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MINI-REVIEW

Functions and importance of mycobacterial extracellular vesicles

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Abstract The release of cellular factors by means of extracellular vesicles (EVs) is conserved in archaea, bacteria, and eukaryotes. EVs are released by growing bacteria as part of their interaction with their environment and, for pathogenic bacteria, constitute an important component of their interactions with the host. While EVs released by gram-negative bacteria have been extensively studied, the vesicles released by thick cell wall microorganisms like mycobacteria were recognized only recently and are less well understood. Nonetheless, studies of mycobacterial EVs have already suggested roles in pathogenesis, opening exciting new avenues of research aimed at understanding their biogenesis and potential use in antitubercular strategies. In this minireview, we discuss the discovery of mycobacterial vesicles, the current understanding of their nature, content, regulation, and possible functions, as well as their potential therapeutic applications.

Keywords Mycobacterium · Vesicles · Siderophores · Iron · Lipoproteins · Vaccines

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Introduction

Mycobacterium tuberculosis (Mtb), the bacterium that causes tuberculosis, has coevolved with humans, who are its only known reservoir. As a result of this coevolution Mtb engages in multiple interactions with the host via cell surface components and by secretion of a variety of molecules into the extracellular environment. Recently, another means by which pathogenic mycobacteria interact with the host was discovered: it was shown that mycobacteria can concentrate and pack various types of macromolecules into membrane vesicles, which are released into the environment. Although the study of mycobacterial microvesicles is in its early stages and much still needs to be learned about their biogenesis and functions, it is clear that microvesicle formation is a regulated process by which Mtb can manipulate host responses and promote bacterial adaptation. This review summarizes what is currently known about mycobacterial microvesicles and discusses possible ways to utilize microvesicles to modulate pathogen-host interactions in ways that lead to positive outcomes for the host.

The discovery of mycobacterial extracellular vesicles

Mycobacterial extracellular vesicles (MEVs) were first discovered embedded in the extracellular matrix of *Mycobacterium ulcerans* biofilms (Marsollier et al. 2007). Subsequently, they were recovered—by differential sedimentation—from the culture supernatants of several mycobacterium species including *M. tuberculosis*, *Mycobacterium bovis* BCG, *Mycobacterium kansasii*, *Mycobacterium avium*, *Mycobacterium smegmatis*, and *Mycobacterium phlei* (*Prados-Rosales et al.* 2011). The observation that fast and

slow growers and virulent and nonvirulent mycobacteria all produce vesicles suggests that this is a conserved phenomenon in the mycobacterium genus. MEVs were observed by transmission electron micrographic (TEM) analysis in the pellets recovered from culture supernatants and were also associated with only live, metabolically active bacteria (Prados-Rosales et al. 2011) (Fig. 1). Furthermore, *M. tuberculosis* (Mtb) and *BCG* associated microvesicles were also observed during intracellular infection of macrophages in culture and in mice, confirming that MEVs production happens not only in vitro but also in vivo (Prados-Rosales et al. 2011).

The biogenesis of MEVs

MEVs vary in size from 60 to 300 nm in diameter, which is similar to the size range of EVs produced by gram-negative bacteria. MEVs are composed of lipids and lipoproteins present in the mycobacterial plasma membrane, indicating their plasma membrane origin (Prados-Rosales et al. 2011). However, while in gram-negative bacteria the vesicles emerge from the outer membrane and are released without any obstruction, mycobacteria have a complex cell wall that surrounds the plasma membrane. This cell wall is composed of peptidoglycan covalently attached to arabinogalactan, which in turn is decorated with mycolic acids and intercalating free lipids, forming the mycomembrane (Daffe 2015). Furthermore, a capsule composed of polysaccharides, proteins and lipids surrounds the cell wall. Thus, one of the most important unanswered questions is how MEVs traverse the cell wall. This question is also relevant to other thick cell wall microorganisms, such as gram-positive bacteria and fungi. The presence of cell wall remodeling enzymes in fungal EVs and peptidoglycan-degrading enzymes in *Staphylococcus aureus* EVs suggests that remodeling of the cell wall may facilitate EV transit across the cell wall (Lee et al. 2009). This and other nonmutually exclusive hypotheses have been proposed to explain the mechanism of vesicle release in mycobacteria; however, there is as yet no definitive information and this remains an area of intense investigation.

The regulation of MEV production by iron limitation

In response to iron limitation, Mtb enhances EV production (Fig. 2) (Prados-Rosales et al. 2014b). This behavior and the increased vesiculation observed in the Mtb strain lacking the vesiculogenesis and immune response regulator (VirR) indicates that MEV production is genetically regulated (Rath et al. 2013). VirR is a cytoplasmic protein that interacts with the plasma membrane and at least one lipoprotein that is included in MEVs. It has been suggested that VirR is part of a high-order protein complex that controls vesicle formation and cargo selection (Rath et al. 2013). However, the precise mechanism by which VirR influences vesiculogenesis remains to be elucidated. Similarly, how iron limitation signals to promote MEV generation and the molecular mechanisms involved in this response are still unknown. For instance, while *virR* expression is downregulated in response to iron deficiency (M. Rodriguez, unpublished), it is still not clear whether reduced *virR* expression is responsible for Mtb's hypervesiculation under iron limitation. Since iron restriction is a natural host response to infection (Weinberg 1984) that frequently signals bacteria to produce virulence factors (Litwin and Calderwood 1993), it is likely that MEVs released in response to iron limitation in vivo are involved in pathogenesis. Characterization of MEVs produced under iron restriction and other conditions that resemble the host environment will aid understanding the function of MEVs in Mtb interactions with the host (see below).

MEV content

In *E. coli*, 0.2–0.5 % of outer membrane (OM) and periplasmic proteins are packed in vesicles (Hoekstra et al. 1976,

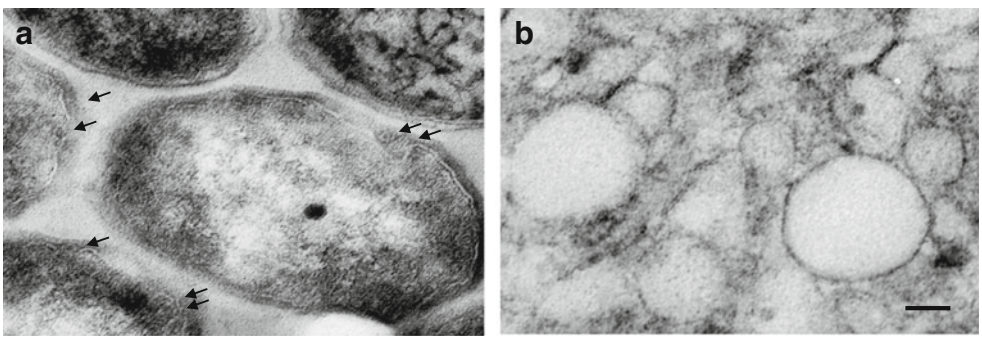

Fig. 1 Microvesicles produced by *Mycobacterium tuberculosis*. Transmission electron micrograph showing vesicles budding from the surface of the bacteria (*arrows*) (**a**) and isolated membrane vesicles (*scale bar*, 50 nm) (**b**). Copyright © 2014, American Society for Microbiology. Journal of Bacteriology. 196(6). 1250–1256. doi:10.1128/JB.01090–13

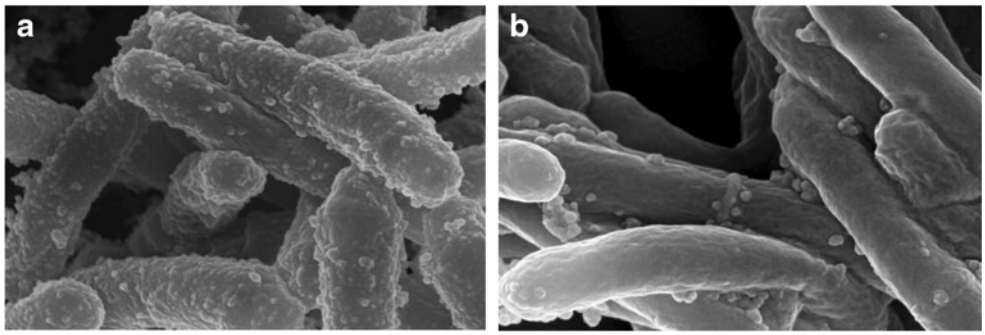

Fig. 2 Microvesicle production is regulated by iron. Scanning electron micrograph of Mtb cultured under iron limiting (**a**) and iron-sufficient conditions (**b**). The number of spherical vesicles associated to the mycobacterial surface is higher in iron deficient cultures. Copyright © 2014, American Society for Microbiology. Journal of Bacteriology. 196(6). 1250–1256. doi:10.1128/JB.01090–13

Kesty and Kuehn 2004), suggesting that vesicle formation is an energy demanding process and thus is likely to serve an important cellular function. In addition to proteins, a large variety of other molecules, including phospholipids, nucleic acids, lipopolysacharide, and periplasmic components, can be found encapsulated in outer membrane vesicles (OMVs) in gram-negative bacteria (Kuehn and Kesty 2005). Because of their potential implications in host-pathogen interactions, vesicles produced by pathogenic bacteria have been most extensively studied and have been shown to deliver a variety of virulence factors like adhesins, toxins, and immunomodulatory effectors (Kuehn and Kesty 2005). In addition, OMVs have been associated with interbacterial transfer of material that contributes to survival and genetic diversity, including antibiotic resistance enzymes and chromosomal, plasmid, and phage DNA (Ciofu et al. 2000, Kolling and Matthews 1999). Analogous to gram-negative bacteria, gram-positive pathogens like *S. aureus* use EVs to deliver cytotoxic material to host cells (Gurung et al. 2011). Similarly, *M. ulcerans* EVs contain the main virulence factor produced by this mycobacterium, the cytotoxin mycolactone (Marsollier et al. 2007). Importantly, mycolactone enclosed in EVs is more potent than isolated toxin, supporting a role of EVs in pathogenicity of *M. ulcerans* (Marsollier et al. 2007).

While it is hypothesized that the quantity, stability, and subcellular localization of a protein might influence its availability for inclusion as vesicle cargo, little is known regarding the determinants of EV cargo inclusion or exclusion. Global protein composition of EVs produced by Mtb, BCG, and *M. smegmatis* (Msmg) cultured in a defined minimal medium, identified 48, 66, and 64 vesicular proteins, respectively (Prados-Rosales et al. 2011). BCG and Mtb EVs were similarly enriched in lipoproteins and proteins belonging to the functional categories of cell wall, membrane function, as well as intermediate metabolism and respiration. BCG EVs contained more proteins classified in the category of lipid metabolism than Mtb and Msmg EVs. The poor representation of lipoproteins in Msmg EVs, despite their similar abundance in the cell, suggests that different species regulate incorporation of EV cargo differently. The lipid content of EVs released by Mtb cultured under conditions of iron sufficiency or deficiency, analyzed by mass spectrometry, showed predominately polar lipids, phosphatidylinositol (PI), acylated phosphatidylinositol dimmannosides (PIM_2), cardiolipin (CL), and phosphatidylethanolamine (PE) in both conditions, consistent with the membrane origin of the vesicles (Prados-Rosales et al. 2011). However, EVs from iron deficient Mtb were also enriched in acylated glicerides, and PE, while acyl trehalose, an important mycobacterial cell wall component, was more abundant in iron-sufficient Mtb EVs. Lipoarabinomanan, an important immunologically active glycolipid released by bacilli replicating within macrophage phagosomes, was also found associated with pathogenic mycobacteria produced EVs. A strong difference between low and high iron EVs was detected in the levels of the lipidic siderophore mycobactin, which was enriched in EVs released by iron limited Mtb (Prados-Rosales et al. 2014b). The finding of mycobactin in Mtb EVs and the stimulation of EV production in response to iron limitation have important implications for pathogenesis as discussed in the next section. DNA was also detected in purified and intact MEVs (R. Prados-Rosales, unpublished) but the implications of this finding are not yet known.

MEVs and host-pathogen interactions

Protein and lipid analysis of MEVs has revealed enrichment of lipoproteins and immunologically active glycolipids known to be ligands of the pattern recognition Toll-like receptor 2 (TLR-2), suggesting that MEVs participate in immune stimulation. Consistent with this idea, macrophage exposure to MEVs in vitro and administration of isolated MEVs to mice induced a TLR-2-dependent pro-inflammatory response distinct and more intense than that elicited by MEVs from nonvirulent Msmg. Notably, naive and BCG vaccinated mice challenged with Mtb aerosols showed acute inflammation and

a higher lung bacillary load when they were also injected intratracheally with BCG MEVs, indicating an overall impairment in control of infection and suggesting a role of MEVs in pathogenesis (Prados-Rosales et al. 2014a). These studies demonstrated that one function of MEVs is to deliver factors that modify the response of host cells to infection in a way that benefits the pathogen.

Iron availability is a critical factor that affects all bacteria living within a host. Because basic cellular metabolic activities require iron, this metal is absolutely essential for growth. However, due to its poor solubility and potential toxicity under aerobic conditions, free iron is not available in the host. Successful pathogens must be able to obtain iron and to adapt their metabolic activity according to iron availability. To obtain iron, Mtb synthesizes and secretes siderophores named mycobactins, which are essential for virulence (De Voss et al. 2000, Reddy et al. 2013). Two forms of mycobactins are produced: carboxymycobactin, an amphiphilic molecule that is secreted into the medium, and mycobactin, a cell surface-associated lipophilic molecule (Ratledge and Dover 2000, Snow 1970, Snow and White 1969, Gobin et al. 1995). Carboxymycobactin effectively sequesters ferric iron from the environment and transfers it to mycobactin (Gobin and Horwitz 1996) or brings it into the cell via the iron-regulated transporter IrtAB (Rodriguez and Smith 2006; Ryndak et al. 2010). The fate of iron bound by mycobactin and its overall contribution to iron uptake are unclear. Mature mycobactin is included in MEVs released by Mtb experiencing iron limitation, whereas mycobactin synthesis intermediates are abundant in iron-sufficient Mtb MEVs (Prados-Rosales et al. 2014b). Also, MEVs produced during iron limitation can deliver iron and support proliferation of iron-deficient bacteria only if they contain mycobactin (Prados-Rosales et al. 2014b). Although the molecular mechanism of MEV-associated mycobactin-mediated iron delivery remains to be characterized, these studies indicate a role for MEVs in pathogenesis.

MEV-mediated iron capture may be critical for Mtb survival during infection, especially in the context of intense iron deprivation in the granuloma (Basaraba et al. 2008). Immune cells forming the granuloma release siderocalin, which binds carboxymycobactin (Holmes et al. 2005), thereby compromising its role in iron acquisition. However, it is possible that vesicular mycobactin is not accessible to siderocalin. If that is the case, by "disguising" mycobactin in the EVs, Mtb may be able to overcome the interference of siderocalin with its iron acquisition. In addition, iron homeostasis and the regulation of macrophages' antimicrobial response are tightly interconnected (Cairo et al. 2011). It is not impossible that MEV-associated mycobactin interferes with macrophage iron homeostasis and thereby perturbs macrophage immune function. Support for this idea comes from the observation of exogenously added mycobactin-containing liposomes in association with phagosomal vacuoles in infected macrophages (Luo et al. 2005). The inclusion of mycobactin in MEVs may also be an example of collaborative Mtb interactions since mycobactin-contained in MEVs can benefit both the bacterium producing the MEVs and its neighbors.

MEV translational applications

Although MEV biogenesis is yet to be fully understood, the accumulation of knowledge, especially in gram-negative bacteria, has opened several avenues for therapeutic MEV applications. For example, vesicles derived from bacterial pathogens have been extensively used in the development of immunogenic vaccine candidates, and vesicles from gram-negative microorganisms have been recently developed into therapeutic vaccines (Acevedo et al. 2014). The vaccine potential of naturally produced MEVs isolated from BCG and Mtb was tested in a mouse model (Prados-Rosales et al. 2014a). Only immunization with Mtb-derived MEVs showed a comparable capacity to control bacterial replication in the lung similar to that of standard BCG vaccination. Analysis of immunogenicity showed a mixed antibody and cellular response directed to lipoproteins and bacterial cell surface components. Although mycobacterial MEVs may represent a promising alternative vaccine, there are still some practical aspects of their production that need to be tailored. For instance, inconsistent protection efficacy of three different preparations of Mtb MEVs produced under the same conditions was observed and composition analysis of multiple preparations showed variable protein cargo (R. Prados-Rosales, unpublished). These observations suggest that naturally produced EVs might not represent an ideal vaccine candidate. Furthermore, although the immune response to Mtb MEVs is directed against lipoproteins and bacterial cell surface components, the MEV-associated components that explain their protection efficacy remain to be elucidated. When this information is in hand, artificial Mtb microvesicles incorporating recombinantly expressed MEV-associated proteins and lipids could be developed and tested as an alternative vaccine.

Because MEVs represent a bacterial compartment with a unique protein composition, they may be useful as an alternative biomarker for tuberculosis. Recently, the human antibody response to naturally produced BCG and Mtb EVs was evaluated to look for novel biomarkers in a small cohort of patients, including smear-positive, smear-negative HIV uninfected pulmonary tuberculosis (TB), and BCG-vaccinated with and without latent TB (Ziegenbalg et al. 2013). A combination of three MEV-associated antigens was clearly recognized by sera from TB patients and not by the control group, encouraging further study of these structures as diagnostic tools. Similarly to the vaccine applications discussed above,

MEVs' natural variable composition may cause inconsistencies in the serology performance. In this scenario, artificially produced microvesicles with uniform content may represent an ideal source of experimental and therapeutic material.

Conclusions

Production of EVs in mycobacteria has now been clearly demonstrated. These vesicles originate from the plasma membrane and are associated with virulence factors and immunomodulators, indicating that they play a role in pathogenesis. MEVs from virulent mycobacteria elicit an immune response that can be protective. These findings suggest that the immunogenic potential of mycobacterial vesicles can be harnessed for vaccine development. Although the study of MEVs has intensified, the mechanisms of vesicle production and release and how these processes are regulated remain poorly understood. Also important to understand are the factors that contribute to cargo selection or exclusion and how environmental signals impact vesicle production and content. Future molecular and immunological studies will likely reveal novel roles of vesicles in mycobacterial physiology and pathogenesis and will stimulate the search of ways to exploit MEVs for antitubercular applications.

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Compliance with ethical standards
All procedures with Mtb-infected animals conducted by the authors and mentioned in this review were approved by the Albert Einstein College of Medicine animal care and use committee.

Conflict of interest The authors declare that they have no conflict of interest.

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