The roles of extracellular vesicles in the immune system

Edit I. Buzas [1,2,3]

Abstract | The twenty-first century has witnessed major developments in the field of extracellular vesicle (EV) research, including significant steps towards defining standard criteria for the separation and detection of EVs. The recent recognition that EVs have the potential to function as biomarkers or as therapeutic tools has attracted even greater attention to their study. With this progress in mind, an updated comprehensive overview of the roles of EVs in the immune system is timely. This Review summarizes the roles of EVs in basic processes of innate and adaptive immunity, including inflammation, antigen presentation, and the development and activation of B cells and T cells. It also highlights key progress related to deciphering the roles of EVs in antimicrobial defence and in allergic, autoimmune and antitumour immune responses. It ends with a focus on the relevance of EVs to immunotherapy and vaccination, drawing attention to ongoing or recently completed clinical trials that aim to harness the therapeutic potential of EVs.

Multivesicular bodies
(MVBs). Membrane-enclosed endosomal organelles with intraluminal vesicles formed by inward budding of the limiting membrane.

Amphisomes
Hybrid organelles formed by the fusion of autophagosomes and MVBs.

Migrasomes
Large extracellular vesicles with numerous small inner vesicles, formed at the tips and intersections of retraction fibres of migrating cells.

[1]*Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest, Hungary.*

[2]*HCEMM-SU Extracellular Vesicles Research Group, Budapest, Hungary.*

[3]*ELKH-SE Immune-Proteogenomics Extracellular Vesicles Research Group, Budapest, Hungary.*

e-mail: buzas.edit@med.semmelweis-univ.hu

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The history of research into extracellular vesicles (EVs) is an example of how a single term can delay the development of an entire scientific field. The commonly used word 'debris' is a nonspecific collective designation of all undefined extracellular particles and its negative tone suggests that all such particles represent cellular waste. For a long time, this connotation discouraged scientists from investigating extracellular particles in depth, thus obscuring the discovery of both EVs and non-EV nanoparticles in this compartment. However, after several decades of sporadic observations of extracellular, membrane-enclosed structures, the early 2000s brought a renewed research focus on these EVs, leading to an exponential development of the field in the past two decades[1,2]. The designation 'extracellular vesicles' was suggested in 2011 as a collective term for lipid bilayer-enclosed, cell-derived particles[3]. EVs are released by all cellular organisms. For example, the release of outer membrane vesicles by Gram-negative bacteria and the more recently described discharge of cytoplasmic membrane vesicles by Gram-positive bacteria and archaea demonstrate that EV production is characteristic of all three domains of life (archaea, bacteria and eukaryota)[4]. The broad term of bacterial extracellular vesicles is increasingly used to refer to all EVs released by bacteria[5].

Since their initial description, a previously unexpected biophysical, biochemical and functional heterogeneity of EVs has been discovered[2,6,7]. Based on their biogenesis, we distinguish two basic types of EV (FIG. 1). Exosomes are of endosomal origin, released upon the fusion of the limiting membrane of multivesicular bodies (MVBs) or amphisomes with the plasma membrane[7–9]. Recent data suggest the involvement of additional endomembranes (such as endoplasmic reticulum[10] and nuclear envelope[11]) in the biogenesis of exosomes. The other basic route of EV biogenesis is the release of plasma membrane-derived EVs (known as ectosomes). However, definitive molecular markers of the different biogenetic routes are not yet available, and operational terms have been suggested to distinguish EV types based on their biophysical or biochemical properties[2]. EVs that are present in the greatest numbers in biological fluids are small EVs with an approximate diameter of 50–150 nm. Medium-sized EVs, with an approximate diameter of 200–800 nm, are present in smaller numbers than small EVs, and large EVs (diameter ≥1 μm; such as migrasomes, exophers, apoptotic bodies, large oncosomes and en bloc-released MVB-like small EV clusters[12]) are the least abundant population of EVs (TABLE 1). The heterogeneity of EVs is a consequence of the variety of types and functional states of the releasing cells as well as of the different biogenetic routes. Of note, EVs also include vesicles generated by different cell death mechanisms (such as apoptosis, necroptosis or pyroptosis). It is also increasingly recognized that EV biogenesis can intersect with viral egress[13], secretory autophagy, the cellular senescence-associated secretory phenotype and the DNA damage response[14].

International guidelines for EV separation and characterization are now available and are regularly updated[2]. However, it should be noted that some studies of EVs, particularly those from earlier periods of EV research, could not benefit from the standards of EV separation

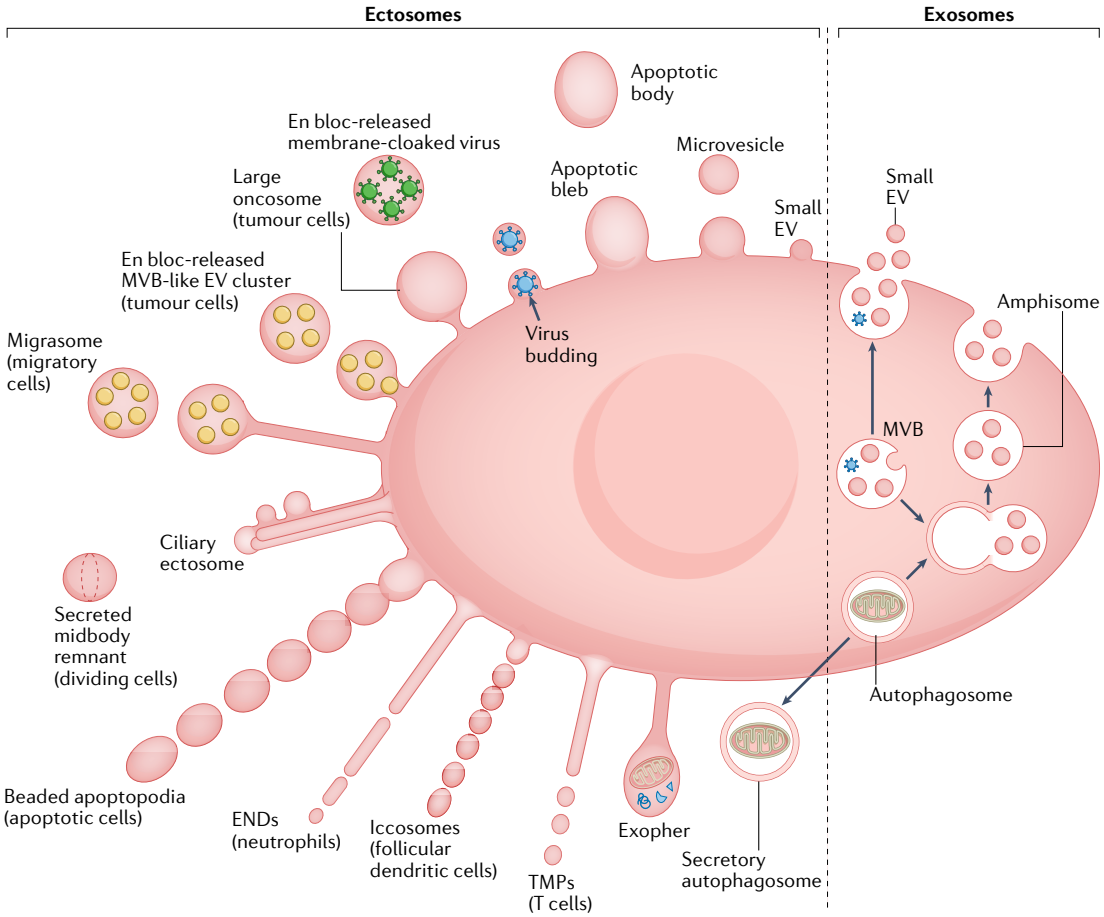

Fig. 1 | **Heterogeneity of extracellular vesicles.** Extracellular vesicles (EVs) are heterogeneous, phospholipid membrane-enclosed structures. Two main types of EV are distinguished based on their biogenesis, known as exosomes and ectosomes. Exosomes are small EVs of endosomal origin released by the exocytosis of multivesicular bodies (MVBs) and amphisomes. Amphisomes are formed by the fusion of autophagosomes and MVBs. By contrast, ectosomes are generated by plasma membrane budding and blebbing. Of note, some ectosomes may also carry endosomal cargo components. Ectosomes include small-sized EVs (such as small ectosomes and arrestin domain-containing protein 1-mediated microvesicles), medium-sized microvesicles and the larger-sized apoptotic bodies. Viruses can also bud from the plasma membrane or can be released from MVBs. En bloc-released virus clusters represent a novel type of large EV similar to the en bloc-released MVB-like EV clusters produced by tumour cells. Oncosomes are large EVs produced by tumour cells. Long protrusions of migrating cells give rise to EVs such as migrasomes, which detach from the end of the long retraction fibres of migrating cells. Secreted midbody remnants are released upon completion of cytokinesis by dividing cells. A special type of ectosome, known as ciliary ectosomes, are shed from the plasma membrane of cilia. Beaded apoptopodia release apoptotic vesicles during apoptosis. Neutrophils rolling on the vascular endothelium leave behind elongated neutrophil-derived structures (ENDs), which later round up. Cytoplasts are large remnants of neutrophils undergoing non-lytic NETosis (not shown). Follicular dendritic cells have long filiform processes from which a beading mechanism gives rise to iccosomes. In the immune synapse, T cell microvilli are fragmented by a similar beading process to give rise to EVs known as T cell microvilli particles (TMPs). Exophers are large vesicles hanging at the end of a stalk that contain damaged organelles and protein aggregates. Secretory autophagosomes are also released by cells. Of note, in the extracellular space, non-EV nanoparticles, such as exomeres[15], supermeres[16] and T cell-derived supramolecular attack particles[45], are also present (not shown). These nanoparticles are distinguished from EVs by their smaller size and by the lack of a phospholipid bilayer membrane surrounding them. The biogenesis of non-EV nanoparticles remains to be explored.

Exophers
Large membrane-enclosed extracellular vesicles released by physiologically normal cells to remove damaged organelles or aggregated proteins to maintain homeostasis.

Large oncosomes
Large (micrometre-sized) extracellular vesicles derived from the plasma membrane of tumour cells.

and characterization that have been defined in the past 5 years[2]. In addition, given the insufficiency of specific inhibitors of the biogenesis of EV subsets and the paucity of in vivo models in which EVs can be tracked in vivo and in situ, it remains challenging to establish the in vivo biological relevance of some of the studies of EVs. Furthermore, EVs should be distinguished from other extracellular nanoparticles such as lipoproteins and the recently discovered exomeres[15] and supermeres[16]. It is important to remember that cells are exposed to extracellular signals resulting from a temporal combination of extracellular soluble mediators, EVs and non-EV nanoparticles.

EVs are involved in several homeostatic processes, including, but not limited to, the rapid removal of unnecessary molecules from cells, enabling cell maturation and quick adaptation to environmental changes, and activation of blood clotting. In addition, they modulate the functions of other cells by delivering intercellular signals[17]. As signalling units, EVs affect the functions

of other cells through their surface proteins, encapsulated cargo molecules (such as proteins and RNAs), and conveyed lipids and glycans. Cytokines and EVs share several functions as mediators of intercellular communication and cytokines can associate with EVs as either internal or external cargo (BOX 1).

This Review demonstrates the essential, ubiquitous involvement of EVs in fundamental immune mechanisms and immune-mediated disease processes, highlighting the key advances and lessons learnt, mostly in the past 5 years of research but also briefly mentioning some of the earlier main findings. Herein, we review published data on the role of EVs in innate and adaptive immunity and the implications for diseases with immune system involvement. This knowledge may be exploited to develop new biomarkers for disease and therapy monitoring as well as new therapeutic tools and delivery vehicles as indicated by recent clinical trials in this area.

Innate immunity and inflammation

All of the immune cell types that participate in inflammation can secrete EVs, which in turn have multiple roles in inflammatory processes. EVs carry arachidonic acid-derived bioactive lipid mediators such as eicosanoids and the enzymes involved in their production, which can have chemotactic effects[18,19]. Furthermore, neutrophil-derived EVs transfer arachidonic acid to platelets, which in turn use cyclooxygenase 1 (COX1) to generate thromboxane A2 from arachidonic acid and induce neutrophil extravasation[20]. Moreover, the arachidonic acid-metabolizing enzyme platelet-type 12-lipoxygenase (12-LO) is transported by platelet-derived EVs. Together with secreted phospholipase A2 type IIA, the EV-associated 12-LO produces 12-hydroxyeicosatetraenoic acid from arachidonic acid, which in turn induces the internalization of platelet-derived EVs by neutrophils, a process that has been implicated in inflammatory arthritis[21]. Given the known ability of EVs to bind to molecules of the extracellular matrix[22], it is also conceivable that EVs secreted by migrating inflammatory cells create stable secondary chemotactic gradients ('trails') in the matrix for other cells[23].

Overall, in sepsis, EVs have been shown to have both pro-inflammatory and anti-inflammatory roles[24]. The effects of EVs depend on the donor cell type and the phase of sepsis in which the EVs are analysed. The pro-inflammatory effects are related to EV-associated cytokines and damage-associated molecular patterns (DAMPs) such as histones, high-mobility group box 1 (HMGB1), heat shock proteins (HSPs) and mitochondrial DAMPs, which induce macrophage polarization to an M1-type phenotype and cytokine secretion, T helper cell differentiation from naive T cells, and leukocyte chemotaxis. By contrast, certain EVs in sepsis have anti-inflammatory effects, which include downregulation of complement factors and acute phase signalling, reduction of leukocyte chemotaxis, reduction in serum pro-inflammatory cytokine levels and reduction in the expression of adhesion molecules on endothelial cells. These anti-inflammatory effects are due, for example, to the release of the lipopolysaccharide (LPS) co-receptor CD14 on macrophage-derived EVs, leading to decreased CD14 levels on the macrophage cell surface and decreased responsiveness to LPS as well as to the downregulation of nuclear factor-κB (NF-κB) activation in LPS-stimulated macrophages by HSPA12B-containing endothelial cell-derived EVs[25].

There is a large body of evidence that EVs generated by either non-inflammatory or inflammatory types of cell death have discordant effects upon uptake by phagocytic cells. Recently, it was shown that uptake of necroptotic cell-derived EVs by macrophages results in the secretion of pro-inflammatory cytokines (tumour necrosis factor (TNF) and CCL2)[26]. Inflammasome activation during pyroptosis induces a marked release of exosomes, and a connection has been established between inflammasome-mediated cleavage of Rab-interacting lysosomal protein (RILP) and the selective loading of microRNAs (miRNAs) into EVs[27]. Thus, inflammasome activation induces the loading of pro-inflammatory miRNAs containing an AAUGC motif (for example, hsa-miR-124-3p, hsa-miR-155-5p and hsa-miR-126-3p) to exosomes. Inflammasome-induced EVs also carry interferon-β (IFNβ), which was suggested to prevent hyperinflammation[28]. The IFNβ carried by EVs induces changes in the expression of interferon response genes in bystander cells and restricts their activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome.

Of note, soluble mediators of innate immunity also have important EV-related functions. EVs can carry the acute-phase protein C-reactive protein (CRP) from the liver and spread it through the circulation. Whereas

Table 1 | **Size-based categories of extracellular vesicles**

Property	Small EVs	Medium EVs	Large EVs
Diameter	~50–150 nm	~200–800 nm	≥1,000 nm
Biogenesis	Endosomal (exosomes) but some small EVs can be derived from the plasma membrane (ectosomes)	Plasma membrane-derived ectosomes	Plasma membrane-derived ectosomes (some of which may carry endosomal small EVs)
Examples	Exosomes, small ectosomes[131], ciliary ectosomes[132], arrestin domain-containing protein 1-mediated microvesicles[133]	Microvesicles, FDC-derived iccosomes, T cell microvilli particles[50], elongated neutrophil-derived structures[85], secreted midbody remnants[134]	Apoptotic bodies, large oncosomes[135], beaded apoptopodia[136], migrasomes[137], exophers[138], en bloc-released virus clusters[13], en bloc-released MVB-like EV clusters[12], secretory autophagosomes[7], cytoplasts[84]

EV, extracellular vesicle; FDC, follicular dendritic cell; MVB, multivesicular body.

Box 1 | **The relationship between extracellular vesicles and cytokines**

Similarly to cytokines, extracellular vesicles (EVs) are ubiquitous conveyors of intercellular messages and can function as intercellular immune mediators. EVs mediate signalling in juxtacrine, autocrine, paracrine and endocrine cell–cell communication. Unlike cytokines, which are well-defined proteins or glycoproteins, EVs are complex molecular structures composed of lipids, nucleic acids, proteins, glycans and metabolites. Both cytokines and EVs function in networks and are characterized by pleiotropic, redundant, synergistic or antagonistic functions. However, unlike cytokines, which function typically through plasma membrane receptors, EVs can signal through both cell surface receptors and intracellular target molecules. There is also evidence that EVs and cytokines can function in combination[139,140]. An additional aspect of the relationship between EVs and cytokines is that EVs can carry various cytokines, either on their surface (for example, tumour necrosis factor (TNF) bound to the TNF receptor, or CCL2 and transforming growth factor-β (TGFβ) bound to glycosaminoglycans)[141] or as their internal cargo[142]. EV-associated cytokines are protected from enzymatic degradation in the extracellular environment and can be delivered to distant cells. EV surface-associated cytokines target EVs to cells expressing cytokine receptors, where cytokine signalling is initiated by the direct binding of EVs to cells. However, the mechanism by which membrane-enclosed cytokines induce signalling is less evident and may require extracellular disintegration of the EV membrane prior to cytokine binding to its receptor. Of note, endosomal SMAD-dependent signalling has been shown to be initiated by TGFβ1 on the surface of EVs during prolonged retention of EVs in the endosomal compartment[143]. Whether other EV-associated cytokines can also initiate signalling via an endosomal cytokine signalling platform[144] remains to be clarified.

most plasma CRP has a pentameric structure, circulating EVs in sepsis carry monomeric CRP, which has pro-inflammatory properties. EV-bound monomeric CRP induces the release of CXCL8 from monocytes, contributing to the chemotaxis of neutrophils and dissemination of inflammation[29]. Moreover, the surface of EVs can bind both complement factors and complement regulatory proteins[22]. Mammalian EVs harbouring antibody-binding epitopes were shown to function as decoys to prevent complement-mediated killing of EV-releasing cells[30].

Taken together, these examples show that EVs can have pro-inflammatory roles by the transfer of mediators (bioactive lipids, acute-phase proteins and cytokines), danger signals, enzymes and RNAs that affect the activation, differentiation or polarization, recruitment, cytokine production, and various other effector functions of innate immune cells. By contrast, EVs have also been shown to have anti-inflammatory properties in some environments. These data highlight the context-dependent activities of EVs in modulating innate immunity and inflammation.

Adaptive immunity

In this section, we focus on the roles of EVs in T cell and B cell development, antigen presentation to lymphocytes, and the immune synapses formed by lymphocytes.

Lymphocyte development. EVs are suggested to have important roles in T cell development, with the majority of thymic EVs being released by thymic epithelial cells. Thymic epithelial cell-derived EVs were shown to carry tissue-restricted antigens to thymic conventional dendritic cells (cDCs) for antigen presentation. In this way, EVs could contribute to the negative selection of T cells with specificity for self-antigens[31]. In addition, thymic epithelial cell-derived EVs have a role in inducing the maturation of single-positive ($CD4^+$ or $CD8^+$) thymocytes by carrying proteins involved in their maturation and thymic egress such as sphingosine-1-phosphatase lyase 1 (SGPL1), Rho GDP-dissociation inhibitor 1 (GDIR1), dedicator of cytokinesis protein 2 (DOCK2) and p21 protein-activated kinase 2 (PAK2)[32].

In the case of B cell development, immature primary bone marrow B cells were shown to release $CD24^+$ plasma membrane-derived EVs upon antibody-mediated engagement of CD24, and an EV-mediated exchange of CD24 was documented between populations of B cells[33]. As CD24 is known to have roles in B cell development and selection in the bone marrow, it was suggested that EVs potentially affect differentiating B cells. Recently, it was reported that stimulation of the B cell receptor (BCR) or CD24 on a mouse B cell lymphoma cell line with anti-IgM or with cross-linking primary and secondary antibodies, respectively, triggered the production of EVs that carried functional BCR and CD24 to recipient B cells[34]. This transfer allowed recipient B cells to respond to novel antigen stimulation by receiving additional BCRs and endowed these cells with sensitivity to CD24-mediated apoptosis. However, the effect of this EV-mediated BCR transfer is likely to be localized both in time and space during B cell development or activation in vivo as only a minority (5–20%) of B cells in the tested cell population were shown to gain new BCR by this mechanism[34].

Antigen presentation. Demonstration of the antigen-presenting capacity of EVs was the first milestone discovery showing that EVs might have important roles in adaptive immunity. In 1996, it was shown for the first time that B cell-derived EVs carry functional peptide–MHC (pMHC) complexes and directly present antigens to T cells[35]. This, as well as other subsequent papers[36–38], provided evidence for the involvement of EVs in antigen presentation (FIG. 2).

The efficacy of antigen presentation is increased if the pMHC-carrying EVs are attached to the surface of dendritic cells (DCs)[39]. In this case, approximately 100 times fewer DC-attached exosomes than free exosomes are required to achieve the same degree of T cell activation. The presumed mechanism is that 'cross-dressed' DCs concentrate a large number of EV-associated pMHCs for immune synapse formation and T cell activation (FIG. 2a). This mechanism is supported by the observation that, similarly to 'cross-dressed' DCs, bead-bound exosomes also induce increased activation of T cells. In addition to these mechanisms for direct and semi-direct (cross-dressing-mediated) antigen presentation by EVs, vesicles carrying pMHC as well as intact antigen can be internalized and processed efficiently by antigen-presenting cells (APCs) for indirect antigen presentation[40].

Recently, the involvement of EVs in cross-presentation has attracted considerable attention. Cross-presentation of exogenous antigens on MHC class I complexes to $CD8^+$ T cells has an important role in immunity against viruses and tumours and in the immune response upon vaccination and tolerance induction. cDCs pulsed with pMHC class I-carrying exosomes could successfully prime naive $CD8^+$ T cells[41]. Furthermore, the synaptic

transfer of vesicles between donor and recipient DCs through a close and sustained cell–cell association was implicated in the cross-priming of tumour-specific CD8⁺ T cells[42] (FIG. 2b). In this case, migratory DCs carrying tumour antigens leave the tumour microenvironment and migrate to the regional lymph nodes. In the lymph nodes, these migratory DCs release EVs carrying pMHC class I complexes that can be synaptically transferred to lymph node-resident DCs. This mechanism is suggested to be a dominant pathway to load lymph node-resident DCs for antigen presentation to CD8⁺ T cells. Recent data have also shown the involvement of plasmacytoid DC-derived EVs in transferring antigen to cDC1 cells for cross-presentation to naive CD8⁺ T cells[43] (FIG. 2c). It remains unclear whether plasmacytoid DC-derived EVs enable antigen presentation by cDCs through a process similar to cross-dressing or through EV uptake and processing for indirect presentation.

Finally, an interesting novel finding is that medium-sized platelet-derived EVs (microvesicles) can function as complete functional units of antigen presentation; they not only carry pMHC class I complexes and co-stimulatory molecules (CD40L, CD40 and OX40L) on their surface but also contain functional 20S proteasomes to enable peptide generation for antigen presentation. Importantly, under experimental conditions, the proteasomes of platelet-derived EVs could process exogenous antigen and load the resulting peptides onto MHC class I molecules, leading to the proliferation of antigen-specific CD8⁺ T cells[44] (FIG. 2d). Whether EV surface-associated enzymes (such as matrix metalloproteases)[22] might also have a role in the extracellular processing of antigens is yet to be established.

Immune synapses. EVs have been shown to be involved in the functions of the immune synapse formed between a lymphocyte and APC. Importantly, EVs in the immune synapse have to be distinguished from the recently identified, CD8⁺ T cell-derived supramolecular attack particles (which are non-EV autonomous killing particles enclosed by a thrombospondin 1 shell) that are also present in the immune synapse[45].

More than 10 years ago, a one-way transfer of T cell MVB-derived EVs and their miRNA cargo to APCs was demonstrated at the site of the immune synapse[46]. Later, antigen-induced, T cell plasma membrane-derived

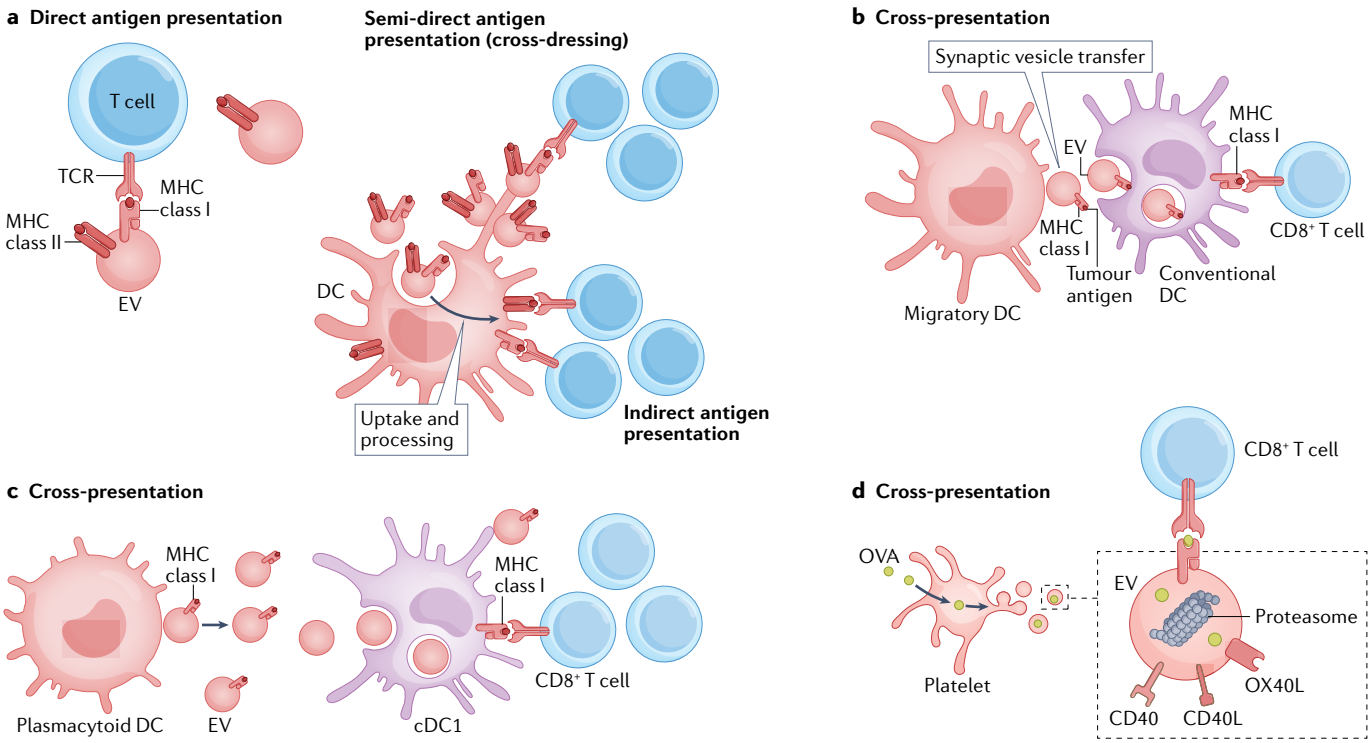

Fig. 2 | **The role of extracellular vesicles in antigen presentation.** **a** | Extracellular vesicles (EVs) can present antigen on their surface MHC molecules directly to T cells. A more efficient form of semi-direct antigen presentation, known as cross-dressing, takes place when EVs attach to (or are possibly recycled to) the surface of dendritic cells (DCs), in which case the DC plasma membrane concentrates a large number of EV-associated peptide–MHC complexes for efficient immune synapse formation. Endocytic uptake of EVs by DCs leads to the intracellular processing of EV-associated antigens and peptides and their indirect presentation by the DC. **b** | Cross-presentation of MHC class I-restricted antigens to tumour-specific CD8⁺ T cells occurs when migratory DCs from the tumour microenvironment migrate to the draining lymph nodes and transmit tumour antigens to conventional DCs (cDCs) in the lymph nodes by synaptic vesicle transfer[42]. **c** | Cross-presentation to CD8⁺ T cells can also be mediated by plasmacytoid DC-derived EVs and requires the uptake of EVs by cDC1 cells[43]. It remains to be clarified if the cross-presentation by cDCs involves a process similar to cross-dressing or if it occurs through EV uptake and processing for indirect presentation. **d** | Platelet-derived EVs carry functional 20S proteasomes that can generate peptides from exogenously delivered proteins such as ovalbumin (OVA); these peptides are subsequently loaded onto the MHC class I molecules of the platelet-derived EVs and cross-presented to CD8⁺ T cells. Platelet-derived EVs can thus function as complex antigen-presenting units[44]. Platelet-derived EVs also have co-stimulatory molecules (CD40, CD40L and OX40L) on their surface. TCR, T cell receptor.

EV secretion was observed at the centre of the synapse, with the released EVs being enriched in T cell receptors (TCRs). Thus, TCR sorting and release in EVs was identified as a characteristic feature of the immune synapse[47,48]. Recent data showed that TCR signalling-induced T cell trans-synaptic vesicles contain greater levels of immune effectors (TCR, CD40L, RNA-binding proteins and miRNAs) than do constitutively released EVs[49].

T cell surface microvilli are found at the T cell–APC contact interface and were originally considered to be structures to screen and/or attach to the surface of APCs. However, recently, it was documented that these microvilli are fragmented into TCR-enriched EVs, known as T cell microvilli particles (TMPs), that are deposited onto the APC surface. $CD4^+$ T cell-derived TMPs carry the TCR complex, co-stimulatory molecules (CD2 and CD28) and cytokines (such as IL-33, IL-4, IL-7 and TNF). TMPs were thus proposed to represent 'immunological synaptosomes', and it was suggested that they enable specific and rapid transfer of cargo molecules between physically interacting T cells and DCs in order to regulate the activation of DCs with minimal bystander effect[50].

B cells can also establish antigen-presentation synapses with T cells. Synaptic exosomal transfer of miRNAs, such as miR-20a-5p, miR-25-3p and miR-155-3p, was documented from T cells to B cells, resulting in silencing of the genes encoding phosphatase and tensin homologue (PTEN) and Bcl-2-interacting mediator of cell death (BIM; also known as BCL-2L11) in B cells. Both BIM and PTEN have crucial roles in B cell biology and in the germinal centre reaction. BIM is required for BCR activation-induced cell cycle entry and for the apoptotic killing of low-affinity BCR-expressing B cells[51,52] whereas PTEN regulates the expression of IgD BCR in mature B cells and controls the germinal centre reaction[53]. Thus, it is suggested that the synaptic transfer of miRNA-containing exosomes from T cells to B cells has a key role in regulating germinal centre formation and antibody production[54]. In addition to forming antigen-presentation synapses with T cells, B cells also establish synapses with follicular dendritic cells (FDCs) for the purpose of antigen capture and/or processing. In vivo, the B cell–FDC synapse contains pMHC class II-carrying exosomes attached to the FDC surface. Given that MHC class II molecules are not expressed by FDCs, it is hypothesized that pMHC class II complexes are transferred to FDCs by EVs released by B cells and that the FDC-associated pMHC class II-carrying exosomes guide antigen-specific T cells for co-stimulation of B cell differentiation in the germinal centre[55]. FDCs also produce EVs known as iccosomes that are formed by the 'beading' of filiform dendrites of FDCs and are later coated by antigen–antibody complexes. These FDC-derived EVs are subsequently endocytosed by B cells and the antigens they carry can be processed for presentation by the B cells[56].

Immune regulation

Numerous molecules known to participate in immune regulation have been identified on the surface of EVs (FIG. 3). These include the immune-checkpoint molecules cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed death ligand 1 (PDL1), the apoptosis-inducing ligand FASL (also known as CD95L), and the ectoenzymes CD39 and CD73, which generate immunosuppressive adenosine from ATP[57]. Regulatory T (T_{reg}) cells release EVs that contribute to the immunosuppressive activity of these cells by various mechanisms such as by surface expression of CD73. In fact, the production of adenosine by CD73 was found to be essential for the immunoregulatory function of EVs generated by T_{reg} cells[58]. Of note, the activity of T_{reg} cell-derived EVs is also mediated by EV-associated miRNAs (such as miR155, Let7b and Let7d). The key role of miRNAs and exosomes in T_{reg} cell-mediated suppression of $CD4^+$ T cell responses was established using Dicer-deficient T_{reg} cells and RAB27A and RAB27B double-deficient T_{reg} cells, in which miRNA and exosome biogenesis were disrupted, respectively[59].

In addition to T cells, DCs are also a target for T_{reg} cell-derived EVs. Specific miRNAs (miR-150-5p and miR-142-3p) associated with T_{reg} cell-derived EVs modulate the cytokine production of DCs following EV uptake, leading to increased IL-10 production and decreased IL-6 production by LPS-exposed DCs. It was suggested that this could be a mechanism by which autoimmunity is prevented by tolerogenic DCs[60]. Also of note in relation to EV-mediated immunosuppression is that tumour cell-derived EVs (exosomes) carry PDL1 and inhibit PD1-expressing $CD8^+$ T cells[61].

The immunoregulatory role of stem cell-derived EVs deserves particular attention. We discuss later how this feature of stem cell-derived and progenitor cell-derived EVs is being exploited in EV-based therapeutic approaches. Stem cell-derived and progenitor cell-derived EVs have been shown to inhibit the functions of a wide range of immune cells (including T cells and B cells, natural killer (NK) cells, DCs, and monocytes and macrophages)[62]. The mechanisms by which stem cell-derived EVs exert these functions include the induction of T cell apoptosis via adenosine A2A receptor, transfer of miR-155-5p to B cells and downregulation of the PI3K–AKT pathway, suppression of NK cells by transforming growth factor-β (TGFβ) mediating downstream signalling through SMAD2 and SMAD3, upregulation of miR-146a expression and downregulation of FAS (also known as CD95) expression in DCs, or miR-182-mediated targeting of the Toll-like receptor 4 (TLR4)–NF-κB–PI3K–AKT pathway in macrophages[62]. There is increasing interest in harnessing these EVs as therapeutic tools in inflammatory diseases.

EV-mediated immune regulation is also implicated in gestational immunology with respect to the maintenance of immune tolerance to the semi-allogeneic fetus. In healthy pregnancy, syncytiotrophoblast-derived EVs contribute to normal immunosuppression at the fetal–maternal interface in multiple ways: they carry ligands for the activating receptor NKG2D on NK cells, such as MICA, MICB and UL16 binding proteins, resulting in the downregulation of NKG2D[63]; they carry the pro-apoptotic proteins FASL and TNF-related apoptosis inducing ligand (TRAIL)[64]; and they promote T_{reg} cell development through HSPE1 and their miRNA cargo[65].

As described above, EVs function in key innate, adaptive and regulatory immune processes, which

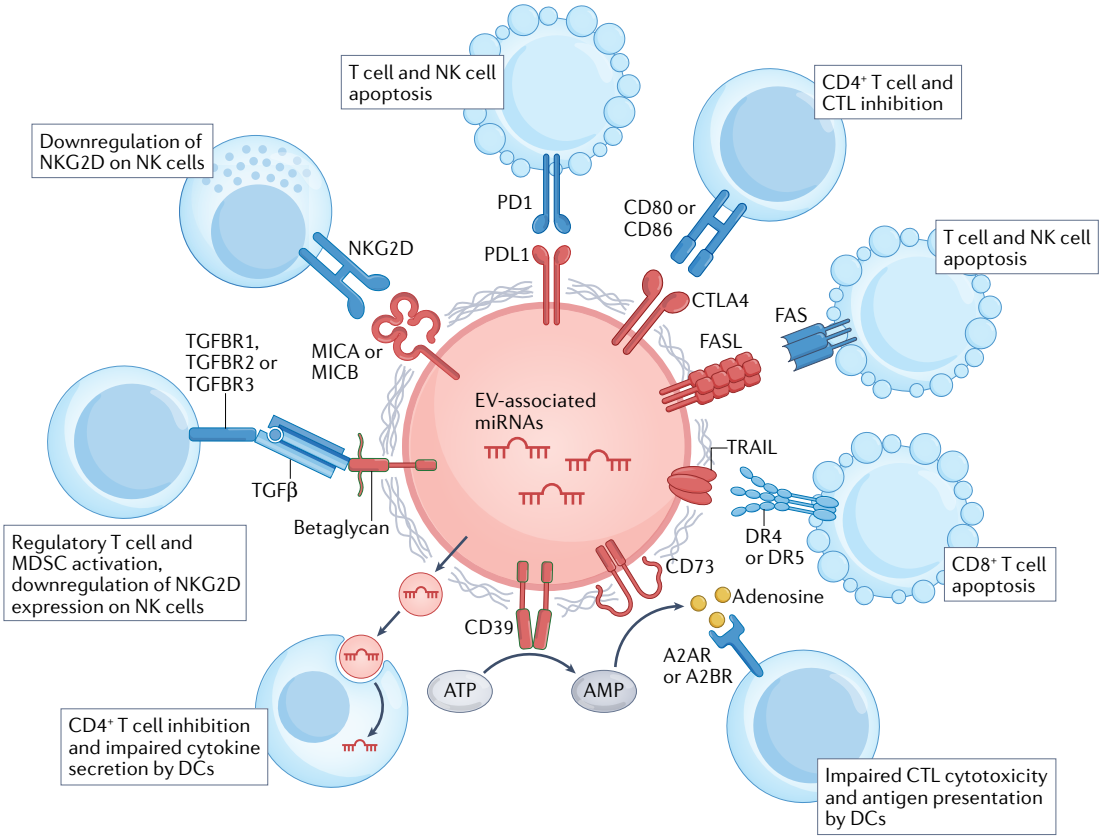

Fig. 3 | **Immunoregulatory functions of extracellular vesicles.** Immunoregulatory molecules on the surface of extracellular vesicles (EVs), including the immune-checkpoint molecules programmed death ligand 1 (PDL1) and cytotoxic T lymphocyte antigen 4 (CTLA4) and the apoptosis-inducing ligands FASL and TNF-related apoptosis inducing ligand (TRAIL), interact with cognate ligands and receptors expressed by T cells and natural killer (NK) cells to inhibit their activity or induce apoptosis. The ectoenzymes CD39 and CD73 generate adenosine from ATP, which impairs cytotoxic T lymphocyte (CTL) responses and antigen presentation by dendritic cells (DCs). Regulatory T cell-derived EVs contain EV-associated microRNAs (miRNAs) that suppress CD4⁺ T cell responses (such as miR-155, Let7b and Let7d) or modulate cytokine production by DCs (such as miR-150-5p and miR-142-3p). The immunosuppressive cytokine transforming growth factor-β (TGFβ), which associates with betaglycan on the surface of EVs, activates regulatory T cells and myeloid derived suppressor cells (MDSCs) and downregulates expression of the activating receptor NKG2D on NK cells. EVs carrying MICA and MICB, which are ligands for NKG2D, can also lead to its downregulation on NK cells.

leads to the question of whether these EV-mediated basic immune functions are involved in protective and pathological immune responses in health and disease. Next, we discuss the implications of these functions in responses to particular types of stimuli and in particular disease processes.

Antimicrobial responses

The release of EVs is evolutionarily conserved from Gram-negative and Gram-positive bacteria, fungi and parasites to humans[66]. Here, we focus on how microbial EVs are recognized by immune cells to activate a host immune response and, conversely, how protection from immune attack can be mediated by microbial EVs. We also discuss the important role of neutrophil-derived EVs in antimicrobial immunity.

Immune activation by microbial EVs. The release of EVs by microorganisms serves their biological interest, for example, by enabling rapid membrane remodelling, providing decoys for infection by bacteriophages, protecting against virulence factors and facilitating bacterial spread. However, microbial EVs can also have pro-inflammatory effects in the infected host. Microbial EVs are carriers of microorganism-associated molecular patterns that activate host innate immune responses through pattern recognition receptors (PRRs)[67]. Overall, the evolutionary costs related to the immunogenicity of microbial EVs must be counterbalanced by benefits to the microorganism of EV emission[68]. Of note, in addition to EVs released by the microorganisms themselves, EVs secreted by infected cells also carry microbial molecules, which could have indirect effects on the immune response[69].

The outer membrane vesicles released by Gram-negative bacteria have, among other functions, roles in quorum sensing and horizontal gene transfer, and they carry toxins and other virulence factors. They can activate various PRRs, such as nucleotide binding oligomerization domain containing protein 1 (NOD1), NOD2 and NLRP3, through their LPS and peptidoglycan molecules[67]. Additional microorganism-associated molecular

Quorum sensing
A process of cell–cell communication by which bacteria share information about cell density and modify their gene expression accordingly.

patterns related to certain bacterial EVs include lipid A, flagellin, PorB and vesicle surface-associated DNA[70].

Despite their thick cell walls, Gram-positive bacteria, mycobacteria and fungi also release EVs that can induce innate immune responses[66]. For example, the Gram-positive bacterium *Staphylococcus aureus* releases EVs that carry DNA, RNA and peptidoglycans, and that are recognized by PRRs such as TLR2, TLR7, TLR8, TLR9 and NOD2. This recognition ultimately leads to the autophagosomal degradation of EVs[71]. The surface glycans of fungal EVs are also PRR ligands, as are the metalloproteinase GP63 and thrombospondin 1 (TSP1) on parasite-derived EVs[70]. EV-associated DNA released by the parasite *Plasmodium falciparum*, which infects red blood cells, can activate the cytosolic stimulator of interferon genes (STING) pathway in monocytes that take up the parasite-derived EVs, thereby activating these innate immune cells[72]. A systemic role for extracellular gut microbiota-derived EVs in peripheral activation of the cGAS–STING–type I interferon axis has recently been identified, whereby DNA associated with commensal-derived EVs is present in the circulation and supports host resistance to infections by DNA and RNA viruses[73].

EV-mediated protection from immune attack. Viruses do not release EVs but can rather hide from the immune system and spread between cells as a cargo of EVs. For example, naked viruses (such as the *Picornaviridae* and *Herpesviridae* families) can be released by cells inside EVs and are thus shielded against extracellular immune recognition[74]. Of note, it is not only single virus particles that can be encapsulated in EVs — the en bloc release of large, membrane-cloaked virus clusters also enables viruses to hide from immune recognition[13].

Another example of EV-based protection of pathogens is related to defence against the complement system. Mammalian EVs have been shown to remove membrane attack complexes from EV-releasing cells to prevent complement-mediated killing of these cells[75,76]. In a similar manner, microbial EVs are also expected to help protect against complement-mediated lysis of the microorganism. Indeed, outer membrane vesicles were shown to protect Gram-negative pathogens against membrane attack complex-mediated lysis[77]. Of note, bacterial EVs can similarly protect against antimicrobial peptides and antibiotics as shown, for example, for *Escherichia coli*-derived EVs in the presence of the antibiotics polymyxin B or colistin (also known as polymyxin E)[78].

Neutrophil-derived EVs in innate immunity. Neutrophils are the most abundant circulating leukocytes and they have a key role in the first line of defence upon infection. Neutrophils release a broad spectrum of EVs[79], the composition of which depends on the environmental conditions at the time of EV production[80]. Of note, the same population of neutrophils can secrete either pro-inflammatory or anti-inflammatory EVs, with clustering of the complement receptor Mac1 (CD11B–CD18) on neutrophils functioning as a switch from anti-inflammatory to pro-inflammatory EV production[81]. Stimulation of neutrophils by opsonized particles induces the release of bacteriostatic EVs, which form large aggregates with bacteria[82]. Furthermore, EVs from neutrophils stimulated with *N*-formylmethionyl-leucyl-phenylalanine, a chemotactic factor produced by bacteria, prime resting naive neutrophils for NADPH oxidase activity and enhance their phagocytic capacity[83]. Upon non-lytic NETosis, which involves the expulsion of nuclear chromatin and granules, anucleated, membrane-enclosed large remnants of neutrophils (cytoplasts) are left behind. In the broad sense, cytoplasts can be considered as very large EVs. However, unlike other EVs, cytoplasts have migratory and phagocytic capacity[84].

Recent research has identified novel types of neutrophil-derived EV. When rolling on endothelial surfaces, neutrophils leave behind tethers that break off and give rise to elongated neutrophil-derived structures (ENDs) that ultimately become spherical. The membrane integrity of these ENDs is increasingly lost over time in vitro, such that they gradually release the S100A8–S100A9 complex, which is highly expressed by neutrophils and is known to stimulate leukocyte recruitment and cytokine secretion. As a result of their delayed release of S100A8–S100A9, ENDs may contribute to the inflammatory process in patients with sepsis, who have approximately 100-fold higher levels of ENDs in plasma compared with healthy controls[85]. Furthermore, a non-conventional exosome biogenesis pathway was identified in activated neutrophils. In these cells, leukotriene B4-containing EVs are generated by budding of the nuclear envelope, which is dependent on activation of neutral sphingomyelinase 1 and the generation of ceramide-rich microdomains in the nuclear envelope[11].

Allergic responses

EVs are implicated in allergic responses both as carriers of allergens and as modulators of the allergic response. EVs can become airborne owing to their small size and, thus, can be inhalable carriers of allergens. For example, allergen-carrying 'pollensomes' are naturally released by pollens of olive and ryegrass[86]. Furthermore, host cell-derived EVs can present allergens to the immune system. DC-derived EVs were shown to present the major cat allergen Fel d 1 and to induce allergic responses[87]. Moreover, EVs in the plasma of individuals with allergic rhinitis carry significantly larger amounts of the house dust mite allergen Der p 1 than healthy controls and plasma EVs from these individuals induce a shift towards a T helper 2 (T_H2) cell response[88]. Early studies showed that B cell-derived EVs can present allergen peptides and induce a T_H2 cell response[89]. Recent data have shown that IL-33, a cytokine known to induce T_H2 cell differentiation, is released by airway epithelial cells on the surface of exosomes[90]. In individuals with asthma, airway epithelial cell-derived EVs and EV-associated contactin 1 were identified as inducers of DC recruitment and activators of monocyte-derived DCs[91].

Mast cells are key players in allergic responses. Mast cell-derived EVs are decorated by FcεRI–IgE complexes[92], and their presence in the circulation is characteristic for individuals with atopy. IgE-activated mast cells secrete EVs that are internalized by IL-33 pre-activated type 2 innate lymphoid cells, leading to the production

Membrane attack complexes
Multiprotein pore-forming complexes generated on target surfaces upon activation of the complement system.

Dermatomyositis
A rare chronic inflammatory disease affecting the skin and the muscles.

of IL-5. This effect of mast cell-derived EVs on type 2 innate lymphoid cells has been attributed to the miRNA miR103a-3p, which is enriched in mast cell-derived EVs. Bone marrow mast cell-derived EVs also induce the differentiation of naive $CD4^+$ T cells to T_H2 cells, in part as a consequence of the ligation of OX40 present on T cells by OX40L on mast cell-derived EVs[93].

Autoimmunity and transplantation

EVs carry numerous autoantigens that are implicated in autoimmune diseases, such as DNA and nucleosomes, DEK, α-enolase, citrullinated proteins, Sjögren syndrome-related antigen A (SSA; also known as Ro or TRIM21), Sjögren syndrome-related antigen B (SSB; also known as La) and Smith antigen (Sm)[94,95]. EVs released by activated or stressed cells or by microorganisms may therefore function as autoimmune triggers. A large amount of data has accumulated to suggest that EVs can activate several inflammatory pathways. For example, circulating EVs isolated from patients with dermatomyositis triggered pro-inflammatory cytokine production, including type I interferon production, through activation of the STING pathway by EV-associated double-stranded DNA[96]. However, it is yet to be clarified to what extent EVs participate in triggering or maintaining progression in different autoimmune diseases. Recently, an EV-mediated pathway by which self-tolerance might be breached was suggested. More than half of patients with sporadic systemic lupus erythematosus associated with nephritis have decreased activity of the deoxyribonuclease DNASE1L3 in the circulation as a result of neutralizing autoantibodies to this enzyme, which leads to reduced clearance of degraded self-DNA. Importantly, these patients also had increased amounts of cell-free DNA associated with EVs, suggesting a potential role of EVs in triggering and stimulating autoimmunity[97]. Moreover, given that EVs can cross biological barriers, such as the blood–brain barrier[98] or the blood–testis barrier[99], and certain EVs have immunoregulatory properties, they are attractive candidates for the therapy of autoimmune diseases.

In addition to their proposed role in autoimmunity, EVs also have an important role in the immune response to transplantation. Donor-derived EVs carrying MHC molecules can induce semi-direct allograft rejection by cross-dressing APCs of the host[100–102]. This process may be of particular importance given that donor APC-derived EVs can leave the graft before it is vascularized and thus before donor APCs can enter the systemic circulation. Thus, EVs may be early inducers of graft rejection. For example, apoptotic exosome-like vesicles can induce autoantibodies to the basement membrane heparan sulfate proteoglycan perlecan (also known as HSPG2) in naive mice; these antibodies are known to be involved in graft rejection and, indeed, increased perlecan-specific antibody production results in allograft inflammation in mice transplanted with MHC-mismatched aortic grafts[103]. Importantly, profiling of circulating EVs may predict transplant rejection[104]. Indeed, the number of donor-derived exosomes in peripheral blood samples could indicate early rejection with high specificity and sensitivity in a mouse model of heterotopic heart transplantation[105].

Overall, these examples illustrate that EVs are strongly implicated in autoimmunity and transplant rejection primarily by carrying self or donor antigens and activating inflammatory pathways.

Antitumour responses

Probably the most highly studied area in the field of EV-associated immune responses is antitumour immunity. A large and rapidly growing body of evidence suggests that tumour cell-derived EVs interact with cells of the immune system in the tumour microenvironment, with an important role for EV-encapsulated miRNAs. We do not discuss the involvement of miRNAs in detail here and, instead, refer readers to a recent review of the topic[106]. Tumour cell-derived EVs mainly suppress antitumour immune responses through their effects on NK cells, T cells, DCs, macrophages, myeloid-derived suppressor cells (MDSCs) and regulatory B cells. Immune cell-derived EVs released by tumour-associated macrophages, DCs, T_{reg} cells, NK cells, B cells and T cells are also involved in antitumour immunity[107].

Tumour cell-derived EVs carry a set of immunoregulatory molecules by which they mediate immunosuppression. They carry FASL and PDL1 and can induce the death of T cells and NK cells through the FAS–FASL and PD1–PDL1 pathways. They also carry the immunosuppressive cytokine TGFβ, which is associated with betaglycan (also known as TGFBR3) on the surface of EVs[22]. T_{reg} cells can be activated by EV-associated TGFβ1 and/or IL-10 (REF.[108]), and MDSCs can be activated by TGFβ and prostaglandin E2 (REF.[109]). Tumour cell-derived EVs also inhibit the maturation of DCs by inducing the production of IL-6 (REF.[110]), inhibit NK cell responses by downregulating NKG2D expression on NK cells via EV-associated TGFβ1 (REF.[111]), and induce apoptosis of $CD8^+$ T cells via EV-associated FASL, TRAIL or PDL1 (REFS.[112,113]). A characteristic effect of tumour cell-derived EVs is the induction of a shift in polarization of macrophages to an M2-type phenotype by EV-associated miR-145 through the downregulated expression of histone deacetylase 11 (REF.[114]). Arginase 1, HSP27, HSP72, macrophage migration inhibitory factor, galectin 9 and several non-coding RNAs also contribute to the immunosuppressive effects of tumour cell-derived EVs[115]. Furthermore, both the EV-associated adenosine-generating ectoenzymes (CD39 and CD73) and the adenosine cargo of EVs are considered key mediators of immunosuppression in the tumour microenvironment[116].

EV-associated immune-checkpoint molecules (such as PDL1) may interfere with antitumour immunity and response to immunotherapy (as reviewed recently[117]). For example, in patients with melanoma, tumour cells led to systemic suppression of immune functions through exosomal PDL1 (REF.[61]). Moreover, there is evidence that tumour-derived EVs may have unexpected effects on the antiviral immunity of patients with cancer by transferring growth factor receptors of the tumour to a select group of leukocytes. In epidermal growth factor receptor (EGFR)-positive lung cancer, tumour cell-derived EVs transfer activated EGFR molecules to host macrophages, in which EGFR activates mitogen-activated

Table 2 | **Recent observational and clinical trials of extracellular vesicles in inflammatory diseases**

Trial identifier (ClinicalTrials.gov)	Trial phase	Condition	Trial purpose or intervention	Start date	Status
NCT05191381	Observational	Critically ill patients with COVID-19, having hypercytokinaemia and lung fibrosis	To characterize the anti-inflammatory and immune modulatory function of MSC-derived exosomes in a whole blood assay	22 December, 2021	Recruiting (estimated completion 31 December, 2022)
NCT04979767	Observational	Bacterial sepsis	To define immune pathways and identify clinically useful biomarkers	15 April, 2021	Recruiting (estimated completion 30 June, 2022)
NCT04850469	Observational	Severe infection in children	To evaluate the application of MSC-derived exosomes	1 January, 2022	Not yet recruiting (estimated completion 31 December, 2024)
NCT05072951	Observational	Kidney transplant	To define a urine biomarker for transplant rejection	October 2021	Not yet recruiting (estimated completion October 2025)
NCT04653610	Observational	HIV-1 infection	To determine the expression profile and content of EVs before and after treatment initiation	27 January, 2021	Recruiting (estimated completion January 2025)
NCT04852653	Observational	Rectal cancer	To detect tumour cell-derived EVs in liquid biopsy	May 2021	Not yet recruiting (estimated completion November 2023)
NCT05061212	Observational	Acute respiratory distress syndrome	To determine the role of EVs containing mitochondrial DNA	1 October, 2021	Not yet recruiting (estimated completion 31 December, 2022)
NCT04892433	Observational	CAR T cell therapy	To study microRNAs derived from EVs and correlate with clinical outcome	14 May, 2021	Recruiting (estimated completion April 2026)
NCT05215288	Early phase I	Abdominal solid organ transplant rejection	Treatment with bone marrow MSC-derived EVs	June 2022	Not yet recruiting (estimated completion December 2022)
NCT04664738	Phase I	Skin graft	Treatment with platelet-derived EVs	16 March, 2021	Enrolling by invitation (estimated completion December 2022)
NCT05116761	Phase I/II	Post-acute COVID-19 or chronic post-COVID-19 syndrome	Treatment with bone marrow MSC-derived EVs	March 2022	Not yet recruiting (estimated completion August 2022)
NCT04798716	Phase I/II	Pneumonia or acute respiratory distress syndrome caused by COVID-19	Treatment with MSC-derived exosomes delivered intravenously	Estimated September 2021	Not yet recruiting (estimated completion 2021)
NCT05127122	Phase I/II	Acute respiratory distress syndrome	Treatment with bone marrow MSC-derived EVs delivered intravenously	March 2022	Not yet recruiting (estimated completion August 2022)
NCT04969172	Phase II	COVID-19	Treatment with exosomes overexpressing CD24	11 July, 2021	Active, not yet recruiting (estimated completion 11 July, 2022)
NCT04902183	Phase II	COVID-19	Treatment with exosomes overexpressing CD24	9 June, 2021	Recruiting (estimated completion 1 September, 2021)
NCT05125562	Phase II	COVID-19	Treatment with bone marrow MSC-derived EVs	7 February, 2022	Not yet recruiting (estimated completion 7 December, 2022)
NCT05216562	Phase II/III	SARS-CoV-2 infection	Treatment with MSC-derived exosomes delivered intravenously	1 July, 2021	Recruiting (estimated completion 30 December, 2022)
NCT04761562	Phase II/III	Chronic otitis media treated with tympanic membrane perforation	Treatment with platelet-rich and EV-rich plasma	14 February, 2021	Recruiting (estimated completion 30 September, 2023)

Recent clinical trials (starting in 2021 and 2022) focusing on EVs in inflammatory diseases were extracted from ClinicalTrials.gov using the search terms "exosomes" and "extracellular vesicles", and filtered for diseases and conditions with an immune-mediated, inflammatory background. CAR, chimeric antigen receptor; EV, extracellular vesicle; MSC, mesenchymal stem cell.

Box 2 | **The potential use of extracellular vesicles to treat COVID-19**

Extracellular vesicles (EVs) are emerging as a potential new therapeutic strategy for SARS-CoV-2 infection. Numerous studies have described strategies to prevent or ameliorate SARS-CoV-2 infection by EVs or to use EVs as predictive biomarkers for disease severity. Patients with COVID-19 have increased levels of circulating EVs that carry the SARS-CoV-2 receptor angiotensin converting enzyme 2 (ACE2). These EVs are very potent in neutralizing SARS-CoV-2 infection by functioning as decoys that compete with cellular ACE2 for binding SARS-CoV-2 and, importantly, they block a wide variety of SARS-CoV-2 variants[145]. EVs can also be engineered to function as drug or vaccine delivery platforms. Engineered EVs carrying ACE2 provided anti-virus protection in vitro and in mice[146,147]. EVs engineered with palmitoylated ACE2 neutralized pseudotyped and authentic SARS-CoV-2 in human *ACE2*-transgenic mice and protected the mice from virus-induced lung inflammation[148]. In mice and in Syrian hamsters, the intranasal administration of a vaccine based on bacterial outer membrane vesicles induced both mucosal and systemic immune responses to SARS-CoV-2 (REF.[149]). Similarly, in another recent study, golden Syrian hamsters were immunized intranasally with outer membrane vesicles from endotoxin-attenuated *Salmonella typhimurium* decorated with the surface-coupled SARS-CoV-2 spike protein receptor-binding domain. The immunization induced neutralizing antibodies to wild type and Delta viral variants and led to less severe lung pathology upon subsequent infection with live virus[150]. As a novel approach, mRNAs encoding SARS-CoV-2 spike and nucleocapsid proteins were loaded into small EVs, which induced immunity to SARS-CoV-2 in mice[151]. Finally, in a prospective, non-randomized, open-label cohort study, the use of an allogeneic bone marrow mesenchymal stem cell-derived EV product was reported to be safe and efficacious for the treatment of severe COVID-19 (REF.[152]).

protein kinase kinase kinase 2 (MEKK2) to negatively regulate the antiviral immune response. This mechanism may partly explain the immunocompromised status of patients with cancer[118].

Tumour-infiltrating T_{reg} cells are regulated by a balance of two endosomal processes in DCs, both being mediated by tumour cell-derived EVs. Binding of EV-derived RNA to endosomal TLR3 in DCs induces IFNβ production, which increases the number of T_{reg} cells and promotes tumour development, whereas EV-associated phosphatidylserine interacts with CD300A of DCs to inhibit TLR3 signalling and reduce the number of T_{reg} cells[119]. Higher expression levels of CD300A on DCs were associated with a decreased number of tumour-infiltrating T_{reg} cells and longer survival time in patients with melanoma. Thus, tumour cell-derived EVs and CD300A of DCs have important roles in the regulation of tumour-infiltrating T_{reg} cells and antitumour immunity.

Of note, under adverse conditions in the tumour microenvironment, such as during hypoxia or nutrient restriction, adaptive 'immunogenic stress' responses of tumour cells are induced (including autophagy, endoplasmic reticulum stress and the DNA damage response), which increase the release of EVs with an altered molecular composition. These tumour cell-derived EVs are carriers of DAMPs such as HMGB1, HSPs, ATP and mitochondrial DNA, which may facilitate immune recognition of the tumour by creating an inflammatory environment. Cancer-derived EVs also carry tumour-associated antigens, which, upon uptake by APCs, may stimulate tumour-specific $CD8^+$ T cells[14].

Therapeutic potential

In the past few years, the therapeutic potential of EVs has been the focus of intense research. Currently, EVs are broadly being considered as potential immune therapies for several clinical conditions having immune or inflammatory components (TABLE 2), including, recently, COVID-19 (BOX 2). In addition to their potential functions as therapeutic tools and delivery vehicles, EVs might also be exploited as biomarkers for disease and therapy monitoring (BOX 3).

The first breakthrough observation that EVs might be used therapeutically was that EVs derived from DCs pulsed with tumour peptides could promote the elimination of established tumours in mice by inducing a $CD8^+$ T cell response[120]. The ability of microbial EVs to induce STING activation inspired a recent cancer therapeutic approach in which engineered EVs carrying the STING agonist cyclic GMP–AMP were successfully used to enhance antitumour immunity and decrease tumour growth[121].

Among the current EV-based immunotherapeutic approaches, stem cell-derived EVs with immunoregulatory effects are taking the lead, mainly involving EVs derived from mesenchymal stem cells. In addition to their tissue repair-promoting properties, these EVs have a broad immunosuppressive potential. As discussed above for tumour cell-derived EVs, EVs derived from stem cells and progenitor cells also inhibit NK cell responses as well as DC maturation and activation, induce M2-type macrophage polarization, promote T_{reg} cell differentiation, and inhibit B cell proliferation and differentiation. Furthermore, their immunotherapeutic potential has been confirmed in a wide range of experimental disease models, for example in experimental models of asthma, graft-versus-host disease, type 1 diabetes, experimental allergic encephalomyelitis, experimental autoimmune uveitis, acute respiratory distress syndrome and collagen-induced arthritis (recently

Box 3 | **Extracellular vesicles in blood plasma and other body fluids may function as biomarkers**

- In patients with melanoma, exosome-associated PDL1 distinguishes responders from non-responders to anti-PD1 therapy[61].
- In patients with COVID-19, increased levels of circulating extracellular vesicles (EVs) that carry the SARS-CoV-2 receptor angiotensin converting enzyme 2 (ACE2) are detected, and the EV-associated coatomer complex subunit β2 (COPB2) predicts COVID-19 severity[153].
- EV-associated molecular signatures are proposed as candidate biomarkers in lung, heart, kidney and liver transplantation, and pancreatic island transplant rejection as reviewed recently[104].
- In human sepsis, levels of elongated neutrophil-derived structures are highly increased in the blood plasma[85].
- In patients with polymyositis and dermatomyositis, plexin D1 on circulating EVs has been identified as a potential biomarker[154].
- Certain EV-associated microRNAs (miR-16, miR-302d-3p, miR-378e, miR-570-3p, miR-574-5p, miR-579 and miR-25-3p) are candidate biomarkers for type 1 diabetes[155].
- Serum EV-associated miR-451a and miR-25-3p and soluble TWEAK (also known as TNFSF12) are candidate biomarkers in early rheumatoid arthritis[156].

reviewed elsewhere[122]). EVs isolated from milk, such as cow milk, also hold promise as future therapeutic agents. Milk-derived EVs have immunomodulatory functions and alleviate dextran sulfate sodium-induced ulcerative colitis in mice by blocking the TLR4–NF-κB and NLRP3 pathways, re-establishing the balance between T_{reg} cells and inflammatory T_H17 cells, and increasing the relative abundance of some beneficial gut bacteria[123].

Importantly, EVs can also be used for vaccine formulation. In particular, the outer membrane vesicles of Gram-negative bacteria are a promising vaccine development platform. These vesicles have an optimal size for uptake by immune cells and carry TLR-activating molecules, such as LPS, to stimulate an innate immune response. Potential benefits of bacterial vesicle-based vaccines include simplicity and low cost of manufacture, potential representation of several antigenic molecules, which reduces the risk of the emergence of escape variants, natural orientation of surface-expressed antigens, stability, and the possibility to be genetically engineered. The immunogenicity of outer membrane vesicles has already been harnessed in two licensed vaccines against *Neisseria meningitidis* serogroup B[124,125]: Bexsero®, developed by Novartis, and VA-MENGOC-BC®, developed by the Finlay institute in Cuba[66]. Another example of how bacterial EVs can be harnessed for therapeutic purposes is the use of probiotic-derived EVs, for example from *Bifidobacterium longum*, *Clostridium butyricum* and *Lactobacillus plantarum* WCFS1. These EVs stimulate innate immune cells to produce TNF and IL-6, suggesting that probiotic EVs might have use as novel vaccine adjuvants[126].

Chimeric antigen receptor (CAR) T cell-derived EVs have also attracted significant attention recently (FIG. 4a).

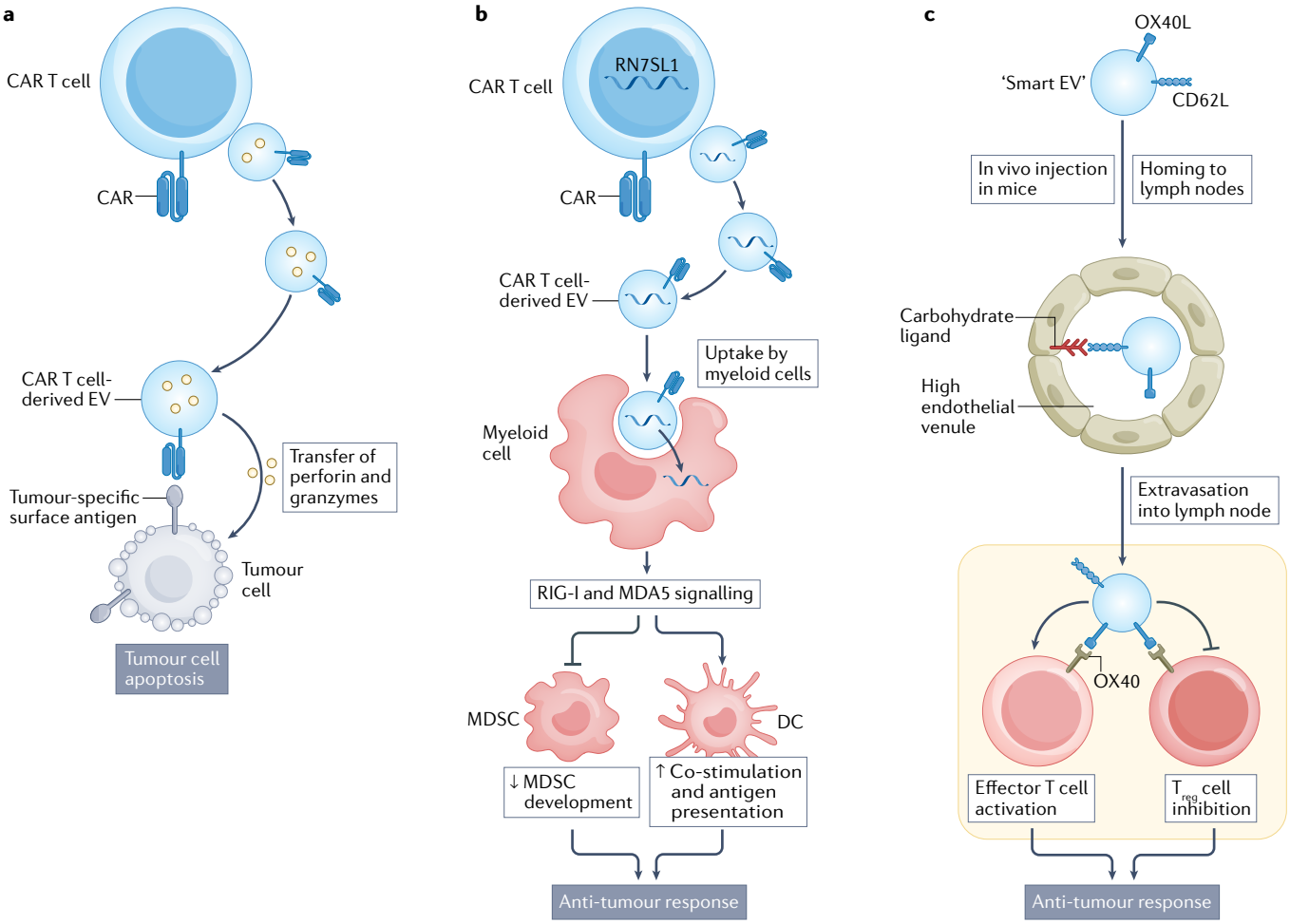

Fig. 4 | **Examples of antitumour effects of extracellular vesicles released by genetically engineered cells. a** | Chimeric antigen receptor (CAR) T cells release extracellular vesicles (EVs) carrying surface CARs. These EVs also contain perforin and granzyme B and can cause tumour cell death upon recognition of the CAR-specific tumour antigen[127]. **b** | CAR T cells can be engineered to express the pattern recognition receptor agonist endogenous RN7SL1 RNA. RN7SL1-containing EVs derived from these CAR T cells are efficiently taken up by myeloid cells, in which RN7SL1 activates signalling through the pattern recognition receptors retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). This inhibits the development of myeloid derived suppressor cells (MDSCs) and increases the co-stimulatory and antigen-presenting capacity of dendritic cells (DCs) in the tumour microenvironment to enhance antitumour immune responses[128]. **c** | 'Smart EVs' were obtained by engineering the producing cells to release EVs with exofacial CD62L (also known as L-selectin), an adhesion molecule for leukocyte homing to lymph nodes, and OX40L, a co-stimulatory molecule that suppresses the differentiation and activity of regulatory T (T_{reg}) cells. These smart EVs home to lymph nodes upon subcutaneous injection into mice, where they interact with lymphatic endothelial cells. They also facilitate the activation of antitumour effector T cells and inhibit T_{reg} cells through OX40–OX40L interactions in the tumour-draining lymph nodes[129].

These EVs express high levels of perforin and granzyme B as well as CARs, and thus can induce the death of tumour cells expressing their cognate antigen. Importantly, they have therapeutic potential even in the case of solid tumours, where the efficacy of CAR T cell therapy is limited by the poor penetration of the tumours by CAR T cells. By contrast, EVs, owing to their small size and their ability to cross biological barriers, can achieve efficient penetration of solid tumours. Unlike CAR T cells, CAR T cell-derived EVs do not have PD1 on their surface and, thus, their antitumour effect is not reduced by recombinant PDL1 therapy[127]. In a recent approach, CAR T cells were engineered to express the endogenous non-coding RNA RN7SL1 at high levels (FIG. 4b), which activates signalling through the PRRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). The efficient uptake of CAR T cell-derived EVs containing RN7SL1 by innate immune cells in the tumour microenvironment resulted in restricted MDSC development and decreased production of immunosupressive TGFβ by myeloid cells as well as in increased co-stimulation by DC subsets. In this study, in addition to the proliferation and effector-memory differentiation of CAR T cells promoted by RN7SL1, the immunostimulatory activity of CAR T cell-derived EVs substantially supported the activity of CAR T cells, resulting in the efficient rejection of solid tumours even with CAR antigen loss[128]. Recently, 'smart EV'-producing cells were generated to release EVs carrying both CD62L and OX40L, which homed to the tumour-draining lymph nodes in mice, activated effector T cells and inhibited T_{reg} cell induction[129] (FIG. 4c). Another recent study generated genetically engineered multifunctional EVs carrying both surface antibodies for activating and directing T cells to kill tumour cells and immune-checkpoint modulators[130]. These examples suggest that EV engineering holds huge potential to develop complex EV-based immunotherapeutics for almost any pathological scenario.

Conclusions and future directions

Research into EVs has long been hampered by conceptual and technological difficulties. Despite the significant progress that has now been made in techniques for the separation and characterization of EVs, particular attention and rigour are required when attributing specific functions to EVs[2]. This is particularly important given that, in the nanoparticle size range, recent research has identified the presence of extracellular non-EV particles. Nevertheless, the available data leave little or no doubt that EVs are ubiquitous key modulators of immune functions that could be exploited for biomarker or therapeutic purposes. However, many questions remain unanswered. Current gaps in our knowledge include the lack of systematic and in-depth information about the relative significance of EV-associated mediators versus soluble mediators and about the contribution of EVs to the epigenetic and metabolic changes in immunity that occur at the single-cell level. The history of immunology shows that the identification of immune cell populations and subsets was enabled by the identification of immune cell markers and marker combinations. Recent technical progress has led to the development of novel platforms by which up to 3–5 different marker molecules per single EV can be detected, which may boost the development of the EV field significantly.

Considering that cells can be genetically engineered to produce EVs that carry targeting and/or therapeutic molecules, and that these EVs can be further modified chemically and/or can be loaded with cargo, there are great opportunities for the biomedical applications of EVs. Current clinical studies reflect high expectations that EVs will be the next generation of immune therapeutics. However, although abundant preclinical data indicate the beneficial effects of EVs, results of human clinical trials are yet to come. Overcoming the current challenges related to large-scale production of EVs in compliance with Current Good Manufacturing Practice regulations will also be required for the introduction of EV products to clinical practice.

Here, we have highlighted some of the key recent advances in our understanding of the roles of EVs in the immune system. Given the complexity of the immune system, it seems likely that there will be many future EV-related discoveries together with the development of novel EV-based biomarker or therapeutic platforms.

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