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Minireview

MINIREVIEW

Mycobacterial extracellular vesicles and host pathogen interactions

Shamba Gupta and G. Marcela Rodriguez*

Public Health Research Institute Center and New Jersey Medical School—Rutgers, The State University of New Jersey, 225 Warren Street, Newark, NJ 07103, USA

*Corresponding author: Public Health Research Institute Center and New Jersey Medical School—Rutgers, The State University of New Jersey, 225 Warren Street, Newark, NJ 07103, USA. Tel: + 973-854-3261; Fax: +973-854-3101; E-mail: rodrigg2@njms.rutgers.edu

One sentence summary: Mycobacterium tuberculosis releases membrane vesicles into the environment; these vesicles contain a broad range of microbial factors important for virulence and molecules that alter host defense systems.

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ABSTRACT

Mycobacteria, like other bacteria, archaea and eukaryotic cells, naturally release extracellular vesicles (EVs) to interact with their environment. EVs produced by pathogenic bacteria are involved in many activities including cell–cell communication, immunomodulation, virulence and cell survival. Although EVs released by thick cell wall microorganisms like mycobacteria were recognized only recently, studies of *Mycobacterium tuberculosis* EVs already point to their important roles in host pathogen interactions, opening exciting new areas of investigation. This minireview will summarize the current understanding of mycobacterial EV biology and roles in pathogenesis and will discuss their potential therapeutic applications.

Keywords: mycobacterium; membrane vesicles; iron; secretion; pathogenicity; immunomodulation

INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound nanoparticles released naturally by prokaryotic and eukaryotic cells. They enable cells to secrete a broad range of biomolecules in a concentrated and protected manner. Because of their prevalence and biological functions, EVs have been extensively studied in eukaryotes and Gram-negative bacteria. Mycobacterial extracellular vesicles (MEVs) were first observed a decade ago, embedded in the extracellular matrix of *Mycobacterium ulcerans* biofilms (Marsollier *et al.* 2007) and subsequently isolated from the culture supernatant of several pathogenic and non-pathogenic mycobacteria (Prados-Rosales *et al.* 2011). In recent studies, release of MEVs has emerged as an important mechanism by which *M. tuberculosis* modulates the host immune response (Athman *et al.* 2015, 2017). This review describes the expanding knowledge of MEVs and their role in microbe–host interactions and pathogenicity. We also note the many aspects of MEVs that remain obscure, including the molecular mechanisms of their biogenesis and regulation. Advancement in this field holds the promise of novel discoveries in *M. tuberculosis* pathogenesis and in diagnostic, preventive or therapeutic applications for MEVs in tuberculosis.

The identification of mycobacterial membrane vesicles

MEVs were first visualized by scanning electron microscopy (SEM). They were found embedded in the extracellular matrix of *M. ulcerans* biofilms and recovered from biopsies of Buruli ulcer-like lesions in infected mice (Marsollier *et al.* 2007). These vesicles contained the sole virulence factor responsible for Bureli ulcer, the lipid toxin mycolactone (George *et al.* 1999) and, accordingly, displayed potent cytotoxic activity. Subsequently, MEVs were recovered by differential sedimentation in culture supernatants of diverse *Mycobacterium* species indicating that

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secretion of EVs is ubiquitous in mycobacteria (Prados-Rosales *et al.* 2011). MEVs recovered from culture supernatants were visualized by transmission electron microscopy and observed by SEM protruding from the surface of intact bacteria (Prados-Rosales *et al.* 2011). EVs were also observed in *M. tuberculosis* and *M. bovis* BCG-infected macrophages and mice (Prados-Rosales *et al.* 2011). Recently, EVs of bacterial origin were isolated from the tissue culture medium of *M. tuberculosis*-infected macrophages, indicating that MEVs are released into the extracellular milieu during intracellular infection (Athman *et al.* 2015). However, the molecular mechanisms involved in MEVs trafficking out of the phagosome and through the macrophage plasma membrane are unknown.

Isolation and characterization of MEVs

MEVs are usually recovered from cell-free culture filtrates by ultracentrifugation (Prados-Rosales *et al.* 2014a; Dauros Singorenko *et al.* 2017). However, given the propensity of lipids to organize into vesicles-like structures, it is important to establish that recovered nanoparticles are not byproducts of cell lysis. For instance, it has been shown that phage-infected *Bacillus subtilis* and *Pseudomonas aeruginosa* release shattered membrane fragments and vesicle-like structures as a result of endolysin-induced peptidoglycan damage (Turnbull *et al.* 2016; Toyofuku *et al.* 2017). Thus, a minimal requirement to characterize secreted membrane vesicles is ensuring that they originate from intact cells. EVs can be isolated by density gradient and ultracentrifugation. In this process, due to their high lipid content, EVs fractionate into lighter density fractions than soluble secreted proteins. However, microscopic visualization is required to confirm the presence of intact vesicles in gradient fractions. Commonly used methodologies to detect and quantify membrane vesicles include lipid metabolic labeling, incorporation of lipophilic dyes into protein-rich fractions from density gradients and immunodetection of known vesicle-cargo proteins (Prados-Rosales *et al.* 2011, 2014a,c; Rath *et al.* 2013; Dauros Singorenko *et al.* 2017). The abundance of cellular surface blebs observed in SEM can also reflect differences in the number of vesicles formed in different environmental conditions or by different bacterial strains (Rath *et al.* 2013; Prados-Rosales *et al.* 2014c). These methods are laborious and require special technology. There is a need for more practical, high-resolution methods for identifying and quantifying MEVs that facilitate high-throughput screens for genetic determinants of MEV biogenesis and function, and accelerate new discoveries in MEVs biology.

Biogenesis of MEVs

Naturally produced MEVs are discrete, closed membrane particles produced by growing cells, not products of cell lysis or cell death. By electron microscopy, MEVs appear spherical, enclosed in a bilayer membrane, with electron-dense luminal content and an average diameter of 60 to 300 nm (Prados-Rosales *et al.* 2011). MEVs contain lipids and lipoproteins characteristic of the mycobacterial plasma membrane, indicating their cytoplasmic membrane origin (Prados-Rosales *et al.* 2011, 2014c). Decades of studies of biogenesis of outer membrane-derived vesicles (OMVs) produced by gram-negative bacteria uncovered multiple mechanisms of OMV biogenesis. These have been reviewed in detail in recent publications (Kuehn and Kesty 2005; Pathirana and Kaparakis-Liaskos 2016). Reduced crosslinking between the peptidoglycan and the outer membrane seems to enable formation and detachment of OMVs without compromising membrane stability. Disruptions in peptidoglycan structure due to altered balance between peptidoglycan breakdown and synthesis and accumulation of periplasmic peptidoglycan fragments leads to increased OMV production. Increase of unfolded proteins in the periplasm and enrichment of the membrane with lipopolysaccharide or other membrane curvature inducing molecules also leads to enhanced OMV production (Kuehn and Kesty 2005; Pathirana and Kaparakis-Liaskos 2016). Thus far, the only known general mechanism of OMV biogenesis is accumulation of phospholipids in the outer leaflet of the outer membrane of Gram-negative bacteria (Roier *et al.* 2016). Whether altered phospholipid content in the cytoplasmic membrane modulates MEVs formation remains to be investigated.

While in Gram-negative bacteria OMVs can be 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 is decorated with mycolic acids and intercalating free lipids forming a lipid bilayer refer to as the mycobacterial exomembrane or mycomembrane (Daffe 2015). A capsule composed of polysaccharides, proteins and lipids surrounds the cell wall. For this reason, a great challenge in the field is to define the mechanisms allowing MEVs to traverse the cell wall. This question is also relevant to other thick cell wall microorganisms that release EVs, such as gram-positive bacteria and fungi. Cell wall altering enzymes are included in fungi and *Staphylococcus aureus* EVs (Brown *et al.* 2015; Lee *et al.* 2009) suggesting that remodeling of the cell wall may facilitate EV transit across the cell envelope.

Mycobacterium tuberculosis cultured in conditions of iron limitation abundantly produces EVs, indicating that MEV biogenesis is regulated by iron availability (Prados-Rosales *et al.* 2014c). Subsequent to this discovery, iron-dependent vesiculogenesis was also reported in *Escherichia coli* (Dauros Singorenko *et al.* 2017). Derepression of a phospholipid translocator repressed by the Ferric uptake repressor (Fur) and the consequent accumulation of phospholipids in the outer leaflet of the outer membrane have also been linked to hypervesiculation in iron-limited gram-negative bacteria, providing a molecular mechanism of iron mediated regulation of OMV biogenesis (Roier *et al.* 2016). It is likely that local membrane or cell wall remodeling underlies augmented MEV production in iron-limited mycobacteria. For instance, iron-limited mycobacteria produce large amounts of the lipidic siderophore, mycobactin, which accumulates on the cell surface (Ratledge, Patel and Mundy 1982; Rao *et al.* 2008; Rodriguez and Smith 2003). Interactions of mycobactin at the cell membrane could directly or indirectly stimulate vesicle biogenesis. Indeed, MEVs produced during iron limitation include mycobactin, lending some support to this theory (Prados-Rosales *et al.* 2014c).

An elevated number of vesicles on the surface of a *M. tuberculosis rv0431* mutant was linked to the Toll-like receptor 2 (TLR-2) dependent hyperinflammatory phenotype of this mutant in macrophages and mice (Rath *et al.* 2013). Given the previously recognized enrichment of TLR-2 agonists in MEVs, the *rv0431* gene product is known as the vesiculogenesis and immune response regulator, VirR. VirR is a cytoplasmic protein that interacts with the plasma membrane and at least one lipoprotein that is included in MEVs (Rath *et al.* 2013). It has been proposed 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. The relationship between VirR and iron-dependent regulation of MEV production also remains to be defined. Expression of *virR* is downregulated in response to iron deficiency (Kurthkoti *et al.* 2017). It is therefore possible that reduced VirR contributes to enhanced *M. tuberculosis* vesiculation under iron limitation.

Hypervesiculation was also observed in a sub-population of short cells resulting from asymmetric cell division in logarithmic cultures of *M. smegmatis* and *M. tuberculosis*, indicating a relationship between MEVs production and active cell growth and division processes (Vijay *et al.* 2017).

Molecular composition of MEVs

Bacterial EVs contain a chemically diverse range of cargo that determines vesicle function. Induction of membrane curvature, quantity, stability and sub-cellular localization of a protein has been hypothesized to influence its availability for inclusion in EVs. However, clear knowledge regarding the determinants of EV cargo inclusion or exclusion is still lacking. Global protein composition analysis of EVs produced by *M. tuberculosis*, *M. bovis* BCG and *M. smegmatis* cultured in minimal medium has identified 48, 66, and 64 vesicular proteins, respectively (Prados-Rosales *et al.* 2011). A subsequent study that employed more sensitive mass spectrometry found over 200 additional proteins in MEV (Lee *et al.* 2015). BCG and *M. tuberculosis* EVs were similarly enriched in lipoproteins and proteins belonging to the functional categories of cell wall, membrane function and intermediate metabolism and respiration. Many of the abundant MEV proteins play a role in pathogen–host interactions (Lee *et al.* 2015). BCG EVs contained more proteins classified in the category of lipid metabolism than *M. tuberculosis* and *M. smegmatis* EVs. The poor representation of lipoproteins in *M. smegmatis* 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 *M. tuberculosis* cultured under conditions of iron sufficiency or deficiency was determined by mass spectrometry and showed predominately polar lipids, phosphatidylinositol, acylated phosphatidylinositol dimmannosides, cardiolipin (CL), and phosphatidylethanolamine (PE) in both conditions (Prados-Rosales *et al.* 2011). This was consistent with the plasma membrane origin of the vesicles (Prados-Rosales *et al.* 2011, 2014c). However, EVs from iron-deficient *M. tuberculosis* were also enriched in acylated glycerides and PE, while acyl trehalose, an important mycobacterial cell wall component, was more abundant in iron-sufficient *M. tuberculosis* vesicles. The lipidic siderophore mycobactin was exclusively present in EVs released by iron-limited mycobacteria (Prados-Rosales *et al.* 2014c) showing that the content of EVs in pathogenic mycobacteria is influenced by environmental factors. In addition, lipoarabinomannan (LAM), an important immunologically active glycolipid released by bacilli replicating within macrophages, was also found associated with EVs produced by pathogenic mycobacteria (Prados-Rosales *et al.* 2011), indicating that based on their content MEVs have the potential to influence host immune responses.

Vesicle cargo delivery

It is well recognized that bacterial EVs are capable of long-distance and direct delivery of their cargo into specific host cells. However, despite extensive studies of interactions of OMVs and host cells, the mechanisms of EVs uptake are still not well understood (O'Donoghue and Krachler 2016). Studies of OMVs indicate more than one route of entry that could vary depending on the type of host cell and purity or size distribution of OMVs. Many studies indicate a role for clathrin- and caveolin-mediated endocytosis and lipid rafts in enabling OMVs entry (O'Donoghue and Krachler 2016). In many cases, endocytosis of OMVs is facilitated by interactions between bacterial ligands and host cell receptors (Kesty *et al.* 2004; Chatterjee and Chaudhuri 2011), and despite the different architecture of the membrane bilayers present in OMVs and host cells, membrane fusion has also been described as a mechanism for OMVs to deliver their cargo (Bomberger *et al.* 2009; Jager *et al.* 2015).

In the Gram-positive model microorganism *B. subtilis*, the lipopeptide surfactin secreted by wild strains disrupts EVs and leads to the release of their cargo into the milieu (Brown *et al.* 2014). EVs produced by *M. tuberculosis* and *Salmonella enterica* infecting macrophages escape not just the phagosome but the infected host cells, demonstrating the ability of bacterial EVs to migrate from the cell of origin. *Mycobacterium tuberculosis*-derived EVs released from infected macrophages can subsequently modulate the host cell immune response (Athman *et al.* 2015) and *Salmonella* OMVs can deliver the genotoxin cytolethal distending toxin to neighboring cells (Guidi *et al.* 2013). It has also been demonstrated that *M. tuberculosis* EVs can transfer immunologically active glycolipids such as lipomannan and LAM to T cells (Athman *et al.* 2017), protein antigens to dendritic cells (Jurkoshek *et al.* 2016) and mycobactin-iron to neighboring bacteria (Prados-Rosales *et al.* 2014c), but the mechanisms involved in MEV interactions with host or bacterial cells are unclear.

MEV functions

In addition to proteins, a large variety of other molecules, including phospholipids, nucleic acids, lipopolysaccharides, and periplasmic components, can be found encapsulated in bacterial membrane vesicles (Kuehn and Kesty 2005). Vesicles produced by the pathogenic bacteria have been most extensively studied and shown to generally function as a mechanism to transport biomolecules from the parent bacterium to distal sites in the host, enabling bacterial communication, transfer of virulence effectors and the maintenance of bacterial communities (Kuehn and Kesty 2005; Brown *et al.* 2015; Domingues and Nielsen 2017). OMVs are also known to function in resistance to endogenous and exogenous surface damage agents such as misfolded proteins and membrane-adhered phage particles. They also can carry enzymes and receptors that may aid in nutrient acquisition and bacterial survival (Kuehn and Kesty 2005). OMVs have been associated with inter-bacterial transfer of material that contributes to survival and genetic diversity, including antibiotic-resistance enzymes and chromosomal, plasmid and phage DNA (Kolling and Matthews 1999; Ciofu *et al.* 2000). Analogous to Gram-negative bacteria, Gram-positive pathogens like *S. aureus* use EVs to deliver cytotoxic material to host cells (Gurung *et al.* 2011) and *M. ulcerans* EVs contain the cytotoxin mycolactone (Marsollier *et al.* 2007). Importantly, mycolactone enclosed in EVs is more potent than isolated toxin, supporting a role of *M. ulcerans* EVs in virulence (Marsollier *et al.* 2007).

Recent studies have clearly established a role of MEVs in immunomodulation (Fig. 1). Protein and lipid analysis of MEVs revealed enrichment of immunologically active lipoproteins, lipoglycans and glycolipids known to be TLR-2 ligands (Harding and Boom 2010). Isolated MEVs activate TLR-2 and induce cytokine production by uninfected macrophages (Prados-Rosales *et al.* 2011). It was also found that MEVs containing immunodominant antigenic proteins could transfer antigens to

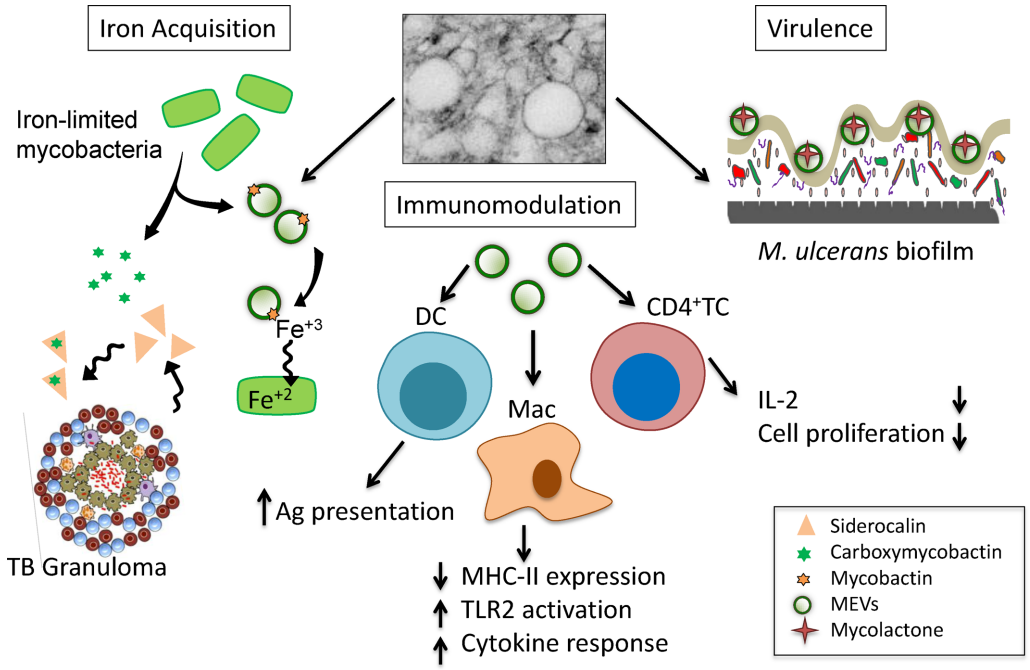

Figure 1. Roles of MEVs in TB pathogenesis: MEVs function in iron acquisition, immunomodulation and virulence. Secretion of mycobactin in MEVs may protect this siderophore from siderocalin and allow *M. tuberculosis* access to Fe available in hydrophobic, distal places. MEVs modulate immune responses in dendritic cells (DC), macrophages (Mac) and T-cells via transfer of microbial antigens, TLR2 agonist and immune active molecules such as LAM. MEVs produced by *M. ulcerans* contain mycolactone and are highly cytotoxic demonstrating a direct role of MEVs in virulence.

dendritic cells for presentation to T-cells (Jurkoshek *et al.* 2016), suggesting that MEVs participate in immune stimulation. It has been demonstrated that EVs released by *M. tuberculosis*-infected macrophages constitute a mixture of macrophage-derived exosomes and MEVs (Athman *et al.* 2015). MEVs isolated from this mix possess the immunostimulatory activity previously assigned to bacterial biomolecules thought to be included into macrophage-released exosomes (Athman *et al.* 2015). These observations suggest that MEVs are the primary means employed by intracellular *M. tuberculosis* to export immunologically active lipoglycans and lipoproteins. Indeed, administration of isolated MEVs to mice induced a TLR-2-dependent proinflammatory response (Prados-Rosales *et al.* 2011). Although activation of TLRs typically promotes immunity, sustained TLR2 signaling by *M. tuberculosis* inhibits MHC-II antigen presentation to CD4+ T cells (Harding and Boom 2010) and is associated with other immune suppressive mechanisms (Richardson *et al.* 2015). Inclusion of lipoglycans such as LAM and LA into *M. tuberculosis* EVs secreted within infected macrophages and their subsequent release into the extracellular milieu has also provided a mechanism for lipoglycans to reach and inhibit T cells, possibly promoting immune evasion (Athman *et al.* 2017). Naive and BCG-vaccinated mice challenged with *M. tuberculosis* aerosols showed acute inflammation and a higher lung bacillary load when they were also injected with BCG EVs, indicating an overall impairment in control of infection (Prados-Rosales *et al.* 2014b). These studies demonstrate that MEVs deliver factors to impair macrophage effector functions, inhibit T cell activation and modify the response of host cells to infection.

Another function of MEVs appears to be iron acquisition (Fig. 1). Iron availability is a critical factor that affects all bacteria living within a host. Because basic cellular metabolic activities require iron, this metal is 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 therefore be able to obtain iron and to adapt their metabolic activity according to iron availability. To obtain iron, *M. tuberculosis* synthesizes and secretes siderophores: carboxymycobactin, an amphiphilic, secreted molecule and mycobactin, a cell surface associated lipophilic molecule (Snow and White 1969; Snow 1970; Gobin *et al.* 1995; Ratledge and Dover 2000). Mycobactin is included in MEVs released by *M. tuberculosis*, and these MEVs can deliver iron and support proliferation of iron-deficient bacteria in a mycobactin-dependent manner (Prados-Rosales *et al.* 2014c). These studies indicate that MEVs may allow *M. tuberculosis* to secrete insoluble mycobactin and obtain iron from distal places. This is analogous to OMVs produced by *P. aeruginosa*, which carry the Pseudomonas quinolone signal (PQS), a highly hydrophobic molecule that binds iron. Using the secreted bridge protein TseF, which interacts with PQS in OMVs and with a siderophore surface receptor, iron-deficient *P. aeruginosa* recruits OMVs for iron acquisition (Lin *et al.* 2017).

MEV-mediated iron capture may be critical for *M. tuberculosis* survival during infection, especially in the context of intense iron deprivation in the granuloma (Basaraba *et al.* 2008; Kurthkoti *et al.* 2017). Immune cells in the granuloma release siderocalin (Kurthkoti *et al.* 2017), which binds carboxymycobactin (Holmes *et al.* 2005), possibly compromising its role in iron acquisition. However, mycobactin in MEVs may be protected from siderocalin. If that is the case, by 'disguising' mycobactin in the EVs, *M. tuberculosis* may be able to overcome the interference of siderocalin with its iron acquisition. Iron homeostasis and antimicrobial response are tightly interconnected in macrophages (Cairo *et al.* 2011). It is possible that MEV-associated mycobactin influences macrophage iron homeostasis to alter immune defence response. The inclusion of mycobactin in MEVs may also be an example of collaborative *M. tuberculosis* interactions, as mycobactin contained in MEVs can benefit both the bacterium

producing the MEVs and neighbouring bacteria (Prados-Rosales *et al.* 2014c).

Possible applications of MEV

Although many aspects of MEV biogenesis and functions are yet to be fully understood, the accumulated knowledge about EVs in Gram-negative bacteria suggests their potential for therapeutic applications. For instance, EVs produced by *Neisseria sp., Salmonella typhimurium, Haemophilus influenzae, Clostridium perfringens* and *Vibrio cholera* elicit humoral and cellular immune responses when administered to mice (Acevedo *et al.* 2014). A membrane vesicle-based vaccine has been recently approved for the prevention of serogroup B meningococcal infection (Gorringe and Pajon 2012; Vernikos and Medini 2014). EVs produced by the Gram-positive pathogen *Streptococcus pneumoniae* have also shown to protect immunized mice against infection with a virulent strain (Olaya-Abril *et al.* 2014). The vaccine potential of subcutaneously injected isolated MEVs produced by BCG and *M. tuberculosis* in culture was tested in a mouse model of tuberculosis (Prados-Rosales *et al.* 2014b). In these studies, only mice immunized with *M. tuberculosis*-derived MEVs showed the capacity to control bacterial replication in the lung similarly to BCG-vaccinated animals. This is in contrast with the impaired control of infection induced by MEVs when injected into mice simultaneously infected with *M. tuberculosis*. This may be explained by heterogeneous content among distinct preparations of purified MEVs in the two studies. Alternatively, MEVs may exhibit distinct immunomodulatory functions when presented to the immune system in isolation or in the context of infection with live bacteria. *Mycobacterium tuberculosis*-derived MEVs were immunogenic, eliciting a mixed antibody and cellular responses directed at lipoproteins and bacterial cell surface components. This study suggested that mycobacterial MEVs may represent a promising vaccine platform. However, heterogeneity in the composition of MEVs produced in culture may lead to variable protective activity. Although the immune response to *M. tuberculosis* MEVs is directed against lipoproteins and bacterial cell surface components, the MEV-associated components responsible for their protection need to be identified so that artificial MEVs incorporating proteins and lipids that induce protective immunity could be generated as a viable platform for vaccine development.

The diagnostic value of MEVs has also been explored. The human antibody response to naturally produced BCG and *M. tuberculosis* EVs was evaluated to identify novel tuberculosis biomarkers in a small cohort of patients, including smear-positive and smear-negative non-HIV infected, tuberculosis patients and BCG-vaccinated individuals with and without latent infection (Ziegenbalg *et al.* 2013). A combination of three MEV-associated antigens was clearly recognized by sera from tuberculosis patients, but not from the control group, encouraging further study of MEV as diagnostic tools.

OUTLOOK

Production of EVs in mycobacteria *in vitro* and *in vivo* has now been clearly demonstrated. These vesicles originate from the plasma membrane and contain 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. It is also important to understand 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 development of ways to exploit MEVs for antitubercular applications.

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