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Classification, Functions, and Clinical Relevance of Extracellular Vesicles

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***Abstract*——Both eukaryotic and prokaryotic cells release small, phospholipid-enclosed vesicles into their environment. Why do cells release vesicles? Initial studies showed that eukaryotic vesicles are used to remove obsolete cellular molecules. Although this release of vesicles is beneficial to the cell, the vesicles can also be a danger to their environment, for instance in blood, where vesicles can provide a surface supporting coagulation. Evidence is accumulating that vesicles are cargo containers used by eukaryotic cells to exchange biomolecules as transmembrane receptors and genetic information. Because also bacteria communicate to each other via extracellular vesicles, the intercellular communication via extracellular cargo carriers seems to be conserved throughout evolution, and therefore vesicles are likely to be a highly efficient, robust, and economic manner of exchanging information between cells. Furthermore, vesicles protect cells from accumulation of waste or drugs, they contribute to physiology and pathology, and they have a myriad of potential clinical applications, ranging from biomarkers to anticancer therapy. Because vesicles may pass the blood-brain barrier, they can perhaps even be considered naturally occurring liposomes. Unfortunately, pathways of vesicle release and vesicles themselves are also being used by tumors and infectious diseases to facilitate spreading, and to escape from immune surveillance. In this review, the different types, nomenclature, functions, and clinical relevance of vesicles will be discussed.**

I. Introduction

A. Cell-Derived Vesicles

Both eukaryotic and prokaryotic cells release vesicles, which are spherical particles enclosed by a phospholipid bilayer. The diameter of vesicles typically ranges from 30 nm to 1 μm (Conde-Vancells et al., 2008), the smallest being some 100-fold smaller than the smallest cells. Because cells release vesicles in their environment, body fluids such as blood and urine, but also conditioned culture media, contain numerous cell-derived vesicles, usually more than 10^{10} per ml (Yuana et al., 2010; Dragovic et al., 2011). It is becoming increasingly clear that most vesicles have specialized functions and play a key role in, for example, intercellular signaling, waste management, and coagulation. Consequently, there is a growing interest in the clinical applications of vesicles. Vesicles can potentially be used for therapy, prognosis, and biomarkers for health and disease. Nevertheless, because of the small size and heterogeneity of vesicles, their detection and classification is challenging (van der Pol et al., 2010). Although different types of vesicles have been identified, widely used terms, such as "exosomes" and "microparticles," are often inconsistent, especially in the older literature. Even if "purified exosomes" are claimed to be measured, it is prudent to remain cautious. Moreover, it should be emphasized that the extent to which vesicles really contribute to processes underlying physiology and pathology is virtually unexplored, and therefore one should also remain cautious to extrapolate results from in vitro studies on vesicles and their functions to the in vivo situation.

In this review, the focus will be on exosome-mediated signaling, although we will also outline recent developments on other vesicles within the relatively novel and rapidly expanding research field. A screening of the literature on cell-derived vesicles provided more than 6000 publications, from which approximately 500 were selected for review, with emphasis on recent publications from high-impact journals.

B. History

The discovery of cell-derived vesicles dates back to 1940, when preliminary studies were performed, addressing the "biological significance of the thromboplastic protein of blood" (Chargaff and West, 1946). Clotting times of plasma were determined after centrifugation at different speeds, and prolonged high-speed centrifugation (150 min at 31,000*g*) was shown to significantly extend the clotting time of the supernatant. Furthermore, when the pellet containing "the clotting factor of which the plasma is deprived" was added to plasma, the clotting times shortened, indicating that cell-free plasma contains a subcellular factor that promotes clotting of blood (Chargaff and West, 1946). More than 20 years later, in 1967, this subcellular fraction was identified by electron microscopy and was shown to consist of small vesicles, originating from platelets and termed

"platelet dust" (Wolf, 1967). These vesicles were reported to have a diameter between 20 and 50 nm and had a density of 1.020 to 1.025 g/ml (Wolf, 1967). One decade later, fetal calf serum was also shown to contain "numerous microvesicles" ranging in diameter from 30 to 60 nm (Dalton, 1975).

Meanwhile, within a completely different line of research, the term "exosomes" was introduced when vesicles were isolated from conditioned culture medium of sheep reticulocytes. These vesicles had several characteristic activities in common with the reticulocyte plasma membrane, including the presence of the transferrin receptor, whereas cytosolic enzyme activities were not detectable. Therefore, it was concluded that "vesicle externalization could be a mechanism for shedding of specific membrane functions, which are known to diminish during maturation of reticulocytes to erythrocytes" (Johnstone et al., 1987). Because these exosomes contained the transferrin receptor but expressed no lysosomal activities, it was also suggested that there may be a common mechanism to segregate and externalize specific plasma membrane proteins (Johnstone et al., 1989). The formation of the transferrin receptor-containing exosomes proved to be a major route for removal of plasma membrane proteins. Because not only mammalian but also embryonic chicken reticulocytes were shown to produce transferrin receptor-containing exosomes, this may be a conserved and common pathway (Johnstone et al., 1991; Grdisa et al., 1993). It was then discovered that exosomes are formed within multivesicular endosomes (MVEs[1]), also known as multivesicular bodies, and are being released when membranes of MVEs fuse with the plasma membrane. This pathway of protein sorting turned out to be highly selective, because other major transmembrane proteins, such as the anion transporter, are fully retained within the mature red cell and are absent within exosomes (Johnstone, 1992). Taken together, these early studies revealed that exosomes might be essential in a sophisticated and specific mechanism to remove obsolete transmembrane proteins.

[1]Abbreviations: BAL, bronchoalveolar lavage; DC, dendritic cell; Dll4, δ-like 4; EBV, Epstein-Barr virus; EGFR, epidermal growth factor receptor; ESCRT, endosomal sorting complex required for transport; FasL, Fas ligand; FFFF, flow field-flow fractionation; hOVA, ovalbumin hydrolysate; HSP, heat shock protein; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; LMP, latent membrane protein; LPS, lipopolysaccharide; MHC, major histocompatibility complex; miRNA, micro-RNA; MVE, multivesicular endosome; NK, natural killer; NKG2D, natural killer cell ligand G2D; NSCLC, non–small-cell lung carcinoma; OMV, outer membrane vesicle; PMN, polymorphonuclear leukocyte; PrP, prion protein; PrP^{sc}, prion protein scrapie; PS, phosphatidylserine; siRNA, short interfering RNA; TEM, transmission electron microscopy; TF, tissue factor; TGF, transforming growth factor; TLR, toll like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VTE, venous thromboembolism; Wnt, Wingless.

C. Nomenclature

Because of the detection difficulties, the multidisciplinary research field, and different ways of classification, there is currently no consensus about the nomenclature of cell-derived vesicles. For example, cell-derived vesicles have often been called after the cells or tissues from which they originate [e.g., dexosomes (dendritic cell-derived exosomes) (Le Pecq, 2005), prostasomes (prostate-derived vesicles) (Stegmayr and Ronquist, 1982), matrix vesicles (vesicles in bone, cartilage and atherosclerotic plaques) (Tanimura et al., 1983), and synaptic vesicles (vesicles from neurons) (Fischer von Mollard et al., 1990)]. However, such names do not provide a clue for classification with regard to the type of vesicles involved.

1. Types of Vesicles in Recent Literature. In four recent reviews, vesicles were classified into between two and six major different types (Cocucci et al., 2009; Théry et al., 2009; Beyer and Pisetsky, 2010; Mathivanan et al., 2010). Two common types were distinguished unanimously [i.e., *exosomes* and *microvesicles* (also called shedding vesicles, shedding microvesicles, or microparticles)], and in three of these reviews, *apoptotic vesicles* (also called apoptotic blebs, or apoptotic bodies) became a separate class (Théry et al., 2009; Beyer and Pisetsky, 2010; Mathivanan et al., 2010). In addition, "ectosomes," "membrane particles," and "exosome-like vesicles" were distinguished on the basis of the physicochemical characteristics of vesicles, including size, density, appearance in microscopy, sedimentation, lipid composition, main protein markers, and subcellular origin [i.e., originating from intracellular compartments (exosomes) or plasma membranes] (Théry et al., 2009). Although this classification is the best and most extensive so far, it is difficult to use in daily practice. For instance, a vesicle with a diameter of 50 nm can be classified either as an exosome, ectosome, membrane particle, exosome-like vesicle, or apoptotic vesicle according to this scheme, and, as correctly mentioned by the authors "in practice, all vesicles preparations are heterogeneous, with different protocols allowing the enrichment of one type over another."

2. Types of Vesicles in This Review. In this review, we propose to distinguish four different types of eukaryotic cell-derived vesicles: 1) exosomes, 2) microvesicles (microparticles), 3) membrane particles, and 4) apoptotic vesicles, thereby omitting "ectosomes" and "exosome-like vesicles," because there is insufficient evidence to support the existence of these types of vesicles (Théry et al., 2009). The main characteristics of these types of vesicles are summarized in Table 1 and Fig. 1 illustrates the various types of vesicles.

"Ectosomes" have been omitted because 1) the reported size of these neutrophil-derived vesicles (50–200 nm) was based partially on flow cytometry using 200 nm beads, a procedure now known to lead to underes-

TABLE 1
Overview of the main characteristics of different types of eukaryotic cell-derived vesicles

	Diameter	Density	Morphology (TEM)	Cellular Origin	Origin	Composition
	nm	*g/ml*				
Exosomes	50–100[a-d]	1.13–1.19[a,c]	Cup-shaped[a–c,e]	Most cell types	Plasma membrane endosomes[f–j]	Biochemical composition known, but most proteins and lipids not unique for exosomes[a,b,e,k–n]
Microvesicles	20–1000[b,n–q]	Unknown	Cup-shaped	Most cell types	Plasma membrane[q–t]	Insufficiently known
Membrane particles	50–80, 600[u]	1.032–1.068[u]	Cup-shaped[u]	Epithelial cells only[u]	Plasma membrane[u]	CD133[u]
Apoptotic vesicles	1000–5000[m,n,v,w]	1.16–1.28[n]	Heterogeneous[w]	All cell types	Plasma membrane endoplasmic reticulum[x]	Histones, DNA[n,v–y]

[a] Escola et al., 1998.
[b] Heijnen et al., 1999.
[c] Raposo et al., 1996.
[d] Trams et al., 1981.
[e] André et al., 2004.
[f] Booth et al., 2006.
[g] Fang et al., 2007.
[h] Harding et al., 1983.
[i] Lenassi et al., 2010.
[j] Pan et al., 1985.
[k] Beyer and Pisetsky, 2010.
[l] Taylor and Gerçel-Taylor, 2005.
[m] Théry et al., 2009.
[n] Turiák et al., 2011.
[o] Dragovic et al., 2011.
[p] György et al., 2011a.
[q] Wolf, 1967.
[r] Allan and Raval, 1983.
[s] Crawford, 1971.
[t] George et al., 1976, 1982.
[u] Marzesco et al., 2005.
[v] Hristov et al., 2004.
[w] Kerr et al., 1972.
[x] Bilyy et al., 2012.
[y] Holmgren et al., 1999.

timation of the diameter and concentration of the vesicles as a result of differences in refractive indexes between the beads and the vesicles (van der Pol et al., 2010; Chandler et al., 2011; Van Der Pol et al., 2012), 2) these vesicles were observed in vitro only, and 3) neutrophils also release microvesicles, which was not known when the term "ectosomes" was introduced (Dalli et al., 2008).

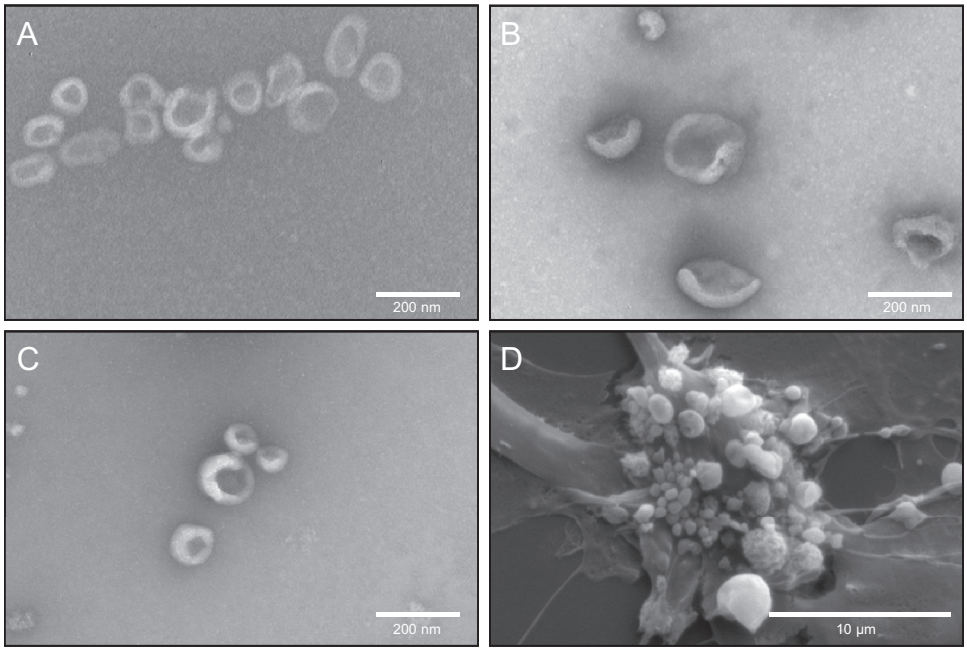

FIG. 1. Different types of eukaryotic cell-derived vesicles. Transmission electron micrographs of cell-derived vesicles isolated by differential centrifugation from plasma (A; exosomes, diameter <100 nm), urine (B; microvesicles, diameter >100 nm), or saliva (C; either exosomes or membrane particles, diameter <100 nm) of a healthy human subject. A scanning electron micrograph (D) showing a human umbilical vein endothelial cell releasing apoptotic vesicles. Please notice the much larger size of apoptotic vesicles compared with the other types of vesicles, and the typical cup shape of vesicles.

The "exosome-like vesicles" were omitted because 1) these vesicles, which were shown to contain the 55-kDa full length from the tumor necrosis factor (TNF)-α receptor, were erroneously reported to be mainly present in the 175,000*g* fraction of conditioned medium of human umbilical vein endothelial cells and bronchoalveolar lavage (BAL) fluid, whereas they are present predominantly in the 100,000*g* fractions (i.e., the fraction in which also exosomes are usually isolated) (Théry et al., 2009), 2) the transmission electron microscopy (TEM) micrographs show damaged and disrupted vesicles, which makes estimation of the real vesicle size difficult, and 3) endothelial-conditioned medium and BAL also contain exosomes (Admyre et al., 2003; Prado et al., 2008; Kesimer et al., 2009; Walker et al., 2009).

D. Current Limitations of Classification

Important criteria for classification with regard to the type of cell-derived vesicles are size, density, morphology, lipid composition, protein composition, and subcellular origin (Théry et al., 2009; van der Pol et al., 2010), which are summarized in Table 1. In the near future, it is expected that also the refractive index, ζ potential, and chemical composition will be accessible from individual vesicles to become novel relevant characteristics. It is important to point out the limitations and problems with the current criteria, which is necessary to fully understand and appreciate the literature about exosomes and other types of cell-derived vesicles.

1. Isolation. Because of the biological complexity of body fluids, isolation of vesicles has proven to be extremely difficult. For instance, isolation of vesicles from blood is affected by venipuncture, time between blood collection and handling, the anticoagulant, the applied separation process, the high viscosity of blood, and the presence of sticky proteins, including fibrinogen and albumin (Yuana et al., 2011). Because of their small size, vesicles are below the detection range of conventional detection methods. Consequently, recovery and contamination of the separation process cannot be reliably quantified, and isolation protocols have not been standardized. The inter-related difficulties of the detection and isolation of vesicles partly explains the differences in classification criteria and clearly exposes one of the main issues to be solved by the research field.

In most studies, vesicles are isolated by differential centrifugation. With centrifugation, the centrifugal force is used for the sedimentation of particulate matter, such as vesicles in solution. Separation of the various sorts of vesicles present in a sample is based on size and density, larger and denser components migrating away from the axis of the centrifuge and smaller and less-dense components migrating toward the axis. Differential centrifugation involves multiple sequential centrifugation steps, each time removing the pellet and increasing the centrifugal force to separate smaller and less dense components than the previous step. Typically, applied centrifugal accelerations are approximately 200 to 1500*g* to remove cells and cellular debris, 10,000 to 20,000*g* to pellet vesicles larger than 100 nm, and 100,000 to 200,000*g* to pellet vesicles smaller than 100 nm.

Besides the size and density of vesicles, the efficiency to isolate vesicles will depend on the shape and volume fraction of the vesicles, the volume, viscosity, and temperature of the fluid in which the vesicles are present, the centrifugation time, and the type of rotor used (fixed angle or swing-out). Because vesicles are heterogeneous in all aspects involved in differential centrifugation, complete separation of vesicles with a certain diameter or density is still utopian. For example, we recently applied differential centrifugation to the best of our ability to separate vesicles smaller than 100 nm from larger vesicles, all present in human saliva, and observed significant cross contamination (~10%) in both fractions (Berckmans et al., 2011).

Centrifugation also raises other problems that have to be taken into account. The removal of all cells from biological fluids can be challenging, for instance in the case of blood, where small platelets and apoptotic bodies overlap in size with large vesicles. When the centrifugal force applied to remove cells is too high, cells may fragment or become activated. Washing of vesicle pellets will often result in the loss of vesicles, resulting in variable yields. For instance, approximately 40 to 60% of platelet-derived vesicles are lost at every washing step, whereas vesicles from erythrocytes are unaffected (M. C. L. Schaap and R. J. Berckmans, personal communication). In addition, a high centrifugal acceleration of 100,000 to 200,000*g* may result in vesicle fusion and contamination of the pellets with proteins, thus hampering TEM and proteomic studies (Bard et al., 2004; Rood et al., 2010; György et al., 2011b). In addition, the functional properties of vesicles may change during isolation. For example, centrifugation of vesicles may increase exposure of phosphatidylserine (PS), thereby enhancing the ability of vesicles to promote coagulation (R. J. Berckmans, personal communication).

Besides differential centrifugation, filtration can be applied to isolate vesicles. Although the pore size of filters is often well defined, increasing forces have to be applied with decreasing pore size, which may result in artifacts.

Alternatively, vesicles can be fractionated by size with 10-nm accuracy using flow field-flow fractionation (FFFF) (Korgel et al., 1998), a technique that fractionates particles based on differences in their diffusion properties without applying forces equally high to differential centrifugation. Although FFFF is successfully applied to isolate exosomes from human neural stem cells (Kang et al., 2008), FFFF is not widely applied because it requires extensive optimization of the settings and is relatively expensive.

2. Size. Because vesicles are assumed to be spherical in their natural state, the size of vesicles given is usually

the diameter. The relation between the range of the diameter of vesicles and their concentration is the size distribution, preferably expressed as number of vesicles per unit particle size and suspension volume. However, because most size determinations of vesicles are based on TEM, the original volume of the suspension from which the vesicles originate cannot be assessed, and a differential size distribution is used, indicating the number of vesicles per unit particle size only. Because TEM is performed in a vacuum, fixation and dehydration are essential preparation steps likely to affect the size and morphology of vesicles (van der Pol et al., 2010). Novel methods have been explored to determine the size and concentration of vesicles directly in suspension, including atomic force microscopy (Siedlecki et al., 1999; Yuana et al., 2010), nanoparticle tracking analysis (Dragovic et al., 2011), and resistive pulse sensing. Typically, size distributions obtained from vesicles have the shape of a Gaussian or log normal distribution, but to what extent this reflects the natural population of vesicles is unknown, because the results are strongly influenced by (pre)analytical variables such as the isolation procedure and the detection limit of the applied detection technique. The (pre)analytical variables together with the choice of statistical parameters used to describe the size distribution (e.g., minimum, maximum, full width at half-maximum, mean, median, and mode diameter) all give rise to a marked range in the reported size for the different types of vesicles.

3. Density. The determination of the density of vesicles is usually based on sucrose gradient centrifugation. Because vesicles are very heterogeneous in all aspects involved in centrifugation, and because observed differences in the densities between types of vesicles are very small, discriminating types of vesicles by density is difficult.

4. Morphology. The morphology of cell-derived vesicles can be assessed with detection methods having subnanometer resolution, such as TEM and atomic force microscopy. For example, the morphology of exosomes has been traditionally described as "typical cup-shaped" after fixation, adhesion, negative staining, and visualization by TEM. However, to what extent this feature is either an artifact due to extensive sample preparation or actually unique for exosomes and does not apply to other vesicles is unknown (Marzesco et al., 2005). The cup-shaped morphology may still be a useful feature to distinguish cell-derived vesicles from similar-sized particles. With atomic force microscopy, the morphology of vesicles adhered to a surface can be studied directly in solution (Siedlecki et al., 1999; Yuana et al., 2010). However, because of the adhesion of vesicles to the surface, their structure changes from spherical to hemispherical or flat, depending on the composition of the membrane (Kanno et al., 2002), which may cause artifacts in the interpretation of both size and morphology.

5. Lipid Composition, Protein Composition, and Cellular Origin. Studies on the cellular origin and intracellular versus plasma membrane origin of vesicles are often based on the measurement of the lipid and protein composition of the *total* population of vesicles that has been isolated (e.g., by Western blotting and mass spectroscopy). Obviously, this approach does not provide information on the presence of contaminants, such as other types of vesicles or copurified proteins. For example, exosomes from malignant pleural effusions isolated by sucrose gradient centrifugation contain immunoglobulins (Bard et al., 2004). By far the most widely used method to establish the cellular origin and phenotype of *single* vesicles is flow cytometry, which is based on the detection of light scattering and fluorescence from labeled vesicles. Nevertheless, the smallest detectable single vesicle by flow cytometry varies between 300 and 700 nm on older generation flow cytometers (Van Der Pol et al., 2012). Modern flow cytometers using high collection angle optics are capable of detecting single polystyrene beads with a diameter as small as 100 nm (Robert et al., 2012), corresponding to vesicles with a diameter larger than 150 nm owing to refractive index differences (Chandler et al., 2011). Because most vesicles have a diameter smaller than 100 nm, it is not surprising that according to recent estimates only 1 to 2% of all vesicles present in biological fluids (e.g., plasma and urine) are actually detected (van der Pol et al., 2010; Yuana et al., 2010; Chandler et al., 2011). One popular solution to this problem has been to use specific capture beads that are sufficiently large to be measurable. The capture beads bind multiple smaller vesicles, thus facilitating their phenotyping by flow cytometry.

In sum, although there is no doubt that different types of cell-derived vesicles do exist, the interrelated difficulties of the detection and isolation of vesicles hamper the development of criteria to distinguish them. Consequently, the classification of vesicles is clearly work in progress. Nonetheless, recent studies have shown that the detection of single vesicles smaller than 100 nm is becoming feasible. Therefore, new developments on the detection of vesicles are likely to improve the criteria for classification (van der Pol et al., 2010; Yuana et al., 2010; Dragovic et al., 2011).

II. Properties of Cell-Derived Vesicles

A. Exosomes

Exosomes are cell-derived vesicles that are present in many and perhaps all biological fluids, including urine, blood, ascites, and cerebrospinal fluid (Pisitkun et al., 2004; Caby et al., 2005; Keller et al., 2006; Vella et al., 2008), fractions of body fluids such as serum and plasma, and cultured medium of cell cultures. The reported diameter of exosomes is between 30 and 100 nm and the density ranges between 1.13 and 1.19 g/ml. Exosomes are usually isolated by ultracentrifugation

(100,000–200,000*g*). The morphology of exosomes has been described as cup-shaped after fixation, adhesion, negative staining, and visualization by TEM. Regarding their biochemical composition, exosomes are surrounded by a phospholipid membrane containing relatively high levels of cholesterol, sphingomyelin, and ceramide and containing detergent-resistant membrane domains (lipid rafts) (Wubbolts et al., 2003; Simons and Raposo, 2009; Théry et al., 2009; Mathivanan et al., 2010). The membrane proteins have the same orientation as the cell. Exosomes are characterized by the presence of proteins involved in membrane transport and fusion, such as Rab, GTPases, annexins, and flotillin, components of the endosomal sorting complex required for transport (ESCRT) complex such as Alix, tumor susceptibility gene 101 (TSG101), heat shock proteins (HSPs), integrins, and tetraspanins, including CD63, CD81, and CD82 (Cocucci et al., 2009; Simons and Raposo, 2009; Théry et al., 2009; Beyer and Pisetsky, 2010; Mathivanan et al., 2010; Bobrie et al., 2011; Chaput and Théry, 2011; Record et al., 2011).

Although all of the aforementioned properties of exosomes are frequently reported and accepted, none of these properties is unique and identifies exosomes. There is increasing evidence that there is overlap between properties previously thought to be unique for exosomes and properties of other types of cell-derived vesicles, suggesting that there is a continuum of vesicle types with overlapping properties present in body fluids.

For example, the typically reported diameter of exosomes may be biased toward smaller particles for two reasons. First, exosomes are often isolated by differential centrifugation, which involves a loss of relatively large vesicles during removal of cells by centrifugation. Second, because in most studies only a limited number of exosomes are visualized by TEM, and because the size distribution of vesicles typically has the shape of a Gaussian or log normal distribution with a peak below 100 nm, as also confirmed by novel detection methods, such an analysis will easily overlook the presence of vesicles larger than 100 nm. Although these larger vesicles represent only a relatively small fraction of the total population, their total surface area or volume and thus their functional contribution may be relatively large.

Identification of exosomes based on their cup-shaped morphology after negative staining and visualization by TEM seems questionable. For example, exosomes and similar-sized vesicles, called membrane particles or prominosomes, both appear with a cup-shaped morphology on the same electron micrographs (Marzesco et al., 2005). Figure 1 shows that not only exosomes but also vesicles larger than 100 nm may appear cup-shaped by TEM. Finally, recent evidence indicates that not all exosomes originate from intracellular MVEs, the "classic pathway" of exosome formation, thereby making identification of exosomes even more complex.

1. Classic Pathway of Exosome Formation. The "classic pathway" of exosome formation is by far the best studied and involves the formation of intraluminal vesicles within MVEs (Fig. 2). In turn, MVEs can fuse with either lysosomes for cargo degradation or with the plasma membrane to secrete the intraluminal vesicles, which are then released as exosomes.

Different intracellular sorting pathways exist in directing proteins toward intraluminal vesicles predestined for either degradation or secretion, thus implicating the existence of different types of MVEs. Redundant transmembrane receptors are sorted to intraluminal vesicles predestined for lysosomal degradation after ubiquitination, a post-translational modification that is executed by ESCRT (Babst, 2005; Michelet et al., 2010).

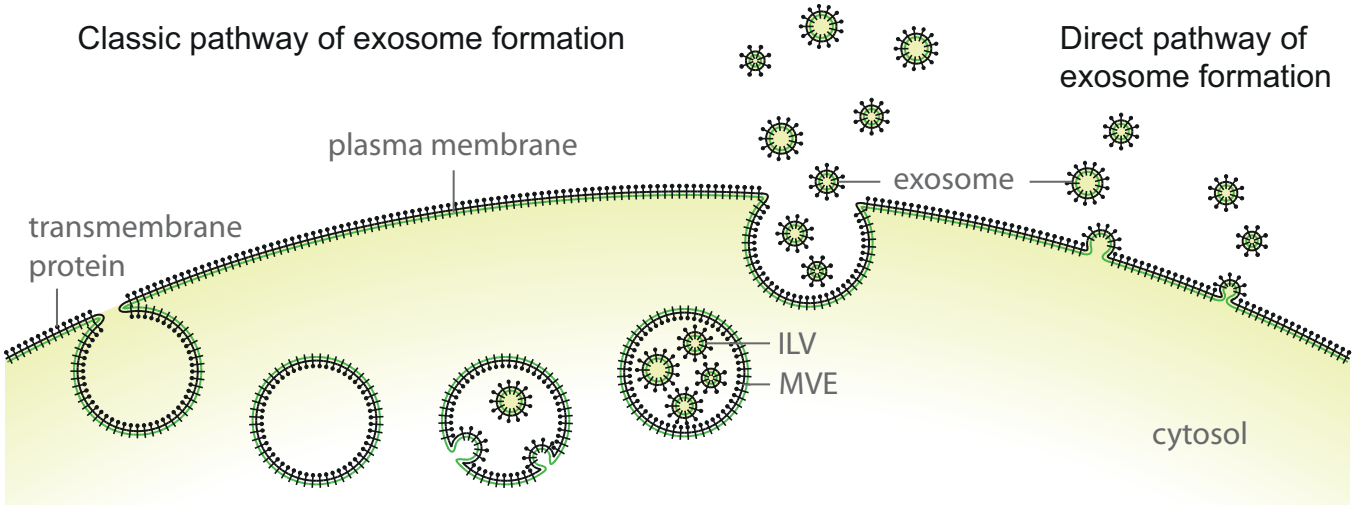

FIG. 2. Pathways of exosome formation. Cells release exosomes via two mechanisms. The classic pathway (left) involves the formation of intraluminal vesicles (ILVs) within MVEs. In turn, the membrane of MVE fuses with the plasma membrane, resulting in the release of ILVs. When secreted, ILVs are called exosomes. Alternatively, the direct pathway (right) involves the release of vesicles, indistinguishable from exosomes, directly from the plasma membrane.

On the other hand, there is no compelling evidence that the ESCRT is involved in the sorting of transmembrane receptors to intraluminal vesicles that are predestined to become secreted as exosomes (Théry et al., 2001; Buschow et al., 2005, 2009; Trajkovic et al., 2008; Simons and Raposo, 2009; Tamai et al., 2010; Bobrie et al., 2011). Although several ESCRT proteins (Théry et al., 2001) and ubiquitinated proteins (Buschow et al., 2005) are present in exosomes, the ubiquitinated proteins are soluble proteins and not transmembrane proteins, suggesting that ubiquitination may have occurred in the cytosol rather than by the ESCRT. Likewise, the sorting of the proteolipid protein to intraluminal vesicles predestined to become secreted as exosomes is independent from the ESCRT but depends on the sphingolipid ceramide (Marsh and van Meer, 2008; Trajkovic et al., 2008). Further evidence for two pathways comes from studies showing that lysobisphosphatidic acid induces the formation of intraluminal vesicles predestined for lysosomal degradation but does not affect the formation of exosomes (Matsuo et al., 2004). This is confirmed by studies in which cholesterol is labeled with perfringolysin O, which reveals perfringolysin O-positive and -negative MVEs in B cells, of which only the cholesterol-containing MVEs fuse with the plasma membrane resulting in the release of exosomes (Möbius et al., 2002). In addition, both epidermal growth factor and the epidermal growth factor receptor (EGFR) travel to exosomes via MVEs not containing the lipids bis(monoacylglycero)phosphate/lysobisphosphatidic acid, whereas the bis(monoacylglycero)phosphate/lysobisphosphatidic acid-containing vesicles are degraded by lysosomes (White et al., 2006). Thus, clearly two intracellular pathways of MVE sorting exist.

Cytosolic domains of proteins (Theos et al., 2006) or lipid domains enriched in the tetraspanins CD9 or CD63 are thought to play a role in the sorting of transmembrane proteins toward intraluminal vesicles (Buschow et al., 2009; Bobrie et al., 2011). Several different types of small GTPases from the Rab family play a role in the intracellular trafficking of MVEs toward either the plasma membrane (Savina et al., 2005; Hsu et al., 2010; Ostrowski et al., 2010) or to lysosomes for degradation (Stenmark, 2009), as well as cytosolic calcium levels (Savina et al., 2003; Fauré et al., 2006; Krämer-Albers et al., 2007), citron kinase, (Loomis et al., 2006), and a still unidentified combination of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors, which is involved in the final fusion of the MVE membrane with the plasma membrane (Rao et al., 2004).

2. Direct Pathway of Exosome Formation. Next to the "classic pathway" of exosome biogenesis, there is a second and much more immediate route of exosome formation (Fig. 2). T cells and erythroleukemia cell lines release exosomes directly from their plasma membrane, both spontaneously as well as upon expression of HIV Gag or Nef, or after cross-linking of surface receptors (Booth et al., 2006; Fang et al., 2007; Lenassi et al., 2010). These vesicles are indistinguishable from exosomes formed by the classic endosomal pathway because they are enriched in classic exosome markers such as CD63 and CD81 and have a similar diameter and density. The extent to which such exosomes are also released from other cells or in vivo (e.g., in biological fluids) is unknown.

B. Microvesicles

Microvesicles, often called microparticles, is a term used for vesicles that are released from the plasma membrane during cell stress. This term is also often used to describe total populations of vesicles isolated from biological fluids (Nieuwland et al., 1997; Heijnen et al., 1999; Beyer and Pisetsky, 2010). In addition, microvesicles are present in most if not all biological fluids, atherosclerotic plaques and conditioned culture medium (Mallat et al., 2000; Berckmans et al., 2002, 2011; Canault et al., 2007; Morel et al., 2011). Although microvesicles are believed to be larger than exosomes and are usually reported to range in size between 100 nm and 1.0 μm in diameter (Théry et al., 2009), there is much confusion on this matter. For instance, with regard to the diameter of vesicles in plasma from healthy human individuals, the following size ranges have been reported: 20 to 50 nm by TEM (Wolf, 1967), 200 to 800 nm by TEM (Turiák et al., 2011), 180 nm (mean) by TEM and atomic force microscopy (György et al., 2011a), and 80 nm (mean) by nanoparticle tracking analysis (Dragovic et al., 2011). Consequently, the size ranges of microvesicles and exosomes may overlap, especially when body fluids are used as a source for isolation of vesicles. In vitro, activated platelets release two clearly distinct populations of vesicles, small (<100 nm) vesicles exposing CD63, exosomes, and large (100 nm–1 μm) microvesicles exposing typical platelet receptors such as glycoprotein Ib (Heijnen et al., 1999). Therefore, when only a single cell type is studied in vitro, both types of vesicles may be distinguishable. The density of microvesicles is unknown, and microvesicles are usually isolated at 10,000 to 20,000*g* by centrifugation (Théry et al., 2009). The term "microparticles," however, have also been used for total populations of vesicles isolated from human plasma at 100,000*g* (Shet et al., 2003) and such populations will contain exosomes (Heijnen et al., 1999). Exosomes have a typical cup shape when studied by TEM (Fig. 1A), but larger vesicles (Fig. 1B) also show this morphological feature. Although exposure of PS is often mentioned as a typical marker for microvesicles (Théry et al., 2009; Beyer and Pisetsky, 2010), this seems questionable at best, because a significant number of microvesicles do not expose PS (Joop et al., 2001; Connor et al., 2010), and because exposure of PS is markedly increased by centrifugation and freeze-thaw procedures (Connor et al., 2010). The mechanisms underlying the formation of microparticles have been re-

cently summarized elsewhere (Morel et al., 2011). Taken together, although exosomes and microvesicles are distinct types of vesicles, neither size, morphology, nor exposure of PS is a sufficient criterion to distinguish both types of vesicles from each other.

C. Membrane Particles

The lumen of the neural tube of embryonic mouse brain contains ventricular fluid, in which two types of prominin-1 (CD133)-exposing vesicles are present (Marzesco et al., 2005), called prominosomes or membrane particles (Marzesco et al., 2005; Théry et al., 2009). One type of membrane particle has a diameter of approximately 600 nm, whereas the other type has a diameter between 50 and 80 nm as determined by TEM. Although the small type of membrane particles is precisely in the size range of exosomes, they showed a slightly lower density (1.032–1.068 g/ml) than exosomes on sucrose gradients, and they do not expose CD63. Because these $CD133^+$ vesicles 1) originate from the plasma membrane of epithelial cells; 2) occur in human body fluids that contact the epithelium, such as saliva, urine, and seminal fluid; and 3) coexist with exosomes in saliva (Marzesco et al., 2005; Berckmans et al., 2011), we assume these $CD133^+/CD63^-$ vesicles to be different from exosomes. The extent to which the larger type of membrane particle is different from microvesicles, however, will need additional studies.

D. Apoptotic Vesicles

When cells are undergoing apoptosis, they release PS-exposing vesicles, often called apoptotic bodies or vesicles. The major difference between apoptotic vesicles and other cell-derived vesicles is their size. In all studies so far, the diameter of apoptotic vesicles is reported to range between 1 and 5 μm (Kerr et al., 1972; Théry et al., 2001; Hristov et al., 2004; Turiák et al., 2011). This is in the precise size range of platelets in the human blood. Because activated platelets or platelets undergoing a process resembling apoptosis also express PS, it may be impossible to resolve apoptotic bodies from platelets based upon size and a PS-positive phenotype. The density of apoptotic vesicles is 1.16 to 1.28 g/ml, which is partly overlapping with the density of exosomes, and their morphology is typically more heterogeneous than other cell-derived vesicles when visualized by TEM.

The inappropriate clearance of apoptotic vesicles is considered to be the primary cause of developing systemic autoimmune disease. Apoptotic cells release at least two immunologically distinct types of apoptotic vesicles. Apoptotic vesicles originating from the plasma membrane contain DNA and histones, whereas apoptotic vesicles originating from the endoplasmic reticulum do not contain DNA and histones but expose immature glycoepitopes (Bilyy et al., 2012).

In general, the process of "membrane blebbing" is thought to precede the release of apoptotic vesicles and microvesicles. The extent to which this assumption is true, however, is unclear. Membrane blebbing requires phosphorylation of myosin light chain and Rho-associated coiled coil kinase I activity, which becomes constitutively active upon cleavage by caspase 3, induces a net increase in myosin light chain phosphorylation and subsequent membrane blebbing (Sebbagh et al., 2001). The key role of caspase 3, one of the executioner enzymes of apoptosis, in membrane blebbing is confirmed by the observation that a human breast cancer cell line, MCF7, which is deficient of functional caspase 3, lacks membrane blebbing (Jänicke et al., 1998). Alternatively, granzyme B cleaves Rho-associated coiled coil kinase II, which produces a constitutively active enzyme, and increases myosin light chain phosphorylation as well as membrane blebbing (Song et al., 2002).

III. Functions of Cell-Derived Vesicles

A. Intercellular Signaling

To summarize and present the extensive literature on exosome-mediated signaling, we have decided to review their functions and processes rather than the classes of molecules involved. As it is increasingly unclear what type of vesicles has been investigated in many studies, we will use the term as mentioned in the original work unless indicated otherwise.

1. Immune Suppression. By transporting ligands and receptors (Fig. 3), exosomes can orchestrate cell growth and development, and modulate the immune system. Activated T cells and peripheral blood mononuclear cells release vesicles exposing Fas ligand (FasL), a death receptor ligand, suggesting that these vesicles play a role in cell death during immune regulation (Martínez-Lorenzo et al., 1999). These vesicles are likely to be exosomes because FasL is stored in MVEs in T cells (Monleón et al., 2001). The sorting of FasL to MVEs and exosomes is regulated by phosphorylation and monoubiquitination (Zuccato et al., 2007), and the secretion of FasL-exposing exosomes is mediated by diacylglycerol

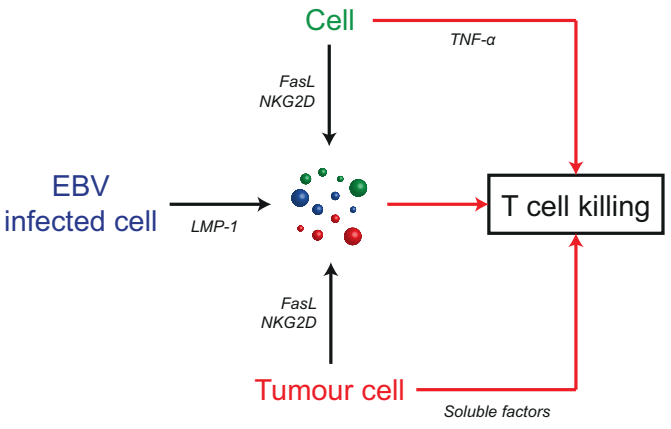

FIG. 3. Immune suppression. Cells, tumor cells, and EBV-infected cells release vesicles exposing proteins (LMP-1) or death receptor ligands (FasL, NKG2D) that induce T-cell apoptosis.

kinase-α (Alonso et al., 2005, 2011). Intraperitoneal injection of FasL-exposing exosomes triggers apoptosis of macrophages in vivo (Hohlbaum et al., 2001).

The ability of vesicles to modulate the immune response is likely to play a role in normal growth and development. For instance, the invading trophoblast is a semiallograft and should in theory be rejected by the mother as "foreign" material. During normal pregnancy, the trophoblast evades the maternal immune system by killing activated T cells that have been sensitized to paternal alloantigens. First-trimester trophoblast cells release exosomes exposing FasL, which are capable of inducing Fas-mediated T-cell death, suggesting that exosomes contribute to this immune privilege (Abrahams et al., 2004; Frängsmyr et al., 2005). Because sera from pregnant women exhibit higher levels of FasL-associated with exosomes at term compared with preterm, it is thought that immune suppression by exosomes may contribute to normal pregnancy and delivery (Taylor et al., 2006). Placenta-derived exosomes also expose several ligands of the natural killer (NK) cell receptor NKG2D, UL-16 binding proteins 1–5, and major histocompatibility complex (MHC) class I chain-related protein, which all down-regulate NKG2D on NK cells, $CD8^+$ cells, and $\gamma\delta$ T cells, thereby all potentially contributing to fetal immune escape (Hedlund et al., 2009).

The ability of exosomes to mediate cell killing at a distance is also used by tumor cells to evade the immune system. FasL is present in MVEs of melanoma cells and a prostate cancer cell line, and FasL-exposing vesicles from these tumor cells kill Jurkat T cells in vitro. Therefore, tumor-derived vesicles may provide an important front-line defense mechanism by inhibiting the homing and antitumor activity of immunocompetent cells (Andreola et al., 2002; Martínez-Lorenzo et al., 2004; Abusamra et al., 2005). In addition, epithelial ovarian cancer cells from ascites release FasL-exposing microvesicles that are capable of inducing T-cell apoptosis (Abrahams et al., 2003). Likewise, FasL-exposing microvesicles isolated from sera of patients with oral squamous cell carcinoma induce T-cell apoptosis, and in these patients, there is a correlation between the microvesicle-associated levels of FasL and tumor burden, suggesting that vesicle-mediated immune suppression (indirectly) promotes tumor growth and development in vivo (Kim et al., 2005a). FasL is not the only mediator of tumor-induced suppression of the immune system, because exosomes from human tumor cell lines and mouse mammary tumor cells inhibit (interleukin-2-induced) proliferation of NK cells, illustrating that tumor-derived exosomes suppress the cytotoxic response to tumor cells (Liu et al., 2006).

The loss of NKG2D, an activating receptor for NK, NKT, $CD8^+$, and $\gamma\delta^+$ T cells, in cancer is also one of the key mechanisms of immune evasion. Exosomes from cancer cell lines or isolated from pleural effusion of patients with mesothelioma down-regulate NKG2D expression of NK cells and $CD8^+$ cells via a transforming growth factor (TGF)-β-dependent mechanism, indicating that NKG2D may be one of the targets for exosome-mediated immune evasion (Clayton et al., 2008). In addition, the high incidence of relapse and fatal outcome in many blood malignancies such as leukemia and lymphoma has been associated with the expression of several NKG2D ligands on exosomes (Hedlund et al., 2011). Alternatively, differentiation of myeloid cells to myeloid-derived suppressor cells, immature myeloid cells that have the ability to suppress T-cell activation and thereby promote tumor progression, induce the expression of TGF-β and prostacyclin E2 within tumor-derived exosomes (Xiang et al., 2009; Liu et al., 2010). In another study, tumor-derived exosomes from mouse cell lines were shown to inhibit immune surveillance by binding to myeloid-derived suppressor cells, thereby increasing the immune suppressive activity of these cells. This interaction was mediated by the exposure of HSP72 on exosomes. Further evidence that tumor-derived exosomes contribute to immune suppression in vivo comes from the observation that inhibition of exosome production by a drug to treat high blood pressure, dimethyl amiloride, enhances the antitumor efficacy of the chemotherapeutic drug cyclophosphamide in mouse tumor models (Chalmin et al., 2010). Finally, local immune responses are inhibited by high levels of extracellular adenosine, which prevent T-cell activation. Exosomes from several cancer cell lines expose potent ATP and 5′AMP-phosphohydrolytic activity, thereby contributing to locally high concentration of adenosine. Exosomes from pleural effusion of patients with mesothelioma contained 20% of the total ATP-hydrolytic activity, indicating that exosomes may contribute to inhibition of T cells in the tumor environment by this mechanism (Clayton et al., 2011). Taken together, exosomes are likely to orchestrate the efficacy of the immune system by a whole set of different mechanisms important not only for normal development but also for tumor development.

Besides healthy and tumor cells, viruses and parasites exploit the ability of exosomes to kill immune cells at a distance to evade the immune system. The latent membrane protein-1 (LMP-1) of Epstein-Barr virus (EBV), which is exposed on exosomes from EBV-infected cells, inhibits the proliferation of peripheral blood mononuclear cells. This mechanism is thought to be relevant in development of EBV-associated tumors, such as nasopharyngeal carcinoma and Hodgkin's disease, by allowing tumor cells to evade the immune system (Flanagan et al., 2003). Cells infected with the intracellular parasite *Leishmania donovani* secrete exosomes that contain and deliver parasite proteins to host target cells, and suppress the immune response by preventing the activation of human monocytes (Silverman et al., 2010a; Silverman et al., 2010b). Exosomes from plasma of mice immunized to keyhole limpet hemocyanin specifically

suppress a keyhole limpet hemocyanin delayed-type hypersensitivity inflammatory response, which is partially dependent on exosomal FasL (Kim et al., 2007b).

Although considerable evidence thus suggests that cell-derived vesicles are involved in immune suppression, there are also studies showing that exosomes may exhibit opposing activity or do not contribute to suppression. For example, exosomes from synovial fibroblasts of rheumatoid arthritis patients expose TNF- α, which delays T-cell activation-induced cell death and thus contributes to apoptosis resistance (Zhang et al., 2006). In addition, soluble factors but not the microvesicles from patients with non–small-cell lung carcinoma (NSCLC) were shown to induce T-cell apoptosis (Prado-Garcia et al., 2008). Furthermore, exosomes from human pancreatic tumor cells inhibit tumor progression by counteracting constitutively active survival pathways, thereby inducing apoptosis of tumor cells rather than apoptosis of immune cells (Ristorcelli et al., 2008, 2009). In sum, the exact role and contribution of microvesicles or exosomes to immune suppression depends on the models studied and is likely to be complex.

2. Antigen Presentation. Vesicles are used by cells not only to suppress the immune system but also to present antigens (Fig. 4). For example, intestinal epithelial cells endocytose dietary proteins, and serum obtained from mice fed with a particular protein can suppress hypersensitivity reactions when this serum is administered to other mice before exposure to this protein. Processing of the antigen in the gut is required to achieve oral (immune) tolerance via serum transfer. Although the true nature of the "tolerogen" in serum is unknown, exosomes are likely candidate tolerogens, because human and mouse intestinal epithelial cells release exosomes exposing MHC class I and MHC class II molecules at both the apical and basolateral sides, suggesting a possible involvement of exosomes in the transcellular transport of antigens from the lumen of the gut to immune cells (van Niel et al., 2001; Van Niel et al., 2003). To test the ability of vesicles to deliver "transcellular information" to the immune system in vivo, mice received peritoneal injection with exosomes from epithelial cells that had been incubated with pepsin/trypsin ovalbumin hydrolysate (hOVA) to mimic luminal digestion. Exosomes from intestinal epithelial cells treated with hOVA and interferon-γ, which is added to increase the secretion of exosomes (Van Niel et al., 2003), expose abundant MHC-class II/OVA complexes. These exosomes, however, induce a specific humoral immune response in vivo, suggesting that these exosomes trigger an immunogenic rather than a tolerogenic response (Van Niel et al., 2003). Because these exosomes preferentially interact with dendritic cells (DCs), they can strongly facilitate antigen presentation to T cells, thereby providing luminal antigenic information to local immune cells and thus facilitating immune surveillance at mucosal surfaces (Mallegol et al., 2007).

In a mouse model of allergic asthma, serum or isolated exosomes from serum of donor mice fed with ovalbumin were transferred to mice followed by intranasal exposure to ovalbumin. Mice receiving serum or isolated exosomes from ovalbumin-fed mice showed tolerance to ovalbumin exposure, because these mice had a reduced concentration of airway eosinophils, lower serum levels of total IgE and ovalbumin-specific IgE, and an increased concentration of activated T cells with a regulatory phenotypethan control animals. Thus, exosomes have the ability to prevent allergic sensitization (Almqvist et al., 2008).

Exosomes, isolated from conditioned medium of B-cell lines originating from patients with birch pollen allergy and loaded with T-cell-activating peptides from a major birch allergen, induce a dose-dependent T-cell proliferation and increase the production of interleukin 5 and 13 by T cells, indicating that B-cell exosomes may contribute to and worsen allergic immune responses (Admyre et al., 2007). In contrast, exosomes from BAL fluid of mice exposed to an olive pollen allergen inhibit the IgE response, cytokine production, and airway inflammation, all signs of allergy, upon exposure to the allergen, show-

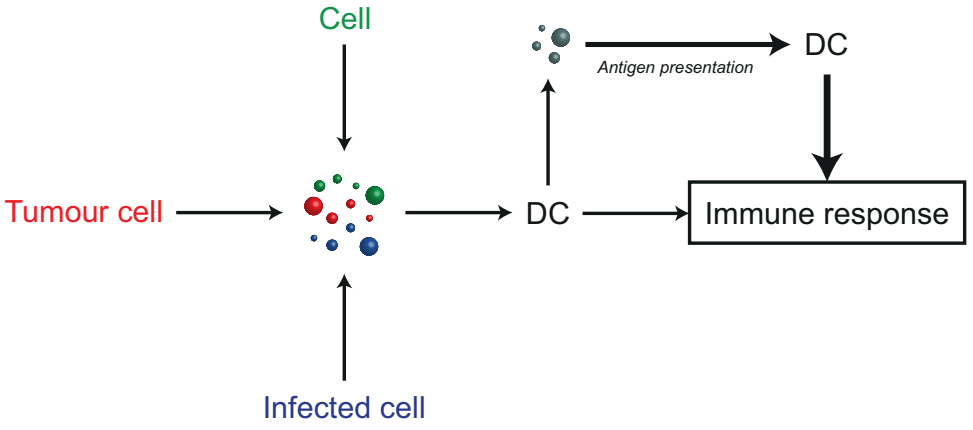

FIG. 4. Antigen presentation. Cells, tumor cells, and infected cells all release vesicles that can be phagocytosed by DCs. DCs induce an immune response but also release antigen-exposing vesicles that present antigens to other DCs, thereby enhancing the immune response.

ing that these exosomes induce tolerance rather than allergic immune responses (Prado et al., 2008).

The initiation of T-cell-mediated antitumor immune responses requires uptake, processing, and presentation of tumor antigens by DCs. Exosomes from mouse tumor cells transfer tumor antigens to DCs in vitro. The binding of these intercellular adhesion molecule-1 (ICAM-1)-exposing exosomes to DCs, which express lymphocyte function-associated antigen-1 (LFA-1) and macrophage 1-antigen as the two major ligands of ICAM-1, is mediated only by LFA-1. Because both mature ($CD8^+$) and immature ($CD8^-$) DCs expose LFA-1, it has been suggested that the interaction between ICAM-1 and LFA-1 plays a role in the exosome-mediated transfer of antigens to DCs (Segura et al., 2007). After binding and uptake of tumor-derived exosomes, DCs are capable of inducing $CD8^+$ T cell-mediated antitumor effects on established mouse tumors in vivo, indicating that tumor-derived exosomes can present tumor antigens to elicit an antitumor immune response (Wolfers et al., 2001).

After uptake of antigens, DCs also secrete exosomes that expose both MHC complexes and T-cell costimulatory molecules. Although an antigen-specific $CD4^+$ T cell activation is induced by injection of antigen- or peptide-exposing exosomes from DCs in vivo, this response only occurs in the presence of mature DCs in vitro, suggesting that the exchange of functional peptide-MHC complexes between (subsets of) DCs by DC-derived exosomes may be a prerequisite to induce an efficient and antigen-specific T-cell response in vivo. As such, this exosome-mediated mechanism may amplify the initiation of the primary adaptive immune response (Théry et al., 2002). Indeed, DC-derived exosomes transfer functional MHC class I/peptide complexes to other DCs, but the presence of mature DCs is essential to efficiently prime cytotoxic T cells in vivo (André et al., 2004).

Mature DCs express MHC class II to present peptide antigens to T cells. Immature DCs, however, have only low surface levels of MHC class II because the peptide-loaded MHC class II complexes are ubiquitinated and predestined for lysosomal degradation. Activation of immature DCs inhibits ubiquitination of the MHC class II-antigen complexes, which results in an increased cell surface exposure of these complexes. In turn, exosomes exposing MHC class II-antigen complexes are secreted from antigen-loaded DCs when these cells contact antigen-specific $CD4^+$ T cells. This secretion is preceded by accumulation of intraluminal vesicles exposing both MHC class II and CD9 in MVEs. The sorting of MHC class II molecules to these intraluminal vesicles requires incorporation into CD9-containing detergent-resistant membrane domains but is independent of ubiquitination, indicating that different intracellular pathways exist for trafficking of MVEs predestined for either lysosomal degradation or secretion (Buschow et al., 2009). It should be pointed out that exosomes from DCs may also be internalized by epithelial cells, which will then produce and release cytokines and chemokines. Thus, exosomes from DCs play a role not only in adaptive immunity but also in innate immunity (Obregon et al., 2009).

Exosomes may also present antigens from microorganisms and allergens. *Toxoplasma gondii* can cause severe sequelae in fetuses of mothers who acquire infection during pregnancy as well as life-threatening neuropathy in immunocompromised patients. Exosomes from a mouse DC cell line incubated with *T. gondii*-derived antigens trigger a strong systemic humoral immune response in vivo and conferred good protection against infection, suggesting that DC-derived exosomes can be used for immunoprophylaxis (Aline et al., 2004; Beauvillain et al., 2007; Beauvillain et al., 2009). Exosomes produced by DCs loaded with protozoan parasite *Leishmania major* antigens provide an effective vaccine against cutaneous leishmaniasis (Schnitzer et al., 2010), and infection with *Mycobacterium tuberculosis* primes macrophages to release increased numbers of exosomes and microvesicles exposing*M. tuberculosis* peptide-MHC class II complexes and HSP70 that enhance antimicrobial T-cell responses (Anand et al., 2010; Ramachandra et al., 2010). Finally, intramuscular immunization of chickens with exosomes from DCs incubated with *Eimeria tenella* (avian coccidiosis) antigens induces protective immunity against avian coccidiosis (Del Cacho et al., 2011). In these examples, presentation of antigens by exosomes induces immune tolerance rather than immune responses. Taken together, presentation of antigens by exosomes affects the immune response at various levels in vitro as well as in vivo. Nevertheless, although exosomes are involved in presentation of specific antigens, they may also play other roles in host defense, for instance, exosomes from human tracheobronchial epithelial cells expose epithelial mucins that immobilize human influenza virus by binding viral α-2,6-linked sialic acid (Kesimer et al., 2009).

3. Transfer of Signaling Components. Cell-derived vesicles also play a role in the intercellular exchange of signaling components. Treatment of microvesicles from platelets and erythrocytes with nonpancreatic secretory phospholipase A_2, which is inactive toward whole cells, generates lysophosphatidic acid, which in turn triggers platelet aggregation (Fourcade et al., 1995). Because secretory phospholipase A_2 and lysophosphatidic acid-containing microvesicles are both present in synovial fluid of inflamed joints, the formation of lysophosphatidic acid is likely to occur in vivo (Fourcade et al., 1995). Likewise, treatment of microparticles from activated platelets with secretory phospholipase A_2 produces intravesicular arachidonic acid, which is transferred and then metabolized by platelets to thromboxane A_2 by cyclooxygenase-1, and by endothelial cells to prostacylin by cyclooxygenase-2 (Barry et al., 1998). The ability of platelet microparticles to increase adhesion of monocytes to endothelial cells is due to the presence of intra-

vesicular arachidonic acid (Barry et al., 1998). Vesicles can evidently transfer lipids between cells for further metabolization, and thus lipid transfer may play a role in atherosclerosis and inflammation.

An elegant example of vesicle-mediated transfer of other signaling elements comes from studies on progesterone-induced Ca^{2+} signals, which play a key role in sperm motility. The progesterone-induced Ca^{2+} signals require fusion with prostasomes, which transfer progesterone receptors, cyclic adenosine diphosphoribose-synthesizing enzymes, ryanodine receptors, and other Ca^{2+}-signaling tools to the sperm. Inhibition of cyclic adenosine diphosphoribose-synthesizing enzymes or depletion of ryanodine receptors from prostasomes reduces sperm motility and fertilization rates, showing that sperm motility and fertilization may depend on the acquisition of Ca^{2+}-signaling tools from prostasomes (Park et al., 2011; Ren, 2011).

Another example comes from the Wnt-signaling pathway in DCs. The Wnt pathway plays an important role in both normal development and cancer. Wnt signaling activity, however, is suppressed by secretion of β-catenin in exosomes, revealing that exosomal packaging and release of cytosolic proteins can affect cellular signaling pathways (Chairoungdua et al., 2010). Exosomes can also contain proteins involved in the apoptotic signaling pathway. For instance, survivin, an inhibitor of caspase activation that promotes proliferation, survival and tumor cell invasion, is also secreted by exosomes (Khan et al., 2011). Thus, it is clear that cells can exchange functional signaling components between cells.

4. Inflammation. Cell-derived vesicles can trigger the production of tissue factor (TF) and proinflammatory cytokines by activating target cells (Fig. 5). For instance, microparticles from *N*-formyl-Met-Leu-Phe-stimulated human polymorphonuclear leukocytes (PMNs) activate endothelial cells by increasing tyrosine phosphorylation of the 46-kDa c-Jun NH_2-terminal kinase (JNK) 1. This activation results in expression and production of TF and interleukin-6 (Mesri and Altieri, 1998; Mesri and Altieri, 1999). Because these vesicles, called "ectosomes," are released at sites of inflammation in vitro and in vivo and expose the complement receptor 1 required for binding to opsonized bacteria, these vesicles have been suggested to represent an "ecto-organelle" designed to focus antimicrobial activity onto opsonized surfaces (Hess et al., 1999). On the other hand, the effects of microparticles from PMNs may be strongly cell-type-dependent, because human macrophages are inhibited upon incubation with PMN-derived microparticles, resulting in production of TGF-β and inhibition of the inflammatory response of macrophages to zymosan or lipopolysaccharide (LPS). This inhibition requires binding of microparticles to macrophages but is independent from their phagocytosis (Gasser and Schifferli, 2004; Eken et al., 2010). Furthermore, PMN-derived microparticles contain the anti-inflammatory protein annexin-1, and intravenous delivery of these microparticles blocks recruitment of PMN in an inflammatory model in an annexin-1-dependent mechanism in vivo, suggesting that PMN-derived microparticles may have both pro- and anti-inflammatory properties at sites of inflammation (Dalli et al., 2008).

Cell-derived vesicles have the ability to affect inflammation by transferring transport receptors and inflammatory mediators. Microvesicles or exosomes are a major pathway for fast secretion of interleukin 1β, which lacks a signal peptide (MacKenzie et al., 2001; Wilson et al., 2004; Bianco et al., 2005). Interleukin 1β was recently shown to be one of the key mediators within microparticles from monocytes, together with other components of the inflammasome, responsible for activation of endothelial cells (Wang et al., 2011). Likewise, mouse macrophages, stimulated with ATP, release exosomes with entrapped interleukin-1β, caspase-1 and other components of the inflammasome (Qu et al., 2007). In addition, microvesicles from P2X7 receptor-stimulated mature DCs contain interleukin-1β (Pizzirani et al., 2007). Furthermore, microparticles contain platelet-activating factor (Watanabe et al., 2003) and can expose the soluble (non–cell-bound) but full-length 55-kDa form of TNF receptor 1, which is also found in human serum and lung epithelial lining fluid (Hawari et al., 2004).

Proteins are not the only biologically active component of vesicles that play a role in inflammatory responses, because the lipid fraction of microvesicles activates macrophage Toll like receptor (TLR) 4. Because the ability of these microparticles to activate TLR4 is impaired by preincubation with an inhibitor of phospho-

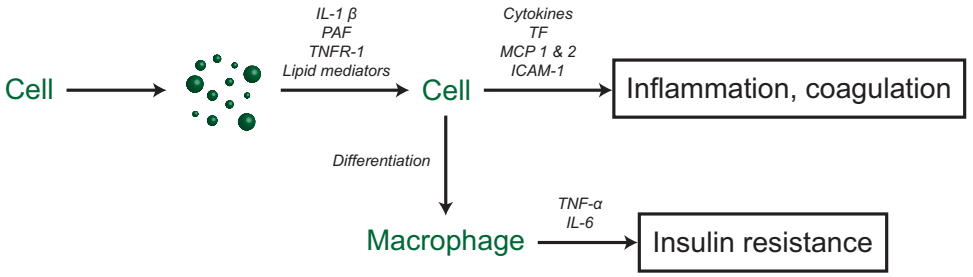

FIG. 5. Inflammation. Cells can transfer inflammatory mediators, including interleukin (IL)-1β, platelet-activating factor (PAF), and TNF receptor-1 (TNFR-1), to cells. Activated cells express and produce proinflammatory cytokines, TF, monocyte chemotactic protein (MCP) 1 and 2, and ICAM-1, which all support inflammation. Vesicles can also induce cellular differentiation of monocytes into macrophages, thereby inducing insulin resistance.

lipase D, which does not affect the release of microvesicles but inhibits hydrolysis of phosphatidylcholine to phosphatidic acid, this further supports the lipid nature of the active component (Thomas and Salter, 2010). In addition, exosomes from DCs and macrophages, and microvesicles from human plasma, contain functional enzymes for synthesis of leukotrienes, which are potent lipid inflammatory mediators (Esser et al., 2010).

The ability of vesicles to modulate the inflammatory response is not limited to blood. Autologous vesicles from synovial fluid and microparticles from T cells, monocytes, and platelets trigger the production and release of interleukins 6 and 8, matrix metalloproteases, monocyte-chemotactic proteins 1 and 2, vascular endothelial growth factor (VEGF), and ICAM-1 by synovial fibroblasts, indicating that these vesicles enhance the destructive activity of these fibroblasts in rheumatoid arthritis (Berckmans et al., 2005; Distler et al., 2005; Beyer and Pisetsky, 2010; Boilard et al., 2010; Distler and Distler, 2010). One of the mechanisms by which microparticles activate synovial fibroblasts is transfer of arachidonic acid from leukocytes to synovial fibroblasts via microparticles (Jüngel et al., 2007). Furthermore, synovial microparticles expose TF and are extremely procoagulant, which could explain the presence of fibrin deposition within inflamed joints (Berckmans et al., 2002). In addition, microparticles from human airway epithelial cells stimulate the production of proinflammatory mediators, thereby possibly enhancing the inflammatory airway response (Cerri et al., 2006).

Peripheral blood of patients with preeclampsia contains elevated concentrations of placenta-derived vesicles compared with normal pregnancy. These vesicles, often called syncytiotrophoblast microparticles, bind to monocytes and the endothelium, thereby triggering the production of proinflammatory mediators. This suggests that syncytiotrophoblast microparticles contribute to the increased systemic inflammatory responsiveness in preeclampsia (Germain et al., 2007; Messerli et al., 2010; Southcombe et al., 2011).

Microparticles from human atherosclerotic plaques have been shown to mediate the functional transfer of ICAM-1 to endothelial cells, thereby promoting the adhesion of monocytes and trans-endothelial migration. The transfer of ICAM-1 from microparticles to endothelial cells results in increased phosphorylation of extracellular signal-regulated kinase 1/2 after ICAM-1 ligation (Rautou et al., 2011). Thus, plaque MPs may further facilitate atherosclerotic plaque progression.

Exosome-like vesicles released from adipose tissue are taken up by peripheral blood monocytes after intravenous injection, which then differentiate into macrophages producing increased levels of TNF-α and interleukin-6. Injection of these vesicles into wild-type C57BL/6 mice results in development of insulin resistance, whereas injection into TLR-4 knockout B6 mice results in much lower levels of glucose intolerance and insulin resistance, pointing to a role for TLR4 in the induction of inflammatory mediators by exosome-like vesicles from adipose tissue (Deng et al., 2009). Evidently, exosomes and other vesicles can influence the inflammatory response in several ways.

5. Tumor Growth, Metastasis, and Angiogenesis. Exosomes can transfer the metastatic activity of highly metastatic Bl6–10 melanoma tumor cells to poorly metastatic F1 melanoma tumor cells in vitro. Mice develop lung metastatic colonies when F1 cells are injected together with exosomes from Bl6–10 exosomes, whereas mice injected solely with F1 cells develop no metastatic colonies (Hao et al., 2006). The ability of exosomes to promote metastasis and angiogenesis can be increased when exosomes are released under hypoxic conditions (i.e., conditions that many tumors encounter when growing) (Park et al., 2010).

One of the molecular mechanisms underlying the intercellular transfer of metastatic activity is the transfer of an oncogenic growth factor receptor or their ligand (Fig. 6). The truncated form of the EGFR vIII is transferred from glioma cancer cells by microvesicles to glioma cells lacking this receptor, in a mechanism involving detergent-resistant membrane domains (lipid rafts). The transferred receptor is fully functional and results

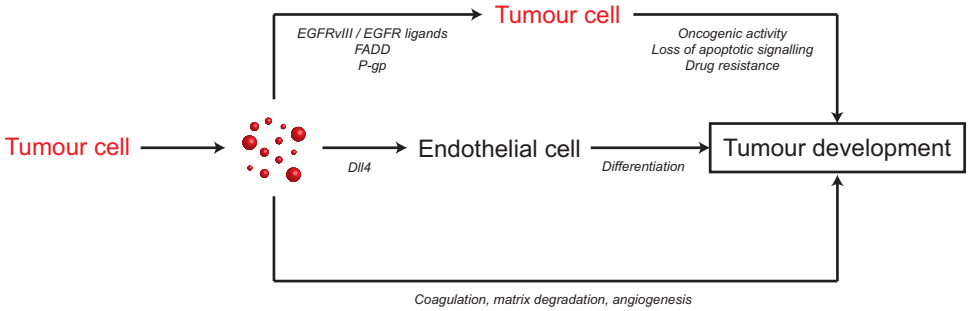

FIG. 6. Tumor growth, metastasis, and angiogenesis. Tumor cell-derived vesicles facilitate tumor development in many ways. The vesicles can transfer oncogenic activity by transferring growth factor receptors (EGFRvIII) or ligands to other tumor cells, vesicles can remove Fas-associated protein with death domain (FADD), so that tumor cells become less susceptible to apoptosis, and vesicles can transfer P-glycoprotein (P-gp), a multidrug transporter, between cells, thereby contributing to drug resistance. Tumor cell-derived vesicles also affect neighboring cells by transferring the Notch ligand Dll4 to endothelial cells, thereby inducing branch formation. Finally, vesicles can support and initiate coagulation, matrix degradation and angiogenesis.

in a full transfer of the oncogenic activity, including activation of the transforming signaling pathways (mitogen-activated protein kinase, AKT), changes in expression of EGFRvIII-regulated genes (VEGF, Bcl-x_L, p27), morphological transformation, and increase in anchorage-independent cell growth capacity (Al-Nedawi et al., 2008). On the other hand, human breast and colorectal cancer cells release exosomes containing full-length, signaling-competent EGFR ligands, and the invasive potential of colon cancer cell lines is related to the concentration of exosomes containing amphiregulin, one of the EGFR ligands, suggesting that ligand exchange contributes to cancer invasiveness and metastasis (Higginbotham et al., 2011).

Vesicles can also contribute to tumor growth and development via several other mechanisms. For instance, cancer cells can release vesicles containing the Fas-associated death domain, a key adaptor protein that transmits apoptotic signals and becomes lost in many cancer cells (Tourneur et al., 2008).

In addition, exosomes may protect tumor cells from entering or accumulation of antitumor drugs, thereby possibly contributing to (multi)drug resistance. For instance, exosomes from HER2-overexpressing breast cancer cell lines, or exosomes present in serum from patients with breast cancer, capture the humanized antibody trastuzumab, thereby reducing the effective concentration of this anticancer drug (Ciravolo et al., 2012). Vesicles may also contribute to drug resistance by exchanging drug transporters between cells. One of the most important drug transporters is P-glycoprotein, a transmembrane protein and member of the ATP binding cassette superfamily, which can be exchanged between cells via vesicles (Gong et al., 2012). P-glycoprotein, a key protein involved in multidrug resistance, is widely expressed at pharmacological interfaces such as the blood-brain barrier, blood-testis barrier, and along the gastrointestinal track (where the influx of carcinogens and harmful chemicals is prevented). Overexpression of P-glycoprotein by cancer cells correlates with anticancer drug failure in many types of cancer. There is also evidence that drugs are sorted, concentrated, and removed from cells via secretion of exosomes (e.g., cisplatin in drug-resistant ovarian carcinoma cells) (Safaei et al., 2005).

Vesicles can also promote vascular development, which is highly relevant for tumor growth. For instance, Notch signaling is an evolutionarily conserved pathway that plays an essential role in vascular development and angiogenesis. δ-like 4 (Dll4) is a Notch ligand that is up-regulated during angiogenesis by endothelial cells and regulates the differentiation between tip cells and stalk cells of the neovasculature. Dll4 is incorporated into exosomes of endothelial cells and tumor cells, and it can be transferred to endothelial cells, where it is incorporated into the plasma membrane. Once incorporated, Dll4 inhibits Notch signaling and induces the loss of Notch receptor, which results in a tip cell phenotype and increases branch formation of vessels (Sheldon et al., 2010).

There are other ways in which vesicles promote tumor growth, including the release of active matrix-degrading enzymes (Hakulinen et al., 2008), stimulation of angiogenesis (Taverna et al., 2011), and production of TF (Uno et al., 2007), but these mechanisms have been summarized excellently elsewhere (Zöller, 2009; Rak, 2010; Peinado et al., 2011). Please see also section III.A.7.

6. Morphogens. The morphogen Wnt is important in development of the mature nervous system. Wnt is transported in vesicles across synapses in *Drosophila melanogaster* larval neuromuscular junctions (Korkut et al., 2009). However, the transport of morphogens is not limited to *D. melanogaster*; it also occurs in higher organisms. For instance, microvesicles from T cells or human blood contain functional Hedgehog proteins, which play a role in development, and these vesicles are capable of inducing differentiation of pluripotent erythroleukemic cells in vitro (Martínez et al., 2006).

7. Genetic Information. Bacteria exchange genetic information via outer membrane vesicles (Yaron et al., 2000). In 2007, it was described that eukaryotic cells can exchange functional genetic information by vesicles as well (Fig. 7). Exosomes from mouse and human mast cell lines as well as exosomes from primary bone marrow-derived mouse mast cells were shown to contain mRNA and miRNA (Valadi et al., 2007). The mRNA of approximately 1300 genes was detectable, many of which were below the detection limit in the cytoplasm of the donor cells. In vitro translation showed that mRNA could be translated, and transfer of mouse exosomal RNA to human mast cells induces expression of mouse proteins. Thus, exosomes have the capacity to facilitate the intercellular exchange of functional genetic information. Exosomes from T cells transfer miRNA to antigen-presenting cells (Mittelbrunn et al., 2011), and exosomes from DCs contain different miRNAs depending on the maturation stage of the DCs. Fusion of these exosomes with acceptor DCs results in the transfer of functional miRNAs,as shown by the reduced expression of target mRNA. Thus, intercellular exchange of miRNAs by vesicles can affect post-transcriptional regulation (Montecalvo et al., 2012). In addition, exosomes from glioblastoma cells transfer functional mRNA, miRNA, and angiogenic proteins to brain microvascular endothelial cells, thus stimulating tubule formation and proliferation of glioma cells (Skog et al., 2008). Several mRNAs and miRNAs characteristic of gliomas were detected in some but not all sera of patients with glioblastoma, suggesting that exosomes contain diagnostic information. Likewise, microvesicles from a colorectal cancer cell line contain mRNA, including 27 mRNAs encoding cell-cycle related proteins. These exosomes triggered proliferation of endothelial cells, again confirming that vesicles can induce angiogenesis and thus promote tumor growth and metastasis (Hong et al., 2009).

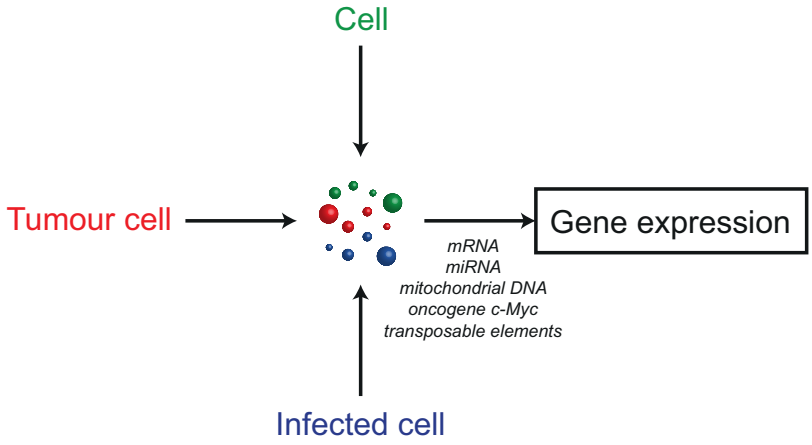

FIG. 7. Genetic information. Vesicles from normal cells, tumor cells, and infected cells release vesicles containing functional genetic information that can be transferred to recipient cells.

Exosomes from astrocytes and glioblastoma cells also contain mitochondrial DNA (Guescini et al., 2010), and microvesicles from several tumors and tumor cell lines have been shown to contain elevated levels of specific coding as well as noncoding RNA and DNA, mutated and amplified oncogene sequences, and transposable elements (Balaj et al., 2011). In addition, serum-derived exosomes from tumor-bearing mice contain the amplified oncogene c-*myc*, suggesting that the genetic information in tumor-derived vesicles can potentially be a unique tumor biomarker (Balaj et al., 2011). It is to be expected that vesicles in biological fluids will contain genetic information, and therefore it is not surprising that exosomes from, for example, human saliva, plasma, and milk all contain detectable levels of RNA, including mRNA (Lässer et al., 2011).

Pathogens have "hijacked" cell-derived vesicles as a transport vehicle to exchange genetic information between cells without the risk of being recognized by the immune system. Exosomes from nasopharyngeal carcinoma cells harboring latent EBV contain viral miRNAs, some of which are enriched compared with intracellular levels. This increased concentration of viral miRNAs may help to manipulate the tumor microenvironment to influence the growth of neighboring cells (Gourzones et al., 2010; Pegtel et al., 2010; Meckes et al., 2010). In addition, human herpes virus 4 or EBV encodes miRNAs that can be transferred from infected to uninfected cells by exosomes. Again, transferred miRNAs are functional because they down-regulate target gene expression in recipient cells (Pegtel et al., 2010). The miRNA-mediated gene silencing is a potentially important mechanism of intercellular communication. Several components of RNA-induced silencing complexes are present in exosomes, suggesting that this complex may be involved in the sorting of miRNAs into exosomes (Pegtel et al., 2011). Taken together, the role of vesicles in the exchange of functional genetic information between cells is not limited to prokaryotes but may also contribute to physiological and pathological processes in eukaryotes.

8. Prions. Prion diseases are infectious and fatal neurodegenerative disorders due to accumulation of, for example, abnormally folded prion protein (PrP) scrapie (PrP^{sc}) in the central nervous system. PrP^{sc} catalyzes the further conversion of normal cellular PrP into PrP^{sc}. Both forms of prion protein, PrP and PrP^{sc}, have been found in exosomes. Because exosomes containing PrP^{sc} are infectious, they are likely to contribute to the spreading of prions (Fevrier et al., 2004; Vella et al., 2007). Likewise, a fraction of β-amyloid peptides is associated with exosomes in Alzheimer's disease. Because exosomal proteins accumulate in plaques of patient brains, exosomes are thought to play a role in the pathogenesis of Alzheimer's disease (Rajendran et al., 2006).

9. Viruses. Viruses use vesicles for infection and survival (Fig. 8). Microparticles transfer receptors that are essential for entry of HIV to cells lacking these receptors. For instance, transfer of the chemokine receptors CCR5 and CXCR4 makes recipient cells susceptible to entry of HIV (Mack et al., 2000; Rozmyslowicz et al., 2003). In addition, uptake of HIV by cells results in camouflage of the virus. Capture of HIV by immature DCs produces viral particles that expose characteristic exosomal proteins as CD63 and CD9, and the presence of such proteins is thought to help the virus particles to escape from recognition by the immune system. These viral particles are more infectious to $CD4^+$ T cells than cell-free viral particles (Wiley and Gummuluru, 2006). This camouflage can be further improved by coating with host-derived glycoproteins, thereby making viral particles even more closely resembling T-cell-derived vesicles (Krishnamoorthy et al., 2009). Because virus particles and cell-derived vesicles are of similar size and have many similarities in composition, this has caused major problems with isolation and purification of the virus particles (Bess et al., 1997; Aupeix et al., 1997; Coren et al., 2008; Park and He, 2010).

One of the earliest and most abundantly expressed viral proteins is Nef. Nef is present in the serum of patients with HIV and interacts with CXCR4, thereby

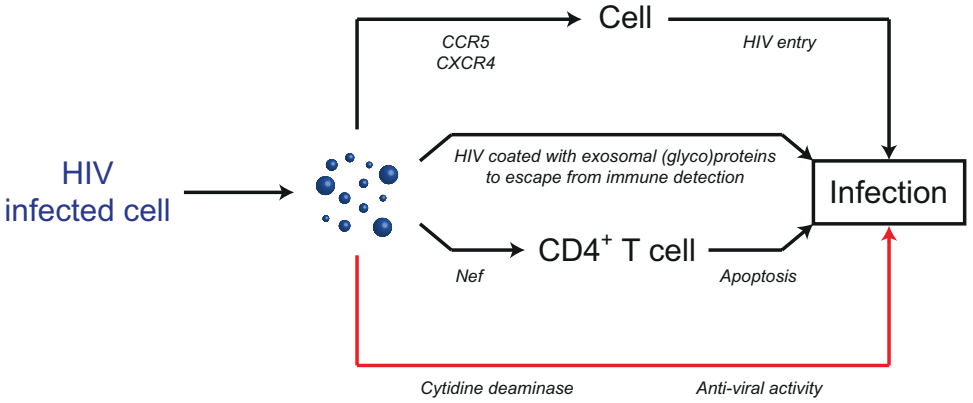

FIG. 8. Viruses. Cells infected with HIV release viral particles exposing exosomal (glyco)proteins, thus escaping from detection by the immune system. Vesicles from HIV-infected cells can transfer chemokine receptors (CCR5, CXCR4) to cells lacking these receptors, thereby facilitating the entry of HIV in these cells. In addition, vesicles from HIV-infected cells expose Nef, which triggers $CD4^+$ T cell apoptosis. Vesicles contain also antiviral activity, in particular cytidine deaminase.

inducing apoptosis and depletion of $CD4^+$ T cells, which is one of the hallmarks of AIDS (Lenassi et al., 2010; Raymond et al., 2011; Shelton et al., 2012). Nef stimulates its own export via secretion of exosomes or CD45-exposing microvesicles. In addition, LMP-1, the major oncogene of EBV, is detectable in serum of infected patients. LMP-1 inhibits T cell proliferation and NK cytotoxicity, although other proteins, such as galectin 9, may be involved as well, thereby facilitating escape from the immune system (Meckes and Raab-Traub, 2011).

Viruses also have other strategies to escape from the immune system. For instance, cells infected with hepatitis B virus, pox viruses, or herpes simplex virus, release empty viral particles exposing viral glycoproteins in concentrations up to 10,000 fold higher than that of the infectious particles, suggesting that these vesicles may act as potential decoys to distract the immune system (Meckes and Raab-Traub, 2011).

Viruses may use the ability of exosomes to kill immune cells at a distance. LMP-1, which is exposed on exosomes and in microvesicles from EBV-infected cells, inhibits proliferation of peripheral blood mononuclear cells. This could be relevant in EBV-associated tumors such as nasopharyngeal carcinoma and Hodgkin's disease, because it allows tumor cells to escape from immune eradication (Flanagan et al., 2003). The underlying sorting mechanism of LMP-1 to exosomes is unknown, but the presence of LMP-1 in exosomes is independent from ubiquitination or detergent-resistant membrane domains (Verweij et al., 2011). Expression of LMP-1 also changes the composition of exosomes by increasing the concentrations of important signaling molecules in cancer, including phosphatidylinositol 3-kinase, EGFR, and fibroblast growth factor-2, suggesting that EBV 1) manipulates and uses the exosomal pathway for tumor growth and development and 2) protects the exosomes from degradation (Ceccarelli et al., 2007; Meckes et al., 2010; Verweij et al., 2011). The uptake of exosomes from EBV-infected cells by epithelial cells indeed results in activation of growth-stimulating signaling pathways, showing that transfer of LMP-1 and other signaling molecules can manipulate the growth characteristics of neighboring cells (Meckes and Raab-Traub, 2011). Exosomes from an EBV-infected B cell line have been shown to bind preferentially to B cells in blood, whereas exosomes from milk, DCs, or noninfected B cells bind to monocytes. The interaction between exosomes from EBV-infected B cells and B cells is mediated by CD21 on the B cells and glycoprotein 350 on the exosomes. It has been hypothesized that exosome-based vaccines can be developed that interfere with this interaction (Vallhov et al., 2011).

Because retroviruses and exosomes have many similarities, including lipid composition and the presence of host-cell proteins, retroviruses have been proposed to have hijacked the pathway of exosome biogenesis for their production to escape from immune detection (i.e., the Trojan exosome hypothesis) (Gould et al., 2003). Although the finding that an inhibitory domain of the HIV Gag protein interferes with the sorting of both viral and exosomal proteins, such as CD63, supports this hypothesis (Gan and Gould, 2011), inhibition of exosome release by blocking ceramide synthesis does not interfere with the release of HIV, suggesting the existence of different routes (Brügger et al., 2006; Jolly and Sattentau, 2007; Sato et al., 2008; Izquierdo-Useros et al., 2009; Meckes and Raab-Traub, 2011).

Exosomes also have anti-HIV-1 activity (Tumne et al., 2009), and the human cytidine deaminase APOBEC3G, which is a part of a cellular defense system against HIV-1 and other retroviruses, is secreted in exosomes and is actually the major exosomal component explaining the anti-HIV-1 activity of exosomes (Khatua et al., 2009, 2010). Furthermore, vesicles from virus-infected cells can present antigens to activate the immune system (Walker et al., 2009; Meckes and Raab-Traub, 2011).

B. Cell Adhesion

Cell-derived vesicles can function as mediators to promote adhesion of a cell to a substrate. For example, microparticles from platelets bind to the endothelial cell matrix at sites of vascular injury in vivo; in turn, the surface-immobilized microparticles support platelet adhesion to that surface, suggesting that the binding of microparticles facilitates thrombus formation at the site of vascular injury (Merten et al., 1999). In addition, B cell exosomes mediate anchorage to the endothelial cell matrix (Clayton et al., 2004).

C. Waste Management and Protection against Stress

Exosomes from reticulocytes contribute to removal of the redundant transferrin receptor from their surfaces (Johnstone et al., 1987, 1989, 1991; Johnstone, 1992; Grdisa et al., 1993). Although it is unknown to what extent cell-derived vesicles contribute to the maintenance of cellular homeostasis by waste removal, it is tempting to speculate that vesicles protect cells from extracellular and intracellular stress (Fig. 9), thereby contributing to cellular survival. This would also explain why most cells release vesicles into their environment and is supported by similar findings from prokaryotes (Kadurugamuwa and Beveridge, 1998, 1999). Platelets incubated with the complement C5b-9 complex, a form of external stress, release vesicles enriched in the C5b-9 complex, presumably to protect the platelets from complement-induced lysis (Sims et al., 1988). In addition, cancer cells release microvesicles enriched in anticancer drugs (Shedden et al., 2003; Safaei et al., 2005). Furthermore, exosomes and microvesicles from different types of cells, as well as microvesicles from human plasma, contain substantial amounts of active caspase 3 (one of the main executioners of programmed cell death) that is not detectable in the releasing cells (de Gassart et al., 2003; Abid Hussein et al., 2005), suggesting that caspase 3, a form of internal stress, is removed from the cytosol to ensure cellular survival. This hypothesis is further strengthened by the observation that caspase 3-enriched vesicles from endothelial cells are virtually devoid of the vitronectin receptor (i.e., integrin $\alpha_v\beta_3$), despite the fact that this receptor is the most abundant endothelial receptor and plays a key role in the generation of adhesion-dependent survival signals. Apparently, endothelial cells are using vesicles to selectively remove the harmful caspase 3, whereas a survival receptor is maintained on the cells. Likewise, exposing B cell lines to heat stress is associated with a marked increase of HSPs in exosomes, suggesting that "alterations in exosome phenotype are a hitherto unknown component of the cellular response to environmental stress" (Clayton et al., 2005).

D. Coagulation

As mentioned, since the 1940s, microvesicles have been associated with the coagulation (Fig. 10) process because vesicles can expose PS, a negatively charged phospholipid to which (activated) coagulation factors can bind and assemble in the presence of calcium ions, thereby promoting coagulation (Chargaff and West, 1946; Wolf, 1967). In the 1980s, it had already been demonstrated that cell-derived vesicles from tumors are strongly procoagulant (Dvorak et al., 1981). This activity was due to the exposure of TF, the initiator of coagulation (Bastida et al., 1984). More recently, tumor-derived vesicles exposing TF were shown to be present in peripheral blood of patients with cancer, where they have been associated with the increased risk of developing venous thromboembolism (VTE) (Tesselaar et al., 2007; Uno et al., 2007; Zwicker et al., 2009; Lima et al., 2011; Owens and Mackman, 2011). An increased concentration of microparticles is associated with an increased incidence of thrombotic events, for instance in patients with paroxysmal nocturnal hemoglobinuria, who are extremely sensitive to complement-induced lysis because of the absence of complement inhibitors (Wiedmer et al., 1993; Hugel et al., 1999; Babiker et al., 2002), and in patients with acute coronary syndromes (Mallat et al., 2000). Conversely, a decreased concentration of microparticles, combined with a defect exposure of PS on activated platelets, is associated with a bleeding tendency, as in patients with Scott syndrome (Dachary-Prigent et al., 1997). Coagulant TF-exposing vesicles have shown to be present in several body fluids and in different clinical conditions, including pericardial blood from patients undergoing open heart surgery or in the circulation of patients with meningococcal septic shock (Nieuwland et al., 1997, 2000), in joint fluid of inflamed joints from patients with arthritis (Berckmans et al., 2002), and in saliva and urine of healthy subjects (Berckmans et al., 2011). TF-exposing vesicles trigger thrombus formation

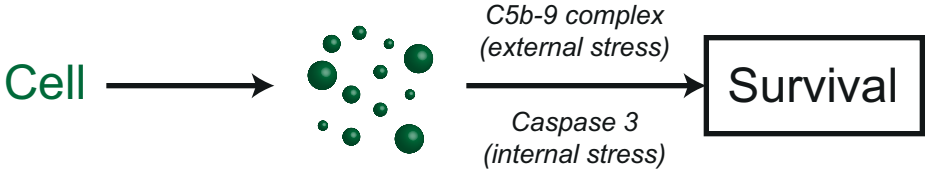

FIG. 9. Waste management and protection against stress. Cells incubated with the lytic complement C5b-9 complex release vesicles enriched in this complex, thereby protecting the cells from external stress. Many cells release caspase 3-containing vesicles. Caspase 3, one of the main executioner enzymes of apoptosis, is not detectable in lysates of releasing cells, suggesting that the release of caspase 3 protects the cells from accumulation of dangerous levels of caspase 3.

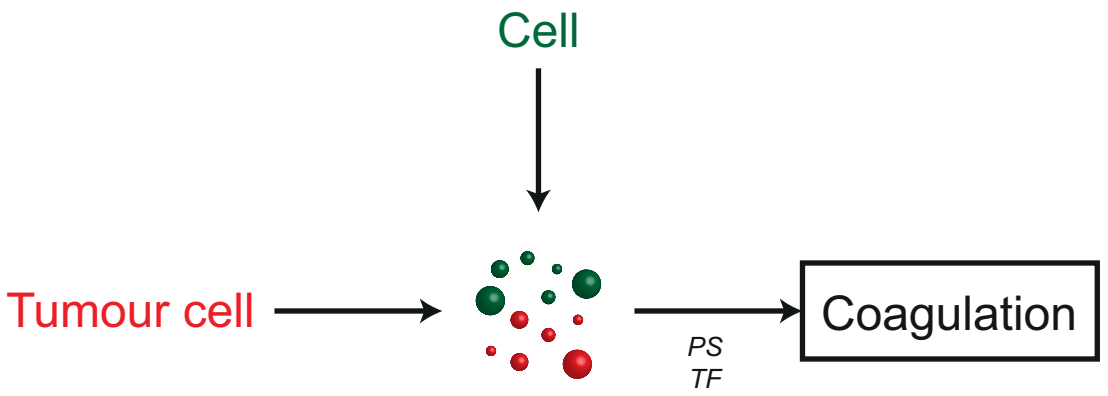

FIG. 10. Coagulation. Cells and tumor cells can release coagulant vesicles exposing PS and/or TF.

in vivo (Biró et al., 2003), are abundantly present in human atherosclerotic plaques (Mallat et al., 1999), and may be deposited at a site of vascular injury by binding to activated platelets (Furie and Furie, 2004; Del Conde et al., 2005; Furie and Furie, 2008). In addition, blood from healthy subjects contains cell-derived vesicles, but these vesicles do not expose coagulant TF and are associated with an anticoagulant rather than procoagulant function (Tans et al., 1991; Dahlbäck et al., 1992; Berckmans et al., 2001).

Direct evidence for involvement of exosomes in coagulation is lacking. Nevertheless, several lines of circumstantial evidence suggest that this may be the case. First, exosomes were reported to expose PS (Théry et al., 2009), although others found no asymmetrical distribution of anionic phospholipids or exposure of PS on exosomes (Heijnen et al., 1999; Laulagnier et al., 2004). Second, biological fluids such as urine and saliva contain exosome-sized vesicles exposing coagulant TF (Berckmans et al., 2011). Third, "TF-bearing microvesicles" from a monocytic cell line were shown to be transferred to activated platelets (Del Conde et al., 2005). The microvesicles in this study, however, were isolated at 200,000*g* and may therefore have been contaminated with exosomes. Fourth, blood from patients with sickle-cell disease was shown to contain TF-exposing microvesicles from monocytes and endothelial cells (Shet et al., 2003). Because these vesicles were isolated at 100,000*g* (i.e., a condition known to pellet exosomes) and these TF-exposing vesicles were not detected in a parallel study in which microvesicles were isolated at 18,000*g* (i.e., a condition unlikely to pellet most exosomes but only most of the larger microvesicles), exosomes exposing TF may be present and contribute to coagulation in vivo (Shet et al., 2003; van Beers et al., 2009).

Although exosomes may be too small to be observed as single vesicles by most laboratory methods presently available, the assays used to quantify the coagulant properties of cell-derived vesicles in biological fluids usually measure the overall coagulant activity of all vesicles present. Thus, the contribution of microvesicles versus exosomes in the coagulation process cannot be distinguished with those activity assays.

E. Vascular Function and Integrity

Apoptotic endothelial cells were shown to release not only apoptotic vesicles but also "nanovesicles." The release of nanoparticles was dependent on caspase 3 activity,and these nanoparticles may contribute to vascular repair because they trigger an extracellular signal-regulated kinase 1/2-dependent antiapoptotic phenotype in vascular smooth muscle cells (Sirois et al., 2011). In contrast, vesicles from LPS-activated monocytes trigger apoptosis of vascular smooth muscle cells by a caspase 1-dependent mechanism (Sarkar et al., 2009), suggesting that vesicles may have beneficial as well as adverse effects on vascular integrity. In addition, several studies have shown that microparticles from plasma of preeclamptic women, endothelial cells, lymphocytes of patients with diabetes or HIV, and T cells all impair endothelium-dependent vasorelaxation in vitro, suggesting that microparticles may also affect vascular function (Vanwijk et al., 2002; Martin et al., 2004).

IV. Clinical Applications

A. Therapy

1. Cancer. Exosomes from antigen-presenting cells, DCs, express functional MHC class I and II molecules and T-cell costimulatory molecules. Because DCs incubated with tumor peptides secrete exosomes that are capable of priming specific cytotoxic T cells to eradicate or suppress growth of established murine tumors in vivo (Fig. 11), exosomes have the potential to be used as vaccines to suppress tumor growth (Zitvogel et al., 1998; Andre et al., 2002). When these DC-derived exosomes were characterized, several proteins were discovered that might explain the ability of exosomes to induce immune responses. The most striking proteins that were discovered are mild fat globule-epidermal growth factor VIII (MFG-E8), also known as lactadherin, and the 73-kDa cytosolic heat shock cognate protein. Lactadherin can target exosomes to antigen-presenting cells as macrophages and DCs, whereas heat shock cognate protein 73 triggers antitumor immune responses in vivo (Théry et al., 1999). Because DCs are potent vaccine carriers

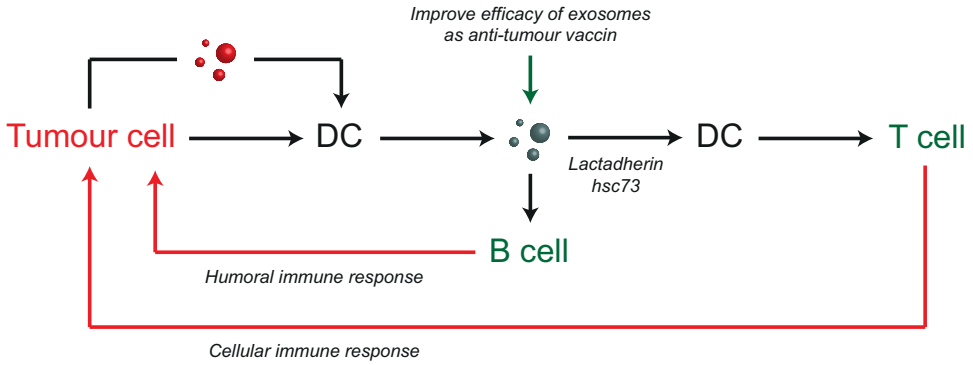

FIG. 11. Cancer therapy. Tumor cells and tumor cell-derived vesicles activate DCs, which in turn release vesicles that activate other DCs. This activation amplifies the cellular (T-cell)-mediated antitumor response. Vesicles from DCs can also induce a humoral (B-cell)-mediated antitumor response. hsc, heat shock cognate protein.

but are short-lived and sensitive to cytotoxic T-cell-mediated elimination in vivo, antigen presentation by exosomes is considered a promising tool. In particular, because functional antigen-presenting exosomes could be recovered from draining lymph nodes in vivo after DCs had disappeared, illustrating that antigen presentation after delivery of DC vaccines persists for longer than expected and indicating that exosomes may have played a previously unrecognized role in presentation of antigens after DC vaccination (Luketic et al., 2007).

Protocols have been developed and optimized to ensure a reliable manufacturing process to harvest DC-derived exosomes (Lamparski et al., 2002; Le Pecq, 2005; Johansson et al., 2008; Viaud et al., 2011) and several strategies have been explored to improve the ability of exosomes to function as immunotherapeutic antitumor agents, including loading of exosomes with oligonucleotides and TLR-3 and TLR-9 ligands (Chaput et al., 2004; Adams et al., 2005), or the priming of DCs with exosomes from tumor cells exposed to heat stress. The heat treatment leads to increased exposure of MHC class I molecules, chemokines, and HSPs, the last of these acting as molecular chaperones having potent adjuvant activity in the induction of antigen-specific T-cell responses and therefore increased immunogenicity (Dai et al., 2005; Chen et al., 2006, 2011). Other protocols include administration of the exosomes after pretreatment with immune-potentiating doses of cyclophosphamide (Taieb et al., 2006), priming of DCs with exosomes from genetically modified tumor cells expressing interleukin-2 or interleukin-12 (Yang et al., 2007; Zhang et al., 2010), or overexpression of fusion proteins consisting of tumor-associated antigens and the C1C2 domain of lactadherin (Hartman et al., 2011; Rountree et al., 2011). Furthermore, exosomes from mature DCs are more efficient in eradicating established tumors than exosomes from immature DCs in vivo, because exosomes from mature DCs express higher levels of MHC complexes and T-cell costimulatory molecules (Hao et al., 2007).

It must be mentioned that exosomes from DCs not only induce a cell-mediated immune response but may also induce a humoral immune response. For example, exosomes from murine bone marrow-derived DCs incubated with intact diphtheria toxoid, or exosomes exposing a glycoconjugate that is cross-reactive with the capsular polysaccharide of *Streptococcus pneumoniae* type 14, induce specific antibody production in vivo (Colino and Snapper, 2006, 2007).

Instead of eradicating or suppressing the growth of already established tumors, also vaccination with exosomes has been tested for its efficacy to prevent tumor growth. Exosomes from plasmacytoma cells protected some 80% of the mice against a challenge with wild-type tumors. This protection is due to generation of specific cytotoxic T cells. The effects were not seen in severe combined immunodeficiency mice, and the immunity was tumor-specific (Altieri et al., 2004).

In several clinical trials, the efficacy of autologous exosome immunotherapy has been tested. In a phase I study, patients with advanced NSCLC with tumor expression of MAGE-A3 or -A4 were included. Patients underwent leukophoresis to produce DCs from isolated monocytes. Exosomes from DCs were isolated and loaded with a variety of MAGE peptides. Administration of exosomes was well tolerated; hardly any adverse effects were observed and several patients showed a long-term stability of disease, and activation of immune effector cells as NK cells and T cells in vivo was detectable in some patients (Morse et al., 2005). In a second study, exosomes incubated with MAGE 3 peptides were administered to patients with stage III or IV melanoma. Again, no adverse effects were observed, but also no MAGE 3-specific T-cell immune responses could be detected in peripheral blood, suggesting that adjuvant administration of exosomes may be of limited therapeutic relevance in this condition (Escudier et al., 2005). In both studies, however, monocyte-derived (immature) DCs were used, and treatment with interferon-γ has been shown to be a suitable maturation agent for these DCs. Exosomes from mature DCs produce exosomes that evoke a much stronger priming of the immune response in vivo than exosomes from immature DCs. At present, the ability of exosomes from mature DCs to trigger an antitumor im-

mune response is tested in a phase II trial in patients with advanced NSCLC (Chaput and Théry, 2011).

Another approach is to use tumor-derived exosomes to raise an antitumor immune response. In a phase I trial, exosomes were isolated from ascites of patients with colorectal cancer. Patients were treated with exosomes plus granulocyte-macrophage colony-stimulating factor or only exosomes. Patients treated with the combination therapy showed beneficial tumor-specific antitumor cytotoxic T lymphocyte response (Dai et al., 2008).

Despite the fact that the different strategies used to explore the clinical use of exosomes as adjuvant therapy in antitumor immune therapy have not been as successful as hoped for, one has to bear in mind that the included patients all had advanced cancers and did not respond well to prior treatments. Therefore, the aim of these studies was not primarily to cure the patients but rather to prolong their survival and to stabilize disease after chemotherapy or radiation therapy (Théry et al., 2009).

2. Passing the Blood-Brain Barrier. The therapeutic potential of RNA drugs requires efficient, tissue-specific, and nonimmunogenic delivery. Delivery of RNA interference to tissues at therapeutic effective doses, however, has proven difficult when using viruses,polycationic polyethylenimine-based nanoparticles, or liposomes(van den Boorn et al., 2011). Exosomes have been used to deliver short interfering RNA (siRNA) into the brain of mice in vivo. Exosomes were produced from autologous DCs, were genetically modified to express lysosomal-associated membrane protein-2b fused to a neuron-specific rabies virus glycoprotein peptide, and were loaded with glyceraldehyde 3-phosphate dehydrogenase siRNA. Intravenously injected rabies virus glycoprotein-targeted exosomes delivered glyceraldehyde 3-phosphate dehydrogenase siRNA specifically to neurons, microglia, and oligodendrocytes and induced a specific gene knockdown. The therapeutic potential of exosome-mediated siRNA delivery was shown by inhibition of more than 50% of expression and production of BACE1, a therapeutic target in Alzheimer's disease (Alvarez-Erviti et al., 2011). This targeted siRNA delivery is clinically very interesting because exosome-mediated drug delivery may overcome important obstacles in drug delivery, such as the blood-brain barrier, which is a major hindrance for treatment of neurological diseases (Lakhal and Wood, 2011). There is only one earlier study in which exosomes were used for drug delivery; in that study, exosomes were shown to transfer curcumin, an anti-inflammatory agent, to activated myeloid cells in vivo (Sun et al., 2010). In sum, because autologous exosomes can evade the immune system, are protected from complement deposition by exposing CD55 or CD59, and expose cell-type specific adhesion receptors, their use for drug delivery is promising.

3. Inflammation and Immune Response. There are alternative strategies to use exosomes for clinical applications. For instance, exosomes from mouse DCs, genetically modified to express interleukin-4, FasL, or the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase, have both immunosuppressive and anti-inflammatory properties when administered systemically in mouse models of experimental arthritis (Kim et al., 2006, 2007a; Bianco et al., 2009). The intra-articular administration of exosomes from DCs transduced with an adenovirus expressing interleukin-10 or from DCs treated with recombinant murine interleukin-10 suppresses delayed type hypersensitivity responses not only in the injected but also in the untreated contralateral joints and suppresses the onset of murine collagen-induced arthritis as well as the severity of arthritis, illustrating that exosomes can efficiently suppress inflammatory and autoimmune responses in vivo (Kim et al., 2005b).

Immature DCs release exosomes exposing lactadherin, which is required to opsonize apoptotic cells for phagocytosis. In an experimental sepsis model, decreased levels of lactadherin in spleen and blood were associated with impaired apoptotic clearance, and administration of exosomes promoted phagocytosis of apoptotic cells and reduced mortality, whereas lactadherin-deficient mice suffered from increased mortality. Thus, exosomes can attenuate the acute systemic inflammatory response in sepsis by enhancing the clearance of apoptotic cells and perhaps apoptotic vesicles via lactadherin interactions (Miksa et al., 2009).

4. Neovascularization. Transplantation of human $CD34^+$ stem cells to ischemic tissues reduces angina, improves exercise time, reduces amputation rates, and induces neovascularization in preclinical models. These beneficial effects of $CD34^+$ stem cells on therapeutic angiogenesis, however, seem to be mediated, at least in part, by exosomes produced by these cells. Exosomes, isolated from $CD34^+$ stem cell-conditioned medium, improved viability of endothelial cells and induced endothelial proliferation and tube formation in vitro and angiogenesis in various in vivo models. Thus, exosomes have an angiogenic activity in vitro and in vivo (Lai et al., 2010; Sahoo et al., 2011).

B. Prognosis

Patients with cancer have an increased risk of developing VTE, which is likely to be explained by the increased TF-initiated (extrinsic) coagulation activation that is observed in many patients with cancer (Kakkar et al., 1995). Because many tumors express and produce TF (e.g., ovarian cancer) and tumors release vesicles exposing coagulant TF, coagulant TF in blood of patients with cancer is likely to originate from the tumor (Uno et al., 2007), (Dvorak et al., 1981; Carr et al., 1985). These tumor-derived and TF-exposing vesicles were shown to be present in peripheral blood of patients with cancer (Zwicker et al., 2009), and tumor-derived vesicles exposing coagulant TF have been associated with patients with cancer who present with VTE (Tesselaar et al., 2007). Because the risk of bleeding is too high to treat all

patients with cancer before the onset of VTE, a study has been initiated in our institute in Amsterdam to predict the development of VTE in patients with cancer. In this study, the ability of blood-borne vesicles to trigger TF-mediated clotting is measured in plasma derived from the blood sample collected when patients enter the hospital for their chemotherapy. On the basis of this assay, patients are classified as either high or low risk for developing VTE. Patients have a 6-month follow-up. At present, eight European hospitals are involved and more than 400 patients have been included. Depending on the outcome of the results of this pilot study, a larger multicenter trial will be started to study the efficacy of prophylactic treatment of patients with cancer identified by the vesicle-based clotting test as having a high-risk for developing VTE.

C. Biomarkers

Cell-derived vesicles may behold a unique source of clinically relevant and noninvasive biomarkers. For example, exosomes in cerebrospinal fluid of patients with Alzheimer's disease contain τ phosphorylated at Thr181, which is an established biomarker of this disease (Saman et al., 2012). Furthermore, prostate-specific antigen and prostate-specific membrane antigen are associated with exosomes in urine of patients with prostate cancer (Mitchell et al., 2009); claudin-containing exosomes in peripheral blood are associated with ovarian cancer (Li et al., 2009); urinary vesicles may provide biomarkers for liver injury in animal models (Conde-Vancells et al., 2010); vesicles from ascites of patients with colorectal cancer may provide information on tumor development (Choi et al., 2011); and VEGF, endostatin, and endothelin-1 are detectable in prostasomes from malignant prostate cancer cell lines but not detectable on seminal prostasomes (Babiker et al., 2010), and prostasomes have been detected in the blood of patients with prostate cancer (Tavoosidana et al., 2011). Nevertheless, the extent to which these biomarkers are of clinical relevance needs further investigation.

V. Relevance of Cell-Derived Vesicles In Vivo

Thus far, most studies on cell-derived vesicles, including exosomes, have been performed in vitro or ex vivo. Despite the fact that we know that the concentration of vesicles, their cellular origin, composition and functional features changes during disease states, the question why cells are releasing vesicles is still unanswered, and we can only speculate on the relevance of vesicles in vivo. The biological activity of cell-derived vesicles will be highly dependent on how long the vesicles are present within their environment. The latter will be a delicate balance between their production and removal, a balance that will be markedly affected by the type of biological fluid in which the vesicles are present. For instance, the release of vesicles by cells in culture will not be affected by clearance, whereas vesicles in blood will be continuously exposed to clearance pressure. In part V of this review, we discuss the clearance.We describe how intercellular communication by vesicles from eukaryotic cells is not unique but closely resembles communication between bacteria and, as such, constitutes a highly conserved mechanisms of exchanging information.

A. Clearance

Cells become susceptible to complement-induced lysis when they lose key complement inhibitors such as CD46, CD55, and CD59. These inhibitors are sorted to microparticles and exosomes, most likely to prevent complement deposition and lysis of these vesicles, thereby increasing their chance of survival in the extracellular environment (Clayton et al., 2003; Elward et al., 2005). Although exosomes can be internalized via phagocytosis by binding to the PS receptor T-cell immunoglobulin and mucin-domain-containing molecule (TIM-4) (Feng et al., 2010), the extent to which exosomes and microvesicles expose PS in vivo remains unclear (Heijnen et al., 1999; Laulagnier et al., 2004; Connor et al., 2010). Phagocytosis of PS-exposing cells and microparticles may be mediated by lactadherin, because lactadherin knockout mice have an increased concentration of (platelet-derived) microparticles, and splenic macrophages from these mice show a decreased capacity to phagocytose these microparticles, resulting in a hypercoagulable state (Dasgupta et al., 2009). Alternatively, vesicles released from immature DCs were shown to expose lactadherin that could shield off PS from detection (Miksa et al., 2009).

Although several studies have reported a fast disappearance of vesicles from blood (Rand et al., 2006; van den Goor et al., 2007), there is also evidence that clearance may be biphasic. Injection of radiolabeled liposomes into patients with cancer resulted in half-life times of approximately 6 min and almost 5 h, suggesting a two-compartment model of distribution; after 24 h, the liposomes were localized in liver, spleen, lung, and bone marrow (i.e., organs rich in reticuloendothelial cells) (Lopez-Berestein et al., 1984). In addition, in guinea pigs, a biphasic clearance of vesicles was observed, with half-life times of 44 min and 11 h (Carr et al., 1985). In most other studies, however, relatively fast (10–30 min) but also much slower (5–6 h) half-life times of vesicle disappearance were reported (Rand et al., 2006; van den Goor et al., 2007; Luo et al., 2009; Rank et al., 2011).The clearance of vesicles will undoubtedly depend on the organism studied, the (clinical) conditions, and whether artificial phospholipid vesicles or cell-derived vesicles were studied. More detailed studies are necessary to establish the clearance of the various vesicle populations.

B. Intercellular Signaling by Vesicles Is an Evolutionary Conserved Mechanism

As described previously, there is a growing interest in the ability of cell-derived vesicles to exchange informa-

tion between eukaryotic cells. However, such a system of advanced intercellular communication is not unique to eukaryotic cells (Fig. 12). Gram-negative and some Gram-positive bacteria, such as *Bacillus anthracis* release vesicles (50–250 nm), most often called OMVs, containing phospholipids, proteins, DNA, RNA, and LPS (Kadurugamuwa and Beveridge, 1995; Yaron et al., 2000; Mashburn-Warren et al., 2008; Rivera et al., 2010). OMVs from the Gram-negative bacterium *Pseudomonas aeruginosa* contain signaling molecules, such as the hydrophobic 2-heptyl-3-hydroxy-4-quinolone, to coordinate group behavior of this bacterium (Mashburn and Whiteley, 2005; Winans, 2005). It is noteworthy, however, that OMVs are used not only to exchange information between bacteria but also to communicate with eukaryotic cells; as such, they are a major cause of disease. For instance, the OMVs of *P. aeruginosa* contain virulence factors, such as phospholipase C, β-lactamase, and proteases, that can be delivered into the cytoplasm of cells after membrane fusion, thereby killing these cells (Kadurugamuwa and Beveridge, 1995; Bomberger et al., 2009). Likewise, OMVs from enterotoxigenic *Escherichia coli*, the prevalent cause of travelers' diarrhea and infant mortality in third-world countries, transfer heat-labile enterotoxin to recipient adrenal and intestinal epithelial cells. These OMVs enter the cells via endocytosis in a mechanism involving detergent membrane-resistant membrane domains, and trigger the production of interleukin-6 via a Nuclear factor-κB-dependent mechanism (Chen et al., 2010; Chutkan and Kuehn, 2011). Delivery of Gram-negative peptidoglycans of *Helicobacter pylori*, *P. aeruginosa*, or *Neisseria gonorrhoea* by OMVs into the cytosol of host cells via adetergent-resistant membrane domain-dependentmechanism results in recognition by the intracytoplasmic pathogen-recognition molecule Nod1 in epithelial cells. When these OMVs are delivered intragastrically to mice, the vesicles evoke both innate and adaptive immune responses via a Nod1-dependent but TLR-independent mechanism, suggesting that OMVs may be a generalized mechanism by which Gram-negative bacteria promote inflammation and pathology in infected hosts (Kaparakis et al., 2010).

OMVs contain also death messages to kill other species of bacteria. These antibacterial factors, such as murein hydrolases, are concentrated in OMVs, which, after binding to target bacteria, degrade the peptidoglycan wall and deliver the vesicular content, followed by bacterial death (Kadurugamuwa and Beveridge, 1996; Kadurugamuwa et al., 1998). Gentamicin, an antibiotic that inhibits bacterial protein synthesis and is often used to treat infections of *P. aeruginosa*, not only increases the release of OMVs but also these vesicles also contain gentamicin, which enhances the "predatory activity" of these OMVs (Kadurugamuwa and Beveridge, 1998, 1999). In fact, these vesicles can be used for delivery of

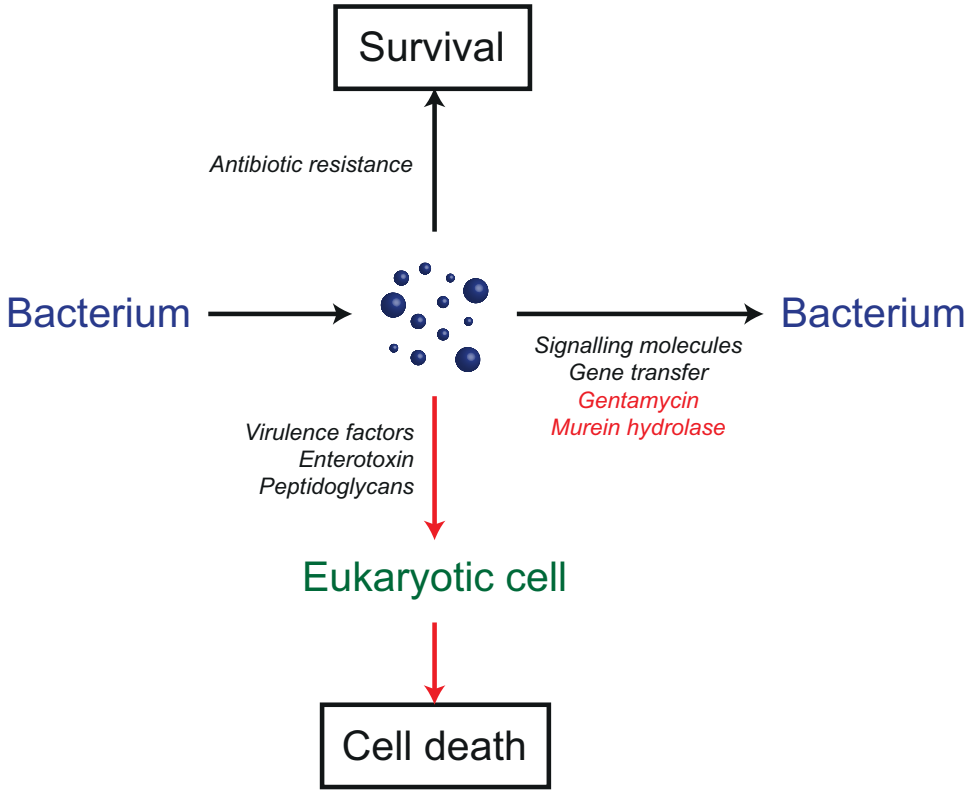

FIG. 12. Bacterial outer membrane vesicles. Outer membrane vesicles facilitate communication between bacteria by transferring, for example, signaling molecules and genes. In addition, bacteria discard antibiotics via outer membrane vesicles, and outer membrane vesicles induce eukaryotic cell death by transferring virulence factors.

antimicrobials to the cytoplasm of infected host cells (Kadurugamuwa and Beveridge, 1998, 1999).

OMVs from food-borne *E. coli* O157:H7 facilitate transfer of genes to and expression by recipient *Salmonella enterica* serovar Enteritidis or *E. coli* JM109. Not only was the cytotoxicity of the transformed bacteria increased, but also the vesicles transferred antibiotic resistance to ampicillin. Thus, not only vesicles from eukaryotic cells but also OMVs are likely to play a increases role in the intercellular exchange of functional genetic information (Yaron et al., 2000).

There has been a long interest in the ability of OMVs to activate the immune system, and OMV-based vaccines have been used in vaccination protocols against serogroup B meningococcal disease (Bjune et al., 1991; Sadarangani and Pollard, 2010; Caron et al., 2011). Classic OMV vaccines have a reduced LPS content because of detergent extraction, but meningococcal strains have been used with genetically detoxified LPS to reduce the endotoxin associated activity (van der Ley and van den Dobbelsteen, 2011). Likewise, OMVs from genetically engineered *Escherichia coli* also provide an easily purified vaccine-delivery system (Chen et al., 2010). Thus, OMVs behold the promise for a new generation of prophylactic and therapeutic vaccines.

The underlying molecular mechanisms by which OMVs activate the immune system are not well known, but stimulation of macrophages and DCs with OMVs from *Salmonella typhimurium* results in a strong proinflammatory and immune response in vitro, including increased expression and production of MHC class II molecules and CD86, TNF-α and interleukin-12. Mice vaccinated with these OMVs generate *S. typhimurium*-specific immunoglobulin (B cell) and $CD4^+$ T-cell responses and protect mice from infectious challenge with live *S. typhimurium* (Alaniz et al., 2007). Not only OMVs of Gram-negative bacteria activate the immune system, but also OMVs from the Gram-positive bacterium *B. anthracis* induce an IgM response in BALB/c mice, and OMV-immunized mice live longer than control mice after *B. anthracis* challenge. Furthermore, part of the toxic components of *B. anthracis* are associated with OMVs and taken up by macrophages, thus allowing a concentrated delivery of toxins to target cells (Rivera et al., 2010).

All aforementioned features of OMVs, including the sorting of specific molecules into these vesicles, the exchange of genetic information, or the killing of other cells at a distance, are therefore not unique for vesicles from eukaryotic cells. The discovery of nanobacteria, fossils of small bacteria-shaped spherical particles and now thought to be fossilized bacterial OMVs but initially described as a new and independent life form, suggest that the system of exchanging messages by vesicles is likely to be at least millions and perhaps billions of years old already (Mashburn-Warren et al., 2008).

VI. Conclusions and Future Directions

The research on cell-derived vesicles from eukaryotic cells is a fast-growing and exciting new field. Although major progress has been made during the last decade, our understanding of the molecular mechanisms underlying the release of vesicles and the sorting of compounds into these vesicles are still incompletely understood, the criteria to identify different types of vesicle have only been partially elucidated so far, and, most importantly, the biological relevance of vesicles in health and disease is poorly understood, especially in vivo.

The finding that vesicles enable the intercellular exchange of biomolecules suggests a new level of communication that may increase our understanding of disease development and progression. Moreover, vesicles may be useful as clinical instruments for prognosis and biomarkers, and they are promising as autologous drug vehicles capable of passing pharmacological barriers. To increase their clinical usefulness, however, novel and dedicated instruments will have to be developed to detect vesicles and to standardize vesicle measurements between laboratories.

Thus far, extracellular vesicles have often been considered either good or bad, but we hypothesize that vesicles may have functions at cellular and environmental levels. For example, when cells release waste-containing vesicles, the waste should be prevented from contacting the extracellular environment. Therefore, vesicles are designed in such a way that they are protected from complement-induced lysis but promote removal by phagocytosis. The exposure of PS, which may be one of the triggers of phagocytosis, also promotes coagulation, which, together with inflammation, protects the organism against external stress. When there is imbalance between the release of vesicles and removal, however, changes in the vesicle levels may affect coagulation, for example, or induce the production of autoantibodies. Likewise, the presence of vesicles that are normally absent, such as vesicles from the placenta or a tumor, may initiate adverse effects contributing to disease development or progression. The answer to the question of whether vesicles are good or bad will depend not only on the level (cell, tissue, organ, organism) and process studied but also on the kind of biological fluid and condition. For example, saliva and urine contain TF-exposing vesicles under normal conditions to facilitate wound healing, whereas blood contains TF-exposing vesicles only under pathological conditions, including surgery, disseminated intravascular coagulation, inflammation, or cancer.

Because vesicles seem to be conserved throughout evolution, more comparative studies will be necessary to gain more insight into similarities, differences, and underlying mechanisms. Taken together, more research on vesicles from eukaryotic cells will be necessary to unravel their complex biology and functions.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: van der Pol, Böing, Harrison, Sturk, and Nieuwland

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