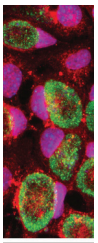

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MINIREVIEW

The carrying pigeons of the cell: exosomes and their role in infectious diseases caused by human pathogens

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This is a thorough review of an important emerging topic in infectious diseases.

Keywords
exosome; infectious diseases; intercellular communication; immune response; pathogenesis; human pathogens.

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Abstract

Exosomes have recently been classified as the newest family members of 'bioactive vesicles' that function to promote intercellular communication. Long ignored and thought to be only a mechanism by which cellular waste is removed, exosomes have garnered a huge amount of interest in recent years as their critical functions in maintaining homeostasis through intercellular communication and also in different types of diseases have been demonstrated. Many groundbreaking studies of exosome functions have been performed in the cancer field and the infectious disease areas of study, revealing the importance and also the fascinating complexity of exosomal packaging, targeting, and functions. Selective packaging of exosomes in response to the type of infection, exosomal modulation of the immune response and host signaling pathways, exosomal regulation of pathogen spread, and effects of exosomes on the degree of pathogenesis have all been well documented. In this review, we provide a synthesis of the current understanding of the role of exosomes during infections caused by human pathogens and discuss the implications of these findings for a better understanding of pathogenic mechanisms and future therapeutic and diagnostic applications.

Introduction

Exosomes are small membrane bound vesicles derived from the late endosome of cells and secreted into the surrounding environment. The term exosome was coined by Johnstone *et al.* (1987) to describe small vesicles being released during reticulocyte development, and later work further postulated that these extracellular vesicles were simply a means of removing cellular waste from the cytosol in maturing reticulocytes (Johnstone *et al.*, 1991). It was not until relatively recently that exosomes were found to play a significant role in cell–cell signaling and implicated to play a role in cancer progression (Luga *et al.*, 2012), HIV particle release (Izquierdo-Useros *et al.*, 2010), host immune responses (Aline *et al.*, 2004; Colino & Snapper, 2007; Giri *et al.*, 2010), and even as carriers of prions (Leblanc *et al.*, 2006). This has led to an explosion in exosome research on a variety of topics and an increased focus on discovering the details of exosome structure and function in a variety of biologic processes.

Exosomes are defined as small extracellular vesicles 50–100 nm in diameter and with a density of 1.23–1.16 g mL^{-1} (Théry *et al.*, 2006). They appear as cup--shaped vesicles by traditional electron microscopy (Luga *et al.*, 2012), although other electron microscopy approaches yield a uniform spherical shape (Conde-Vancells *et al.*, 2008), indicating that the cup-shaped morphology may be an artifact of the imaging process. Exosomes originate from the late endosomes and contain specific populations of mRNA, microRNA (miRNA), lipids, and proteins (Fig. 1). They are released when a multivesicular endosome fuses with the plasma membrane (Harding *et al.*, 1984). Although individual exosomal content may vary based on a variety of factors, there are common exosomal proteins that serve as markers: CD63, Alix, Rab-5, and Lamp-1 amongst others (van Niel *et al.*, 2006; Logozzi *et al.*, 2009). Secreted exosomes can be internalized by recipient cells through endocytosis (Tian *et al.*, 2010) where the release of exosomal contents can trigger a variety of responses in the target cell (Fig. 1). The downstream effects of exosomes are of

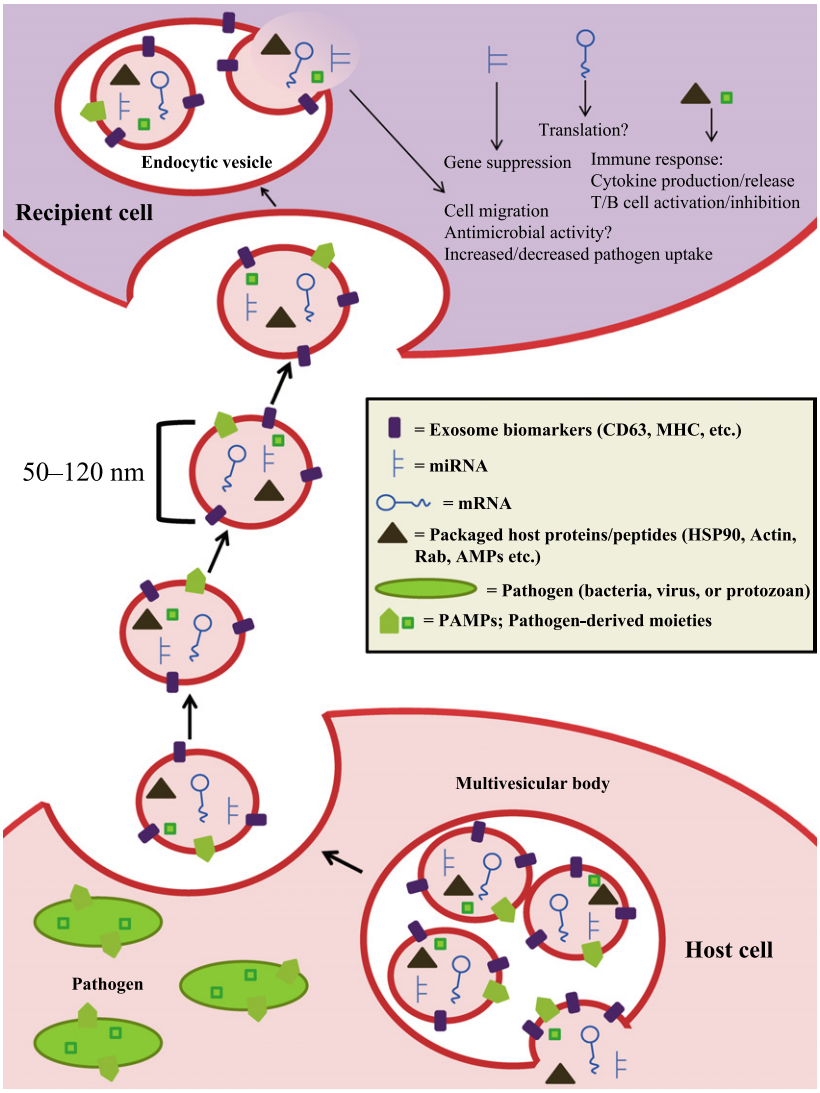

Fig. 1 Diagram of exosome-mediated intercellular communication during infection. A model is presented for exosomal packaging and release, uptake by recipient cells, and known/potential effects of released exosomal contents on recipient cells during the course of pathogenic infection. Exosomes released by infected host cells carry a variety of host cargo molecules, including exosomal biomarkers, miRNA, mRNA, and antimicrobial peptides (AMPs). Pathogen-derived molecules (as indicated in inset) have also been shown to be incorporated into exosomes. Released exosomes are endocytosed by recipient cells for depositing their cargo. Released cargo molecules induce a variety of effects on the recipient cells, which vary depending on the pathogen causing the infection and the recipient cell type. These can include cytokine production, T-/B-cell activation/inhibition, cell migration and increased or decreased pathogen uptake. Additionally, studies have indicated the possibility for antimicrobial activity and translation of packaged mRNAs.

particular interest with respect to their impact on the progression of diseases and have been shown to decrease host susceptibility in some cases (Aline *et al.*, 2004; Colino & Snapper, 2007) but not in others (Coppieters *et al.*, 2009). Some of these observed differences may be rooted in release of distinct subpopulations of exosomes by the cells. Recently, our laboratory has investigated the variation in vesicle subpopulations derived from THP-1 cells infected with *Yersinia pestis*. Briefly, microvesicles (MVs) were fractionated on an OptiPrep (Sigma-Aldrich, St. Louis, MO) gradient, and fractions were tested for the presence of exosomal markers CD63, HSP60, and acetylcholinesterase (AChE) activity. Although there is some overlap between the fractions that test positive for the three markers, each marker clearly identifies a distinct subpopulation of vesicles (Fig. 2). This indication of the existence of exosomal subtypes has clear implications for categorization of exosomes with respect to their functional characteristics and needs further in-depth research as different investigators have used different markers to confirm the presence of exosomes and test for the efficacy of their purification designs. To date, a number of informative review articles on exosomes have been published, such as reviews of the emerging understanding of exosomal biogenesis (Pant *et al.*, 2012), the role of exosomes in cancer (Kharaziha *et al.*, 2012), the role of exosomes as pharmacological effectors (Record *et al.*, 2011), and the general role of exosomes during viral infections (Meckes & Raab-Traub, 2011). In this review, we provide a synthesis of the recent findings on the role of exosomes during the infectious processes of bacterial, viral, and parasitic human pathogens and summarize the current findings on variation of exosomal contents when comparing different infection processes. We also discuss the potential application of exosomes in disease prevention and therapy and suggest future directions of study.

Lessons from cancer studies

Exosomes have proved to be of particular interest in analysis of various types of cancer (Kharaziha *et al.*

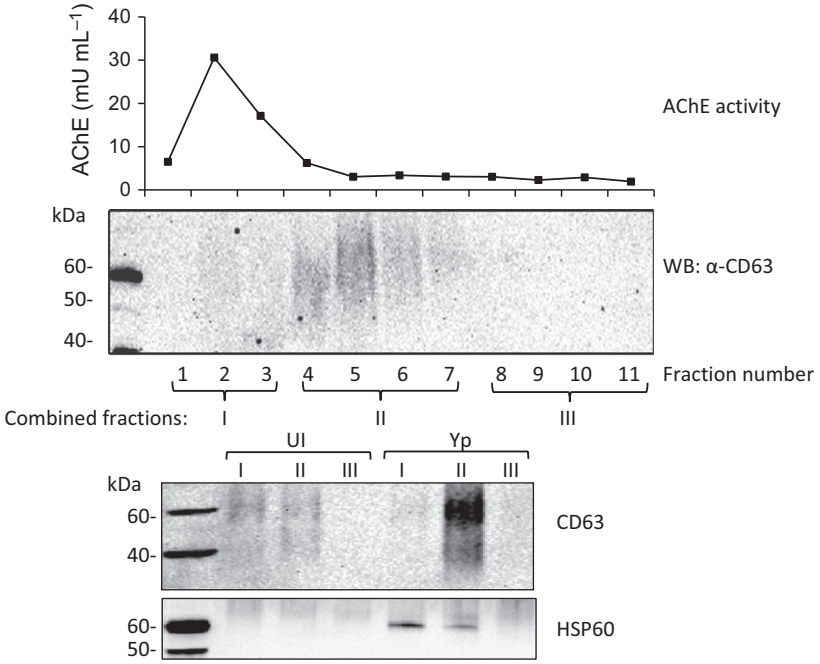

Fig. 2 Characterization of exosomes derived from THP-1 cells infected with *Yersinia pestis* or *Bacillus anthracis*. Exosomes derived from THP-1 cells infected with *Y. pestis* were collected by differential centrifugation and purified by fractionated over a 6–18% OptiPrep gradient (Sigma-Aldrich). Exosome containing fractions were identified by acetylcholinesterase (AChE) activity and by Western blot analysis for the presence of the exosomal protein marker CD63. Exosome marker analysis revealed distinct subpopulations; AChE activity was primarily present in fractions 2 and 3, whereas CD63 was detected in fractions 4–7. Gradient fractions for both uninfected control (UI) and *Y. pestis* infection (Yp) were subsequently combined into three samples designated I, II, and III, and following microvesicle recovery by Exoquick were reprobed for CD63 and the exosomal marker HSP60. The analysis revealed a highly enriched CD63 population in fractions from sample II, whereas HSP60 was more prominently present in sample I, further indicating the presence of distinct exosomal subtypes.

(2012). Studies of the roles of exosomes in cancer can provide important insights and guide the infectious disease research efforts in this arena. From a diagnostic perspective, *in vivo* studies have indicated that the overall exosome load can be indicative of disease progression; circulating exosome titers are increased in patients with cancer and in mice with cancer, and this increase is directly correlated with tumor size (Logozzi *et al.*, 2009). As further exosome research findings for various infectious diseases accumulate, it will be interesting to observe whether exosome load can also serve a similar indicator function in infectious diseases. Peinado *et al.* (2012) identified melanoma-specific signatures in the exosomes produced in patients with cancer, suggesting another potential diagnostic application for exosomes. If similar types of exosomal signatures can be verified for infectious diseases, it will present an interesting and novel possibility of using exosome sampling for infectious disease diagnostics.

Exosomes derived from tumor cells have been implicated in stimulating tumor cell migration (Epple *et al.*, 2012; Luga *et al.*, 2012) and conferring invasion ability to noncancerous cells (Xiao *et al.*, 2012), highlighting the integral role of exosomes in determining the course of disease progression. A mechanism by which cancerous cells can utilize exosomes to enable disease progression is in the preparation of distal tissues for their metastatic colonization. One study demonstrated that exosomes released from melanoma cells are preferentially taken up by sentinel lymph nodes and subsequently, free melanoma cells are recruited to the lymph nodes that have taken up the cancer-derived exosomes (Hood *et al.*, 2011). This exosome-mediated recruitment involves several genes that regulate cellular recruitment, extra-cellular matrix (ECM) modification, and angiogenesis. In subsequent studies, it was further shown that melanoma exosome activation of Met signaling in bone-marrow-derived cells drives them toward an angiogenic and pro-metastatic phenotype requisite for subsequent melanoma colonization of tissues beyond the primary tumor (Peinado *et al.*, 2012). It was demonstrated that a significant portion of the signaling in the recipient cells was caused by the direct transfer of Met from melanoma-derived exosomes. Exosomes may play a parallel role during infectious diseases, regulating the extent and timing of microbial dissemination processes.

Cancer studies of exosomal role have also demonstrated that tumor-derived exosomes can bind to and degrade ECM via integrated proteases, giving tumor cells yet another mechanism by which they can leverage exosomes to enhance motility and invasiveness into distal tissues (Mu *et al.*, 2013). The release of growth factors and cytokines within the ECM leads to increased anti-apoptotic effects and proliferation of the invading tumor cells, yet another benefit of exosomal activity on metastatic spread. In summary,

exosomes released from cancer cells provide a multifaceted tool by which cancer cells can establish a pre-invasive niche at future metastatic sites and facilitate their colonization of secondary tissues. As investigation into the effect of exosomes in infection biology continues, it will be interesting to observe whether many of these same roles frequently appear, such as the priming of uninfected tissues *in vivo* toward increased infectivity or spread of the pathogen.

Pathogen signatures within host-derived exosomes

Multiple databases exist to annotate the proteins and other molecules found in exosomes [EVPedia (Kim *et al.*, 2013), Exocarta (Mathivanan *et al.*, 2012), Vesiclepedia (Kalra *et al.*, 2012)]. For example, EVPedia has catalogued more than 49 000 proteins, 164 000 mRNAs, and 13 000 miRNAs identified in eukaryotic membrane vesicles. In addition, lipids are now being identified and catalogued. The most common proteins identified in eukaryotic-derived exosomes include the well-known exosome marker proteins, including Alix, CD63, CD81, and CD9 (Schorey & Bhatnagar, 2008). Interestingly, 89% of proteomic studies listed in an exocarta-based study identified HSP70 as a constituent in exosomes (Mathivanan *et al.*, 2012) and HSP90, and various forms of annexins are also frequently identified. Despite the growing interest in the molecular signatures of pathogens associated with host-derived exosomes, we have been unable to find a database for annotation of pathogen moieties identified in eukaryotic exosomes and their functional roles during exosome-mediated intercellular communication. Based on a search of the current literature, we have begun to collate such a list in support of researchers in this emerging field (Fig. 3).

Early suggestions of the presentation of pathogen-derived molecules on exosomes came from the work of Beatty *et al.* (2000) who demonstrated that lipid-containing moieties of the mycobacterial cell wall such as lipoarabinomannan and phosphatidyl-myo-inositol mannosides could be identified in the cellular multivesicular bodies (MVBs). Subsequently, proteomic examination of exosomes derived from *Mycobacterium tuberculosis*-infected cells has demonstrated the presence of more than 40 mycobacterial proteins (Giri *et al.*, 2010). Studies have demonstrated that vesicles containing mycobacterial-derived molecules are trafficked to uninfected

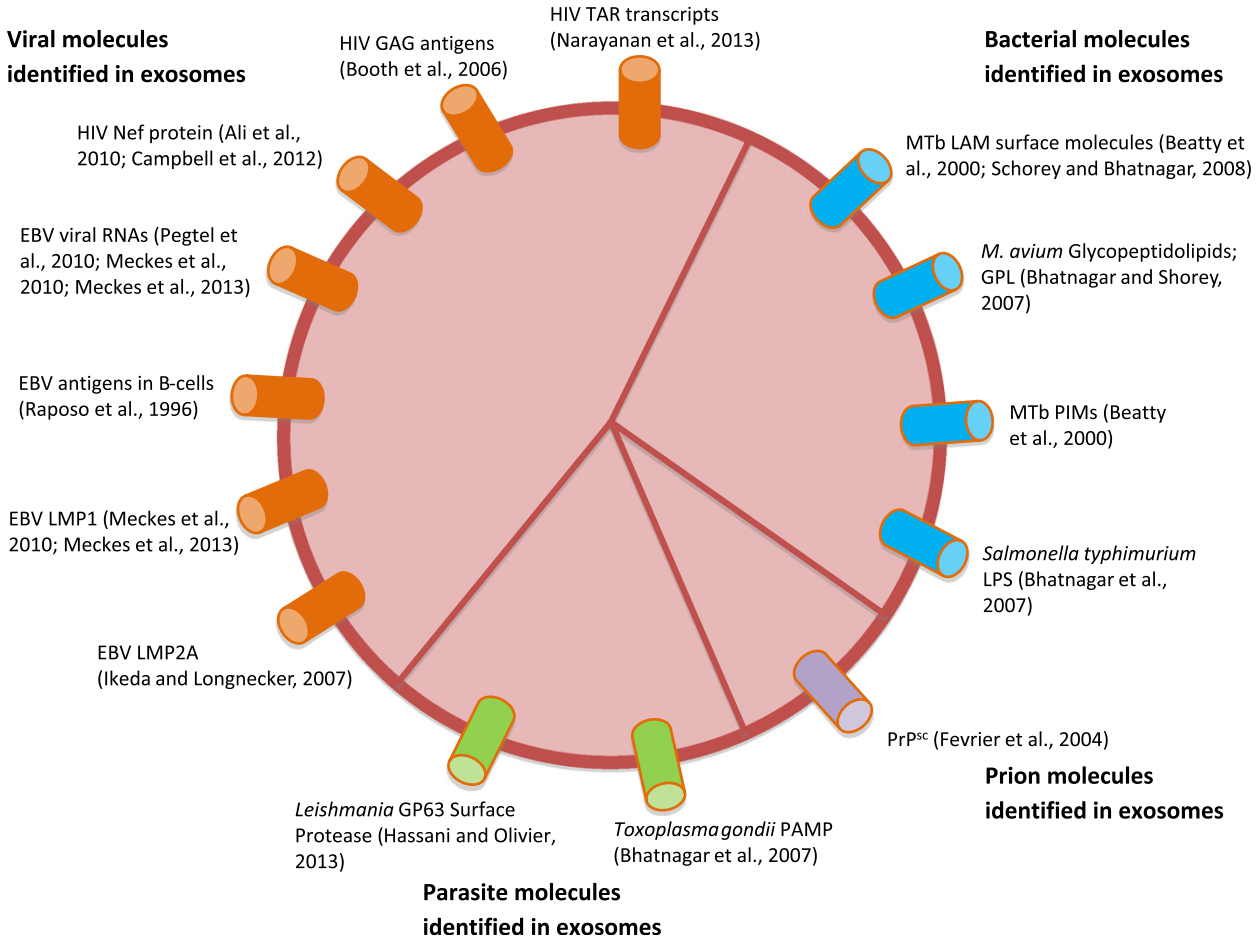

Fig. 3 Pathogen-derived molecules identified in exosomes. A summary diagram of human pathogen moieties reported to be associated with exosomes is provided. In addition to microbial proteins and RNAs, lipid and carbohydrate containing molecules are represented. The pathogen-derived exosome-associated molecules have been grouped and color coded according to the type of pathogen.

bystander cells (Beatty *et al.*, 2000, 2001; Rhoades *et al.*, 2003) and that these pathogen molecule-containing vesicles affect the host cells at a distance and *in vivo* (Rhoades *et al.*, 2003; Bhatnagar *et al.*, 2007). In the context of dendritic cells (DCs), these molecules may function as pathogen associated molecular patterns (PAMPs), and exosomes carrying these PAMPs may act as toll-like receptor (TLR) ligands for uninfected bystander cells (O'Neill & Quah, 2008). In the study of exosomes from cells infected with *Salmonella typhimurium*, it was found that *Salmonella* LPS is present, a bacterial PAMP that is likely responsible for the ability of these exosomes to stimulate a proinflammatory response in uninfected cells (Bhatnagar *et al.*, 2007). Another interesting and illuminating finding has been made for pneumococcal infections. A glycoconjugate that is cross-reactive with the capsular polysaccharide of *Streptococcus pneumoniae* type 14 has been identified on exosomes derived from DCs, and it has been demonstrated that this cross-reactive antigen induces protective responses against pneumococcal infection *in vivo* (Colino & Snapper, 2007). In other kingdoms, parasites such as *Toxoplasma gondii* have also been demonstrated to contribute PAMPs to exosomes (Bhatnagar *et al.*, 2007), and exosome-associated *Leishmania* GP63 surface protease has also been identified (Hassani & Olivier, 2013). Together, these studies illustrate the diversity of pathogen molecules associated with host-derived exosomes and point to their functional importance during infection.

Bacterial human pathogens

Exosomal content

As discussed above, both pathogen-derived and host-derived molecules are packaged within exosomes, although the mechanisms and specificity of the packaging remain largely obscure. Many studies are being published regarding the nature of the host mRNA and miRNA within exosomes derived from virally infected or cancer cells, and on the potential biologic effects of exosomal RNA, including transfer of functions of exosomal mRNAs and miRNAs between cells (Valadi *et al.*, 2007). We have undertaken a study of exosomal miRNA content and function for bacterial pathogens. Remarkably, in our studies of the miRNA populations within exosomes derived from THP-1 cells infected with either *Y. pestis* or *Bacillus anthracis*, we have found that not only the relative amounts of specific host miRNAs are different compared with exosomes from uninfected cells, but that there is also differential specificity of packaging that is dictated by the type of infection (Fig. 4). Thus, while some exosomal miRNAs show similar profiles between the two infections, many miRNAs with altered abundance in exosomes derived from *Y. pestis* infected cells do not show the same profile for *B. anthracis* infection (Fig. 4). The alteration of miRNAs in exosomes derived from bacterially infected host cells is currently uncharacterized and remains to be undertaken in a comprehensive manner.

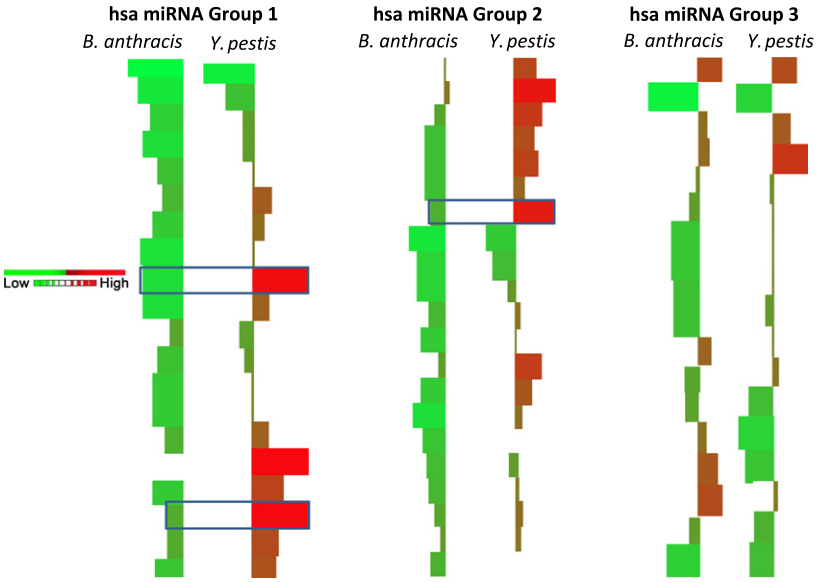

Fig. 4 Changes in the relative abundance of exosomal miRNA population in response to infection. Exosomes were derived from both uninfected THP-1 cells and THP-1 cells infected with either *Bacillus anthracis* or *Yersinia pestis*. The exosomal miRNA cargo was purified and analyzed using Human miRNA Array III and Human miRNA Array IV from Signosis (Santa Clara, CA). Each colored column represents an individual exosomal miRNA, and both the color intensity and heights of the columns are indicative of the degree of relative change in miRNA levels compared with the exosomes obtained from uninfected control cells. Red color signifies miRNA increase in exosomes derived from infected cells relative to the uninfected control, while green color signifies miRNA decrease. Groupings were performed to present observed changes in the levels of the same miRNAs for both *Y. pestis* and *B. anthracis* infections. The miRNA profiles for the two pathogens were distinct from each other and in many instances showed differential packaging between *Y. pestis* and *B. anthracis* infections (examples designated by blue boxes). Blank spaces indicate the absence of detectable difference compared with uninfected control.

With respect to the analysis of host or pathogen moieties packaged into exosomes derived from bacterially infected cells, one well-studied example is the *M. tuberculosis* (MTb) infection. For instance, exosomes from MTb-infected macrophages have been shown to contain major histocompatibility complex (MHC) class II molecules that can function in antigen presentation (Ramachandra *et al.*, 2010). However, no proteomic analysis of altered host protein incorporation within the exosomes from MTb-infected macrophages has yet been published. The first report of this type of analysis has been provided by Hassani & Olivier (2013) and illustrates that exosomes derived from J774 murine macrophages undergo significant proteomic changes when the cells are stimulated with bacterial LPS. Also, the LPS effect was compared with the effect of exposing the cells to *Leishmania mexicana* promastigotes. The analysis found 137 proteins in exosomes from untreated cells, 173 proteins in exosomes from LPS-treated cells, and 200 proteins in exosomes from cells exposed to *Leishmania*. While a high percentage of the discovered proteins were in common between the three samples (a total of 107 proteins), their abundance varied between the LPS and the *Leishmania* treatments. Furthermore, 19 proteins unique to the untreated sample, 18 proteins unique to the LPS-treated sample, and 44 proteins unique to the sample for *Leishmania* exposure were identified, with 11 proteins shared only between the untreated and the LPS-treated sample and 37 proteins shared only between the LPS-treated and the *Leishmania* exposed samples (Hassani & Olivier, 2013). When comparing the untreated and LPS-treated samples, significant differences were found in exosomal proteins associated with different molecular functions, biologic processes, and cellular compartments, indicating that signaling by bacterial PAMPs such as LPS can significantly alter the incorporation of host proteins into exosomes (Hassani & Olivier, 2013). Another example of proteomic profiling of exosomes from bacterially infected cells is for pro-coagulant MVs that are released in increased numbers in response to *Streptococcus pyogenes* infection. This study showed a dramatic change in the protein composition of pro-coagulant exosomes released from human peripheral blood mononuclear cells (PBMCs) compared with control exosomes, including a significant up-regulation of fibrinogen-binding integrins CD18 and CD11b and the enrichment of antimicrobial functions such as lysozyme and neutrophil defensin 1 in pro-coagulant exosomes (Oehmcke *et al.*, 2013).

Host response and effects on pathogenesis

In response to bacterial infection, exosomes derived from cells infected with *Mycobacterium* species, *S. typhimurium*, *S. pneumoniae* and from cells exposed to diphtheria toxoid have all been shown to induce proinflammatory responses (Colino & Snapper, 2006, 2007; Bhatnagar & Schorey, 2007; Bhatnagar *et al.*, 2007; Singh *et al.*, 2012). Further, exosomes from cells infected with bacteria have been shown to induce antigen-specific T-cell activation (Giri *et al.*, 2010), B-cell activation (Quah & O'Neill, 2007), and macrophage chemotaxis (Singh *et al.*, 2012). In addition to cytokine production and immune cell activation, mice injected with infection-derived exosomes have been shown to have an increase in immunoglobulin (Ig) titer and to have developed protective immunity from infection against *S. pneumoniae* (Colino & Snapper, 2007). However, Singh *et al.* (2011) showed that while exosomes derived from *M. tuberculosis*-infected cells do not interfere with cytokine production, they can inhibit cellular response to cytokine stimulation, including inhibition of MHC II expression and inhibition of macrophage activation. Yang *et al.* (2012) recently showed that exosomes derived from tumor cells infected with *Mycoplasma* simultaneously induce B-cell activation and inhibit T-cell responses. The potential for infection-derived exosomes to both induce expression of immune-stimulating cytokines and reduce the effectiveness of the cytokines on target cells suggests a much more complex picture for the role of exosomes in bacterial infection than has been investigated thus far and implies that the specific roles of exosomes may be tailored according to the type of bacterial infection.

Cell signaling and gene activation events are also modulated by exosomes. Once within the target cells, exosomes derived from bacterially infected cells can activate MAPK signaling pathways and NFκB signaling pathways through TLR interactions (Bhatnagar *et al.*, 2007). These two pathways play key roles in the regulation of a number of immune response genes and are thus inherently of interest in any infection process. The study by Singh *et al.* (2011) has shown that exosomes derived from *M. tuberculosis*-infected cells down-regulate immune genes normally activated by IFN-γ, such as nitric oxide synthase, PGE2 synthase, and HSP70. Interestingly, they also showed that the exosomes derived from infected cells affected a largely different subset of genes compared to infection with the bacterium itself or IFN- γ activation alone, demonstrating that exosomes play a distinct role in affecting gene regulation apart from the effects of cytokines or bacterial cells. Collectively, these studies point to a multifaceted functional role for exosomes during bacterial infection, including the ability to induce cytokine secretion and to modulate host cell signaling and gene expression at both a transcriptional and translational level. This variety of functional modes is not surprising given the complex nature of exosomes (Fig. 1) and reveals a wide potential for the effect of exosome secretion on infection outcomes.

Viral human pathogens

Exosomal content

While still a relatively new field of study, experiments performed in regard to the effect of viral infections on host exosomes have begun to delineate the alterations of exosomal composition and determine the biochemical mechanisms driving variation in exosomal content. A recent proteomic analysis of B cells infected with either Kaposi's sarcoma herpes virus (KSHV), Epstein–Barr Virus (EBV), or a dual infection showed major changes to the exosomal protein composition as compared to exosomes from the uninfected parental cell line (Meckes *et al.*, 2013). Specif-

ically, a total of 345 proteins were identified by mass spectrometry to be uniquely incorporated into the exosomes released from the infected cells. Additionally, the up-regulation of exosomal proteins in EBV-infected cells was correlated with the incorporation of the viral latent membrane protein 1 (LMP1). Many of the cellular pathways affected by the unique exosomal proteins derived from either EBV or KSHV infection differed, indicating divergent functionalities of the altered exosomes. Specifically, the pathways affected by the KSHV exosomal proteins were primarily related to metabolism, protein translation, and cellular migration, while those pathways affected by EBV up-regulation of exosomal proteins affected interferon and NF-κB signaling, membrane and protein trafficking, lipid raft organization, and cellular-vesicle binding. These observations are reminiscent of the exosome discoveries for bacterial pathogens, suggesting that the functional roles of exosomes are tailored by the type of viral infection.

While it is important to identify which proteins are selectively incorporated into exosomes of virally infected cells, it is also imperative to understand how these proteins are packaged. The human immunodeficiency virus (HIV) viral protein Negative factor (Nef), which has been found to be packaged and secreted in exosomes from infected cells, was shown to contain several conserved N-terminal motifs that are required for its exosomal secretion (Ali *et al.*, 2010; Campbell *et al.*, 2012). Use of a peptide mimetic for one of these novel Nef domains, termed the secretion modification region, disrupted the interaction of Nef with the cellular protein mortalin, thereby blocking exosomal incorporation of Nef, as well as virion budding (Shelton *et al.*, 2012). This dual inhibition also indicates a shared cellular protein trafficking mechanism between exosome packaging and viral production. The novel secretion motifs identified within Nef can be used to screen other exosome incorporated proteins for homologous structures to determine whether this is a common mechanism for exosome trafficking.

Another mechanism of exosome incorporation has been studied with the EBV viral protein LMP2A, which has been shown to be integrated into exosomes and secreted from infected cells (Ikeda & Longnecker, 2007). Mechanistically, it was found that cholesterol depletion from the cellular membranes using the drug methyl-beta-cyclodextrin (MCD), which disrupts lipid rafts, caused significant increases in overall cellular LMP2A as well as exosome-integrated LMP2A. It was further demonstrated that the MCD treatment caused marked decreases in both phosphorylation and ubiquitination of LMP2A in cellular lysates. However, for the exosome-integrated LMP2A, only phosphorylation was inhibited while the level of protein ubiquitination remained high. This indicates that the ubiquitin modification of LMP2A may be a key mechanism for sorting of this protein into secreted exosomes, while phosphorylation may signal retention within the cell. Based on this experimental evidence, the ubiquitination and phosphorylation states of selectively incorporated exosomal proteins should be examined as a potential mechanism for cellular sorting into the secreted vesicles.

Beyond proteins, varied RNA compositions have also been identified in exosomes released from virally infected cells. Exosomes from EBV-infected B cells were tested and shown to contain viral RNAs, with an enrichment of smaller 15–40 nt RNA molecules (Pegtel *et al.*, 2010). Furthermore, exosomes from EBV-infected cells contained a relative abundance of mature EBV miRNAs. More importantly, transfer of these mature EBV miRNAs into co-cultured uninfected monocyte-derived DCs was shown to down-regulate luciferase reporter genes fused with the miRNAs target 3′ UTR sequences. Non-B cells were also shown to have significant levels of mature EBV miRNAs in 60% of asymptomatic HIV-EBV co-infection patients who had elevated EBV loads. In a separate study, it was demonstrated that several EBV viral miRNAs were selectively enriched in the exosomes of infected cells at up to fourfold higher concentrations as compared to intracellular levels and that the viral miRNAs could successfully transfer into and function within uninfected recipient cells to regulate their target genes (Meckes *et al.*, 2010).

In addition to large gamma herpes viruses altering RNA content, HIV-1 infections have also been found to vary the RNA molecules packaged within exosomes. Our laboratory recently reported that the nascent viral trans-activating response (TAR) transcripts from the integrated provirus are incorporated within exosomes from HIV-1-infected cell lines (Narayanan *et al.*, 2013). The TAR RNAs were found in exosomes from the serum of patients on highly active antiretroviral treatment, as well as from long-term nonprogressors. The TAR RNAs were not found to be associated with the RNA interference (RNAi) component protein Argonaute 2 (Ago2) although the miRNA biogenesis proteins Drosha and Dicer were found to be incorporated at elevated levels in the exosomes secreted from HIV-1-infected cells. Our laboratory has recently found that in contrast to HIV-1-infected cell lines, exosomes from cell lines infected with the retrovirus human T-lymphotropic virus 1 (HTLV-1) do not carry detectable viral RNAs although they do contain viral proteins such as Tax (Jaworski J, Narayanan N, Duyne RV, Iordanskiy I, Saifuddin MS, Das R, Afonso RV, Sampey GC, Chung M, Popratiloff A, Shrestha S, Vertes A, Mahieux R, Kashanchi F). Additionally, the composition of RNAi proteins varies between HIV-1 and HTLV-1 manipulated exosomes, with HTLV-1 exosomes containing Ago2 along with cellular miRNAs but integrating only limited amounts of Drosha and Dicer (unpublished data). The increased levels of Ago2 in the presence of cellular miRNAs suggests that, unlike HIV-1 altered exosomes, HTLV-1 manipulated exosomes can rapidly control mRNA translation in recipient cells upon exosome uptake. Although the specific mechanisms for the exosomal enrichment of select viral and host proteins or RNAs has yet to be elucidated, the data demonstrate that viruses can influence host cells by commandeering the cellular exosome secretion machinery.

Host response and effects on pathogenesis

The altered composition of exosomes from virally infected cells confers numerous novel functionalities such as immunomodulation, enhanced infectivity, and induced pathogenesis. Several studies have demonstrated immunomodulation

as the primary cellular process impacted by viral-induced changes to exosomes. An initial study demonstrated that LMP1-enriched exosomes produced during EBV infection also contained increased levels of galectin 9 and that both proteins contributed to reduced proliferation of recipient peripheral T cells, with the immunosuppressive functionality of the exosomes primarily attributable to LMP1 itself (Keryer-Bibens *et al.*, 2006). A subsequent study of LMP1-negative exosomes derived from a different EBV-infected nasopharyngeal carcinoma (NPC) cell line showed that galectin 9 significantly increased apoptosis in EBV-reactive cytotoxic CD4+ T cells isolated from healthy EBV carriers (Klibi *et al.*, 2009). These two mechanisms of action by exosomal LMP1 and galectin 9 may indicate a multifaceted immunosuppressive defense orchestrated by EBV.

Another complex DNA virus that has been shown to alter the immune response via exosome manipulation is the herpes simplex virus 1 (HSV-1). Specifically, the HSV-1 glycoprotein B (gB) has been shown to associate with the MHCII surface receptor human leukocyte antigen DR (HLA-DR) and alter its trafficking through the secretory pathway (Neumann *et al.*, 2003; Temme *et al.*, 2010). The association of gB with HLA-DR in a stably transfected gB melanoma cell line was demonstrated to occur in post-Golgi membrane compartments where antigen (Ag) loading occurs (Temme *et al.*, 2010). Furthermore, gB prevented Ag peptide loading into the HLA-DR complex, as well as expression of HLA-DR on the plasma membrane. In contrast to the normal cell surface expression of HLA-DR in melanoma cells, in the presence of gB protein, HLA-DR was alternately trafficked through the exosomal secretory pathway and released from the cells in exosomes. This altered antigen loading and vesicular trafficking of HLA-DR represents a novel mechanism by which HSV-1 can evade activation of CD4+ T-helper cells and attenuate the adaptive immune response to the virus.

While most of the immunomodulation studies involving viral alteration of exosomes have been focused on changes beneficial to the virus, virus induced changes in exosomes that can benefit the host immune system have also been observed. In one study, hepatitis C virus (HCV) RNA packaged into exosomes and released from an HCV-infected hepatocarcinoma cell line was found to activate co-cultured plasmacytoid DCs (pDCs), thereby up-regulating interferon α (IFN-α) production (Dreux *et al.*, 2012). Furthermore, the sphingomyelinase inhibitors GW4869 and spiroepoxide, which inhibit exosome release, drastically reduced the levels of IFN-α secreted by the co-cultured pDCs, indicating the necessity of exosomal release for pDC activation. This study demonstrates a mechanism by which virally infected cells can take advantage of exosomes as part of a defense strategy, activating the innate immune response by transferring viral RNA to responsive immune cells.

In addition to altering immune surveillance, exosomes released from virally infected cells have been shown to assist in viral spread. In one study, exosomes from HCV-infected hepatoma cells were shown to carry virions in addition to viral RNA and proteins (Ramakrishnaiah *et al.*, 2013). Incubation of these exosomes could establish fully productive infection in uninfected recipient hepatoma cells, and the exosome-mediated infection was partially resistant to HCV-neutralizing antibodies, demonstrating a mechanism for evading the humoral immune response. This packaging of fully infectious material into exosomes therefore served two primary purposes: increasing viral spread and avoiding immune clearance. In another example of enhanced infectivity, HIV-1-infected monocyte-derived macrophages (MDMs) were shown to increase the number of exosomes and MVs secreted from the host cells, with some virions shed from the infected MDMs associated with aggregates of secreted exosomes (Kadiu *et al.*, 2012). The secreted exosomes were also shown to contain cytokines that induce cellular migration and the release of pro-inflammatory cytokines that enhance HIV-1 infectivity. Compared with purified virus, the HIV-1 virions entrapped within aggregates of exosomes showed enhanced infectivity toward CD4+ target cells, demonstrating a beneficial role of these aggregates for the virus. Similar to these findings, our laboratory has shown that pre-incubation of recipient cells with exosomes from chronically infected T-cell lines enhanced the infectivity of subsequent HIV-1 exposure (Narayanan *et al.*, 2013). Overall, studies of exosomes secreted from HIV-1-infected cells demonstrate a mechanism by which uninfected cells could become more susceptible to subsequent exposure to the virus, thereby enhancing the overall spread of infectivity within the host.

Beyond eliciting immunomodulation and enhancing infectivity, the alteration of exosomes by viral infection also contributes to the virus-associated pathogenesis. One aforementioned study on EBV-altered exosomes showed that LMP1-positive exosomes also boosted the incorporation of epidermal growth factor receptor (EGFR) into exosomes (Meckes *et al.*, 2010). Functionally, exposure of bystander cells to the LMP1 and EGFR-enriched exosomes leads to the cellular uptake of these vesicles and causes activation of the Erk1/2 and Akt1 signaling cascades in the recipient human umbilical vein epithelial cells. Activation of these pathways is indicative of cellular proliferation and, therefore, could enhance tumor growth associated with EBV infection. Beyond direct viral alterations of the protein or RNA composition of exosomes, an indirect mechanism of action involving exosomes has also been identified by which the virus may influence uninfected bystander cells. One example has been shown in relation to the activity of the HIV-transactivating protein Tat, which is toxic to human neurons and therefore a significant contributor to HIV-associated neurological disorders. The study showed that astrocytes exposed to a combination of Tat and opiate drugs, which potentiate Tat neurotoxicity, secreted exosomes with elevated levels of miR-29b (Hu *et al.*, 2012). When human neurons were subsequently exposed to the miR-29b-fortified exosomes, the target gene, platelet-derived growth factor-B (PDGF-B), was repressed and neuronal viability was decreased. These results demonstrate the potential for indirect exosomal-mediated neurotoxicity associated with a retroviral infection, obviating the necessity of direct viral infection to induce the associated pathology. As a related concept, based on published studies, the 'Trojan Horse'

hypothesis has been proposed for indirect modes of retroviral infection through exosomes (Gould *et al.*, 2003), stating that the pre-existing exosome biogenesis pathway can be manipulated for the formation of infectious particles that allow an independent mode of infection through the regular exosomal uptake machinery.

While most of the exosome studies to date have focused on a relatively few viruses, the insights gained from these findings, as well as inferences from the more established paradigms in the cancer field, open numerous avenues for future experimentation and novel scientific discovery in this field. Future findings regarding viral manipulation of host exosomes may clarify several questions in relation to viral life cycles and pathogenesis, yielding potential novel strategies for therapeutic intervention.

Eukaryotic parasites

Host response and effects on pathogenesis

The subject of exosome release in response to eukaryotic parasitic infection is a fascinating one, as exosomes or exosome-like vesicles have been shown to be released by the eukaryotic parasites themselves as a part of their pathogenicity (Torrecilhas *et al.*, 2012). For the purposes of this review, we will focus only on host-derived exosomes released in response to parasitic eukaryotic infections. Host exosome release has been studied for infections caused by *T. gondii* (Aline *et al.*, 2004; Beauvillain *et al.*, 2009), *Cryptospiridium parvum* (Hu *et al.*, 2013), and *Leishmania major* (Schnitzer *et al.*, 2010). The conference of protective immunity against these three pathogens has been observed when pretreating cells, or injecting mice, with exosomes derived from antigen-pulsed DCs. Exosomes derived from DCs pulsed with *T. gondii* antigens induced robust cytokine production when injected into mice and protected the mice from infection (Beauvillain *et al.*, 2007), although in the case of *T. gondii,* a decrease in the levels of IL-10 and IL-5 has also been observed, which are associated with anti-inflammatory response and eosinophil activation, respectively (Pestka *et al.*, 2004). Exosomes derived from cells infected with *T. gondii* have also been shown to increase antiparasite IgG levels in the circulating blood of mice (Aline *et al.*, 2004; Beauvillain *et al.*, 2009); parenthetically, increased IgG and IgA responses to poultry parasitic pathogens from the *Eimeria* genus have also been observed in chickens immunized with exosomes derived from *Eimeria* parasite antigen-loaded DCs (del Cacho *et al.*, 2012). An intriguing aspect of exosomal function has been demonstrated in a study of *C. parvum* infection by Hu *et al.* (2013). They demonstrated that exosomes derived from infected cells have direct anti-*C. parvum* activity after binding to the surface of *C. parvum* cells in a lectin-mediated manner and sporozoites exposed to these exosomes exhibited decreased infectivity. In addition, they showed that exosomes released from infected cells carry the miR-98 miRNA and the antimicrobial peptides LL-37 and HBD2. miR-98 induces increased exosome release through suppression of SNAP23, which plays an important role in innate immune defense through TLR-4 activation (Chen *et al.*, 2005). Overall, the immune response modulation by exosomes released from cells infected with eukaryotic parasites is one in favor of the host; cytokines are induced, protective immunity occurs, and B-cell responses are increased. The capacity of exosomes to act directly upon the parasites as shown by Hu *et al.* (2013) is quite intriguing and at the moment seems to be unique to eukaryotic pathogens. It also adds a layer of complexity to consider when analyzing the effects of exosomes on the progression of eukaryotic infection.

The question of how released or injected exosomes find their targets within an organism has been examined in cancer studies as well as in studies of protozoan pathogens. Indeed, two of the aforementioned studies on *T. gondii* infection have addressed this issue by tracking labeled exosomes after injection into mice (Aline *et al.*, 2004; Beauvillain *et al.*, 2007). Both studies found that a majority of DC-derived exosomes were transferred to the spleen following intravenous injection into mice, which is consistent with the role of spleen in filtering the blood and serving as an immune organ (Swirski *et al.*, 2009). Aline *et al.* (2004) utilized exosomes derived from *T. gondii*-pulsed cells, whereas Beauvillain *et al.* utilized exosomes from uninfected DCs. Both studies found that in addition to homing to the spleen, the DC exosomes also had an initial high presence in the intestine of the mice before decreasing in levels over time, which is significant given the role of intestine in the progression of *T. gondii* infection. The increased duration of intestinal exosomes derived from infected cells provides an interesting notion of targeted exosomal delivery to tissues relevant to the course of infection. However, more rigorous study is needed, and indeed, the topic of exosomal homing is of great interest and a focus of the ongoing research in the exosome field (Lakhal & Wood, 2011).

Disease prevention and therapeutic approaches

In contrast to the induction of protective immunity provided by exosomes from cells infected with bacteria or *T. gondii*, in several cases, exosomes derived from virally infected cells either played no significant role in infection (Coppieters *et al.*, 2009) or had the opposite effect, enhancing viral infectivity and cell–cell transmission (Kadiu *et al.*, 2012; Narayanan *et al.*, 2013). Thus, the potential for direct application of exosomes from infected cells as a general prophylactic strategy against infection seems most promising for bacterial and eukaryotic infections, although future exosome research is needed to analyze the effects for additional pathogens and for advances toward reaching the stage of clinical trials. With regard to the role of exosomes in the progression of viral infections, interference with the capacity for exosome secretion or the ability of the virus to influence packaging of exosomes appears to be the most promising avenues for exosome-centered treatments. More in-depth research into the potential of using exosomes as therapeutic tools has been conducted in the cancer field, with exosome treatment of patients with advanced nonsmall cell lung cancer showing promise in phase I trials (Morse

et al., 2005). Further research in the direct use of exosomes as therapy for infectious diseases should look to such studies as models for the development of treatment practices and appropriate protocols for isolation of exosomes for clinical purposes.

Exosome-based treatments need not be limited to simply transferring exosomes derived from infected cells into patients. Xiu *et al.* (2007) tethered a staphylococcal superantigen to the surface of exosomes and found that the tethered exosomes decreased tumor growth and increased survival rates in mice, demonstrating that engineered exosomes may also serve as effective tools for therapy. Furthermore, modification of the contents of exosomes produced by cells has also shown promise in increasing the antitumor efficacy of exosomes (Yang *et al.*, 2007). In this study, it was found that IL-2 genetic modification of tumor cells leads to the production of IL-2 containing exosomes that have enhanced antitumor effects. This observation suggests the possibility of using transgenic cells to generate custom-packaged, stable, and effective exosomes to treat infectious diseases. While clinical treatment possibilities are only now beginning to be explored (Morse *et al.*, 2005), as the understanding of the role of exosomes in the infection process evolves so too will the possible avenues of utilizing exosomes to combat infection, either through interference, modulation, or vaccination.

Concluding remarks and future directions

The examination of the role of exosomes in infectious diseases is a burgeoning field, and while important progress has been made, much remains that requires investigation. One important aspect is developing a deeper understanding and specific definitions of the exosomal subtypes and how they differ with respect to content, general characteristics, and functional capabilities. In this regard, the development of efficient methods for purification and characterization of different subtypes is important and will provide some of the necessary tools. A great deal also remains to be studied with regard to the different aspects of the varied exosomal functions. While many studies have identified an observable differential response of recipient cells to infection-derived exosomes, few have studied exosome homing mechanisms and the mechanisms that facilitate recipient cell responses. More in-depth studies of exosome packaging, release and uptake are also needed, especially considering the variation in observable host cell response to exosomes arising from different types of infection.

Another area of future exploration that holds much promise is the use of exosomes as novel diagnostic tools for infectious diseases. Potential venues are provided by the disparity that exists in the projected role for exosomes in bacterial and parasitic infections compared with viral infections, as well as variations in exosomal subtypes and the response of host cells to different pathogens. In addition, exosome-associated biomarkers specific for infectious agents have been identified from a variety of bodily fluids (Bhatnagar & Schorey, 2007; Simpson *et al.*, 2009), further raising the possibility of developing novel and rapid exosome-based diagnostics.

Potential roles of host exosomes in response to many other pathogens also remain to be investigated and should yield further wealth of invaluable knowledge. Remarkably, beyond the infectious diseases covered here, exosomes have also been shown to carry the prion protein scrapie (PrPsc) and transport the infectious protein to recipient cells (Fevrier *et al.*, 2004; Fig. 3), further emphasizing the relevance of host exosomes regardless of the origin of the disease. We anticipate that the fascinating field of exosome research will continue to remain extremely active and viable in the foreseeable future, with the next several years witnessing exciting progress and significant new breakthroughs.

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