Membrane vesicles as conveyors of immune responses

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Abstract | In multicellular organisms, communication between cells mainly involves the secretion of proteins that then bind to receptors on neighbouring cells. But another mode of intercellular communication — the release of membrane vesicles — has recently become the subject of increasing interest. Membrane vesicles are complex structures composed of a lipid bilayer that contains transmembrane proteins and encloses soluble hydrophilic components derived from the cytosol of the donor cell. These vesicles have been shown to affect the physiology of neighbouring recipient cells in various ways, from inducing intracellular signalling following binding to receptors to conferring new properties after the acquisition of new receptors, enzymes or even genetic material from the vesicles. This Review focuses on the role of membrane vesicles, in particular exosomes, in the communication between immune cells, and between tumour and immune cells.

Nibbling
The ability of dendritic cells to physically strip large membrane fragments from live cells on close contact without inducing death of the donor cell.

Trogocytosis
The transfer of plasma membrane fragments from one cell to another without cell death induction. This process is mediated by receptor signalling following cell–cell contact.

Nanotube
A membranous channel of 50–200 nm in diameter that connects cells over long distances.

Membrane vesicle
A spherical or approximately spherical structure limited by a lipid bilayer, which encloses soluble cargo.

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The exchange of membrane-derived proteins between cells of the immune system was first described almost 30 years ago on the basis of studies of bone marrow chimeras, in which it was observed that donor thymocytes could acquire host-derived MHC molecules[1]. Many other reports have since described how portions of cell membranes can be transferred between cells, either after direct cell–cell contact (through the recently proposed mechanisms of nibbling, trogocytosis and nanotubes) or through the secretion of membrane vesicles. The functional consequences of such membrane transfers include the induction, amplification and/or modulation of immune responses, as well as the acquisition of new functional properties by recipient cells, such as migratory or metastatic abilities. In addition, a recent study has described the presence of mRNAs and microRNAs in secreted membrane vesicles, and this has raised the exciting possibility that the transfer of genetic material might affect the function of recipient cells. Together, these studies have led to the idea that membrane transfer is a common mode of intercellular communication. A review focusing on membrane transfer following direct contact between immune cells has been published recently[2], and so this process will not be discussed further. Instead, in this Review we provide a comprehensive overview of the mechanisms and consequences of long-range membrane exchange involving secreted membrane vesicles, with a specific emphasis on the functional outcomes during immune responses. In addition, we aim to clarify the confusion created by the heterogeneity of membrane vesicle terminology used in the literature. We therefore classify membrane vesicles on the basis of their intracellular origin.

Biology of secreted membrane vesicles

Classification of secreted membrane vesicles. Membrane vesicles are spherical structures that are limited by a lipid bilayer (of similar structure to that of cell membranes) and that contain hydrophilic soluble components. In eukaryotic cells, the transfer of components between intracellular compartments involves carrier vesicles that bud from the membrane of a donor compartment and travel in the cytoplasm before fusing with the membrane of an acceptor compartment. These carrier vesicles contain material from the lumen of the donor compartment and expose the cytoplasmic side of this compartment at their outer surface (FIG. 1). All these vesicles remain strictly intracellular. In addition, cells can generate membrane vesicles that are secreted into the extracellular space; such vesicles can form either at the plasma membrane or in the lumen of internal compartments. Irrespective of their origin, these vesicles contain cytosol and expose the luminal (that is, extracellular) side of the membrane they form from at their outer surface. Because their membrane orientation is the same as that of the donor cell, they can be considered to be miniature versions of a cell.

There are various types of secreted membrane vesicles that have distinct structural and biochemical properties depending on their intracellular site of origin, and these

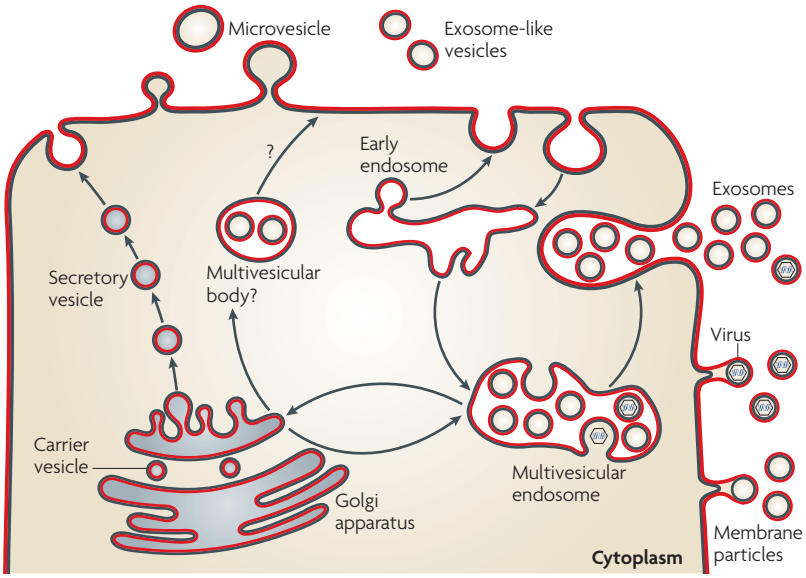

Figure 1 | **Different types of secreted membrane vesicles.** Intracellular trafficking either between subcellular compartments or towards the plasma membrane for secretion of soluble proteins occurs through carrier and secretory vesicles that contain intraluminal components. By contrast, secreted membrane vesicles contain cytoplasmic components. Secreted membrane vesicles can form at the plasma membrane by direct budding into the extracellular space, giving rise to microvesicles, envelopped viruses (such as retroviruses) and membrane particles. Alternatively, secreted vesicles can form inside internal compartments from where they are subsequently secreted by fusion of these compartments with the plasma membrane. Vesicles generated in multivesicular endosomes are called exosomes once secreted. Some viruses, such as retroviruses, hijack the exosomal machinery and also bud inside multivesicular endosomes and are subsequently secreted. Finally, exosome-like vesicles are also thought to originate from internal multivesicular compartments (or multivesicular bodies), although the nature of these compartments is not clear.

features probably affect their function (FIG. 1). TABLE 1 provides an overview of the main characteristics of each type of vesicle that is released by live cells. Membrane vesicles are also released by dying and/or apoptotic cells, and vesicle preparations obtained from cultures containing unhealthy cells can therefore be contaminated by apoptotic cell-derived vesicles. These vesicles have different features to those derived from live cells (TABLE 1) and are not discussed further in this Review.

Large membrane vesicles (>100 nm diameter) that are secreted by budding or shedding from the plasma membrane of platelets[3], tumours[4,5], neutrophils[6] and dendritic cells (DCs)[7] have been described; such vesicles have been referred to as microvesicles, ectosomes, microparticles and exovesicles. Other types of membrane vesicles that are also secreted from the plasma membrane include smaller (<100 nm) membrane particles, which arise from the shedding of prominin 1-enriched membrane subdomains[8]. Enveloped viruses that bud from the cell surface or from internal compartments, such as HIV, can also be considered as another type of secreted membrane vesicle[9] (FIG. 1).

Membrane vesicles can also be secreted following the fusion of internal compartments that contain intraluminal vesicles with the plasma membrane (FIG. 1). Such fusion events involving late endocytic compartments, known as multivesicular endosomes (MVEs), were first observed more than 20 years ago[10,11], and the term exosome has been used since 1987 to refer to these exocytosed internal vesicles of endosomal origin (BOX 1). However, typical exosomes are sometimes referred to as microvesicles[12–14], or were initially described as plasma membrane vesicles[5] and later characterized as exosomes[15]. True exosomes share key biophysical and biochemical characteristics with the internal vesicles of MVEs (TABLE 1, FIG. 2), in particular their size (50–100 nm in diameter), which distinguishes them from other small vesicles that have different biophysical characteristics and have therefore been called nanoparticles[16] or exosome-like vesicles[17] (TABLE 1). The exact nature of the intracellular compartments from which exosomes derive is still unclear. A recent study suggests that in oligodendrocytes the formation of intraluminal vesicles that are destined for either secretion as exosomes or degradation in lysosomes involves different molecular machineries[18]. This observation, if it holds true for other cell types, suggests that there might be different types of MVE or different types of vesicle within MVEs. In addition, it is possible that other multivesicular compartments (maybe not part of the endosomal system) can generate other exosome-like vesicles.

Constitutive and inducible vesicle secretion. The secretion of microvesicles or ectosomes by blood cells is regulated by various stimuli that lead to increased intracellular calcium levels, which induces plasma membrane remodelling and vesicle shedding[19]. Such stimuli include the activation of $P2X_7$ receptor (purinergic receptor P2X, ligand-gated ion channel, 7) by ATP on monocytes and neutrophils[20], thrombin receptor on platelets[3] and Toll-like receptor 4 by lipopolysaccharide (LPS) on DCs[7].

By contrast, the secretion of exosomes can be spontaneous or induced depending on the cell type. Reticulocytes[11], T cells[21,22], mastocytes[23] and resting B cells[24–27] secrete detectable levels of exosomes only following the activation of a cell surface receptor. By contrast, Epstein–Barr virus (EBV)-transformed B cells[28], DCs[29] and macrophages[30] constitutively secrete exosomes *in vitro*, as do most tumour cell lines. In the case of DCs, the extent of exosome secretion varies during their life cycle: LPS-matured DCs secrete fewer exosomes than immature DCs (25–75% reduction)[31–33]. In addition, secretion by immature DCs is transiently increased following cognate interactions with T cell clones[145], suggesting that some T cell-derived stimuli can induce exosome secretion by DCs. Finally, deleterious treatments such as radiation or senescence induction increase the secretion of exosome-like membrane vesicles by tumour cells[34,35], fibroblasts and DCs[36]. In these DNA-damaging conditions, activation of the p53 transcription factor leads, among many other physiological changes, to increased expression of the transmembrane protein tumour suppressor-activated pathway 6 (TSAP6), which is required for increased exosome secretion[36]. Exosome secretion is therefore modulated in different cell types by various environmental changes, such as ligand encounter or stress conditions, and could be one of the means used by tissues to adapt to these changes.

MicroRNA
A small RNA molecule that regulates the expression of genes by binding to the 3′-untranslated regions of specific mRNAs.

Carrier vesicle
An intracellular membrane vesicle that buds from internal compartments (such as the endoplasmic reticulum, Golgi apparatus and endosomes) into the cytoplasm. Carrier vesicles contain cargo from the lumen of the donor compartment.

Table 1 | **Physicochemical characteristics of different types of secreted vesicle**

Feature*	Exosomes	Microvesicles	Ectosomes	Membrane particles	Exosome-like vesicles	Apoptotic vesicles
Size	50–100 nm	100–1,000 nm	50–200 nm	50–80 nm	20–50 nm	50–500 nm
Density in sucrose	1.13–1.19 g/ml	ND	ND	1.04–1.07 g/ml	1.1 g/ml	1.16–1.28 g/ml
Appearance by electron microscopy‡	Cup shape	Irregular shape and electron-dense	Bilamellar round structures	Round	Irregular shape	Heterogeneous
Sedimentation	100,000 g	10,000 g	160,000–200,000 g	100,000–200,000 g	175,000 g	1,200g , 10,000 g or 100,000 g
Lipid composition	Enriched in cholesterol, sphingomyelin and ceramide; contain lipid rafts; expose phosphatidylserine	Expose phosphatidylserine	Enriched in cholesterol and diacylglycerol; expose phosphatidylserine	ND	Do not contain lipid rafts	ND
Main protein markers	Tetraspanins (CD63, CD9), Alix and TSG101	Integrins, selectins and CD40 ligand	CR1 and proteolytic enzymes; no CD63	CD133; no CD63	TNFRI	Histones
Intracellular origin	Internal compartments (endosomes)	Plasma membrane	Plasma membrane	Plasma membrane	Internal compartments?	ND
Main reference	142	3	143	8	17	144

*The listed features of vesicles secreted by live cells are based on observation of preparations of 100% pure vesicles. However, in practice, all vesicle preparations are heterogeneous, with different protocols allowing the enrichment of one type over another, and they can be classified according to the presence of several (but not necessarily all) of the listed features. ‡Appearance by electron microscopy is only an indication of vesicle type and should not be used to define vesicles, as their microscopic appearance can be influenced by the fixation and phase contrast techniques used. CR1, complement component receptor 1; ND, not determined; TNFRI, tumour necrosis factor receptor I; TSG101, tumour susceptibility gene 101.

Follicular DC
A specialized non-haematopoietic stromal cell that resides in the follicles and germinal centres. Follicular dendritic cells (DCs) have long dendrites but are not related to DCs and carry intact antigen on their surface.

In addition to p53 (REFS 34,35), several intracellular proteins, such as diacylglycerol kinase α[37] and brefeldin A-inhibited guanine-nucleotide exchange protein 2 (BIG2; also known as ARFGEF2)[38], have been proposed to have a role in the secretion of exosomes or exosome-like vesicles. However, these molecules are also involved in more general physiological processes: p53 is involved in the cellular response to stress, diacylglycerol kinase-α in signal transduction by lipid phosphorylation and BIG2 in protein trafficking in the secretory pathway. The small GTPase RAB11 promotes exosome secretion in an erythroleukaemia cell line[39], but whether it acts directly on MVEs or by modulating other endosomal compartments (such as recycling endosomes) is not clear. The specific molecular mechanisms underlying exosome secretion are therefore still unknown, and we currently lack the means to promote or prevent secretion without affecting the general cell physiology.

Box 1 | **History of exosome discovery**

The term exosome was first used in 1981 to describe microvesicles that contain a 5′ nucleotidase activity and that are secreted by neoplastic cell lines[127]. A few years later, the groups of Stahl and Johnstone[10,11] reported the secretion of vesicles of endocytic origin by cultured reticulocytes. These small vesicles (50–100 nm in diameter) originated from large multivesicular endosomes and contained the transferrin receptor. Indeed, using electron microscopy they observed the fusion of multivesicular endosomes with the plasma membrane, resulting in the secretion of these internal vesicles in the extracellular medium. These vesicles were purified by ultracentrifugation of the culture supernatant and termed exosomes. These results were controversial because at the time late endosomes were considered to be transitional compartments, the content of which was destined for degradation by lysosomes and not for recycling to the cell surface. In 1989, Peters *et al.*[21] showed the existence of fusion profiles between late endosomes and the plasma membrane in cytotoxic T cells, which could explain the secretion of cytotoxic granules. But it was not until 1996, when the secretion of the internal vesicles of multivesicular endosomes by Epstein–Barr virus-transformed B cells was described[28], that the functionality of exosomes began to be widely studied. Indeed, B cell-derived exosomes formed in MHC class II-containing compartments and contained peptide–MHC class II complexes that could be presented to antigen-specific T cells. These results were then extended to dendritic cells[29,31]. In addition, the possibility that exosomes were involved in several pathological conditions, such as in antitumour immune responses, the pathogenesis of infectious diseases caused by viruses (such as HIV and herpes simplex virus)[128] and prions[129], and the pathogenesis of Alzheimer's disease[130], has stimulated further research on these enigmatic vesicles.

***Existence of secreted membrane vesicles* in vivo.** Although there have been numerous descriptions of membrane vesicles that are secreted by cells cultured *in vitro*, determining whether they are generated *in vivo* has proved challenging and continues to be a subject of debate. Current methods to address this issue are limited by the fact that *ex vivo* purified vesicles are artificially generated during the manual dissociation of tissues (for example, arising from the plasma membrane of damaged or dying cells, or from ruptured intracellular compartments). So, any reports claiming that exosomes can be isolated after mechanical dissociation of tissues must be interpreted with caution; even if the protein composition of these vesicles resembles that of exosomes, they could correspond to internal vesicles of ruptured multivesicular compartments rather than to secreted exosomes.

Electron microscopy studies of tissue sections have shown the presence of nanometre-sized vesicles bearing MHC class II and tetraspanin molecules in tonsil germinal centres, where they seem to be attached to the surface of follicular DCs[40]. As follicular DCs are non-haematopoietic cells and therefore do not express MHC class II molecules, this observation suggests that follicular DCs capture MHC class II-bearing exosomes from the surrounding B cells. In addition, membrane vesicles

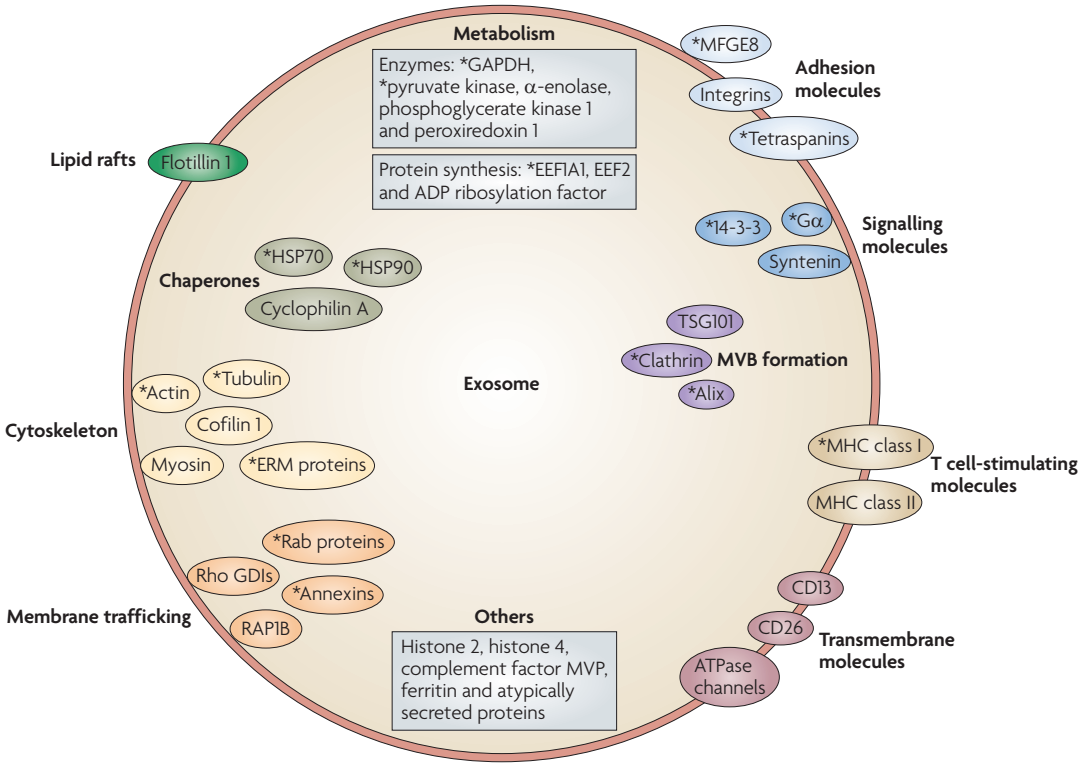

Figure 2 | **Protein composition of a canonical exosome.** This schematic showing the composition of a typical exosome is based on data from 15 proteomic analyses carried out on exosomes purified from cultured cells[106,129,131–140] and from biological fluids[46,48,141]. Proteins found in at least 30% of different exosomes are listed and proteins present in at least 50% of exosomes are indicated by an asterisk. EEF, eukaryotic translation elongation factor; ERM, ezrin, radixin and moesin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase-activating protein; HSP, heat shock protein; MFGE8, milk fat globule EGF factor 8 protein; MVB, multivesicular body; MVP, major vault protein; RAP1B, RAS related protein 1B; Rho GDI, Rho GDP dissociation inhibitor; TSG101, tumour susceptibility gene 101.

and/or exosomes have been purified in the absence of any mechanical dissociation from several body fluids, such as human plasma[41], serum[42,43], bronchoalveolar fluid[44,45], urine[46], tumoral effusions[15,47], epididymal fluid[48], amniotic fluid[49] and milk[50,51]. These exosomes contain proteins that are expressed by epithelial, tumour and haematopoietic cells, which suggests that exosomes could be released from several cell types and tissues *in vivo*. Tumour cell-derived vesicles have been described in the serum of patients with tumours, with increased amounts in patients with advanced cancers[43], showing that secreted vesicles can travel far from secreting cells.

Interaction of membrane vesicles with neighbouring cells. Visualizing exosomes following encounter with recipient cells *in vitro* or *in vivo* has proven to be a difficult task. This is because of their small size, which makes exosomes undetectable by regular confocal microscopy and only reliably visualized by electron microscopy. Improvements in current imaging techniques will be required to determine the full extent of exosome production and their fate *in vivo*. Nevertheless, several types of interaction between exosomes and recipient cells have been proposed based on indirect evidence and *in vitro* studies (FIG. 3). These include adhesion of vesicles to the recipient cell surface through lipids or ligand–receptor interactions, internalization of whole vesicles into endocytic compartments mediated by various receptors and fusion of vesicles with the plasma membrane or possibly with internal endosomal membranes.

All membrane vesicles of any cellular origin express adhesion molecules on their surface, which could favour their capture by recipient cells. Co-incubation of exosomes and DCs with blocking antibodies specific for various integrins, adhesion molecules or tetraspanins reduced exosome capture by DCs by 5–30% (REF. 52), but the contribution of exosomal or cellular receptors to this process was not determined in this study. More recently, it has been shown that intercellular adhesion molecule 1 (ICAM1)-bearing exosomes derived from mature DCs are captured by binding to lymphocyte function-associated antigen 1 (LFA1; a ligand for ICAM1), on the surface of $CD8^+$ DCs[53] and activated T cells[54], implicating a role for this ligand–receptor interaction in exosome capture. Retroviruses might have evolved to hijack this mechanism to favour dissemination: indeed, HIV virions express on their surface ICAM1 that is derived from the plasma membrane of infected T cells and can therefore interact with other T cells that express LFA1 (REFS 55,56). Alternatively, β1 and β2 integrins on exosomes might bind to ICAM1-expressing cells[57] and extracellular matrix proteins such as fibronectin[57,58].

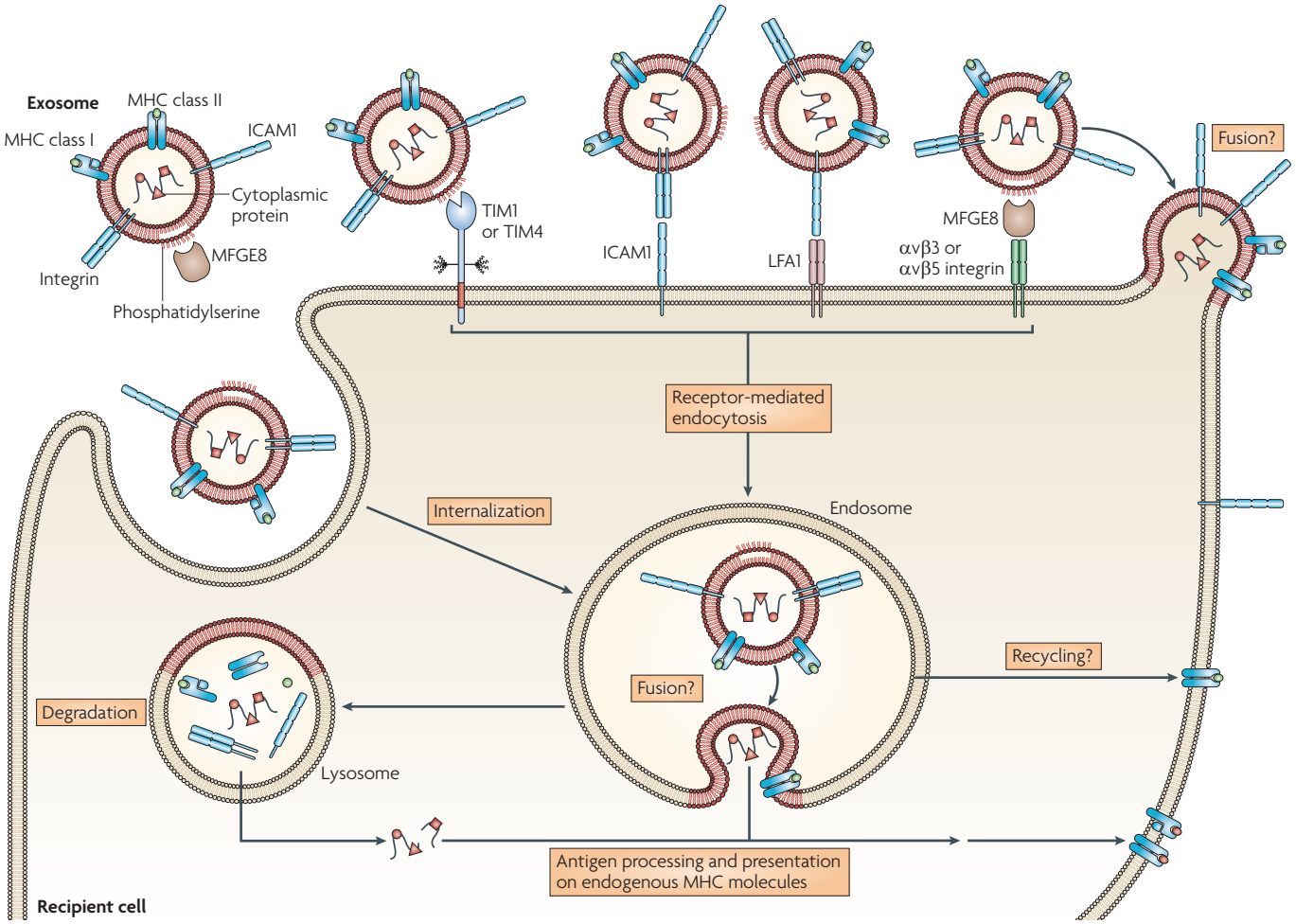

Figure 3 | **Interaction of secreted membrane vesicles with recipient cells.** The binding of secreted vesicles to the surface of a recipient cell involves interactions between exosomal molecules and cellular receptors. These include intercellular adhesion molecule 1 (ICAM1) binding to lymphocyte function-associated antigen 1 (LFA1), phosphatidylserine binding to T cell immunoglobulin domain and mucin domain protein 1 (TIM1) or TIM4 and possibly milk fat globule EGF factor 8 protein (MFGE8) binding to αvβ3 or αvβ5 integrins. Other unknown receptors might also participate in this process. After interacting with molecules on the recipient cell surface, exosomes might directly fuse with the recipient plasma membrane, leading to the incorporation of proteins from the exosomal membrane into the plasma membrane and the release of exosome contents into the cytoplasm of the recipient cell. Alternatively, endocytosed exosomes could fuse with the limiting membrane of the endosome, leading to the release of exosome contents in the cytoplasm and incorporation of exosome membrane proteins into the endosome membrane, which could then be recycled to the cell surface. Finally, exosomes internalized through receptor-mediated endocytosis or phagocytosis could be degraded in the endocytic or phagocytic pathway. This degradation can lead to the production of antigenic peptides for loading onto MHC class II and class I molecules.

Exosomes and microvesicles also expose phosphatidylserine on their surface and therefore could be captured by phosphatidylserine receptors. For example, T cell immunoglobulin domain and mucin domain protein 1 (TIM1; also known as HAVCR1) and TIM4 (also known as TIMD4), which have recently been described to be phosphatidylserine-binding molecules, are expressed on the surface of activated lymphocytes or phagocytes, respectively, and can mediate the capture of membrane vesicles such as exosomes and larger vesicles[59]. In addition, MFGE8 (milk fat globule epidermal growth factor (EGF) factor 8 protein; also known as lactadherin) binds to phosphatidylserine exposed on the surface of apoptotic cells and secreted membrane vesicles through its carboxy-terminal factorV/VIII-like domain[51,60,61]. The amino-terminal domain of MFGE8 that is exposed on the surface of MFGE8-bearing apoptotic bodies binds to αvβ3 and αvβ5 integrins[51], thereby promoting their phagocytosis by macrophages[60]. It is possible, therefore, that MFGE8 is also involved in the capture of membrane vesicles by αvβ3 integrin- or αvβ5 integrin-expressing phagocytes. However, MFGE8-deficient exosomes are captured efficiently by mouse bone marrow-derived DCs that express little or no αvβ3 or αvβ5 integrins *in vitro*[33,61], indicating that MFGE8-independent mechanisms can also account for vesicle capture.

The fate of exosomes after binding to the surface of recipient cells is not known. Recent evidence suggests that exosomes might fuse with recipient cell membranes. Indeed, stem cell-derived microvesicles have been shown to transfer RNA to haematopoietic cells, leading to reprogramming of haematopoietic cell differentiation[62], and microvesicles from glioblastoma cell lines can transfer reporter RNA to normal endothelial cells[63]. These findings suggest that in both cases the RNA is directly delivered into the cytoplasm of the recipient cells. Similarly, RNA present in mouse mast cell-derived exosomes can be transferred and transcribed into mouse proteins in human mast cells[64]. Another recent report[4] showed that tumour microvesicles can transfer an oncogenic form of EGF receptor (EGFR) to cells expressing a wild-type receptor, which leads to aberrant intracellular signalling by the oncogenic receptor and subsequent transformation of the recipient cell. Whether exosome fusion occurs on the surface of the recipient cell or after endocytosis in internal compartments is still unclear.

Functions of membrane vesicles *in vitro*

In this section we summarize the proposed roles of membrane vesicles in various stages of an immune response, as suggested by studies carried out *in vitro* with membrane vesicles purified from body fluids or cultured cells. The effects of membrane vesicles on immune cells are schematically represented in FIG. 4. Because exosomes are the best studied of all secreted membrane vesicles, we focus on their functions, but this does not mean that exosomes are physiologically more important than other vesicles.

Membrane vesicles and antigen presentation. The finding that secreted membrane vesicles carry both antigenic material and peptide–MHC complexes generated interest by immunologists on their possible roles in triggering immune responses (FIG. 4a). Membrane vesicles released by live cells are a possible source of exogenous antigens for antigen-presenting cells (APCs). Exosomes purified from cultured tumour cell lines[65] or from ascites of patients with tumours[47] contain tumour antigens and can induce the activation of antigen-specific T cells *in vitro* in the presence of recipient DCs that have otherwise not encountered the antigen. Antigens that are commonly present in tumour-derived exosomes include transmembrane proteins from the donor cell surface (such as human EGFR2 and carcinoembryonic antigen (CEA), which are found in various carcinomas), or from the donor cell endosomal compartments (such as MART1 (also known as MLANA), gp100 and tyrosinase-related protein 1 from melanosomes of melanocytes). Moreover, exosomes derived from macrophages infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis*, which reside in endosomal compartments, contain pathogen-derived antigens[66], and exosomes derived from endothelial cells infected with cytomegalovirus also transfer virus-derived antigens to DCs for activation of $CD4^+$ T cells[67]. By contrast, exosomes secreted by DCs infected with lymphocytic choriomeningitis virus (LCMV) do not bear LCMV antigens and are not involved in the induction of immune responses against this virus[68].

In addition to whole or partially processed antigens, secreted vesicles also display preformed peptide–MHC complexes on their surface that can, in certain circumstances, be directly presented to T cells. Exosomes and microvesicles derived from virtually any cell type bear MHC class I molecules that could potentially induce $CD8^+$ T cell activation. Indeed, several groups have reported that DC-derived exosomes could induce the activation of $CD8^+$ T cell clones, either alone[69–71] or when incubated with DCs that express allogeneic MHC class I molecules[72,73], which indicates that preformed peptide–MHC class I complexes from exosomes are functional. T cell activation was more efficient when exosomes were purified from mature rather than immature DCs, suggesting that mature DC-derived co-stimulatory molecules that are present on exosomes participate in co-stimulating the T cells[70]. Nevertheless, tumour-derived exosomes only seem to activate $CD8^+$ T cells when incubated in the presence of recipient DCs that express the right MHC haplotype[47,65]. This could be due to the absence of co-stimulatory molecules on the surface of tumour cells and tumour cell-derived exosomes. However, exosomes bearing a mismatched MHC haplotype can still induce T cell activation in these conditions, so the relevant antigenic entity carried by exosomes from tumours seems to be the antigens (which have to be processed by the recipient APCs) and not preformed peptide–MHC complexes.

APC-derived exosomes also contain large amounts of MHC class II molecules[28,31] because they originate from late endocytic compartments, in which MHC class II molecules reside. APC-derived exosomes can directly activate cognate T cell clones or lines[28,70], or pre-activated $CD4^+$ T cells[27], but they need to be captured by recipient DCs to activate naive $CD4^+$ T cells[27,33,74]. In these conditions, DC-derived exosomes can transfer preformed peptide–MHC class II complexes to MHC class II-deficient DCs, thereby enabling them to activate antigen-specific $CD4^+$ T cells[33,74]. As observed with $CD8^+$ T cells, exosomes derived from mature DCs induce more efficient $CD4^+$ T cell activation *in vitro* than those from immature DCs[33,75], and exosome-derived MHC molecules can also be presented by B cells, which alone poorly activate naive $CD4^+$ T cells[33]. Recipient DCs can also process MHC molecules from exosomes of allogeneic DCs to generate peptides to load onto their own MHC molecules[75]. In addition, in MHC class II-expressing intestinal epithelial cells[76] or macrophages[66], MHC class II molecules must be present on both exosomes and recipient DCs, suggesting that following capture of exosomes by DCs, peptide is extracted from the groove of exosomal MHC molecules and reloaded onto MHC molecules of the recipient DC.

Similarly to exosomes, microvesicles released from the plasma membrane of mature DCs can also transfer MHC molecules to recipient DCs for the activation of allogeneic T cells[7]. Exosomes and/or microvesicles are therefore probably responsible for the transfer of MHC class II molecules between DCs in culture that was observed 10 years ago[77].

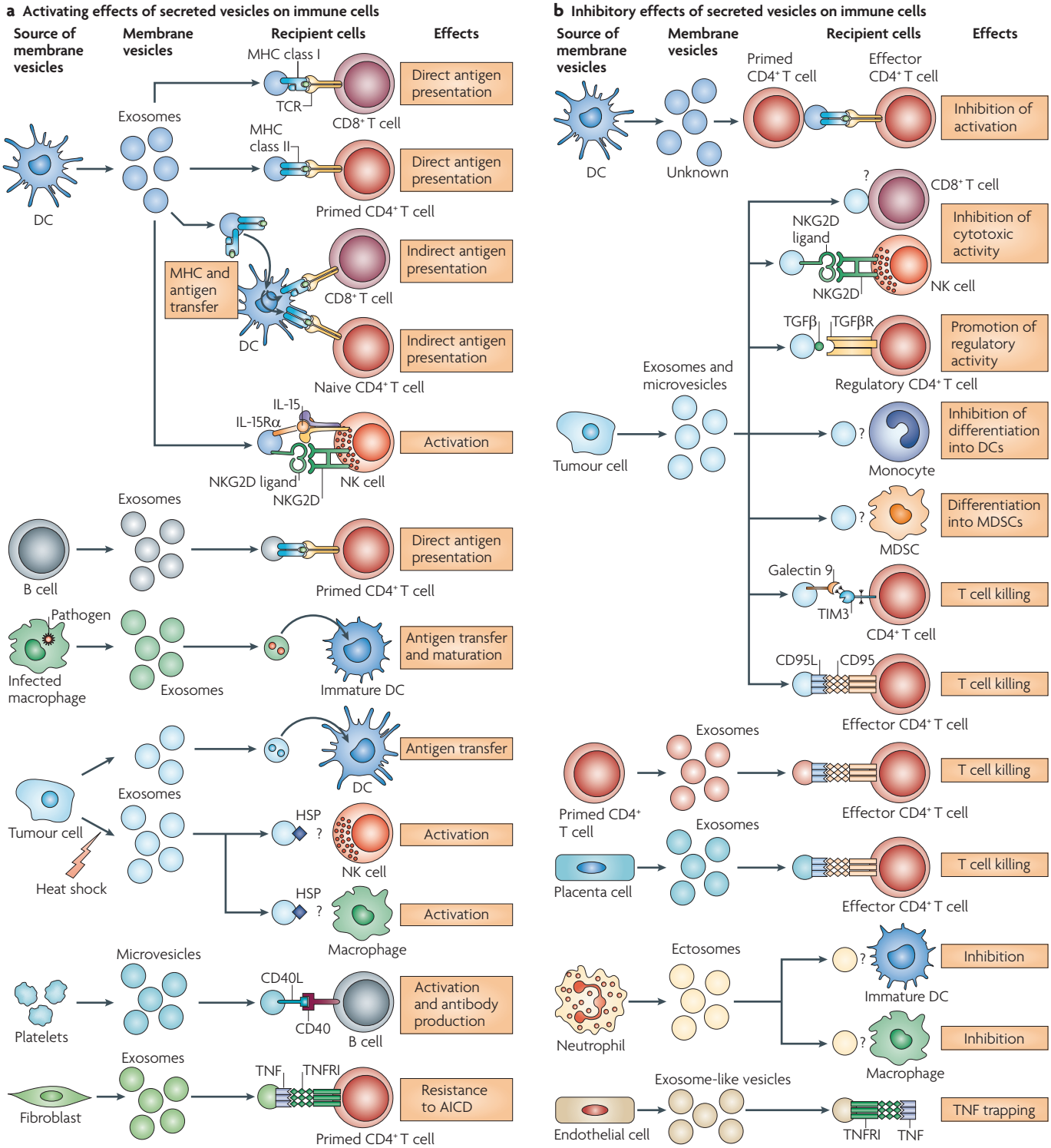

Figure 4 | **Involvement of secreted vesicles in interactions of immune cells.** This figure summarizes the main effects of secreted vesicles on immune cells observed *in vitro*. **a** | Vesicles secreted from various cellular sources have numerous activating effects on immune cells, including direct peptide–MHC complex presentation to T cells, antigen and/or peptide–MHC complex transfer to dendritic cells (DCs) leading to indirect antigen presentation, activation of natural killer (NK) cells, macrophages and B cells and maturation of DCs, and tumour necrosis factor (TNF)-dependent protection of T cells against activation-induced cell death (AICD). **b** | Inhibitory functions of vesicles derived from various cellular sources include: inhibition of T cell activation, inhibition of NK cell and $CD8^+$ T cell cytotoxicity, promotion of regulatory T cell activity inhibition of monocyte differentiation into DCs and promotion of monocyte differentiation into myeloid-derived suppressor cells (MDSCs), T cell killing through ligation of CD95 by CD95 ligand (CD95L) or T cell immunoglobulin domain and mucin domain protein 3 (TIM3) and TNF trapping. HSP, heat shock protein; IL-15Rα, IL-15 receptor α-chain; NKG2D, NK group 2, member D; TGFβR, transforming growth factor-β receptor; TNFRI, TNF receptor superfamily, member I.

Antigen-independent roles in immune responses. In addition to MHC molecules, secreted membrane vesicles bear proteins that can inhibit or promote immune responses in various antigen-independent ways (FIG. 4).

Pioneering studies in the 1980s suggested that tumour-derived membrane vesicles have immunosuppressive properties[5]. Subsequent studies showed that microvesicles or exosomes derived from tumour cell lines or tumour-bearing patients could induce T cell apoptosis *in vitro* through CD95 ligand (CD95L; also known as FASL)[12,13] or galectin 9 (REF. 78). In addition, tumour-derived exosomes can inhibit interleukin-2 (IL-2)-induced T cell proliferation by promoting the function of regulatory T (T_{Reg}) cells at the expense of other T cell subsets[79]. Furthermore, tumour-derived exosomes can reduce the cytotoxic capacity of natural killer (NK) and $CD8^+$ T cells[80,81], impair the differentiation of myeloid precursors into DCs[82] and induce the generation of myeloid-derived supressor cells (MDSCs)[83] (FIG. 4b). The capacity of tumour-derived exosomes to promote T_{Reg} cell function and inhibit NK cell cytotoxicity could be ascribed partly to the presence of NK group 2, member D (NKG2D; also known as KLRK1) ligands on exosomes (which induce NKG2D downregulation on $CD8^+$ T cells and NK cells), but mainly to the presence of membrane-bound transforming growth factor-β (TGFβ) on these vesicles[79,81]. Two recent articles[84,85] have also proposed a TGFβ-dependent T_{Reg} cell- and MDSC-promoting activity for vesicles isolated from mouse tissues (thymus[84] or growing mammary tumours[85]) but, as stated earlier, exosome-like vesicles that are isolated from dissociated tissues can not strictly be considered as true secreted vesicles. In summary, tumour-derived vesicles seem to have pleiotropic actions that subvert antitumour immune responses.

Vesicles derived from immune cells also have immunosuppressive properties. Similar to some tumour cells, activated T cells secrete exosomes bearing CD95L, which induces apoptosis of bystander T cells, thereby participating in activation-induced cell death (AICD)[14]. Ectosomes derived from neutrophils and erythrocytes inhibit the secretion of IL-8 and tumour necrosis factor (TNF) by macrophages and the maturation of DCs[86], thereby dampening inflammation (FIG. 4b).

Vesicles purified from some body fluids, rather than from *in vitro* cultured cells, can also have immunosuppressive activities. Exosomes in milk and colostrum inhibit T cell activation and increase the number of T_{Reg} cells *in vitro*[50]. Similarly, exosomes or microvesicles in the plasma of pregnant women bear CD95L and cause a decrease in T cell CD3ζ expression and consequently a reduction in T cell responsiveness[42]. Finally, exosome-like vesicles secreted by endothelial cells or purified from bronchoalveolar fluid bear TNF receptor I and therefore might bind and sequester TNF, thereby limiting its activity *in vivo*[17] (FIG. 4b).

By contrast, immune-activating properties of secreted vesicles have also been reported (FIG. 4a). For example, microvesicles released by thrombin-activated platelets stimulate the proliferation, survival and chemotaxis of haematopoietic cells[87], activate monocytes to secrete pro-inflammatory cytokines[88] and activate B cells through CD40L, thereby bypassing the need for $CD4^+$ T cell help[89]. Macrophages infected by intracellular pathogens (such as *M. tuberculosis*, *M. bovis*, *Salmonella enterica* subspecies *enterica* serovar Typhimurium and *Toxoplasma gondii*) release exosomes that contain pathogen-derived pro-inflammatory molecular determinants that induce the secretion of pro-inflammatory cytokines by recipient macrophages[30,90]. Notably, cultured cells that are infected by *Mycoplasma arginini* and *Mycoplasma orale* also release pro-inflammatory exosomes that can induce the polyclonal activation of B and T cells[91]. This observation calls for careful consideration of studies describing B cell mitogenic or DC maturation activities of exosomes because of the potential for contamination of the donor cells with *Mycoplasma* species.

Recently, ligands for NK cell-activating receptors on exosomes derived from immature human DCs were shown to promote NK cell activation *in vitro*. This occured through HLA-B-associated transcript 3 (BAT3; a ligand for natural cytotoxicity triggering receptor 3 (NCR3), an NK cell cytotoxicity receptor) on exosomes secreted by DCs following heat shock[92] or through UL16 binding protein 1, MHC class I polypeptide-related sequence A (MICA) and MICB (which are ligands for NKG2D) on exosomes secreted by DCs from healthy volunteers or patients with melanoma[93]. The ability of these exosomes to activate NK cells (rather than inhibit them, as do NKG2D ligand-bearing tumour exosomes[81]) was apparently due to the simultaneous presence of the IL-15 receptor α-chain (IL-15Rα) on the DC-derived exosomes, which could bind to exogenous IL-15 and thereby promote NK cell proliferation[93].

Immune-activating functions of tumour-derived exosomes have also been reported, but they mainly occur when the tumour cells are exposed to stress conditions. For example, exosomes or membrane vesicles derived from heat-shocked tumour cells bear heat shock protein 70 (HSP70) on their surface and can promote NK cell activity[94] and TNF secretion by macrophages[95]. Exosomes derived from heat-shocked lymphoma or carcinoma cells have also been shown to be more immunogenic than those from control cells[96].

Finally, fibroblasts obtained from the synovial fluid of patients with rheumatoid arthritis secrete exosomes bearing active membrane-bound TNF, which binds to T cells and renders them resistant to AICD[97], thereby potentially participating in the development of this disease (FIG. 4a).

Functions of membrane vesicles *in vivo*

In vivo *immune responses induced by membrane vesicles purified* in vitro. As discussed above, numerous studies have shown that membrane vesicles have pleiotropic effects on individual immune cells *in vitro*. But are these properties maintained *in vivo* and which of them dominate when exosomes are transferred to an *in vivo* context?

Several studies support the idea that DC-derived exosomes are immunogenic: injection of DC-derived exosomes bearing tumour peptide–MHC class I complexes into mice with established tumours led to tumour rejection[29]. As expected from *in vitro* studies, the efficiency

Regulatory T (T_{Reg}) cell
A specialized type of $CD4^+$ T cell that can suppress the responses of other T cells. These cells provide a crucial mechanism for the maintenance of peripheral self tolerance, and a subset of these cells is characterized by expression of CD25 and the transcription factor forkhead box P3.

of this process seems to be increased if exosomes are co-injected with mature DCs or chemical adjuvants that promote DC maturation[73]. Injection of exosomes derived from antigen-pulsed mature (but not immature) DCs from male mice into female mice promotes rapid rejection of male skin grafts, indicating *in vivo* priming and differentiation of activated $CD4^+$ T cells into effector $CD4^+$ T cells[33]. Exosomes secreted by DCs exposed to antigen can also induce humoral immune responses to the same antigen, even if intact antigen is barely detectable in the exosomes[32]. For example, when DCs are exposed to *T. gondii* antigens, the humoral and T helper 1 (T_H1) cell responses induced by their exosomes lead to protection against acute infection with the parasite[98]. Antigen-independent effects of DC-derived exosomes have also been described recently. In mice, DC-derived exosomes induce NK cell recruitment to and proliferation in draining lymph nodes, and in patients with melanoma they restore NKG2D surface expression on NK cells and T cells, and therefore their cytotoxic activity[93].

By contrast, tolerogenic (rather than activating) effects of immature DC-derived exosomes have been described: they can promote graft survival[99] and reduce inflammation in a model of septic shock[100] by mediating MFGE8-dependent phagocytosis of apoptotic cells by macrophages. Exosomes derived from CD95L- or IL-10-expressing DCs also reduce inflammation in a model of arthritis[101,102].

With regard to exosomes secreted by tumour cells, work by several groups suggests that tumour cell-derived exosomes do not spontaneously support the induction of effector immune responses *in vivo*. Whether they induce tolerogenic immune responses, as suggested by the increased mammary tumour growth in mice vaccinated with tumour-derived exosomes in the absence of inflammatory signal[80], is still not established for other tumour models but remains a possibility. However, when tumour-derived exosomes are administered together with strong adjuvants, such as Toll-like receptor ligands[103], and/or are purified from heat-shocked[96,103] or transgenic cells expressing IL-2 or IL-18 (REF. 104), they promote efficient antitumour immune responses *in vivo*.

Finally, exosomes secreted by epithelial cells have tolerogenic effects. For example, vesicles derived from ovalbumin (OVA)-peptide-pulsed intestinal epithelial cells or from the serum of OVA-fed rodents induce a tolerogenic immune response in the recipient animals, such that the development of an OVA-specific allergic response to oral antigen is prevented; these vesicles were termed tolerosomes[105]. Similarly, exosomes (or exosome-like vesicles) secreted by lung tissue and purified from bronchoalveolar lavage of allergen-tolerized mice could transfer protection from allergy induction to other mice[45]. By contrast, exosomes secreted by interferon-γ (IFNγ)-exposed, OVA-pulsed intestinal epithelial cells induce OVA-specific humoral immune responses *in vivo*[106], suggesting that they induce an effector rather than a tolerogenic response; the reasons for the discrepancy between the two studies is not clear. Microvesicles secreted by platelets also promote humoral immune responses through CD40L-mediated B cell activation[89].

So, similarly to *in vitro* findings, it seems that exosomes can have either activating or inhibitory effects *in vivo*. The nature of the exosome-induced immune response may depend on the physiological state of the donor cells, which could result in different exosome composition. Specifically, tolerogenic exosomes would comprise molecules such as CD95L or TGFβ, whereas T cell-promoting exosomes would comprise molecules such as co-stimulatory molecules, adjuvant factors and molecules from stressed cells. Molecular comparison of exosomes secreted by tumour cells with or without exposure to stressors could help to address this issue. Alternatively, discrepancies between studies may be linked to different experimental methodology, such as the route of administration of the exosomes, the dose of transferred antigen or the *in vivo* microenvironment. These considerations will be especially important when designing therapeutic strategies involving exosomes (see later).

***Potential physiological roles for membrane vesicles secreted* in vivo.** Although exosomes purified *in vitro* affect immune responses *in vivo*, the role of exosomes secreted *in vivo* in physiological immune responses remains to be fully determined. One study showed a correlation between the presence of membrane vesicles and a physiological outcome *in vivo*[42]. Placenta-derived vesicles bearing CD95L and MHC class II molecules were found at higher levels in the serum of women delivering at full term than those delivering pre-term. *In vitro*, these vesicles inhibited T cell responses in a CD95L-dependent manner, and greater inhibition was observed with vesicles obtained from the serum of women delivering at term. These results suggest that membrane vesicles which are produced by the placenta and are present in the serum could participate in the prevention of maternal immune responses against the fetus.

Given the opposing activities described for *in vitro* (or *ex vivo*)-purified tumour-derived vesicles, the roles of these vesicles when secreted by tumour cells *in vivo* is still a matter of debate. A few groups have suggested that vesicle secretion by tumours inhibits antitumour immune responses[15,81,107]. By contrast, we have recently generated tumour cells that secrete a model antigen exclusively on the surface of membrane vesicles and can induce efficient activation of transferred $CD8^+$ T cells *in vivo*, leading to tumour rejection[108]. Thus, in this mouse carcinoma model, secretion of membrane vesicles bearing an antigen promotes the induction of effector, and not tolerogenic, immune responses. In this experimental system, in which we used a foreign antigen that was not subject to tolerance, we may have tilted the balance in favour of the antigen-carrying functions of exosomes rather than their potential tolerogenic activities. In patients with cancer, the level of tumour-derived exosomes in the serum increases with the tumour burden[43], suggesting that, if immune responses are induced *in vivo* by tumour-derived exosomes, they are not sufficient to eliminate the tumour permanently. Determining whether these *in vivo*-released exosomes allow transient control of tumours or favour tumour progression will

only be made possible by the generation of tools that specifically inhibit exosome secretion. Finally, the recent finding that mRNAs and microRNAs are present in exosomes and vesicles that have been purified from ascites or serum from cancer patients[43,63] and can transfer new physiological properties (such as metastatic ability) to recipient cells *in vitro*[63] suggests another mechanism for pro-tumoural activity of tumour-secreted vesicles. The net result of the opposing activities of tumour-derived vesicles might change depending on the general immune state of the patient and/or the stage of tumour progression.

As discussed, DC-derived vesicles could be involved in amplifying immune responses *in vivo*, by transferring peptide–MHC complexes from DCs that have been exposed to an antigen to other DCs that have not been in contact with the same antigen. Indeed, antigen transfer between different DC subpopulations *in vivo* has been shown in mouse models of autoimmune gastritis[109] and in experimental models of infection of the intestine with reovirus[110], of the skin with herpes simplex virus[111] and of the lungs with influenza virus[112]. In these studies, antigen acquired in the periphery by migrating DCs was transferred to lymph node-resident $CD8^+$ DCs. The mechanisms involved remain unclear, but exosomes could have a role in this process, especially given that we have shown that DC-derived exosomes are preferentially captured by lymphoid-resident $CD8^+$ DCs *in vivo*[53].

In the context of organ transplantation, host DCs that invade the grafted tissue have recently been proposed to migrate to the spleen, where they transfer allopeptides captured from the graft to other host DCs through exosomes[75]. In this mouse model, peptide–MHC class II complexes present on exosomes could participate in host DC recognition of alloantigen, but the outcome of this in terms of graft tolerance or rejection was not addressed.

Similarly, transfer of peptide–MHC complexes and/or other molecules between intestinal epithelial cells or bronchial cells and DCs in the absence of any stress signal may be involved in the development of tolerance to food or airway allergens[105,113]. In addition, transfer of peptide–MHC complexes can occur between DCs and T cells[54,114], which could be mediated by secreted membrane vesicles. The resulting antigen-presenting T cells have recently been shown to impair the recruitment of antigen-experienced T cells following re-exposure to the antigen, without affecting naive T cells[115]. This negative feedback loop could therefore prevent the induction of more restricted immune responses and favour the development of a broad T cell repertoire.

Membrane vesicles as therapeutic agents

Pioneering work carried out in a mouse model showing rejection of established tumours by exosomes from autologous DCs loaded with tumour peptides[29] prompted investigation into the use of exosomes in anti-tumour immunotherapy approaches as an alternative to the use of DCs loaded with tumour antigens. The use of exosomes instead of DCs as an antigen-presenting unit may address current limitations of DC-based therapies, such as controlling the percentage of live DCs after freeze–thaw protocols and the unknown behaviour or fate of DCs after *in vivo* injection (for example, maturation of DCs after injection could modify the range of MHC-bound peptides displayed on their surface).

Two Phase I clinical trials have been carried out with exosomes, and these involved patients with advanced stage melanomas[116] or non-small cell lung carcinomas[117] expressing melanoma-associated antigen (MAGE). These trials required the establishment of good manufacturing procedures to obtain clinical-grade exosomes from patients' DCs[118] and protocols to ensure efficient peptide loading on exosomal MHC class I and class II molecules[72]. These studies proved that it is feasible to produce enough exosomes from each individual for at least four injections and that they are safe to give to human subjects, the only side effects being mild localized reactions at the site of injection and mild fever in a few patients. The clinical outcomes were encouraging, showing transient stabilization of the disease in 3 out of 6 patients in the melanoma trial and 3 out of 9 patients in the lung carcinoma trial. Immune responses to the tumour peptides were detected in 1 out of 6 and 3 out of 9 patients, respectively.

A Phase I clinical trial using exosomes derived from tumours has also been recently published[119]. In this study, increasing doses of CEA and exosomes purified from ascites of patients with colorectal cancer were injected with or without granulocyte/macrophage colony-stimulating factor (GM-CSF). The exosome vaccine in the presence of GM-CSF induced the activation of CEA-specific cytotoxic T cells in more than 75% of the patients (compared with 20% for exosomes alone), and minor clinical benefit was observed in 2 out of the 20 treated patients.

In these trials, the clinical and immune responses were limited because the Phase I clinical studies involved patients with heterogeneous characteristics in terms of their response to prior treatments and with advanced (stage III and IV) cancers. However, exosome-based therapies still seem to be a promising route to follow when the objective is to prolong disease-free survival in patients with stabilized disease after chemotherapy or radiation therapy, rather than to cure patients that did not previously respond to conventional treatment. Preclinical studies in mouse models suggest ways in which exosome-based approaches could be improved. These include co-treatment with adjuvants that promote DC maturation[73,120] and drugs that inhibit T_{Reg} cell responses[121], purification of pro-immune exosomes from mature DCs[33,70], heat-shocked[96] or genetically modified tumour cells expressing cytokines[104] and the use of exosomes that have superantigens tethered to their surface[122]. The next generation of exosome-based treatments may therefore be best combined with several already established therapies that enhance immune responses. Indeed, a new Phase II clinical trial is about to be initiated in France that combines the administration of exosomes from IFNγ-treated DCs, which express NKG2D ligands and IL-15Rα, with T_{Reg} cell-inhibiting approaches to patients with non-small cell lung cancer that has been stabilized by chemotherapy[93].

Because secreted vesicles can induce immune responses against the antigens they carry, another potential therapeutic approach is to generate tools to induce the secretion of immunogenic vesicles *in vivo*. For example, virus-like particles secreted from the plasma membrane and containing virus antigens could be obtained by transfecting cells or tissues with retroviral vectors encoding Gag and Env proteins[123]. These vectors were used for a DNA vaccination approach, and virus-like vesicles secreted *in vivo* induced efficient antiviral immune responses, leading to protection against the virus.

Another strategy has been to take advantage of the fact that secreted vesicles are enriched in lipid rafts[124] (TABLE 1) and therefore in glycosylphosphatidylinositol (GPI)-linked proteins[125]. The use of fusion protein constructs that encode a GPI-linked cytokine led to the production of membrane vesicles that display active cytokines on their surface[126], although the *in vivo* effects of such vesicles have not been assessed. Finally, we have recently targeted an antigen to secreted membrane vesicles using the phosphatidylserine-binding domain of MFGE8 (REF. 108) and shown that vaccination of mice with plasmid DNA encoding this modified antigen induced antigen-specific $CD8^+$ T cell activation *in vivo*, leading to protection against subsequent tumour growth. Therefore, genetic engineering of membrane vesicles is a new approach to target antigens that are not normally found in exosomes, such as cytoplasmic or nuclear proteins, to secreted vesicles.

On the other hand, tolerogenic effects of exosomes could be used to treat pathologies that result from overexuberant immune responses. Such potential applications include the induction of donor-specific tolerance to support allograft survival[99], anti-inflammatory effects of exosomes from modified DCs in arthritis[101,102] and anti-allergenic effects of exosomes derived from bronchoalveolar lavage fluid[45]. As exosomes are more stable than DCs and are therefore more likely to remain tolerogenic after administration *in vivo*, they could be more suitable for the treatment of these diseases.

Concluding remarks

In this Review, we describe the extensive published literature that supports the idea that secreted vesicles are vehicles for cell–cell communication. This is a particularly exciting prospect given that intercellular communication is a crucial requirement for the functioning of any multicellular organism. However, this concept has yet to be shown definitively *in vivo*. Addressing this question poses an important challenge, one that requires the development of techniques to manipulate vesicle secretion *in vivo*. To meet this challenge, we need to better understand the molecular mechanisms that are involved in the secretion of membrane vesicles. Another major challenge to our understanding of the physiological function of exosomes is the need to determine precisely how and with what types of cell secreted vesicles preferentially interact *in vivo*. Nevertheless, our growing knowledge of the effects of secreted vesicles on immune responses and their potential use as therapeutic agents in various conditions provide exciting lines of investigation for the future.

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DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
CD95L | ICAM1 | LFA1 | MFGE8 | NKG2D | TGFβ | TIM1 | TIM4 | TNF

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