliminary studies showing that plant and human host-derived extracellular vesicles can deliver natural antimicrobial cargos to invading fungal and bacterial pathogens are captivating. Further, modification of host extracellular vesicle populations may ultimately lead to enhanced killing and serve as a starting point for the development of more advanced therapeutic options, especially against difficult to treat pathogens. Despite the rapid pace of growth surrounding extracellular vesicle biology, many questions remain unanswered. For example, the heterogeneity of vesicle populations continues to be a confounding factor in ascribing clear functions to a vesicular subset, and the molecular cargos responsible for specific antimicrobial actions of extracellular vesicles during infection remain especially poorly described. In this short review, we will summarize the current state of affairs surrounding the antimicrobial function, and potential, of host-derived extracellular vesicles.

## INTRODUCTION

Extracellular vesicles were originally described in 1967 as 'platelet dust,' which was physically separable from platelets by ultracentrifugation (Wolf 1967). In the intervening years, research into these small particles has grown rapidly, in large part due to their involvement in a wide array of diseases and their obvious potential as biomarkers (Hargett and Bauer 2013). The term 'extracellular vesicle' was a later addition to the field, but now broadly encompasses a heterogenous group of cell-derived, lipid-bilayer-enclosed particles important for both intercellular and inter-organismal communication in all domains of life (Colombo, Raposo and Thery 2014; van Niel, D'Angelo and Raposo 2018). In prokaryotes, extracellular vesicles are abundantly produced by both Gram-negative and Gram-positive bacteria and typically referred to as outer membrane vesicles or more broadly bacterial extracellular vesicles, respectively (MacDonald and Kuehn 2012; Toyofuku, Nomura and Eberl 2019). In the Eukaryota, exosomes, microvesicles, and apoptotic cell-derived extracellular vesicles (frequently referred to as apoptotic bodies) are the terms most-widely accepted to describe the three major groups based on their biogenesis through multivesicular bodies, membrane shedding, and apoptosis, respectively (Lorincz et al. 2015). However, it is clear that even these terms are too simple for the description of the eukaryotic extracellular vesicle population in its entirety, as additional layers of complexity can be observed with further purification (Jeppesen et al. 2019). In general, extracellular vesicles carry a combination of lipids, nucleic acids, proteins, and metabolites, and the particular cargo found within a subset appears to be tightly regulated and highly dependent on cell of origin and isolation method (Kolonics et al. 2020b; He et al. 2021). Thus, in an effort to standardize research into extracellular vesicles, the International Society for Extracellular Vesicles (ISEV) has released several position statements, most recently in 2019, to define the minimal requirements to report findings on extracellular vesicles (Thery et al. 2018; Russell et al. 2019). The community is slowly implementing these guidelines, and the quality of reporting and reproducibility appears to be generally improving.

Even with these new guidelines, the size distributions of extracellular vesicles are still contested in the literature. The majority of researchers agree that exosomes are typically less than 150 nm in diameter (Fig. 1; Russell et al. 2019) and formed by invagination of late endosomal membranes to create intra-luminal vesicles, within a structure known as a multivesicular body (Table 1; Latifkar et al. 2019). The multivesicular body then fuses with the plasma membrane to release the exosomes into the extracellular space. We now know that biogenesis and cargo loading of exosomes are linked through the action of the Endosomal Sorting Complex Required for Transport (ESCRT) family of proteins, which are important for membrane bending/budding (Juan and Furthauer 2018). However, the ESCRTs are not the only factors that contribute to exosome cargo loading, as more recently, ESCRT-independent mechanisms of cargo selection have also been described, including mechanisms reliant on lipid raft formation or dependent on ceramide-mediated recruitment (Li et al. 2018). Microvesicles, typically greater than 200 nm in diameter, are shed directly from the plasma membrane after distinct, localized changes in lipid content (Haraszti

et al. 2016; Tricarico, Clancy and D'Souza-Schorey 2017). Interestingly, microvesicles also exhibit selective enrichment for certain cargo, despite budding directly from the plasma membrane. Apoptotic cell-derived extracellular vesicles, a heterogeneous fraction of 50–5000 nm diameter vesicles, are less well defined but are again formed during the highly regulated cellular process of apoptotic cell disassembly (Caruso and Poon 2018). This diverse class of extracellular vesicles also includes more specific subsets, including the apoptotic bodies (1000–5000 nm in diameter; Table 1) and apoptotic microvesicles (50–1000 nm in diameter). Although the biogenesis of each subset of extracellular vesicles appears to be biochemically distinct, many features are shared across subsets, including a high level of regulation and the specificity of cargo selection.

Nearly every cell-type tested has been shown to produce extracellular vesicles, and many different functions have been ascribed to this diverse category of small particles. In addition to intercellular signaling, extracellular vesicles have also been shown to be important in microbial communication, disease, and host pathogenesis (Buck et al. 2014; Bielska et al. 2018; Genschmer et al. 2019; Munhoz da Rocha et al. 2020). The function of extracellular vesicles during infectious disease depends on the particular host and pathogen. In many cases, extracellular vesicles have been shown to play a role in propagating the immune response against invading pathogens (Rybak and Robatzek 2019; Kolonics et al. 2020b). The contribution of extracellular vesicles to pathogenesis is not one-sided, as many pathogens including bacteria, fungi, and parasites also depend on activities encased in extracellular vesicles to exploit their host (Kim et al. 2015; Ofir-Birin, Heidenreich and Regev-Rudzki 2017; Kuipers et al. 2018; Liu et al. 2018; Bielska et al. 2019; Ofir-Birin and Regev-Rudzki 2019). Interestingly, extracellular vesicles are even relied upon by the host to control viral infection, as vesicles containing the RNA editing enzyme APOBEC3G are known to help the host fight against HIV infection (Khatua et al. 2009). Bacteria have been observed on several occasions to increase production of outer membrane vesicles as cellular decoys in defense against phage infection or even to counteract attacks by the host complement system (Manning and Kuehn 2011; Roier et al. 2016; Reyes-Robles et al. 2018; McNamara and Dittmer 2020). Collectively, these examples highlight just a few of the many roles of extracellular vesicles in mediating the outcome of infection.

In this short review, we focus not on the pathogen or the propagation of host immunity by extracellular vesicles, but instead on how extracellular vesicles may function as discrete containers to deliver antimicrobials to their competitors and enemies. We intend to define the common antimicrobial theme present in each of these systems as we provide an outlook for the future development of antimicrobial therapeutics based on knowledge gained from the study of extracellular vesicles.

## HUMAN HOSTS PRODUCE ANTIMICROBIAL EXTRACELLULAR VESICLES AGAINST BACTERIAL PATHOGENS

Although hypothesized earlier (Hess et al. 1999; Gonzalez-Cano et al. 2010), the first reported experimental demonstration of an antibacterial function for host-derived extracellular vesicles
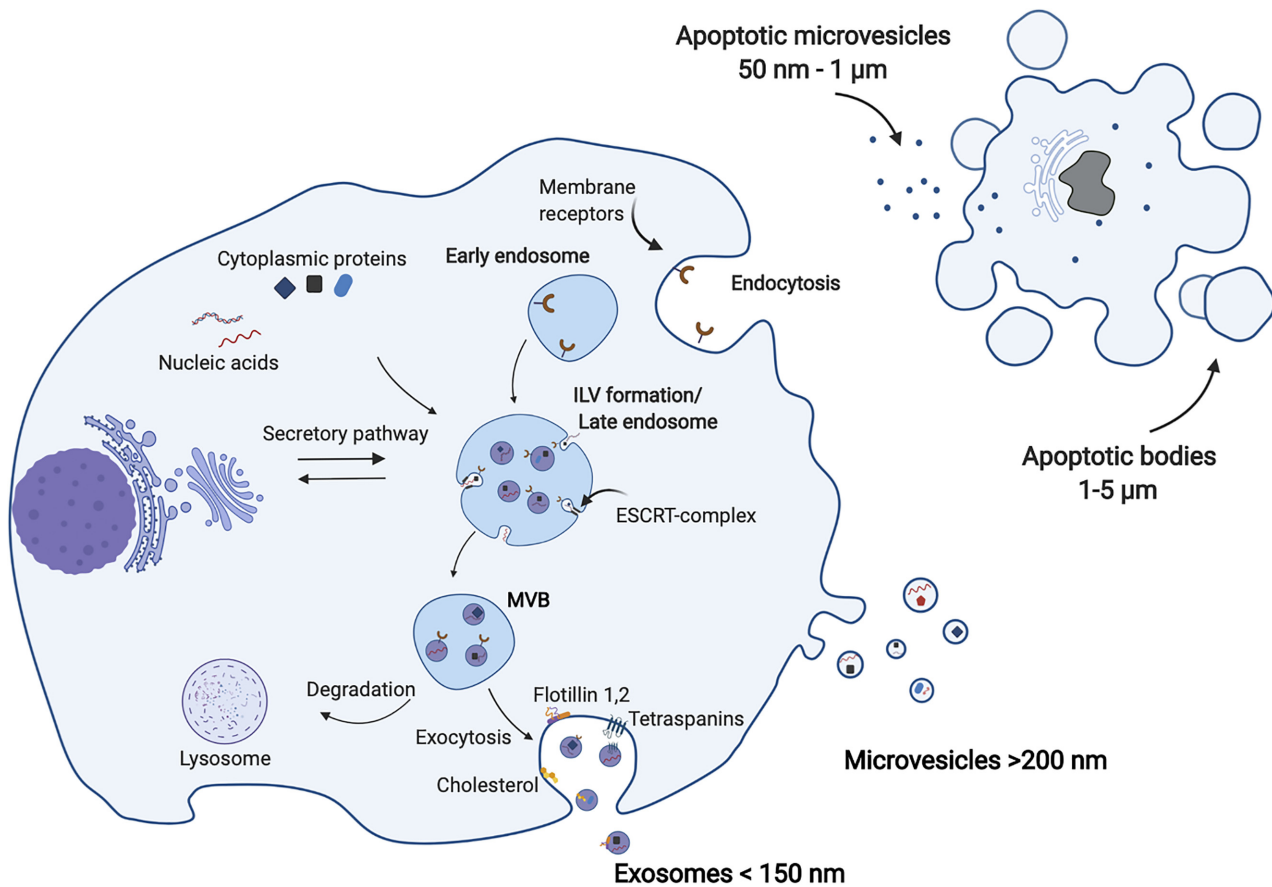
**Figure 1.** Biogenesis of extracellular vesicles. Extracellular vesicles are produced through a variety of different cellular processes, including budding of plasma membrane (microvesicles), release of intraluminal vesicles (ILV) from multivesicular bodies (exosomes), and apoptotic cell disassembly (apoptotic cell-derived extracellular vesicles). Created with BioRender.com.

**Table 1.** Nomenclature and origin of commonly described extracellular vesicle populations.

| Extracellular vesicle category | | Characteristic diameter (nm) | Cellular origin | Reference |
|---|---|---|---|---|
| Mammalian-derived | Exosome | <150 | Multivesicular body | Russell *et al.* (2019) |
| | Microvesicle | >200 | Plasma membrane | Tricarico, Clancy and D'Souza-Schorey (2017) |
| | Apoptotic cell-derived extracellular vesicles | >50 | Broad biogenesis derived from apoptotic cells (includes apoptotic bodies) | Caruso and Poon (2018) |
| | Apoptotic microvesicles | 50–1000 | Biogenesis unclear | Caruso and Poon (2018) |
| | Apoptotic bodies | 1000–5000 | Apoptotic cell plasma membrane blebbing | Caruso and Poon (2018) |
| Plant-derived | Exosomes/Exosome-like (PEN1-, TET8- and EXPO-positive subsets) | <500 | Multivesicular body, exocyst-positive organelles (EXPO) | Rutter and Innes (2017); Cui *et al.* (2020); He *et al.* (2021) |
| Microbe-associated | Bacterial outer membrane vesicles | 20–400 | Budding of outer membrane of Gram-negative bacteria | Toyofuku, Nomura and Eberl (2019) |
| | Gram-positive bacterial extracellular vesicles | 10–400 | The biogenesis remains disputed | Liu *et al.* (2018) |
| | Fungal-derived extracellular vesicles | 50–1000 | Diverse, poorly understood class; similarities to higher eukaryotes | Joffe *et al.* (2016) |
| | Protozoa-derived extracellular vesicles | 30–500 | Diverse pathways; similarities to higher eukaryotes | Ofir-Birin and Regev-Rudzki (2019) |

came from the study of microvesicles released from human neutrophilic granulocytes in response to infection with *Staphylococcus aureus* (Fig. 2; Timar *et al.* 2013). The antibacterial activity of microvesicles was shown to depend on opsonization of the bacteria via activation of opsonin receptors, PLC$\gamma$2 and extracellular calcium, in a manner distinct from that of spontaneously released extracellular vesicles (Lorincz *et al.* 2015, 2019). Further investigation revealed that the Mac-1 integrin complex, also known as Complement Receptor 3, is essential to initiate the production of these antibacterial extracellular vesicles (Lorincz *et al.* 2020). Surprisingly, the activation of pathogen recognition receptors or immunoglobulin binding Fc receptors was not sufficient for production of antibacterial extracellular vesicles.

The antimicrobial activity observed in response to *S. aureus* infection was not limited to *S. aureus* alone, as *Escherichia coli* was also inhibited by neutrophil-derived vesicles (Timar *et al.* 2013). In contrast, these antibacterial extracellular vesicles produced in response to opsonized *S. aureus* infection had no antibacterial activity against *Proteus mirabilis*, suggesting some level of specificity (Timar *et al.* 2013). Extracellular vesicles that were formed spontaneously by neutrophils, or in response to treatment with phorbolmyristate acetate, showed less antibacterial activity. The antimicrobial activity of extracellular vesicles was not reliant on toxic reactive oxygen intermediates or the opsonization status of the target bacteria, but instead required an intact cytoskeleton and metabolic activity within the extracellular vesicles (Timar *et al.* 2013). Since these initial studies were only performed using chemical inhibitors, further studies will be required to definitely determine the function of the cytoskeleton and active metabolism in antibacterial extracellular vesicles.

The inability of extracellular vesicles to produce superoxide like their parent neutrophils was a bit of a surprise, as superoxide radicals are a major mechanism known to target many pathogens (Lorincz *et al.* 2015). One possible explanation of this phenotype is that extracellular vesicles act indirectly by promoting the inflammatory response in neighboring cells. In fact, it was shown that opsonized zymosan particles induced neutrophils to produce extracellular vesicles that facilitated neutrophil interleukin 8 secretion and reactive oxygen species production (Kolonics *et al.* 2020a). This explanation agrees with studies of *Mycobacterium tuberculosis* pathogenesis, where pathogen-induced extracellular vesicles from neutrophils were shown to increase superoxide production by macrophages (Alvarez-Jimenez *et al.* 2018). Interestingly, extracellular vesicles released spontaneously from macrophages led to a stronger reduction of bacterial load than *M. tuberculosis*-infected macrophages, suggesting an active inhibition of antimicrobial activity of extracellular vesicles by the bacteria (Garcia-Martinez *et al.* 2019).

Importantly, there appeared to be no sensitization of neutrophils to extracellular vesicle induction, as repeated exposure to opsonized *S. aureus* led to additional production of antibacterial extracellular vesicles (Timar *et al.* 2013). Of potential relevance to future therapeutic design, the antimicrobial function of neutrophil-derived extracellular vesicles over time was shown to depend on storage temperature (Lorincz *et al.* 2014). At $-80°$C the activity was quite stable, whereas at 4°C or 20°C, the activity rapidly declined.

The *in vivo* relevance of extracellular vesicles has been an important question from the start of the field. In terms of an antibacterial function, early studies showed that extracellular vesicles derived from neutrophils could be found in the circulation (Timar *et al.* 2013). However, when extracellular vesicles from the murine macrophage cell line J774A.1 were applied

exogenously to *M. tuberculosis*-infected mice, there was no major change in survival, despite a reduction in bacterial load (Garcia-Martinez *et al.* 2019). Additional experiments using extracellular vesicles from patient samples are needed to fully understand the *in vivo* relevance during infection.

The exact mechanism of action for antibacterial extracellular vesicles remains unknown, but we are starting to have an idea of what is possible. In fact, a recent study showed that extracellular vesicles derived from *M. tuberculosis*-infected macrophages contained molecules derived from the pathogen itself (Cheng and Schorey 2019). In this case, bacterial RNA was transferred from the macrophages in extracellular vesicles and activated microtubule-associated protein 1A/1B-light chain 3 (LC3)-associated phagosome maturation via a retinoic acid-inducible gene I (RIG-I)/mitochondrial antiviral-signaling protein (MAVS)-dependent signaling pathway in recipient cells. This sharing of bacterial RNA between cells ultimately led to increased killing of the intracellular bacteria.

A similar strategy is also employed by the host to transfer cytokines for immune activation in response to infection. In particular, extracellular vesicles from dendritic cells carried the proinflammatory cytokine TNF-$\alpha$ and were able to activate epithelial cells, which resulted in the release of additional inflammatory mediators (Obregon *et al.* 2009). More recent work showed that the induction of proinflammatory cytokines by host-derived extracellular vesicles can further activate antibacterial immunity (Radomski *et al.* 2019). In this study, *Chlamydia psittaci*-infected dendritic cells were shown to release vesicles capable of inducing IFN-$\gamma$ production in natural killer-cells via a TNF-$\alpha$/TNF receptor interaction. Interestingly, in this case, the extracellular vesicles produced by infected dendritic cells contained no discernable bacterial material, suggesting a process mediated by host factors alone. Ultimately, it was shown that dendritic cell-derived extracellular vesicles worked in concert with natural killer cell-derived IFN-$\gamma$ and TNF-$\alpha$ to limit the growth of *C. psittaci* in infected epithelial cells. These studies highlight the complicated interplay of extracellular vesicles with other well-described immune reactions and highlight that much work remains to fully understand the antibacterial activity of extracellular vesicles and their role in propagating the immune response.

## PLANT HOSTS PRODUCE EXTRACELLULAR VESICLES AND EFFECTOR SMALL RNAS AGAINST PATHOGENS

In plants, extracellular vesicles were only first isolated in 2009 from water-imbibed seeds of the sunflower, *Helianthus annuus* (Regente *et al.* 2009). This is despite the initial ultrastructural observations from carrot cell culture having occurred more than 50 years prior (Halperin and Jensen 1967). The antimicrobial nature of these plant-derived extracellular vesicles was not immediately obvious, and remains, like the entire field of antimicrobial extracellular vesicles, a work in progress (Rutter and Innes 2020). The first hints of an importance for extracellular vesicles during plant infection came from the discovery that paramural vesicles of barley were enriched around sites of infection of the powdery mildew fungus *Blumeria graminis* (An *et al.* 2006). Paramural vesicles are produced from multivesicular bodies, similar to the mammalian exosomes, and reside between the plant cell membrane and cell wall. It was therefore hypothesized that the vesicles that accumulate around the pathogen might in some instances be released from the cell wall to serve
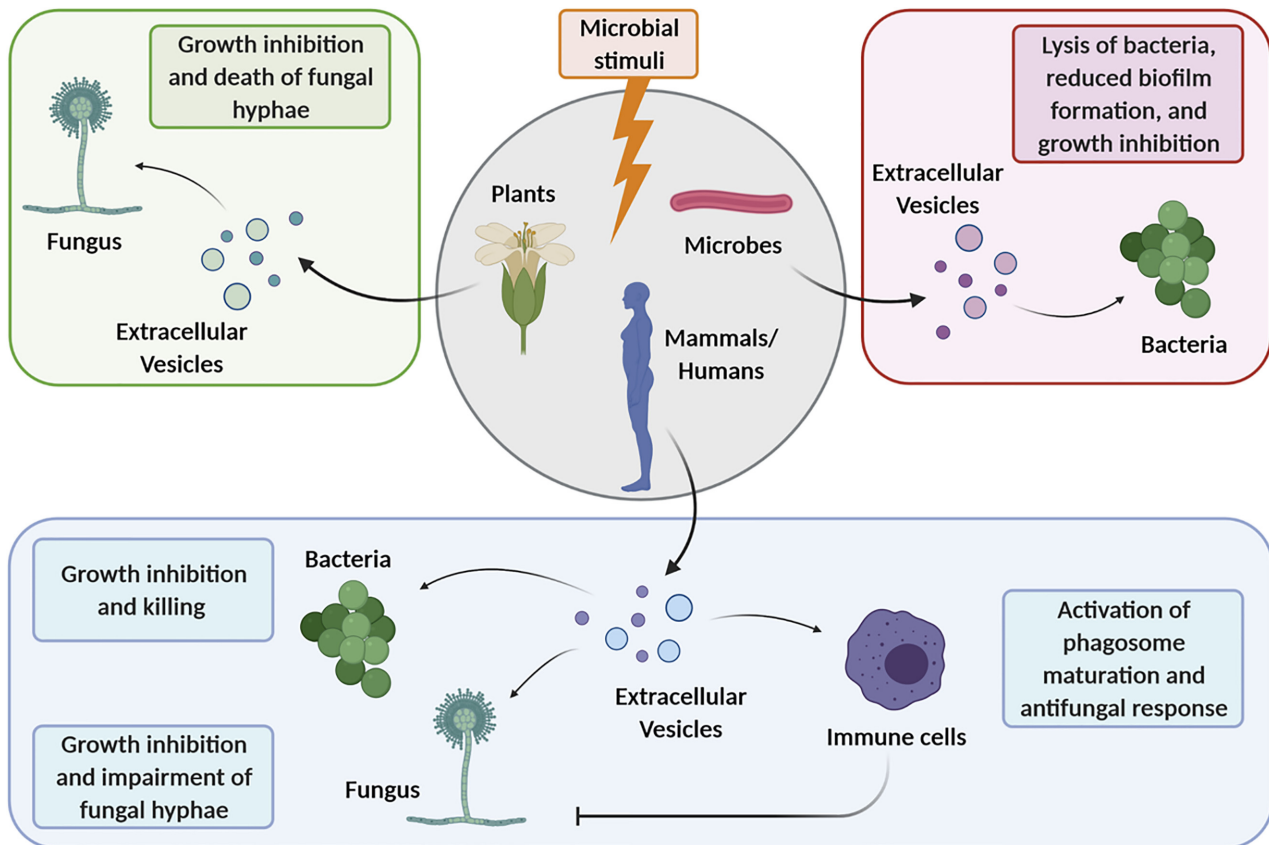
**Figure 2.** Antimicrobial functions of extracellular vesicles. Extracellular vesicles with antimicrobial cargo are routinely produced as a response to microbial competitors or in response to infection of a host. Extracellular vesicles produced by plants influence the outcome of infection by fungi and oomycetes. Humans use a similar strategy to control fungal and bacterial infections. Finally, microbes often produce extracellular vesicles to modulate neighbors and gain an advantage over competitors. Created with BioRender.com.

as antimicrobial extracellular vesicles in response to infection (An, van Bel and Huckelhoven 2007; Samuel *et al*. 2015). Another clue came from the first experimental isolation of the extracellular vesicles derived from *Arabidopsis thaliana* apoplastic fluid (Rutter and Innes 2017). These extracellular vesicles were enriched for proteins involved in host defense and stress adaptation, including proteins important for reactive oxygen species signaling and bioactive metabolite transport, again suggesting a role in plant immunity. Consistent with the suspected role in host defense, the syntaxin, PENETRATION-1 (PEN1)-specific extracellular vesicle fraction was shown to increase in abundance in response to the bacterial plant pathogen, *Pseudomonas syringae*. Surprisingly, the composition of the extracellular vesicles did not change after infection, suggesting that some organisms may constitutively produce antimicrobial extracellular vesicles to ward off potential pathogens.

The antimicrobial nature of plant-derived extracellular vesicles was demonstrated soon thereafter, when extracellular vesicles again derived from seedlings of the sunflower, *H. annuus*, were demonstrated to be antifungal against the phytopathogenic fungus, *Sclerotinia sclerotiorum* (Regente *et al*. 2017). In this case, extracellular vesicles released during infection were enriched in defense proteins and cell wall remodeling enzymes. Upon confrontation, host-derived extracellular vesicles were taken up by *S. sclerotiorum* spores and hyphae, which resulted in growth inhibition and potentially death of hyphae as observed by incorporation of the cell impermeable dyes propidium iodide

and Evans blue. In this case, the exact source of the antifungal activity remains to be uncovered.

Perhaps the most compelling examples of antimicrobial extracellular vesicles come from studies of bidirectional cross-kingdom RNA interference. In this phenomenon, small RNAs produced by both the host and the pathogen are thought to be transferred to the opposing organism to modulate the outcome of infection. Most small RNAs are produced by cleavage of a double-stranded RNA precursor by a dicer or a dicer-like protein before loading into an argonaute protein for gene silencing (Wilson and Doudna 2013). The small RNA/argonaute complex is the core component of the RNA induced silencing complex that recognizes target genes based on sequence complementarity between the small RNA and target to drive mRNA degradation or translational repression. Cross-kingdom RNA interference takes advantage of the conserved nature of the RNA interference machinery across eukaryotes to facilitate silencing between organisms after transfer of small RNAs.

An early example of cross-kingdom RNA interference came from the study of the grey mold, *Botrytis cinerea*, which is capable of infecting a wide array of plant species. The small RNAs of *B. cinerea* were identified during infection of two plant hosts, *A. thaliana* and *Solanum lycopersicum*, where they were shown to be transferred from the fungus to the host to silence plant immunity genes (Weiberg *et al*. 2013). Deletion of either the fungal dicer-like proteins or the *A. thaliana* argonaute proteins resulted in diminished virulence, suggesting that the small RNAs were

produced in the fungus, but capable of trafficking to and hijacking the plant RNA interference machinery for virulence. Follow-up work indicated that the host plant also uses a similar approach to fight back against fungal infection. It was demonstrated that tetraspanin 8 (TET8) and 9 (TET9)-positive exosome-like extracellular vesicles were used by the plant as a vehicle for delivery of plant-derived effector small RNAs into the fungus (Cai *et al.* 2018). These endogenous small RNAs produced by *A. thaliana* are transferred to *B. cinerea* to target the mRNAs of conserved vesicle pathways important to virulence, thus limiting the virulence potential of the organism upon knockdown (Cai *et al.* 2018). Although there has been some discussion over the exact subsets of extracellular vesicles, or possibly even ribonucleoprotein particles, that are responsible for small RNA transfer (Rutter and Innes 2020), a recent publication provided support for small RNAs at least being contained within the TET8-positive extracellular vesicle fraction (He *et al.* 2021).

In the study from He *et al.* (2021), proteomic analysis revealed that the TET8-positive extracellular vesicle subset was enriched for diverse RNA binding proteins, including the argonaute protein AGO1, the DEAD-box RNA helicases RH11 and RH37, as well as the annexins ANN1 and ANN2. Within the cell these RNA-binding proteins colocalized with multivesicular bodies, likely to facilitate their loading into the plant exosome-like extracellular vesicles. Investigation of the RNA binding capacity uncovered the selective binding of AGO1, RH11, and RH37 to small RNAs previously found to be enriched in TET8-positive extracellular vesicles, indicating their involvement in the specific sorting of RNA molecules into plant extracellular vesicles. The abundant annexin proteins on the other hand were hypothesized to play a role in the stabilization of RNA within the extracellular vesicle compartment. Particularly compelling were the findings that plants lacking these RNA binding proteins displayed lower levels of small RNAs loaded into their extracellular vesicles and a higher susceptibility to *B. cinerea* infection. Several other examples also support the use of small RNAs by plants to target fungal pathogens. In particular, cotton plants were shown to export microRNAs to inhibit the virulence of *Verticillium dahliae* (Zhang *et al.* 2016), and wheat plants to suppress the invasion of *Fusarium graminearum* again using microRNAs (Jiao and Peng 2018). It remains unknown if extracellular vesicles are the true transporters of small RNAs in all cases, but it seems clear that small RNAs play a role in equipping extracellular vesicles with an antimicrobial capacity in some instances.

In response to infection by the evolutionarily distinct oomycetes, plants again seem to rely on small RNAs in extracellular vesicles to target pathogen virulence genes. For example, study of the oomycete, *Phytophthora capsici* has revealed that *A. thaliana* responds to infection with production of extracellular vesicles capable of delivering small interfering RNAs to potentially silence pathogen genes (Hou *et al.* 2019). *P. capsici* suppressed this host-induced gene-silencing reaction using the effector molecule PSR2, which blocks biogenesis of secondary small interfering RNAs. The strategy of delivering antimicrobial RNAs seems to be conserved against different oomycetes, as *Hyaloperonospora arabidopsidis* infection is also inhibited by *A. thaliana* using small RNAs (Bilir *et al.* 2019), in an interaction that provides another example of bidirectional cross-kingdom RNA interference (Dunker *et al.* 2020). Not surprisingly, and despite these clear examples of plant-derived antifungal extracellular vesicles, there are also cases of host-pathogenesis where extracellular vesicles do not seem to play an obvious role, as shown during the interaction of wheat plants and the *Zymoseptoria tritici* fungus (Ma, Wiedmer and Palma-Guerrero 2019). In the future,

additional work will be required to better define the subsets of extracellular vesicles involved in the transfer of small RNAs into invading pathogens. It seems likely that both extracellular vesicle-dependent and -independent mechanisms will be discovered to facilitate transfer.

## MAMMALIAN HOSTS PRODUCE EXTRACELLULAR VESICLES AGAINST FUNGAL PATHOGENS

In contrast to the studies in plants, the role of mammalian host-derived extracellular vesicles in combating fungal infections remains relatively unexplored. However, recent studies have started to shed light on the host response to two important human fungal pathogens, *Aspergillus fumigatus* and *Candida albicans*. In the first case, neutrophils were recently shown to produce antifungal extracellular vesicles in response to *A. fumigatus* infection *ex vivo* (Shopova *et al.* 2020). These extracellular vesicles limited the growth of fungal conidia when applied *in vitro* and were also able to bind, enter and seemingly kill fungal hyphae. LC-MS/MS proteomics analysis of these antifungal extracellular vesicles revealed an abundance of antifungal cargo proteins like cathepsin G and azurocidin. Surprisingly, a mutant strain of *A. fumigatus* lacking dihydroxynaphthalene-melanin, an important polymer and virulence factor in the spore cell wall, induced production of some extracellular vesicle subsets and a similar protein cargo, but these vesicles were no longer antifungal, again suggesting some level of host specificity against particular fungal pathogens. More work is required to fully understand the breadth of this specificity in the control of fungal pathogens. Finally, it was shown that inducible, heterologous overexpression of the human extracellular vesicle proteins cathepsin G or azurocidin by the fungus was toxic upon induction. Albeit artificial, this finding provided a proof-of-principle that the presence of antifungal peptides inside of a fungus, as would be delivered by an extracellular vesicle, could prove toxic in sufficient concentrations. These results also suggest that different cargo molecules might be responsible for the antimicrobial action of extracellular vesicles depending on the subset and doses delivered. In agreement, it was recently shown that opsonized fungal zymosan particles induced neutrophils to produce pro-inflammatory extracellular vesicles, whereas resting and apoptotic neutrophils produced anti-inflammatory and coagulation-promoting extracellular vesicles, respectively (Kolonics *et al.* 2020a).

In response to *C. albicans* infection, extracellular vesicles from primary monocytes appear to serve yet another function. It was recently shown that soluble $\beta$-glucan released from *C. albicans* binds to Complement Receptor 3 to induce the release of TGF-$\beta$1-transporting vesicles that are anti-inflammatory in nature (Halder *et al.* 2020). Rather than serving an antimicrobial function as in *S. aureus* or *A. fumigatus* infection, these extracellular vesicles seem to facilitate niche establishment for *C. albicans*; a difference that might reflect the frequent role of *C. albicans* as a human commensal.

## MICROBES PRODUCE EXTRACELLULAR VESICLES AGAINST OTHER MICROBES

The antimicrobial features of some extracellular vesicles are not only found in eukaryotes. In fact, many examples of antimicrobial extracellular vesicles come from studies of microbe–microbe communication. Outer membrane vesicles, a particularly well-studied class of extracellular vesicles derived from

Gram-negative bacteria have been well-studied in this regard (Olsen and Amano 2015; Lynch and Alegado 2017). As early as the late 1990s, naturally produced outer membrane vesicles of Gram-negative bacteria were shown to have an antibacterial activity due in large part to hydrolytic enzyme cargo (Kadurugamuwa and Beveridge 1996; Li, Clarke and Beveridge 1998). In particular, it was shown that *Pseudomonas aeruginosa*, an opportunistic lung pathogen, can release extracellular vesicles containing autolysin (Kadurugamuwa and Beveridge 1996). This 26 kDa protein can lyse a wide range of Gram-negative bacteria and, surprisingly, also some Gram-positive bacteria, including the genus of *Mycobacterium* (MacDonald and Beveridge 2002). Interestingly, when *P. aeruginosa* was treated with an antimicrobial agent such as gentamicin, a small amount of the molecule was encapsulated in extracellular vesicles, which further enhanced the growth inhibition of bacteria. Later on, it was made clear that the antibacterial activity of these extracellular vesicles was specific, relying on the peptidoglycan content in the outer membrane and the absence of O-acetylation (Kadurugamuwa and Beveridge 1996; Li, Clarke and Beveridge 1998; Moynihan and Clarke 2011).

The ability of extracellular vesicles to encapsulate antimicrobial agents opened a wide range of possible applications, one of which was to facilitate bacterial biofilm disassembly. In agreement, it was shown that outer membrane vesicles play a fundamental role in the biofilm formation of some bacteria, like *P. aeruginosa* and *Helicobacter pylori* (Yonezawa *et al.* 2011; Murphy *et al.* 2014), making the outer membrane vesicle machinery a potential target for future therapeutics. Studies based on *P. aeruginosa* hypothesized that outer membrane vesicles produced from one species in a biofilm may also be able to lyse neighboring bacteria, thus releasing nutrients for growth and DNA for the biofilm matrix (Beveridge *et al.* 1997). The experimental evidence for this hypothesis is still somewhat lacking, but the ability of outer membrane vesicles to influence neighboring bacteria has been strengthened over time. For example, bacterial-secreted vesicles can be deadly to other bacteria as shown by the study of the Gram-positive bacteria *Lactobacillus acidophilus*. Vesicles secreted from this bacterium contain and can deliver bacteriocin peptides to the opportunistic pathogen *Lactobacillus delbrueckii*. The final effect is to inhibit *L. delbrueckii* growth and compromise membrane integrity (Dean *et al.* 2020). A recent report extended the idea of biofilm dissolution further by showing that outer membrane vesicles from nonpathogenic *Burkholderia thailandensis* are loaded with antimicrobial and antibiofilm compounds such as 4-hydroxy-3-methyl-2-(2-nonenyl)-quinoline that can limit the growth and biofilm formation of drug-sensitive and drug-resistant bacteria like *S. aureus*, but have no effect on closely related *P. aeruginosa* (Wang *et al.* 2020). Many groups are now focused on trying to leverage outer membrane vesicles as bio delivery containers for small interfering RNA or antimicrobial molecules (Gujrati and Jon 2014; Gerritzen *et al.* 2017; Jan 2017). This approach may ultimately provide a strategy for promoting biofilm disassembly via targeting by specialized outer membrane vesicles; however, further research is still required to reach these goals.

In some cases, bacteria can also utilize outer membrane vesicles to kill and prey on other bacteria as a nutrition source. This is the case for the soil bacterium, *Myxococcus xanthus*, which relies on outer membrane vesicles to kill *E. coli* prey, again using hydrolytic enzymes, in addition to proteases, phosphatases and secondary metabolites (Evans *et al.* 2012). These outer membrane vesicles were shown to fuse with the outer membrane of Gram-negative bacteria, in a process that was further facilitated by the presence of glyceraldehyde-3-phosphatase dehydrogenase, an enzyme actively secreted by *M. xanthus*. The inherent antibacterial activity of Myxobacteria outer membrane vesicles makes them intriguing leads for the development of potential delivery systems for antibacterial therapeutics (Schulz *et al.* 2018). One of the major problems with the development of antimicrobials remains the inability to distinguish between pathogen and commensal. Properly targeted extracellular vesicles may eventually offer a strategy for targeting a particular antimicrobial cargo to a pathogen of interest in a sick patient.

There are a number of other intriguing examples of antimicrobials delivered by extracellular vesicles during bacterial communication. *Chromobacterium violaceum* delivers violacein, a hydrophobic antibiotic, to other microbes in membrane vesicles (Choi *et al.* 2020; Wettstadt 2020). Not only do the extracellular vesicles provide a transport mechanism, they also offer a clever solution to the problem of solubility of hydrophobic compounds like violacein, a bisindole with antibiotic capacity. Similarly, the linearmycin family of polyketides, with antifungal and antibacterial activity, are also known to be packaged in extracellular vesicles and released by bacteria of the genus *Streptomyces* (Stubbendieck and Straight 2015; Stubbendieck *et al.* 2018). The linearmycins were even found to be important for the biogenesis of the vesicle, and the disruption of linearmycin production led to the inhibition of vesicle biogenesis (Hoefler *et al.* 2017). There is great potential for the identification of new therapeutics for treatment of human disease from the antimicrobial extracellular vesicles produced by bacteria. Future studies into this realm will likely continue to reveal novel mechanisms important to extracellular biology as well as intriguing new options for the development of therapeutics.

## CONCLUSIONS: THE FUTURE OF EXTRACELLULAR VESICLE-BASED THERAPEUTICS

The examples provided in this short review show a reality where extracellular vesicles are used throughout the kingdoms of life to manage interkingdom communication. Extracellular vesicles produced by plants are able to contribute to defense against highly destructive fungi, humans rely on extracellular vesicles to manage bacterial and fungal infections, and microbes even use extracellular vesicles to deter invading competitors. All of these examples point to the fantastic potential of extracellular vesicles as future therapeutic delivery systems for the treatment of human infectious diseases. In fact, these systems are already exploited for the protection of crops and in human medicine. For example, in host-induced gene silencing, the expression of small RNAs in plants that are capable of targeting common virulence gene-encoding mRNAs of pathogens resulted in extracellular vesicle-mediated silencing of pathogen genes (Cai *et al.* 2018). Recent work showing the importance of the TET8-positive extracellular fraction in small RNA delivery pushes us further towards a mechanistic understanding of this phenomenon (He *et al.* 2021). Another interesting case is the antifungal drug formulation, AmBisome (amphotericin B liposomal), which relies on lipid-enclosed Amphotericin B to maximize uptake and limit off target effects (Walker *et al.* 2018). Although AmBisome is not technically an extracellular vesicle in the classical sense, it is a strong proof-of-principle that approaches based on lipid-encapsulation of drugs offer a promising path forward. With the additional specificity granted by a better understanding of

the surface receptors of extracellular vesicles, we will hopefully be able to directly target extracellular vesicles to pathogens of interest in the near future. Another approach in this vein will be to coat nanoparticles with extracellular vesicle membrane constituents, as has been used in the fight against *S. aureus* (Gao *et al.* 2019; Witwer and Wolfram 2021).

We are also learning a lot about the antimicrobial potential of extracellular vesicles through detailed analyses of the cargo of extracellular vesicles. Advanced proteomics and RNA sequencing technologies are now allowing for elucidation of components found at even fleeting amounts in these populations (Cheng and Schorey 2019; Shopova *et al.* 2020). As our understanding of the cargo within extracellular vesicles increases, so will our ability to leverage these cargos for therapeutic design. In many of the examples described throughout this review, the exact molecules resulting in the observed antimicrobial activity remain elusive, likely due to a combined action of many different factors. We like to think of extracellular vesicles as a toolbox, capable of using many different tools to address many different situations. For the development of therapeutics, we will likely have to increase the relative abundance of particular tools, cargo, in order to create a consistent treatment option against infectious diseases. We suspect that small RNAs might be the easiest species to manipulate for this purpose, as they can be easily and cheaply synthesized for therapeutic purposes (O'Brien *et al.* 2020).

Extracellular vesicles are not an easy class of particles with which to work. Their low abundance and small size make tracking their biogenesis and distribution difficult. This becomes increasingly complex in situations with more than one or two partners; for example, amongst the gut microbiota or during the coordinated immune response by multiple immune cell types. Understanding the complex interaction between pathogens and hosts in the context of the entire environment during infection will be a challenge for the future. As our ability to identify and track extracellular vesicles improves, so too will our understanding of the role of these small particles in transferring information between organisms. New systems will likely have to be established to better understand how host-derived extracellular vesicles produced in response to large consortia of microbes target specific pathogens to influence the outcome infection.

There are many other unanswered questions surrounding the antimicrobial function of extracellular vesicles. For example, in many cases we still do not know whether extracellular vesicle abundance, cargo, or surface receptor specificity play a pivotal role in function. Even, the delivery of specific cargo to extracellular vesicles remains a mystery in many cases. However, there is an emerging body of data that suggests the loading of extracellular vesicle cargo is a highly tailored process. For example, in the work described above, the Mac-1 receptor mediates the activation of a tailoring process in neutrophils that ultimately results in antibacterial extracellular vesicles being produced in response to *S. aureus* infection (Lorincz *et al.* 2020) and proteins like AGO1, RH11, and RH37 are important for antifungal RNA loading into TET8-positive extracellular vesicles in plants (He *et al.* 2021). We expect that examples of precision-loading will prove to be widespread throughout host pathogenesis.

We also find the hypothesis compelling that extracellular vesicles of hosts and pathogens are likely under strong evolutionary pressure to adapt to the near-constant evolutionary arms race of host-pathogenesis, as has been postulated previously (Correa *et al.* 2020). It remains to be seen whether pathogens influence or take advantage of the production of extracellular vesicles by the host or commensals to their benefit to promote pathogenesis. Ultimately, we believe that there is a wealth of information to gain from increased scrutiny of the role of extracellular vesicles during infection. The coming years will likely see a surge in the use of extracellular vesicles as both diagnostics for cancers, metabolic diseases, and infectious diseases, but also as starting points for the development of novel therapeutics.

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