The role of bacterial extracellular vesicles in chronic wound infections: Current knowledge and future challenges

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Abstract
Chronic wounds are a significant global problem with an increasing economic and patient welfare impact. How wounds move from an acute to chronic, non-healing, state is not well understood although it is likely that it is driven by a poorly regulated local inflammatory state. Opportunistic pathogens such as Staphylococcus aureus and Pseudomonas aeruginosa are well known to stimulate a pro-inflammatory response and so their presence may further drive chronicity. Studies have demonstrated that host cell extracellular vesicles (hEVs), in particular exosomes, have multiple roles in both increasing and decreasing chronicity within wounds; however, the role of bacterial extracellular vesicles (bEVs) is still poorly understood. The aim of this review is to evaluate bEV biogenesis and function within chronic wound relevant bacterial species to determine what, if any, role bEVs may have in driving wound chronicity. We determine that bEVs drive chronicity by both increasing persistence of key pathogens such as Staphylococcus aureus and Pseudomonas aeruginosa and stimulating a pro-inflammatory response by the host. Data also suggest that both bEVs and hEVs show therapeutic promise, providing vaccine candidates, decoy targets for bacterial toxins or modulating the bacterial species within chronic wound biofilms. Caution should, however, be used when interpreting findings to date as the bEV field is still in its infancy and as such lacks consistency in bEV isolation and characterization. It is of primary importance that this is addressed, allowing meaningful conclusions to be drawn and increasing reproducibility within the field.

KEYWORDS
biofilm, chronic wound infection, exosome, extracellular vesicle, outer membrane vesicle, Pseudomonas aeruginosa, Staphylococcus aureus

1 | INTRODUCTION

Wound healing is a complex and highly regulated process, typically divided into four stages: haemostasis, inflammation, proliferation and remodelling. A proportion of wounds are slow to progress beyond the inflammation stage and as such are classified as chronic. An absolute definition of a chronic wound has yet to be agreed although generally wounds are typically classed as chronic if they have failed to show significant closure within a “reasonable” time frame, typically 12 weeks. By their very definition, chronic wounds are long-term health problems. Guest et al reported that 39% of the wounds within their study had not fully healed within the 12 month study period, similarly Posnett and Franks reported that one-third of wounds are unresolved within 6 months, with a fifth of wounds taking 12 months or more. Within the United Kingdom, chronic wounds cost the national health service GBP £5.3 billion per annum, equivalent to 4%
of the total National Health Service (NHS) annual budget. In the United States, chronic wounds are estimated to cost US $ 9.7 billion, with a combined (direct and societal) cost per individual of US $ 13,334, raising to US $ 33,499 per individual if amputation is required. Infection within these chronic wounds is a complicating factor, leading not only to increased patient distress but also prolonged treatment and increased financial burden. As the global population ages, becomes increasing sedentary and co-morbidities such as obesity and type two diabetes rise, so too do chronic wounds. As such it is imperative that we gain a better understanding of not only the chronic wound itself but also how to successfully treat them. In this review, we have evaluated the evidence that extracellular vesicles (EVs), from both the host and bacterial species (hEVs and bEVs respectively), are able to contribute towards chronic wound pathogenesis and evaluated potential avenues for EV-based interventions. In particular, we focus on bEVs within the chronic wound as significantly less investigation of bEVs has been carried out to date.

2 | INFECTION WITHIN CHRONIC WOUNDS

Because of the presence of a complex microbial community upon the skin, keeping chronic wounds sterile is an unrealistic goal, and many wound infections originate from the patient’s own microbiome. There is a continuous spectrum from microbial contamination of a wound, through colonization and finally infection, where colonization has become persistent and clinical signs of infection are present. It is likely that the majority of chronic wounds are colonized; however, diagnosis of infection is based on recognition of clinical infection indicators. Prompers et al reported that in their European study, 50% of diabetic foot ulcers were infected upon first presentation, whereas a US study reported a more conservative figure of 15%. Metagenomic studies have shown that decreased microbial diversity and increased temporal stability of the microbial wound community are linked to delayed healing and poorer outcome. Frequently microbes associated with chronic wounds originate from the patient or their environment. Such species are referred to as “opportunistic pathogens” because they are able to colonize the body as a commensal but can take advantage of wound sites to establish an infection and proliferate uncontrollably. Opportunistic species commonly associated with chronic wounds include Staphylococcus epidermidis, a skin commensal, Staphylococcus aureus, a colonizer of the nasal passages, Pseudomonas aeruginosa, an environmental species found in soil and water, and Escherichia coli, a member of the gut community. P. aeruginosa and S. aureus are frequently identified as dominant microbial wound community members and their presence linked to poor outcome. Both species are important opportunistic pathogens, identified by the World Health Organization as priority pathogens, for which novel therapeutics are urgently required.

Microbial communities within chronic wounds exist as mixed species biofilm communities. Biofilms are defined as communities of microbes that are surrounded by and embedded within a self-produced extra-poly saccharide matrix (EPS). Their presence within wounds is a significant complication and debridement has little long-term effect on the microbial community. The biofilm’s lifestyle limits bacterial clearance by phagocytosis, drives the production of pro-inflammatory cytokines and reduces bacterial susceptibility to antibiotics. EVs are hypothesized to play a significant role in not only interactions between bacterial species within the biofilm, but also between bacteria and the host.

3 | BACTERIAL AND HOST EXTRACELLULAR VESICLES

EVs are particularly difficult to define definitively, but in general terms are small (<500 nm) membrane bounded vesicles, produced by conserved pathways. Multiple EV types have been identified within both mammalian and bacterial populations. Morphological heterogeneity is commonly observed within EV populations, even when considering a single well-defined and homogeneous parent cell population. Vesicles also harbour complex sets of molecular constituents, which are heterogeneously distributed amongst the EV population.

Normally investigators working with mammalian cells describe their EV preparations as being “enriched” for specific EV types, rather than claiming pure populations, although isolation strategies exhibiting higher resolutions are beginning to be increasingly utilized for more selective sub-population isolations. Diverse mammalian vesicles have been extensively investigated in terms of their biophysical and molecular nature and their varied contributions to pathogenic processes.

Bacterial EVs are much less well studied, although interest within the field is increasing. Markers are yet to be determined for bEVs, with identification currently relying on microscopic observations and size measurements, but some protein profiling studies have also been undertaken. Within bacterial cultures, the term outer membrane vesicles (OMV) is used to describe vesicles produced by Gram-negative bacteria and membrane vesicle (MV) for Gram-positive vesicles. Herein, the term bacterial EV (bEV) will be used to refer collectively to bacterial OMVs and MVs, while the term hEV refers to host EV. This review focuses predominantly on the role of bEVs produced by S. aureus and P. aeruginosa, respectively, Gram-positive and negative bacteria, because these are not only commonly wound pathogens but are also linked to adverse tissue healing outcomes.

4 | bEV CONCENTRATION AND COMPOSITION VARIES IN RESPONSE TO GROWTH CONDITIONS AND GENETIC BACKGROUND

4.1 | Isolation and characterization techniques used within bEV studies would benefit from standardization, identification of markers and minimum essential guidance

A brief analysis of the bEV-focused manuscripts referenced within this work provided basic information about the methodologies used across the studies (Figure 1 and Table S1). Many of the studies
referenced used ultracentrifugation, either alone or in combination with density gradients, for the purification of EVs. In contrast to hEV analysis, production and characterization of the bEV isolates across studies shows little consistency in both techniques presented and the data reported, with minimal vesicle-defining analyses taking place. For the majority of research papers analysed within this study, no more than two characterization assays were performed to determine the quality, concentration and composition of the bEV preparations obtained (Figure 1(A)). We hypothesised that this is due to a lack of bEV standardization and minimal information guidelines, such as have been published for hEV studies in 2014 and 2018. Following purification, many groups measured protein concentration, typically via Bradford Assay, and considered this as an indicator of the concentration of their EV preparations (Figure 1(B)). Many studies also supported their characterization by carrying out several techniques, most commonly transmission electron microscopy (TEM) and particle analysis, to give further information about the size, density and purity of the preps (Figure 1(B)). Nanoparticle tracking analysis (NTA) is a method of interrogating the particle sizes and particle concentration of EV preparations. Commonly, NTA is carried out using NanoSight instruments, which allow visualization of EVs in suspension as they travel along a capillary tube using a microscope and camera. Although commonly used in EV studies, it was not frequently used in the bEV studies referenced in this review. NTA systems measure whole particle numbers, with no distinction able to be made between EV particles and other debris in the suspensions. As such care must be taken to measure the particle sizes and concentration of the vehicle used to both prepare EVs and for dilution prior to NTA. This non-specificity is particularly problematic when carrying out NTA using complex and undefined samples, for example, from EV preparations originating from body fluids or cultures growing in rich broths or serum. Recent work indicated that repeated measures using a single machine and operator on one day were reproducible but results varied when machines in two separate geographical locations were used, even when used by the same operator and matched software settings. Similarly, Bachurski et al demonstrated that both NanoSight and ZetaVeiw, both NTA tools, showed poor accuracy in estimating particle concentration and size, respectively. These studies highlight that although NTA can be a rapid and useful tool to assess quality of EV preparations, the data generated by NTA should be interpreted with care. Bachurski et al concluded that TEM, a tool used in many of the studies described here, was superior to NTA in accurately determining EV particle size and preparation purity.

The majority of the data discussed within this manuscript focuses on the activity of P. aeruginosa and S. aureus bEVs within chronic wounds, and as such the majority of the studies referenced focus on these two species. It is, however, interesting that that majority of studies to date have focused on using lab strains of bacteria, rather than clinical isolates, the latter being more representative of chronic wound isolates. The bEV analysis of P. aeruginosa was dominated by two well commonly used laboratory strains: PA01 and PA14, with greater variation in isolate choice shown within the S. aureus bEV studies. The chosen isolates are genetically tractable, well adapted to laboratory growth and genetically and phenotypically well characterized, making their selection understandable; however, caution must be used when comparing studies and extrapolating clinical significance from the obtained data. Prolonged growth of bacterial isolates, including P. aeruginosa, within the laboratory is known to lead to genetic and phenotypic variations, with differences in antimicrobial susceptibility, biofilm formation and virulence between different clinical and laboratory isolates also widely reported. As such, within this manuscript, caution has been used to interpret how findings obtained from bEV cultures derived from in vitro, single species and planktonic bacterial cultures may translate to bEV activity within the complex, multi-species environment of the chronic wound. Details of each study using bEVs along with bacterial isolates, growth conditions, bEV isolation and characterization techniques are shown in Table S1.

4.2 Culture conditions and genetic background of bacterial isolates are important considerations

Originally bEVs were thought to be a mechanism of clearing misfolded and damaged components from bacterial cells but there is now significant evidence that clearance is not their only role. Conditions in which fundamental bacterial process are inhibited have been shown to increase bEV biogenesis in both P. aeruginosa and S. aureus. For example, oxidative stress but not temperature stress was shown to increase bEV production in P. aeruginosa and the presence of ciprofloxacin, an antibiotic that inhibits DNA replication, increases S. aureus bEV biogenesis.

The production of bEVs by bacteria is a dynamic process influenced by several factors. A comparison of two P. aeruginosa strains, PA01 and PA14, reported that PA14 produced significantly more bEVs than PA01 under the same growth conditions, indicating that the genetic background is relevant. However, the bEV production rate of PA01 could be increased by culturing in nutrient-rich medium, highlighting an additional role for environmental factors.
Gentamicin also appears to increase bEV release from *P. aeruginosa*. Although Gentamicin's activity is primarily via inhibition of protein synthesis, it is able to destabilize the cells wall of *P. aeruginosa*, likely contributing to elevated vesicle output. Both *S. aureus* and *P. aeruginosa* have been reported to alter bEV production during different growth phases, with peak bEV production reported during the stationary phase.

Much of the bEV field to date has focused on collecting vesicles from stationary phase planktonic cultures, with little investigation of bEVs produced during biofilm growth. Where the few studies have compared planktonic and biofilm bEV population differences have been described. Biofilm bEVs exhibit increased lipopolysaccharide (LPS) content, proteolytic activity and were approximately half the size of planktonic bEV (45 and 86 nm diameter respectively). In contrast, the *P. aeruginosa* planktonic and biofilm bEV populations isolated by Toyofuku *et al* did not show a difference in size; however, proteomic analysis revealed multiple differences in the protein composition of biofilm and planktonic bEV preparations. Measurement of total protein indicated that planktonic bEVs contained 2.5-fold more protein than their biofilm bEV counterparts. Proteomic analysis highlighted that biofilm bEVs lacked cyttoplasmic proteins and virulence factors (including LasA, LasB and alkaline phosphatase), but included 18 proteins not found within planktonic bEV. Four of the proteins unique to biofilms were involved in iron acquisition and three were highly abundant. Taken together, these findings suggest that *P. aeruginosa* can not only vary bEV-associated proteins depending on its lifestyle but may produce bEVs that are focused less on virulence and more on persistence when in a biofilm community.

The current lack of biofilm bEV-focused studies is a significant limitation in fully understanding how bEVs might contribute to pathogenicity within chronic wounds. As described above, several researchers have reported fundamental differences in the characteristics of bEVs from biofilm or planktonic populations, leading to the assumption that their activity could also differ fundamentally. These studies, combined with the report by Florez *et al* that nutrient availability can also distinctly alter the number of EVs produced by genetically identical bacterial populations, suggest that only when bEVs are collected in chronic wound relevant conditions can their exact function within the chronic wound be accurately determined. Techniques and growth mediums have now been developed to allow investigation of chronic wound relevant biofilms, raising the exciting possibility that chronic wound relevant bEVs will soon be able to be investigated in greater detail.

### 5 | BIOGENESIS OF *P. aeruginosa* AND *S. aureus* bEV TAKES PLACE VIA BOTH ACTIVE AND PASSIVE MECHANISMS

#### 5.1 | *P. aeruginosa* bEV biogenesis occurs via binding and deformation of the outer membrane

Gram-negative bEV and outer membrane preparations, including those produced by *P. aeruginosa*, have similar protein profiles, suggesting bEV originate from the outer membrane. Several *P. aeruginosa* bEV biogenesis mechanisms have been proposed, the best described of which utilizes the Pseudomonas quinolone signal (PQS) pathway. PQS plays an important role in population density sensing and is particularly important for stationary phase transition, biofilm formation and maintenance. *P. aeruginosa* bEV production peaks during stationary phase growth and several groups have confirmed that PQS plays a role in *P. aeruginosa* bEV biogenesis. Inactivation of genes involved in PQS, such as *pqsH* and *pqsA*, significantly reduce *P. aeruginosa* bEV production while supplementation with exogenous PQS restores bEV production. Interestingly, this was still observed in isolates with defective PQS downstream signalling due to deletion of the transcriptional regulator *mvfR*, hinting at the possibility that the action of PQS is not due to regulation, but acts as a mechanical factor for biogenesis (Figure 2, upper panel). As such, *P. aeruginosa* bEV production is related to the exogenous, not internal, PQS molecule concentration. Interestingly, this effect is not species specific as exogenous *P. aeruginosa* PQS molecules were reported to increase bEV production in *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*.

#### 5.2 | Passive mechanisms of bEV biogenesis may occur in both Gram-negative and positive populations

It is important to note that small amounts of bEVs are produced by *pqsA* inactivated mutants, suggesting that PQS is not solely responsible for *P. aeruginosa* bEV biogenesis. Recently, the *P. aeruginosa* explosive cell lysis required to release eDNA within biofilms was demonstrated to also form membrane vesicles between 110 and 800 nm in diameter, independent of PQS (Figure 2, lower panel). Vesicle formation was due to recircularization of membrane fragments, trapping local cell cytoplasm components within them.

As described for *P. aeruginosa*, sub-populations of *S. aureus* lysis during the early stages of biofilm formation, also providing a source of eDNA within the biofilm EPS. Although no link has yet been made between *S. aureus* lysis during biofilm formation and bEV genesis, it is interesting to speculate that this may also contribute to bEV populations within biofilms. Certainly, cell wall disruption within planktonic cultures contributes to *S. aureus* bEV production. The presence of the β-lactam antibiotics flucloxacillin and ceftaroline, which perturb Gram-positive cell walls causing lysis, increased *S. aureus* bEV formation, as did the presence of lysogenic phases.

#### 5.3 | Biogenesis of bEVs by *S. aureus* requires modification and loosening of the bacterial cell wall

The cell walls of Gram-negative bacteria are composed of two thin layers separated by a periplasmic space, whereas the Gram-positive cell wall comprises only a single thick peptidoglycan layer. This difference means the cell wall is more rigid and inflexible and therefore resistant to vesicle formation by budding. No definitive method of bEV release has yet been demonstrated for Gram-positive bacteria;
FIGURE 2 Both active and passive bEV biogenesis mechanisms are utilised by the Gram-negative chronic wound pathogen P. aeruginosa. Within P. aeruginosa two mechanisms of biogenesis are shown: genesis via membrane deformation and budding following PQS molecule binding (upper panel) and biogenesis via explosive cell lysis and recircularization of membrane fragments (lower panel). The former mechanism has been demonstrated to be selective, with currently undescribed mechanisms sorting and transporting cargo to the budding membrane, which is deformed, circularised, and eventually separated into a vesicle via binding of PQS molecules to the membrane. In contrast, the latter mechanism of explosive cell lysis is a random process where cargo is packaged only due to its close proximity to the vesicle at the time of recircularization. During this process the membrane ruptures, releasing cell membrane fragments and the cell contents. Membrane recircularization occurs passively and randomly, creating vesicles of varying size and contents. The blue, green and red triangles represent the range of bacterial components which are able to be packaged, both actively or passively, into the Gram-negative bEVs (i.e. siderophores, enzymes, virulence factors, etc.)

FIGURE 3 The S. aureus bEV biogenesis mechanism is fundamentally different to that of P. aeruginosa. Only one mechanism of S. aureus biogenesis has been described in detail to date and is presented here. As with P. aeruginosa PQS dependent biogenesis, S. aureus bEV biogenesis is known to involve selective packaging of components, via a currently unidentified transport mechanism. The process appears to be controlled by the accessory gene regulator (agr) which has a role in controlling many nongrowth functions of S. aureus biology. Vesicle formation occurs via enzymatic loosening of the cell wall to allow membrane blebbing and budding through the newly created cell wall gaps. The blue, green and red triangles represent the range of bacterial components which are able to be packaged into the Gram-positive bEV (i.e. siderophores, enzymes, virulence factors, etc.)
nevertheless, it is indisputable that bEV production occurs.\textsuperscript{70} Thus far, three models of release have been proposed, including (i) forcing of bEV through the cell wall by turgor pressure, (ii) enzymatic loosening of the cell wall to allow bEV passage and (iii) transport of EVs through channels in the cell wall\textsuperscript{71} (Figure 3).

\textit{S. aureus} bEV proteomic analysis identified enzymes with cell wall components activity, including N-acetylmuramoyl-l-alanine amidase, lipoteichoic acid synthase and penicillin binding proteins\textsuperscript{34}, suggesting that loosening of cell wall bonds could assist bEV release. Phenol soluble modulins (PSM) that have surfactant properties and are able to disrupt membrane integrity\textsuperscript{72–74} are also important in \textit{S. aureus} bEV release.\textsuperscript{75} Inactivation of single or multiple PSM genes reduced bEV production, whereas overexpression increased bEV numbers.\textsuperscript{75} Similarly, inactivation of other peptidoglycan degrading enzymes, Sle1 and Atl, decreased \textit{S. aureus} bEV production.\textsuperscript{75} Taken together, these findings indicate that \textit{S. aureus} bEV biogenesis occurs when membrane, containing EV components, is forced through enzymatically created cell wall breaks, forming membrane blebs that bud off and form vesicles (Figure 3).

\textit{S. aureus} bEV biogenesis has been proposed to be controlled by the accessory gene regulator, agr. As is the case with PQS within \textit{P. aeruginosa}, agr is responsible for regulating multiple \textit{S. aureus} functions including virulence factor production and biofilm formation.\textsuperscript{76} Deletion of \textit{agrA} decreased bEVs to an undetectable level although some bEV related (antibiofilm) activity was still present, suggesting that perhaps bEV production, although undetectable, was not completely lost.\textsuperscript{77} In contrast, Wang \textit{et al.}\textsuperscript{78} did not see different bEV production in their inactivated agr strain, although the bEVs produced by the mutant showed significantly reduced cytotoxicity to, and ability to, stimulate a pro-inflammatory response in a macrophage cell line (THP1).

### 5.4 The bEV genesis mechanism may influence both composition and role within the chronic wound

The extent of the differences in the composition and properties of the bEVs formed by different biogenesis mechanisms is still largely unexplored, yet is essential to understanding bEV roles during wound colonization. Preliminary data suggest that inclusion of proteins and genetic material within passively created \textit{P. aeruginosa} bEVs was random, with no enrichment of specific molecules.\textsuperscript{66} DNA sequencing of bEVs created by explosive lysis showed they contained DNA representative of the whole genome.\textsuperscript{66} In contrast, bEVs produced by active mechanisms appear to be more selective in specific components, with multiple reports showing that both the protein and genetic profiles of \textit{P. aeruginosa} and \textit{S. aureus} bEVs differ from both whole cell and cell membrane profiles.\textsuperscript{48,53,64,70,75,79–82} Both \textit{P. aeruginosa} and \textit{S. aureus} bEVs are enriched in toxins like haemagglutinin\textsuperscript{53,80} (\textit{P. aeruginosa}) and \(\alpha\) haemolysin (\textit{S. aureus})\textsuperscript{81} and \(\beta\) lactamase\textsuperscript{70,83} and nutrient sequestering proteins.\textsuperscript{53,84} Proteomic analysis of \textit{P. aeruginosa} bEVs showed vesicle-associated proteins are enriched in outer membrane localized proteins, whereas \textit{S. aureus} bEV contain a greater mix of cytoplasmic and cell wall/membrane proteins.\textsuperscript{34,70,79,84} Investigation of the “core proteome” of several \textit{S. aureus} isolate bEVs showed 36\% of bEV related proteins were virulence-associated.\textsuperscript{84} This is proportionally much higher than would be expected if bEV packaging had simply been random, strongly suggesting the existence of processes for virulence protein selection, inclusion and hence function in the context of an expelled EV. As a comparator, \textit{S. aureus} protein isolates showed that 102 out of a total of 1135 non-redundant peptides were virulence-associated (comprising only 11\% of the total whole cell lysate).\textsuperscript{85}

### 5.5 The role of bEVs, and their components, in driving chronicity of wounds

bEVs appear to have multiple functions throughout the biofilm life cycle (see Table 1 for a summary). The bEVs of the oral pathogen Porphyromonas gingivalis stimulate aggregation of \textit{S. aureus} with Streptococcus spp. and the yeast pathogen Candida albicans, all three of which are also found within chronic wound biofilm communities.\textsuperscript{93} \textit{P. gingivalis} is a poor biofilm former, typically integrating into pre-existing oral biofilms rather than creating \textit{de novo} biofilms.\textsuperscript{92} As such it is interesting to speculate that this pro-aggregation property of \textit{P. gingivalis} bEVs develops a favourable environment for the organism, increasing its survival. In contrast, the bEVs of \textit{S. aureus} decreases the attachment of competitor species, in particular Acinetobacter baumannii, to abiotic surfaces.\textsuperscript{77}

Biofilm EPS typically comprises LPS, eDNA and protein\textsuperscript{96} and bEVs have a role in providing EPS materials. Transmission electron microscopy showed that \textit{P. aeruginosa} bEVs were present in \textit{in vitro} and \textit{in vivo} biofilms and a major source of EPS associated LPS.\textsuperscript{52} Between 20\% and 30\% of the total \textit{S. aureus} and \textit{P. aeruginosa} biofilm EPS protein content originates from their bEVs.\textsuperscript{53,80} Both \textit{P. aeruginosa} and \textit{S. aureus} bEV also provide a structural role. \textit{P. aeruginosa} bEVs bind biofilm eDNA\textsuperscript{93} and \textit{S. aureus} bEVs are incorporated into \textit{S. aureus} biofilms. In the case of one methicillin resistant \textit{S. aureus} isolate, the presence of the antibiotic ceftazidime decreased EPS production; as such bEVs became the primary EPS component and provided structural support.\textsuperscript{99} These studies highlight that bEVs therefore contribute to EPS, and are likely to form important structural elements, which are highly dynamic in responses to therapeutic agents.

### 5.6 Proteins associated with bEVs protect biofilm communities and aid nutrient acquisition

Iron is an important nutrient for bacteria, and microbial chronic wound communities must be able to obtain free iron from the host environment or nearby microbes. In turn, the host restricts iron availability, reducing colonization.\textsuperscript{99} \textit{P. aeruginosa} and \textit{S. aureus} bEV each contain several proteins with iron sequestering function.\textsuperscript{84} PQS, which is so
important in the production of *P. aeruginosa* bEV, remains associated with bEV after thier formation and has iron chelating activity. In turn it is able to interact with vesicle-associated TseF, a type VI secretion system effector, to deliver iron to *P. aeruginosa* cells via the outer membrane proteins OprF and FptA.

**Table 1** Summary of the roles bEV play in biofilm formation, maintenance and supporting the biofilm inhabitants

| Activity | Effect | Benefactor | References |
|----------|--------|------------|------------|
| Component delivery | Delivery of lytic toxins | Local bacteria are lysed, limit their population and releasing nutrients. | Originating bacterial species | 48, 64, 84, 86, 87 |
| | Delivery of “scavenging” proteins | bEV inclusion of siderophores protects them from degradation and allows collection of nutrients (both extracellular and released following lysis). | Originating bacterial species | 84, 88–91 |
| | Inclusion of β-lactamase enzymes | Extracellular presence of β-lactamase degrades penicillin class antibiotics. | All local bacterial species | 34, 70, 83, 92 |
| | Delivery of quorum sensing molecules | Inclusion of quorum sensing molecules (i.e. PQS) allows communication over greater distance and limits rapid diffusion and/or degradation. | Originating bacterial species | 91 |

| Influencing biofilm formation | Increasing aggregation | The presence of bEVs promotes attachment and aggregation of disparate bacterial species to surfaces. | Originating bacterial species | 93 |
| Decreasing attachment | The presence of bEVs limits attachment and aggregation of other bacterial species to surfaces. | Originating bacterial species | 77 |
| Modification of bEV activity | Species living within biofilms reduce the cytotoxicity of their bEVs. | All local bacterial species | 62 |
| Use of bEV components within EPS | LPS from bEV is utilized to produce biofilm extracellular matrix | All local bacterial species | 53, 80, 94 |
| Structural biofilm support | bEVs can be physically included within biofilm matrix, providing structural support where typical EPS is not available | All local bacterial species | 95, 96 |

*Benefactors are dependent on the molecules associated with bEV. To the best of the authors’ knowledge, to date only PQS has been shown to be bEV associated. However, if universal quorum sensing molecules (i.e. Autoinducer-2) are also present then multiple species may be able to sense and respond to bEV associated signals.*

*Bacterial species other than the originating species may also benefit from either increased or decreased attachment and biofilm formation; however, to date no evidence exists that this effect is intended to benefit organisms other than the originating species.*

5.7 | bEVs as a source of mobile genetic elements within chronic wound biofilms

Alongside AMR enzymes, the presence of AMR genes within bEVs is of concern as they could lead to the spread of AMR genes throughout biofilm populations. Bacterial genetic competence is the ability of bacterial species to bind and uptake DNA from donors or the environment and incorporate it into their genetic repertoire. Competence can either be naturally present (referred to as naturally competence) or induced in vitro by chemical or electrical means. Once the DNA is
incorporated into a bacterium’s genetic repertoire, the cell is referred to as “transformed.”\textsuperscript{101} \textit{P. aeruginosa} has recently been shown to be naturally competent within biofilms,\textsuperscript{102} allowing uptake of naked DNA that hypothetically could be contributed by bEV. Because of the presence of several restriction modification systems within \textit{S. aureus},\textsuperscript{103} natural transformation is less common, although minimal genetic mutation does permit it\textsuperscript{104} and the bacterium is able to accept and transfer plasmids via conjugation.\textsuperscript{105} Genetic material is reportedly associated with bEVs,\textsuperscript{35,66,82,106} although to date there is little evidence that bEVs have a role in transmitting genes. Renelli \textit{et al}\textsuperscript{25} reported that following genetic manipulation of \textit{P. aeruginosa}, the shuttle cloning vector was detected within the transformants bEVs. Despite the vector being readily taken up by both \textit{P. aeruginosa} and \textit{E. coli} when exogenously added to bacterial cultures, bEV-associated shuttle vector could not be transmitted to either \textit{P. aeruginosa} or \textit{E. coli},\textsuperscript{35} suggesting association of DNA with bEVs limits the capacity for uptake and transformation.

This raises important questions around the capacity for bEV involvement in horizontal gene transfer. \textit{P. aeruginosa} bEVs are associated with both chromosomal and plasmid DNA, although only plasmid DNA was found within vesicle lumen in this study.\textsuperscript{35} In contrast, Yaron \textit{et al}\textsuperscript{107} described bEV-mediated transfer of a gene encoding green fluorescent protein among \textit{E. coli} isolates. Transformation was achieved following DNase I treatment of the purified \textit{E. coli} bEV, indicating that the plasmid was protected from the enzymatic activity by inclusion within the bEV lumen.\textsuperscript{107} Worryingly it was demonstrated, utilizing the same experimental conditions, that \textit{A. baumannii} could be transformed with \textit{bla}_{OXA-24}, a \beta-lactamase gene encoding penicillin resistance, and hence implicate bEVs as a modality to transmit therapy resistant genes to neighbouring bacteria.\textsuperscript{108} 

Although multiple studies have shown an association of genetic material with Gram-negative bEVs, less evidence is available for Gram-positive species such as \textit{S. aureus}. Lee \textit{et al}\textsuperscript{83} reported that although bEVs contained functioning \beta-lactamase enzymes, the relevant genes were not associated with bEVs. An early study by Dorward and Garon\textsuperscript{109} also reported that neither chromosomal or plasmid DNA was associated with bEVs of four Gram-positive species tested (\textit{S. aureus, Bacillus cereus, B. subtilis} and \textit{Streptococcus sanguis}); however, genetic material was present in all 14 Gram-negative species, including \textit{P. aeruginosa}. It should, however, be noted that these experiments were carried out using exponential phase cultures rather than stationary phase and as shown previously, growth phase influences bEV biogenesis of both \textit{S. aureus} and \textit{S. sanguis}. In contrast, Rodríguez and Kuehn\textsuperscript{110} reported both DNA and RNA were associated with \textit{S. aureus} bEVs isolated from mid-exponential phase cultures. Similarly, Kliveau \textit{et al}\textsuperscript{111} reported that bEVs from the Gram-positive gut bacterium \textit{Ruminococcus albus} contained chromosomal DNA and a bEV enriched supernatant was able to restore crystalline cellulose degradation function in deficient recipient cells. Within this study, no DNase I treatment of bEVs was carried out, meaning intraluminal DNA delivery was not conclusively demonstrated and that DNA transmitted to the donor cells may be from exogenous sources.\textsuperscript{111}

Although many studies have conclusively shown the DNA is present in bEV, there is less persuasive evidence showing that this bEV-associated DNA is a widely utilized mechanism of transformation within biofilm communities, particularly compared with other methods such as conjugation. It must be noted that natural transformation via bEV is likely to be species and DNA dependent, reflected by the contrasting evidence from the studies of Gram-negative species, and further investigation is required before it is known to what extent transformation via bEV contributes to the overall quantity of genetic exchange. To the authors’ knowledge, although multiple bEV biogenesis mechanisms are now described, there is still little understanding of the mechanisms involved in bEV vesicle uptake and unpackaging by recipient bacterium. Even naturally competent bacterial species contain multiple restriction modification systems, which must be overcome before foreign DNA incorporated into the recipient’s genome. Although incorporation into bEV may protect DNA from degradation while within the extracellular milieu, it is not yet known if bEV incorporation also provides a similar advantage once taken up by recipients. As such, although several studies have presented evidence that hints that AMR spread can be facilitated by bEV-associated DNA, further investigation is required before this can be confirmed to be a widespread phenomenon, utilized by multiple clinically relevant bacterial species.

5.8 The role of bEVs in promoting competition between bacterial species

The majority of biofilm inhabitants participate in synergistic and/or antagonistic interactions with other community members, creating a high degree of spatial organization.\textsuperscript{112} The interactions between \textit{P. aeruginosa} and \textit{S. aureus} are usually reported to be antagonistic, with \textit{P. aeruginosa} dominating cystic fibrosis lung communities.\textsuperscript{113–115} This paradigm has recently been shown to be more nuanced (for a review see Reference 116), particularly within chronic biofilm infections, such as those found in wounds. In the chronic wound, \textit{S. aureus} and \textit{P. aeruginosa} have been shown to co-exist in vivo, although they are spatially separated within the wound.\textsuperscript{117} The presence of \textit{S. aureus} biofilm may increase the biofilm biomass of \textit{P. aeruginosa}\textsuperscript{118} and it has been shown that \textit{P. aeruginosa} presence can promote \textit{S. aureus} colonization within an \textit{in vivo} mouse lung infection model, even when \textit{in vitro} testing suggested an antagonistic relationship.\textsuperscript{119} The role of bEVs within these biofilm interactions is still largely unknown; however, evidence from planktonic studies strongly suggests a role in promoting competition and co-operation.

\textit{P. aeruginosa} bEVs appear to have significant bactericidal activity, with greater protease, phospholipase C and alkaline phosphatase activity than whole cell lysates. In total, 50% of exogenous alkaline phosphatase activity was linked to \textit{P. aeruginosa} bEVs.\textsuperscript{88} This activity contributes to the ability of \textit{P. aeruginosa} bEVs to lyse multiple Gram-negative species, although lysis activity was less evident against Gram-positive organisms, with no ability to lyse \textit{Staphylococcus} sp. observed.\textsuperscript{87} In contrast, Mashburn and Whiteley\textsuperscript{84} reported the
inhibitory activity of *P. aeruginosa* bEVs against *S. aureus* and also commonly identified within chronic wounds. Activity against Gram-positive organisms was also shown by Kadurugamuwa et al who demonstrated lytic activity of *P. aeruginosa* bEVs against *Bacillus stearothermophilus* (now *Geobacillus stearothermophilus*) and *Aneurinibacillus thermoanophilus*. These are both environmental species not relevant to chronic wounds but possibly encounter *P. aeruginosa* in natural habitats. The above study linked the antimicrobial activity to the high quinolone content of *P. aeruginosa* bEVs, rather than presence of other lysins or toxins. Quinolones, including the *P. aeruginosa* PQS molecules, are potent antimicrobials alongside the other functions described earlier. Interestingly, Cooke et al reported that *P. aeruginosa* biofilm bEVs showed decreased bactericidal activity compared with planktonic EVs. This raises the interesting possibility that *P. aeruginosa* can move between competitive and co-operative positions as it transitions from an activity growing planktonic lifestyle towards existence within biofilms.

Evidence of the role of *S. aureus* bEVs in suppressing competition is less widely reported. *S. aureus* bEVs demonstrate antibiofilm, but not lytic, activity against *Acinetobacter baumannii*, *Enterococcus faecium* and *Klebsiella pneumonia*, but not *P. aeruginosa*. The mechanisms of action were likely due to inhibition of initial surface attachment of the pathogens, rather than dispersal of biofilms once formed. Proteomics show *S. aureus* bEVs contain phenol soluble modulins, which, in *S. epidermidis*, have bactericidal activity. Association of components such as these suggests that *S. aureus* bEV may have antimicrobial activity; however, further investigation is required to confirm this.

### 5.9 The contribution of bEVs to chronic wound tissue damage and inflammation

*S. aureus* and *P. aeruginosa* bEVs and their associated components are immunostimulatory and have been show to bind to cholesterol rich areas of the cell membrane with disruption of these areas, for example, by sequestering cholesterol, attenuating binding. Associated bEV pro-inflammatory components include LPS and unmethylated CpG DNA, which are detected by Toll like receptor four (TLR4) and nine (TLR9), respectively. As such, bEVs can elicit a strong inflammatory response, potentially exacerbating this feature so characteristic of chronic wounds. Application of *S. aureus* bEV to in vitro cultures of human dermal microvascular endothelial cells increased expression of the pro-inflammatory cytokine Interleukin (IL) 6 and the adhesins E-selectin, Intracellular Adhesion Molecule 1 and Vascular Adhesion Molecule 1, via TLR4. This in turn increased recruitment and attachment of THP1 monocytes, further driving a pro-inflammatory response. Wang et al also reported increased expression of pro-inflammatory cytokines IL6 and IL1β upon THP1 exposure to *S. aureus* bEVs. Similarly, in murine studies, *S. aureus* bEVs stimulated a pro-inflammatory response when either inhaled into the lung or applied directly to tape stripped skin. It must also be noted that evidence is emerging to indicate that the host is also able to degrade bEVs and package components into hEVs (for an overview, see Schorey et al). This is important for the immunosurveillance of intracellular pathogens, a lifestyle demonstrated by both *S. aureus* and *P. aeruginosa*, although to the best of the authors' knowledge the presence of bEV components from these bacterial species within hEVs has not yet been determined (Figure 4 upper panel).

The bEVs from some *S. aureus* isolates also have a cytotoxic effect on host cells (Figure 4 lower panel). This is not unexpected as many *S. aureus* bEV factors, including α, δ, γ and ρ-haemolysin, leukocidin D, exfoliative toxin C, exfoliative toxin A and α and β-class phenol soluble modulins, have potent cytotoxic activity. Jeon et al demonstrated that the bEVs purified from the supernatant of four *S. aureus* isolates had varying levels of cytotoxicity to human epithelial cells (Hep-2). Two isolates showing no cytotoxicity and a fourth (*S. aureus* MO60) potentiating significant cytotoxic effect even at low (≥10 μg/ml) concentrations. Proteomics revealed that despite the inconsistent cytotoxic activity, the bEVs of all four isolates contained haemolysins, leukocidin D and exfoliative toxin C. However, only *S. aureus* MO60 contained exfoliative toxin A, suggesting that perhaps this compound was responsible for the observed cytotoxicity. In contrast to *S. aureus*, there are few reports of *P. aeruginosa* bEV cytotoxicity, probably due to a lack of relevant studies rather than activity. One preliminary study has reported cytotoxicity to airway epithelial cells, suggesting that further studies are likely to confirm that *P. aeruginosa* has similar cytotoxicity described for *S. aureus*. Both *P. aeruginosa* DNA and protein can be detected within the lumen of lung epithelial cells incubated with purified *P. aeruginosa* bEVs, highlighting their ability to merge with host cells and release their contents.

Although evidence of *P. aeruginosa* bEV cytotoxicity may be lacking, the pro-inflammatory properties of *P. aeruginosa* bEVs are in no doubt. In vitro addition of *P. aeruginosa* bEVs to the RAW267.4 macrophage cell line increased both mRNA and protein levels of the pro-inflammatory factors monocyte inhibitory protein 2, tumour necrosis factor α (TNFα) and IL1β. Similarly, the bEVs of four *P. aeruginosa* strains from environmental and clinical sources were all able to increase IL8 concentration in a lung epithelial cell line (A549) and primary human bronchial epithelial cells. *P. aeruginosa* bEVs' pro-inflammatory activity was demonstrated in vivo by internal administration in mice, increasing infiltration of neutrophils, macrophages, dendritic cells, natural killer cells, CD4+ and CD8+ T lymphocytes into lung tissue and elevating IL1β, TNFα, IL6 and interferon gamma (IFNγ) levels. The inflammatory response in this study was partly dependent on bEV stimulation of TLR2 and TLR4, as mice lacking either TLR2 or TLR4 showed decreased inflammatory response, while HEK 293 cells overexpressing either receptor had elevated IL8 levels following bEV stimulation.

Importantly, pro-inflammatory molecules associated with intact bEVs, as opposed to free extracellular molecules, exert greater potency. Wang et al reported that IL6 levels increased much more rapidly when THP1 cells were exposed to *S. aureus* bEVs (4 h) compared with whole cells (48 h for a comparable response). Similarly,
apoptosis of a Hep-2 cell line was increased in the presence of whole S. aureus bEVs compared with lysed preparations. Park et al. also demonstrated an elevation of IL 1β, TNF α and IFN γ within mouse bronchoalveolar lavage fluid in the presence of 10 μg/ml P. aeruginosa bEVs than with either 10^6 or 10^7 CFU P. aeruginosa. Finally, the cytotoxic activity of P. aeruginosa bEVs against lung cells was greater than the activity observed when cells were treated with recombinant versions of their toxins. These investigations therefore identify the importance of an intact bEV structure in mediating biological responses in mammalian host cells and suggest the sensing of bEV by mucosal surfaces and inflammatory cells is highly sensitive and evolutionary conserved.

6 | UTILIZING THE ANTIMICROBIAL PROPERTIES OF BOTH bEVs AND hEVs IN CONTROLLING CHRONIC WOUND MICROBIAL POPULATIONS

The complex and multi-mechanistic properties of bEVs can modulate microbial populations in model systems. This property could also be harnessed to control wound colonization or shift wound microbiomes towards a community more associated with rapid healing. Probiotic species, in particular members of the genus Lactobacillus, are known to inhibit growth of multiple opportunistic pathogens including S. aureus and P aeruginosa, without generating the pro-inflammatory response described above. Inhibitory activity is found in bacterium free supernatants, indicating that secreted factors, including bEVs, are responsible for antimicrobial activity. When fed with vancomycin resistant S. aureus, survival of Caenorhabditis elegans was increased by supplementation with Lactobacillus plantarum bEVs. Similarly, daily oral administration of L. plantarum bEVs decreased S. aureus bEV-induced skin inflammation in a mouse atopic dermatitis model. Keratinocytes and macrophages incubated with L. plantarium bEVs showed reduced S. aureus bEV-induced IL 6 expression, indicating that L. planetarium bEVs not only control pathogen populations but dampen the host inflammatory response.

Although Lactobacillus sp. are one of the most commonly investigated probiotic species in relation to human health and disease, they are by no means the only bacterial species capable of modulating wound healing. Recent investigation of the industrially relevant cyanobacterium Synechococcus elongatus PCC 7942 showed it was able to produce bEVs with a size range of 80–250 nm. Uptake of the bEVs by human microvascular endothelial cells and their perinuclear localisation was shown in vitro. The bEVs were able to stimulate statistically significant increases in proliferation and migration of the cell line in vitro. This activity was statistically significantly decreased in the presence of GW4869, a compound impairing bEV release. Both bEV preparations and bacterial cultures were also shown to also have activity in vivo, improving wound closure, cell proliferation and decreasing scar formation in a mouse burn wound model. Interestingly, the bEV preparations had enhanced activity compared with the
bacterial cultures, highlighting their potency. The pro-wound healing mechanism of activity was suggested to be based on the photosynthetic ability of the bacterium and the improved oxygenation this brought to damaged tissue. However, in vivo healing was also reported in mice kept in dark conditions, suggesting that the mechanism is likely to be multi-factorial.

There has also been significant interest in the potential of hEVs, particularly from mesenchymal stromal cells (MSC), as chronic wound therapeutics. MSC and their EVs can contribute to the resolution of chronic wounds by modulating the pro-inflammatory response, driving progression towards cell proliferation and tissue remodelling and suppressing bacterial colonization. Multiple MSC therapies are under development, with some undergoing clinical trials, but safety concerns have been raised. As such non-viable MSC derivatives, including MSC EVs, are a promising alternative to whole cell therapeutics. Studies have shown that MSC hEVs are able to improve wound healing via simulation of Wnt/β-catenin signalling. Loading of hEVs with a miRNA miR-21 mimic allowed delivery of the miRNA to target cells to improve Wnt/β-catenin controlled migration of keratinocytes in vitro and wound healing in vivo using a diabetic rat model.

The secretome of MSC cultures exerts antimicrobial and antibiofilm activity against both Gram-positive and -negative bacterial species, including S. aureus and P. aeruginosa (for a review of MSC antimicrobial peptides see Alcayaga-Miranda et al). Impregnating wound dressings with a combination of silver nanoparticles and MSC derived hEV increased wound closure, angiogenesis and alpha smooth muscle actin presence in an murine wound model, while also showing in vivo and in vitro activity against P. aeruginosa and broad in vitro antimicrobial activity against E. coli, S. aureus and Candida albicans.

Although MSC hEV studies to date have shown significant promise in stimulating chronic wound healing both in vitro and in vivo, several limitations need to be overcome before therapeutics can be developed efficiently. Increasing the scale of hEV production and improving product purity and constancy must be addressed (for an overview of the challenges see ). The use of 3D tissue culture, rather than monolayer culture, has been shown to improve hEV yield as has exposing cell lines to low intensity ultrasound radiation. Alterations in EV generation must be carried out with great care, however, as alterations in the conditions originating cells are exposed to are reported to alter the composition of both bEV (discussed above) and hEV. As such, care must be taken to show that changes to EV generation and purification processes do not impact the specific components of interest.

The wound environment itself also provides challenges for therapeutic development. Chronic wounds are typically very moist, with liquids continually being flushed across the wound site. Because of this, topical wound treatments must be able to resist both dilution and rinsing from the wound. To overcome these effects, several groups have recently investigated the feasibility of loading therapeutic EVs into hydrogels. Incorporation of hEVs from adipose derived stem cells in an alginate hydrogel showed improved closure of in vivo full thickness wounds compared with both hydrogel only and untreated wounds. Similarly, a chitosan hydrogel incorporating endometrial stem cell hEVs improved closure of mouse full thickness wounds and enhanced fibroblast migration in vitro. As well as improving in vivo wound closure, in vitro antimicrobial activity has been reported for hEV impregnated hydrogels, although not all the hydrogel types tested were antibacterial, suggesting that both the hEV and gel type may be important in determining the final activity of the product.

6.1 | Bacterial EVs as a potential vaccine candidate

Identifying suitable vaccine candidates for S. aureus and P. aeruginosa has proved difficult due to both genetic variability across the species and the transcriptional flexibility of conserved antigens. Since the bEVs of P. aeruginosa and S. aureus can elicit an immune response and are enriched in both highly conserved and antigenic targets, they have become of interest as a vaccine candidate. Wang et al demonstrated that inoculation of mice with S. aureus bEVs containing attenuated proteins was protective against several S. aureus strains. Chen et al further demonstrated that injection of bEVs alone could stimulate an immune response and vaccinated animals showed both a reduction in lesion size and S. aureus wound colonization. Promising results were also reported for P. aeruginosa bEV-based vaccine, with vaccinated mice showed both reduced P. aeruginosa lung colonization and subsequent tissue damage.

7 | CONCLUSIONS AND FUTURE PERSPECTIVES

The field of bEV research is still in its infancy. Knowledge of the role bEVs have in establishing and supporting biofilm infections is still limited. However, from the current understanding, some interesting themes are emerging. For example, bEVs appear to have a significant role in securing nutrients for the originating microbial population, accomplished by competing effectively with the surrounding microbial species or obtaining nutrients directly from the host. The methods utilized appear to be specific to the originating organism and their “normal” environmental niche. In turn, the host surveils bEV populations, instigating a pro-inflammatory response when bEVs from opportunistic pathogens such as P. aeruginosa and S. aureus are detected.

Much however, still remains to be discovered; fundamental mechanisms of bEV biogenesis such as the targeting and packaging of bEV contents is still poorly understood, yet as described above the contents of bEVs can be selective and can play a significant role in their toxicity. Similarly, bEV classification and characterization is poor at present. Identification of bEVs within the studies mentioned here relied primarily on transmission electron microscopy, protein concentration measurement and basic proteomic analysis. Many studies report a large size range of bEVs within their preparations and it is likely that further investigation will further divide bEV populations into sub-classifications with distinct activity, as for Eukaryotic vesicles.
Finally, there is a lack of bEV markers. Although we recognize that the intra- and inter-species diversity of prokaryotes will likely make identification of “universal” bacterial markers impossible, it would be helpful for classification purposes to identify molecular factors to help define bEV subsets. The standardization and classification of human vesicle types, as described in the MISEV2018 guidelines, have been essential to increase the robustness of the Eukaryotic EV field, and the bEV field would benefit greatly from a similar approach. Markers of EV allowing differentiation between bacterial genus and/or species would be particularly useful, especially when attempting to investigate the complex hEV and bEV populations that are found within human infections, including chronic wounds. Currently, although it is possible to isolate whole EV populations from chronic wounds, it is not possible to separate bEV and hEV. Markers specific for bEV might make this possible in the future. An approach similar to that applied to shotgun metagenomics might prove useful in this instance. Shotgun metagenomics harvests and sequences the total DNA from any source. Computational analysis of the sequencing reads then allows the community to be interrogated and separated into host and DNA from various microbial sources. This allows identification of bacterial, fungal/yeast and viral isolates to the species level and highlights the presence of AMR and virulence genes within the community. Multi-omics methods, combining analysis of DNA, gene and protein expression are now being used to investigate biofilm communities and such approaches could be applied to EV populations in the future. Unfortunately, all ‘omics tools’ are heavily reliant on the availability of well curated databases and knowledge of markers that allow components to be linked to likely originating species or genus. Since databases are not yet widely available for EVs, the focus must currently be on not only identifying markers and components, but creating freely available and curated databases, such as are now available for DNA and protein analysis. Biofilm communities are very rarely composed of a single species, and the interactions within these communities are important for persistence. As we demonstrate in this review, it is abundantly clear that bEVs have a critical role in these interactions, but a better understanding of bEV biology and type is required before these findings can be confidently tested.

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