Exosomes and other extracellular vesicles in host–pathogen interactions

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Abstract

An effective immune response requires the engagement of host receptors by pathogen-derived molecules and the stimulation of an appropriate cellular response. Therefore, a crucial factor in our ability to control an infection is the accessibility of our immune cells to the foreign material. Exosomes—which are extracellular vesicles that function in intercellular communication—may play a key role in the dissemination of pathogen- as well as host-derived molecules during infection. In this review, we highlight the composition and function of exosomes and other extracellular vesicles produced during viral, parasitic, fungal and bacterial infections and describe how these vesicles could function to either promote or inhibit host immunity.

Introduction

The ability of the immune system to recognize and respond to pathogenic organisms is essential for the body's ability to control an infection. Divided into the innate and acquired branches, immune responses elicit protection against most pathogenic invaders. A number of mechanisms for how the immune system accomplishes this protection have been defined. Cytokines and chemokines, as well as other inflammatory mediators produced by infected or resident immune cells, clearly recruit and activate leukocytes and other cells, culminating in the elimination of the invading organism. Pathogen-derived products are major drivers of both the innate and acquired immune response. The microbial components that trigger the innate immune response do so either directly by inducing the production of immune effector molecules like reactive oxygen species or indirectly through stimulating the production of cytokines and chemokines. These microbial components that promote the innate immune response are generally defined as PAMPs (pathogen-associated molecular patterns). PAMPs are structurally diverse molecules found across many species of non-pathogenic and pathogenic organisms and include lipids, proteins, carbohydrates and genetic material. Pattern recognition receptors (PRRs), such as TLRs, present on leukocytes and various non-immune cells, can bind to PAMPs, thereby initiating cell-signaling cascades. This ultimately leads to the activation of an immune response against the pathogen. The importance of PRRs in the recognition and response to pathogens has been shown in both animal infection models and human studies [1]. Given their role in immunity, it is not surprising that pathogenic organisms have evolved methods to modulate the binding and/or signaling through the PRRs as mechanisms to promote their virulence and evade surveillance by the immune system [2,3].

The mechanisms for exposure of PAMPs to PRRs differ between intracellular and extracellular pathogens. For extracellular pathogens, PAMPs are released through an active process or through shedding or death of the organism. The released factors can then directly bind PRRs on immune cells, stimulating or inhibiting host responses. In contrast, intracellular pathogens, such as *Mycobacteria*, *Salmonella* and *Toxoplasma*, produce PAMPs which—like the pathogens they are derived from—have limited exposure to the immune system. Nevertheless, the host does respond to these PAMPs, likely through multiple mechanisms. An obvious source of interaction is the cell invasion process [4–6]. Moreover, a pathogen may be present in the extracellular milieu between host cell entries, allowing for release of PAMPs. This is likely a particularly important mechanism for viral proteins and viral RNA/DNA. The ability of HIV to invade host cells is known to be heterogeneous depending on the interactions with its cellular receptors; a slower invasion leads to increased phagocytosis of the virion by phagocytic cells [7]. However, some intracellular pathogens like *Listeria monocytogenes* can go from cell to cell without being exposed to the extracellular environment [8]. Debris from necrotic infected cells or release of apoptotic bodies from infected cells can also disseminate pathogenic components to surrounding cells/tissue [9]. However, what has become increasingly clear is that exosomes and other extracellular vesicles released from infected cells, as well as from the pathogens, likely play an important role in this dissemination process [10–12]. These components not only include PAMPs, but also T- and B-cell antigens, as well as pathogen-derived toxins.

In this review, we briefly introduce exosomes and how they are generated, as well as their role in non-infectious diseases, with an emphasis on their immune modulatory activity. We then focus in-depth on the production and activity of exosomes and other

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Glossary

APC	antigen-presenting cell	**LFA-1**	lymphocyte function-associated antigen 1
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	**LMP1**	latent membrane protein 1
BALF	bronchoalveolar lavage fluid	**LPS**	lipopolysaccharide
BAT3	HLA-B-associated transcript 3	**M.tb**	*Mycobacterium tuberculosis*
BCG	bacillus Calmette–Guérin	**MAP kinase**	mitogen-activated protein kinase
BMDCs	bone marrow-derived dendritic cells	**MC/9**	*Mus musculus* mast cell line
CCR5	C-C chemokine receptor type 5	**MCP-1**	monocyte chemotactic protein 1
CD	cluster of differentiation	**mDC**	mature DC
CFP	culture filtrate protein	**MFG-E8**	milk fat globule-EGF factor 8 protein
CIA	collagen-induced arthritis	**MHC**	major histocompatibility complex
CMV	cytomegalovirus	**miRNA**	microRNA
CTL	cytotoxic T lymphocyte	**MVB**	multivesicular bodies
CXCR4	C-X-C chemokine receptor type 4	**MyD88**	myeloid differentiation primary response gene (88)
DC	dendritic cell	**NF-κB**	nuclear factor kappa-light-chain-enhancer of activated B cells
DT	diphtheria toxin	**NK**	natural killer
DTH	delayed-type hypersensitivity	**NLRP3**	NACHT, LRR and PYD domains-containing protein 3
EBA-175	erythrocyte binding antigen 175	**NPC**	nasopharyngeal carcinoma
EBV	Epstein–Barr virus	**PAMP**	pathogen-associated molecular pattern
EM	electron microscopy	**PBMC**	peripheral blood mononucleated cells
ESCRT	endosomal sorting complexes required for transport	**PIM**	phosphatidylinositol mannoside
FACS	fluorescence-activated cell sorting	**PRR**	pattern recognition receptor
Fas/FasL	Fas and Fas ligand	**RANTES**	regulated on activation, normal T cell expressed and secreted
HCV	hepatitis C virus	**RBC**	red blood cell
HEK	human embryonic kidney	**RILP**	rab-interacting lysosomal protein
HIV	human immunodeficiency virus	**RNP**	ribonucleoprotein
HLA-DR	major histocompatibility complex, class II, DR beta 1	**ROS**	reactive oxygen species
HMC-1	human mast cell line-1	**SNARE**	SNAP (soluble NSF attachment protein) REceptor
HSP	heat-shock protein	**TAR**	trans-activation response
HSV	herpes simplex virus	**TB**	tuberculosis
ICAM1	intercellular adhesion molecule 1	**TCR**	T-cell receptor
iDC	immature DC	**TF**	tissue factor
IFN	interferon	**Tim4**	T-cell immunoglobulin mucin protein 4
IL	interleukin	**TLR**	toll-like receptor
ILVs	intraluminal vesicles	**TNF**	tumor necrosis factor
JNK	c-Jun N-terminal kinase	**VAMP7**	vesicle-associated membrane protein 7
LAM	lipoarabinomannan	**WT**	wild-type
LBPA	lysobisphosphatidic acid		

extracellular vesicles during infection, and how these vesicles could benefit the host immune response but also be used to promote pathogen survival. Finally, we discuss their therapeutic potential, including their use as vaccines and diagnostic tools.

Extracellular vesicles

Extracellular vesicles are broadly defined as membrane-bound vesicles released from cells. Those produced during an infection can be pathogen or host derived. The former include, for example, outer membrane vesicles from gram-negative bacteria and membrane vesicles from gram-positive bacteria. The content and function of these bacteria-generated vesicles has recently been under intensive investigation and excellently reviewed elsewhere [13–15]. Although these vesicles likely play an important role during the course of an extracellular bacterial infection, their role in intracellular pathogen infections is less clear, as mechanisms to transport the vesicles outside the host cell are not known. Parasitic and fungal pathogens also release extracellular vesicles, which may function in modulating the immune response [16,17].

Host-derived vesicles are present during viral, bacterial, parasitic and fungal infections. These vesicles have different origins and composition and, based on their biogenesis, are divided into three main categories: apoptotic bodies, exosomes and microvesicles. All three of these cell-derived vesicles are enclosed by a lipid bilayer, but vary in size (from 30 to 2,000 nm in diameter), as well as in composition. In contrast to microvesicles, which are generated by budding from the plasma membrane [18], exosomes are derived from the endolysosomal pathway and have a unique lipid and protein makeup. Exosomes have been the most studied in the context of infection. An important note, however, is that exosome purity was not always analyzed in these studies, and therefore, the vesicle population may have consisted of both exosomes and microvesicles, which overlap in size and density. Nevertheless, we will use the terminology as defined in the original studies when discussing the results.

Exosomes

Exosomes are formed through the fusion of multivesicular bodies (MVBs) with the plasma membrane and subsequent release of
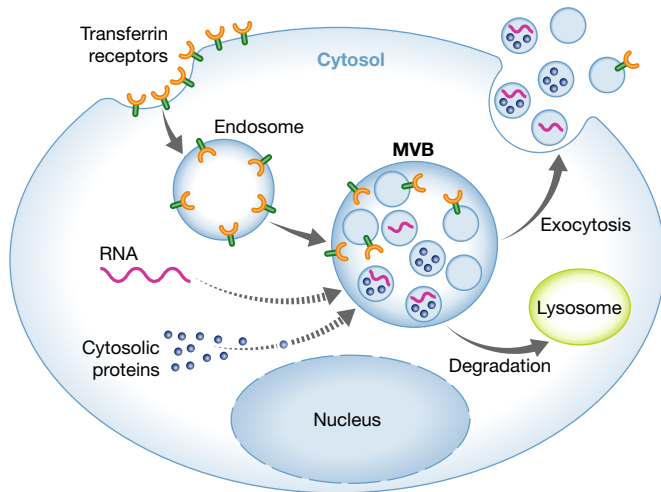
Figure 1. Exosome biogenesis.
Lipids, proteins and nucleic acids are transported to MVBs and onto or into the intraluminal vesicles, which upon fusion of the MVB with the plasma membrane are released as exosomes. Originally identified as a way to release transferrin receptor from maturing reticulocytes, other plasma membrane proteins have been shown to be targeted to MVBs through various mechanisms and released on exosomes. RNA and cytoplasmic proteins are also transported to MVBs, although the mechanisms mediating this transport are less understood (indicated by dashed line). See Glossary for definitions.

intraluminal vesicles (ILVs) as exosomes (Fig 1). Exosomes are 30–100 nm vesicles, surrounded by a lipid bilayer, that have a density of 1.13–1.19 g/ml. Biophysically, exosomes are equivalent to cytoplasm enclosed in a lipid bilayer with the external domains of transmembrane proteins exposed to the extracellular environment. EM studies have demonstrated the fusion of the limiting membrane of MVB with the plasma membrane, as well as the release of ILVs, in different cell types of hematopoietic origin, such as Epstein–Barr virus (EBV)-transformed B cells [19], mastocytes [20], DCs [21,22], platelets [23], macrophages [10] and cells of non-hematopoietic origin such as neurons and epithelial cells [24–26]. Exosomes can act locally or circulate through various bodily fluids, including blood and lymph, resulting in a systemic response [27]. Exosomes were first identified in the culture media of reticulocytes [28,29]. However, over the past two decades, the study of exosomes has extended to most cell types, and they have been isolated from different organisms—including unicellular eukaryotes—suggesting that this is an evolutionarily conserved mechanism of cell–cell communication. The advantage of using exosomes for cell–cell communication stems from their complex composition, which allows more control over the communication process. Moreover, the presence of signaling lipids, proteins and various species of RNA within a single structure can lead to rapid and profound changes in the target cell, enabling a swift response to cellular perturbations, which can be local or systemic. These changes may be induced under physiological or pathological conditions. Although the complexity of exosomes has clear benefits to the organisms that produce them, it has made the study of their function exceedingly difficult, as the effect of an exosome or pool of exosomes is a result of all the different components within them, including lipids, proteins, carbohydrates and RNA. Moreover, the tools available to modulate exosome production and composition

in vitro and *in vivo* are severely limited, hampering our ability to define exosome function in normal and diseased states. Nevertheless, we have gained important insights into exosome biogenesis, composition and function over the past decade, a decade that has seen a rapid expansion in publications on this type of extracellular vesicle.

Exosome biogenesis
A major mechanism for down-regulating and degrading plasma membrane receptors is through their endocytosis and trafficking to an MVB, which can subsequently fuse with the lysosome to mediate protein degradation [30]. However, at least a subpopulation of MVBs can also fuse with the plasma membrane, resulting in the release of the intraluminal vesicles (ILV) as exosomes. Despite their discovery nearly three decades ago, the mechanism for MVB biogenesis and exosome release is still being defined. Several models have been suggested as a mechanism for ILV formation. Initial studies in yeast demonstrated a role for the ESCRT proteins [31]. Although the ESCRT machinery has primarily been studied for its role in the endosomal sorting and degradation of ubiquitinated proteins, it has also been implicated in mediating membrane invagination [32,33]. Through its ubiquitin-interacting domains, ESCRT-0 clusters ubiquitinated proteins for delivery into MVBs [34]. ESCRT-0 subsequently recruits ESCRT-1 to the endosomal membrane, which in turn recruits the remaining members of the ESCRT machinery, ESCRT-II and ESCRT-III [35,36]. Through the formation of polymeric filaments mediated by ESCRT-III, membrane invagination results in ILV formation [37] (for a recent review, see [38]). Indeed, various studies support a role for the ESCRT machinery in exosome formation. Proteomic analysis of exosomes has demonstrated the presence of ESCRT machinery within exosomes, and knockdown of key components of ESCRT machinery can abrogate ILV formation and exosome release [39], although this is likely cell type-specific [40,41]. While this general model for MVB biogenesis has been well characterized, it is unclear whether this constitutes the major mechanism of MVB formation. A number of studies suggest there are ESCRT-independent mechanisms for MVB biogenesis and exosome release. In oligodendroglial cell lines, exosome formation is driven by the production of ceramide, rather than the ESCRT machinery [41]. Stuffers and colleagues found that depleting specific subunits from the four ESCRTs complexes did not completely inhibit MVB formation [40]. Furthermore, a mechanism independent of both ESCRTs and ceramide has been proposed. Studies by van Niel and colleagues found that the tetraspanin CD63, which is present on exosomes in high abundance, mediates cargo sorting and ILV formation [42]. Additionally, CD81 has been demonstrated to mediate cargo sorting of tetraspanin ligands, such as Rac GTPase, although knockdown of this tetraspanin does not appear to alter MVB morphology or exosome secretion [43]. These different observations suggest that the mechanism for exosome biogenesis and protein sorting may be cell type-specific or specific to different subpopulations of MVBs within a cell. In support of the latter, Stoorvogel and colleagues have shown that within immature DCs, the MHC molecules are targeted to MVBs that are low in cholesterol but enriched for lysobisphosphatidic acid, which are destined for lysosomal degradation. However, in mature DCs, MHC molecules are sorted into MVBs that are enriched in CD9 and cholesterol, which are targeted for fusion with the plasma membrane [44].

Once MVBs are formed, their fusion with the plasma membrane is mediated by the cytoskeleton, fusion machinery—such as the

SNARE proteins—and molecular switches (such as small molecular weight GTPases) [45]. Rab GTPases are members of the Ras GTPase superfamily and are known to regulate four steps in membrane trafficking: vesicle formation, trafficking, tethering and fusion with target organelles. Almost 70 different Rab GTPases have been identified to date in mammalian cells [46]. Several of these have been found on exosomes, including Rab5, Rab11, Rab27 and Rab35. Some of these Rab effectors have been experimentally shown to function in exosome release. Early studies suggested that Rab11 might function to promote MVB fusion with the plasma membrane in the K562 erythroleukemic cell line [47]. More recent studies have implicated Rab35 in mediating MVB docking to the plasma membrane in neuralgia cells, where depletion of Rab35 resulted in a significant loss in exosome release [48]. Rab27a and Rab27b were also shown to have different, but sometimes redundant, roles in MVB biogenesis, with Rab27a more implicated in mediating MVB docking to the plasma membrane [49]. Although the Rab GTPases have been implicated in MVB trafficking and fusion, their role in the process is still under investigation and will likely be cell type-dependent, as well as dependent on the physiological/pathological state of the cell.

Exosome composition

Exosomes contain all types of biomolecules, including proteins, carbohydrates, lipids and nucleic acids. Their lipid and protein composition has been extensively analyzed by various techniques, including Western blotting, FACS, immuno-EM and mass spectrometry. Exosome composition will vary depending on the cell type of origin, its physiological/pathological state and even the cell site of origin, as seen in epithelial cells. Epithelial exosomes have different composition if they are released from the apical or basolateral surfaces [50]. Cell type-specific markers can help define the exosome cellular origin; for example, the presence of T-cell or B-cell receptors is indicative of T-cell and B-cell origin, respectively. The exosome protein composition can also be informative of the existence of a pathology, as they can, for example, carry tumor antigens or inflammatory mediators. In addition, exosomes contain a number of common proteins, including Tsg101, Hsc70 and various tetraspanins [51], as well as proteins that participate in vesicle formation and trafficking, such as the LBPA-binding protein, Alix [52]. Exosome lipid composition has also been well characterized. As for proteins, the ratios of the different lipids can vary between exosomes released from different cellular origins. In general, exosomes are enriched in lipids such as sphingomyelin, phosphatidylserine, gangliosides and cholesterol, as compared to plasma membranes and other intracellular membranes [53]. A number of reviews have highlighted the protein and lipid content of exosomes [54,55], and various databases have cataloged the protein, lipid and RNA content of exosomes (ExoCarta, http://www.exocarta.org/, Vesiclepedia, http://microvesicles.org/).

Most recent studies have focused on exosomal RNA; the types of RNA and their nucleotide sequence, their ability to be transferred between cells, their function once transferred and the mechanism by which they are trafficked to MVBs and into exosomes. Pioneering studies by Valadi and colleagues showed that exosomes are enriched in mRNA and miRNA [56]. More recent studies have identified other non-coding RNAs in exosomes, but limited amounts of DNA or ribosomal RNA [57]. The exosomes derived from a human (HMC-1) and mouse (MC/9) mast cell lines were found to transport mRNA to neighboring mast cells. This mRNA was subsequently translated, indicating that it is biologically active [56]. Exosomes released by immune cells have been shown to contain a selective repertoire of miRNAs that can be functionally transferred to recipient cells [58,59]. The source of these exosomes/extracellular vesicles were cultured cells [60] and body fluids [61,62]. Together, these data suggest that exosomes function as carriers of genetic information and that this genetic material plays a role in cell–cell communication. However, the exosomal RNA content differs both in quantity and in composition depending of the cellular origin and cellular environment. Eldh and colleagues found that the exosomes released by mast cells differ in their mRNA content after exposure to an oxidative stress, and oxidative stress resistance was induced in recipient cells [63]. These results indicate that the incorporation of RNA into vesicles is a regulated event leading to selective packaging of RNA into exosomes and other extracellular vesicles [64,65]. The mechanism(s) responsible for the targeted loading of RNA into exosomes is still being defined and remains an active area of investigation.

In addition to host components, a number of pathogen-derived components have been found on exosomes after cell or animal infection (Table 1). Unfortunately, we know very little about how these diverse pathogen-derived proteins, glycolipids, etc. are sorted to MVBs and onto exosomes (see Sidebar A). Much of our current understanding stems from studies of viruses, where viral assembly and exosome biogenesis share many similarities. For example, HIV assembly and release from infected cells depend on both ESCRT machinery and tetraspanin-rich lipid domains [66,67]. The presence of viral proteins in exosomes and the similarities in biogenesis and assembly suggest that a degree of 'crosstalk' or 'hijacking' could be responsible for sorting the viral proteins into exosomes. However, some viral proteins—such as the HIV protein Nef—may contain necessary signals to mediate their direct sorting into exosomes [68]. For other types of pathogens, even less is understood. Some intracellular bacterial pathogens, such as *Mycobacterium tuberculosis*, are also known to interfere with host machinery implicated in exosome biogenesis, such as ESCRTs [69], although the extent to which this contributes to protein sorting during exosome biogenesis is unclear. Based on our observations, sorting of mycobacterial proteins seems to be independent of cell entry mechanisms, as mycobacterial proteins are found on exosomes whether added as free protein, and therefore taken in through an endocytic route, or expressed in mycobacteria, which enters by phagocytosis [11]. This finding suggests that these mycobacterial proteins have the necessary 'signal' to be trafficked to the MVB during exosome biogenesis. However, further investigation is needed to shed light on potential sorting mechanisms.

Viral RNAs have also been found within exosomes. HCV viral RNA transport to exosomes was found to be dependent on the ESCRT machinery and on Annexin A2, an RNA-binding protein involved in membrane vesicle trafficking [70]. Similarly, EAP30—a subunit of ESCRT-II—controls HIV-1 RNA trafficking and gene expression through a complex formed by HIV-1 Gag, ESCRT-II and Staufen-1 [71]. The mechanism by which EAP30/ESCRT-II facilitates HIV-1 genomic RNA trafficking remains unclear, although—considering the roles for ESCRT-II in the nucleus [72]—EAP30/ESCRT-II is likely part of the RNP complex that mediates the nuclear export of viral RNA. Other partners of EAP30, such as EAP45, have an RNA-binding domain that is likely conserved [73,74], and RILP

Table 1. Pathogen components present on exosomes/extracellular vesicles released from infected cells.

Pathogen	Vesicle contents	References
HIV	Gag proteins	119
	Nef protein	68,121,123,124
	TAR transcripts	120
EBV	Viral RNAs	134
	LMP1	129
	LMP2a	130
CMV	Glycoprotein B	138
Hepatitis C virus	Viral RNAs	70
	Viral RNA/proteins	141,142
	Envelope glycoprotein E2	139
HSV	Viral tegument proteins and various glycoproteins	146
Toxoplasma gondii	PAMPs	10
Leishmania mexicana	GP63	156
	Proteomic analysis	156
Leishmania major	GP63	157
Leishmania donovani	GP63	158
	Proteomic analysis	155
Plasmodium yoelii	Proteomic analysis	160
Plasmodium falciparum	EBA-175, EBA-181	162
	Proteomic analysis	162
	PfPTP2	163
Mycobacterium tuberculosis	LAM, PIM	10,180,181
	19 kDa lipoprotein	10
	Proteomic analysis	11
	mRNA	J.S. Schorey, unpublished data
Mycobacterium avium	GPLs	182
Salmonella typhimurium	LPS	10
Mycoplasma	Lethal factor	195
	Proteomic analysis	195

—which associates with EAP30—can also have an effect on the localization of viral RNA in the cytoplasm. EAP30/ESCRT-II could also contribute to the stability of cellular factors that are required for viral RNA trafficking.

Exosomes as modulators of the immune response

Most studies of exosomes and their effect on the immune response are in the context of cancer and autoimmunity. These studies have: (i) defined the host molecules that facilitate exosome transfer; (ii) characterized the presence of tumor antigens on exosomes and

mechanisms by which these antigens can promote T-cell activation; (iii) defined the mechanisms by which some exosomes can induce T-cell anergy and deletion; and (iv) defined the RNA content within the exosomes, as well as characterized their transcriptional and translational effect on recipient cells [75]. Other studies have characterized exosomes as drivers of an innate immune response, although significantly less work has been done in this area [76].

Innate immunity

Dendritic cells produce exosomes constitutively and have been implicated in the activation of the innate immune response (Fig 2). Exosomes from both immature and mature DCs contain multiple TNF superfamily members—such as TNF, FasL and Trail—on their surface, which directly bind to the surface receptors on NK cells to enhance their cytotoxic activity. However, the activation of NK cells is significantly stronger in response to exosomes released from activated DCs [77]. Similarly, exosomes from DCs express BAT3 (HLA-B-associated transcript-3) and thus are recognized by the NK surface receptor, NKp30, leading to NK-cell activation [78]. DCs activated by lipopolysaccharide (LPS) release extracellular vesicles that can stimulate epithelial cells to secrete chemokines—such as IL-8 and RANTES—which may be an important component in the pathogenesis of sepsis [79]. DC-derived microvesicles have also been shown to induce NF-κB activation in microglia cells, which may play a role in the inflammatory response observed in the CNS during experimental autoimmune encephalomyelitis (EAE) [80]. IL-1β, a major driver of the innate immune response, is produced as a pro-form and converted to its active form through cleavage by the inflammasome. The mature form is released by activated macrophages and DCs through a non-classical secretion pathway. Qu and colleagues propose that trafficking via exosomes may be one mechanism for IL-1β release, although this was not definitively proven because exosomes containing the mature IL-1β could not be isolated after ATP stimulation of bone marrow-derived macrophages [81,82]. In the presence of oxidized low-density lipoprotein (oxLDL)-conjugated immune complexes, macrophages release exosomes containing IL-1β, as well as increased levels of acid sphingomyelinase and HSP70, and these exosomes may promote the propagation of atherosclerotic plaques [83]. These and other studies clearly demonstrate a role for exosomes/extracellular vesicles in regulating inflammatory and innate immune responses. As described below, their role is likely more pronounced in the context of an infection, as these exosomes could carry both host and pathogen components.

Acquired immunity

The presence of MHC class I and II, as well as T-cell co-stimulatory molecules, on the surface of macrophage- and DC-derived exosomes has attracted the interest of immunologists, as exosomes may be an important mechanism of antigen presentation. Since exosomes can carry foreign antigens in the context of an infection, defining the mechanisms by which this antigen presentation occurs is important to understanding the immune response to the pathogen. There are currently three proposed models for exosome-mediated antigen delivery to T cells (Fig 2).

1) *Cross-dressing pattern*. In this model, DCs capture extracellular exosomes and directly present antigenic peptide–MHC complex to CD4$^+$ or CD8$^+$ T cells, suggesting that exosomes shuttle peptide–MHC II complex between DC populations [84–87].
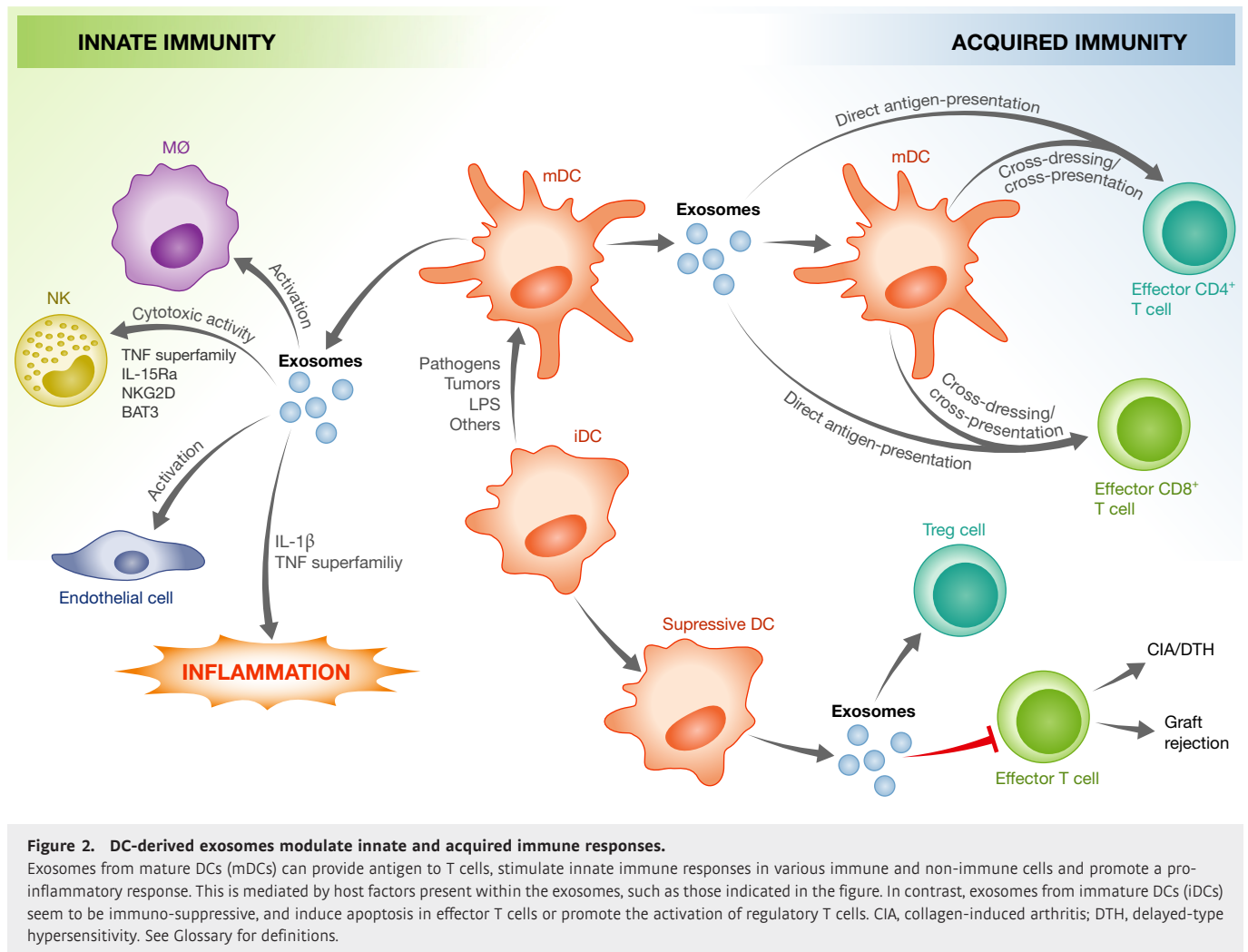
Figure 2. DC-derived exosomes modulate innate and acquired immune responses.
Exosomes from mature DCs (mDCs) can provide antigen to T cells, stimulate innate immune responses in various immune and non-immune cells and promote a pro-inflammatory response. This is mediated by host factors present within the exosomes, such as those indicated in the figure. In contrast, exosomes from immature DCs (iDCs) seem to be immuno-suppressive, and induce apoptosis in effector T cells or promote the activation of regulatory T cells. CIA, collagen-induced arthritis; DTH, delayed-type hypersensitivity. See Glossary for definitions.

2) *Cross-presentation pattern.* In this scenario, bystander DCs capture the exosomes containing the proteins/peptides and then present these exosome-delivered antigens on their endogenous MHC class I and II molecules for subsequent activation of antigen-specific T cells [88,89]. Both of these mechanisms require the capture of exosomes by recipient cells, and thus the receptors and ligands that facilitate this interaction have been the object of study. Several exosome surface ligands and adhesion molecules have been identified, including MFG-E8/lactadherin, tetraspanins, ICAM1 and phosphatidylserine [22,90,91]. MFG-E8 binds to the integrins αvβ3 and αvβ5, which are constitutively expressed by human DCs and macrophages, but also recognizes phosphatidylserine on the cell surface through its phosphatidylserine-binding domain [22,90]. Exosomal phosphatidylserine may also interact with DC surface protein Tim4 [92,93]. The uptake of DC-derived exosomes is not limited to bystander DCs, as exosomes can transfer peptide–MHC I complex and co-stimulatory molecule CD80 to non-specific CD4$^+$ T cells, which then acquire the capacity to activate antigen-specific/naïve CD8$^+$ CTLs *in vivo* and *in vitro* [94]. Furthermore, upon the formation of an immunological synapse between mature DCs and T lymphocytes, exosomes released from DCs specifically bind to activated T cells

in a LFA-1-dependent manner and could potentiate T-cell activation [95].

3) *Direct exosome-induced T-cell activation.* Since DC-derived exosomes contain MHC class I and II, as well as co-stimulatory molecules, they have the potential to directly activate CD4$^+$ or CD8$^+$ T cells [87,96–98]. Although this mechanism has mostly been observed for memory or previously activated T cells [99,100], some studies have shown exosome-mediated activation of naïve T cells. Using transfected *Drosophila* APCs and mouse CD8$^+$ T cells, Hwang and colleagues demonstrated that APC-derived extracellular vesicles directly activate naïve CD8$^+$ T cells *in vitro* [101]. This activation requires the presence of ICAM1 on the vesicles, which likely functions to promote adhesion between the exosomes and T cells. The presence of the co-stimulatory molecule B7 on the vesicles was also necessary. Direct stimulation of naïve PBMC-derived CD8$^+$ T cells was also detected in the presence of viral MHC I-specific peptide-loaded exosomes [91]. Although direct activation of naïve T cells has been observed, studies suggest that T-cell stimulatory activity by vesicles alone is 10- to 20-fold less efficient than when presented by an APC [19,102], suggesting that direct exosome–T-cell interaction may not be a major mechanism of naïve T-cell activation *in vivo*. Nevertheless, both *in vitro* and *in vivo* data suggest that

DC-derived exosomes likely function in antigen presentation to T cells. This ability to stimulate T cells may depend on the activation state of the DC, as exosomes from activated DCs have more MHC class II, ICAM1 and costimulatory molecules than exosomes from immature DCs [91]. Unfortunately, we do not yet have the tools to specifically block exosome-mediated antigen presentation *in vivo* and, therefore, the contribution of the exosome to T-cell activation in animal models is still unclear.

In contrast to exosomes from mature DCs, those derived from immature DCs have been shown to promote T-cell anergy/deletion as well as to promote the activation of regulatory T (Treg) cells. Thus, exosomes have been evaluated for the potential treatment of autoimmune diseases (Fig 2), a topic that has recently been the subject of several excellent reviews [75,103,104]. Exosomes are also released from lymphocytes, such as $CD4^+$ and $CD8^+$ T cells, which constitutively secret exosomes containing the TCR/CD3 complex. However, the production of these exosomes increases when they are activated through TCR ligand binding [105]. The function of these exosomes will depend on the activation status and tissue microenvironment of the T cell and can broadly be classified into two main categories based on their immunological functions, either activating or suppressive.

1) *Immuno-activation.* Exosomes from activated T cells may potentiate an immune response through effects on resting autologous T cells. For example, when activated with anti-CD3 and IL-2, human peripheral $CD3^+$ T cells release exosomes that stimulate cytokine secretion and proliferation of $CD8^+$ T cells *in vitro* [106].

2) *Immuno-suppression.* $CD4^+CD25^+FOXP3^+$ Treg cells release exosomes containing CD73, an ecto-5′-nucleotidase enzyme that converts extracellular adenosine-5-monophosphate to adenosine, which is an anti-inflammatory mediator, thereby inhibiting $CD4^+$ T-cell proliferation [107]. Exosomes from activated effector $CD4^+$ T cells can interact with DCs through specific peptide–MHC I/TCR and CD54/LFA-1 interactions and inhibit the ability of the recipient DCs to stimulate $CD4^+$ T-cell proliferation, as well as inhibit an *in vivo* $CD8^+$ CTL response [108]. Moreover, $CD8^+CD25^+$ Tregs release exosomes that could inhibit a $CD8^+$ T-cell antitumor response [109]. Exosomes released from $CD4^+$ T cells containing FasL induce apoptosis in recipient T cells [110,111]. Further, human B-cell-derived lymphoblastoid cell lines (LCL) constitutively produce FasL-positive exosomes that can induce apoptosis in $CD4^+$ T cells [112].

The balance between exosomes that promote T- and B-cell activation and those that inhibit lymphocyte function is thought to be an important component in the pathogenesis of many diseases including cancers, cardiovascular diseases and autoimmune diseases. As reviewed below, during an infection, exosomes also function in immune modulation, including activation and inhibition of T cells, which likely has an important role in disease pathogenesis.

Exosomes and other extracellular vesicles in infectious disease

Exosomes and other extracellular vesicles, both pathogen- and host-derived, have been isolated and characterized in all known pathogen classes, including viruses, bacteria, fungi and parasites (Table 2). As one would expect, the composition and activity of these exosomes

Table 2. Exosome production by host cells upon infection.

Pathogen	Host resources	References
Viruses		
Human immunodeficiency virus (HIV)	DCs, T cells	68,71,119–121, 124,127
Epstein–Barr virus (EBV)	B cells	129–131,133–135
Cytomegalovirus (CMV)	Endothelial cells	137,138
Herpes simplex virus (HSV)	Melanoma cells	144–146
Hepatitis C virus (HCV)	Hepatocytes, Serum	70,139–142
Bacteria		
Chlamydia trachomatis	Fibroblast	198
Chlamydia pneumoniae	ECV304 cells	197
Mycobacterium tuberculosis	Macrophages, Plasma	10,11,180,181,186, 187,190,191
Mycobacterium bovis BCG	Macrophages, Plasma, BALF	186,189
Mycobacterium smegmatis	Macrophages	184
Mycobacterium avium	Macrophages	182–184
Salmonella typhimurium	Macrophages	10
Mycoplasma spp.	Tumor cells	195
Bacillus anthracis	Retinal pigment epithelial cells	196
Protozoa		
Leishmania donovani	Macrophages	156
Plasmodium vivax	Plasma	161
Plasmodium berghei	Plasma	164
Plasmodium falciparum	Erythrocytes	162–164
Plasmodium yoelii	Reticulocyte, Plasma	160
Toxoplasma gondii	Macrophages	10
Trypanosoma cruzi	Macrophages	165
Fungi		
Malassezia sympodialis	DCs, Plasma	176

vary significantly between the different taxa and even between pathogens in the same genus. Other factors such as the animal model used, the experimental design, the cell types chosen for the infection and which recipient cells are targeted can affect the observed immune response. We classify our current knowledge according to pathogen class and use specific examples to illustrate the major points.

Viruses

As mentioned above, a number of studies have recognized commonalities between the assembly and release of viruses and exosomes, and identified key host components that are used in both viral and exosome biogenesis. The readers are referred to recent reviews that have highlighted these interesting studies [113,114]. During the course of a viral infection, host cells release exosomes and other extracellular vesicles carrying viral and host components, which can modulate the immune response.

Exosomes and other extracellular vesicles generated during HIV infection have been extensively studied. Most studies have addressed the origin of viral budding (plasma membrane or exosomes) and the role of host proteins such as the ESCRTs in this process [115,116], although a few have looked at the role of host-derived extracellular vesicles in the pathogenesis of HIV. Exosomes and other extracellular vesicles released from infected PBMC or megakaryocytes and platelets contain CCR5 and CXCR4, respectively. In both cases, the chemokine receptors could be transferred to target cells, enhancing their susceptibility to HIV infection [117,118]. The HIV membrane protein Gag has been found on exosomes from Jurkat T cells, and this transport seems to be dependent on its oligomerization [119]. Pathogen-associated RNAs, including viral TAR RNA, have been detected in exosomes isolated from the supernatants of HIV-1-infected T cells and from patient sera. TAR RNA can down-regulate apoptotic signals, and therefore, recipient cells might support enhanced HIV replication [120]. Additionally, the HIV virulence factor Nef was detected in exosomes released from HIV-infected cells, the function of which has been extensively studied [121]. Mortalin, a member of the HSP70 family, appears to interact specifically with Nef and may play a role in its targeting to MVBs [122]. Work by Campbell and colleagues showed that Nef is present in exosomes secreted from transfected HEK 293 cells, which upon fusion with uninfected Jurkat T cells restores infectivity to Nef (-) HIV virions [121]. Nef-containing exosomes released from transfected SupT1 and Jurkat T cells can induce CD4$^+$ T-cell apoptosis *in vitro*, pointing to their possible role in the T-cell depletion inherent to HIV pathogenesis [68]. The effect of Nef may not be limited to T cells, as endothelial cell exposure to Nef results in an increased production of ROS and MCP-1 and increased endothelial cell apoptosis [123]. Nef expression also appears to affect the cellular miRNA content within exosomes, potentially limiting the effects of RNA interference in recipient cells [124]. In contrast to the immune inhibitory effects, exosomes secreted from CD8$^+$ T cells suppress HIV-1 transcription within infected cells in a protein-dependent but antigen-independent and MHC-unrestricted manner [125]. Furthermore, host-derived exosomes containing APOBEC3G, a cytidine deaminase that functions in cellular anti-retroviral activity, can inhibit HIV replication in the recipient cells [126]. These results suggest that exosomes and other extracellular vesicles can either promote or inhibit HIV replication, and the balance of these two functions will depend on the cells releasing the vesicles, the target cells and likely many other as yet undefined factors.

Human gamma herpesviruses, such as the Kaposi sarcoma-associated virus and EBV, have complex effects on the immune system, leading not only to viral infection but also, in some cases, to cancer, such as nasopharyngeal carcinoma (NPC). Most research on exosomes from herpesvirus-infected cells has focused on their role in cancer pathogenesis, more than on viral pathogenesis and immune responses [127,128]. Nevertheless, a few key studies have shed light on the immunology of EBV infection in the context of extracellular vesicles. Dukers and colleagues discovered that LMP1, a signal transduction protein important in EBV infection, could block the proliferation of T cells and inhibit NK-cell cytotoxicity [129]. LMP1 is present on exosomes from EBV-infected cells, suggesting that they could be a vehicle for the immunosuppressive effects of LMP1 during EBV infection. LMP2a was also found on

exosomes, but its function within these extracellular vesicles has not been explored [130]. Galectin-9, which is present in exosomes released from EBV-infected cells, induces apoptosis of EBV-specific CD4$^+$ T cells through its interaction with the T-cell immunoglobulin mucin-3. This receptor is known to negatively regulate both macrophage and T-cell activation [131]. However, exosomes from EBV-infected cells have also been shown to contain the dUTPase, which can induce NF-κB activation and cytokine release from primary DCs and PBMC, driving a pro-inflammatory/anti-viral response [132]. In addition, EBV encodes for a surprising number of miRNAs—44 have been currently identified [133,134]. These miRNAs not only modify the transcriptome of the infected cells, but also that of uninfected cells through the transfer of the viral miRNA through exosomes. Pegtel and colleagues demonstrated that EBV-infected B cells release exosomes containing EBV miRNAs that induce miRNA-mediated repression of confirmed EBV target genes, including *CXCL11*, an immunoregulatory gene involved in antiviral activity [135]. The ability of EBV miRNAs to be transferred from infected B cells to non-infected T cells and monocytes suggests that exosomal transport of viral miRNAs could contribute to EBV persistence in humans. This hypothesis is supported by the observation that EBV miRNA present in exosomes can target the miR-223 binding site in the 3′-untranslated region of the NLRP3 inflammasome, inhibiting production of IL-1β [136].

Exosomes released from CMV-infected human endothelial cells can stimulate memory CD4$^+$ T cells isolated from CMV-infected donors, likely through the transfer of antigen to allogenic DCs [137]. In the case of the human CMV, microvesicles released by infected cells contain soluble DC-SIGN, a C-type lectin family molecule, in complex with the CMV glycoprotein B. The transport of this complex through microvesicles increases the susceptibility of recipient cells to CMV [138] Similarly, in HCV-infected patients, the association of cellular membrane protein CD81 with HCV envelope glycoprotein E2, and the subsequent release of this complex within microvesicles, increases the infectivity of previously naïve recipient cells [139]. HCV structural proteins have been found in exosomes isolated from the sera of HCV-infected patients [139], and exosome-like vesicles have been purified from infected cells [140]. Purified exosomes isolated from HCV-infected human hepatoma cells contain intact viral particles with full-length viral RNA and protein, and these exosomes can transfer infectivity to naïve hepatoma cells, resulting in a productive infection [141,142]. Dreux and colleagues reported that exosomes released from HCV-infected cells can induce IFN-α release from uninfected plasmacytoid DCs due to the viral RNA present within the exosomes [70]. These results suggest that export of viral RNA may serve both as a viral strategy to evade pathogen sensing within infected cells and as a host strategy to induce an innate response in bystander cells. Similar findings were reported by Li and colleagues, who showed that IFN-α-treated liver non-parenchymal cells release exosomes that contain a number of host molecules with antiviral activity, and this activity can be transferred to hepatocytes that were previously permissive to hepatitis B virus infection [143]. HSV may divert transport of HLA-DR to MVBs and exosomes, thus limiting the amount of peptide–MHC complex on the cell surface of the HSV-infected cells, allowing the virus to evade detection by the immune system [144]. HSV also releases exosome-like vesicles, previously known as L particles, which are of
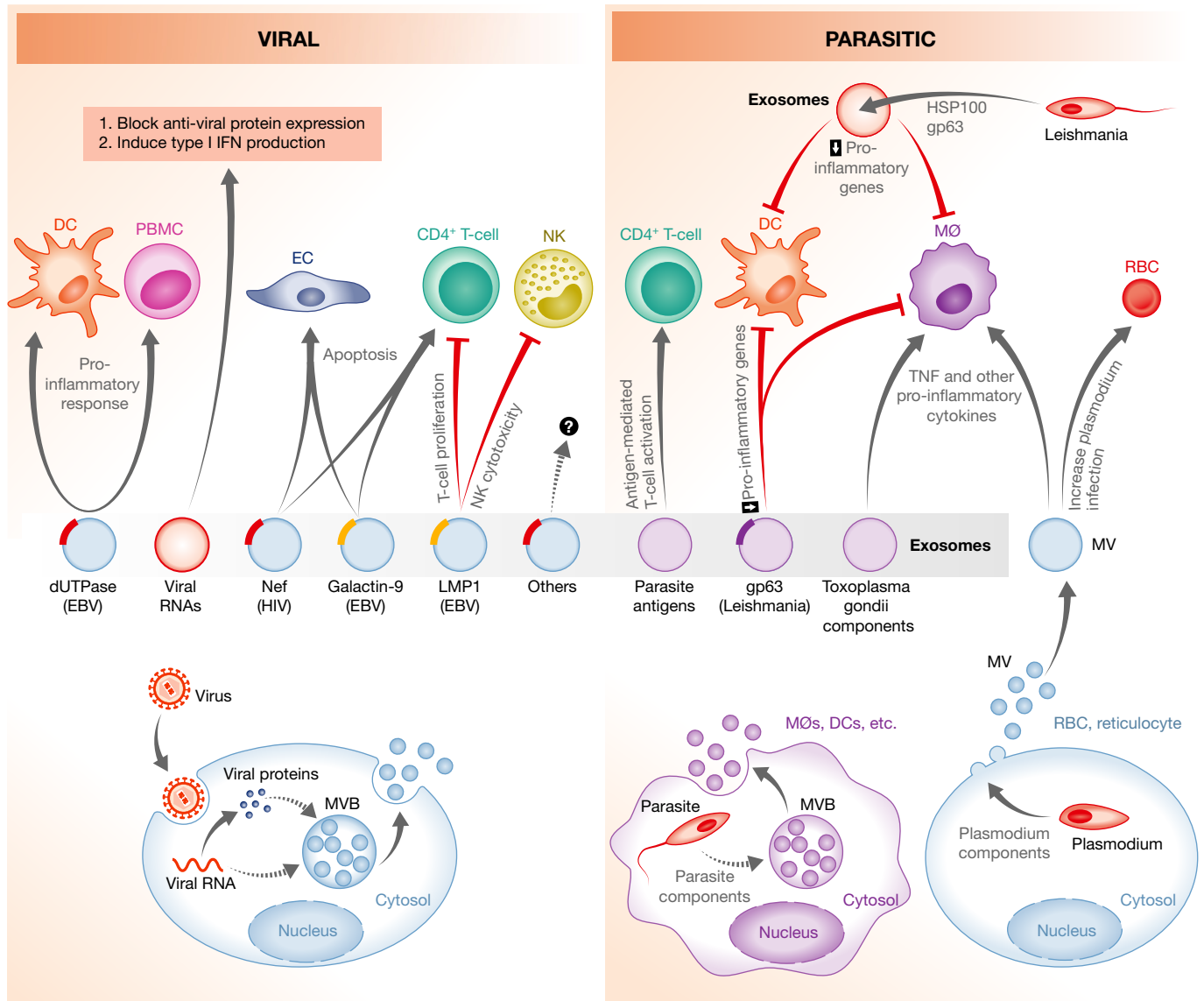
Figure 3. Modulation of host immunity by exosomes during a viral or parasitic infection.
Virus- or parasite-infected cells, or the parasites themselves, release exosomes or microvesicles that can stimulate T-cell activation by providing antigens to APCs. In contrast, exosomes containing microbial molecules, such as HIV Nef or *Leishmania* GP63, can block T-cell activation or induce the apoptosis of immune effector cells. Extracellular vesicles released from virus- or parasite-infected cells can modulate both the innate and acquired immune response. In some cases, this is to the benefit of the pathogen, whereas in others, this is to the benefit of the host. Dashed lines indicate unknown mechanisms. See Glossary for definitions.

similar size to exosomes and originate on an internal membrane, but are not exosomes [145]. These vesicles contain viral tegument proteins and glycoproteins, including transcription factors that can facilitate the replication of other viral particles in recipient cells [146]. The generation of a recombinant Coxsackie virus expressing 'fluorescence timer' protein (Timer-CVB3) has recently allowed to detect increased shedding of microvesicles containing virus in partially differentiated infected progenitor cells *in vivo*, suggesting their role in virus dissemination [147].

Extracellular vesicles have thus been implicated in the pathogenesis of many different viruses (Fig 3). Upon release, exosomes and other extracellular vesicles are 'captured' by cells and the transfer of host and viral proteins and/or RNA could enhance viral infection

and replication in recipient cells, as observed for HIV and other viruses. Furthermore, as highlighted above, exosomes released from virus-infected cells can inhibit both CD4^{+} and CD8^{+} T-cell activation. Other studies further suggest that infected cell-derived extracellular vesicles can promote the innate and acquired immune response through cytokine production and antigen presentation [143,148]. Additional studies are clearly needed to unravel how and when these extracellular vesicles promote or limit anti-viral immunity.

Parasites

Toxoplasma gondii was the first non-viral pathogen to be studied in the context of exosomes. In studies by Dimier-Poisson and

colleagues, DCs were pulsed with *T. gondii* proteins, and the exosomes released from the DCs could stimulate a protective immune response against acute and chronic *T. gondii* infection when adoptively transferred to mice. This response was antigen specific and included both cellular and humoral immunity [149]. When used as a vaccine, these exosomes were also associated with the development of fewer brain cysts in *T. gondii*-infected CBA/1 mice [150]. Exosomes isolated in a similar manner were shown to be an effective vaccine for preventing congenital toxoplasmosis when given to mice before pregnancy [151]. We found that exosomes released from the human monocytic cell line THP-1 after infection with *T. gondii* could stimulate non-infected THP-1 cells to produce TNF-α and other pro-inflammatory mediators [10], although the exosomal component responsible for this activity remains undefined. *T. gondii* resides within a vacuole with only limited contact with the endocytic network [152], so how the parasite components are transported to the MVB and onto exosomes is unclear. However, *T. gondii* may be in contact with the endosomes, at least transiently, through a microtubule network [153].

Leishmania spp. have also been well studied in the context of exosomes [16]. The initial studies by Reiner and colleagues established that pathogen-derived exosomes are a vehicle for *Leishmania* protein secretion and uptake by target macrophages [154]. Exosomes were proposed to be a mechanism to deliver *Leishmania* molecules directly into macrophages. *Leishmania* exosomes were also found to suppress the immune response and the heat-shock protein 100 (HSP100) to have an important role in the packaging of the parasite's proteins into exosomes, as its absence from exosomes resulted in different cargo and a different (pro-inflammatory) effect on immune cells [155]. A similar effect was seen *in vivo*, as mice treated with *Leishmania major* and *Leishmania donovani*-released exosomes prior to infection had higher parasite load compared to untreated mice, likely due to the suppression of the immune system by the exosomes [155]. Moreover, proteomic analysis of exosomes from *Leishmania mexicana*-infected macrophages identified parasite and host proteins, which differed between exosomes from uninfected and infected cells [156]. GP63—an important *Leishmania* virulence factor—is present in exosomes from infected cells, which can trigger the expression of genes related to the immune system in recipient cells. This includes signaling molecules such as MAP kinases (except JNK) and transcription factors like NF-κB. However, the overall effect is the down-regulation of pro-inflammatory genes and suppression of macrophage activation, which would promote parasite survival. This suppression may stem, at least in part, from the expression of GP63 within infected macrophages, as cells infected with a WT and GP63-deficient *L. major* released exosomes with significantly different host and microbial protein content, including proteins known to function in immune modulation [156,157]. Moreover, exosomes containing GP63 released from *L. donovani*-infected macrophages target the pre-miRNA processor Dicer1 in hepatocytes to prevent miRNP formation and block production of the miR122, resulting in altered serum cholesterol concentration and higher parasite burden [158]. In contrast, Schnitzer and colleagues showed that DC-derived exosomes containing *Leishmania* antigens, when used as a vaccine, provide protective immunity against cutaneous leishmaniasis [159].

The study of exosomes in the context of a *Plasmodium* infection is relatively new and has focused primarily on host-derived versus pathogen-produced vesicles. These studies suggest that host exosomes can modulate the host immune response or parasite survival (Table 2). In work by Martin-Jaular and colleagues, exosomes isolated from the blood of *P. yoelii*-infected BALB/c mice were found to contain parasite proteins and, when used as a vaccine in naïve mice, provided protection against subsequent *P. yoelii* infection [160]. These exosomes stimulated the production of IgG antibodies that recognized *P. yoelii*-infected red blood cells (RBC), decreased the level of parasitemia, allowed infected animals to survive longer and resulted in a preferential infection of reticulocytes over other RBC developmental stages. The production of exosomes during a *P. falciparum* infection may be more limited than during infections with the parasites described above, which replicate inside macrophages, as *Plasmodium* primarily infects RBCs, which lack MVBs. However, like *P. vivax* [161], *P. falciparum* can also infect reticulocytes and these infected cells could produce exosomes during a *P. falciparum* infection. In addition, *P. falciparum*-infected RBCs release exosome-like vesicles and microvesicles, which may enhance infectivity, as their number increases during an infection and they contain parasite components, including proteins that promote pathogen invasion of RBCs, such as EBA-175 and EBA-181 [162]. Interestingly, a recent study found exosome-like vesicles carrying the *P. falciparum* protein PfPTP2 released from infected RBCs, which promoted the sexual differentiation of a subset of parasites [163]. Couper and colleagues demonstrated that microvesicles isolated from the plasma of malaria-infected, but not naïve, mice induce potent, TLR4-dependent activation of macrophages *in vitro*, as measured by CD40 up-regulation and TNF-α production [164]. Conversely, host-derived microvesicles released from both *Trypanosoma cruzi*- and *P. falciparum*-infected cells limit host immune surveillance, leading to increased parasite production and transmission [165,166]. These studies suggest that microvesicles released from *Plasmodium*-infected host cells may play a prominent role in modulating the immune response. Most data suggest that they enhance the pro-inflammatory response, thereby linking microvesicles to disease pathology [167]. This conclusion is supported by the timing associated with microvesicle release, which peaks late during schizogony—a few hours prior to parasite egress—and therefore may partly drive the strong cytokine response associated with the 72-h *P. falciparum* infection cycle [166].

Exosomes have also been identified during other parasitic infections, including those by *Eimeria* spp., which are responsible for avian coccidiosis. Del Cacho and colleagues observed that DCs pulsed with *Eimeria* antigens or exosomes released from the antigen-pulsed DCs could be used as a vaccine against *Eimeria* infection in chickens [168]. Infection with the gastrointestinal parasite *Cryptosporidium parvum* was shown to increase exosome release from intestinal and biliary epithelial cells into the lumen of the gastrointestinal tract [169]. These exosomes also carried antimicrobial peptides from the epithelial cells, the export of which was increased through TLR4 activation by the parasite. The antimicrobial exosomes were shown to have a negative effect on the parasites *in vitro* and *ex vivo*, decreasing their ability to survive and infect host cells [169].

The study of exosomes and other extracellular vesicles in the context of a parasitic infection is complicated by the fact that both the pathogen and host make and release vesicles into the extracellular environment and both likely play a role in disease pathogenesis (Fig 3). Despite significant interest in exosomes in the context of

these infections, we have only begun to characterize their role in pathogenesis. Future studies should develop mechanisms to inhibit/modulate both pathogen- and host-derived vesicles.

Fungi

Most of the studies of extracellular vesicles in fungal infections have focused on exosome-like vesicles released directly by the fungi. Casadevall and colleagues demonstrated the export of exosome-like extracellular vesicles from the fungus *Cryptococcus neoformans* and found they could react with sera from patients infected with the fungus, indicating the presence of fungal antigens in/on vesicles [170–173]. As foreign materials, these exosome-like vesicles activate macrophages, leading to increased production of cytokines, such as TNF-α, and other anti-microbial molecules, thereby restricting fungal infection [172]. In contrast, other studies suggest that these vesicles may promote fungal virulence. Panepinto and colleagues showed that interfering with the export of exosomes from *C. neoformans* by knocking down *Sec6* (a gene involved in the fusion of exocytic vesicles with the cell membrane) resulted in decreased virulence of the fungi *in vivo* [174]. Knockdown of *Sec6* completely inhibited extracellular vesicle production in *C. neoformans*, including that of fungal exosomes, and blocked the export of a major virulence factor, laccase. This enzyme is required for the synthesis of melanin, an important molecule for fungal virulence [171]. The importance of melanin in fungal pathogenesis is suggested by the recent observation that fungal cell walls harboring melanin promote fungal infection by blocking macrophage phagocytosis [175]. A number of studies have shown the release of extracellular vesicles from other fungal species, such as *Malassezia sympodialis* and

Paracoccidioides brasiliensis, but they have provided limited insight into the influence of the vesicles in pathogenesis [176–179]. The role of extracellular vesicles in fungi has been comprehensibly reviewed recently [17].

Bacteria

Exposure of the immune system to bacterial and other microbial components is key to both control of the pathogen and subversion of the immune system by the pathogen. Many of the bacterial components known to be involved in the activation/subversion of the immune response are secreted or released from the bacteria during an infection. Understanding how these bacterial components disseminate is important to our understanding of the disease and how our immune system responds to the infection. As highlighted above for viral and parasitic infections, exosomes could play a vital role in this dissemination process. Much of our information regarding exosome production and function during a bacterial infection stems from work on mycobacteria, which will be discussed first, followed an analysis of exosomes in other bacteria.

Russell and colleagues observed that *M. tuberculosis* PAMPs, such as LAM and PIM, are transported from the phagosome to the MVB during a macrophage infection. These PAMPs are also found in extracellular vesicles released by infected macrophages, and their content can be detected inside neighboring uninfected cells [180]. These vesicles have markers of a late endosomal/lysosomal compartment and are released through calcium-dependent exocytosis [181], implying they are exosomes.

Our studies have expanded on these original observations (Fig 4). We determined that *Mycobacterium avium*-infected macrophages
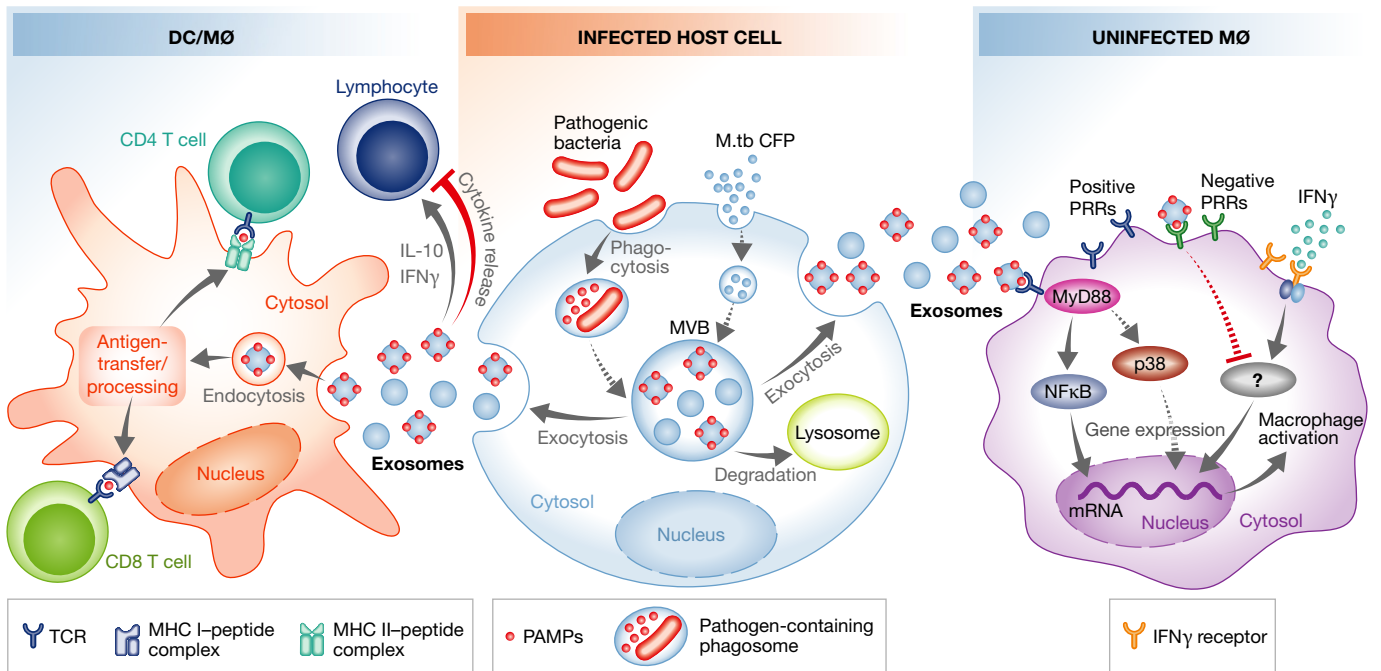

Figure 4. Bacteria-infected cells release exosomes that modify T-cell and macrophage function.
Exosomes from bacteria-infected macrophages release exosomes containing antigens that induce cross-priming to activate antigen-specific CD4^{+} and CD8^{+} T cells. In contrast, some exosomes released from infected cells inhibit cytokine production by T cells. Exosomes from infected cells also contain PAMPs that stimulate macrophage production of pro-inflammatory mediators like TNF-α, or limit the macrophage response to IFN-γ stimulation. Dashed lines indicate unknown mechanisms. See Glossary for definitions.

release vesicles that can stimulate a pro-inflammatory response in non-infected or 'bystander' macrophages [182]. Similar results were reported by Wang and colleagues [183]. Anand and colleagues observed increased exosome production in macrophages infected with *M. avium* and *M. smegmatis* compared to uninfected cells, as well as increased levels of the host protein HSP70, a protein they found could activate macrophages *in vitro* [184]. Exosomes released from *M.tb*- or *M. bovis* BCG-infected macrophages were also shown to be pro-inflammatory [10]. The mycobacterial 19-kDa lipoprotein present on exosomes that are released from *M. tuberculosis*-infected cells was later shown to be a primary driver of this inflammatory response, which depends on the TLR/MyD88 pathway [185]. Moreover, exosomes isolated from the bronchoalveolar lavage fluid (BALF) of *M. bovis* BCG-infected mice contained mycobacterial components, including the 19-kDa lipoprotein, and were pro-inflammatory *ex vivo*. In addition, exosomes from *M. bovis* BCG- or *M.tb*-infected macrophages could stimulate a pro-inflammatory response *in vivo*, as intranasal injection of mice induced TNF-α and IL-12 production, as well as recruitment of macrophages and neutrophils to the lung [10]. Macrophages treated with exosomes from *M.tb*-infected macrophages secrete chemokines that induce naïve macrophage and T-cell migration *in vitro* [186]. Together, these results suggest that exosomes from mycobacterial-infected cells can promote both recruitment and activation of immune cells *in vitro* and *in vivo* and may play a role in promoting the innate immune response upon a mycobacterial infection. However, the mycobacterial components present on/in exosomes could also function to suppress the immune response. In recent studies, we observed that exosomes from *M.tb*-infected cells could partially suppress the ability of recipient macrophages to respond to IFN-γ, inhibition that was dependent on macrophage expression of TLR2 and MyD88 [187]. Additional studies are needed to define the receptors and signaling responses induced upon exosome–macrophage interaction and how these interactions/responses change as the exosome composition is modified during an infection. In addition to their effect on macrophage activation, exosomes from *M.tb*-infected cells have also been shown to contain a member of the PE family (Rv1818c) that can induce apoptosis in Jurkat T cells [188], suggesting that the inhibitory effect of these exosomes may extend to T cells.

However, exosomes released from *M.tb*- or *M. bovis* BCG-infected cells, or from *M.tb* culture filtrate protein (CFP)-treated macrophages, can also activate antigen-specific CD4^{+} and CD8^{+} T cells *in vivo* and promote the activation and maturation of bone marrow-derived dendritic cells (BMDCs) [189]. These exosomes induce a Th1 immune response, as defined by antigen-specific T-cell production of IFN-γ [187,189]. Moreover, vaccination of mice with exosomes released from macrophages treated with CFP protects mice against a low-dose aerosolized *M.tb* inoculation, equivalent to BCG-vaccinated mice [190]. The release of mycobacterial antigens from infected macrophages is not limited to exosomes. Ramachandra and colleagues observed that infection with *M.tb* or *M. bovis* BCG resulted in increase in both exosome and microvesicle release, and both vesicles could stimulate an antigen-specific T-cell response [191]. Together, these results suggest that exosomes and perhaps other extracellular vesicles are a source of antigen to stimulate the acquired immune response. However, other mechanisms for antigen delivery during a mycobacterial infection have been proposed, including necrotic cells, apoptotic bodies and release of free antigen [192–194]. Unfortunately, our ability to test the relative importance of exosomes in antigen delivery is limited, due to the lack of molecular tools to block exosome production in macrophages without affecting other aspects of vesicular transport and without blocking exosome production by other cell types.

In addition to mycobacteria, exosomes from *Salmonella*-infected macrophages are also pro-inflammatory, increasing TNF-α production by human monocytes (Fig 4) [10]. These exosomes contain LPS, a known PAMP present on *Salmonella* and other gram-negative bacteria. Exosomes from cells infected with *Mycoplasma* induce a mixed cytokine response, including production of both IFN-γ and IL-10 from B cells. However, these exosomes appear to be primarily inhibitory, at least in the context of T-cell activation [195]. Exosomes can also be carriers of toxins, as shown by Abrami and colleagues, who found lethal factor—a well-characterized toxin produced by *Bacillus anthracis*—packaged into intraluminal vesicles and released on exosomes when expressed in a human epithelial cell line [196]. Ettelaie and colleagues reported that 'microparticles' released from *Chlamydia pneumoniae*-infected cells contain TF, a blood coagulation protein, which has also been associated with cell proliferation, migration and apoptosis. The TF-positive microparticles activate NF-κB, the transcription factor that partially regulates TF expression in endothelial cells. *C. pneumoniae* elementary bodies were also proposed to be released in microparticles, pointing to a potential role in the dissemination of the infection through the bloodstream. These findings have implications not only for the control of the infection by the host, but also for the potential cardiovascular consequences in relation to inflammatory conditions such as atherosclerosis [197]. Although the *C. pneumoniae* vesicles were referred to as microparticles, the isolation procedure used would enrich for exosomes. Several cytotoxic and secreted proteins were also associated with host vesicles released from *Chlamydia trachomatis*, which might function in the delivery of virulence factors [198].

Additional studies have analyzed the presence of bacterial antigens within host exosomes, with an eye toward developing cell-free vaccines against bacterial pathogens. Colino and colleagues treated BMDCs with diphtheria toxin (DT), isolated exosomes from the cells and injected them into mice. The exosomes stimulated an IgG response specific for DT [199]. Similar results were obtained with *Streptococcus pneumoniae*. Exosomes from BMDCs pulsed with *S. pneumoniae* capsular polysaccharide 14 antigen (Cps14) were enriched in Cps14 and could stimulate a protective IgM and IgG response against *S. pneumoniae* when injected into naïve mice [200].

Summary

Together, the results discussed above suggest that microbial and host components can spread beyond the infected cell through exosomes to either activate or suppress immune responses. We hypothesize that exosomes will be involved in multiple steps during the infection process, including formation/modification of infection loci, discrimination of antigens during the initial stages of infection, source of antigens for activation of T cells and B cells and modulation of immune cell function. In addition, the exosomes released from infected cells could also interact with non-immune cells such as fibroblasts and endothelial cells, influencing matrix deposition, vascular permeability, etc., all of which could impact the outcome of an infection (see Sidebar A).

Exosomes: good or bad for the host?

Whether exosomes activate or suppress the immune response depends on multiple factors, as discussed above. One of them is the source of the exosomes, that is, the cell type from which the exosomes were derived and/or the bodily fluid from which they were isolated. For example, exosomes from *Leishmania*-infected DCs were found to be immune stimulatory, making them an effective vaccine against the parasite [159]. In contrast, exosomes from infected macrophages were found to suppress the immune response to the parasite, promoting its survival in the host [155,156]. These different responses stem from changes in exosome composition, including their internal cargo (proteins and RNA) and their surface markers. Differences in the composition of exosomes would, in turn, lead to different effects on the recipient cells. A proteomic analysis of exosomes released from *M.tb*-infected or CFP-treated macrophages identified 41 and 29 mycobacterial proteins present in the exosomes from infected and CFP-treated macrophages, respectively [11]. Many of the proteins identified had been previously characterized as dominant antigens [201,202]. There was also significant overlap between the mycobacterial proteins present in the two populations of exosomes. In contrast, a proteomic analysis of exosomes from *Leishmania*-infected macrophages revealed few parasite proteins and limited differences in host proteins between exosomes from infected compared to uninfected macrophages [156]. One of the few differences was the presence of GP63 on exosomes from infected cells. GP63 is immune modulatory, but not pro-inflammatory, and contributes to parasite survival by down-regulating the immune response of the host. Another example of the significant effect that the presence or absence of a single protein can have on the immune response includes work by Miksa and colleagues in a rat septic model, where exosomes with or without MFG-E8 (milk fat globule epidermal growth factor 8) had a significantly different effect on removal of apoptotic bodies and survival of the rats. MFG-E8-containing exosomes down-regulated the inflammatory response, reducing TNF-α and IL-6 production [203]. In this setting, in which increased immune response to bacteria could be detrimental (by leading to sepsis), the exosomes were able to dampen the immune response due to the presence of MFG-E8 within the vesicles.

Understanding the beneficial or harmful effects of exosomes in the context of an *in vivo* infection is a key goal that will require additional insight into exosome biogenesis, and new tools to specifically block exosome release in a cell-specific manner. At present, targeting Rab GTPases or the various ESCRT proteins does not afford the control and specificity we need to answer these questions. Other factors, such as adaptor proteins that are specific for the tethering and fusion of MVBs with the plasma membrane, would be logical choices. Unfortunately, although SNAREs such as VAMP7 have been implicated in the MVB–plasma membrane fusion [204], this SNARE is also involved in other intracellular membrane fusion processes and therefore not a useful target for blocking exosome biogenesis.

Exosomes as vaccines

The concept of using exosomes as vaccines has its origin in the cancer field. Exosomes released from dendritic cells pulsed with tumor antigens have been tested in clinical trials as tumor 'vaccines': a treatment that aims to mobilize a patient's immune system to recognize and destroy the tumor cells. More recently, the potential use of exosomes as vaccines against infectious diseases has been assessed. Specific examples have been discussed in the different pathogen sections above. There are a number of potential advantages to using exosomes as vaccines against pathogens. These include: (i) more stable conformational conditions for the proteins; (ii) improved molecular distribution due to the ability of exosomes to circulate in bodily fluids and reach distal organs; (iii) more efficient association with the antigen-presenting cells, due to the expression of adhesion molecules on exosomes; and (iv) the fact that exosomes are one of the body's 'natural' mechanisms for transporting antigens between cells and one that likely plays a role in cross-priming.

However, although the use of exosomes allows for a cell-free-based vaccine, there are both conceptual and practical issues that need to be addressed before this potential application can become a reality. These include obtaining exosomes with the correct mix of antigens that provide protection, being able to reproducibly generate exosomes with this correct antigen composition, the risks of introducing 'non-self' human molecules into a vaccinated individual, among other issues. This latter point is particularly important, as unlike the use of exosomes as vaccines in cancer patients—which uses exosomes obtained from autologous cells—a vaccine against a particular pathogen will likely be derived from a human cell line and therefore will have proteins and other molecules specific to this cell line. The effect of these 'foreign' antigens on the recipient's immune response is unknown, and additional experiments will be required before performing any clinical studies. Nevertheless, the available data indicate that exosomes may provide a unique approach to vaccine generation against various pathogens, and this area will likely grow significantly in the coming years.

Exosomes as a source of diagnostic markers

Exosomes have been isolated from many different body fluids, including serum, bronchoalveolar lavage fluid, urine, saliva and several others [205–207]. A number of studies have shown quantitative and qualitative differences in exosome composition between healthy individuals and those with underlying diseases, including cancers and renal diseases [208]. These differences, combined with their easy accessibility, make exosomes excellent biomarker candidates. The use of exosomes to diagnose infectious diseases has been less studied, but shows great promise, as the markers could be both host and pathogen derived. Some support for this idea comes from the fact that serum exosome levels are significantly elevated in *M. bovis* BCG-infected mice compared to uninfected controls, and exosome concentration correlates with bacterial load [186]. Moreover, the exosomes isolated from the bronchoalveolar lavage fluid of *M.tb*-infected mice contain mycobacterial proteins, some of which are present in exosomes throughout a 112-day infection [209]. Finally, we have isolated exosomes from TB patient serum and identified a number of mycobacterial proteins, some of which were consistently found in the patient population under study, suggesting that they could be viable biomarkers for disease [210]. Surprisingly, we have also found that macrophages infected with *M.tb* release

exosomes containing mycobacterial RNA (J.S. Schorey, unpublished data), which suggests that exosomal RNA may be a useful marker for active TB. Further experiments are warranted to determine whether RNA from other intracellular pathogens is also present in exosomes.

From the perspective of host markers, Welker and colleagues found the exosomal protein CD81 to be elevated in the serum of patients with chronic hepatitis C infection compared to healthy controls. There was a correlation between serum CD81 elevation, higher ALT levels (a measure of liver inflammation) and more severe liver fibrosis. This suggests that measuring CD81 in the exosomal fraction of patient serum could be useful in the diagnosis or in following the course of chronic hepatitis C infection [211]. These examples illustrate the excellent potential for exosomes and other extracellular vesicles in the diagnosis and prognosis of infectious diseases.

Conclusion and future directions

Our understanding of the relevance of exosomes in host–pathogen interactions is still at an early stage relative to other fields of study, particularly cancer, on which most studies on exosomes have focused. Nevertheless, there are interesting and compelling results regarding the importance of exosomes and other extracellular vesicles during infection, and continued growth in this area will allow for a better understanding of virulence mechanisms and immune responses, as well as the development of new diagnostics and vaccines. However, more work is clearly needed on defining exosome composition and function during the course of an infection. This should include defining the cell types that produce the exosomes, the exosome recipient cells, the intracellular signaling pathways affected by exosomes, etc. Critical to this work will be the development of methods to specifically block exosome production and evaluate disease outcome, as a way to determine whether exosome production benefits the host or is used by the pathogen to subvert the immune response.

Acknowledgements

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