ANNUAL REVIEWS

Annual Review of Immunology

Antigen Presentation by Extracellular Vesicles from Professional Antigen-Presenting Cells

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Annu. Rev. Immunol. 2018. 36:16.1–16.25

The *Annual Review of Immunology* is online at immunol.annualreviews.org

https://doi.org/10.1146/annurev-immunol-041015-055700

Keywords

exosomes, microvesicles, MHC molecules, dendritic cells, B cells, T cells

Abstract

The initiation and maintenance of adaptive immunity require multifaceted modes of communication between different types of immune cells, including direct intercellular contact, secreted soluble signaling molecules, and extracellular vesicles (EVs). EVs can be formed as microvesicles directly pinched off from the plasma membrane or as exosomes secreted by multivesicular endosomes. Membrane receptors guide EVs to specific target cells, allowing directional transfer of specific and complex signaling cues. EVs are released by most, if not all, immune cells. Depending on the type and status of their originating cell, EVs may facilitate the initiation, expansion, maintenance, or silencing of adaptive immune responses. This review focusses on EVs from professional antigen-presenting cells, their demonstrated and speculated roles, and their potential for cancer immunotherapy.

INTRODUCTION

Adaptive immunity is required when innate immunity alone is insufficient to eliminate invading pathogens or to eradicate pathogen-infected or neoplastic cells. At the same time, healthy cells, commensal bacteria, and other nonhazardous environmental compounds should be tolerated. Discrimination between good and bad requires a high level of selectivity in antigen determination, which is dictated by diverse layers of communication between different immune cells. Communication mechanisms include direct cell-to-cell contact, as exemplified by the formation of immune synapses between antigen-presenting cells (APCs) and T cells. Within the immune synapse, major histocompatibility complex (MHC) molecules that are loaded with peptide antigen (pMHC) may bind to cognate antigen-specific T cell receptors (TCRs). Depending on local availability of costimulatory membrane proteins and cytokines, antigen-specific pMHC-TCR interactions may then either stimulate or inhibit T cell proliferation and differentiation. A novel and perhaps even more complex mode of immune regulation is provided for by extracellular vesicles (EVs) that are released by dendritic cells (DCs), B cells, T cells, and other cell types that function within the adaptive immune system. EVs are released by most, if not all, cell types, and depending on their origin they display a plethora of functions (1). First, it was reported in 1996 that human B cell–derived EVs could effectively present MHC class II (MHC-II) peptide complexes (pMHC-II) to CD4 T cells in vitro (2). Later, Théry and coworkers (3) found that injecting mice with pMHC-II-bearing EVs also induced in vivo effects, resulting in the activation of antigen-specific naive CD4 T cells. Zitvogel and coworkers (4) demonstrated that murine bone marrow–derived DCs secreted EVs carrying MHC-I, MHC-II, and T cell costimulatory molecules. When MHC-I molecules on EVs from cultured DCs were loaded with tumor-specific peptides, they could prime tumor-specific cytotoxic T lymphocytes (CTLs) and suppress tumor growth in vivo. Together these three landmark studies have set the stage for many researchers to investigate the role and possible applications of EVs in immunotherapy. Realization that EVs have complex functions in adaptive immunity was further reinforced by observations that selective sets of RNA, including miRNA, are present in EVs from immune cells (5, 6). However, it should be noted that many miRNAs that are found extracellularly are not incorporated in EVs and that probably only a restricted subset of EVs contains biologically significant amounts of miRNA (7). During the last two decades, and after some initial skepticism, it has been increasingly recognized that EVs play a role not only in antigen presentation but also at other levels of communication between immune cells, as illustrated by the >1,000 published research articles on the subject. Although the importance of EVs in antigen presentation is now generally acknowledged and some mechanistic aspects of antigen presentation by EVs have been disclosed, many regulatory functions of EVs and their discriminatory roles in adaptive immunity remain to be resolved.

EVs comprise at least two classes: microvesicles, which bud directly from the plasma membrane, and exosomes, which are secreted as a consequence of the fusion of multivesicular endosomes (MVEs) with the plasma membrane (**Figure 1**). Exosomes have diameters ranging 30–150 nm and are on average smaller than microvesicles (100 nm to >1 μm), but their overlapping size distributions and the absence of discriminatory molecular markers often hinder the assignment of isolated EVs to one of these classes in experimental settings (1, 8, 9). Furthermore, the precise molecular machineries that drive EV formation or incorporation of their contents have not been fully resolved (10). As a consequence, studies on the distinctive physiological roles of exosomes and microvesicles are hampered by limited availability of molecular intervention methods. Nevertheless, great progress has been made in recent years. In this review, we use the term EV unless the classification of microvesicle or exosome is evident. We do not aim to extensively evaluate current knowledge on generic mechanisms for molecular sorting into EVs or EV release,

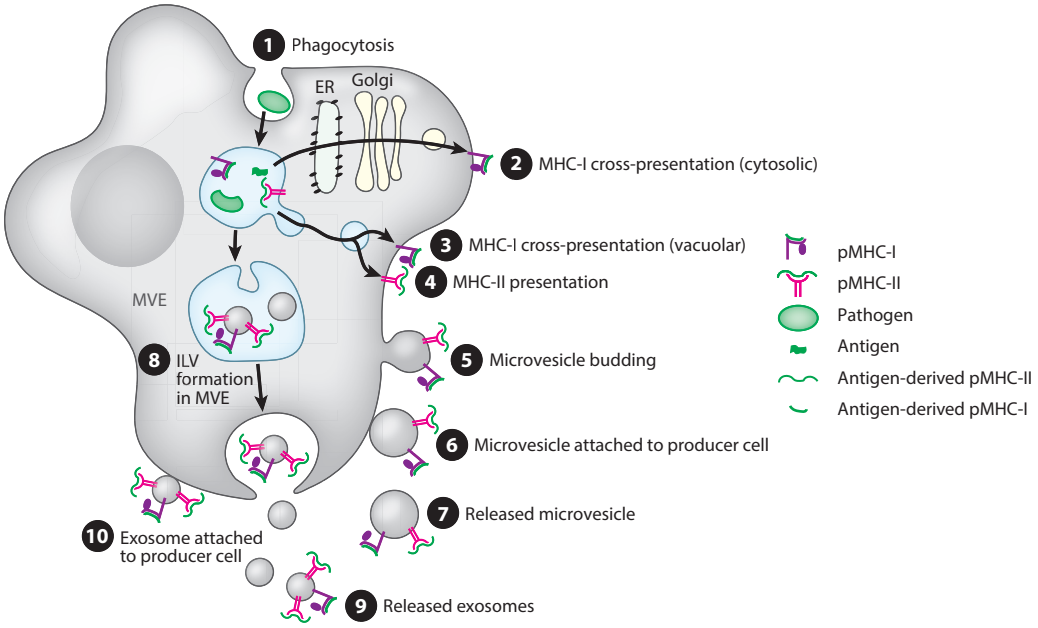


Figure 1

MHC-I and MHC-II pathways in DCs. ❶ Phagocytosis and receptor-mediated endocytosis of extracellular material. ❷ Cytosolic pathway for pMHC-I cross-presentation at the plasma membrane. ❸ Vacuolar pathway for peptide–MHC-I cross-presentation at the plasma membrane. ❹ pMHC-II complex formation in endosomes and transfer to the plasma membrane. ❺ Microvesicles carrying peptide–MHC-I and peptide–MHC-II complexes budding from the plasma membrane. ❻ After budding, microvesicles may remain attached or rebind to the plasma membrane. ❼ Alternatively, microvesicles are released in the extracellular milieu. ❽ ILVs carrying peptide–MHC-I and peptide–MHC-II complexes are formed within MVEs. ❾ Exosomes carrying peptide–MHC-I and peptide–MHC-II complexes are released upon fusion of MVEs with the plasma membrane. ❿ Alternatively, exosomes may remain attached or rebind to the plasma membrane after being released by MVEs. Abbreviations: DC, dendritic cell; ER, endoplasmic reticulum; ILV, intraluminal vesicle; MVE, multivesicular endosome.

as these have been reviewed elsewhere (1, 11). Instead we expand on excellent previous reviews (12, 13), focusing on currently available data on the role of EVs in antigen presentation by professional APCs, and finish by discussing the potential of EVs isolated from DCs in immunotherapy against cancer.

INTERCELLULAR TRANSFER OF MHC MOLECULES

The ability of T cells to acquire immune-relevant membrane proteins from APCs, including MHC molecules and costimulatory molecules, both in vitro and in vivo, was reported in the early 1980s (14–16). The transfer of intact pMHC complexes from a donor cell to the cell surface of an acceptor cell is referred to as cross-dressing (17). In contrast to cross-presentation, cross-dressing does not involve loading of acquired antigen onto MHC molecules of the acceptor cell but rather the acquisition of entire pMHC complexes. For example, DCs can acquire pMHC-I complexes from dead or apoptotic tumor cells and present these to naive CD8 T cells, supplementing the cross-presentation pathways (18). Similarly, DCs that were cross-dressed with pMHC-I complexes

from virus-infected cells effectively activated previously primed but not naive CD8 T cells, both in vitro and in vivo (19). However, cross-dressing also occurs between DCs, as clearly demonstrated by in vitro experiments in which mouse DCs acquired substantial amounts of pMHC-I and pMHC-II complexes from cocultured allogeneic DCs (3, 20). Although cross-dressing refers exclusively to the acquisition of pMHC complexes, other membrane-associated molecules can also be transferred in the process (3, 21, 22).

One mechanism for transfer has been proposed to involve transient fusions between plasma membrane extrusions of neighboring cells (23, 24). These so-called tunneling nanotubes (TNTs) are continuous plasma membrane bridges, with lengths of up to several cell diameters and thicknesses ranging between 50 nm and 0.5 μm. Depending on the connecting cell types, membrane extensions of opposing cells may either just stick together or be truly fused, connecting the cytosolic compartments of the cells (23, 25). Based on the observation of transfer of fluorescence-tagged MHC-I between HeLa cells, it was proposed that such plasma membrane bridges are important for exchange of MHC-I between cells (26). In a later study, TNTs were shown to develop in a CD40L-dependent manner between human mature DCs, as well as between DCs and CD4 T cells (27). The authors who reported this study proposed a function of TNTs in MHC cross-dressing, which may indeed explain how nonmigratory lymph node–resident DCs can acquire pMHC molecules from migratory DCs (28, 29). In this scenario, integral membrane proteins such as MHC-I and MHC-II would be transferred by lateral diffusion along a continuous intercellular nanotubular membrane. Alternatively, transfer could occur by means of EVs that after their release remain associated with the plasma membrane of the donor cell and "surf" along the outside of the nanotube to an adjacent acceptor cell. Such a process would be analogous to the transport of membrane viruses such as influenza, which also surf along the exoplasmic side of TNTs for spreading to neighboring cells (30, 31). Indeed, this could explain the punctate rather than diffuse pattern of cross-dressed MHC on acceptor cells, as observed using fluorescence microscopy (26).

An alternative mechanism for cross-dressing is trogocytosis, a process in which cells "gnaw" parts of the plasma membrane from adjacent cells (32). For example, trogocytosis was described for basophils acquiring pMHC-II complexes from cocultured DCs, enabling them to stimulate peptide-specific CD4 T cells (33). Separation of these two cell types by porous filters intervened with transfer; hence, it was concluded that direct cell-cell contact was required. The alternative explanation, however, that EVs were released as a consequence of cell-cell contact and subsequently transferred to the acceptor cell, has not been ruled out. Indeed, many cell types release EVs that carry immune regulatory molecules in response to immune synapsis (5, 8, 34–37), and in some occasions EVs have been demonstrated to be captured directly from the immunological synapse (36, 38). EVs that are released into the immune synapse may constitute either plasma membrane–derived microvesicles (37) or exosomes that are delivered by polarized fusion of multivesicular endosomes with the plasma membrane at the synapse domain (5, 39). This pathway is consistent with, and parallels, targeted secretory delivery of cytokines into the immune synapse (40). Alternatively, EVs may be released outside the immune synapse, as suggested by increased release of EVs into the culture medium of APCs upon stimulation by cognately interacting T cells (34, 41). In conclusion, transcellular acquisition of membrane (proteins) involves distinct mechanisms that may act in a complementary manner.

EXTRACELLULAR VESICLES FROM DENDRITIC CELLS

Mouse bone marrow–derived DCs (BMDCs), human primary monocyte-derived DCs (moDCs), and DC cell lines have all been reported to release EVs (3, 4, 34, 42). In a recent study, moDC-derived EVs were described to be very heterogeneous in size, with diameters ranging from 50 nm

to > 400 nm, and they could be fractionated in populations with different but overlapping protein compositions (9). MHC molecules and proteins classically described as exosome markers, such as flotillin 1 and 70-kDa heat shock proteins, were found to be equally present in all isolated EV subclasses, while many other proteins were more selectively enriched to particular subclasses of isolated EVs. The authors who published the study identified exosomes by the high enrichment of the endosomal markers CD63, CD81, syntenin 1 and TSG101, and their relative homogeneous, small size of 50–150 nm. Plasma membrane–derived microvesicles, on the other hand, appeared to contain little if any syntenin 1 and TSG101 and to vary in diameter from 50 nm to >500 nm. Contributions of exosomes are perhaps most convincingly demonstrated by static immuno–electron microscopy images of human and mouse DCs, showing MVE–plasma membrane fusion profiles releasing 60- to 100-nm exosomes carrying MHC molecules and exosome-characteristic tetraspanin molecules such as CD63, CD81, and CD9 (4, 34).

Both immature and activated DCs release EVs, but their number and cargo composition are dictated by the status of the DC and interactions with T cells. For example, EVs from mature DCs are relatively enriched for CD86 and intercellular adhesion molecule 1 (ICAM-1), whereas EVs from immature DCs carry more milk fat globule–epidermal growth factor–factor VIII (MFG-E8) (43). These differences are partly due to changing protein expression profiles, but also to changing subcellular protein distributions during DC maturation. For instance, MHC-II is redistributed from endosomes to the plasma membrane during DC maturation (44), and its relative abundances in exosomes versus microvesicles can thus be expected to shift accordingly. Moreover, the relative contributions of exosomes and microvesicles to the total EV population are likely to change during DC maturation (9). In response to cognate interactions with CD4 T cells, activated DCs develop a mechanism by which MHC-II is sorted independently of β-chain ubiquitination to the intraluminal vesicles of a distinct subclass of MVEs, to be secreted in association with CD9-carrying exosomes (34). Consistent with this observation, sorting of MHC-II in MVEs to exosomes appears to rely on only a few components of the endosomal sorting complexes required for transport (ESCRT) machinery, including TSG101 (45, 46). The ESCRT mechanism is also required for formation and pinching off of microvesicles at the plasma membrane, however, and intervention with this complex can therefore not be used to discriminate exosomes from microvesicles (13, 39, 46). Exosome secretion was more specifically inhibited by knocking down the GTPases Rab27a and Rab27b (47). Interestingly, the release of EV-associated MHC-II by immature DCs from Rab27a/Rab27b double knock-out mice was hardly affected (47), and this may indicate that the majority of EV-associated MHC-II from immature DCs is associated with plasma membrane–derived microvesicles rather than with exosomes. In contrast to immature DCs, DCs that were activated by lipopolysaccharide (48–50) or through cognate interactions with CD4 T cells (34) released significantly more EVs compared to immature DCs, and these EVs have the characteristics of exosomes (34). Together, these types of experiments are indicative of two distinct MVE pathways, one for lysosomal targeting and the other for exosome secretion, with the first being particularly dominant in immature DCs and the second in activated DCs. Activated DCs, however, also release microvesicles, as exemplified by the release of IL1-β-containing microvesicles by lipopolysaccharide-activated DCs, in response to stimulation of the P2X7 receptor by extracellular ATP (51).

DC-derived EVs are reported to have either immune-inhibitory or -stimulatory functions, depending on the status of the donor DC (discussed in detail below). A complication of the molecular and functional analyses of DC-derived EVs is that it is extremely difficult, if not impossible, to obtain EVs from populations exclusively comprising only immature DCs or only activated DCs. Cultures of nonstimulated DCs always contain some activated DCs, as determined by the expression of activation markers. Conversely, DC stimulation with Toll-like receptor (TLR) ligands

usually results in asynchronous and incomplete activation of cells within the population. It is thus very likely that EVs isolated from either immature DCs or activated DC cultures are always contaminated to some extent by EVs from DCs with the opposite status. Therefore, it should be kept in mind that such contaminating EV types, even when present in relatively low quantities, may significantly influence functionality tests.

Human blood has been estimated to contain $\sim 10^5$ EVs per microliter, and most of these derive from platelets or erythrocytes (52). Multiple EV markers can be detected in EVs isolated from blood, but the concentration of MHC molecules is very low (53) (X. Zhang & W. Stoorvogel, unpublished data), suggesting either that APCs release relatively low amounts of EVs into circulation or that EVs are rapidly removed by other cells after release. When EVs isolated from DCs or B cells were injected into mice, they were able to interact with cells far away from the injection site (54, 55). Nevertheless, it is likely that most EVs secreted in situ by APCs interact with nearby cells, immediately after their release. This idea is supported by the demonstration that migratory DCs home to lymphoid organs, where locally released EVs can be efficiently recruited by interacting or nearby lymph node–resident DCs (28, 56). In contrast, EVs that were isolated from cultured DCs and introduced into the circulation were inefficient in stimulating T cell responses in vivo (56), supporting the notion that efficient immune responses to EVs from migratory DCs require local secretion in lymphoid tissues.

ANTIGEN PRESENTATION BY MHC-I AND THE ROLE OF DENDRITIC CELL–DERIVED EXTRACELLULAR VESICLES

MHC-I molecules function on all nucleated cells to bind and display protein-derived peptides to CD8 T cells. When cells are infected or transformed, a fraction of their MHC-I molecules will display foreign peptides, and recognition of such complexes by CD8 CTLs via peptide-specific cognate TCRs results in selective killing of these infected or transformed cells. The majority of peptides that bind to MHC-I are generated from proteins that are endogenously expressed in the cytosol. Cytosolic proteins can be degraded by the proteasome and the resulting peptides trimmed by aminopeptidases. After being translocated from the cytosol into the endoplasmic reticulum (ER) by a transporter associated with antigen processing (TAP), peptides can be loaded onto newly synthesized MHC-I molecules. The resulting pMHC-I complexes are then transported along the secretory pathway toward the plasma membrane for display to CD8 T cells. When cells are infected with viruses or bacteria, or transformed by mutation, their MHC-I molecules will present, in addition to endogenous peptides, peptides from pathogen-encoded or tumor cell–specific proteins, thus enabling recognition and eradication by antigen-specific CTLs. To stimulate the generation of CTLs with specificities toward relevant antigens, cognate naive CD8 T cells need to be activated by DCs. DCs have the unique ability to present peptides derived from proteins of exogenous origin that are acquired by endocytic processes. This process of cross-presentation of exogenously acquired antigen involves both vacuolar and cytosolic pathways (steps 2 and 3 in **Figure 1**), and their relative contributions may differ between DC lineages (17, 57). In the cytosolic pathway, endocytosed proteins are translocated across the endosomal membrane into the cytosol for degradation by proteasomes. For loading onto MHC- I molecules the resulting peptides are transported by TAP, either into the lumen of the ER or, uniquely for DCs, into the lumen of endosomes or phagosomes. In the alternative vacuolar pathway, endocytosed proteins are directly degraded by lysosomal proteases to generate peptides that are loaded onto MHC-I in the endosomal or phagosomal lumen (17, 57–59). When resting DCs present antigens in the absence of costimulatory molecules, corresponding cognate CD8 T cells may become anergic (60). The contribution of DCs to the generation of self-tolerance has been termed cross-tolerance (61).

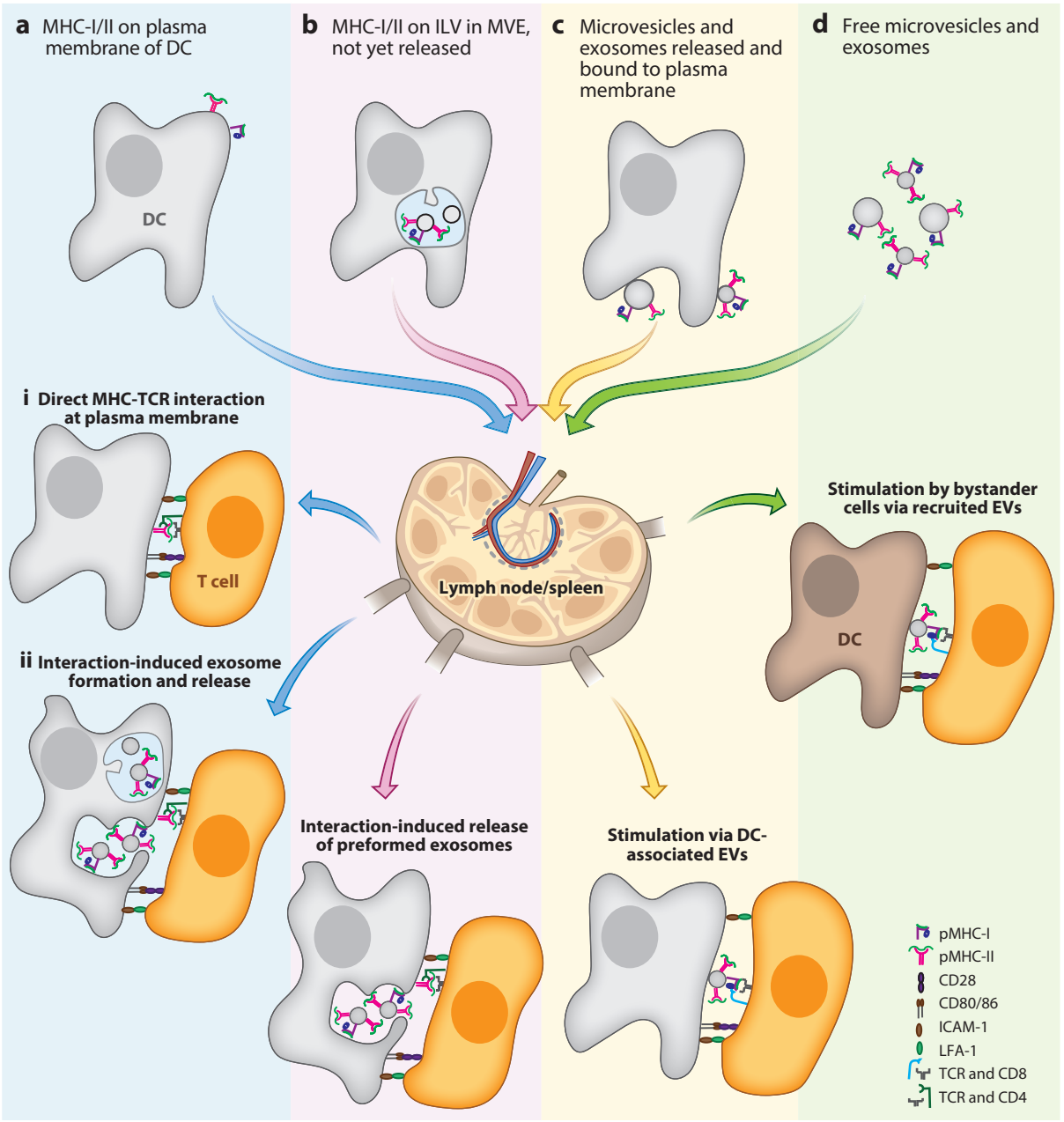
In the presence of danger signals, however, cross-presentation by activated DCs is considered to represent a major pathway for the activation of naive CD8 T cells (57). Thus, DCs are essential to initiate and control proper cellular adaptive immunity through selective activation of pathogen- or tumor-specific naive CD8 T cells.

In their early study, Zitvogel and colleagues observed that EVs isolated from cultured immature mouse DCs could prime CD8 T cell responses, both in vitro and in vivo (4). EVs isolated from activated human moDCs could stimulate peripheral CD8 T cells in vitro, independently of target DCs, demonstrating an intrinsic stimulatory capacity of EVs (62). However, it should be noted that in the absence of acceptor DCs, EVs may activate only primed T cells and not naive T cells. In vitro activation of mouse CD8 T cells by EVs carrying cognate pMHC-I complexes was greatly supported by bystander DCs. For example, the immunological efficacy of cross-dressing by EVs was shown in experiments where pMHC-I complexes transferred by EVs isolated from cultured human DCs could provide naive DCs with the capacity to efficiently prime melanoma-specific CD8 T cells in HLA-A2 transgenic mice (63). Cross-dressing of MHC-I by means of intercellular transfer of EVs can be expected to occur most effectively at sites with a high density of active immune cells, such as in the spleen and lymph nodes. Indeed, primary MHC-I-restricted CD8 T cell responses were most efficient when DC-derived EVs were transferred in vivo onto mature DCs in lymphoid tissues, in combination with CpG adjuvants (64). But the status of the EV-producing DC is also important, as EVs from mature DCs could more strongly stimulate CD8 T cell proliferation in vitro and in vivo than could EVs from immature DCs, and more efficiently induce antigen-specific CTL responses, CD8 T cell memory, and antitumor immunity (65, 66). In vivo mouse experiments demonstrated that transfer of pMHC-I complexes predominantly occurs in a unidirectional manner from migratory DCs toward lymph node– or spleen-resident DCs (28). Cross-dressed resident DCs were in fact more efficient in activating CD8 T cells than the pMHC-transferring migratory DCs (29), indicating a prominent role for cross-dressing. Together, these findings are consistent with the idea that activated DCs endocytose antigen in peripheral tissues, cross-present antigens onto MHC-I molecules, and migrate to lymphoid tissues, where they release EVs carrying pMHC complexes and costimulatory molecules for cross-dressing of interacting or nearby lymphoid tissue–resident DCs (**Figure 2*a–c***).

ANTIGEN PRESENTATION BY MHC-II AND THE ROLE OF DENDRITIC CELL–DERIVED EXTRACELLULAR VESICLES

In contrast to MHC-I, MHC-II is constitutively expressed only by professional APCs, including DCs, B cells and macrophages, and some epithelial cells. Newly synthesized MHC-II molecules are transported from the ER to endosomes with their peptide-binding groove occupied by the molecular chaperone invariant chain (CD74). Within endosomes, the invariant chain is degraded and the resulting class II–associated invariant chain peptide (CLIP) is subsequently displaced, allowing other peptides to bind to MHC-II. MHC-II-bound peptides mostly derive from proteins that are degraded by lysosomal proteases within the endocytic pathway. The originating proteins are either expressed by the APC itself and transported to endosomes and lysosomes via the vacuolar pathway or derived from endocytosed exogenous molecular complexes or apoptotic cells (fragments) (**Figure 1**). MHC-II-bound peptides can, however, also derive from proteins that are originally expressed in the cytosol, encoded by the APC itself or by an infectious pathogen, and have entered the lysosomal pathway through (micro)autophagy (67). In the absence of danger signals, pMHC-II complexes are ubiquitinated and as a consequence sorted by the ESCRT machinery to intraluminal vesicles of MVEs. When such MVEs fuse with lysosomes, their contents, including the pMHC-II-containing intraluminal vesicles, will be degraded (68). In activated DCs,

however, ubiquitination of MHC-II is abrogated, thus allowing efficient redistribution of newly formed pMHC-II complexes from endosomes to the plasma membrane (69). Surface-expressed pMHC-II can then be recognized by naive CD4 T cells that express antigen-specific TCRs (**Figure 2*a***). Similar to CD8 T cells, peptide-specific CD4 T cells will be activated only when proper costimulation is provided by the APC. Naive T cells that are triggered by cognate pMHC-II



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Transfer and interaction of DC-derived EVs with T cells. (*a*) Peripheral DC presenting pMHC complexes at its plasma membrane. (*a*, *i*) After traveling to a lymph node or the spleen, plasma membrane peptide-MHC is presented directly to T cells. (*a*, *ii*) DC–T cell interactions in lymphoid organs may induce the formation and release of EVs (only exosomes and not microvesicles are shown), which may then interact with the T cell or bystander cells. (*b*) Peripheral DC packing peptide-MHC complexes into multivesicular endosomes (MVEs), which release their preformed intraluminal vesicles (ILVs) as exosomes only after the DC reaches a lymph node or the spleen and interacts with a target cell. (*c*) A peripheral DC that has released exosomes and/or microvesicles that remained associated with the plasma membrane of the producing DC while traveling to a lymph node or the spleen. Here the membrane-associated EVs can either present their peptide-MHC complexes directly to T cells or be released and transferred to bystander DCs. (*d*) Free microvesicles that are released by peripheral DCs travel toward a lymph node or the spleen. Here they can activate T cells, either directly or after being recruited by bystander APCs.

complexes on nonactivated DCs in the absence of costimulatory signals may enter a state of unresponsiveness, or anergy (70). Alternatively, antigen presentation by immature (or resting) DCs may also induce the expansion and differentiation of CD4 T cells toward an immunosuppressive regulatory phenotype (Treg), to control peripheral T cell tolerance and autoimmunity (71). In this case, recognition and trans-endocytosis of DC-encoded costimulatory molecules CD80 and CD86 by CD4 T cell–encoded CTLA-4 plays a major role (21), a process that may involve intercellular transfer of these membrane molecules via EVs.

When stimulated by cognate pMHC-II in the presence of costimulatory signals, naive CD4 T cells will differentiate into CD4 T helper (Th) cells, which can provide assistance either in the cellular immune response (Th1) by stimulating the activation of macrophages or cytotoxic CD8 T cells, or in the humoral immune response (Th2) by activating B cell class switching. As described above, the outcome of an interaction between DCs and T cells carrying a cognate TCR depends on the activation status of the DC. DC recognition of conserved pathogen-associated molecular patterns or damage-associated molecular patterns induces the expression of costimulatory plasma membrane proteins such as CD80 and CD86 (the ligands of the T cell costimulatory receptor CD28) and CD70 (the ligand for the T cell costimulatory receptor CD27) as well as secretion of proinflammatory cytokines, but also (as discussed below) the release of immune regulatory EVs. Indeed, CD27 costimulation of CD8 T cells appears to be a key pathway of CD4 T cell help in CTL responses (72). Alternatively, pMHC-II- and pMHC-I-presenting DCs can be stimulated by cognately interacting, activated CD4 T cells to upregulate CD40 ligand, licensing the DCs to activate CD8 T cells carrying both CD40 and representative TCRs (73). While MHC-I and MHC-II present different sets of peptide antigens to activate CD8 and CD4 T cells, respectively, a shared source of peptides could thus ensure presentation by the same DC, helping the differentiation of effective CD8 T cell responses (74).

Transfer of MHC-II molecules between allogeneic DCs plays a major role in primary mixed leukocyte reactions (75), demonstrating the potency of MHC-II cross-dressing in antigen presentation. After allogeneic DCs were injected into acceptor mice, the majority of draining lymph node–resident DCs expressed donor MHC-II molecules, and vice versa, demonstrating bidirectional transfer of MHC-II molecules between migratory and resident DCs (76). In vitro and in vivo experiments demonstrated that EVs isolated from antigen-loaded DCs could stimulate both primed CD4 T cells and CD4 T cell clones (3, 42) but were less effective in stimulating naive T cells (3, 77, 78). Activation of naive CD4 T cells was dependent on the additional presence of mature bystander DCs, and costimulation by CD80 and CD86 on these DCs (3, 19, 43, 56, 63, 79), indicating that DC-derived EVs can amplify CD4 T cell responses through cross-dressing of DCs that have not encountered the antigen directly. In vivo studies confirmed that EVs from antigen-pulsed mature DCs were more effective in providing protection against infections than EVs from antigen-pulsed immature DCs (80, 81). Activated, antigen-loaded, migrating DCs

were shown to transfer EVs to spleen-resident DCs, which could then activate CD4 T cells (56) (**Figure 2*d***). EVs from activated DCs are highly enriched in ICAM-1, a ligand for the integrin LFA-1 on DCs and T cells, explaining why they are much more effective in priming T cell responses than EVs from immature DCs (42, 56, 77, 78). CD8 DCs in lymphoid tissue express higher levels of LFA-1 than peripheral $CD8^-$ DCs, facilitating their role as EV recipient in vitro and in vivo (78). DC-derived EVs carry complement (82) and therefore could potentially also be recruited by the complement receptor CD21 on follicular DCs (FDCs). This idea is supported by the observation, reported by the Gabrielsson laboratory, that CD8 T cell activation in response to immunization with EVs from whole protein antigen–loaded, but not from antigen peptide–loaded DCs, was entirely dependent on B cells and complement activation (83). These authors proposed that B cells may recruit EVs loaded with MHC-I- and MHC-II-antigen complexes from DCs to help their transfer to FDCs, facilitating both CD4 and CD8 T cell responses. Later, however, the same laboratory reported that similar immune responses could be obtained with EVs isolated from protein antigen loaded MHC-I knock out DCs (84), indicating independence of the presence of MHC-I on EVs in this setting, and that isolated EVs may also serve as adjuvants. Either way, DCs that were pulsed with intact protein antigen could also stimulate in vivo antigen-specific immunoglobulin responses, both in naive and primed recipients, demonstrating their capacity to induce humoral immunity (48).

In vivo studies have demonstrated that DC-derived EVs can induce humoral immune responses (81, 85). Others reported that injected DC-derived EVs were as effective as their originating antigen-pulsed DCs in inducing both protective humoral and cellular immune responses against the parasite *Toxoplasma gondii* (86, 87). That exosomes secreted in vivo also play a role is supported by a recent study in which *Mycobacterium tuberculosis*–infected DCs from Rab27a-deficient mice were found to be limited in their ability to present antigen to CD4 T cells, suggesting that Rab27a-dependent secretion of exosomes by these DCs is important to acquire adaptive immunity against tuberculosis (88).

Upon transfer to acceptor DCs, EVs may either remain associated with the plasma membrane or fuse with the acceptor cells, integrating the membrane proteins and cytosolic constituents of the EVs into the respective compartments of the acceptor cells. The latter was perhaps most convincingly demonstrated by the transfer of EV-contained miRNAs that could repress target mRNAs in acceptor DCs (89). Indeed, distinct sets of miRNAs are incorporated into EVs from immature versus mature DCs and transferred to recipient DCs to modulate immune responses (89, 90). After endocytic uptake of recruited EVs, peptides may be eluted from associated MHC-II and transferred to endogenous MHC molecules (cross-presentation). This process was found to enable acceptor DCs to activate CD4 T cells after the acquisition of EVs from alloreactive DCs (54, 56). It is also possible that a significant proportion of released EVs remain associated with the plasma membrane of the producing cell itself, either by retention or recapture [**Figures 1** (steps 6–9), **2*c***], although this has not been investigated for DCs. Recapture is likely to occur, as this would be equivalent to the demonstrated recapture by bystander DCs involving ICAM-I binding to LFA-1 (3, 77). Retention is also possible via other mechanisms. For example, exosomes secreted by HeLa cells remained largely bound to its cell surface with the aid of tetherin (BST2) (91). Analogously, the spread of certain enveloped viruses, including HIV, is limited by tetherin-mediated restriction of the virions at the plasma membrane of the virus-producing cell (92). Interestingly, tetherin knockout DCs were compromised ex vivo in their capacity to stimulate antigen-specific CD4 T cells (93) and in vivo to induce an adaptive cell-mediated immune response against retrovirus infection (94). Depending on the stimulus, the expression of tetherin is increased in activated DCs (95). This implies, although direct evidence is lacking, that EVs generated by activated DCs, but less so by immature DCs, may be retained at the plasma membrane of the producing DC

by tetherin. Hence it is possible, although entirely speculative at this time, that those EVs that remain associated with the producing DC serve as signaling platforms to DC-interacting cells, for example, through physical clustering of MHC and/or costimulatory molecules. It is also possible that such surface-associated EVs dissociate only in response to DC-interacting cells, ensuring targeted delivery.

TRANSFER OF EXTRACELLULAR VESICLES FROM DENDRITIC CELLS TO OTHER CELL TYPES AND IMMUNE TOLERANCE

EVs derived from DCs with an immunosuppressive phenotype have been shown to decrease inflammation systemically (96–99). IL-10-treated tolerogenic DCs, and their EVs, have a low expression of costimulatory and coregulatory molecules compared to mature DCs (97–99). Importantly, EVs from IL-10-treated DCs were shown to delay onset of arthritis and reduce the severity of the disease systemically via an MHC-II-dependent mechanism (99). In transplantation studies, infusion of donor-derived EVs resulted in the induction of regulatory B and T cells, increasing survival of the allograft (100, 101). Also the course of autoimmune disease can be influenced by EVs, for instance in systemic lupus erythematosus, where autoantigen-presenting EVs have been shown to play a role (102, 103).

DCs can cross-dress CD4 T cells with pMHC complexes and costimulatory molecules during antigen-specific immune synapse formation (34, 36, 38, 42). In vitro, the release of MHC-II-carrying EVs from antigen-loaded DCs was stimulated by cognately interacting CD4 T cells (34). A significant proportion is recruited to the DC-interacting CD4 T cells (33, 34, 41, 42). Like for DCs, transfer to CD4 T cells depended on integrin binding, with LFA-1 on the acceptor cells and ICAM-1 on the EVs (42, 49, 77, 78, 104). The functions of such antigen-presenting T cells have not been completely clarified. One proposed role is that the transferred pMHC and costimulatory molecules help to sustain the activated status of the T cells, thereby releasing the requirement for further activation by DCs (105). Alternatively, they can signal to other cells. For example, CD4 Th1 cells that were cross-dressed with both MHC-I- and MHC-II-antigen complexes were shown to prime effector antigen specific CD8 T cells in a CD80 dependent manner (38). Furthermore, T cells that were cross-dressed with DC-derived pMHC, costimulatory molecules, and CD40 have been demonstrated to establish CD4 and central memory CD8 T cell responses (106–108). Alternatively, cross-dressed CD4 T cells have been proposed to function in a negative-feedback loop that inhibits further expansion of antigen-experienced CD4 T cells, thereby limiting clonal expansion and allowing recruitment of other naive T cells (109). Also, presentation by CD4 T cells of cross-dressed pMHC-II complexes in the absence of costimulatory molecules may induce apoptosis and hyporesponsiveness of antigen-specific T cells (110). Activated human, but not mouse, CD4 T cells have the ability to endogenously express MHC-II, and the peptide repertoire associated with MHC-II on human CD4 T cells overlaps with that of professional APCs (111). MHC-II/antigen presentation by T:T cell interactions induces anergy, and this pathway has been proposed to function in limiting the expansion of self-reactive CD4 T cells (112). Consistent with the recruitment of MHC-II-carrying EVs from cultured DCs to cognately interacting CD4 T cells (34, 42), it is possible that contributions of cross-dressed pMHC-II, in addition to endogenously expressed pMHC-II complexes, also contribute in tolerance induction by antigen-presenting CD4 T cells. Cross-dressing of T cells may also suppress immune reactions by enabling T cells with APC capacities to kill both antigen-presenting DCs and other antigen-presenting T cells (106). Th1 APCs that acquired pMHC-II could induce apoptosis in cognate memory CD4 T cells while leaving the naive CD4 T cells unaffected, thus establishing a more varied TCR repertoire (109). In an experimental autoimmune encephalomyelitis rat model, transfer of EV-associated pMHC-II

complexes from APCs to antigen-specific T cells was associated with the induction of peripheral self-tolerance (113).

Self-reactive T cells that escape thymic negative selection can also become anergic or be deleted by peripheral tolerance mechanisms that involve lymph node stromal cells. Lymph node stromal cells include lymphatic endothelial cells, blood endothelial cells, and fibroblastic reticular cells, and all of these are thought to ectopically express peripheral tissue antigens as well as MHC-I and MHC-II (114). The outcome of MHC-antigen presentation is determined by the presence or absence of costimulatory molecules, including CD80, CD86, and programmed death 1 (PD-1). Owing to the absence of costimulatory molecules on lymph node stromal cells, antigen presentation by endogenously expressed MHC can induce deletion of both self-reactive CD4 T cells and self-reactive CD8 T cells. Cross-dressing of lymph node stromal cells with pMHC-II-carrying EVs from DCs also promotes cognate CD4 T cell unresponsiveness and deletion (115). The relative contributions of endogenously expressed MHC-II and cross-dressed MHC-II to lymph node stromal cell–driven CD4 T cell deletion are unclear (reviewed in 116).

ROLE OF EXTRACELLULAR VESICLES FROM B CELLS IN MHC-II ANTIGEN PRESENTATION

EVs isolated from both human and murine B cell lines carried pMHC-II complexes, as well as costimulatory and adhesion molecules, and could induce pMHC-II-restricted T cell responses in vitro, thus demonstrating for the first time the antigen-presenting capacities of EVs (2). Electron microscopy analysis of Epstein-Barr virus (EBV)-transformed B cells revealed MVEs fusing with the plasma membrane, thus classifying the released EVs as exosomes. pMHC-II in B cell exosomes is incorporated into detergent-resistant protein complexes that also contain CD63 and other members of the tetraspanin family (2, 117–119). It has been proposed that proteins incorporated into a CD63-containing tetraspanin network can be recruited at MVEs by the ESCRT machinery via the cytosolic adaptors syntenin and ALIX for incorporation into exosomes (10, 120–122).

To generate antibody-secreting plasma cells, resting immature B cells in germinal centers of secondary lymphoid organs need to be activated consecutively by antigen-carrying FDCs and CD4 T cells to undergo the processes of isotype switching and affinity maturation, proliferation, and differentiation (123) (steps 1 and 3 in **Figure 3**). Antigens that form complexes with antibodies and/or complement are retained by FDCs by their FcRIIb or complement receptor CD21/35 (124). B cells can engage FDC-displayed antigens via their plasma membrane–bound immunoglobulin, the B cell receptor (BCR). Subsequently, this association is enforced by integrin-receptor interactions, establishing an immune synapse between the B cell and the FDC. Antigens can then be released directly from the FDC membrane by the action of lysosomal proteases that are locally secreted into the synapse by the B cell, and be endocytosed by the BCR and transported to endosomes and lysosomes for further degradation (125) (step 1 in **Figure 3**). Resulting peptides are then loaded within the endocytic compartments onto MHC-II, after which pMHC-II complexes can be displayed at the plasma membrane. Such primed B cells are subsequently activated when their surface-displayed pMHC-II complexes are recognized by cognate TCRs on antigen-specific CD4 T cells (126) (step 3 in **Figure 3**). Full B cell activation, however, also requires binding of B cell CD40 to CD40 ligand (CD40L), which is only displayed by CD4 T cells when they have previously been activated by DCs (step 2 in **Figure 3**). In this way, licensing of CD4 T cells by DCs ensures selective activation of antigen-specific B cells to proliferate and differentiate into high-affinity antibody-producing plasma cells. The release of EVs by primary B cells is induced by pMHC-II ligation with cognate TCR on CD4 T cells (41, 127–129) (step 3 in **Figure 3**) and can amount to as much as 12% of all cellular MHC-II per day (41). T cell–induced release of

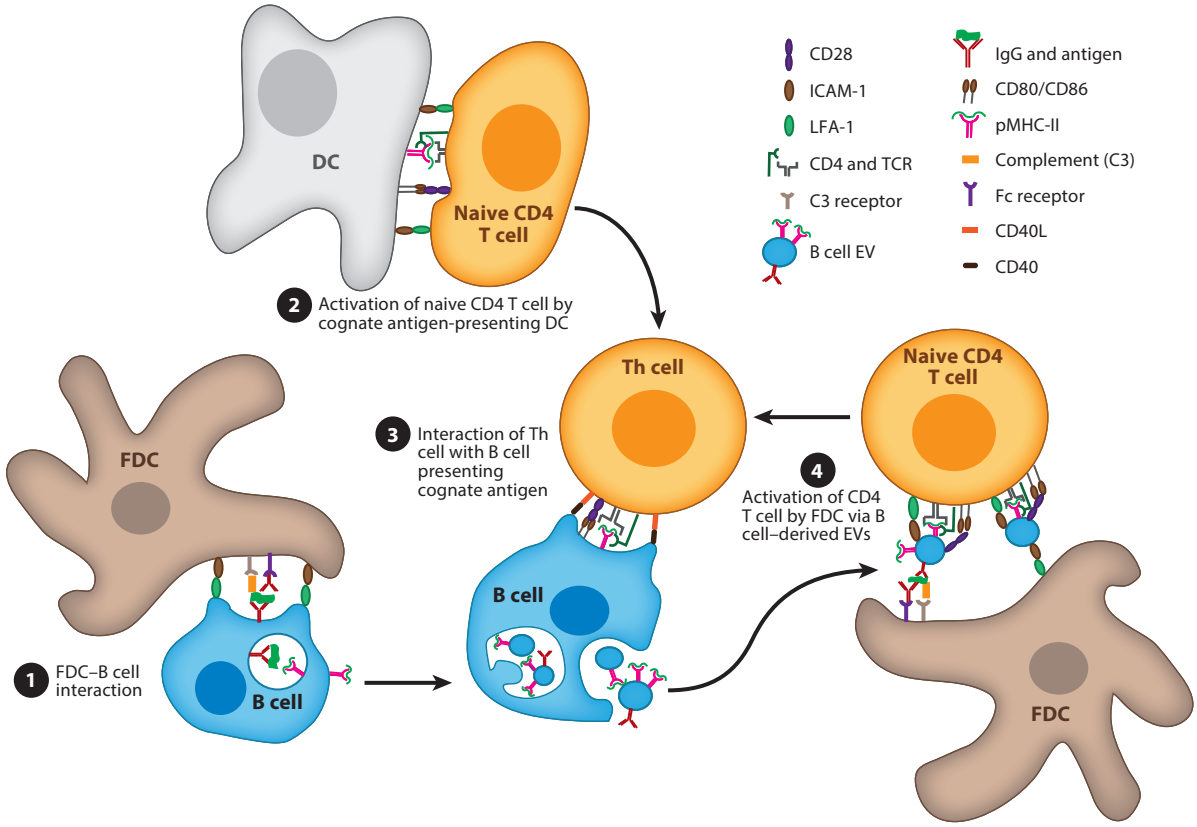


Figure 3

Hypothetical model for the role of B cell exosomes. ❶ A B cell interacts with a follicular DC (FDC) from which it obtains opsonized antigens via BCR-mediated endocytosis. Endocytosed antigen is processed into peptides that are presented by MHC-II on the B cell plasma membrane. ❷ A naive CD4 T cell is activated by a DC presenting cognate peptide–MHC-II complexes, resulting in T cell proliferation and differentiation into Th cells. ❸ A Th cell encounters a B cell presenting cognate peptide–MHC-II complexes, resulting in the B cell releasing exosomes carrying peptide–MHC-II. ❹ These exosomes are recruited by FDCs, and in association with these cells they present peptide–MHC-II to CD4 T cells, for further CD4 T cell expansion, differentiation, or silencing.

B cell–derived EVs also requires CD40 and IL4 signaling, and relies on NF-κB (127, 128), sharing the classical signaling pathways required for T cell–induced B cell proliferation. IgG-mediated BCR cross-linking in an immortalized B cell line also stimulated EV release but only to a minor extent (130), suggesting that the TCR–pMHC-II interaction is the major determinant for EV release by B cells. Exosome release is constitutive by human B cells that are infected by EBV (128), explaining high exosome secretion by EBV-transformed cell lines, with an equivalent of up to ∼10% of all cellular MHC-II being released in 24 h (2). The EBV oncogene-encoded latent membrane protein 1 (LMP1) mimics downstream signaling via CD40 by constitutively activating NF-κB, explaining how EBV transformation induces exosome secretion by B cells (131). A pathological role for LMP1-carrying exosomes from Burkitt lymphoma B cells is indicated by the observation that they supported in vitro B cell–proliferation, differentiation, and immunoglobulin class switching (132). Exosomes from EBV-infected B cells were also found to contain EBV-encoded miRNAs that could be functionally transferred to noninfected DCs, possibly interfering with the development of adaptive immunity (133).

One of the potential targets of B cell–derived EVs is FDCs in lymphoid follicles. FDCs do not synthesize MHC-II themselves nor do they have MHC-II incorporated into their plasma membrane, but they are abundantly decorated at their plasma membrane with MHC-II-carrying EVs (134, 135). The notion that these EVs may be largely acquired from B cells was supported by the observation that isolated B cell exosomes were efficiently and preferentially recruited in vitro by FDCs from tonsil lymphoid tissue, as compared to other cell types within the same preparation (135). FDCs are nonphagocytic cells; hence, the residency time of recruited EVs on the plasma membrane may be quite long. How B cell–derived EVs are recruited by FDCs is unknown. However, B cell EVs carry complement component 3 (C3) fragments (136) as well as BCR-antigen complexes (128, 130), and C3 fragments on these EVs were shown to stimulate T cell response in the presence of suboptimal antigen concentrations (136). Therefore, it is likely that FDCs recruit B cell exosomes via their FcR and/or complement receptors. Such binding might be enforced, for example, by integrin $\alpha_4\beta_1$, which is also highly enriched and functional on B cell exosomes (118, 137) and known to play an important role in B cell–FDC interactions in germinal center responses (138). Exosomes from B cells, but also from DCs, are protected from complement-mediated lysis by incorporated glycosylphosphatidylinositol-anchored CD55 and CD59 (139), potentially contributing to their long residency time on FDCs. As explained above, antigen-specific CD4 T cells are essential drivers of B cell affinity maturation and development of memory B cells (140), and maintenance of DC-primed antigen-specific T cells requires constant stimulation, a task that could be performed by FDC-associated B cell exosomes (step 4 in **Figure 3**). This scenario is supported by the observation that isolated, antigen-loaded B cell exosomes can directly stimulate primed, but not naive, CD4 T cells (41, 141). Exosomes that were isolated from cultured primary B cells and injected intravenously or subcutaneously into mice were captured in the subcapsular sinus of lymph nodes by macrophages, involving binding of exosome-linked α2,3-linked sialic acids to CD169 in the marginal zone of the spleen and lymph nodes (55). In $CD169^{-/-}$ mice, such exosomes were not efficiently retained in the subcapsular sinus and penetrated deeper into the paracortex, increasing the response to antigen-pulsed exosomes (55). These data suggest that functional B cell exosomes are normally released locally within follicles in response to B cell–T cell interactions, and that B cell exosomes released outside the lymphoid tissue are eliminated by macrophages to control immune responses. Another level of control is dictated by upregulation of FasL on exosomes from activated B cells and EBV-transformed B cells (142). The presence of FasL induced apoptosis of antigen-specific CD4 T cells (143), suggesting a negative-feedback loop to restrain T cell–mediated responses.

ANTIGEN PRESENTATION BY MACROPHAGE-DERIVED EXTRACELLULAR VESICLES

Macrophages clear their environment from pathogens through phagocytosis and play essential regulatory roles in innate and adaptive immune responses. Macrophages also function in anti-tumor immunity by cross-presenting dead cell–associated antigens to initiate CD8 T cell responses (144). CD8 T cells were much better activated when cocultured together with both splenic macrophages and CD8 DCs, as compared to macrophages or DCs alone, suggesting a role for either cross-presentation or cross-dressing (145). In response to phagocytic uptake of pathogens such as mycobacteria, salmonella, or toxoplasma, macrophages increase the release of EVs that are apparently formed as exosomes within phagosomes (146, 147). These EVs carry MHC-I, MHC-II, and costimulatory molecules such as CD86 and have the capacity to prime naive T cells (147, 148). EVs isolated from macrophages infected with pathogens stimulated CD4 and CD8 T cell responses in vitro, particularly in the presence of DCs, as well as in vivo (147–149),

indicating a role for cross-dressing. Consistent with a role of exosomes, Rab27a-deficient mice had a diminished capacity to control an infection with *M. tuberculosis*, and macrophages from these mice were compromised in releasing EVs, as well as in their capacity to stimulate T cells (88).

ANTIGEN PRESENTATION BY EXTRACELLULAR VESICLES FROM INTESTINAL EPITHELIAL CELLS

Although intestinal epithelial cells express MHC-I and MHC-II, the basement membrane impedes direct contact with T cells. However, basolaterally released pMHC-carrying EVs can overcome this barrier. Cultured intestinal epithelial cells released MHC-II-carrying EVs that could efficiently activate cognate CD4 T cells when cross-dressed onto DCs (150, 151). The A33 antigen is specifically expressed by intestinal epithelial cells but could also be detected in EVs from these cells and in mouse mesenteric lymph nodes, suggesting a role for EV transfer to lymphoid tissues (150). Microscopy revealed high concentrations of MHC-II and A33 antigen within MVEs of intestinal epithelial cells, suggesting that EVs from these cells are exosomes (152). EVs that were isolated from the serum of antigen-fed mice could transfer antigen-specific CD4 T cell tolerance in syngeneic recipient mice, depending on MHC-II expression in intestinal epithelial cells, providing in vivo evidence for a role of MHC-II/antigen presentation by EVs from these cells in generating oral tolerance (153). Similarly, EVs isolated from bronchoalveolar fluid of mice that were intranasally inoculated with antigen prevented allergic reactions in recipient mice (154).

T CELL–DERIVED EXTRACELLULAR VESICLES

T cells, like the professional APCs they interact with, release EVs with heterogeneous characteristics (6, 155). CD4 T cells that are activated via their TCRs release microvesicles from their plasma membrane into immune synapses in a TSG101- and VPS4-dependent manner (37, 155), indicating an active and specific process. T cell–derived EVs can be targeted to different types of immune cells and modify their function. In the absence of a synapse, EVs were isolated from antibody- and IL-2 -stimulated T cell cultures, and these stimulated in vitro proliferation of autologous resting CD8 T cells (156). On the other hand, EVs from activated T cells carried bioactive membrane-associated FasL and TNF-related apoptosis-inducing ligand (TRAIL), and these EVs have been proposed to help prevent potential autoimmune damage by eliminating activated T cells after a cellular immune response has taken place (157, 158). Apparently, two populations of EVs are released by DC-activated CD4 T cells, with the first (presumably microvesicles) being transferred to interacting DCs before T cell activation and the second (presumably exosomes) being transferred in response to T cell activation (159). While the first group might facilitate a productive DC–T cell interaction, the second group could play an important immunomodulatory role in intraclonal competition of T cells (159). Similarly, CD4 T cell–released exosomes were found to inhibit CTL responses and antitumor immunity (160). Yet, others observed that T cell–derived EVs could activate cognate B cells (37, 155), possibly through transfer of miRNA (5). Delivery of CD4 T cell help to B cells is dependent on cognate MHC-II/antigen complexes and CD40 signaling by the B cells. When Th1 cells recognize antigen-presenting B cells, CD40L is upregulated on the T cells to interact with and activate CD40 on the B cells. In a recent study it was found that CD40L is transferred to and endocytosed in association with CD40 by the B cell (161). In this way, EV-mediated transfer of CD40L from T cells to B cells may sustain signaling cues that promote B cell proliferation and differentiation without the requirement of continued intercellular contact.

POTENTIAL OF DENDRITIC CELL–DERIVED EXTRACELLULAR VESICLES IN IMMUNOTHERAPY AGAINST CANCER

Given their central role as initiator and regulator of the adaptive immune response, DCs are essential for T cell–mediated cancer immunity. Hence, many efforts are being made to develop DC-based vaccination therapies against cancer, but so far with only limited success (reviewed in 162). Current vaccination strategies are mostly based on injection of peptide, protein, or nucleic acids that can be captured by DCs in vivo. DCs have also been isolated from patients, expanded, activated, and loaded ex vivo with tumor-associated antigen, and then reintroduced into patients. Although such approaches often resulted in the expansion of circulating tumor-specific CD4 and CD8 T cells, clinical efficacy remained low (reviewed in 163). One major problem is imposed by counteracting immune-suppressive signals from the tumor, for example, by the presence of preexisting regulatory T cells or myeloid-derived suppressor cells. As an alternative, DC-derived EVs have been developed toward a clinical grade product and tested in phase 1 and phase 2 clinical trials for their efficacy in targeting cancer. In two initial studies, tumor antigen–loaded EVs from cultured autologous immature DCs were used as vaccine (164, 165). Minimal antigen-specific T cell responses were observed in only one of these studies (165), while both reported stability of disease in some of the immunized patients, a proposed consequence of the stimulation of natural killer cells. In a more recent clinical trial, tumor antigen–loaded EVs were derived from IFN-γ-stimulated DCs, but these also could not induce strong adaptive immune responses (166). One possible limitation is that the EVs used in these clinical trials were loaded with selected peptides and therefore could be expected to activate T cells with a very limited repertoire in antigen specificity. In mice, CD8 T cell responses improved on immunization with EVs from protein-loaded, as compared to peptide-loaded, DCs (83). Interestingly, in this setting, CTL responses were also dependent on CD4 T cells as well as on complement activation and antigen shuttling by B cells. It has been proposed that antigen-specific B cells may recruit antigen-loaded DC–derived EVs to facilitate their transfer to FDCs. EVs isolated from cultured DCs indeed carried complement (82) and thus may be recruited by complement receptor CD21 on FDCs.

In conclusion, to be effective in future applications in cancer immunotherapy, DC-derived EVs should preferably contain costimulatory molecules as well as relevant tumor-specific antigens loaded onto both MHC-I and MHC-II. This idea is consistent with awareness that DNA and RNA vaccines, intact proteins, and synthetic long peptides that deliver antigen to both MHC-I and MHC-II are more effective in cancer immunotherapy than short-peptide antigens that are restricted to MHC-I (reviewed in 74). A potential caveat is that in vitro–generated EVs that are used for cancer immunotherapy should preferably be produced by 100% pure immune-stimulatory DCs. Indeed, prior activation of the EV-producing DCs improved the antitumor immunity elicited by the EVs in a murine model of melanoma (167). Conversely, EVs from immature DCs can display immune-tolerizing capacities (98, 99, 101–103, 113). Primary human DCs are difficult to isolate to 100% homogeneity, and their activation is often asynchronous and incomplete. Isolated immunostimulatory EVs can therefore easily be contaminated with tolerogenic EVs, with the latter worsening the outcome of cancer immunotherapy. Furthermore, large-scale production of autologous moDCs is laborious and costly. To circumvent these problems, artificial EVs have been generated that consist of liposomes coated with pMHC-I complexes and a range of ligands for adhesion, early activation, late activation, and survival TCRs (168). In vitro, these artificial exosomes specifically triggered human antigen-specific CD8 T cells, but only in the presence of APCs, in a similar manner as natural DC-derived EVs do. As a future perspective, a homogeneous population of artificial EVs, loaded with tumor-specific pMHC-I and pMHC-II complexes and equipped with immune-activating costimulatory molecules and integrins, may prove to be effective

in cancer immunotherapy. In situ, activated DCs mature and travel to lymph nodes or the spleen, where they should release their EVs, to optimize recruitment by resident DCs (28). Therefore, the activity of the therapeutic EVs is likely to be influenced by the site of administration. While systemic administration might result in clearance of EVs by macrophages and hepatic Kupffer cells (13), direct administration into the lymphatic system should enhance proper delivery and functionality (169).

FUTURE PERSPECTIVES

The immune system is very complex given the vast number of distinct cell types involved, and novel subtypes of each are being discovered at a fast pace. For example, many distinct subsets of DCs with different functional specializations in regulating immunity have been described. Realization that all these immune cells can exchange membrane proteins, cytosolic proteins, lipids, and nucleic acids (including miRNA) by means of EVs has complicated the picture even further. First, transfer of what were previously thought to be unique marker proteins complicates the analysis and identification of specific cell types. Second, transfer by EVs of signaling molecules, such as kinases, signaling lipids, and miRNA, affects the behavior and characteristics of the receiving cells. Many immunologists were or still are wary of accepting a role for EVs in immune regulation, as this phenomenon would complicate research on the role of different immune cells in immunity even further. However, overwhelming and ever-increasing evidence for significant functions of EVs in immune regulation has been gathered over the last two decades and can no longer be neglected. Likely only a small fraction of the functions of EVs in immune regulation has been discovered. Further progress in this area would be boosted by the development of additional molecular tools for in vitro and in vivo intervention of EV release or incorporation of specific cargo therein.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We apologize to all authors whose work was not cited or discussed in depth because of the length limitation. This work has been supported by project number 022.004.018a, which is financed by the Netherlands Organisation for Scientific Research (NWO).

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