Pathogens, Parasites Exploit Exosomes as Delivery Vehicles

Viruses, parasites, and fungal pathogens use exosomes as “virulence bags” for delivering factors to harm host cells and suppress immune responses

Shannon Weiman

Exosomes, 30- to 100-nm vesicles that eukaryotic cells release, serve as carriers of critical messenger packets between cells. Under ordinary circumstances, exosomes cart proteins, mRNA, and microRNA (miRNA) molecules from one healthy cell to another in paracrine fashion, modifying signal transduction cascades and epigenetic programming in recipient cells.

During the past few years, researchers began to realize that some pathogens and parasites take advantage of host exosomes, sometimes using them to convey virulence factors and undertake other activities that promote the growth and survival of pathogens at the expense of their hosts. Various types of microorganisms, including viruses, protozoan parasites, and fungi, can exploit host exosomes, according to researchers who described different ways in which specific microbes commandeer exosomes during the symposium, “Virulence Bags: Extracellular Vesicles that Modify Host-Pathogen Interactions,” convened as part of the 2012 Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), held in San Francisco, Calif.

Finding pathogen-derived, virulence factor-carrying exosomes opens another chapter into how pathogens and parasites manipulate their hosts. Exosomes provide a means for delivering a wide array of molecules—signal transducing proteins, immune-modulating polysaccharides and lipids, and gene-silencing miRNAs—whose effects might be synergistic. Importantly, within those exosomes, the contents are protected against proteolytic cleavage during transit, allowing for safe delivery of both soluble and membrane-bound factors to host cells. Hence, exosomes derived from a single cell type can have far-reaching effects throughout the host. Developing a fuller understanding of this delivery mechanism may guide the development of novel strategies to combat viral, parasitic, and fungal diseases, including those that resist efforts to develop new drugs and vaccines.

Latent Epstein-Barr Virus Harnesses Host Exosomes

The Epstein Barr Virus (EBV), also called human herpesvirus 4, latently infects about 90% of the human population. While latent, the viral genome does not replicate, and the main part of its genome remains silent, according to Nancy Raab-Traub of the University of North Carolina, Chapel Hill. However, genes encoding oncogenic proteins and miRNAs are expressed even while the virus appears to be idle. Moreover, those viral gene products can induce various cancers in the host, including Burkitt’s lymphoma, nasopharyngeal carcinoma (NPC), T cell lymphoma, salivary gland carcinoma, Hodgkin’s lymphoma, and gastric carcinoma, she says.

Raab-Traub and other researchers are investi-
gating how genes expressed by latent EBV can so profoundly affect such a variety of host tissues. A key to this versatility, she says, is that host exosomes distribute these virally encoded oncogenic agents during latency. "Microvesicle-mediated communication allows the virus to respond to or control the cellular microenvironment in the absence of viral replication," she explains. "Through exosomal secretion and uptake, a human tumor virus can induce the transfer of viral oncoproteins, signal transduction molecules, and virus-encoded miRNA into multiple cell types and activate cell-signaling pathways."

Indeed, exosomes can modulate transformation, tumor growth, tumor microenvironment remodeling, invasion, angiogenesis, cell migration, metastasis, immune evasion, and response in the context of various nonviral cancers, Raab-Traub continues. Further, nasopharyngeal carcinoma cells (NPC) that harbor latent EBV can produce what she calls "oncosomes"—specialized exosomes containing in this case latent membrane protein 1 (LMP1), which is a constitutively active member of the tumor necrosis factor (TNF) receptor family. When delivered to a neighboring cell, LMP1, which is a transmembrane receptor, activates several growth-stimulating signaling molecules, including nuclear factor-κB (NF-κB), that alter the expression of genes involved in host cell-cycle progression, proliferation, apoptosis, and migration. "Thus, EBV can manipulate the growth characteristics of neighboring cells," she says.

LMP1 also induces neighboring cells to secrete growth factors, including epidermal growth factor (EGFR) and fibroblast growth factor 2 (FGF-2), both of which can be oncogenic. Thus, uninfected host cells are subject to growth signaling molecules, further amplifying the oncogenic capacity of EBV. "Release of LMP1 from a rare expressing cell could have wide-ranging effects on the entire cell population," Raab-Traub says. "LMP1 is not always detected within every tumor cell or sample, but exosomal transfer of LMP1 and other signaling molecules could impact growth control of other cells within a tumor."

Other susceptible host-cell types include human umbilical vascular endothelial cells, which play a major role in vascularization and metastasis, and epithelial cells, a major target of EBV-induced cancers.

**Some Exosomes Suppress Host Immune Responses, Others Involved in Gene Silencing**

Exosomes contribute to immune evasion, enabling NPC tumors, which may become heavily infiltrated with T lymphocytes, to resist immune clearance. "The emergence of a malignant process producing several immunogenic viral proteins in a context of local inflammation and heavy leukocytic infiltration is one major paradox of NPC pathogenesis," says Pierre Busson of the University of Paris, France. The actions of "immunosomes," which inhibit T cell activation and proliferation, help to explain this paradox, he says.

These immunosuppressive exosomes contain the protein galectin-9, which induces apoptosis in Th1 lymphocytes (CD4+, T-helper cells), Busson continues. Galectin-9 is an inducible host protein that ordinarily is responsive to cytokines. However, EBV highjacks the protein as part of an immune evasion scheme. "NPC exosomes induce massive apoptosis in EBV-specific CD4+ cells," he says. Without these CD4+ T-helper cells, cytotoxic T-killer cells (CD8+) fail to act against EBV-infected cells. Thus, despite infiltrating such tumors, they remain quiescent. Immunosomes can act locally, within the tumor, or systemically by traveling through the bloodstream to lymph nodes and other tissues of the immune system, he points out.

Busson is evaluating whether blocking interactions between galectin-9 and its receptor on Th1 cells, Tim-3, can restore immune responses against EBV and associated tumors. Monoclonal antibodies neutralize the Tim-3 binding domain of galectin-9 in vitro, blocking the immunosuppressive effects of NPC exosomes on T cells, he says. "Blocking galectin-9/Tim-3 interaction in vivo might sustain antitumor T cell responses in patients and/or enhance long-term survival of CD4+ EBV-specific T cells infused into NPC patients." This approach may be applicable to other EBV-associated cancers such as Hodgkin’s lymphoma, which also expresses galectin-9.

On another front, exosomes package and deliver miRNAs from EBV, "silencing" molecules that epigenetically reprogram target cells, according to Jaap Middeldorp of VU University Medical Center in Amsterdam, the Netherlands, and his collaborators. For example, exosomes in EBV-infected B cells contain miRNA that, when re-
leased, can be taken up by uninfected dendritic cells. Even though these recipient cells cannot be directly infected by EBV, which is B cell tropic, the miRNA-carrying exosomes enable this virus to manipulate otherwise uninfected cell types. Dendritic cells are particularly important recipients of EBV genetic material because they are involved in both T cell-mediated immunity to EBV and EBV-driven transformation.

Once inside such cells, exosomes are directed to late endosomes, the subcellular compartment from which miRNA-mediated gene silencing emanates, according to Middeldorp. Exosomes also deliver other essential components of the RNA silencing machinery such as GW182. “GW182, required for miRNA function through its association with argonaute 2, is dramatically enriched in exosomes,” says Raab-Traub. Thus, EBV target genes are repressed in uninfected recipient dendritic cells. One such target gene is CXCL11, which is downregulated in EBV-associated lymphomas, tying these in vitro studies to clinical disease pathology.

“It is likely that other viruses that establish
long-term, latent, or chronic infections also modulate exosomes to enhance their persistence,” Raab-Traub says. Indeed, HIV-infected cells release exosomes containing the viral protein Nef, and exposure to these exosomes impairs humoral immunity in neighboring uninfected B cells. These exosomes also induce apoptosis in CD4+ T helper cells, which may contribute to pathogenesis and progression to AIDS.

**Leishmania Exosomes Deliver Virulence Factors**

Protozoan parasites also depend on exosomes to deliver virulence factors into host cells and to dampen host immune responses, according to Neil Reiner of the University of British Columbia (BC) in Vancouver, BC, Canada, and his collaborators. They are studying this phenomenon in *Leishmania*, a protozoan parasite that occupies host macrophages, giving rise to a chronic, non-resolving disease in humans. Leishmaniasis is typically found in the tropics, and can lead to disfiguring lesions and damage to haemopoetic organs.

*Leishmania* release exosomes from the flagellar pocket, and these exosomes contain multiple virulence factors, Reiner and his collaborators find. These include soluble macromolecules such as heat shock proteins and elongation factor-1α (EF-1α) as well as a membrane-bound protease called GP63. Because these soluble virulence factors lack secretion signals and GP63 is membrane-bound, how they exert effects on host cells long remained a mystery. In fact, few *Leishmania* proteins carry N-terminal secretion signals, he says. “The exosome proteome accounted for over half (52%) of the total secretome, and several individual exosomes overlapped with the secretome by more than 70%. The high protein overlap suggests that these vesicles are the primary mechanism of protein release from *Leishmania*.”

Exosomes have two potential routes of entry into macrophage cells. They may be released by *Leishmania* prior to phagocytosis for macrophage cells to take up, or they may be released within phagolysosomes of infected macrophages, and then released into the cytoplasm. Within the cytosol, these virulence factors interfere with signaling pathways involved in immune activation. “[Virulence factors] access host cell cytosol and activate multiple host protein tyrosine phosphatases, negatively regulating interferon (IFN)-γ signaling and preventing effective expression of the macrophage microbicidal arsenal, including tumor necrosis factor (TNF)-α and nitric oxide,” Reiner says.

IFN-γ-mediated activation of macrophage cells is crucial for clearing *Leishmania* infections. However, when macrophage cells are treated with exosomes, their responses to IFN-γ are blunted, and they produce lower levels of the proinflammatory cytokines TNF-α and interleukin (IL)-8, and produce more anti-inflammatory IL-10, according to Reiner. “Given that IL-10 is a potent anti-inflammatory cytokine involved in mediating immune suppression during *Leishmania* infection, enhanced production of IL-10 as a result of exposure to exosomes suggests a significant role for these vesicles in pathogenesis,” he says.

**Leishmania Exosomes also Suppress Host Immune Responses**

*Leishmania*-derived exosomes also exert immunosuppressive effects on monocyte-derived dendritic cells (moDCs), inhibiting production of proinflammatory IL12p70 and TNF-α, Reiner continues. “HLA-DR expression was also significantly attenuated, suggesting the possibility that specific functional properties might be impaired,” he says.

One role of moDCs is to induce naïve CD4 T cells to form Th1 helper cells, which produce IFN-γ and activate other immune effector cells. However, *Leishmania* exosomes prevent moDCs from driving Th1 differentiation, leading to a deficit in this important cell population. These differentiated Th1 cells are particularly critical in the control of *Leishmania* infection, according to Reiner. “Th1 cells producing IFN-γ have recently been shown to be the dominant suppressor cell in *Leishmania* infections,” he says. When mice are treated with such exosomes, CD4 Th1 cells are reduced in number while IL-10 levels in spleen and draining lymph nodes rise.

The immunosuppressive effects of *Leishmania* exosomes on host macrophage cells and moDCs enhances progression of disease, according to Reiner. When mice are vaccinated with *Leishmania* exosomes, their parasitic load increases eight-fold compared to untreated animals, he says. “*Leishmania* exosomes secreted upon initial in-
fection are capable of delivering effector cargo to naïve target cells wherein the cargo primes the host for permissive infection by interfering with host cell signaling pathways, biasing the immune response to create a proparasitic environment.”

Further, conditions that mimic early infection induce exosome release, Reiner continues. *Leishmania* parasites experience “heat shock” when they are delivered to mammalian hosts, whose body temperature is 37°C, by biting sandflies, whose internal temperature is 26°C,” he says. In experiments, *Leishmania* upregulate exosome release threefold within 4 hours of being heat shocked, suggesting that during infection, exosomes are released from the parasites before they infect host cells, priming immune cells for permissive infection.

*Leishmania* parasites also are exposed to a more acidic pH when entering mammalian hosts from sandflies. Once ingested by mammalian macrophage cells, the change is even more dramatic, as the phagolysosome attempts to digest its contents. Reiner finds that *Leishmania* modify their exosomes in response to these more-acidic conditions. “Not only was there specific packaging of individual proteins, we also detected specific functional enrichments based on changes in both temperature and pH,” he says. Indeed, lowering the pH enriched exosomes for survival proteins such as TRYP1 that enable *Leishmania* to endure acidic conditions within the phagolysosomes.

The contents of exosomes and their impact on the host may vary by strain. “Our preliminary data show that *Leishmania* isolates of the same species, but causing divergent disease phenotypes clinically, display distinct exosome proteomic profile,” Reiner says. “This suggests that exosome-based secretion contributes to different disease phenotypes.”

The proteins within exosomes are of critical importance to the pathogenicity of *Leishmania*, according to Reiner. Heat shock protein 100 (Hsp100) helps to control what goes into the exosomes, and Hsp100 knockout mutants of *Leishmania* produce exosomes that lack key virulence factors such as GP63, EF-1α, and kinetoplast membrane protein 11, he says. The exosomes from such mutants are immunostimulatory rather than immunosuppressive, enhancing the production of TNF-α and IL-10 from macrophages, whereas wild-type exosomes blunt these responses. “Hsp100 mediates some essential packaging function, without which a mature anti-*Leishmania* Th1 response effectively clears the infection,” he notes. Such immunostimulating exosomes from mutant parasites might provide a means for vaccinating and perhaps partly protecting hosts—at least in mice.

These findings about the role of *Leishmania* exosomes might also apply to other intracellular parasites such as *Toxoplasma* and plasmodia, both of which chronically infect hosts, according to Reiner. Exosome-based vaccination strategies may turn the tables on these or other persistent, intracellular parasites.

**Several Types of Fungi Rely on Exosomes**

A diverse variety of fungal species produces exosomes, including *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Candida albicans*, *Candida parapsilosis*, and *Sporothrix schenckii*. Cargo proteins include virulence factors and immune modifiers, including enzymes such as superoxide dismutase, heat shock proteins, and signal transcription regulators. For example, laccase in *C. neoformans* exosomes interferes with host metabolism of prostaglandin, which modulates B and T lymphocyte proliferation and macrophage function, says Leonardo Nimrichter of Federal University, Rio de Janeiro, Brazil.

Fungal exosomes also contain immune-modifying polysaccharides. Glucuronoxylomannan, (GXM), an essential component of the *C. neoformans* capsule, is delivered to the exterior of the cell via exosomes and potently inhibits immune response, according to Anna Vecchiarelli, of the University of Perugia in Italy. “Effects ascribed to GXM include inhibition of the humoral response, T cell proliferation, development of Th1, chemotactic activity, production of inflammatory cytokines such as TNF-α, and induction of inhibitory factors such as IL-10,” she says.

The lipids phosphatidylcholine and phosphatidylserine, which account for the bulk of fungal membranes, also help to modulate host-fungal interactions, according to Nimrichter. “Liposomes carrying phosphatidylserine can modulate cytokine production, decrease microbial killing, and inhibit nitric oxide production by macrophages,” he says. Glucosylceramide, found in exosomes from *C. neoformans*, is an important virulence factor.

Though the contents of fungal exosomes sug-
gest the potential to inhibit host immune responses, their impact in vivo is under debate, and, in some cases, they may activate instead of suppress immune responses. C. neoformans exosomes induce phagocytosis by macrophage cells as well as production of proinflammatory cytokines and nitric oxide, Nimrichter says. Dendritic cells are similarly induced to increase proinflammatory cytokines and MHC class II and costimulatory molecules. Whether these processes contribute to pathogenesis or instead help to clear this fungus is not known.

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