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Exosomes and their roles in immune regulation and cancer

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Abstract

Exosomes, a subset of extracellular vesicles (EVs), function as a mode of intercellular communication and molecular transfer. Exosomes facilitate the direct extracellular transfer of proteins, lipids, and miRNA/mRNA/DNAs between cells *in vitro* and *in vivo*. The immunological activities of exosomes affect immunoregulation mechanisms including modulating antigen presentation, immune activation, immune suppression, immune surveillance, and intercellular communication. Besides immune cells, cancer cells secrete immunologically active exosomes that influence both physiological and pathological processes. The observation that exosomes isolated from immune cells such as dendritic cells (DCs) modulate the immune response has enforced the way these membranous vesicles are being considered as potential immunotherapeutic reagents. Indeed, tumour- and immune cell-derived exosomes have been shown to carry tumour antigens and promote immunity, leading to eradication of established tumours by $CD8^+$ T cells and $CD4^+$ T cells, as well as directly suppressing tumour growth and resistance to malignant tumour development. Further understanding of these areas of exosome biology, and especially of molecular mechanisms involved in immune cell targeting, interaction and manipulation, is likely to provide significant insights into immunorecognition and therapeutic intervention. Here, we review the emerging roles of exosomes in immune regulation and the therapeutic potential in cancer.

1 Introduction

The immune system embodies ordered biological processes and structures that serve to recognise and respond to the surrounding extracellular environment. In the context of cancer, precancerous and malignant cells can provoke an immune response that abolishes transformed and/or malignant cells; a process known as immune surveillance [1]. In both adaptive and innate immunity, several immune pro-tumour effector mechanisms are dysregulated, leading to the hypothesis that inflammation in certain instances can facilitate carcinogenesis and tumour progression by modulating immunoregulation [2]. Further, in some cancer types, an inflammatory microenvironment restricts the immune system from rejecting malignant cells, and promotes the development of tumours [3]. Throughout the tumour microenvironment, multiple immune cell types, including T lymphocytes, B lymphocytes, macrophages, and natural killer (NK) cells regulate tumourigenesis depending on the tissue composition and cellular stimuli [4,5]. Recently, the role of extracellular vesicles (EVs), in particular, endosome-derived exosomes, secreted by tumour cells in modulating the immune response has been highlighted [6-12]. As focal mediators of intercellular communication and immunological function, exosomes can enhance opsonisation, regulate antigen presentation, and induce immune activation and immune suppression. Further, there is increasing evidence highlighting that exosomes may facilitate cancer progression by regulating different immune cell types towards a pro-tumourigenic environment [13].

Exosomes are homogenous membrane vesicles (40-150 nm diameter), derived from the exocytosis of intraluminal vesicles (within multivesicular bodies, MVBs), and released into the extracellular space when fused with the plasma membrane [14,15]. Most cell types

release exosomes through this mechanism including hematopoietic cells, reticulocytes, B- and T-lymphocytes, dendritic cells, mast cells, platelets, intestinal epithelial cells, astrocytes, neurons and tumour cells [16]. Specific characteristics associated with exosomes include their composition (bilipidic layer), size, density (1.09-1.13g/mL) and protein content, including endosome-derived (endosomal sorting complex required for transport proteins; ESCRTs, such as Alix and Tsg101), sorting- and trafficking-related (endosomal Rab GTPases) and cell membrane-derived (tetraspanins, CD63, CD81 and CD82) [17,18]. In addition to protein constituents, exosomes are also comprised of various lipids and lipid-raft-associated proteins originating either at the plasma membrane or from early/late endosome compartments, including cholesterol, sphinogmyelin, and flotillins [15,19,20]. Exosomes are involved in the transfer of various mRNAs and microRNAs (miRs) to neighbouring cells for translation [21,22]. Pancreatic cancer cell-derived exosomes have recently been shown to contain double-stranded DNA of mutated *KRAS* and *p53* [23], the most frequent genetic mutations in human pancreatic cancer. Interactions between tumour-derived exosomes and recipient cells are mediated through direct signalling interactions via surface-expressed molecules or by transfer of exosomes and/or their cargo [24,25]. Tumour-derived exosomes are released both locally and into the circulation to interact with an assortment of target cells, including other tumour, stroma, and immune cells. The precise mechanism of exosome internalisation is still unclear, however, receptor-mediated endocytosis (e.g., LFA1, TIM1 and TIM4), phagocytosis, and direct plasma membrane fusion have been proposed [26,27]. It has been demonstrated that the low pH of the tumour microenvironment is essential for exosome uptake by human metastatic melanoma *in vitro*, that is suggested to be related to elevated stability and lipid/cholesterol content of exosomal membranes in an acidic environment [28].

Exosomes adopt many distinct roles that vary depending on their cellular origin, from modulating immune function, enhancing tumour-cell invasion, and intercellular communication. In an effort to understand immune-related functions of exosomes, recent studies have investigated specific components of exosomes that directly or indirectly regulate the tumour immune response [11,29-31]. These components have been shown to include peptide-bound MHC class I and II, T-cell stimulatory molecules (B7.2, ICAM-1), and other immune molecules such as MFG-E8, FasL, galectin-9, TGF-β, TNF-α, or NKG2D ligand [32-44]. Exosomes derived from cancer cells and virally infected cells have been shown to influence the tumour extracellular environment [35,36,45-48]. Tumour cells secrete immunologically active exosomes, capable of inhibiting tumour growth through anti-tumour immune responses [49] and promotion of tumour growth by inhibiting anti-tumour immunity [50] or enhancing the metastatic process [51,52]. [35,36,45-48] This review will summarise the role of exosomes in immunoregulation, including antigen presentation, activation and suppression of the immune response, and expression of cell surface opsonins and complement factors as a means of immune surveillance and immunorecognition in the context of cancer.

2 Modulating the immune response: The role of exosomes

The immune response is the collective process of innate and adaptive immunity [53]. These responses are regulated by many different components, including proteins, RNAs, and lipids; a process known as immunomodulation. In cancer, complex networks are mediated by tumour cells or pro-inflammatory cell constituents, to facilitate immunomodulation and result in the repression of adaptive immunity against cancer cells [54-56]. With their diverse, and often contrasting immune-related functions, exosomes have recently gained attention during tumourigenesis, in particular on cancer immune surveillance and tumour escape [11].

responses at many levels, thus promoting tumour progression. Exosomes facilitate angiogenesis [57-60], directly suppressing cytotoxic T lymphocytes and NK-cells' anti-tumour responses, and inducing activation of immune suppressor cell subsets, leading to loss of tumour immune surveillance [9]. Furthermore, the suppressor activity of tumour vesicles seems to involve induction of the generation, expansion, and suppressive function of human regulatory T cells [61,62]. The molecules expressed on exosomes involved in regulating the immune response include FasL, TRAIL, membrane-bound TGF-β, CD73, and galectins [36,43,44,63-67]. Tumor-derived exosomes can also suppress immune-cell responses by inhibiting the cytotoxicity of CD8+ T cells and NK cells [63,68-71]. This inhibition is mediated predominantly through NKG2D down-regulation [68-70] and TGF-β [71] on the tumour-derived exosome surface. The inhibition of cytotoxic T-cell function has also been suggested to be induced in part by the increased T-cell ROS content mediated by melanoma-derived exosomes [72].

In addition to their direct roles in the extracellular tumour environment, exosomes have been shown to be significant in the regulation of immune responses during cancer immunotherapy [33,73-84]. Exosomes carrying MHC-peptide complexes and antigens are crucial in initiation and amplification of an immune response. Pioneering work by Raposo and co-workers demonstrated that B lymphoblast-derived exosomes that bear MHC class II-peptide complexes were capable of activating human and mouse antigen specific T cell clones [33]. The presentation of these complexes to T cells identified a possible role of exosomes in the modulation of the adaptive immune response. Zitvogel and colleagues demonstrated that exosomes derived from dendritic cells (DCs) pulsed with antigenic peptides induced potent anti-tumour $CD8^+$ T cell responses in murine mastocytoma P815 and mammary carcinoma TS/A tumour models, resulting in the regression of established tumours [78]. Presentation of

MHC-peptide complexes to naïve T lymphocytes and the priming of cytotoxic T lymphocytes (CTLs) by these exosomes were shown to selectively promote the anti-tumour immune response [78]. Further, this study highlighted that DC maturation accompanied exosome production. These results and subsequent studies discussed in this review further highlight the importance and often contrasting functional effects of exosomes in mediating and influencing the immune surveillance.

2.1 Exosomes and their roles in antigen presentation and T cell activation

Antigen-presenting cells (APCs), such as dendritic cells (DCs), present antigenic peptides (namely MHC-peptide complexes) to T cells. Exosomes derived from various cell types have been shown to play crucial roles in carrying and presenting functional MHC-peptide complexes to modulate antigen-specific $CD8^+$ response [43] through direct presentation and cross-presentation as illustrated in **Figure 1A/B**. Direct presentation occurs when MHC-peptide complexes on the exosomes are directly engaged by antigen-specific T cells leading to T cell activation. In cross-presentation, APCs acquire antigens carried by exosomes, further process these antigens and present their peptides to $CD8^+$ T cells. Moreover, cross-presentation can occur whereby antigenic peptide-MHC complexes are together transferred onto DCs and then presented to T cells (termed cross-dressing) [85]. In the context of exosome function, MHC-peptide complexes from exosomes have been attributed to cross-dressing of APCs to activate allo-reactive T cells [37] but yet to be confirmed as a mechanism as exosome-mediated T cell cross-priming [86,87]. Thery and colleagues highlighted exosomes secreted by DCs, in addition to inducing naïve T cell stimulation *in vivo* and *in vitro*, could further stimulate T cells in the presence of MHC class II-deficient DCs (without representing H-Y antigen or peptide) [40]. In the absence of intact antigen,

exosomal peptide-MHC (pMHC) and H-Y-peptide complexes could stimulate specific T cell response *in vitro* in the presence of mature DCs. Exosomes therefore mediate the transfer and cross-dressing of pMHC complexes between different DC populations. Further, Wakim and Bevan peptide-pulsed H–2K^{bm1} DC-derived exosomes which resulted in OT-I T cell division, indicating that DCs can use exosomes as an antigen source by reloading endogenous antigenic peptide-MHC class I complexes [87]. This study indicated that exosomes are not the means by which intact antigenic peptide-MHC class I complexes are cross-dressed onto DCs, but may serve as an effective means to transfer peptide antigen between cells. Clearly, with these opposing data, the issue of exosome-mediated cross-dressing of DCs requires further investigation.

Exosomes carrying native tumour antigen or functional MHC-peptide complexes have been widely investigated and shown to play a critical role in antigen cross-presentation during cancer surveillance (**Figure 1A**) [33,77-79,88]. In an *in vitro* system, Wolfers et al., [77] demonstrated that dendritic cells activated tumour-specific CD8$^+$ T cells following uptake of human melanoma exosomes and that these exosomes are enriched with HSP70 and full-length tumour antigens. In mouse tumour cells, these authors further demonstrated that exosomes induced CD8$^+$ T cell cross priming and tumour rejection *in vivo* [77]. Monocytes-derived dendritic cells (MoDCs) pulsed with exosomes from melanoma ascites induced Mart1/Melan A-specific, HLA-A2 restricted CD8$^+$ T cell responses *ex vivo* [88]. Further, bulk lymphocytes from these melanoma patients stimulated with MoDCs loaded with ascites-derived exosomes expanded into tumour specific cytotoxic T lymphocytes. The authors therefore proposed that such ascites-derived exosomes could be a suitable tumour antigen source for exosome-based vaccines against cancer [88].

Exosomes have also been shown to directly induce T cell activation in the absence of APCs as they carry MHC-peptide complexes, sometimes even co-stimulatory molecules, that include exosomes isolated from viral infected MoDCs [89]. Utsugi-Kobukai et al., [41] demonstrated that exosomes secreted from chicken ovalbumin (OVA)-pulsed BmDCs, presented $OVA_{257-264}$ peptide to its specific $CD8^+$ T cell hybridomas. Interestingly, exosomes secreted by $OVA_{257-264}$ peptide- or OVA protein-pulsed mature BmDCs stimulated T cell hybridomas more efficiently than the immature counterparts likely due to higher abundance of co-stimulatory molecules on the mature BmDC derived exosomes [41]. *In vivo*, exosomes have been shown to also transfer functional MHC-peptide complexes to DCs leading to priming of $CD8^+$ and $CD4^+$ T cells [48,90,91] (**Figure 1B**). Furthermore, DC-derived exosomes from knock-out mice lacking MHC class II-peptide complex and ICAM-1 expression were not able to prime naïve T cells directly [39]. Therefore, changes in protein composition and priming abilities of exosomes reflect maturation signals received by DCs.

Although exosomes also transfer class II-peptide complexes, their capacity to directly activate $CD4^+$ T cells is less well understood. It was reported that 50% of MHC class II-peptide complexes are lost on activated B cells every day including 12% loss as a result of exosome release [38]. These exosomes were shown to directly stimulate primed, but not naïve, $CD4^+$ T cells. Interestingly, recycled MHC II-peptide complexes were also able to directly activate $CD4^+$ T cells [38]. Mallegol et al., [92] demonstrated *in vitro* that exosomes, secreted by HLA-DR4-expressing intestinal epithelial cell line T84 and pulsed with human serum albumin peptide $HSA_{64–76}$, could not directly activate HLA-DR4-restricted $HSA_{64–76}$-specific $CD4^+$ T-cell hybridoma. However, the same T-cell hybridoma was activated by human MoDCs loaded with these exosomes, even more efficiently than the MoDCs loaded with the soluble antigen. In addition, intestinal epithelial exosomes were shown to contain

MHC class II-peptide complexes, and tetraspanins CD9, CD81 and CD82 [93] which may enhance local antigen presentation. These results indicate that exosomes carrying MHC II-peptide complexes may directly activate $CD4^+$ T cells and MoDCs loaded with peptide-pulsed exosomes could be more efficient than MoDCs loaded with soluble antigen, arguing that exosomes may enhance the immune surveillance in the mucosal surface [94] and in other locations.

In transplantation, indirect allorecognition initiated by recipient DCs presenting donor allogeneic antigens is one of the mechanisms responsible for transplant rejection [95]. Using Thy1.1 congenic, TCR transgenic 1H3.1 $CD4^+$ T cells specific for the IA^b-$IE\alpha_{52–68}$ complex, Montecalvo et al., [37] demonstrated that donor DC-derived exosomes mediated the transfer of functional allogeneic IA^b-$IE\alpha_{52–68}$ complexes to Balb/c recipient DCs which in turn initiated the indirect allorecognition. The authors therefore proposed that donor DC-derived exosomes may amplify allorecognition during organ transplantation [37,40]. Such studies have raised controversy regarding the feasibility of direct presentation by exosomes, as indirect presentation might be a possibility and has not been excluded. Some studies proved the need of indirect presentation by DCs for exosomes stimulated T-cells [37,96], while other studies have demonstrated direct functional presentation through exosomes themselves [39,97].

Cell surface and integral membrane/ adhesion proteins on exosomes are important in mediating associated cell recognition, adhesion, antigen uptake, and recipient cell function [24,27,28,98-100]. Taken together, functional MHC/peptide complexes and tumour antigens such as, Mart-1, gp100, Her2/Neu and CEA present in exosomes have been shown to be a promising anti-tumour therapeutic vaccines in clinical intervention via antigen presentation

and T cell activation [43,44,101]. Other ligands, including lactadherin/ milk fat globule E8 (MFGE8), tetraspanins and externalized phosphatidylserine, are also present on exosomes. These ligands and adhesion molecules participate either directly or indirectly in the binding of exosomes to APCs [102-104]. Moreover, intestinal epithelial cell-derived exosomes were shown to contain enriched transmembrane proteins CD9, CD81, CD82 and glycoprotein A33 antigen that were capable of binding human serum albumin (HSA). HSA bound exosomes preferentially interact with DCs, thereby enhancing antigen presentation to T cells [92].

2.2 Exosomes: Promoting immune responses

Exosomes have recently been shown to be involved in the pro-inflammatory response and are capable of promoting immunity (**Figure 1C**). Exosomes derived from bacteria-infected macrophages have been shown to be immunomodulatory and stimulate macrophages and neutrophils to secrete pro-inflammatory mediators, including TNF-α and RANTES (up-regulating iNOS expression) [105,106]. Further, exosomes released from macrophages infected with intracellular pathogens stimulate a pro-inflammatory response *in vitro* and *in vivo* [106]. Pathogen-associated molecular patterns (PAMP) on these exosomes may also play critical roles in enhancing immune surveillance [105,106]. Whilst its been well established that T cells secrete exosomes, distinct exosome populations have been identified from $CD4^+$ T cells, of which one vesicle type increases upon T-cell activation depending on the level of co-stimulation [90]. This suggests that T-cell activation differentially regulates the release of distinct exosome subpopulations.

Mature DC-derived exosomes can activate an immune response through the TNF-α pathway. Exosome-derived TNF-α-induced epithelial cells have been demonstrated to secrete pro-

inflammatory cytokines (MCP-1, IL-8, TNF-α, RANTES), indicating a key role for exosomes in immunity [107]. Moreover, NK cells incubated with tumour-derived Hsp70-positive exosomes were induced to release granzyme B that initiated apoptosis in human pancreatic/colorectal tumours [30]. Therefore, pro-immunogenic potential was identified as a key property of tumour-derived exosomes. In response to stress the assimilation of Hsp70 within the lipid bilayer has been demonstrated to modulate exosomes release into the extracellular milieu [108]. Interestingly, Vega and colleagues identified TNF-α secretion by macrophages in membranous structures that constitute Hsp70. They suggest that stress-induced membrane translocation of Hsp70 stimulates an immunogenic response. Qazi et al., [109] observed enrichment of exosomes in bronchoalveolar lavage fluid from patients with sarcoidosis. Bronchoalveolar lavage fluid-derived exosomes induced epithelial cells to produce interleukin (IL)-8, a neutrophil chemotactant, and peripheral blood monocytes to produce IFN-γ and IL-13, factors important for activating T cell responses. It was reported that exosomes derived from synovial fibroblasts of rheumatoid arthritis individuals contained a membrane form of TNF-α [32]. Interestingly, these exosomes were demonstrated to be internalised by activated T cells, induce Akt and NF-κB pathway activation and therefore delay activation-induced cell death. Therefore, exposing tumour cells to stress may cause derived exosomes to be significantly more immunogenic [110,111]. These activities require the active participation of DCs to process and cross-present delivered antigens to T cells. It is important to emphasize that the cancer exosome content, which is under the influence of the microenvironment, is important for these immune functions.

DC-derived exosomes also induced anti-tumour responses by activating other immune effector cells (**Figure 1B**). For example, mouse BmDC-derived exosomes harbouring functional membrane bound IL-15Rα (NKG2D ligand) were shown *in vivo* to promote IL-

15Rα- and NKG2D-dependent NK cell activation and proliferation, resulting in tumour suppression [112]. Similarly, human MoDC-derived exosomes also carried functional NKG2D ligands and are able to restore the number and NKG2D-dependent function of NK cells in exosome-based vaccine clinical trial patients [112]. This capacity of DC-derived exosomes to suppress/ activate the immune system indicates that exosomes are an important component of the immune network, however the influence of the tumour microenvironment is an important contributing factor to these immune functions. Together, these studies highlight the fundamental roles of exosomes in the initiation and amplification of pro-inflammatory immune responses.

2.3 Exosomes: Inhibiting immune responses

A large body of evidence points towards the established role of tumour-derived exosomes in promoting a pro-tumourigenic phenotype and facilitating immunosuppression (extensively reviewed in [113]). Several studies further show that these exosomes have the potential to hijack mechanisms enlisted by cancer cells to influence the efficacy of therapeutic agents [114,115]. Therefore, dissection of tumour exosomes stimulated pathways leading to immune suppression will provide valuable insights into identification and/or selection for cancer therapies.

Suppression of the immune response by tumour-derived exosomes has been shown to suppress T-cell and NK cell activity, and stimulate myeloid-derived suppressor cells (MDSCs) (**Figure 1D**) [12,116]. Seminal work by Poutsiaka and colleagues [117] showed that membranous vesicles released by murine B16 melanoma cells repressed the IFN-γ-dependent class II expression on murine macrophages, which may affect antigen presentation

to $CD4^+$ T cells. Moreover, many studies have also shown that tumour-derived exosomes can induce T-cell apoptosis that facilitate evasion of immune surveillance. Andreola and colleagues demonstrated that in melanoma cells, Fas ligand (FasL) was restricted to MVBs that contain melanosomes [118]. These melanosome-positive MVBs were demonstrated to further release FasL-containing exosomes that induce apoptosis in Jurkat and lymphoid cells. Therefore, neoplastic modulation involving release of FasL-positive exosomes promotes immune escape. exosomes expressing FasL and TNF were also shown to regulate T-cell apoptosis in human colorectal cancer (CRC) [64]. Exosomes from OVA-specific $CD8^+$ T cells have been observed to be recruited by DCs through LFA-1-ICAM-1 interaction, leading to down-regulation of OVA MHC-peptide expression, induction of apoptosis in OVA-loaded DCs, leading to inhibition of OVA-specific $CD8^+$ CTL responses in murine cancer models [119]. T-cell activation and fate can therefore be regulated by tumour-derived exosomes that facilitate evasion of the immune response and tumour progression.

Liu et al., [120] showed that pre-treatment of mice with exosomes derived from TS/A or 4T.1 murine tumour cells, led to enhanced tumour cell growth in syngeneic and nude mice. Additionally, in response to tumour-derived exosomes, expression of NK cell cytotoxic molecules was diminished and IL-2 induced NK cell proliferation was repressed, contributing to tumourigenesis. Ashiru et al., [68] observed truncated MHC class I-related chain (MIC) A (allele MICA*008) in exosomes. The authors showed that exosomes comprising truncated MICA*008 induced down-regulation of its ligand NKG2D leading to decreased NK cytotoxicity. MICA and MICB are crucial for the induction of the NK activating receptor NKG2D, with expression of these molecules in sera associated with compromised immune response facilitating tumour escape from immune surveillance. Clayton and colleagues reported that exosomes isolated from various cancer cell lines and pleural effusions from

patients with mesothelioma, mediated attenuation of NKG2D expression by NK cells and $CD8^+$ T cells [69]. Induction of TGF-β by exosomes was also shown to mediate down-regulation of NKG2D. Hedlund et al., [121] showed that exosomes bearing NKG2D ligands were secreted by the human placenta, possibly preventing immune targeting of the foetus. These exosomes decreased the surface expression of NKG2D receptor on NK cells and $CD8^+$ T cells in peripheral blood mononuclear cells (PBMC) from healthy donors. Further, Admyre et al., [122] showed that exosome-like vesicles repressed IL-2 and IFN-γ production in PBMCs. Together, these studies highlight exosome-mediated suppression of NK cells as an important aspect during immune evasion and neoplastic progression.

Recently, it has been suggested that cooperation between MDSCs and tumour-derived exosomes from various murine cell lines promotes MDSC-mediated repression of T cells. Chalmin et al., [7] showed that the *in vivo* anti-tumour efficacy of the chemotherapeutic drug cyclophosphamide in various murine models was consistent with the involvement of exosomal Hsp72 in potentiating MDSC activity. Xiang et al. [123] reported that murine TS/A and 4T-1 breast tumour cell-derived exosomes induced MDSC morphology and activity in bone marrow myeloid cells (BMMCs), with a concomitant increase in tumour development and growth. In addition, TGF-β and prostaglandin E2 (PGE2) were found to be crucial for tumour exosomes to mediate neoplastic progression through MDSCs. Liu and colleagues showed that MyD88, a cytoplasmic adaptor molecule that is crucial for the propagation and integration of signals generated by the TLR family, is important for exosomes derived from metastatic lung tumour cells in promoting IL-6, TNF-α, and chemokine CCL2 production and induction of MDSCs [124]. Treatment of MyD88 KO mice with such exosomes attenuated induction of IL-6 and chemokine CCL2, and enhanced $CD8^+$ T cell cytotoxicity, demonstrating an important role for exosome-mediated expansion of MDSCs and tumour

metastasis. Taken together, these studies highlight the various strategies employed by tumour-derived exosomes in suppressing tumour immune responses.

2.4 Opsonins and complement regulating factors as a means of immune surveillance (Immunorecognition)

Immunorecognition is a fundamental process, important for identification and targeting of foreign elements that influence the extracellular environment. The complement system is composed of abundant proteins that react with each other to opsonize pathogens and induce a series of inflammatory responses [125]. Clayton and colleagues investigated the modulation of CD55 and CD59, two glycophosphatidylinositol (GPI)-anchored complement regulator proteins that prevent formation of the complement membrane attacking complex, on exosomes derived from APCs [126]. Exosome lysis was enhanced by CD55 and CD59 blocking antibodies, demonstrating the role of CD55 and CD59 on exosomes for their stability in the extracellular environment. Enrichment of cell surface CD9 and MHC class II-peptide complex was also found in DC-derived exosomes [44]. The transmembrane tetraspanin CD9 was suggested to facilitate direct membrane fusion, circumventing the endosome-to-lysosome fusion [127]. In addition, Morelli and colleagues demonstrated that exosomes are targeted, internalised and processed by recipient splenic DCs *in vivo* [103]. Ligand-mediated targeting of exosomes towards DCs resulted in their internalisation and antigen presentation to $CD4^+$ T cells. The targeting of exosomes to recipient DCs is postulated to involve a suite of cell surface and membrane proteins including milk fat globule EGF factor VII (MFGE8), CD11a, CD54, CD9, and CD81 [44,103].

Recently, the role of exosomes in presenting opsonins has been demonstrated (**Figure 1E**). Opsonins are characterised as molecules that target antigens or pathogens for phagocytosis, or destruction through the action of NK cells [125]. A recent study showed that exosomes, derived from immature DCs, attenuated the inflammatory response by opsonising apoptotic cells for phagocytosis [128]. The authors further observed that MFGE8 protein that was released by immature DCs was crucial for efficient apoptotic cell clearance through *in vivo* experimental sepsis models. Mice with MFGE8 deficiency showed diminished survival whereas administration of both immature DC-derived exosomes and treatment with recombinant MFGE8 enhanced their survival. The authors suggested that opsonisation of apoptotic cells induced by exosomes resulted in a reduced systemic inflammatory response during sepsis.

3 Exosomes, immune regulation and the tumour microenvironment

The ability to evade immune recognition, suppress immune reactivity, and permit a chronic inflammatory response is crucial during the progression of cancer [129,130]. A significant complication is that there is no clear association between the presence of any individual adaptive or innate immune cell type and a defined outcome in terms of malignancy or prognosis across a range of different tumours [4,5,127]. Tumour-derived exosomes promote tumour growth by inhibiting anti-tumour immune responses and stimulating tumour proliferation and metastasis. The immune system functions by a coordinated response of many cell types that exchange information through complex communication networks. Various studies have shown that exosomes are composed of mRNAs, miRs, DNAs, proteins and lipid components that act on target cells [17]. This exchange between immune cells and other cell types is possibly achieved by packaging RNAs and DNAs (including single- and

double-stranded) into exosomes and selectively targeted and internalised by specific cell surface motifs [13,23,131]. The delivered RNA molecules are proposed to be functional, and mRNAs can be translated, while miRs target host mRNAs to modulate translation in the recipient cell [22,59].

Tumour-derived exosomes are important in conferring intercellular signals to these various immune cell types to modulate immunosurveillance in the tumour microenvironment [132,133]. Various cancer cell types are described in releasing exosomes capable of inducing apoptosis in activated T cells by the transfer of FasL and TRAIL [34,134]. miRNA transported by tumour exosomes may act like ligands by binding to Toll-like receptors and trigger an inflammatory response. In fact, oncogene miR-21 and -29a secreted from lung cancer cell-derived exosomes were shown to bind TLRs to murine (TLR7) and human (TLR8), leading to TLR-mediated NF-κB activation and secretion of pro-metastatic inflammatory cytokines TNF-α and IL-6 [135]. Both TLR7 and TLR8 bind to and are activated by 20-nt-long ssRNAs, which represent physiological ligands for these receptors [136], located in intracellular endosomes. Further, exosomes derived from human colorectal and melanoma cells impair the differentiation of peripheral blood monocytes to functional dendritic cells, instead transforming them towards MDSCs [137]. This mechanism of tumour-immune system communication is important in understanding regulators of the tumour microenvironment.

Various exogenous factors associated with the tumour microenvironment regulate the biological function of exosomes and their role in immunity. These factors include heat treatment, adjuvant components and neoplastic microenvironment pressure. Cho et al., [138]

demonstrated that tumour-derived exosomes enriched in Hsp70 were capable of enhancing tumour immunogenicity in comparison to tumour-derived exosomes that were independent of external stimulus. Hsp70-enriched exosomes stimulated Th1-immune responses, characterised by elevated production of IFN-γ and IgG2a in murine models. Moreover, exosomes derived from activated $CD8^+$ T cells have been shown to express bioactive FasL, Fas and APO2 ligands to promote activation-induced cell death which may be required for an immune response [91,139]. FasL-expressing exosomes were shown to activate ERK and NF-κB in B16 murine melanoma cells, leading to increased matrix metalloproteinase 9 (MMP-9) expression and invasive ability of Fas-resistant B16 tumour cells as shown by increased lung metastasis, which may provide a novel view for understanding tumour escape from the immune system [140].

Dai et al., [110] reported that exosomes derived from heat-stressed carcinoembryonic antigen (CEA)–positive tumour cells (CEA+/HS) have enhanced immunogenicity. In transgenic mice, CEA+/HS exosomes contained more HSP70 and MHC-I, induced DC maturation, and enhanced immune response (both *in vivo* in HLA-A2 transgenic mice, and CEA-specific CTL response *in vitro*). Further, Adams and colleagues utilised tumour exosomes in conjunction with TLR3 agonist and chemotherapy in advanced ovarian cancer patients [141] to induce an effective antigen-specific T cell immune response. Collectively, these and other such studies emphasize the role of tumour-derived exosomes in enhancing tumour immunogenicity [142]. Exosomes have also further been shown to induce anti-tumour immunity in the absence of adjuvants or heat treatment. Graner et al. [143] demonstrated that exosomes derived from glioblastoma cells induced protective immunity and anti-tumour immune responses in syngeneic mice. In addition, proteomic assessment of exosomes from glioma-derived patient sera revealed enrichment of EGFRvIII and TGF-β in comparison to normal, patient-matched

sera. This study highlights brain tumour-derived exosomes are capable of inducing effector immune responses, allowing exosomes to escape the blood-brain-barrier to elicit potential immune response in the recipient.

Summary & Future Perspectives

Exosomes function as a fundamental mode of intercellular communication and molecular transfer. Of key interest, tumour cells secrete exosomes that can both inhibit tumour growth by eliciting anti-tumour immune responses [49] and promote tumour growth by inhibiting anti- tumour immunity or enhancing angiogenesis and/or metastases [52,144]. Tumour- and DC-derived exosomes have been shown to carry tumour antigens and promote immunity [145], leading to the eradication of established tumours [146] by $CD8^+$ T cells [102] and $CD4^+$ T cells [147], as well as having the capacity to directly suppress tumour growth and increase resistance to malignant tumour development [148]. These roles are most likely important during the immune surveillance of early tumour development. During the later stages of tumour development, immune surveillance in patients could be less efficient or actively suppressed by various tumour-derived mechanisms including tumour exosomes. There is increasing evidence indicating that exosomes contribute and support cancer progression by transporting oncoproteins/mRNAs/miRs/DNAs and immune suppressive molecules to modulate a pro- tumourigenic phenotype. Understanding recipient cell function and regulation by exosomes will certainly focus on specific mechanisms of targeting and delivery, uptake and transfer, including modulation of key signalling pathways in various recipient cells both *in vitro* and *in vivo*. The recent impact of tumour exosomes on pre-metastatic organ and tissue colonisation, highlight the importance of exosomes as a promising therapeutic avenue. Indeed, by regulating the availability of circulating tumour exosomes through pharmacological intervention, it could be speculated that multiple

functions of cancer immunity could be simultaneously recovered or amenable to control. Further, the intrinsic ability of exosomes to traverse biological barriers and to naturally transport functional small RNAs/DNAs between cells, represents an exciting delivery vehicle for the field of translational therapy. The importance of tumour-derived exosomes in tumour progression has further been highlighted by their ability to sequester tumour-reactive Abs, inhibiting anti-tumour cytotoxicity [149] and reducing effectiveness of Ab-based anti-cancer drug intervention [150]. As tumour-specific markers, tumour-derived exosomes could be excellent systemic candidate biomarkers, containing an abundance of components involved in cell-cell communication and membrane exchange, tumour antigens, and cell-specific markers, or otherwise as evaluating responses to therapy [151-153]. Finally, coupling exosomes and other types of EV with nanotechnology will most likely form the basis where novel nanoscale cancer vaccines will be developed in the near future [142,154].

The propensity of exosomes to direct, promote or suppress immune activity is contingent upon the host cells, state of the host cells, recipient cells and the microenvironment these interactions take place in. The fate of (intravenously) injected EVs is still under discussion. It has been described that the expression of integrins, adhesion molecules, lipids, and other molecules on EVs con- tribute to the attachment and fusion of the injected vesicles to "acceptor" cells [8,103,155-157]. As a first step towards defining a context-dependent exosomal response, an in-depth biophysical characterisation of exosomes and appropriate biological responses are crucial. Different EV types from different cellular origins, or even the activation state of the cell-derived vesicle population, will produce a specific response on target cells. Recently, the regulatory effect of exosomes (and other EVs) from different cell origins in the immune system has been extensively reviewed [11]. Further, the transfer of molecules between cells during cognate immune cell interactions has been reported, and

recently a novel mechanism of transfer of proteins and small RNA between T cells and APCs has been described, involving exchange of exosomes during the formation of the immunological synapse [158]. However, their regulatory effects *in vivo* remain largely unknown, especially in humans.

Nonetheless, the prospect of enlisting exosomes as immuno-therapeutic agents remains an attractive one, with the advent of vesicle–mediated therapeutic platforms that present an efficient and targeted mode of delivery [159]. In addition to exhibiting distinct advantages over a cell based approach [160], vesicle therapeutics also features the ability to cross tissue barriers (e.g blood brain barrier) and exhibit non-tumourigenic potential [161]. Given these various potential outcomes, it is tempting to speculate that vesicle-based therapy may be crucial during tolerance induction in particular in organ transplantation. This review has focused on the importance of exosomes in immunoregulation, mechanisms including modulating antigen presentation, immune activation, immune suppression, immune surveillance, and intercellular communication throughout the extracellular environment. Further understanding of these areas of exosome biology, and especially of molecular mechanisms involved in immune cell interactions, is likely to provide significant insights into the phenomenon of tumour-related immune suppression and therapeutic intervention, including immunorecognition during malignancy.

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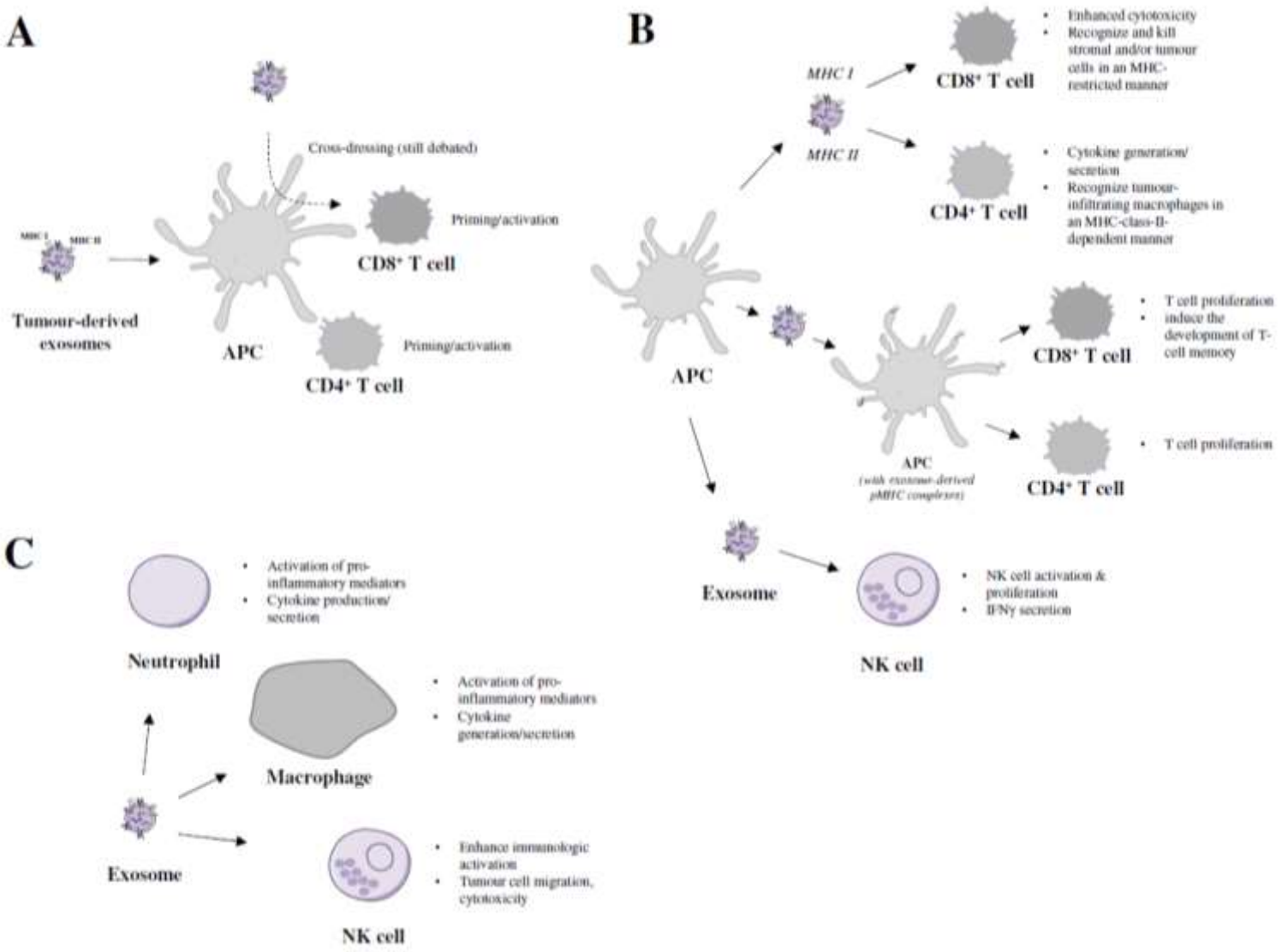
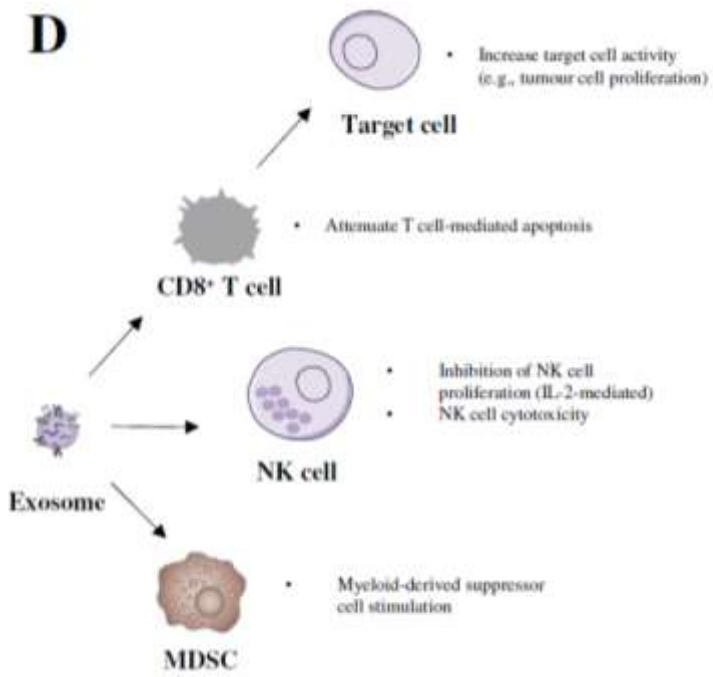
Figure legend

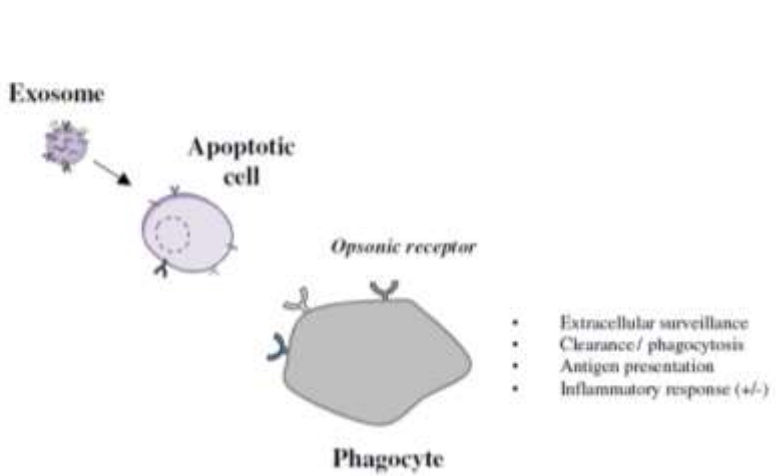
Figure 1. The role of exosomes in modulating the immune response: (A) Tumour-derived exosomes internalised by or fused with antigen-presenting cells (APCs) have been shown to prime $CD8^+$ and $CD4^+$ T cells. These exosomes express tumour antigens (e.g., as MHC class I- and class II-peptide complexes and as intra-exosomal and membrane-bound antigens). These exosomes also express some receptor ligands and adhesion molecules (e.g., LFA1, MFGE8, TIM1/4, tetraspanins) participating either directly or indirectly in APC binding. In addition, antigen cross-presentation through MHC-peptide complex "cross-dressing" APCs have been shown to be potentially mediated by exosomes to induce T cell activation. It is however still debated. (B) APC-derived exosomes can directly modulate the antigen-specific response of $CD8^+$ and $CD4^+$ T cells and activation of NK cells. DC-derived exosomes, when used as potential vaccines, have been demonstrated to transfer functional MHC class I- and class II-peptide complexes to host DCs to prime $CD8^+$ and $CD4^+$ T cells, respectively. These exosomes also exhibit comparable efficacy as mature DCs to stimulate antigen-specific T cell activation *in vivo*. Exosomes therefore are vehicles for MHC-peptide complexes (pMHC) and that require co-stimulatory molecules expressed by recipient DCs to stimulate T cells. Exosomes derived from APCs loaded with specific peptides and/ or antigens are also capable of inducing an immune response, including NKG2D ligand, IL-15/IL-15Rα-mediated activation of NK cells. (C) Exosomes have been demonstrated to have more general immune

stimulatory roles, including activation of pro-inflammatory mediators (TNF-α, RANTES), cytokine/chemokine generation/secretion (CCL2), and enhancing immune activation through Hsp70. (D) Exosomes repress immune responses through different mechanisms including attenuation of T cell-mediated killing (exosomes enriched for CD95L, TRAIL or galectin 9, which promotes T cell apoptosis), NK cell cytotoxicity (exosomes suppress CD3 ζ-chain expression by T cells, blocking NKG2D-dependent cytotoxicity of NK cells and $CD8^+$ T cells), and activation of myeloid-derived suppressor cells (MDSCs) (exosomes contain PGE2, TGF-β, HSP72 in tumour-derived vesicles). These mechanisms facilitate immune escape, and tumour invasion and metastasis. (E) Exosomes carrying and transferring opsonins and complement factors enhance extracellular surveillance, apoptotic cell phagocytosis/clearance, antigen presentation, and modulating the inflammatory response. CD55 and CD59 on exosomes derived from immature DCs, have been shown to attenuate the inflammatory response by opsonising apoptotic cells (through opsonic receptors, e.g., iC3b) for phagocytosis. Further, targeting of circulating exosomes to DCs has been shown mediated by MFG-E8/lactadherin, CD9, CD11a, CD54, CD81, and phosphatidylserine on exosomes, and CD11a and CD54 on DCs.

Figure 1


A
Cross-dressing (still debated)
Priming/activation
CD8⁺ T cell
Tumour-derived exosomes
APC
Priming/activation
CD4⁺ T cell
B
MHC I
MHC II
CD8⁺ T cell
Enhanced cytotoxicity
Recognize and kill stromal and/or tumour cells in an MHC-restricted manner
CD4⁺ T cell
Cytokine generation/ secretion
Recognize tumour-infiltrating macrophages in an MHC-class-II-dependent manner
APC
CD8⁺ T cell
T cell proliferation
induce the development of T-cell memory
APC (with exosome-derived pMHC complexes)
CD4⁺ T cell
T cell proliferation
Exosome
NK cell activation & proliferation
IFNγ secretion
NK cell
C
Activation of pro-inflammatory mediators
Cytokine production/ secretion
Neutrophil
Activation of pro-inflammatory mediators
Cytokine generation/secretion
Macrophage
Exosome
Enhance immunologic activation
Tumour cell migration, cytotoxicity
NK cell


D
Increase target cell activity
(e.g., tumour cell proliferation)
Target cell
Attenuate T cell-mediated apoptosis
CD8+ T cell
Inhibition of NK cell
proliferation (IL-2-mediated)
NK cell cytotoxicity
NK cell
Exosome
Myeloid-derived suppressor
cell stimulation
MDSC


E
Exosome
Apoptotic
cell
Opsonic receptor
Extracellular surveillance
Clearance / phagocytosis
Antigen presentation
Inflammatory response (+/-)
Phagocyte

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