Exosomes from B cells and Dendritic cells: Mechanisms of formation, secretion and targeting

If you want to go fast, go slow
-Anonymous-

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Exosomes from B cells and Dendritic cells: Mechanisms of formation, secretion and targeting

Exosomen van B cellen en dendritische cellen: mechanismen voor vorming, secretie en overdracht
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Abbreviations

APC	antigen presenting cell
BCR	B cell receptor
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid
CLR	C-type lectin receptors
CSLM	confocal scanning laser microscopy
DC	dendritic cell
DRM	detergent resistant microdomain
DUB	de-ubiquitination enzyme
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
FACS	fluorescent activated cell sorter
FDC	follicular dendritic cell
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSP	heat shock protein
HRP	horse radish peroxidase
IEM	immuno-electron microscopy
IS	immune synapse
LE	late endosome
LPS	lipopolysaccharides
LV	luminal vesicle
MHC	major histocompatibility complex
MVB	multi vesicular body
PAMP	pathogen associated molecular pattern
PRR	pattern recognition receptor
RE	recycling endosome
SE	sorting endosome
SIV	simian immunodeficiency virus
SMAC	supra-molecular activation complex
TAP	transporter associated with antigen presentation
TCR	T cell receptor
TEM	tetraspanin enriched microdomain
TGN	trans-Golgi network
Th	T helper cell
TLR	Toll like receptor
Treg	regulatory T cell
Ub	ubiquitin
UIM	ubiquitin interacting motif
VSV	vesicular stomatitis virus

Chapter 1

General Introduction

Exosomes are small 50-100nm vesicles secreted by cells of many origins (reviewed by (1-4)). The release of exosomes has been described for the first time over 2 decades ago for maturing reticulocytes which use this pathway to dispose off proteins that have become superfluous during maturation, such as the transferin receptor, acetylcholinesterase and the integrin VLA4 (5, 6). Since then the release of exosomes has been observed for a multitude of other heamatopoietic cells, including B cells (7), dendritic cells (DC) (8), mast cells (9), and T cells (10) but also for cells from non-heamatopoietic origins like intestinal epithelial cells (11), tumor cells of various origins (12) and kidney cells (13). Exosomes originate from a ubiquitous cellular organelle, the multi vesicular body (MVB) (7), making it conceivable that perhaps all cell types are capable of secreting exosomes and the list of known exosome secreting cells is thus likely to be far from complete.

Multi vesicular body/exosome biogenesis

Exosomes are equivalent to the luminal vesicles (LV) of MVB, which are late endosomal compartments (Fig. 1). Exosomes are secreted when MVB fuse with the plasma membrane therewith releasing their content in the extracellular milieu (7). Endosomes are intracellular organelles that communicate by means of vesicular traffic with the plasma membrane and other organelles (e.g. the trans Golgi network (TGN) and lysosomes) (reviewed by (14)) (Fig. 1). After endocytic uptake proteins are transferred by means of transport vesicles to the sorting endosome (SE). The relative low pH at the SE causes dissociation of many ligands from their cognate transmembrane receptors, after which the latter may recycle back to the plasma membrane, either directly or via recycling endosomes (RE). In contrast, soluble proteins and transmembrane proteins destined for lysosomal degradation remain associated with SE, which gradually mature into a late endosomes (LE) (15). During maturation, SE change their protein composition and lower their intraluminal pH. In addition, the limiting membrane of SE may bud into the endosomal lumen, forming internal vesicles. As a result SE become MVB.
Once sorted at MVB, proteins await three possible fates. First, after incorporation into LV, plasma membrane receptors such as ligand associated EGF receptor, can be targeted to lysosomes as a consequence of fusion of MVB with lysosomes (16). Therewith ligand induced signaling is terminated and LV and receptor are broken down by lysosomal enzymes. Second, the MVB can serve as a “ready to use” storage compartment, as is described for major histocompatibility complex class II (MHC class II) in immature DC (17). In resting DC, MHC class II is targeted to LV but upon DC activation LV may fuse back with the delimiting membrane. From there MHC class II can be transported to the cell surface (17). Third, the

MVB can fuse directly with the plasma membrane resulting in secretion of the LV as exosomes (7).

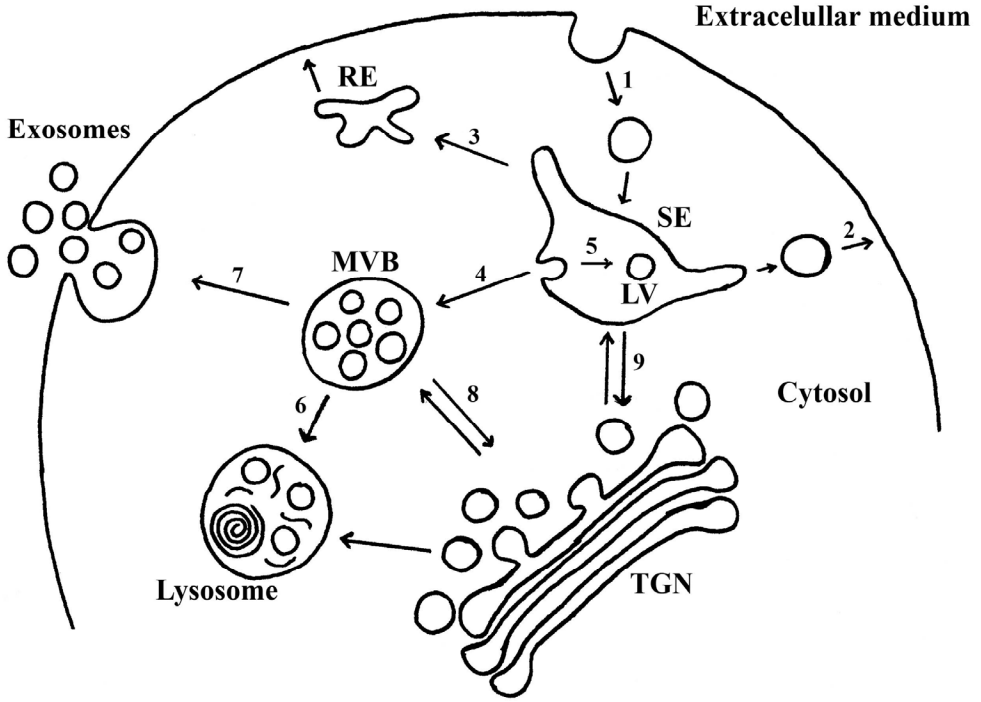

Figure 1: Endosomal pathways After endocytosis (1) membrane proteins are targeted to sorting endosomes (SE) from where they can be recycled back to the plasma membrane either directly (2) or via the recycling endosome (RE)(3). Other proteins remain in the SE that, in time matures into a late endosome/MVB (4). This process includes the formation of LV that are formed by inward budding of the endosomal delimiting membrane away from the cytosol (5). MVB can fuse with a lysosome (6) resulting in the degradation of MVB resident proteins or fuse directly with the plasma membrane (7) therewith releasing LV as exosomes. Proteins at the MVB delimiting membrane may be transported to and from the TGN (8, 9) by means of transport vesicles. Note that all indicated processes are sorting events involving the formation of transport vesicles, except the maturation of SE into MVB (4).

Sorting of most membrane proteins at MVB and for some proteins also endocytic uptake, is dependent on their ubiquitination, the covalent attachment of one or more ubiquitin (Ub) moieties (reviewed by (18)). At the plasma membrane, ubiquitinated proteins may be recognized by Ub-binding proteins that mediate

internalization and sorting at MVB (see below). A multitude of cell surface receptors, including receptor tyrosine kinases (RTK, e.g. the EGF receptor, TGF-β receptor, TCR and CD4) but also MHC class I & II, several ion channels and many other membrane proteins have been described to use the ubiquitin mediated sorting pathway (18-21).
Ub is a 76 amino acid polypeptide that can be covalently attached to lysine residues of other proteins and, besides its function in sorting, is also used by the cell in many other cellular processes, including proteasomal degradation of cytosolic proteins and transcriptional regulation (22, 23). Depending on the cellular process involved, either a single or multiple Ub moieties may be conjugated to a protein. In yeast, the attachment of one ubiquitin moiety to membrane proteins (mono-ubiquitination) is sufficient for internalization and sorting to the vacuole, the yeast equivalent of a lysosome. Mammalian membrane proteins may either be mono- or poly-ubiquitinated for internalization and sorting at MVB (19, 24). Ligation of ubiquitin is catalyzed by the sequential actions of three enzymes: a Ub-activating enzyme E1, a Ub-conjugating enzyme E2, and a Ub-ligase E3 (18). Many different E3 ligases exist, each with substrate specificity. The CBL family of E3 enzymes is involved in the ubiquitination and down-regulation of several RTK, including the EGF-R, PGDF-R, the T Cell Receptor (TCR) and B Cell Receptor (BCR) (See (25, 26)). Whereas the MARCH family of E3 enzymes is important for regulating surface expression of many membrane proteins that are part of the immune system (20).

At the plasma membrane ubiquitinated proteins are recognized by adaptor proteins, including Eps15 and Epsin. Such adaptor proteins contain ubiquitin interaction motifs (UIM) and link to the clathrin-mediated endocytosis machinery (19). After internalization removal of the ubiquitin moiety by de-ubiquitination enzymes (DUB) may prevent further sorting into the late endocytic/MVB pathway and lead to receptor recycling (27). Membrane proteins that remain ubiquitinated are sorted at MVB by the combined actions of a group of conserved proteins that were originally identified as Vacuolar protein sorting (Vps proteins) in *Saccharomyces cerevisiae* (Fig. 2, reviewed in (28, 29)). At the endosome ubiquitinated proteins are bound by UIM on Vps27/HRS, which, possibly with the help of clathrin, concentrates cargo on the endosomal membrane and recruits the ESCRT-I complex (composed of Tsg101, VPS28, VPS37 and EAP30). ESCRT-I activates ESCRT-II (EAP25, EAP45, CHMP2A/B), which in turn recruits the ESCRT-III complex (CHMP 3, 4, 6). Together these events result in the recruitment of selected membrane proteins in distinct patches at the MVB limiting membrane. Many components of the sorting machinery itself, including Eps15, epsin, Hrs and tsg101 are ubiquitinated. When sorting is complete both cargo and sorting machinery are

de-ubiquitinated, in yeast by the enzyme DOA4 and in mammals by the recently identified DUB AMSH (30, 31) and/or UBPY (32). Finally, the ESCRT-III complexes are dissociated by the AAA-ATPase VPS4/SKID1, liberating components of the sorting machinery for a new round of sorting.

Even though the importance of the ESCRT machinery for MVB sorting is clear, parallel mechanisms may also drive MVB sorting. Some cargo does not require ubiquitination, or is independent of ESCRT for its sorting at MVB (33-35). These proteins may either rely on attachment to other cargo, use other protein intermediates to link to ESCRT complex components or even use yet undefined alternative mechanisms.

Other machineries that participate in MVB sorting may include the family of annexins, as is illustrated by the inhibition of EGF-induced EGFR down-regulation and in annexin 1-deficient cells (36). Basal MVB formation was normal in these cells suggesting that the function of annexin 1 may be confined to cargo selection. Using RNA interference annexin 2 was also found to play an undefined role in MVB biogenesis (37).

Because of their abundant presence in exosomes (see below) (38), it is speculated that proteins of the tetraspanin family may assist in MVB biogenesis (39). These proteins form large protein networks and organize with neighboring transmembrane proteins in cholesterol and ganglioside rich membrane microdomains distinct from lipid rafts, designated tetraspanin enriched microdomains (TEM) (reviewed in (40)). Proteins that do not make use of the ESCRT machinery, may be directed to LV by adhering to tetraspanins and/or by their preferential partitioning into lipid microdomains (41). Exosomes are also enriched in cholesterol, sphingomyelin, and ganglioside GM3, lipids that are typically enriched in detergent-resistant membranes (39), indicating also lipid microdomains may provide an alternative route of sorting into LV of MVB.

The mechanisms for the inward budding and vesicle formation at MVB have not been resolved. The ESCRT interacting protein Bro1/Alix has been proposed to drive inward vesiculation through its interaction with the membrane phospholipid LBPA, which is able to spontaneously form MVB like structures from liposomes in vitro (42). LBPA is indeed found on MVB/lysosome like structures but is absent on less mature MVB (43) and B cell derived exosomes (39). Furthermore it was recently shown that both LBPA containing and LPBA-negative MVB subpopulations may exist, suggesting LBPA is not a prerequisite for LV formation or that several parallel mechanisms of internalization may exist. Other phospholipids like PI(3)P and PI(3,5)P may also function in MVB sorting/ vesicle formation, as inhibition or ablation of the kinases necessary for their formation (Vps34 and Fab1 respectively), abrogate MVB formation (44-46).

The release of exosomes requires fusion of MVB the plasma membrane. To date not much is known about signals that trigger and mechanisms responsible for this process. In mast cells exosome release can be triggered by IgE immune complexes and a subsequent rise in cytosolic Ca^{2+} (9). Also in reticulocytes cytosolic Ca^{2+} seems to play a role, possibly in concert with Rab 11 (47, 48). A rise in cytosolic Ca^{2+} is associated with the downstream signaling of many different cell surface receptors and several of those may thus induce exosome secretion.

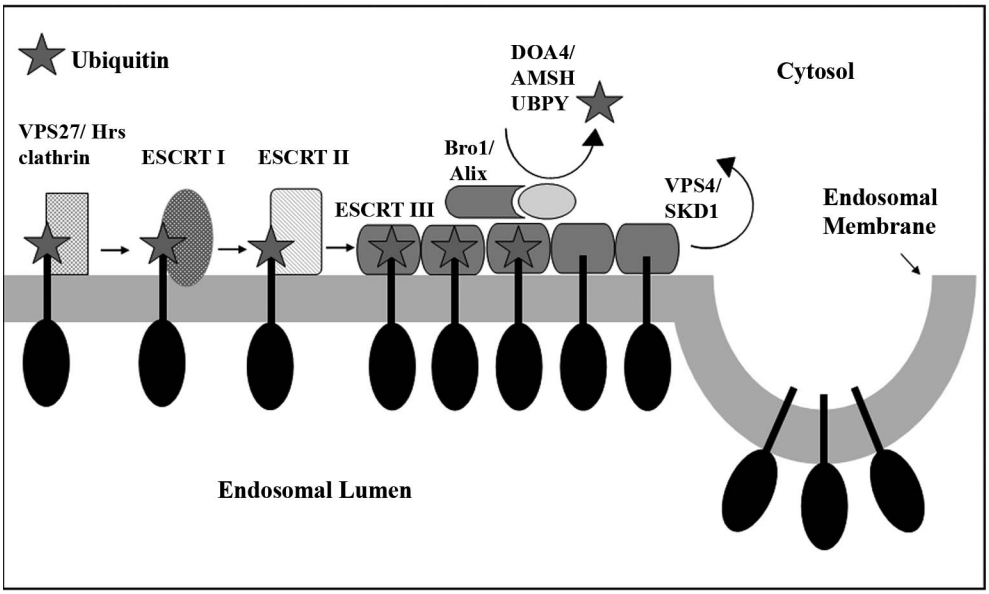

Figure 2: Model for ubiquitin mediated sorting of membrane proteins at MVB At MVB ubiquitinated cargo is recognized by the VPS 27/Hrs complex which associates with clathrin and recruits the ESCRT I complex from the cytoplasm. ESCRT I activates ESCRT II, which in turn may initiate recruitment of the ESCRT III complex that further concentrates recruited cargo on the endosomal limiting membrane. Prior to vesicle fission ubiquitin is removed from the cargo protein by the action of DUB (DOA4/ AMSH or UBPY) and ESCRT complexes are released by the AAA-ATPase VPS4/ SKD1. Both yeast and mammalian homologues of proteins involved are indicated. For details see main text. Based on a model in (28).

Exosome content

The protein composition of exosomes from many different sources has been documented. Exosomes contain cell type specific proteins (discussed below) as

well as ubiquitous proteins. The content of exosomes provides us with clues on both exosome function and the mechanism of MVB sorting.
Regardless of their source, members of the tetraspanin family of proteins are persistently found in exosomes (e.g. CD9, CD37, CD53, CD63, CD81, CD82) (8, 10, 11, 38, 49). The molecular chaperones HSP70 and HSP90, MHC class I, adhesion receptors, metabolic enzymes, cytoskeleton associated proteins (e.g. actin, tubulin, ERM (ezrin, radixin, moesin) family proteins) and protein associated with MVB biogenesis (e.g. Tsg101, Alix) are also ubiquitous (reviewed in (2, 50).
Tetraspanins belong to a family of transmembrane proteins that span the membrane 4 times and contain 2 small exoplasmic loops, conserved CCG motifs and conserved cysteines that form two intra-molecular disulfide bonds (reviewed in (40, 51)). In addition, nearly all tetraspanins contain membrane proximal cysteines that undergo palmitoylation. Proteins belonging to this family have regulatory functions in cell adhesion, motility, cell-cell or virus-cell fusion or signaling (reviewed in (52)). Tetraspanins organize into large protein networks, so called tetraspanin enriched microdomains or TEM, at the plasma membrane as well as in the endocytic tract. Within TEM, tetraspanins interact with each other as well as with integrins, immuno-globulins, other cell-surface receptors and intracellular signaling molecules and such microdomains have been proposed to provide a scaffold for the transmission of external signals (e.g. ligand binding) to the intracellular signaling machinery. In addition to proteins, also lipids (e.g. cholesterol, gangliosides) associate with TEM in detergent resistant microdomains (DRM) (40).The function of tetraspanins in exosomes is not clear but they may have a role in the sorting of tetraspanin associated proteins into the LV of the MVB (see above) and binding and/or fusion of exosomes with target cell membranes.
Most of the other ubiquitous proteins in exosomes are cytoplasmic of origin. These may be incorporated aspecifically as a consequence of the inclusion of a small volume of cytosol during MVB formation. Alternatively, cytosolic proteins may target to exosomes by adherence to membrane proteins or lipids that enter the MVB pathway. Many proteins involved in cargo selection and MVB biogenesis, such as Tsg101 and Alix, dissociate from the cargo before the LV are actually formed (see above, reviewed in (28)). However, dissociation may not be very efficient as such proteins have consistently been found in exosomes. Alternatively, dissociation may occur in concert with vesicle fission, leaving little time to diffuse away, resulting in incorporation into LV/exosomes. Similarly other, non-relevant, cytoplasmic proteins may also be incorporated into exosomes (e.g. metabolic enzymes).
Heat shock proteins (HSP) are also found in exosome preparations from many cells. HSP are ubiquitously expressed and chaperone the folding of newly synthesized or stress-denatured proteins (53) but also aid other cellular processes.

In exosomes from maturing reticulocytes Hsc70 is proposed to target the transferrin receptor (TfR) to exosomes (54). Hsc70 also binds and disassembles clathrin, which is necessary for the budding of many cellular transport vesicles and in this role may also have a function in MVB formation/ sorting (55). Indeed Hsc73 associates with late endocytic/lysosomal compartments (8). In addition, upon starvation, complexes containing Hsp40, Hsp70 and Hsp90 can translocate cytoplasmic proteins across lysosomal membranes in a process called chaperone-mediated autophagy (56). Besides a structural role in MVB dynamics, HSP may also have implications for immunological functions of exosomes, including spreading of antigens. These aspects of HSP will be discussed below when in relation with tumor-derived exosomes.

Functional implications of exosomes

Since exosomes/LV are formed by budding away from the cytosol their membrane proteins have the same topology as plasma membrane proteins, with their exoplasmic side facing the outside. Membrane proteins on exosomes are thus exposed for binding cognate ligands, providing exosomes with the potential to directly modulate target cell function. This is exemplified by MHC class I, MHC class II and integrins (ICAM-1), which equip exosomes from antigen presenting cells (APC; see below), to exert an effect on recipient T cells in vitro (1, 57, 58). Other proteins, such as CD55 and CD59, allow exosomes to survive in the extracellular milieu by protecting them from complement-mediated lysis (59, 60).
Although evidence is mounting for an important role of exosomes in modulating immune responses, physiological functions of exosomes have not yet been demonstrated directly. In addition to ubiquitous proteins, exosomes are equipped with cell type specific molecules and are therefore likely to have functions representative for the cell of origin. The current knowledge and ideas on exosome functions are therefore discussed best separately for specific cells. The studies presented in this thesis concentrate on exosomes derived from DC and B cells, which are both professional APC. The functions of DC and B cells are discussed prior to their exosomes.

DC & DC exosomes

Introduction into DC biology
Dendritic cells are professional APC and essential for the initiation of adaptive immune responses (reviewed by (61) (62)). In peripheral tissues, DC continuously

sample their environment for pathogens and infected or malignant cells. DC internalize these by receptor-mediated endocytosis or phagocytosis. Once inside, endocytosed protein complexes are degraded into peptides, which may associate with MHC molecules that are presented by DC to T cells (Fig. 3).

Two types of MHC molecules have evolved to present peptides to T cells, MHC class I and MHC class II (for overview see (63) and (64)). MHC class I is expressed by all cells of the body and predominantly serves to present peptides derived from infected or malignant cells to T cells (reviewed by (65)), such peptides mostly derive from proteins that are degraded by the proteasome in the cytosol. Thus formed cytosolic peptides are transported into the ER by the TAP complex (transporter associated with antigen presentation) for loading onto MHC class I.

In contrast to MHC class I, MHC class II is expressed only by so called "professional antigen presenting cells" (APC). These cells endocytose proteins for degradation by lysosomal enzymes and resulting peptides are loaded onto MHC class II within the endocytic tract (reviewed by (66)) Because of their specialized function in APC, endocytic structures in these cells have also been referred to as the MHC class II loading compartment or MIIC.

MHC class I and MHC class II that are complexed to peptides, are recognized by different subsets of T cells (see (63)). MHC class I is recognized by T cells carrying a CD8 T cell co-receptor. After binding the peptide-MHC class I complexes together with the appropriate co-stimulatory factors these cytotoxic T cells can kill target cells through the release of lytic granules. In contrast, peptide-MHC class II complexes are recognized by T cells carrying the CD4 co-receptor. These "T helper cells (Th)" cells can either provide "help" in the cellular immune response (Th1) by helping in the activation of macrophages or cytotoxic CD8+ T cells, or in the humeral immune response (Th2) by helping in the activation and class switching of antibody producing B cells.

In general, MHC class I and MHC class II carry antigen specific information from different sources and activate CD8+ or CD4+ T cells respectively. The source of peptides loaded onto MHC class I and MHC class II, however, is not as strict as indicated above. Peptides derived from endocytosed proteins may also be presented by MHC class I in a process called "cross-presentation" (reviewed in (65, 67-69). Cross-presentation is mainly performed by DC and required for the APC mediated activation of cytotoxic T lymphocytes, at locations (e.g. the lymph node) remote from the site of infection particularly when antigens that are needed to trigger an immune response cannot be expressed by DC themselves (e.g. tumor antigens and cell type specific viruses that do not infect DC). Loading of endogenously expressed peptides onto MHC class I usually occurs in the ER, which is difficult to reach for peptides from endocytosed proteins. Cross-presentation has been

demonstrated to require both cytosolic proteasomes and ER resident TAP complexes, suggesting that endocytosed proteins may be released from endosomes or lysosomes into the cytosol. Other pathways have also been proposed: Recently, it was suggested that the ER might be recruited to the phagocytic cup and it was proposed this would bring all components needed for cross-presentation together (70). Others, however, failed to find evidence for such a mechanism (71). Other potential explanations include the intercellular transfer of peptides via GAP-junctions, exchange of peptides of endocytosed MHC class I in endosomes, transport of peptides via a retrograde vesicular route from the endosomal system to the ER, transport of peptides into the cytosol over the endosomal membrane. Finally, exosomes derived from infected cells might be recruited by DC, which may thus present exogenous peptide-MHC I complexes derived from these cells (See (65, 67-69)).

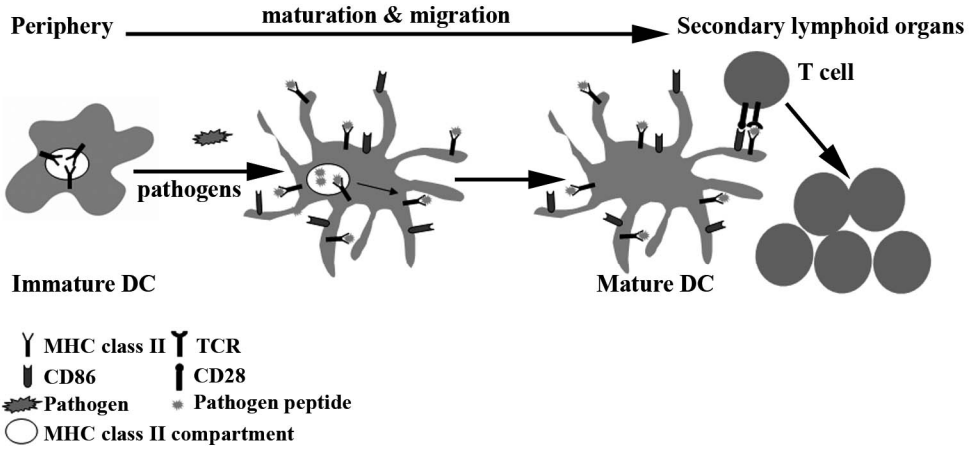

Figure 3 DC present pathogen derived peptides on MHC class II to T cells. In peripheral structures DC recognize and internalize pathogens. As a result DC mature and migrate to the lymph node. Meanwhile DC process internalized pathogens into peptides and load these onto MHC class II in the endosomal/lysosomal MHC class II loading compartment. Subsequently, peptide-loaded MHC class II complexes are transported to the plasma membrane for presentation to CD4+ T cells. Concomitantly, DC elevate the surface expression of co-stimulatory molecules such as CD86. In lymphoid organs mature DC activate cognate T cells to proliferate and trigger an adaptive immune response

The state of maturation of the DC that present peptide-MHC complexes determines whether the outcome of the induced immune response is tolerogenic (immunosuppressive) or immunogenic (see (72)). Resting or immature DC are thought to induce tolerance as opposed to mature DC that rather trigger immunity. DC are activated and mature in response to danger signals that accompany inflammation and infection. Danger signals can be either endogenous (e.g. cytokines, CD40-Ligand) or exogenous (e.g. microbial constituents) (61).
To detect the presence of pathogens, DC are equipped with so called "pattern recognition receptors (PRR)". These receptors, mainly represented by the family of Toll-like receptors (TLR) and C-type lectins (CLR), recognize well conserved pathogen-associated molecular patterns (PAMP) (reviewed by (73, 74)). CLR (e.g. mannose receptor, DEC205, DC-SIGN) bind glycosylated antigens and internalize these for processing and antigen presentation. Ligation of TLR on the other hand leads to maturation of the DC, reflected by increased cytokine and chemokine secretion, migration of the DC to the lymph nodes, enhanced antigen processing and loading on MHC, and elevated surface expression of co-stimulatory molecules (e.g. CD80, CD86). Together with peptide-MHC complexes these co-stimulatory molecules and cytokines are necessary to activate T cells in an adaptive immune response (Fig. 3).
In the absence of inflammation, peptides from degraded self-proteins are presented by immature DC. These immature DC lack co-stimulatory molecules that are needed for T cell activation. Therefore they do not induce an effective immune response but instead maintain T cell tolerance (reviewed by (72)). The presentation of self peptides by immature DC results in apoptosis or induces a state of unresponsiveness called anergy in responding cognate T cells. Antigen presentation by immature DC may also induce the expansion and differentiation of immunosuppressive/ regulatory T cells (Treg) (reviewed by (75, 76). Treg play an important role in the negative regulation of immune responses by tuning down the activity of other T cells, APC and natural killer (NK) cells. Treg may either derive from the thymus (e.g. naturally occurring Treg) or develop in the periphery after immune activation (e.g. adaptive/ induced Treg). Treg can suppress other immune cells in either an antigen dependent or an antigen independent fashion and may require direct cell-cell contact (mainly natural occurring Treg) or exert their effect trough inhibitory soluble factors such as TGF-β and IL-10 (mainly adaptive T reg).

When DC interact with T cells in a cognate manner, an immune synapse (IS) is formed between these cells (reviewed by (77, 78). The first antigen-independent contact of DC with T cells is largely driven by the integrins ICAM-1 on DC and ICAM-3 on T cells which bind to LFA-1 on T Cells and DC-SIGN on DC respectively (reviewed by (79)). Subsequently, when specific peptide-MHC

complexes on the DC are recognized by TCR, both the DC and T cell may rapidly organize their cell surface receptors and form a "mature" synapse. On the T cells, supra-molecular activation complexes (SMAC) are formed: A central cSMAC lined by a ring or peripheral pSMAC. On the T cells the cSMAC contains the TCR, the co-stimulatory receptor CD28 and associated signaling molecules. The pSMAC contains the integrin LFA1 and associated molecule Talin. Analogously, on the DC a central accumulation of MHC and co-stimulatory molecules is surrounded by a ring of ICAM-1. The exact function of the high organization of the mature synapse is not yet known. It is postulated that TCR signaling is initiated at microclusters in the periphery of the contact site between T cells and APC (80, 81). Upon migration of engaged TCR into the cSMAC, signaling is attenuated by TCR internalization and subsequent lysosomal degradation (reviewed in (82)). In support of this scenario, it has recently been found that the E3-ubiquitin ligase responsible for TCR ubiquitination, c-Cbl, is enriched in the immune synapse (83). Ubiquitination of the TCR triggers its endocytosis and sorting to lysosomes (reviewed by (26)). In general the IS is likely to be an important site for local containment of factors released and exchanged between cognate interacting cells. For example, polarized secretion of lytic granules by cytotoxic CD8+ T allows selective killing of the target cell and not neighboring cells. Recently, also some cytokines like IL-2 and IFN-γ, but not IL-4 or TNF, were found to be secreted in a polarized fashion into the IS (84) between CD4+ Th cells and APC. Notably, not only soluble factors but also transmembrane receptors such as the TCR and the INF-γ receptor are directionally delivered to the contact site between T cell and APC (85, 86). Likewise, DC may deliver MHC complexes and co-stimulatory molecules in a directional fashion to the IS. Upon T cell engagement the MHC class II compartments may form tubules that polarize towards the DC/T cell contact site (87). Previously, Kleijmeer and colleagues had shown that LPS stimulation of DC triggers the formation of long tubular organelles as consequence of fusion of the MVB LV with the delimiting membrane of this compartment (17). Consistent with this observation, T cell directed tubulation in DC also requires TLR ligation (88).
Together, these findings suggest that the IS allows concentrated and contained transfer of signals from one participating cell to the other, thus facilitating specific and optimal signal delivery to target cells.

DC exosomes

Because DC have such a prominent regulatory role in the immune system, exosomes from these cells have been well studied. Although, a physiological function for DC exosomes has yet to be demonstrated, they have already been used in clinical trials addressing their potential use for anticancer therapy (see below).

DC exosomes contain many proteins essential for DC function, including MHC class I, MHC class II, the co-stimulatory molecule CD86 and adhesion molecules like ICAM-1 and CD11a/b/c ((8, 89, 90). Exosomes isolated from cultured DC contain functional MHC class I and MHC class II and have been shown to stimulate both CD4+ T cell and CD8+ T cell responses in vivo (1, 57). In vitro, isolated DC exosomes were a source of peptide-MHC class II complexes but required the concomitant presence of mature DC for efficient activation of T cells (1, 57). The mature recipient DC was found to deliver the co-stimulatory signals CD80 and CD86 that are needed for T cell activation. Based on these studies it is thought that DC exosomes spread antigen-specific signals to neighboring DC, thereby amplifying primary adaptive immune responses (1). The requirement for acceptor DC, however, was recently demonstrated not to be compulsory, as exosomes containing specific peptide-loaded MHC class I molecules were capable of activating CD8+ T cells (91, 92). The first observations on DC exosome immunocompetence were all based on studies using exosomes produced by immature DC. Later it became clear that exosomes from mature DC bear a much greater capacity to stimulate naïve CD4+ T cells, compared to those from immature DC (93). Mature DC exosomes contain more MHC II, CD86 and ICAM-1 than their immature counterparts. The increased amount of ICAM-1, but not CD86 was responsible for their elevated immunocompetence. Exosomes from mature DC still required the presence of mature recipient APC, but at high concentrations, these exosomes were able to induce T cell proliferation by themselves, albeit not very efficient (93). ICAM-1 is a ligand for LFA-1 and MAC-1, which are both expressed on DC, T cells and B cells. Thus the high concentration of ICAM-1 on mature exosomes could support exosome binding to recipient DC as well as to T cells.

After binding to the cell surface of a recipient cell, exosomes may either remain associated or fuse with the acceptor plasma membrane. Alternatively, exosomes may be endocytosed and fuse with membranes in the endocytic tract or be targeted to and degraded in lysosomes. DC efficiently internalize exosomes and process and present exosome derived peptides to T cells (94). Peptides can be presented either on endogenous MHC class II of acceptor DC (94) or on exogenous MHC class II present on the exosome (1). Fusion of endocytosed exosomes may occur in endocytic compartments in analogy to the back-fusion of the LV with the MVB limiting membrane in the LPS stimulated DC (17)(see above). In conclusion, our current knowledge on the characteristics of DC exosomes suggests that they may have a physiological role in the initiation of adaptive immune responses but their precise function remains elusive.

Clinical applications of DC & DC exosomes

Because of their key role in the initiation of both innate and adaptive immune responses and in the maintenance of peripheral T cell tolerance, DC are of major interest for cancer, transplantation and autoimmune disease related immunotherapies (reviewed in (95-98). Therapies that make us of the DC's ability to process antigens and present resulting peptides to T cells, are currently being tested. For example, mature DC presenting tumor antigens could be used for tumor eradication. On the other hand the ability of immature DC to induce tolerance to allogeneic antigens and auto-antigens may be used to prolong allograft survival and break autoimmunity respectively. Although several case reports indicate that vaccination with DC in humans may result in immune responses and even tumor regression (reviewed in (95), success rates are still limited. A great number of variables (e.g. DC type, route of administration, choice of antigen and adjuvant, maturation state of the DC, dose and timing) influence the outcome of DC induced immune responses and complicate optimization and standardization of DC vaccination protocols.

Exosomes on the other hand are highly stable, easily stored and contain a distinct set of proteins making them more defined and easier to manipulate than DC themselves. The ability of DC exosomes to bind to other DC and in this way amplify immune responses (see above) may further increase their suitability for immunotherapy. Several reports already indicate that DC exosomes loaded with tumor-specific antigen can be used to trigger an anti-tumor immune response in mice and humans (reviewed by (99)). In mice, DC exosomes loaded with tumor derived peptides induced potent CD8+ T cell-mediated immune responses, resulting in eradication of established tumors (49). Particularly in combination with TLR 3 and 9 ligands, exosomes efficiently induced an anti-tumor responses in mice (100). These promising results in mice led to the development of preparation techniques for the clinical testing of tumor antigen loaded DC exosomes in human advanced cancer patients (101). To date two clinical trials have been performed in humans with advanced staged melanoma and non small cell lung cancer respectively (102, 103). These phase I studies demonstrate that exosome production from ex vivo cultured autologous DC is feasible, that administered exosomes were well tolerated and that in some cases stabilization of disease occurred.

In addition to cancer immunotherapy, it was recently shown that exosomes also can serve as a cell-free vaccine for protective immunity against invading pathogens, such as Toxoplasma gondii ((104), and may help to prevent sepsis (105).

DC exosomes are not only effective in inducing immunity. Allogeneic exosomes can decrease or even prevent allograft rejection when administered prior to

transplantation (106). This effect may be explained by the presentation of donor MHC that induce allograft tolerance in the recipient ((106) and references therein). Similar to what is shown for DC, therapies using DC exosomes should be carefully designed, as the conditions at which exosomes induce immunity or tolerance are not yet fully clear. As can be expected, however, the maturation state of the DC secreting the exosome (93) and the concomitant presence of TLR ligands (100) may ultimately determine the outcome of immune responses.

B cells & B cell exosomes

Introduction into B cell biology

B lymphocytes or B cells, like DC, are professional antigen presenting cells capable of activating naïve CD4 + T cells (reviewed by (107)). Their main function, however, lies within the humoral immune response, fighting extracellular pathogens through the secretion of antibodies (for a detailed overview see (63, 108)). Only activated B cells, designated plasma cells, secrete antibodies. Resting B cells need two signals for their differentiation into plasma cells. The first signal involves antigen recognition by the B cell receptor (BCR). The BCR is a membrane-bound immunoglobulin that associates with specific antigens. Ligation of the BCR is followed by internalization of the BCR and associated antigen and its subsequent targeting to MIIC for protein processing and loading of the resulting peptides onto MHC class II. In this way the B cell displays on its cell surface, in complex with MHC class II, a repertoire of peptides derived from the ingested antigen and associated molecules (e.g. viruses, bacteria). The initial interaction with T cells is specified through the association of TCR with peptide-MHC class II complexes. Full B cell activation, however, requires a second signal involving the ligation of B cell CD40 to CD40 ligand (CD40L; CD154) on the surface of DC activated CD4+ T cells. In some cases the second signal for B cells activation may also be provided by TLR ligation and such antigens are designated “thymus independent (TI) antigens”. However B cells activated by TI antigens secrete less potent antibodies and no memory B cells are formed.

B cells that are fully activated with the help of specific T cells proliferate and migrate into the B cell area of the lymph node where they form a germinal centre (reviewed by (108, 109)). Inside this germinal centre the activated B cells, again with the help of CD4+ T cells also reside there, proliferate further while undergoing the processes of isotype switching and affinity maturation. These processes highly increase the effectiveness of the produced antibodies. This so called germinal centre reaction results in the generation of plasma cells that secrete large amounts of high affinity antibodies.

B cell exosomes

Cultured human EBV transformed B cells secrete as much as 10% of their total MHC class II through exosomes over a period of 24 hours (7), in contrast to DC that typically secrete only 0.1% of their MHC class II in 24 hours (8). Because of their high steady state exosome production EBV transformed B cells are ideal to study exosome characteristics (39). B cell exosomes contain many proteins, including tetraspanins (CD37, CD63, CD81, CD82), HSP70 and HSP90, actin and tubulin (38, 39) that are ubiquitously found in exosomes from other sources. Like DC exosomes, B cell exosomes also contain proteins involved in antigen presentation, including MHC class I, MHC class II and CD86, and have been described to elicit antigen specific T cells responses in vitro (7, 38). At the B cells plasma membrane MHC class II is clustered with the co-stimulatory molecule CD86 and tetraspanins (CD9,CD81, CD82) in TEM which may provide a scaffold for efficient antigen presentation of specific peptides (110, 111) or downstream signaling events (112). These clusters at the plasma membrane are not included into DRM (39). In contrast, MHC class II in both exosomes and at MVB is associated with tetraspanin (CD81, CD82, CD63) containing DRM (39). MHC class II may associate with DRM already prior to peptide loading when entering the MVB from the biosynthetic route (113), providing one explanation for the association of MHC class II with DRM on exosomes. Alternatively, ligand binding may lead to association of MHC class II with DRM. as has been demonstrated to occur after the stimulation of APC with MHC class II specific antibodies (112) and for the recruitment of MHC class II to lipid rafts at the IS (114). MHC class II ligation also triggers downstream signaling with the help of CD38 and CD9 (112) and concomitantly recruitment of these molecules into DRM may facilitate that process. Ligand-induced receptor cross-linking and/ or raft association may also lead to internalization of the receptor and its subsequently targeting to late endosomes/MVB and thus explain the presence of DRM associated MHC class II on exosomes. Regardless of the circumstances during which these TEM and DRM are formed, their presence on exosomes, like on the plasma membrane (110, 111), may promote antigen presentation and T cell stimulation.

The integrins VLA-4 and ICAM-1 are also found in B cells exosomes and these may enable B cell exosomes to bind to acceptor cells and extracellular matrix components (39, 58). Exosome were shown to bind follicular dendritic cells (FDC) from human tonsils in vitro (115). In vivo, FDC carry numerous exosome resembling MHC class II positive microvesicles at their cell surface but do not synthesize MHC class II themselves. These vesicles probably represent B cell exosomes from MHC class II expressing B cells that are abundantly present in the B cell follicle. It cannot be excluded, however, that they derive from other MHC class II expressing cells, such as macrophages or DC.

Implications of exosomes produced by other immune cells

Mast cells secrete MHC class II and HSP containing exosomes and these can activate B and T lymphocytes and DC (116-118). Possibly, mast cell exosomes amplify immune responses by binding to and activating DC and in this way may present antigens derived from mast cell exosomes to T cells. Mast cell exosomes contain high levels of HSP as compared to exosomes from other cells and have been proposed to activate DC trough the adjuvant activity of HSP (117). The binding of mast cell exosomes to DC is dependent on the HSP receptor, CD91.
Yet another cell that has been proposed to use exosomes to confer antigenic information to DC, is the intestinal epithelial cell IEC. Although IEC are non-professional APC, they express MHC class II, which can be loaded with food-derived peptides. IEC secrete exosomes that contain functional MHC class II peptide complexes but lack co-stimulatory molecules at their basolateral surface (119). It has been postulated that IEC transfer antigens from the intestinal lumen to DC that, depending on a local immune stimulatory or inhibiting environment, can trigger immunogenic or tolerogenic T cell responses respectively (119).
T cells secrete exosomes as well. In response to TCR ligation, both human CD4+ and CD8+ T cells secrete exosomes, which contain the TCR, LFA-1, MHC and CD2 but not CD28 and CD45 (10). T cell-derived exosomes can associate to APC and this may alter the functional properties of the APC (120). Both activated Treg and effector T cells donate molecules to APC and, analogous to the function of the donor T cell, acceptor APC are endowed with tolerogenic or T cell stimulatory properties respectively (120). Cytotoxic CD8+ T cells contain secretory lysosomes/MVB that hold perforin and granzymes, which are used for target cell killing. Upon association with a target cell MVB from CD8+ T cells fuse with the plasma membrane releasing their lethal content and LV (121) towards the target cell.

Immunological effects of exosomes from tumor cells

Many neoplastic cells secrete vesicles, some of which are exosomes (reviewed by (3)). Tumor antigens can be efficiently transferred by tumor derived vesicles to DC, which may subsequently present these to T cells. Both anti-tumor (12, 89) and tumor promoting (122, 123) effects of tumor-derived exosomes have been described. The tumor promoting effect is attributed to the expression of Fas ligand on tumor exosomes, that may trigger apoptosis of CD8+ T cells (122, 123) and this might be part of the immune evasion strategy of tumors. These results imply that

the use of tumor derived exosomes for cancer treatment should be addressed with care.

Exosomes from many sources, including tumor-derived exosomes, contain HSP (see above). HSP efficiently bind a multitude of proteins, including those that are formed as a result of proteolysis. When released into the extracellular space either by secretion or cell lysis, HSP-peptide complexes may be taken up by APC, processed and presented by MHC. In addition, certain HSP can activate immune cells through ligation to TLR (reviewed in (124)). HSP are well conserved during evolution and microbial and mammalian HSP are highly homologous. Mammalian HSP associate with TLR on DC and are implicated in many autoimmune disorders. HSP are released in the extracellular space by necrotic rather than apoptotic cells and necrosis is associated with tumor growth and tumor treatment. Released HSP is associated with tumor-derived antigens and when taken up by an APC may induce specific anti-tumor immune responses. The presence of HSP in DC exosomes may also be relevant for the observed anti-tumor effect of tumor-peptide loaded DC exosomes (8, 49). HSP in the lumen of endocytosed exosomes may be released into the endosome/lysosome as a consequence of membrane destabilization/degradation and subsequently may be loaded onto MHC class II. Alternatively, the content of endocytosed exosomes may perhaps be released into the cytosol as a consequence of the fusion of exosomes with the endosomal limiting membrane. In the latter situation the antigens delivered to the cytosol may be cross-presented on MHC class I.
Three recent reports demonstrate that incorporation of cytosolic HSP into B cell, T cell, peripheral blood mononuclear cell and B lymphoma cell exosomes is greatly increased when cells are exposed to heat, and that this is accompanied by a modest increase in exosome secretion (125-127). The immunostimulatory capacity of exosomes from heat shocked B cells was limited however, as they were not capable of inducing DC maturation. HSP that were targeted to exosomes in heat-shocked cells were mainly found in the exosomal lumen and thus unable to bind to DC TLR directly. In contrast, exosomes produced by heat-shocked B-lymphoma cells were found to be highly immunogenic (127). However, in the latter situation, exosomes displayed, in addition to HSP, increased levels of MHC complexes and co-stimulatory molecules. Therefore it remains unclear whether HSP were responsible for the increased immunogenic capacity of exosomes from heat shocked B lymphoma cells.

Exosomes and virus budding

The budding of enveloped viruses like human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and hepatitis C virus (HCV) can occur either at the plasma membrane or at MVB (128-130) reviewed by (131). Virus budding at the plasma membrane is topologically identical to budding into the lumen of the MVB. Irrespective of the site of budding these viruses make use of the ESCRT machinery, which is also required for the formation of MVB LV (reviewed in (132) and (133)). Retroviral GAG protein associates with ESCRT components, thereby recruiting this machinery to the site of budding. Several other viruses, including filo viruses (e.g. Ebola), rabdo viruses (e.g. VSV, vesicular stomatitis virus) and influenza viruses, also use the ESCRT machinery for their budding, indicating a wide involvement in virus assembly (133). In macrophages, HIV has been found to coexist with LV in tetraspanin and MHC II containing MVB (129). HIV was even found to contain host proteins normally associated with LV in MVB and exosomes, such as MHC complexes, integrins and tetraspanins (reviewed by (134). Like exosomes, viruses might use these components for attachment to and/or fusion with target cells. These findings have resulted in speculations concerning the relation between exosomes and viruses (135). In the “Trojan exosome hypothesis” it was proposed that viruses have hijacked the pre-existing non-viral exosome biogenesis pathway for their formation, release and uptake (135). Although there are many similarities between retroviral budding, secretion and uptake and corresponding processes for exosomes, others think these speculations extend to far (133). In this respect, particularly the question whether virus budding occurs predominantly at MVB or the plasma membrane is debated. Nonetheless, the similarities are intriguing and predict that for further elucidation of exosome behavior and function, important lessons may be drawn from virus behavior.

Thesis outline

Although extensively studied over the past decade, to date physiological functions for DC or B cell exosomes have not yet been demonstrated. Furthermore, many mechanistic aspects of exosome formation and targeting remain unclear. To shed some light on these topics we studied the targeting of MHC class II to exosomes and the regulation of exosome secretion in DC. In addition we used B cell exosomes to study the exosome proteome and protein-protein interaction in exosomes.
In the second chapter of this thesis we report on the presence of poly-ubiquitinated proteins in B cell and DC exosomes and discuss the potential implications for the targeting of cytoplasmic proteins to exosomes. In chapter 3 we performed a comprehensive analysis of the proteome of B cell derived exosomes and demonstrate that MHC class II on these exosomes is associated to large protein complexes that contain many distinct components and may have implications for exosome formation, targeting and function.
In chapter 4 we describe for the first time that release of exosomes can be triggered in response to cognate cell-cell contact. We demonstrate that upon cognate interaction with a CD4+ T cell, DC secrete and transfer exosomes to the interacting T cell and we discuss the potential functional implications of this process.
In chapter 5 we show that during DC maturation, ubiquitination of the MHC class II is decreased, allowing its recruitment to the cell surface. Furthermore, in agreement with the need for ubiquitination for targeting of MHC class II to MVB, we demonstrate that ubiquitination of MHC class II is also required for its sorting into exosomes. A Cognate interaction between DC and T cells results in translocation of MHC class to the cell surface, as well as increased release of MHC class II carrying exosomes. The differential regulation of these processes in DC in response to T cells suggests that separate MVB may exist for storage of “ready to use” cell surface proteins and for secretion of exosomes.

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Chapter 2

Exosomes contain ubiquitinated proteins

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Abstract

Multi vesicular bodies (MVB) are endosomal compartments that contain multiple vesicles, which derive from a delimiting membrane by inward budding. Incorporation of membrane proteins into the luminal vesicles requires, at least for some model proteins, mono-ubiquitination of their cytoplasmic domain. The ubiquitin tags are recognized by a sorting machinery, of which some components are also mono-ubiquitinated. The ubiquitin tags and the sorting machinery are both removed before the vesicles bud into the MVB lumen. MVB vesicles are therefore not expected to contain mono-ubiquitinated proteins. The MVB content is degraded upon fusion of MVB with lysosomes. In many cell types, however, MVB can also fuse with the plasma membrane, resulting in secretion of their luminal vesicles into the extracellular milieu. Such secreted vesicles are termed exosomes, and their protein composition should, due to their origin, be identical to that of MVB luminal vesicles. We here demonstrate that exosomes contain poly-ubiquitinated proteins, many of which are not integrated into the membrane and relatively enriched as compared to total cell lysates. These results suggest that a subset of poly-ubiquitinated cytoplasmic proteins is incorporated into the MVB pathway. The potential cell biological relevance of this observation is discussed. Furthermore, these data indicate that ubiquitinated proteins can serve as markers for exosomes.

Introduction

Endosomal compartments receive proteins via the endocytic and the biosynthetic pathways and distribute them to distinct destinations, including the trans-Golgi network (TGN), the plasma membrane (domains) and lysosomes. The endocytic pathway comprises early sorting endosomes, recycling endosomes, late endosomes and lysosomes. Early sorting endosomes are the major entry site for endocytosed material. Late endosomes derive from early endosomes through a maturation process that involves a gradual change in contents and access for plasma-membrane-derived and TGN-derived transport vesicles (1). Multi vesicular bodies (MVB) have been described by electron microscopic studies already in the 1950s and 1960s, but it was not until the early 1980s that they were recognized as an intrinsic component of the endocytic tract (2, 3). The formation of MVB is initiated at the early endosomal state as a result of the inward budding of the endosomal delimiting membrane. During the maturation of early endosomes to late endosomes, tens or even hundreds of 60–80 nm vesicles accumulate in their lumen (1, 4), hence, the name MVB. Only recently, it was demonstrated that the MVB luminal vesicles (LV) are truly free vesicles that have completely dissociated from the endosomal delimiting membrane (4). Proteins that are not incorporated and thus retained at the MVB limiting membrane are either recycled to the TGN and plasma membrane or are delivered to the limiting membrane of lysosomes. The LV potentially have three distinct fates. First, their earliest acknowledged function is to target incorporated proteins to lysosomes for degradation, a process that requires either direct fusion of MVB with lysosomes (5, 6) or a poorly understood complex maturation process. Many membrane proteins employ this pathway for their degradation. For example, several growth factor receptors (7), e.g. the epidermal growth factor receptor (8, 9), are sorted at MVB for proteolytic degradation after ligand induced endocytosis. Second, MVB can also serve as temporal storage compartments; recently, we demonstrated that immature dendritic cells (DC) store major histocompatibility complex (MHC) class II at LV (10).Upon DC activation, the LV fuse with the MVB limiting membrane, from where subsequent transfer of MHC class II to the plasma membrane can proceed. The third possible fate of MVB occurs when their limiting membrane fuses with the plasma membrane. This process results in secretion of the LV, which are now termed exosomes (11, 12). This pathway was first discovered in maturing reticulocytes (13, 14) and observed later for a large group of different cell types, including B lymphocytes, DC, T cells, mast cells, platelets and epithelial cells. The physiological role of exosomes is still unclear, but it is likely that they function in intercellular communication. Particularly, exosomes from DC have drawn lots of attention. These bear functional MHC class I– and class II–peptide complexes and have been shown to

induce activation of specific T cells in vitro and in vivo (12, 15-17). Similarly, exosomes derived from human and murine B lymphocytes induced antigen specific MHC class II-restricted T cell responses in vitro (18). Together, these data suggest a physiological role for exosomes in antigen presentation. Incorporation of membrane proteins into the LV of MVB is a selective and regulated process; mono-ubiquitination of the cytoplasmic domain of membrane proteins can serve as a signal for their sorting at MVB (2, 3, 19). The formation of MVB LV and the sorting of cargo protein therein depend on the function of at least 18 conserved proteins that were originally identified in the yeast Saccharomyces cerevisiae (3, 19). Vps27/HRS contains two Ubiquitin Interaction Motifs (UIM) and is thought to act as an adaptor protein that binds both mono-ubiquitinated transmembrane proteins and clathrin and recruits mono-ubiquitinated membrane proteins to characteristic flat clathrin lattices on endosomal vacuoles (20, 21). Vps27/HRS also interacts with ESCRT I (Endosomal Sorting Complex Required for Transport), a cytosolic protein complex that is transiently recruited to the endosomal membrane and functions in the sorting of ubiquitin-tagged transmembrane proteins into the MVB pathway. Whether the flat clathrin coat is strictly required for the interaction with ESCRT-I has not yet been established. After the interaction with ESCRT-I, two other protein complexes, ESCRT-II and ESCRT-III, sequentially associate with ubiquitinated membrane proteins, resulting in further concentration of cargo proteins (22, 23). After sorting has been completed, ESCRT-III recruits the de-ubiquitinating enzyme Doa4, which removes the ubiquitin tag from the cargo transmembrane proteins prior to their incorporation into newly forming MVB vesicles (24). A multimeric AAA-type ATPase, Vps4, dissociates the ESCRT-III complex before the LV are formed. Several proteins of the MVB sorting machinery, including TSG101, a subunit of ESCRT-I, and HRS, are, like the cargo, being mono-ubiquitinated. As both the ubiquitinated components of the sorting machinery and the ubiquitin tags of the cargo are removed before MVB luminal vesicles are pinched off from the delimiting membrane, mono-ubiquitinated proteins are not expected to accumulate in MVB vesicles.
Since exosomes are secreted MVB vesicles, one would not expect them to contain mono-ubiquitinated proteins either. We report here, however, that exosomes do contain poly-ubiquitinated non-integral membrane proteins and speculate on their relevance.

Materials and methods

Cells

RN, an EBV-transformed human B-cell line RN (HLADR15), was cultured as described (18). D1, a mouse immature splenic dendritic cell line, was cultured in 35% conditioned medium from R1 cells as described (25). To remove exogenous exosomes from the culture media of RN and D1 cells, both fetal calf serum and R1 culture supernatants were ultracentrifuged at 140,000g for 60 min prior to use.

Antibodies, SDS-PAGE and Western blotting

Mouse monoclonal anti-human MHC class II (CR3/43) was from DakoCytomation (Glostrup, Denmark), mouse monoclonal anti-ubiquitin (P4D1) and mouse anti-poly-ubiquitin (FK1) were purchased from Santa Cruz Biotech (Santa Cruz, CA) and Biomol International, L.P. (Exeter, UK), respectively. Rabbit polyclonal anti-mouse MHC CLASS II-β was generously provided by Dr. Barois (26). For the analysis of exosomes and cell fractions by SDS-PAGE and Western blotting, samples were incubated for 15 min at 65°C in urea-containing sample buffer (5% SDS, 9 M urea, 10 mM EDTA, 120 mM Tris–HCl, pH 6.8) (27). For the detection of ubiquitinated proteins, pre-heated sample buffer, supplemented with 0.1% TX-100, was added directly to cells or exosomes. Proteins were separated on 12.5% (MHC class II) or 10% (Ubiquitinated proteins) polyacrylamide gels (SDSPAGE). For Western blotting, proteins were transferred from polyacrylamide gels to Immobilon-P membrane (Millipore, Bedford, MA). The membranes were blocked and probed with antibodies in PBS containing 5% (w/v) non-fat dry milk (Protifar plus; Nutricia, Zoetermeer, The Netherlands) or 0.5% gelatin from cold water fish skin (Sigma-Aldrich, St. Louis, MO) and 0.1% (w/v) Tween 20. Primary antibodies were probed with horseradish peroxidase conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories Inc., Westgrove, PA) and detected by Supersignal west pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

Exosome isolation

As a first isolation step, exosomes were collected from the cell culture medium by differential centrifugation, as described (18). In short, cells were removed by centrifugation for 10 min at 200g. Supernatants were collected and sequentially centrifuged twice for 10 min at 500g, once for 30 min at 10,000g and once for 60 min at 70,000g using a SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). Exosomes pelleted at the final centrifugation step and were resuspended in PBS

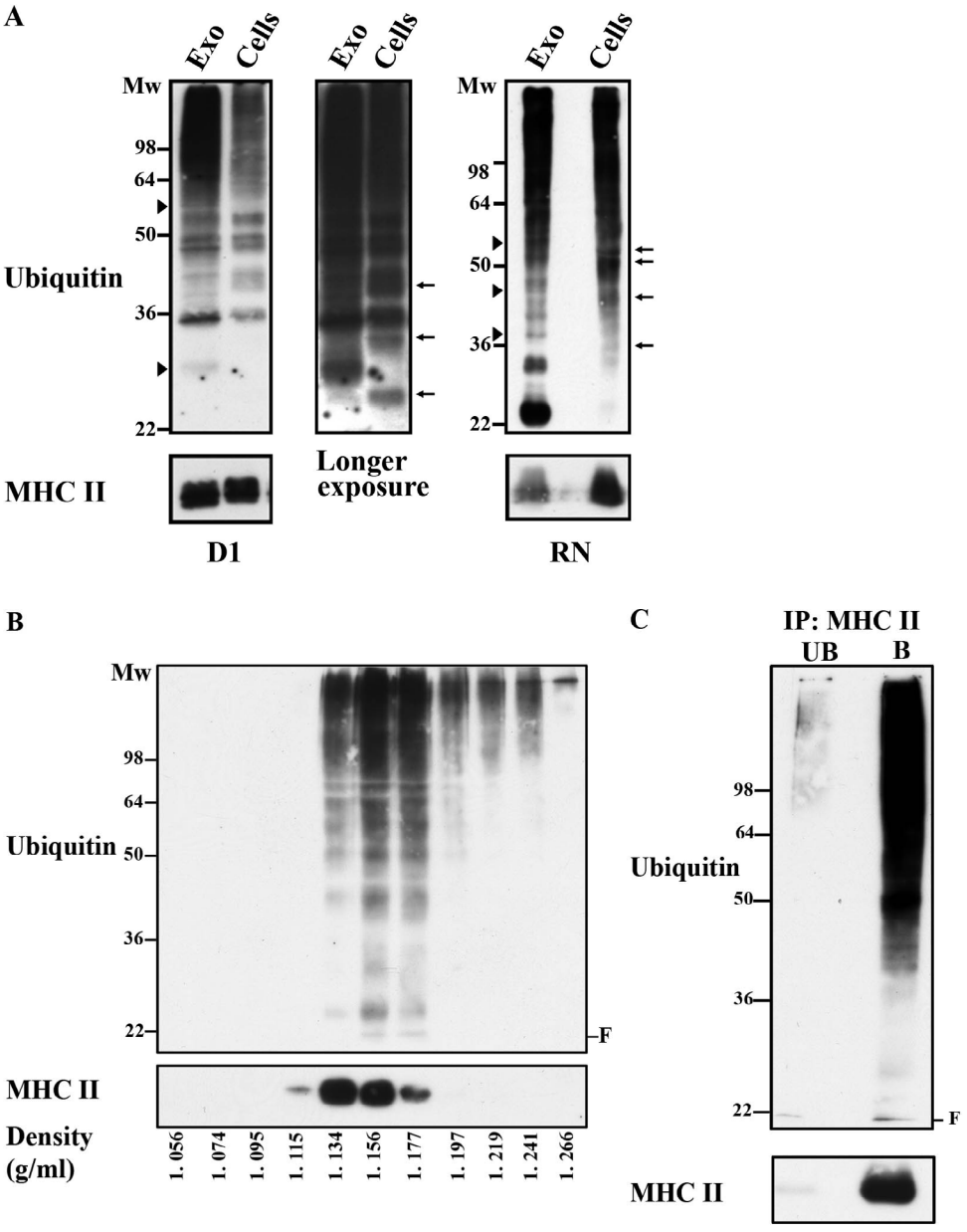
and re-pelleted at 70,000g. PBS-washed exosomes were either taken up directly in urea sample buffer for analysis by Western blotting or, when indicated, resuspended in 0.5 ml of 2.5 M sucrose, 20 mM Tris–HCl, pH 7.2, and floated into an overlaid linear sucrose gradient (2.0–0.4 M sucrose, 20 mM Tris–HCl, pH 7.2) by centrifugation in a SW40 tube for 16 h at 270,000g. Gradient fractions of 1 ml were collected from the bottom of the tube and analyzed directly for the presence of MHC class II by Western blotting. For detection of ubiquitinated proteins, gradient fractions were diluted 1:2 with PBS, centrifuged for 60 min at 110,000g in a TLA-55 rotor (Beckman Instruments, Inc., Fullerton, CA) and the pellets were analyzed by Western blotting. For immunoabsorption of exosomes, Dynabeads M-450 (8 x 10^7 beads) coated with monoclonal mouse anti-human MHC class II (Dynal Biotech, Oslo, Norway) were washed, resuspended in PBS containing 1 mg/ml bovine serum albumin and added to pooled exosome-containing gradient fractions 1:4 (V/V). Samples were rotated end-over-end for 16 h at 4°C after which the beads were collected and washed twice with PBS using a magnet (Dynal Biotech, Oslo, Norway). Non-adsorbed membranes were diluted with PBS and collected by centrifugation at 110,000g for 60 min in a TLA-55 rotor.

Carbonate treatment of exosomes

Pelleted exosomes, as obtained by differential centrifugation, were resuspended either in 1 M Na2CO3, pH 11.5, or, for control samples, in 20 mM Tris–HCl, pH 7.4, and incubated for 30 min at 37°C. In Na2CO3, closed vesicles are converted to open membrane sheets, and content proteins and peripheral membrane proteins are released in soluble form (28). Subsequently, membranes were pelleted at 100,000g for 1 h at 4°C, washed with 20 mM Tris, pH 7.4 by centrifugation and taken up in sample buffer for analysis by Western blotting.

Results and discussion

In this study, we used two cell types, D1, a growth factor dependent long-term mouse DC culture (25), and RN (18), a human B-cell line. Cell culture media were first cleared from non-exosomal cell debris by differential centrifugation steps up to 30 min at 10,000g. Subsequently, exosomes were pelleted by centrifugation for 60 min at 70,000g (18). In line with previous observations (17, 18), exosomes from both sources, like the cells that secrete them, contained MHC class II (Fig. 1A). Within cells, MHC class II is abundantly present at the internal vesicles of MVB (10, 29), consistent with the concept of exosome formation and secretion. Cell lysates and exosomes were probed for the presence of ubiquitinated proteins by Western blotting using P4D1, an antibody that binds both mono- and poly-ubiquitinated proteins. Both for cells and exosomes, the upper part of the gels revealed smears of label, indicative for heavily poly-ubiquitinated proteins, which are poorly resolved by SDS-PAGE. The low molecular weight range of the blots (64–22 kDa), however, also showed discrete bands, indicative for mono- or oligo-ubiquitination. Although the composition of ubiquitinated proteins in exosomes may overlap with that of total cell lysates, striking differences were observed, suggesting that ubiquitinated proteins are incorporated selectively into the MVB pathway. Exosomes display a characteristic buoyant density of 1.15 g/ml in sucrose density gradients, in contrast to many other membranes, such as plasma membrane fragments shed from (apoptotic) cells (30). Ubiquitinated proteins floated to the same fractions of sucrose density gradients as MHC class II (Fig. 1B), consistent with their presence in exosomes. To determine this unambiguously, floated exosomes were purified further by immunoadsorption using magnetic Dynabeads, coated with antibodies directed against MHC class II. Ubiquitinated proteins were adsorbed with equal efficiency as MHC class II (Fig. 1C), indicating their presence in exosomes.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50
36
22
Ubiquitin
F
MHC II

Fig. 1: Exosomes from B cells and DC contain specific ubiquitinated proteins.
(A) MHC CLASS II and ubiquitinated proteins from D1 or RN cells and exosomes isolated from cell culture media were detected by Western blotting as indicated. For D1, exosomes loaded were secreted in 24 h by 300x the amount of cells loaded. For R1, exosomes loaded were secreted in 5 days by 1000x the amount of cells loaded. The ubiquitin blot of the D1 samples is shown at two distinct exposure times for optimal comparison. In addition to MHC CLASS II, many distinct ubiquitinated proteins are detected in exosomes from both RN cells and D1 cells. The composition of ubiquitinated proteins in exosomes (as exemplified by arrows) is distinct from that in cells (as exemplified by the arrowheads). Molecular weight markers are indicated in kDa. (B) Exosomes were collected from isolated RN cell culture media by differential centrifugation as in panel A and subsequently floated into a sucrose density gradient. Ubiquitinated proteins and MHC CLASS II were detected by Western blotting in the same fractions of the gradient (densities indicated in g/ml). Molecular weight markers are indicated in kDa, the front of the gel by F. (C) Exosomes from RN cells were isolated by floatation as in panel B and subsequently immuno-adsorbed to anti-MHC CLASS II-coated Dynabeads. Like MHC CLASS II, ubiquitinated proteins were efficiently adsorbed to the beads (B) as compared to the unbound (UB) fraction. Molecular weight markers are indicated in kDa, the front of the gel by F.

Ubiquitinated proteins in exosomes are not membrane-integrated

To determine whether ubiquitinated proteins are intrinsic membrane proteins, exosomes were treated with alkaline carbonate (Fig. 2). This condition is known to convert sealed membrane vesicles to open sheets and release their contents except for integral membrane proteins (28). As expected, actin, a cytoplasmic protein, but not MHC class II, a transmembrane protein complex, was extracted by carbonate. Ubiquitinated proteins, except for some heavily ubiquitinated proteins migrating in the upper part of the gel, were released from exosomes by the carbonate treatment, indicating that they were not integrated into the membrane.

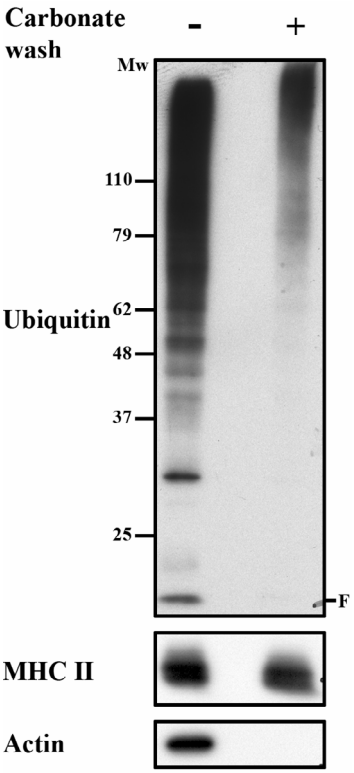

Fig. 2: Ubiquitinated proteins can mostly be extracted from exosomes.
Exosomes from RN cells were collected by differential centrifugation and incubated in 1 M carbonate pH 11 to expose their contents and allow dissociation of non-integral membrane proteins. Carbonate stripped exosomes were collected by centrifugation at 100,000g (+) and compared with an equivalent sample of non-treated exosomes (-) by Western blotting for ubiquitin, MHC CLASS II β and actin, as indicated. The membrane protein MHC CLASS II β remained associated with carbonate-stripped exosomes. In contrast, actin, as a representative of cytosolic proteins, as well as most of the ubiquitinated proteins dissociated from carbonate-treated exosomes. Molecular weight markers are indicated in kDa, the front of the gel by F.

Many proteins in exosomes are poly-ubiquitinated

To distinguish poly-ubiquitinated proteins from mono-ubiquitinated proteins, we used FK1, a monoclonal antibody that binds poly-ubiquitinated proteins only (31), and compared its labeling pattern on Western blots with that for P4D1, which binds both poly- and mono-ubiquitinated proteins (Fig. 3). For total cell lysates, some overlap in labeling pattern was observed, although P4D1 labeled a number of discrete bands that were absent in the FK1 blots. Such bands likely indicate mono-ubiquitinated proteins. For exosomes, a clear signal was observed using FK1, indicating many poly-ubiquitinated proteins. Although the labeling pattern for FK1 with that for P4D1 showed differences in relative labeling intensities, we did not observe proteins that were exclusively labeled with P4D1. We conclude that many of the ubiquitinated proteins in exosomes are poly-ubiquitinated but do not exclude the possibility that mono-ubiquitinated proteins are also present. Poly-

ubiquitination is traditionally associated with degradation of cytoplasmic proteins by proteasomes. Mono-ubiquitination on the other hand has been associated with diverse proteasome-independent cellular functions, including delivery of transmembrane proteins into the MVB pathway. De-ubiquitination itself is not mandatory for receptor sorting as ubiquitin can confer sorting after being fused in frame with a cargo protein (32). Thus, although the majority of ubiquitinated proteins may be de-ubiquitinated after sorting at MVB and prior to their actual incorporation into luminal vesicles, some may escape de-ubiquitination and end up in exosomes. Membrane proteins such as the epidermal growth factor and platelet-derived growth factor receptors are driven for sorting at MVB, however, by mono-ubiquitination at multiple sites rather than by poly-ubiquitination (31). Moreover, we observed that the majority of the ubiquitinated cargo is not integrated into the exosome membrane. What then could be the physiological relevance of our observation that poly-ubiquitinated non-integral membrane proteins are associated with exosomes? Poly-ubiquitinated proteins (as stained with FK1) were previously shown to accumulate on endosomes in response of EGF induced EGF-R down-regulation by immunofluorescence microscopy [9]. Another study demonstrated the presence of poly-ubiquitinated proteins on the same endosomal microdomains as HRS (33), consistent with a role for poly-ubiquitinated proteins in MVB sorting. Yet, another indication is the requirement of proteasomes; proteasome inhibitors prevent mono-ubiquitinated receptors from entering the MVB pathway without interfering with the formation of MBV themselves (34, 35). This is not due to a reduction of the pool of free ubiquitin since ubiquitination of the EGF-R is efficient even in the presence of proteasome inhibitors (35). Finally, hepatocyte growth factor-dependent Met receptor sorting and phosphorylation of HRS are inhibited by proteasome inhibitors, and this is overcome by overexpression of ubiquitin but not K48R ubiquitin, a mutant that interferes with poly-ubiquitination (36). Together, these observations could be explained with the hypothesis that poly-ubiquitinated proteins regulate MVB sorting. Incorporation of regulatory poly-ubiquitinated proteins into the MVB pathway would explain their presence in exosomes. Alternatively, it cannot be excluded that poly-ubiquitinated proteins in exosomes reflect cytoplasmic cargo, possibly in majority to be degraded in lysosomes. Given the specific composition as compared to total cell lysate (Fig. 1), such cargo might be incorporated specifically. Cytosolic poly-ubiquitinated non-integral membrane proteins are, however, generally thought to be degraded by proteasomes. Nevertheless, the presence of poly-ubiquitinated proteins in exosomes may reflect uptake by micro-autophagy. That ubiquitination may play a role in micro-autophagy has been suggested previously by the observation that ubiquitin–protein conjugates are highly enriched in primary lysosome-related granules as compared to the cytosol (37). In conclusion, our data indicate that ubiquitinated proteins can

serve as valid markers for exosomes. Understanding of the physiological relevance of the presence of cytoplasmic poly-ubiquitinated proteins in exosomes requires further research.

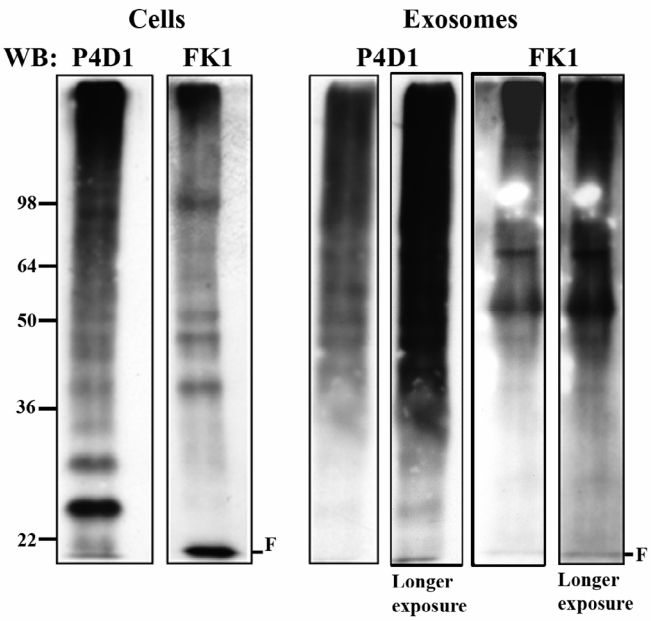

Fig. 3: Most ubiquitinated proteins in exosomes are poly-ubiquitinated.
Exosomes isolated from RN culture media were analyzed by Western blotting for ubiquitinated proteins using two distinct antibodies, P4D1 (detects mono- and poly-ubiquitinated proteins) and FK1 (detects poly-ubiquitinated proteins only). P4D1 and FK1 display overlapping labeling patterns, indicating that ubiquitinated proteins in exosomes are largely poly-ubiquitinated. Molecular weight markers are indicated in kDa, the front of the gel by F. Exosome blots are shown at two distinct exposure times for optimal comparison.

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Chapter 3

A Comprehensive analysis of the proteome of B cell exosomes and MHC class II-associated proteins: implications for exosome biogenesis and function

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Manuscript in preparation

Abstract

We here present a detailed proteomic analysis of B cell exosomes using highly sensitive and accurate mass spectrometry. In total 681 proteins were identified, including known and novel constituents. Many of the exosome-associated proteins are involved in sorting at multi vesicular bodies, illustrating the role of this intracellular compartment in exosome biogenesis. Furthermore, using a quantitative proteomics approach, we found that MHC class II in exosomes is in large protein complexes, which in addition to MHC class II, contain at least 24 other proteins. The potential function(s) of these protein complexes may relate to protein sorting at multi vesicular bodies or, alternatively, clustering of membrane receptors and integrins may facilitate targeting to and signaling at exosome recipient cells.

Introduction

Multivesicular bodies (MVB) are endosomal compartments which, as they mature, accumulate up to hundreds of luminal vesicles (LV) that derive from the delimiting membrane by inward budding (1). Proteins that are recruited from the MVB delimiting membrane to the LV potentially have distinct fates. Membrane proteins such as the EGF-receptor can, after ligand induced endocytosis and sorting at MVB, be targeted to and degraded in lysosomes (2). Similarly, MHC class II in dendritic cells (DC) and B cells may be targeted to lysosomes for degradation, therewith reducing its half-life. Alternatively, MVB may serve as temporal storage devices for MHC class II (3). Finally, MVB can fuse with the plasma membrane resulting in the release of LV, which are then called exosomes, into the extracellular milieu (1). Recently, exosomes gained interest because of their potential role as cell-free, membrane-associated intercellular transport and signaling devices (4, 5), their similarity to HIV and other viruses with respect to their formation (6), and their potential application as cancer vaccines (7-9).
Exosomes are secreted by various cell-types, including epithelial cells, B cells and DC (1, 4, 10, 11). In contrast to intracellular organelles, exosomes can be isolated to exceptional high purity from cell-culture media or extracellular body fluids such as urine, lymph, semen and blood (3, 12-15). Although physiological functions of exosomes remain to be elucidated, in recent years many *in vitro* and *in vivo* effects of isolated exosomes have been reported, in particular for exosomes from antigen presenting cells (APC).
For example, DC-derived exosomes can serve as vehicles for the transfer of peptide-MHC class II complexes between DC (16). DC-derived exosomes have also been demonstrated to stimulate adaptive MHC-restricted immune responses *in vivo* and have been used successfully in mouse tumor immunotherapy assays (7). Furthermore, the first phase-I clinical trials using autologous DC-exosomes for vaccination against human cancers have recently been published (17). Also B cells, when activated, secrete large amounts of exosomes, which have been suggested to bind to follicular dendritic cells (FDC) in the lymph node (4).
The molecular composition of exosomes from several sources has been studied in order to understand their physiological role. Analysis of exosomes from DC (18), semen (19) and urine (20) revealed a relatively non-complex proteome. Among the proteins found so far, many may be involved in immune functions, such as antigen presentation (e.g. MHC class I and II, co-stimulatory molecules and adhesion molecules). Interestingly, many proteins with a known function in protein sorting at MVB, such as components of the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, are also present in exosomes. A comprehensive analysis of the exosome proteome is therefore not only relevant to elucidate the

function of exosomes, but may also yield clues on the molecular mechanism of MVB formation and protein sorting at MVB. Previously we identified 21 of the most abundant proteins on B cell derived exosomes (21). In addition, we found that some of these proteins, including MHC class II and tetraspanins, associated with detergent resistant microdomains (DRM) on exosomes but not on the plasma membrane (21).
Here we report on a comprehensive analysis of highly purified B cell derived exosomes and of MHC class II- associated proteins. We discuss the putative involvement of these protein complexes in MVB sorting and/or exosome function.

Methods

Cell culture

The EBV transformed human B cell line RN (HLA-DR15) was cultured as described (22). The culture medium was supplemented with fetal calf serum that had been depleted from bovine exosomes and other potential large contaminants by centrifugation for 60 min at 100,000*g* (SW28 rotor, Beckman).

Exosome isolation

As a first isolation step, exosomes were collected from the medium by differential centrifugation, as described (22). In short, cells were removed by centrifugation for 10 min at 200*g*. Supernatants were collected and centrifuged sequentially twice for 10 min at 500*g*, once for 30 min at 10,000*g*, and once for 60 min at 70,000*g* using a SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). Exosomes were pelleted at the final centrifugation step and resuspended in 0.5 ml 2.5M Sucrose, 20 mM TRIS HCl pH 7.4 and floated into an overlaid linear sucrose gradient (2.0-0.25 M sucrose, 20 mM Tris-HCl, pH 7.2) in a SW40 tube for 16 h at 190,000*g*. Gradient fractions of 1 ml were collected from the bottom of the tube and analyzed for the presence of MHC class II by Western blotting. As a final purification step, exosomes from pooled exosome-containing gradient fractions were immuno-adsorbed to Dynabeads M-450 coated with the monoclonal mouse anti-human MHC class II antibody clone HKB-1 (Dynal Biotech, Oslo, Norway), or to CNBr-activated sepharose 4B that had been coupled to mouse monoclonal anti-MHC class II clone L243 was kindly provided by Dr. Neefjes (NKI, Amsterdam, the Netherlands) either in the absence or presence of CHAPS. Both HKB-1 coated Dynabeads and L243-coated sepharose beads were first extensively washed with and resuspended in PBS supplemented with 5 mg/ml bovine serum albumin before

addition to the exosome pool. For adsorption, samples were rotated end-over-end for 16 h at 4 °C. The Dynabeads beads were collected with the aid of a magnet (Dynal Biotech, Oslo, Norway) and washed once with PBS. Sepharose beads were collected by centrifugation for 1 minute at 300g. After washing proteins were eluted from the beads in urea-containing exosome sample buffer (5% SDS, 9 M urea, 10 mM EDTA, 120 mM Tris-HCl, pH 6.8) and incubated for 15 min at 65 °C prior to separation on an SDS gel.

Western blotting

Proteins were separated on 12 % polyacrylamide gels (SDS-PAGE). For Western blotting, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked and probed with antibodies in PBS containing 5% (w/v) non-fat dry milk (Protivar; Nutricia, Zoetermeer, The Netherlands) and 0.1% (w/v) Tween 20. Mouse monoclonal anti-MHC class II (CR3/43) and horseradish peroxidase-conjugated Goat anti-mouse antibodies were all purchased from DAKO (Glostrup, Denmark). Mouse monoclonal anti-CD81 (JS64) was from Immunotech (Marseille, France). For detection on film, enhanced chemiluminescence (Pierce Supersignal West Pico Chemiluminescent Substrate) was used.

Mass Spectrometry

Proteins from isolated exosomes were separated on a 4-12% Tris/Glycine NuPAGE gradient gel (Invitrogen). Gels were fixed in 5% Acetic acid/30% methanol and stained using GelCode Blue reagent (Pierce). After o/n incubation in MilliQ water, gel-lanes were cut into 24 equally-sized slices which were subjected to in-gel trypsin digestion as described (23). After digestion, 20 to 80% of the supernatants was used for LC-MS/MS analysis on a ThermoFinnigan FT-ICR equipped with a 7 Tesla magnet coupled to an Agilent Series 1100 binary pump system (Agilent Technologies, Waldbronn, Germany). Peptide mixtures were trapped on an in-house packed 5 cm x 100 μm AquaTM C18 reversed phase column (Phenomenex) at a flow rate of 5 μl/min. Peptide separation was achieved on an 15 cm x 75 μm AquaTM C18 reversed phase column using a gradient of 0 to 70% solution B (solution A = 0.1 M acetic acid; solution B = 80% [v/v] acetonitrile, 0.1 M acetic acid) in 60 min. at a constant flow rate of 200 nl/min.

Data analysis

Finnigan *.raw files were converted to *.dta files using BioWorks software, version 3.1 SR1 (Thermo Electron Corporation). For this process the program was set to track the scan limits automatically and calculate for peptides with a mass from 300 to 5000 AMU, automatically detecting the charge state and MSn level. The threshold was set to 100 counts. Subsequently, Mascot generic files were generated through in-house developed software. These files were used to search the IPI_Human 3.10 database on an in-house Mascot server (24), allowing up to 2 missed cleavages, a peptide mass tolerance of 5 ppm and a fragment mass tolerance of 0.5 Da. Peptide modifications used in the searches were Carbamidimethyl on cysteine (fixed) and oxidation on methionine, tryptophan and histidine (variable). Next, Mascot *.dat files were imported into the Scaffold program (Proteome Software, Inc, Portland, OR, USA), simultaneously analyzing the data with the X!Tandem database search algorithm (25-27) incorporated into this program. For this analysis the same settings were used as for the Mascot searches, except for the peptide mass tolerance, which was set to 0.008 AMU. Protein identifications displaying a minimal protein confidence of 99% and matched with least two peptides with individual peptide confidence scores higher than 95% in the Scaffold analysis were assumed significant. For protein identifications based on two or less (unique) peptides, spectra were investigated manually.

O^{18}/O^{16}-based quantitative mass spectrometry

Two samples (total exosome preparation and CHAPS-treated exosomes) were loaded onto a 12 % SDS polyacrylamide gels. Gels were run until stacked proteins had just entered the separating gel. For each sample a single band containing all proteins was cut out of the top of the running gel and subjected to in gel tryptic digestion as described (23). After digestion, buffer was collected and 100 µl acetonitrile was added to the gel pieces for 10 minutes at RT for additional peptide extraction. After 10 minutes, the supernatant was combined with the digestion buffer and the total sample was dried in a vacuum centrifuge. Prior to the labeling procedure, water from 60 µl samples of digestion buffer (50 mM Tris-HCl, 50 mM $CaCl_2$) was evaporated in a vacuum centrifuge and reconstituted in normal MilliQ (O^{16}) or 98% H_2O^{18} (Spectra Stable Isotopes, Colombia, MD, U.S.A.), respectively. Dried peptide samples were reconstituted in 50 µl O^{16} or O^{18} containing digestion buffer, and added to dried immobilized trypsin beads (10 µl starting volume; Pierce). Acetonitrile was added to a final concentration of 20 % and the mixture was incubated o/n at RT while shaking at 1,200 rpm. After o/n labeling, trypsin

beads were removed by centrifugation at maximum speed for 1 minute in an eppendorf centrifuge and the supernatant was collected. Residual trypsin activity was eliminated by adding 0.2 M dithiotreitol and incubating samples for 60 minutes at 37°C. Subsequently samples were added to dried urea/iodoacetamide (6M/ 0.75mM final concentration) and incubated o/n at RT in the dark. O^{16} or O^{18} labeled samples were pooled, cleaned up using the STAGE tip procedure (28), dried in a vacuum centrifuge and reconstituted in 0.1% TFA before analysis by LC-MS/MS as described above. Results from LC-MS/MS analysis were used to search the IPI human database as described above, including the double O^{18} label as a variable modification. Raw data files and Mascot html results pages were loaded into the MSQuant program (29) adapted for O^{16}/O^{18}-based quantitative analysis. All quantified peptides were checked by manual inspection of the spectra used for quantification.

Results

Identification of 681 proteins in exosomes

To analyze the proteome of exosomes excreted by human B cells, exosomes were purified from tissue culture medium of RN cells using a previously established three-step purification protocol, yielding highly purified exosomes (21). First, exosomes were isolated from the B cell culture supernatant by differential centrifugation. Subsequently, exosomes were floated into a sucrose gradient by ultracentrifugation. Finally, exosomes were immuno-adsorbed from exosome containing gradient fractions using anti-MHC class II coated Dynabeads. The presence of exosomes during each purification step was monitored by Western blotting (not shown).

To analyze the proteome of isolated exosomes, proteins were first separated by SDS-PAGE. For each sample, the entire lane was cut into 24 equally sized gel-pieces which were subjected to in-gel tryptic digestion. Resulting peptides were separated by liquid chromatography and analyzed by highly accurate and sensitive MS/MS using an LTQ-FT. This method resulted in the identification of in total 681 proteins in human B cell derived exosomes that were reproducibly recovered in three independent experiments. These proteins were grouped into putative cytosol or membrane localization (Fig 1) and into functional classes (table 1 and supplementary data table 1).

The vast majority of identified proteins are of cytoplasmic of origin (508 proteins; 75% of all proteins) and 23% are integrated or associated to membranes. Most identified proteins are involved in general homeostatic processes, including protein

synthesis (13.4%), protein folding (chaperones; 4.85%), metabolism (metabolic enzymes, 10. 9%), signal transduction (16.8%), membrane traffic (10.4 %) or are part of or associated with the cytoskeleton (8.8%). Proteins that are thought to be involved in MVB/exosome formation were also identified and grouped separately (2.4 %). The latter group includes ubiquitin, the ubiquitin activating enzymes E1 and E2, several human homologues of yeast ESCRT complex components, accessory proteins involved in MVB biogenesis, such as Alix and a Bro1 domain containing protein, and several proteins with yet unclear implications for ubiquitin mediated sorting. Furthermore, we identified a large number of proteins with known functions in intercellular adhesion (4.1%) or immune regulation (8.5%) that may have important implications for exosome targeting and function, including integrins, immunoglobulins, tetraspanins, several T cell co-stimulatory molecules and MHC molecules (table 1).

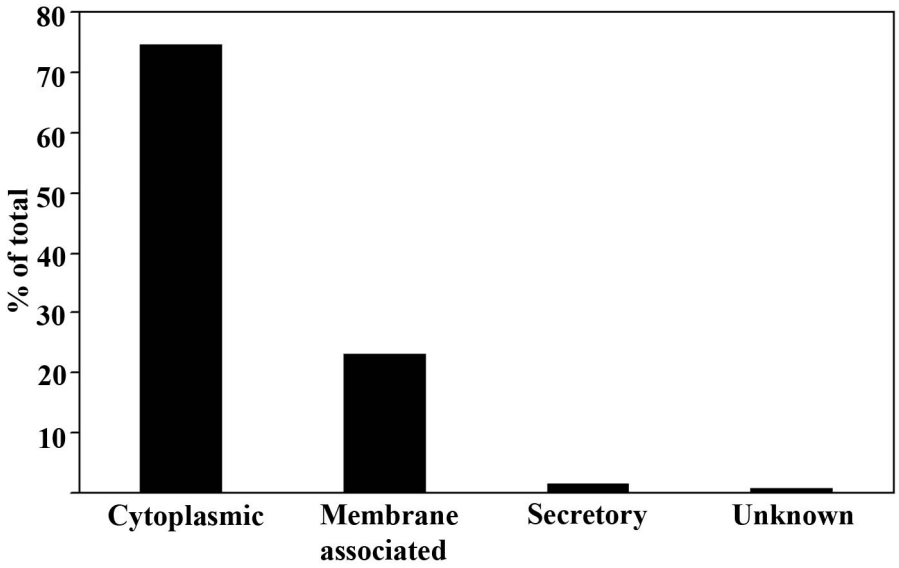

Fig. 1: Subcellular distribution of identified proteins.
Proteins from table 1 were classified according to their subcellular distribution: cytoplasmic, membrane associated or secretory. The class of cytoplasmic proteins includes all proteins that localize to the cytoplasm. Also proteins that (transiently) associate with membranes by means of protein-protein interactions are classified "cytoplasmic". The class of membrane associated proteins includes all transmembrane proteins and those that directly associate with membranes via lipid anchors. Proteins that preferably reside in the lumen of the endocytic/secretory tract are denoted as "secretory".

MHC class II associates with large protein complexes on exosomes.

Among the proteins identified are several tetraspanin proteins. In a previous study we demonstrated that, from exosomes but not from the plasma membrane, MHC class II and tetraspanins were resistant to solubilization in the detergent CHAPS. To test whether tetrapanins and/or other exosome proteins associate with MHC class II in DRM, exosomes were first immuno-isolated using anti-MHC class II coated Dynabeads. Bead adherent exosomes were subsequently incubated in the absence or presence of 1% CHAPS for 1 hour at 0°C. Subsequently beads were washed and adherent proteins were analyzed by Western blotting. Not only MHC class II but also the tetraspanin CD81 remained associated with the beads after CHAPS solubilization (Fig. 2A), indicating that MHC class II and CD81 were associated with each other. Analysis of the immunoprecipitated complexes on silver stained SDS gels revealed that several other proteins also remained adherent to the beads after CHAPS treatment, while others were completely lost. Control IgG coated beads did not bind any proteins (Fig. 2).
Previously, using floatation experiments, we had already observed that only about 30% of both MHC class II and CD81 was associated with DRM after CHAPS treatment (21). The present data, however, show that virtually all CD81 remained associated with MHC class II after CHAPS solubilization of exosomes, suggesting that MHC class II containing protein complexes may exist independently of associated lipids. Possibly, MHC class II and CD81 were initially associated with DRM which gradually lost their lipid content after prolonged solubilization.

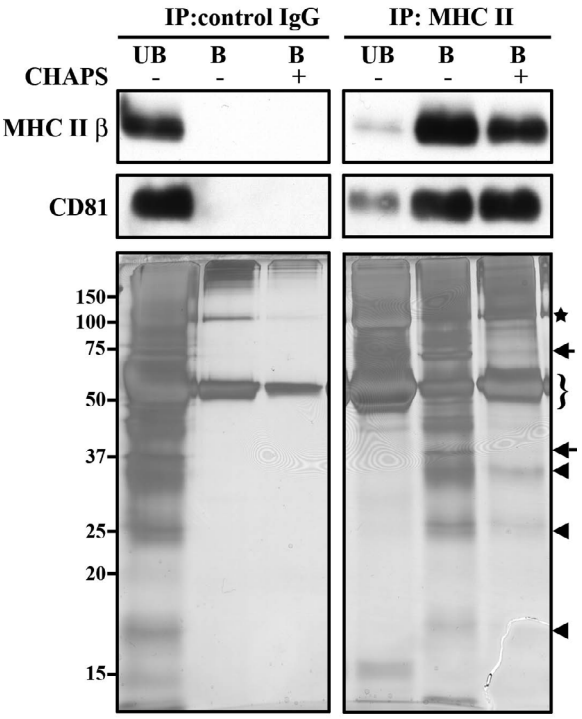

Fig. 2: Association of proteins with MHC class II after detergent extraction.
Exosomes were isolated by differential centrifugation followed by floatation into an overlaid sucrose gradient. Floated exosomes were allowed to bind anti-MHC class II (Mab HKB1) coated dynabeads or control IgG coated dynabeads. Bead associated exosomes were collected using a magnet. Unbound exosomes were collected from the bead supernatant by centrifugation (UB). Immuno-adsorbed exosomes (B) were solubilized (+) or not (-) by CHAPS for 1 hour at 4 ºC. Proteins that remained associated to the beads were analyzed by SDS-PAGE followed by Silver staining (lower panel) or Western blotting for MHC class II β and CD81 as indicated. Arrows and arrowheads in the lower panel indicate proteins that were lost during CHAPS treatment or remained, respectively. The asterisk and brace indicate the positions of IgG and BSA respectively.

To investigate this further we solubilized exosomes in CHAPS and allowed DRM to float into a sucrose gradient by ultracentrifugation for either 4 hours or 16 hours (Fig. 3A). After CHAPS solubilization, about 30% of exosomal MHC class II and CD81 floated to low density into an overlaid sucrose gradient during 4 hour ultracentrifugation, consistent with earlier observations (21). In contrast, after centrifugation for 16 hours most MHC class II and CD81 was recovered in fractions at the bottom of the gradient and a minor amount in fractions at

intermediate densities (Fig. 3A). This indicates that MHC class II/ CD81 containing protein complexes gain density during the course of centrifugation, probably reflecting loss of lipids from the complexes, suggesting that the protein complexes were stable independent of associated lipids. This was further illustrated by the observation that even after overnight solubilization in CHAPS, nearly all MHC class II and CD81 could be pelleted by ultracentrifugation (Fig. 3B). Together, these findings suggest that CD81 and MHC class II and most likely other proteins associate with each other by means of (direct or indirect) protein-protein interactions rather than by lipid-mediated interactions.

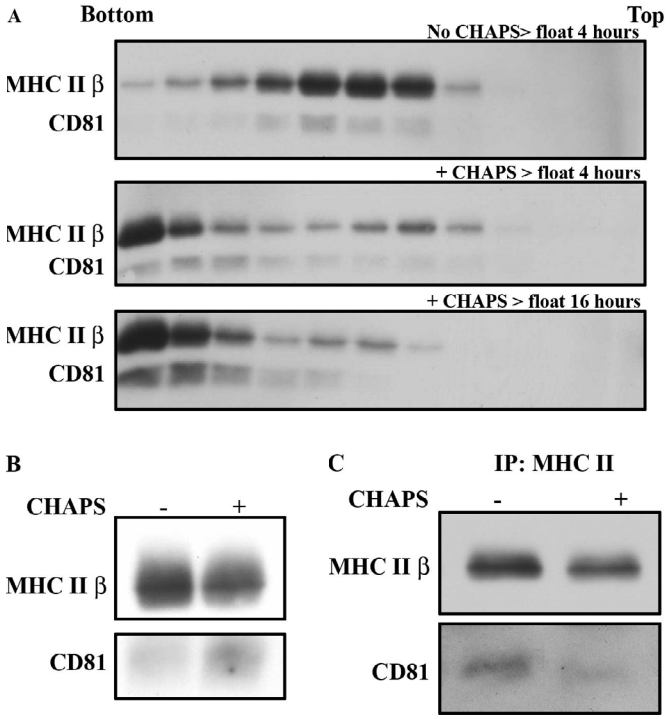

Fig. 3: MHC class II and CD81 in exosomes are associated with large protein complexes, independent of lipids

A: Exosomes were isolated by differential centrifugation, incubated for 30 minutes in the presence or absence of CHAPS as indicated and overlaid with a sucrose gradient and ultracentrifuged in parallel either for 4 or 16 hours as indicated. Gradient fractions were analyzed for the presence of MHC class II and CD81 by Western blotting. B: Isolated exosomes were incubated overnight at 4 ºC in the presence or absence of 1% CHAPS and subsequently ultra-centrifuged at 100,000xg. Pellets were analyzed for MHC class II-β and CD81 by Western blotting. C: MHC class II from isolated exosomes

was immuno-adsorbed in the absence or presence of 1% CHAPS for 16 hours. Adsorbed proteins were analyzed by Western blotting for MHC class II-β and CD81.

To investigate the composition of these protein complexes, MHC class II was immuno-isolated from CHAPS solubilized exosomes and associated protein complexes were analyzed by MS. Both MHC class II and CD81 were immuno-adsorbed with almost equal efficiency in the presence of CHAPS as compared to intact exosomes (Fig. 3C). To analyze the total proteome of solubilized MHC class II-containing complexes, we performed O^{18}-based quantitative MS (30, 31), a method for the quantitative comparison of two protein samples, in which protein direved peptides are labeled either with O^{18} or O^{16} at their C-terminus. Peptides from equally sized samples of MHC class II, immuno-adsorbed in the presence or absence of CHAPS, were labeled with either O^{16} or O^{18}. Subsequently O^{16} and O^{18} labeled samples were mixed and submitted to LC-MS/MS. Using this approach, in total 200 proteins were identified (data not shown), which is significantly less compared to the 681 proteins identified using the previous approach. The main reason for this is in the latter approach samples were not segregated by SDS-PAGE prior to LC-MS/MS. Analysis of the unmixed samples showed that the incorporation of O^{18} was 98% efficient, matching the purity of the H_2O^{18} water used for labeling (data not shown).
In accordance with the Western blot analysis (Fig. 3C), quantatative MS showed that 59% of MHC class II was precipitated from CHAPS solubilized exosomes relative to intact exosomes. As expected, a range of proteins was co-immuno-isolated with MHC class II. To validate these data and to exclude non-specific binding, we repeated the experiment using another antibody against MHC class II (L243), which was coupled to sepharose rather than Dynabeads. A similar set of proteins was precipitated using HKB-1 coated Dynabeads as compared to L243 coated sepharose beads (data not shown). In table 2 we set the relative amount of MHC class II (HLA DRβ) that was precipitated in the presence of CHAPS at 100 % and expressed the amount of co-precipitated proteins relative to this value. We included only those proteins that in the presence of CHAPS, were co-immunoprecipitated with MHC class II using HKB-1/ Dynabeads with an efficiency of at least 20% as compared to MHC class II and for which association was confirmed using L243-sepharose. According to these criteria we found 24 proteins that associate with MHC class II (table 2). As expected, MHC class II-α (HLA-DRα) which forms a heterodimeric complex with MHC class II-β (HLA DRβ), was precipitated with equal efficiency (126 ± 35 %).

Table 2: Proteins in MHC class II containing complexes
MHC class II-associated proteins were immuno-isolated from intact and CHAPS solibilized exosomes as in figure 3B. Precipitated proteins were analyzed by O^{18}-based quantitative MS. The amount of each protein that remained associated with MHC class II in CHAPS solubilized exosomes was compared to the amount found in intact exosomes. Association was expressed relative to the recovery efficiency of MHC class II (HLA DR β), which was set at 100% ± standard deviation (SD). The SD relates to differences between recovered peptides. Only those proteins that remained associated for at least 20% with HKB1 Mab in the presence of CHAPS are shown. All proteins shown in this table also associated with L243 Mab coated beads in the presence of CHAPS.

protein name	accession number	% associated with HLA DR beta 1
HLA class II		
HLA DR beta 1	IPI00449584	100 +/- 32
HLA DR alpha	IPI00005171	126 +/- 35
HLA DP	IPI00103082	27 +/- 3
HLA DQ	IPI00640031	34 +/- 19
Other proteins		
LFA-1 alpha/ CD11A	IPI00025380	24 +/- 6
peroxiredoxin-1	IPI00000874	30 +/- 26
Rap1b	IPI00015148	32 +/- 9
HLA class I alpha	IPI00472800	34 +/- 7
HSP90	IPI00334775	38 +/- 4
EF2	IPI00186290	39 +/ 7
Basigin/ CD147	IPI00019906	40 +/- 10
CD70/ TNFSF7	IPI00031713	40 +/- 14
HLA class I alpha	IPI00472855	42 +/- 27
HLA class I alpha	IPI00472592	44 +/- 8
B2M/ HLA class I beta	IPI00004656	49 +/- 31
CD98/ 4F2	IPI00027493	50 +/- 7
CAPZA1	IPI00005969	52 +/- 4
Rho GDI	IPI00003817	55 +/- 36
Na/K ATP ase alpha	IPI00006482	56 +/- 17
Hsc 71	IPI00003865	56 +/- 27
TfR	IPI00022462	63 +/- 9
EHDC4	IPI00005578	72 +/- 8
CD20	IPI00007880	81 +/- 13
Na/K ATP ase beta	IPI00179529	92 +/- 21
Tubulin	IPI00011654	93 +/- 35
HBA	IPI00410714	106/ +/- 38

Among the associated proteins are HLA DQ and HLA DP, which are recognized only by the HKB-1 antibody but not by the L243 antibody. Their presence in both immuno-isolations thus indicates that they associate in protein complexes with HLA DR. Other MHC class II associated proteins, include MHC class I, CD20, LFA-1 and CD70, all of which are involved in immune regulation. However, also proteins involved in more diverse cellular processes were found to associate with MHC class II. These include, tubulin, transferrin receptor (TfR), CD98, CD147, Na^+/K^+ ATPase, heat shock proteins (HSP), small GTPases and others (table 2).

Discussion

We have identified 681 proteins in highly-purified B cell-derived exosomes using a combination of highly-sensitive LC-MS/MS and two database search algorithms, Mascot and X!Tandem. Furthermore O^{18} based quantitative MS led to the identification of 24 proteins that associate with MHC class II in large protein complexes on exosomes.

LV of MVB and thus also exosomes form by membrane budding away from the cytosol into the endosomal lumen. As a result, both membrane-associated proteins and cytosolic proteins are incorporated. We here identified 508 cytosolic proteins and 157 membrane-associated proteins, which we categorized according to their known or proposed functions.
Sorting of membrane proteins into LV is tightly regulated as will be discussed below. Cytosolic proteins on the other hand may reach the LV merely because of their presence in the cytosol, explaining the large diversity of cytosolic exosome-associated proteins (Fig. 1). Alternatively, some cytosolic proteins may be actively targeted to exosomes by associating to membrane proteins. Finally, cytosolic proteins involved in MVB biogenesis and dynamics may locally be highly concentrated at the time of LV formation, resulting in their incorporation into exosomes.

Exosomes were isolated from the culture media of B cells, and because exosomes are the sole organelles that are excreted by cells, impurities from other cellular constituents are expected to be limited. However, it cannot be excluded that cellular debris from lysed or apoptotic cells is also present in the culture medium. The three-step exosome purification procedure, however, including differential centrifugation, floatation and immune precipitation, highly reduces contamination of our samples with non-relevant proteins. The identification of precursors of nuclear and mitochondrial proteins in our exosome preparation can be explained by

the notion that they are synthesized in the cytosol and subsequently targeted to their final destinations (32, 33). A strong argument against the presence of mitochondria in our samples is that only nuclear encoded proteins and none encoded by the mitochondrial genome were found.

Proteins involved in exosome biogenesis

Formation of MVB is orchestrated by the sequential actions of a multitude of proteins that drive protein sorting, vesicle budding and vesicle fission. It is therefore not surprising that we here identified not only cargo proteins, but also elements involved in MVB biogenesis or membrane transport from other organelles to MVB. Among the latter are the clathrin heavy chain protein (vesicle coat), SNARE proteins, such as SNAP23 and syntaxins (involved in docking and fusion of vesicles), several Rab proteins (regulating the directionality and extent of vesicle traffic) (34) and components of the adaptor protein complexes 1 and 2. ESCRT proteins were already identified in exosomes from urine (20) and could also be indentified in our experiments. These proteins dissociate from the selected cargo prior to the fission of the nascent LV (35), but some may be incorporated due to local high concentrations in the cytoplasm. Their identification in exosomes may thus illustrate the inclusion of locally active cytoplasmic proteins into exosomes.
Raft-based sorting has been proposed as an alternative to ESCRT mediated sorting at MVB (36). Indications for such a process are the presence of the GPI-anchored acetylcholinesterase in reticulocyte-derived exosomes (37) and high concentrations of cholesterol, sphingomyelin and ganglioside GM3 in B cell exosomes as well as the presence of DRM (21). Here we identified several proteins known to associate with lipid rafts, including flottilin-1 and –2, phospholipid scramblase 1 and various members of the Arp2/3 complex (38-40), further suggesting a role for lipid rafts in MVB sorting.
The Arp2/3 protein complex, which is essential in the polymerization of actin and associates with Wiskott-Aldrich syndrome protein (WASP) family members (41), is also found in exosomes, possibly as a consequence of its role in lipid raft-mediated and clathrin-mediated sorting processes (42, 43).
Sorting of membrane proteins into LV may also involve members of the tetraspanin family of proteins, which are consistently found in exosomes regardless of their origin (20, 44-46). Also in our present analysis several members of this family were identified, including CD9, CD37, CD53, CD81, CD82 and CD59. Tetraspanins are associated with a variety of functions, such as cell motility, membrane fusion and signaling (47, 48). They have the property to form so-called tetraspanin webs, or tetraspanin enriched microdomains (TEM) (47, 48). These complexes are distinct from DRM or raft-domains, as they do not rely on the

presence of lipids. Our observation that the association of tetraspanins with exosome-derived protein complexes persisted after 16 hours in CHAPS, a condition that allowed dissociation of lipids, is consistent with the definition of TEM (48). In addition to tetraspanins, TEM are comprised of a network other proteins involved in signaling and adhesion. On intact B cells, TEM containing CD9, CD37, CD53, CD81, CD82 as well as other proteins that are also identified in the present study, such as the tetraspanin-like protein CD20, MHC class II and integrins, have been described (45, 49-51). Although the composition of such microdomains may vary depending on their subcellular location, our observations suggest that microdomains exist in exosomes and that incorporation into TEMs could even be a prerequisite for sorting of (some) proteins into exosomes.

Proteins with implications for exosome function and targeting

Although several studies, including the study presented here, have shed light on the molecular composition of exosomes, their biological function remains unclear. The presence of immuno-regulatory proteins in exosomes from APC has led to many studies addressing their potential role in antigen presentation. Isolated exosomes from DC have been shown capable of activating T cells, both *in vitro* and *in vivo* (52, 53). Similarly, exosomes from both human and murine B cells induced antigen-specific MHC class II-restricted B cell responses (22). In addition to MHC-peptide complexes, exosomes also harbor a variety of heat shock proteins (HSP) ((21, 44) and this paper), which may bind antigenic proteins and provide exosomes with immune-stimulatory capacities. Recently it was proposed that the exosomal pathway might represent a distinct secretory pathway for heat shock proteins that is induced in response to cellular stress (54).
For the first time we here show Notch-1 on exosomes. This cell surface receptor is implicated in the cell fate decisions and activation of immune cells (55). Notch-1 in exosomes could potentially bind to cognate ligands on target cells and induce subsequent downstream signaling (56).
Denzer et al., have demonstrated the presence of MHC class II containing exosome-like vesicles on the surface of FDC in situ and showed in vitro binding of B cell exosomes to isolated FDC, suggesting a role for B cell exosomes in the germinal center reaction (4). Here we identified several proteins that may be involved in targeting of B cell exosomes to FDC, including 6 integrin subunits (β2, β3, β7 and α4, α5, αL) that can form the integrin couples α4/β7 (LPAM-1), α5/β3 (vitronectin receptor) and αL/β2 (LFA-1). Other lymphocyte adhesion molecules we identified are ICAM-1 and 3, LFA-3, CD9, CD22, CD43, CD44, CD48 and CD166. It has previously been demonstrated that integrins on exosomes can be functional and may allow exosomes to bind extracellular matrix or cells (46).

Moreover, an increased concentration of ICAM-1 on DC exosomes, in response to DC maturation has been shown to greatly enhance their immunogenicity (57). Integrins on B cell exosomes potentially could serve a similar role.

Although speculative, it is an appealing thought that exosomes may be capable of fusion with target cells, either directly with the plasma membrane or with endosomal membranes after endocytic uptake. Interestingly, two of the tetraspanins identified in this study have been proposed to play a role in membrane fusion events and therefore may promote exosome fusion with target cells. CD9 was proposed to enhance sperm-egg fusion (58-60) and both CD9 and CD81 were shown to promote muscle cell fusion (61) and are instrumental for certain viruses to promote cell-cell fusion (62). Both proteins are also implicated in virus-cell fusion as CD9 is the putative receptor for feline immunodeficiency virus and CD81 is implicated in hepatitis C entry (63). We also identified CD98, an integrin binding amino acid transporter that is ubiquitously expressed in all human cells (reviewed in (64). Among other functions, CD98 has been implicated in syncytium formation and virus-cell fusion. The capacity of CD98 to bind and activate β1 and β2 integrins may in part be responsible for these effects, but the precise mechanisms are not yet clear (65, 66). It has been suggested, however, that integrin avidity may be enhanced through CD98-induced clustering of integrins into complexes (67). The identification of both CD98 and integrins on B cells exosomes suggests CD98 may increase integrin activity and avidity on exosomes and thus promote the binding and/or fusion of these exosomes with target cells. Finally, we identified CD147 (also named Basigin or EMMPRIN) on exosomes which, in concert with CD98 and in analogy to its role in virus-cell or cell-cell fusion (68), may be important for exosome targeting.

MHC class II-associated protein complexes

In the present study we show that MHC class II in exosomes is incorporated into large protein networks. Previously we have already shown that part of exosomal MHC class II and tetraspanins reside in DRM (21). Here we demonstrate these DRM increase in density with time, probably as a result of loss of lipids. Nevertheless, the protein complexes remain intact suggesting that lipids are not required for inter-molecular linkages. Indeed 24 different proteins remained associated to MHC class II for at least 16 hours in the presence of CHAPS, with efficiencies ranging from 24-100% (table 2). Although we restricted our report to proteins of which at least 20% remained associated with MHC class II, we cannot exclude that exosomal proteins with a lesser association to or higher dissociation rate from MHC class II may be of importance as well. Specific associations with

MHC class II were illustrated by co-isolation using two different MHC class II antibodies and by the lack of precipitation using control beads (Fig. 1).
To our surprise we found hemoglobin A (HBA) derived peptides highly associated to exosome derived MHC class II (106 %). HBA is not synthesized by B cells, but B cells and other APC may constitutively present peptides derived from HBA in the medium on MHC class II (69). All peptides we used for identification were conserved in Bovine and Human HBA, suggesting that peptides derived from bovine serum HBA were loaded onto MHC class II. Together with the complete co-isolation of MHC class II-α, this finding validates the quantitative aspect of the method used.

Among the identified MHC class II-associated proteins, Hsp70 and Hsp90 draw our special interest. As indicated above, HSP are involved in immune regulation as they can potentially bind and transfer antigenic proteins via exosomes to target cells. Furthermore, HSP have been shown to be involved in cellular sorting events. In exosomes from maturing reticulocytes, Hsc70 is proposed to target the transferin receptor (TfR) to exosomes for its removal from the cell (70-72). Hsc70 also binds to and disassembles clathrin-lattices, which are important for intracellular protein-transport processes, including MVB formation/sorting (73). In addition Hsc73, associates with cytosolic misfolded proteins (44) and addresses these to lysosomes (74). Both Hsc70 secretion through exosomes and its association with DRM are induced in stressed cells (54, 75), suggesting that targeting of HSP to exosomes may involve DRM. Together with the present identification of HSP in large MHC class II-containing protein complexes these observations may point to a role for HSP in the targeting of proteins to MVB/exosomes via DRM/ lipid-raft-based sorting.
Also the TfR was found to associate with MHC class II containing protein complexes. In contrast to maturing reticulocytes which remove TfR from the cell via exosomes (37), other cell types, including B cells, efficiently recycle endocytosed TfR to the plasma membrane and only little is present on MVB or exosomes (22). The quantitative method we used here, however, was not designed to measure the enrichment of proteins in exosomes versus plasma membrane, but rather to determine how much of exosomal TfR is associated with MHC class II. Thus, although little TfR is targeted to exosomes, approximately 60% of exosomal TfR was associated with MHC class II, possibly through a direct interaction with Hsc70.
The EH domain containing protein 4 (EHD4) was highly associated to the MHC class II protein complex (72%). EHD4 is a member of the EHD family of proteins that is involved in the modulation of endocytic processes (reviewed in (76)). Recently, its rat homologue pincher was implicated in macro-endocytosis of

neurotrophin receptors in neurons and its subsequent targeting to MVB (77). The presence of EDH4 in MHC class II protein complexes in exosomes, suggests a more general role in MVB sorting.

Several proteins involved in ubiquitous cellular functions, including cytoskeletal organization and organelle motility, were found to associate with MHC class II complexes (e.g. tubulin, CapZ, Rho GDI) in exosomes. Their association may reflect a potential role of the cytoskeleton in MVB formation and cargo selection.

At the surface of the human B cell line JY, the tetraspanin and B cell marker CD20 has previously been found to associate with MHC class II, MHC class I and tetraspanins, including CD53, CD81 and CD82 (49). Therefore, its presence in large MHC class II-containing protein complexes is not surprising. On the plasma membrane, clustering of MHC class II, tetraspanins and co-stimulatory molecules may enhance efficient antigen presentation and T cell stimulation (49). Likewise, the here reported clustering of MHC class II with tetraspanins, integrins, MHC class I and T cell interacting molecules, such as CD20 and the co-stimulatory molecule CD70, may enhance the immunostimulatory capacity of exosomes. In addition, association of co-stimulatory molecules and MHC class II with TEM may be a prerequisite for their incorporation into exosomes (see above). Tetraspanins such as CD81 (Fig. 3) and CD63 (data not shown) were not detected in the complexes by MS but could be readily identified as part of the complex on Western blots. The lack of identification of these (and possibly other) tetraspanins in the protein complexes using MS may reflect a low abundance as compared to other identified proteins.

Another protein identified in the MHC class II containing complex is CD98 (see above). Interestingly also other proteins described to interact with CD98 in a multimolecular complex regulating monocyte cell-cell and HIV-cell fusion were found in the complex, including vimentin of which only 17 % was associated (data not shown) and Hsc70 (78) as well as CD147 (68). CD98 binds to and mediates the activation of intergrins including β2 intergrins like LFA-1 (66). Intriguingly, also the α-L subunit of LFA-1 and downstream effector Rap1b were found to associate, suggest that CD98 may function as a scaffold for clustering high avidity activated integrins on exosomes. The importance of integrins for exosome function was recently demonstrated for ICAM-1 on exosomes from activated DC, which greatly enhance their potential for T cell stimulation (57).

Finally, both the α and the β subunits of the Na^+/K^+ ATPase, a ion-pump involved in cellular ion homeostasis, were found to associate with MHC class II containing complexes. Apart from its function as an ion-pump, Na^+/K^+ ATPase may have a signaling function through an interaction with other membrane proteins, including SRC, thereby activating cell-growth and gene expression (reviewed in (79). Cell surface expression of the Na^+/K^+ ATPase is regulated by the binding of ligands,

including ouabain and dopamine, which induce its endocytosis and subsequent lysosomal targeting (80-83). Endocytosis and sorting of Na^+/K^+ ATPase to exosomes may involve its incorporation into DRM/rafts, caveolea and/or clathrin coated vesicles but also 14-3-3 proteins and the ubiquitin system (80-83).

In summary, we here describe a comprehensive analysis of the proteome of highly purified B cell exosomes using sensitive and accurate mass spectrometry. Many of the identified proteins are potentially involved in MVB dynamics or immuno-regulation. The identification of multi-component protein networks containing MHC class II, provided us with many clues towards potential functions of its components. The constituents of these complexes will be the main focus of future research as they might help us to unravel unknown aspects of MVB/exosome biogenesis and function.

Table 1: The proteome of B cell exosomes.
B cell exosomes were isolated using a three-step purification method that included differential centrifugation, floatation into sucrose density gradients and immuno-isolation. Highly purified exosomes were analyzed using LC-MS/MS. Identified proteins were grouped according to their main (putative) function. Numbers between brackets refer to the protein numbers in table S1 (on added CD).

Adhesion
CD11A (Integrin alpha-L; 73.1);CD18 (Integrin beta-2; 48.1);
CD18 (Integrin beta-2; 48.1);
CD31 (PECAM-1; 391.1);
CD36 (SCR B; 138.1);
CD37 (149.1);
CD43 (162.1);
CD44 (54.1)
CD49D (Integrin alpha-4; 90.1)
CD50 (ICAM-3; 178.1)
CD51 (Integrin alpha-5; 253.1)
CD53 (CD53; 183,1)
CD58 (LFA-3; 291.1)
CD61 (Integrin beta-3; 289.1)
CD9 (465.1)
CD97 (89.1)
CD98 (4F2; 29.1)
CD147 (Basigin; 73.1)
CD316 (EWI2; 124.1)
CD321(JAM-1; 255.1)
DEL1 (EDIL3; 449.1)
ESAM (460.1)
fibronectin (448.1)
Galectin-1 (333.1)
ICAM-1 (71.1)
Integrin beta 7 (72.1; 72.2)
ITG3 (457.1)
Semaphorin 4A (572.1)
Immune Response
CD19 (245.1)

Chaperones
Calnexin (584.1)
Calreticulin (568.1)
CDC37 (455.1)
cyclophilin A , B(44.1; 44.2; 313.1)
DNAJA1, 2 (531.1; 498.1)
Endoplasmin (230.1)
Hsp10 (286.1)
Hsp60 (126.1)
Hsc70 (12.1; 12.2)
Hsp70 (104.1; 200.1; 239.1; 550.1)
Hsp90 (11.1; 11.2; 11.3)
Hsp105 (308.1)
Hsp70 interacting protein (330.1)
prefoldin subunit 2 (522.1)
TBCA (431.1)
TCP1 (62.1; 80.1; 127.1; 132.1;146.1; 147.1; 167.1; 254.1)
Cytoskeleton
Actin (2.1; 2.2; 157.1)
Actin like protein 2 (209.1)
Actin like protein 3 (105.1)
AIP1/ NORI-1 (76.1; 76.2)
CAPG (510.1)
CAPZ alpha1,2 (187.1; 187.2)
CAPZ beta (82.1)
Coaactosin like 1
257.1
Cofilin (39.1)
Cofilin-2 (39.2
Coronin 1A (15.1)

CD20 (23.1)
CD21 (CR2; 434.1)
CD22 (388.1)
CD23 (67.1)
CD26 (100.1)
CD30 (282.1)
CD40 (TNFR5; 195.1)
CD46 (346.1)
CD48 (SLAM F2; 64.1)
CD59 (163.1)
CD70 (70.1)
CD74 (Invariant chain; 229.1)
CD81 (173.1)
CD82 (125.1)
CD85 (LILRB1; 524.1)
CD86 (262.1)
CD101 (359.1)
CD150 (SLAM F1; 111.1)
CD166 (367.1)
CD225 (284.1; 284.2)
CD300 (CMRF35; 220.1)
HLA class I alpha (5.1-5.7;6.1-6.3; 432.1)
HLA class I (B2M; 94.1)
HLA class I, E (218.1)
HLA class II DR alpha (43.1)
HLA class II DR-beta (14.1-14.5; 595.1)
HLA class II DQ (28.1-28.3; 91.1)
HLA class II DP alpha (212.1)
Alpha 2 macroglobulin (396.1)
BTF3 (593.1)
Dermcidin (268.1)
FKBP4 (256.1)
Iga (85.1)
IGLC1 (233.1)
LAT2 (227.1)
SLAM F6 (307.1)

Membrane trafficking

ALG-2 (267.1)
Annexin 2 (20.1; 20.2)
Dynein (318.1)
EPB41L2 (160.1)
ERM binding protein 50 (424.1)
Ezrin (10.3;10.4)
Fascin (55.1)
Filamin A (68.1)
Formin-like protein 1 (420.1)
L-plastin (8.1)
Lymphocyte specific protein 1 (438.1)
MAPRE1 (551.1)
MARCKS (123.1)
Moesin (10.1; 10.2)
Myosin 9, 10, 11, 14 (74.2)
Myosin 1E, 1G (365.1)
Myosin 1G (45.1)
Myosin light chain (222.1)
Myosin 9 (74.1)
Myosin 1C (93.1)
Myosin regulatory light chain (445.1)
Radixin (236.1)
Septin 9 (375.1)
Spectrin (95.1; 95.2; 192.1)
Stathmin (349.1)
Talin-1 (77.1)
TPT-1 (582.1)
Transgelin 2 (119.1
trombospondin 4 (47.1)
Tropomyosin 3 (225.1)
Tubulin (16.1; 16.2; 19.1; 19.2; 51.1;
URP2 (148.1)
VASP (295.1)
Vimentin (75.1)
Vinculin (172.1)
Profilin-1 (34.1)

Lipid rafts

Flottilin-1 (129.1)
Flotillin-2 (129.1)
raftlin (66.1)

Annexin 4 (26.1)	**Metabolism; enzymes**
Annexin 5 (87.1)	6PGD (269.1)
Annexin 6 (1.1; 1.2)	6-phosphofructokinase type C (191.1)
Annexin 7 (81.1)	6-phosphogluconolactonase (481.1)
Annexin 11 (56.1)	Acetyl-CoA acetyltransferase (519.1)
AP-1 (m) (504.1)	ACSL4 (408.1)
AP-2 (a) (494.1)	Acylamino-acid-releasing enzyme (387.1)
ARF 1,3,4, or 5 (102.1-102.3)	ADE2 (177.1)
ARF6 (213.1)	Adenine phosphoribosyltransferase (352.1)
ARP 2 (209.2)	Adenosylhomocysteinase (197.1)
ARP2/3 (159.1; 196.1; 204.1; 224.1; 329.1; 456.1)	Adenylosuccinate synthetase 2 (490.1)
aSNAP (581.1)	ADP-sugar pyrophosphatase (314.1)
Clathrin heavy chain (50.1)	Aldo-keto reductase family 1 (314.1)
Copine-1 (143.1)	Aldolase C (69.1)
COPZ1 (558.1)	Aldose reductase (407.1)
Coronin 7 (469.1)	Aspartate aminotransferase (370.1)
CYFIP2 (109.1)	Aspartate aminotransferase (398.1)
EHD1 (78.1)	ATP synthase alpha chain (518.1)
EHD4 (24.1)	ATP synthase beta chain (315.1)
GCP16 (235.1)	ATP-citrate synthase (247.1)
Grancalcin (500.1)	Biliverdin reductase A (505.1)
LIN 7A (451.1)	C-1-tetrahydrofolate synthase (106.1)
LIN 7C (515.1)	Citrate synthase (430.1)
M6PRBP1 (TIP47; 560.1)	Dipeptidyl-peptidase III (412.1)
Munc18B (474.1)	dUTPase (348.1)
Munc18C (281.1)	Farnesyl diphosphate synthase (323.1)
NIPSNAP1 (585.1)	FASN (201.1)
Rab2A (276.1	Flavin reductase (566.1)
Rab 5A, 5B, 5C (184.1-184.3)	Fumarate hydratase (357.1)
Rab 6A (65.3)	Gamma-glutamyltranspeptidase 1 (416.1)
Rab 7 (98.1)	GFPT1 (GFPT1)
Rab 8A (65.2)	Glucose-6-phosphate isomerase (GPI; 84.1)
Rab 9A (383.1)	Glutathione reductase (567.1)
Rab 10 (65.5)	GRHPR (557.1)
Rab 11B (112.1)	GTP:AMP phosphotransferase (535.1)
Rab 14 (65.4)	HPRT1 (402.1)
Rab 21 (443.1)	IMPDH2 (210.1)
Rab 22A (574.1)	Isochorismatase hydrolase (484.1)
Rab 35 (65.1)	Isocitrate dehydrogenase (NADP; 471.1)

Rab GDI (110.1)
Ral-A (141.1)
Rap 2A, 2B, 2C (174.1-174.2)
Ral-A (141.1)
RasGAP (133.1)
SCAMP3 (415.1)
Sec24C (483.1)
Septin-2 (Nedd5; 299.1)
SNAP23A (107.1)
SNARE protein Ykt6 (397.1)
syntaxin 7 (421.1)
Syntaxin-4 (190.1)
TMP21 (503.1)
TRAPD (556.1)
VAMP2 (380.1)
VAMP3 (533.1)
VAMP5 (215.1)
VAMP8 (271.1)
WASP (491.1)
WASP 2 (395.1)
VCP (57.1)
Metabolism; protein synthesis
AARS (151.1)
DARS (280.1)
ECH1(452.1)
EEF1A1 protein (32.1)
32.1
EF1 gamma (237.1)
EF1 delta (234.1)
EF2 (22.1)
eIF2 (447.1; 459.1)
eIF3 (378.1; 482.1; 546.1; 569.1; 594.1)
eIF4A (152.1; 152.2)
eIF4E (547.1)
eIF5A (583.1)
Elongation factor Tu (414.1)
FARSLB (552.1)
GART (325.1)
HNRKP (290.1)
Malate dehydrogenase (142.1; 161.1)
MCT4 (467.1)
NAPRTase (354.1)
Nit protein 2 (553.1)
PCBD (563.1)
phosphofructokinase (191.2)
Phosphoglucomutase 1 (580.1)
Phosphoglycerate mutase 1 (114.1)
Phosphoserine phosphatase (554.1)
Ppase (479.1)
PURH (108.1)
Putative adenosylhomocysteinase 2 (464.1)
Quinone oxidoreductase (265.1)
Serine hydroxymethyltransferase (228.1)
Sorbitol dehydrogenase (521.1)
TALDO1 (393.1)
Transketolase (199.1)
Triosephosphate isomerase 1 (53.1)
UGP2 (488.1)
ALDO A (17.1)
Alpha enolase (13.1)
Beta enolase (13.2)
GADH (9.1; 9.2)
Gamma enolase (13.3)
LDH (25.1-25.3)
peroxiredoxin (37.1-37.3)
PGK1 (30.1)
Pyruvate kinase (4.1-4.3)
MVB formation
AIP1/ Alix (135.1)
Calcyclin-binding protein (538.1)
CAND1/ TIP120 (389.1)
CHMP1b (539.1)
CHMP2a (337.1)
CHMP4b (444.1)
CHMP5 (489.1)
CHMP6 (487.1)
E1 ubiquitin activating enzyme (96.1)
E2 ubiquitin activating enzyme (311.1;411.1)

IARS (373.1)	Neddylin 492.1
60 S ribosomal protein (386.1; 273.1; 390.1; 540.1; 317.1; 501.1; 261.1; 194.1; 279.1; 361.1; 399.1; 275.1; 294.1; 246.1; 310.1; 475.1; 203.1; 417.1; 586.1; 466.1; 406.1)	Novel protein (BRO1 homology) (513.1)
40 S ribosomal protein (301.1; 137.1; 208.1; 241.1; 232.1; 168.1; ;217.1; 182.1; 258.1; 206.1; 155.1; 185.1; 221.1; 520.1; 422.1; 340.1; 410.1; 334.1; 529.1; 561.1; 321.1; 362.1; 463.1; 328.1	Ubiquitin (27.1; 72.2)
LISCH (181.1)	VPS28 homolog (549.1)
MARS (404.1)	
NACA (379.1)	**Signaling**
NME1 (83.20	Rap 2A (174.1)
NME2 (83.1)	Rap 2C (174.2; 174.3)
NSEP1 (377.1)	Ras (131.4)
nucleolin (461.1)	IQGAP (36.1)
P2 (324.1)	14-3-3 (331.-33.5)
PABP4 (453.1)	ADAM10 (122.1)
PARS (418.1)	Adenylate kinase isoenzyme 2 (341.1)
PC4 (589.1)	BID (577.1)
PCBP (144.1)	BPNT1 (565.1)
PDIA6 (485.1)	Cab39 (374.1)
PFAS (309.1)	Calgizzarin (384.1)
PSAT1 (274.1; 274.2)	Calmodulin (285.1)
PTBP1 (480.1)	CAP1 (165.1)
SARS (351.1)	Casein kinase II beta subunit (548.1)
SNP1 (306.1)	CD317 (BST2) (117.10
SNRPD2 (381.1)	CD45 (18.1; 136.1)
TART (381.1)	CD95 (FAS; 136.1)
VARS (364.1)	Cdc2 (590.1)
WARS (288.1)	Cdc42 (40.1; 40.2)
	CIP1 (277.1)
Transmembrane proteins	Copine3 (41.1)
ABCE1 (478.1)	Copine-8 (429.1)
Adiponectin (512.1)	CSK (c-SRC kinase; 486.1)
ASCT1 (169.1)	DDAH (506.1)
CD39 (ENTPD1; 170.1)	DJ-1 (PARK7; 158.1)
CD71 (TfR; 63.1)	DOCK10 (240.1)
CD98 (4F2/ LAT1; 121.1)	Dock2 (409.1)

CD107A (LAMP-1)	EBP1 (441.1)
CD220 (IR; 176.1)	FKBP1 (532.1)
CD228 (Melanotransferrin; 171.1)	G protein (21.3; 31.2; 31.3; 21.4; 544.1; 134.1; 369.1; 21.1; 31.1; 21.2)
CD232 (plexin c1; 508.1)	Grb2 (347.1)
Choline transporter-like protein 1 (150.1)	HCLS1 (473.1)
Choline transporter-like protein 2 (156.1)	HINT1 (425.1)
CLIC1 (42.1)	Hornerin (145.1)
CLIC4 (115.1)	H-Ras (131.3)
CXCR4 (336.1)	Inositol monophosphatase (576.1)
ENT1 (175.1)	K-Ras (131.2)
Ephrin type-B receptor 1 (322.1)	LAP4 (358.1)
EVI2B protein (363.1)	LCK (128.3)
EVIN2 (345.1)	Major Vault Protein (278.1)
GLUT1 (101.1)	MAP4K4 (293.1)
GLUT3 (242.1)	MARCKS-like 1 (231.1)
HEM-1 (189.1)	MOB1 (343.1)
MCT1 (140.1)	MOB3A (331.1)
MCT2 (555.1)	Neurocalcin (477.1)
Na/ K ATP ase (3.1; 3.2; 113.1; 188.1; 433.1; 433.2)	N-Ras (131.1)
Notch1 (564.1)	NT5 (303.10)
PMCA4 (86.1)	PDT004 (468.1)
PZR1b (591.1)	PEBP (205.1)
SLC12A (442.1)	Peflin (545.1)
SLC1A5 (92.1)	Phosphatidylinositol 4-kinase alpha (450.1)
SLC38A1 (305.1)	PI(4,5)K type II alpha (319.1)
SLC43A3 (226.1)	PILRalpha (439.1)
TMC6 (283.1)	PKACB (534.1)
TMED9 (507.1)	PKA-RII alpha (496.1)
TMEM16F (297.1)	PLCG2 (320.1)
TMEM2 (371.1)	plexin B2 (79.1)
Tetraspanin 33 (497.1)	PP2A, subunit A (495.1)
VDAC1 (423.1)	PRK (128.4)
	Prohibitin (526.1)
Other	Protein kinase C (312.1)
other	PTP-1C (139.1)
Agrin (264.1)	PTPA (296.1)
AHSG (270.1)	Puromycin-sensitive aminopeptidase (339.1)
ALB protein (7.1)	RAC (35.2)

Apo E (385.1)	RAC2 (35.1)
Bax (428.1)	RACGAP1 (493.1)
CAD (251.1)	RACK1 (427.1)
Carbonic anhydrase 4 (592.1)	Rap (65.6; 38.3)
CGI-150 (578.1)	Rap 1A (38.1)
CNDP dipeptidase 2 (259.1)	Rap 1B (38.2)
CYFIP1 (120.1)	Ras suppressor protein 1 (517.1)
CytoChrome b reduCtase 1 (499.1)	Rheb (446.1)
DDX3 (462.1)	Rho G (59.1)
Desmoplakin (292.1)	Rho GDI 1 (219.1)
DIP2 (130.1)	Rho GDI 2 (58.1)
DRP2 (186.1)	RhoA (118.1; 118.2)
FABP5 (372.1)	r-Ras (88.1; 88.2)
FAM49b (179.1)	RSG14 (436.1)
Glutathione S-transferase P (103.1)	RSG19 (454.1)
Glutathione synthetase (516.1)	SAP97 (435.1)
Glutathione transferase omega 1 (405.1)	Septin-7 (368.1)
Hemoglobin A (52.1)	SIT1 (166.1)
Hemoglobin Z (52.2)	Sorcin (154.1)
Histone 2 (202.1; 332.1)	SRC (SRC)
Histone 3 (248.1)	STAT1 (476.1)
Histone 4 (223.1)	STK10 (392.1)
HNRPA1/B (244.1)	Syntenin-1 (400.1)
HNRPA2/B1 (243.1)	Thioredoxin (263.1)
HNRPC (353.1)	Thioredoxin-like 5 (525.1)
importin beta (207.1)	TNFAIP3 (338.1)
Importin beta-3 (575.1)	TRAF1 (571.1)
ITIH2 (413.1)	TYK2 (180.1)
LAP3 (266.1)	Yes (128.1)
Lipocalin-1 (VEGP; 570.1)	
MPP6 (216.1)	
MYG1 (587.1)	
NAP1 (401.1)	
Nicastrin (356.1)	
Nucleophosmin (588.1)	
NUDC (542.1)	
PA28 alpha (198.1; 238.1)	
PCMT1 (543.1)	
PCNA (cyclin; 511.1)	
PDCD10 (342.1)	
PDCD5 (536.1)	

PDGDH (99.1) Peroxiredoxin 6 (394.1) phospholipid scramblase 1 (573.1) Plasminogen activator inhibitor 1 (437.1) PLAT protein (49.1)	
proteasome alpha subunit (272.1; 272.2; 350.1; 250.1; 304.1;302.1; 193.1; 300.1)	
proteasome beta subunit (214.1; 316.1; 298.1; 327.1; 360.1; 530.1; 260.1; 366.1; 382.1)	
PSMD2 (249.1) PSMD3 (541.1) PSMD7 (509.1) Purine nucleoside phosphorylase (440.1) RAN (116.1) RANBP1 (502.1) RBMXL1 (537.1) Ribonuclease UK114 (426.1) RNH1 (419.1) RuvB-like 1 (403.1) RuvB-like 2 (252.1) SerpinB9 (287.1) SH3BGRL (514.1)	
Similar to metallo-beta-lactamase superfamily protein (470.1)	
Small VCP/p97-interacting protein (344.1) SNRPD3 (527.1) SSBP1 (355.1) Superoxide dismutase (472.1) Translin (523.1) HSPC300 (326.1)	

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Chapter 4

Dendritic cells secrete and target MHC class II carrying exosomes to cognate T cells

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Abstract

Exosomes are small vesicles that are secreted due to fusion of multivesicular bodies with the plasma membrane. Exosomes isolated from the culture medium of dendritic cells contain major histocompatibility complexes and can exert immunomodulatory effects in vitro and in vivo. If and how secretion of exosomes by dendritic cells is regulated was not known. We here show that mouse dendritic cells secrete exosomes into the immune synapse upon engagement with cognate CD4+ T cells. Secreted exosomes were efficiently transferred during T cell activation and remained associated with the T cell plasma membrane after cell dissociation, indicating that CD4+ T cells engaged in a cognate interaction with dendritic cells are a target for dendritic cell exosomes. These results resolve a long standing question of how major histocompatibility complex class II and other membrane constituents are transferred from antigen presenting cells to T cells.

Introduction

Dendritic cells (DC) regulate the initiation of adaptive immune responses (reviewed in (1)) Pathogens that invade peripheral tissues are taken up by DC via endocytic mechanisms and transferred to endosomes/lysosomes for proteolytic processing, after which resulting peptides can be loaded onto Major Histocompatibility Complexes (MHC) class II. In addition, associated pathogens activate DC trough pattern-recognition receptors, resulting in DC maturation. Maturation of DC is a complex process, involving increased expression of MHC class II and co-stimulatory molecules, transport of peptide-MHC class II complexes to the plasma membrane and migration of DC from peripheral tissues to secondary lymphoid organs. Here, mature DC display peptide-MHC class II complexes and may stimulate cognate CD4+ T cells, ultimately resulting in the initiation of primary antigen-specific immune responses.

Peptide-loading of MHC class II occurs in endosomes and lysosomes and such compartments are therefore also collectively referred to as MHC class II-peptide loading compartment or MIIC (2). These include multivesicular bodies (MVB), a specialized endosomal compartment that is composed of a single delimiting membrane surrounding multiple luminal vesicles (LV). The LV are formed by inward budding from the MVB delimiting membrane and are a major storage site for MHC class II in immature DC (3). Membrane proteins that are incorporated into LV potentially have three distinct fates: (i) They can be targeted for degradation as a consequence of fusion of MVB with lysosomes. This process may explain the abundant presence of MHC class II (degradation products) in lysosomes (4) and the relative short half-life of MHC class II in immature DC (5). (ii) MHC class II can be stored temporarily at LV. We previously demonstrated that in pathogen-stimulated DC, LV may fuse back with the MVB-limiting membrane. From there subsequent transport of MHC class II to the plasma membrane occurs by means of tubular/vesicular intermediates (3). (iii) The MVB delimiting membrane can fuse with the plasma membrane, resulting in the release of LV, which are now termed exosomes (reviewed by (6, 7)).

Exosomes are vesicles with a diameter of ~100 nm which, as a consequence of their formation by budding away from the cytosol into the lumen of the MVB, are filled with cytoplasmic proteins and expose the exoplasmatic site of transmembrane proteins at their outside. Exosomes are secreted by many cell types *in vitro*. *In vivo*, exosomes have been found in body fluids (see (8)), including serum, seminal plasma and urine as well as in association with the plasma membrane of follicular dendritic cells in human tonsils (9). DC derived exosomes

abundantly display MHC class II as well as other molecules that are involved in antigen presentation, including MHC class I, co-stimulatory molecules and integrins (10, 11). Exosomes isolated from the culture media of antigen-loaded DC have been demonstrated to elicit both CD4+ and CD8+ T cell responses *in vivo* and *in vitro* (11-13). Furthermore, exosomes isolated from tumor peptide-pulsed DC could eradicate certain previously established murine tumors (12). DC that are activated by lipopolysaccharides (LPS) secrete fewer exosomes as compared to immature DC (14), but the immunocompetence of exosomes from mature DC is much greater compared to those from immature DC (11). In vitro, MHC-restricted antigen presentation to T cells, by exosomes from both immature and mature DC, required the presence of recipient DC. Therefore it was suggested that DC exosomes may help to spread antigen to neighboring DC. Physiological targets of DC exosomes, however, remain unknown.

We reasoned that the physiological function of DC exosomes might be reflected by triggers for their secretion and hence searched for environmental factors that induce exosome secretion by DC. We here show that cognate DC/T cell interactions trigger DC to release exosomes into the immune synapse. In this manner MHC class II carrying exosomes are efficiently transferred from DC to peptide-specific $CD4^+$ T cells. Furthermore, we demonstrate that transfer is efficient only after T cell activation. Cell-cell contact dependent transfer of membrane proteins is a general feature displayed by several cell types (for reviews see (15, 16)) and transfer of membrane proteins to T cells was described already in the 1980s (17-19). Contact dependent transfer of MHC and co-stimulatory membrane proteins from APC of different origins to CD4+ and CD8+ T cells has been observed, *in vitro* and *in vivo* (20-25). To date, however, the mechanism for transfer has not been clarified. Transfer has been proposed to occur either by transient fusions between the plasma membranes of neighboring cells (26, 27), through the recruitment of shed plasma membrane from the DC (25, 28) or by transfer of exosomes (23, 29, 30). We here demonstrate that directed transfer of DC exosomes to cognate T cells via the immune synapse is the principle mechanism behind the transfer of membrane constituents.

Methods

Cell culture

D1 is an immature CD8- splenic DC line from C57Bl/6 mice. D1 cells were cultured on plastic non-coated bacterial dishes (Greiner) (31) in IMDM

(Biowhitaker) containing 10% heat inactivated FCS (Sigma), 100 IU/ml penicillin/100μM streptomycin (Gibco), 2 mM Ultraglutamine (Biowhitaker), 50 μM 2-mercaptoethanol (Sigma), and 35% conditioned medium from R1 cells (31). Prior to use FCS was depleted from bovine exosomes by ultracentrifugation for 60 min at 100,000g.
The p53 specific CD4+ T cell clone (KO4C1) was generated in p53 knockout mice. P53 derived peptides corresponding to amino acids 62-91 and 78-107 of murine p53 were used to immunize mice. Splenocytes from immunized mice were restimulated in vitro and CD4+ T cell clones were generated by limiting dilution. Clone KO4C1 was found to produce high levels of IFN-gamma and IL-2 upon p53 peptide stimulation. KO4C1 cells were cultured in IMDM (Biowhitaker) supplemented with 10% FCS, 100 IU/ml penicillin/ 100 μM Streptomycin, 2 mM Ultraglutamine and 30 μM 2-mercaptoethanol (Sigma) and re-stimulated every 2-3 weeks with irradiated B6 spleen cells in the presence of p53 peptides. P53 Peptides were used in combination, at a concentration of 1 μg/ml for each peptide. After 4-5 days of re-stimulation, T cells were isolated using a Ficoll gradient and grown in the presence of 15 IU IL-2/ ml (Roche).
For DC/T cell co-cultures, DC cells were pre-loaded with 1 μg/ml of p53 peptides for two hours prior to adding T cells. Unless stated otherwise, 10^7 T cells were added in pre-warmed fresh medium to approximately $5x10^6$ DC and the co-cultures where maintained at 37°C, 5% CO_2 as indicated.

Antibodies and reagents

Rabbit polyclonal antibody directed against the cytoplasmic domain of the MHC class II β-chain was obtained from Dr. Barois (University of Oslo, Norway). Rat monoclonal anti-mouse MHC class II (M5.114-APC), rat monoclonal anti-CD40-APC (1C10) and isotype controls were all from Southern Biotech (Birmingham, AL, USA). Monoclonal anti-CD86-APC (GL1) and isotype control and Syrian hamster monoclonal anti-CD28 clone 37.51 were purchased from Becton Dickinson and company (NJ, USA). Armenian hamster monoclonal anti-CD3 (145-2C11) was a kind gift from Peter van Kooten (dept. of Immunology, faculty of Veterinary Sciences, Utrecht University, The Netherlands). Cy3-conjugated goat anti-rat-IgG was from Jackson Immunoresearch (Soham, UK). Horseradish peroxidase (HRP)–conjugated secondary antibodies were from Pierce Biotechnology Inc (Rockford IL, USA), carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) from Invitrogen. Tissue culture quality lipopolysaccharide from

E. coli, (LPS) was obtained from Sigma-Aldrich (St Louis, MO, USA) and used at 10 μg/ml.

SDS-PAGE and Western blotting

Proteins were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, USA) that were blocked and probed with primary antibodies followed by HRP-conjugated secondary antibodies in PBS containing 5% (w/v) non-fat dry milk (Protifar plus; Nutricia, Zoetermeer, The Netherlands) and 0.1% (w/v) Tween 20. Labeled proteins were detected on films using Supersignal west pico chemiluminescent substrate from Pierce Biotechnology (Rockford, USA). For quantification films were scanned using a Bio-Rad GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules CA, USA) and analyzed using Quantify One software.

Exosome Isolation

As a first isolation step, exosomes were collected from DC culture or DC/ T cell co-culture medium by differential centrifugation, principally as described before (32). In short, cells were removed by centrifugation for 10 minutes at 200g (2x). Supernatants were collected and centrifuged subsequently two times for 10 minutes at 500g, 30 min at 10,000g and 60 min at 70,000g using an SW40 rotor (Beckman Instruments, Inc., Fullerton, CA). Exosomes were pelleted at the final centrifugation step and either collected directly in Laemli sample buffer for Western blot analysis or, when indicated, resuspended in 0.5 ml of 2.5 M sucrose, 20 mM Tris-HCl, pH 7.2. The latter were overlaid in a SW40 tube with a linear sucrose gradient (2.0–0.4 M sucrose, 20 mM Tris-HCl, pH 7.2) and floated to equilibrium density into the gradient by centrifugation for 16 hours at 270,000g. Gradient fractions of 1 ml were collected from the bottom of the tube and analyzed for the presence of MHC class II by Western blotting. For exosome binding studies, culture supernatants were depleted of cells and debris as above and the 10,000g supernatant was incubated with T cells. Exosome containing medium was added together with T cells either to regular tissue culture coated flasks (Corning) or, for activation of T cells, to the same flasks that had been coated overnight at 4ºC with 10 μg/ml of monoclonal antibodies directed against CD3 and CD28. T Cells and exosomes were cultured together for 24 hours after which T cells were labeled for MHC class II and analyzed by flow cytometry.

Flow cytometry

Cells were detached in PBS containing 2 mM EDTA on ice, fixed in 2% paraformaldehyde (PFA) in PBS for 1 hour and free aldehyde groups were quenched with 50 mM NH_4Cl in PBS. Cells were immuno-labeled in PBS, 2% BSA, 0.02% azide and analyzed using a FACS Calibur and CellQuest Software. To allow sorting of T cells from DC/T cell co-cultures, T cells were CFSE labeled in 0.5 μM CFSE for 15 minutes at 37°C prior to the start of co-culture. Single T Cells were sorted from the T cell/DC mixture using a FACSVantage system and CellQuest software.

Immuno-fluorescence microscopy

Cells were directly grown on glass cover slips or, in case of sorted T cells, spotted onto poly-L-Lysine coated cover slips and allowed to adhere for 30'on ice. Cells on cover slips were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 1 hour. Fixed cells were washed with PBS and free aldehyde groups were quenched with 50 mM NH_4Cl in PBS. Cells were permeabilized and immuno-labelled in PBS containing 2% BSA and 0.1% saponin (Riedel-de Haën), according to standard procedures.
For the exosome internalization assays, cells were fixed in 4% PFA as above and surface-labeled with M5.114 in PBS, 2% BSA, lacking saponin, followed by Cy3-conjugated goat anti rat IgG. Subsequently, the cells were extensively washed with PBS 2% BSA, fixed again in 4% PFA, permeabilized with PBS containing 2% BSA and 0.1% saponin, and labeled with APC-conjugated M5.114. Cells were imaged using a Leica TCS-SP1 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Electron microscopy

Cells were fixed for 2 hours in 0.1M phosphate buffer, 2% PFA, 0.2% glutaraldehyde. Fixed cells were scraped from culture dishes using a rubber policeman and collected by centrifugation at 500g. FACS sorted T cells were pelleted at 1000g. Free aldehyde groups were quenched with 50 mM glycine in PBS. Cells were embedded in 10% Gelatin and prepared for ultrathin cryosectioning and immunogold labeling (33). For whole mount analysis of secreted exosomes, pelleted exosomes (see above) were resuspended in PBS, spotted onto Formvar, carbon-coated copper grids and absorbed to the grids overnight at room temperature. Exosomes were immuno-labeled and thereafter fixed in 1% GA in 0.1 M phosphatebuffer pH 7.4 for 1 hour. Grids were washed

extensively and contrasted/embedded in 0.4% Uranyl acetate, 1.8% methylcellulose. Sections and whole mount exosomes were observed and photographed in a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV.

Results

DC increase the secretion of MHC class II carrying exosomes in response to cognate interactions with CD4+ T cells

To study the effect of T cells on exosome secretion by DC, a growth factor-dependent long term culture of splenic mouse DC was used. These D1 cells behave indistinguishable from freshly isolated DC (31). The advantages of D1 cells include the absence of contaminating cells and cellular debris from dying or deleted cells, factors that may interfere with studies on exosome secretion. As a T cell model system, we used KO4C1, a murine CD4+ T cell clone with TCR specificity for a p53 epitope. Unlike human T cells, mouse T cells do not synthesize MHC class II upon activation ((34), see also below). In addition to pathogen related signals, CD4+ T cells can act as activators of DC through ligation to MHC, CD40, FAS and/ or OX40L. DC that are activated in this way and have migrated to secondary lymphoid tissues are capable of priming other T cells, such as CTL (1). The ability of DC to engage in a cognate interaction with K04C1 T cells is shown by the maturation of peptide-pulsed DC during a 24 hour co-culture with T cells (Fig. 1). In response to T cells peptide loaded DC increased their surface expression of the activation markers MHC class II, CD86 and CD40 (Fig. 1A). T cells also increased MHC class II peptide loading in DC, as indicated by the appearance of SDS-resistant peptide-MHC class II complexes (Fig. 1B). Surface exposure and SDS-resistance of MHC class II occurred only in the concomitant presence of T cells and relevant peptides, indicating that DC maturation relied on the cognate interaction of TCR with peptide-MHC class II complexes.

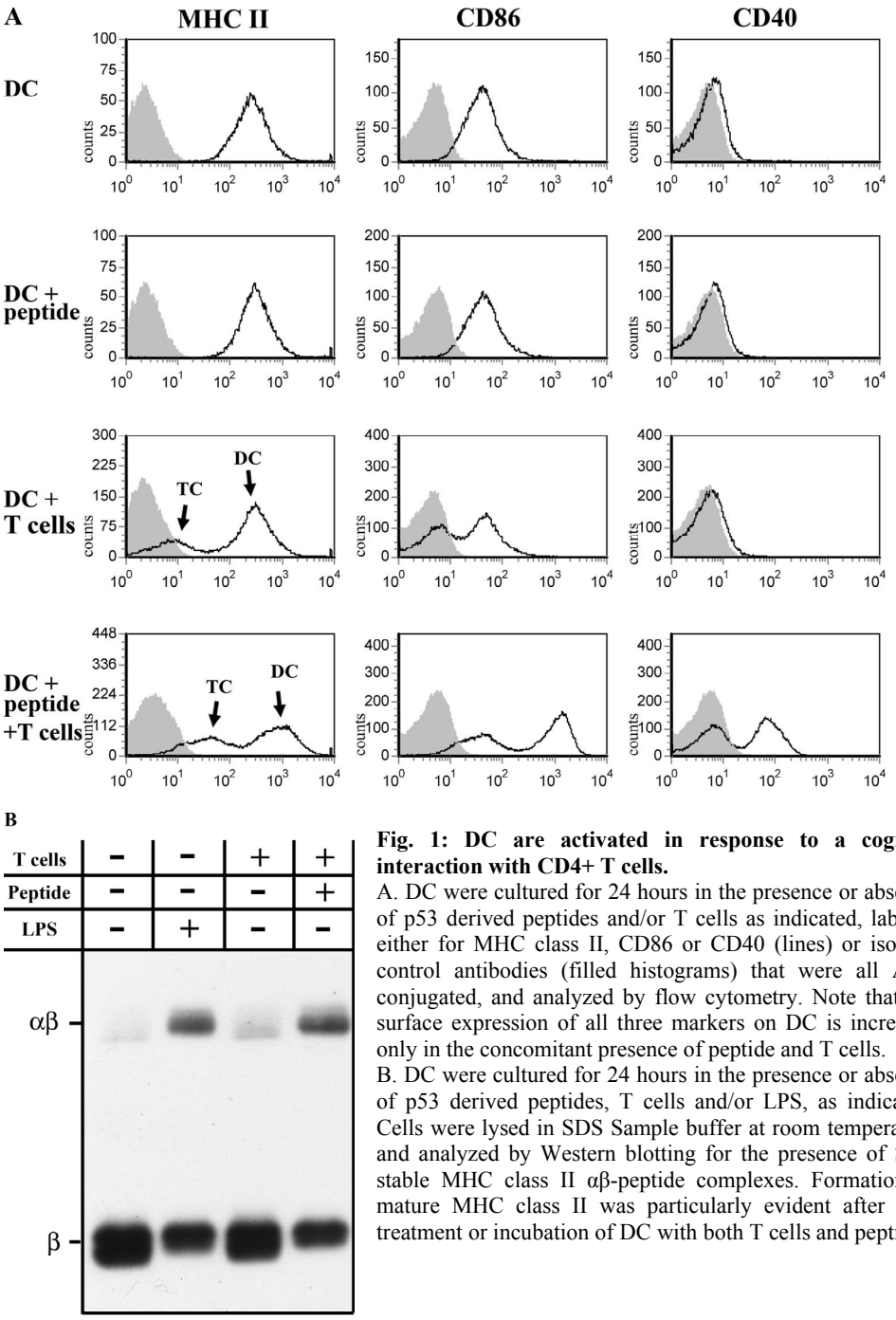


Fig. 1: DC are activated in response to a cognate interaction with CD4+ T cells.

A. DC were cultured for 24 hours in the presence or absence of p53 derived peptides and/or T cells as indicated, labeled either for MHC class II, CD86 or CD40 (lines) or isotype control antibodies (filled histograms) that were all APC conjugated, and analyzed by flow cytometry. Note that the surface expression of all three markers on DC is increased only in the concomitant presence of peptide and T cells.

B. DC were cultured for 24 hours in the presence or absence of p53 derived peptides, T cells and/or LPS, as indicated. Cells were lysed in SDS Sample buffer at room temperature and analyzed by Western blotting for the presence of SDS stable MHC class II αβ-peptide complexes. Formation of mature MHC class II was particularly evident after LPS treatment or incubation of DC with both T cells and peptide.

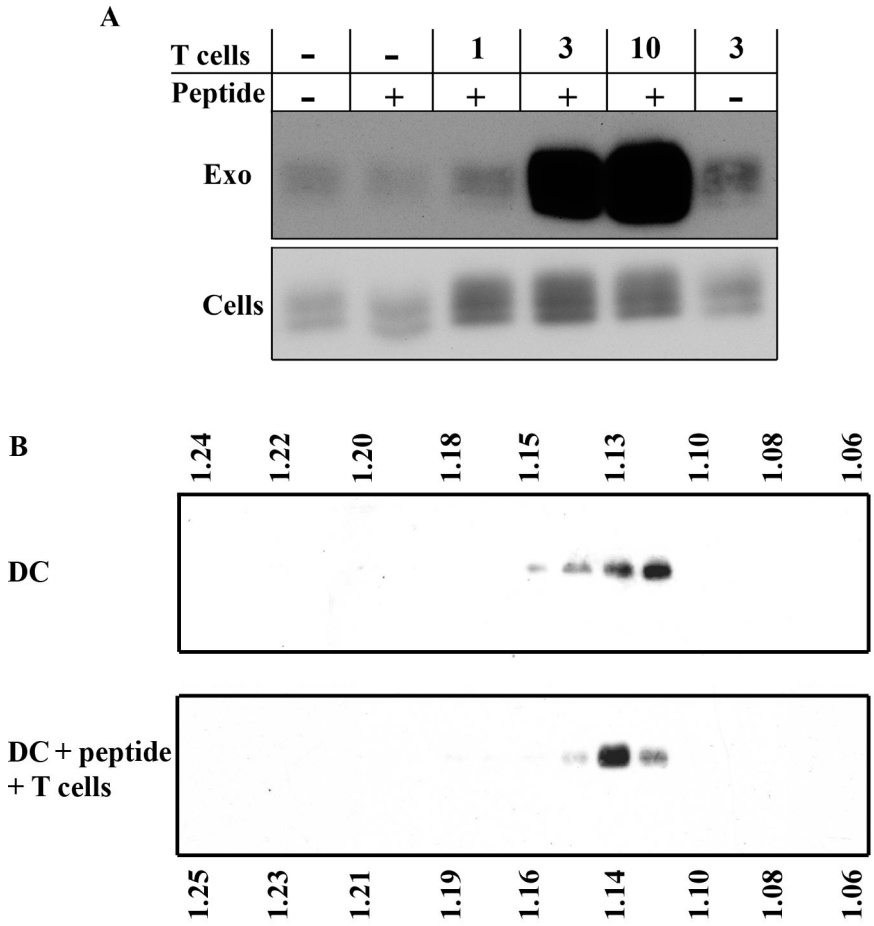


Fig. 2: Secretion of MHC class II is enhanced by T cells.
A. 5.10^6 DC were cultured for 24 hours either in the absence or presence of $1x10^6$, $3 x10^6$ or 10^7 T cells and in the absence or presence of peptide as indicated. Exosomes were isolated from the culture supernatant by differential centrifugation. Both cells and exosomes were lysed in SDS sample buffer at 100ºC to disrupt MHC class II αβ-peptide complexes and analyzed for the presence of MHC class II β-chain by Western blotting.
B. DC were cultured for 24 hours, either alone or in the presence of both peptide and T cells. Exosomes were collected from the culture media by differential centrifugation. Pelleted exosomes were floated into an overlaid sucrose density gradient by ultracentrifugation. The presence of MHC class II in gradient fractions was analyzed by Western blotting. The density of the gradient fractions is indicated at the bottom of each blot.

Next, we tested whether a cognate interaction with T cells triggered DC to release exosomes. Exosomes were isolated from the culture medium by differential centrifugation and MHC class II content of exosomal pellets was analyzed as an indication for the amount of secreted DC exosomes. The amount of exosome-associated MHC class II that was released by $5x10^6$ DC increased 9 fold (S.D. ± 5; result of 7 independent experiments) by the concomitant presence of p53 peptide and 10^7 T cells (Fig. 2). Addition of fewer T cells (10^6 or $3x10^6$) gave intermediate results, suggesting that MHC class II release depended on the frequency of T cell/DC interactions. When DC were incubated with either peptide or T helper cells alone, only a minor increase in MHC class II secretion was observed, indicating that cognate interactions between peptide-MHC class II complexes and TCR are required.

To test whether secreted MHC class II was indeed associated with exosomes, MHC class II carrying membranes were collected from the culture medium by differential centrifugation and subsequently floated into sucrose density gradients by ultracentrifugation (Fig. 2B). Consistent with earlier observations (14), MHC class II carrying exosomes from DC cultured in the absence of T cells floated into the sucrose gradient to a characteristic buoyant density of 1.14g/ml. MHC class II carrying membranes collected from the media of co-cultured cells displayed identical characteristics (Fig. 2B). Vesicles that may have shed from the plasma membrane in response to T cells are expected to be relatively large and, in contrast to exosomes, would have been removed by earlier centrifugation steps. Furthermore, plasma membrane derived vesicles would float in sucrose gradients at a density of 1.24-1.28 g/ml (10) and no signal for MHC class II was obtained at this position in the sucrose gradients (Fig. 2B), confirming association of MHC class II with exosomes

As expected, the amount of total cellular MHC class II increased during DC maturation (Fig. 2). In contrast to exosome associated MHC class II, which mounted progressively with increasing numbers of T cells, the expression of cellular MHC class II was already maximal in the presence of 10^6 T cells. Thus the increase of cellular MHC class II cannot explain the observed increase of MHC class II secretion. This is also apparent from the observation that activation of DC by lipopolysaccharide (LPS) decreased, rather than increased, the secretion of exosome associated MHC class II (Fig. 3, see also (14)). DC that had been pretreated with LPS for 4 hours responded to T cells by increasing MHC class II secretion, albeit not as strongly as non LPS-treated DC (Fig. 3). Similar results were obtained when cells were pre-matured for 14 hours with LPS (data not shown).

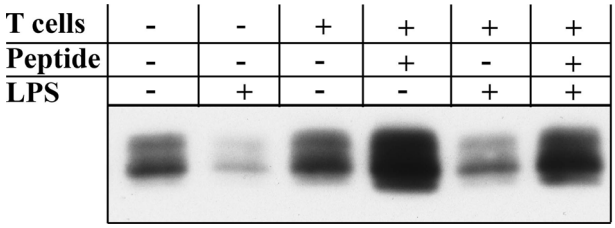


Fig. 3: In response to T cells, LPS pre-activated DC release fewer exosomes as compared to immature DC. Immature DC or DC that had been pre-matured for 4 hours with LPS prior to the start of the co-culture, were incubated for 24 hours in the presence or absence of peptide and/ or 10^7 T cells as indicated. Exosomes were isolated from the culture supernatant by differential centrifugation, lysed in SDS sample buffer at 100ºC to disrupt MHC class II αβ-peptide complexes and analyzed for the presence of MHC class II β-chain by Western blotting.

The increase of MHC class II secretion upon cognate interaction with T cells could either result from increased sorting of MHC class II into exosomes during their formation at MVB or could be due to up-regulated exosome secretion. We could not quantify the amount of DC derived exosomes by total protein nor by non-DC specific exosome markers because exosomes secreted by T cells (35, 36) are likely to contribute to the total pool of exosomes in the culture medium. As an alternative, we determined the relative amount of MHC class II on individual exosomes using immuno-electron microscopy (IEM). Exosomes were mounted on grids and labeled for MHC class II. As expected, MHC class II was associated with ~100 nm vesicles (Fig. 4A), irrespective whether DC were co-cultured with T cells, further demonstrating its association with exosomes. Exosomes from DC/T cell co-cultures on average contained 1.5 fold more MHC class II compared to exosomes from mono-cultured DC (Fig. 4B). This small increase cannot account for the 9 fold increase of MHC class II secretion in response to T cells (Fig. 2) and rather may reflect the increase in MHC class II expression by DC. We thus conclude that T cells induced the secretion of exosomes by DC.

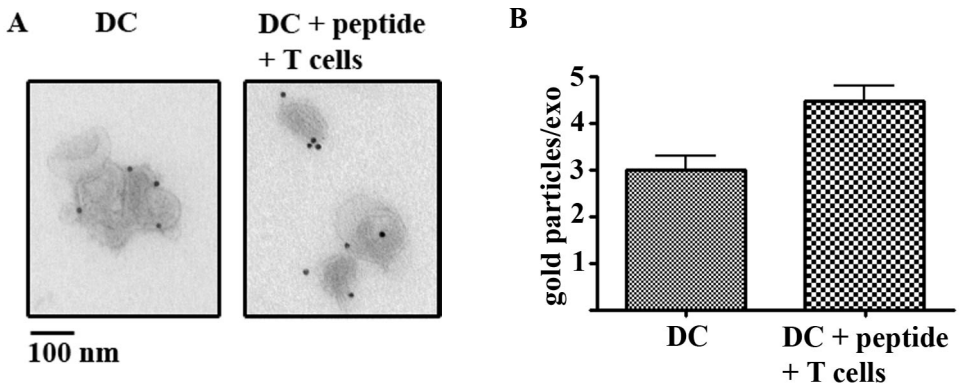


Fig. 4: The amount of MHC class II is only slightly increased in response to cognate T cells
A. Pelleted exosomes were mounted onto grids, immuno-labeled for MHC class II using colloidal gold and analyzed by electron microscopy. Each panel shows 3-4 individual exosomes.
B. The average number of gold particles per exosome was determined (average ± SEM) for n=101 (DC alone) and n=107 (DC + peptide + T cells) exosomes.

DC exosomes are efficiently transferred to activated T cells.

We next asked whether DC derived exosomes associated to T cells. Indeed, T cells acquired MHC class II during co-culture with peptide loaded DC, as measured by flow cytometry (Fig. 5). T cells might have obtained MHC class II indirectly by recruiting DC derived exosomes from the culture medium. Alternatively, DC may secrete exosomes straight into the immune synapse, allowing direct targeting to T cells. The latter possibility is supported by IEM of ultrathin cryosections of the synapse, showing the presence of MHC class II containing 100 nm vesicles reminiscent of exosomes (Fig. 6). MVB were often seen in close proximity to the DC/T cell contact site, suggesting directional secretion towards the T cell.
To test the efficiency of transfer, T cells were isolated by flow cytometry from a 24 hour co-culture and analyzed for MHC class II by Western blotting (Fig. 7A). In the same experiment, exosomes were isolated from an equivalent amount of co-culture medium. A similar amount of MHC class II was associated with exosomes from the culture medium compared to that associated with T cells, indicating rather efficient exosome transfer.

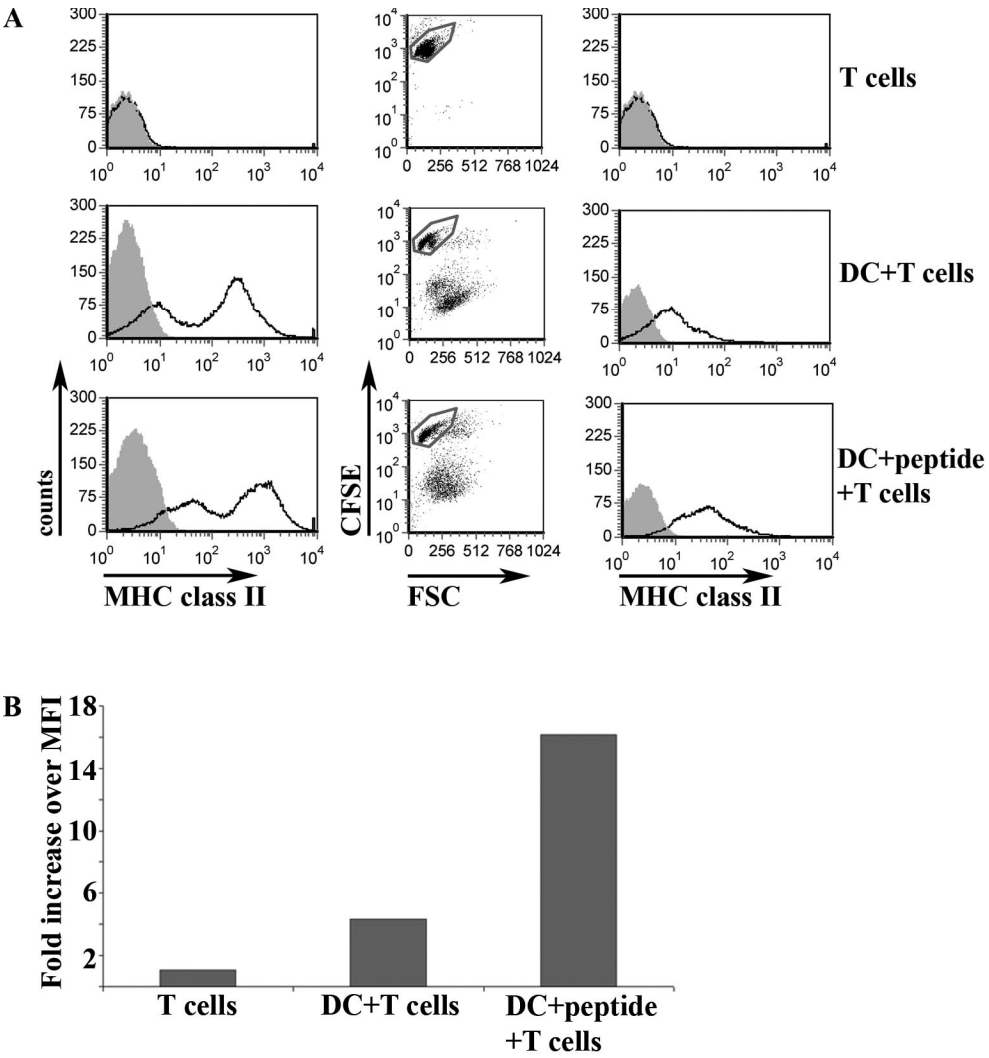


Fig. 5: Exosomes are transferred to T cells

A. T cells were first labeled with CFSE and subsequently cultured for 24 hours in the presence or absence of DC and peptide as indicated. Subsequently, cells were labeled for MHC class II and analyzed by flow cytometry (histograms on the left). CFSE positive single T cells were gated (encircled populations in dotplots) and analyzed for MHC class II (histograms on the right).

B. Mean fluorescent intensities of CFSE positive cells from A as compared to isotype controls are depicted.

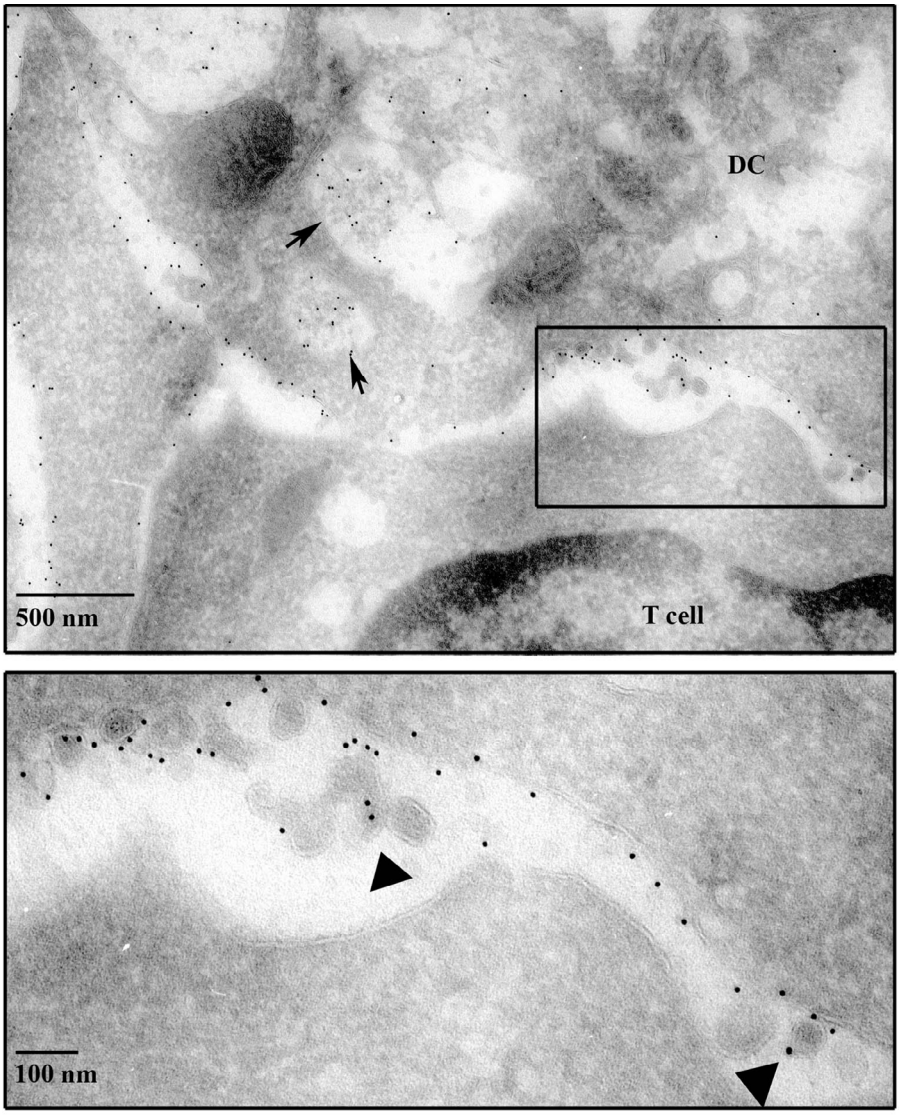


Fig. 6 MHC class II containing exosomes are released in the immune synapse.
DC were pre-loaded with peptide for 2 hours and co-cultured with T cells for another 2 hours. Cells were fixed, sectioned and prepared for IEM analyses. An enlargement of the boxed area in the upper image is depicted in the lower image. MHC class II is associated with MVB (arrows) and the plasma membrane of DC but absent on the T cell plasma membrane. In addition, MHC class II positive vesicles (arrowheads) are found in the synapse between the DC and T cell. An enlargement of the boxed area in the upper image is depicted in the lower image.

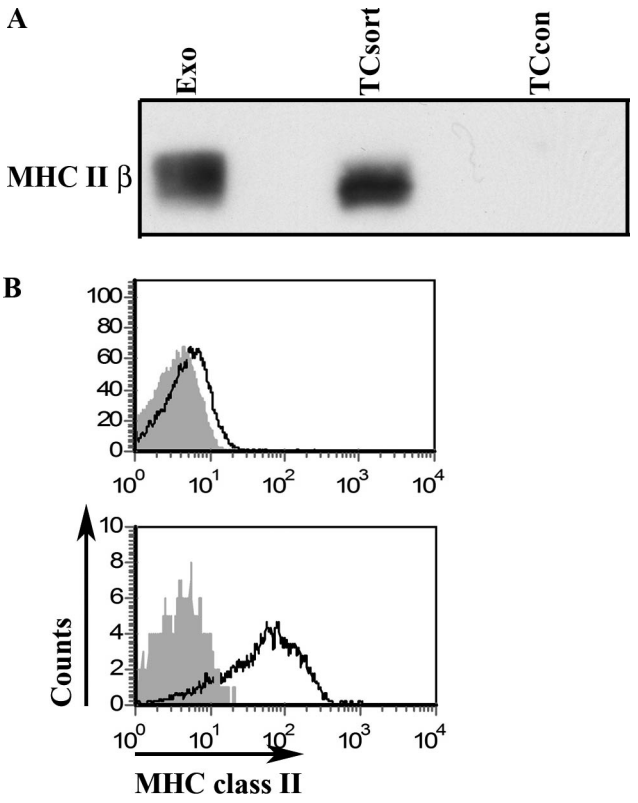


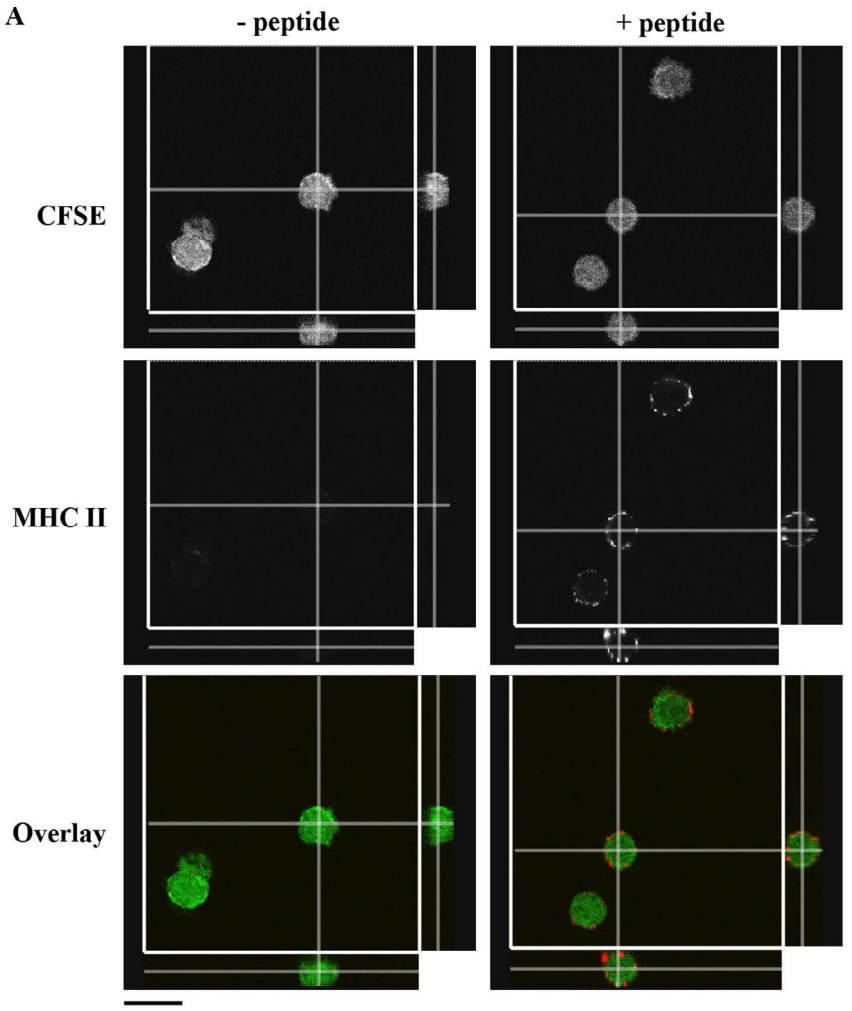
Fig. 7: DC exosomes are recruited by activated T cells.
A. CFSE labeled T cells were incubated in the presence or absence of peptide-loaded DC for 24 hours. T cells that had not been co-cultured with DC (TCcon) and T cells from the co-culture (TCsort) were isolated by flow cytometery and the acquired MHC class II was compared directly to that from an equivalent amount of exosomes that were collected from the co-culture medium (Exo) by Western blotting. The amount of exosome-associated MHC class II that was released into the medium was about equal to that associated to T cells.
B. CFSE-labeled resting T cells (upper panel) or activated T cells (lower panel), were incubated for 24 hours in the presence of exosome containing medium from a previous peptide containing DC/ T cell co-culture. The T cells were labeled with an Allophycocyanin (APC)-conjugated MHC class II antibody or isotype control antibody (filled histograms) and analyzed by flow cytometry. Only activated T cells acquired MHC class II efficiently.

To distinguish between these possibilities, the culture medium from DC/T cell co-cultures was collected, depleted from cells and debris (10,000g supernatant) and added to a fresh batch of mono-cultured T cells (Fig. 7B). In this setup, T cells acquired only very little MHC class II. However, these T cells were not activated while T cells in co-culture with DC were. To study the effect of T cell activation on exosome binding without the concomitant presence of APC, T cells were activated with antibodies directed to CD3 and CD28. In contrast to resting T cells, activated T cells efficiently recruited exosomes from the culture medium (fig. 7B), suggesting that also in cognate DC/T cell co-cultures exosomes may have been selectively recruited to T cells indirectly via the culture medium. Control activated T cells incubated in the absence of DC exosomes did not gain any MHC class II (data not shown), confirming that mouse T cells cannot express MHC class II themselves upon activation. Together, these results indicate that exosomes from DC are directionally transferred to interacting activated T cells and that this may be achieved either directly via the immune synapse or indirectly via the surrounding medium.

Transferred exosomes are retained at the T cell plasma membrane

Next, we focused on the fate of transferred exosomes. After binding, DC derived exosomes may either be internalized through endocytosis or be retained at the T cell plasma membrane. To study the precise location of transferred exosomes, T cells were labeled with CFSE, co-cultured with DC, purified from the co-culture by flow cytometry and analyzed by confocal scanning laser microscopy (CSLM). MHC class II was detected in distinct small spots on or near the surface of the T cells (Fig. 8A). Occasionally, relatively large MHC class II positive structures adhered to the T cells, which may represent DC plasma membrane fragments. To investigate the possibly that such fragments might have been sheared from the DC during harvesting or flow cytometery of co-cultured cells, we also investigated co-cultures that were grown directly on cover slips (Fig. 9). Indeed when T cells were not ripped from the DC only small MHC class II positive spots could be seen at the T cell surface. IEM analysis of sectioned cells revealed numerous exosome-like MHC class II containing vesicles adhering to the T cells surface (Fig. 8B). Notably, MHC class II was entirely absent from the T cell plasma membrane itself, confirming that the T cells did not synthesize MHC class II. In addition to plasma membrane associated exosomes, we also observed MHC class II containing compartments that appeared to be intracellular. Based on their morphology we suspected that these might represent cross-sectioned plasma membrane invaginations rather than true intracellular compartments. To discriminate between intracellular and cell surface exposed MHC class II, cells were immuno-double

labeled for MHC class II subsequently before and after permeabilization using the same antibody conjugated to distinct fluorophores and examined by CSLM (Fig. 9). As a control for this procedure we used immature DC which store most of their MHC class II intracellularly. In DC, intracellular MHC class II was indeed detected only after permeabilization. In contrast, all MHC class II containing structures on T cells were accessible to the antibody already before permeabilization, demonstrating that the T cells do not endocytose significant amounts of transferred exosomes.



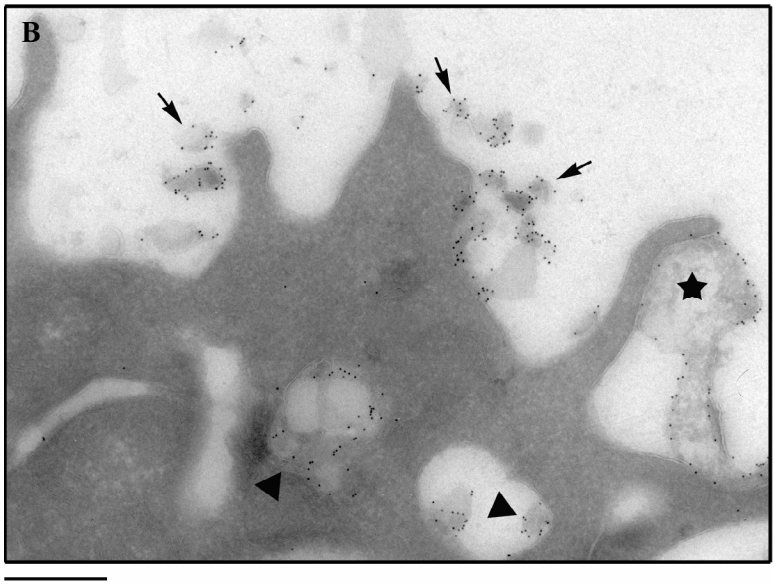


Fig. 8: Transferred MHC class II is on exosomes.
An and B. T cells were labeled with CFSE, cultured with DC for 24 hours either in the presence (B) or absence (A) of peptide and sorted from the co-culture by flow cytometry. Sorted cells were attached to poly-L-lysine coated cover slips, fixed, labeled for MHC class II and analyzed by CSLM. Confocal X-Y sections are indicated in the boxed area. X-Z and Y-Z- stacked images at the indicated sections are projected at the bottom and right side of each figure. Bar 10 μm. MHC class II is associated with the cell periphery in discrete spots (B).
C. T cells were labeled with CFSE, cultured with peptide loaded DC for 24 hours, sorted from the co-culture by flow cytometry, sectioned and analyzed by IEM. MHC class II is found on distinct ~ 100 nm vesicles that associated with the T cell plasma membrane (arrows) and occasionally with larger membranes (asterisk) that may represent sheared DC plasma membrane fragments. Furthermore, MHC class II was found on vesicular membranes which appear to be in the lumen of intracellular compartments but that, out of the plane of the section, may have been continuous with the plasma membrane (arrow heads). Note that the T cell plasma membrane itself is negative for MHC class II. Bar 500 nm.

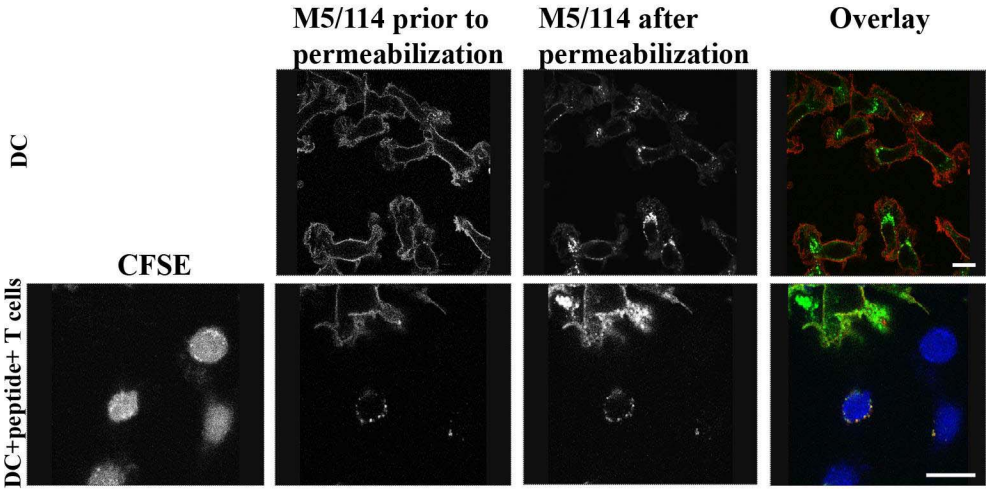


Fig. 9: Exosomes are associated with the T cell surface
DC alone or DC/T cell co-cultures were grown on cover slips, fixed and labeled for MHC class II (M5/114) followed by Cy3-conjugated secondary antibody. After labeling the cells were fixed again, permeabilized and labeled with APC-conjugated M5/114. For clarity, CFSE is falsely colored in blue and APC in green. Note the absence of large MHC class II positive membrane fragments and the perfect overlap of MHC class II labels on the T cells before and after permeabilization. In contrast, for immature DC most MHC class II labeled only after permeabilization. Bar 10 μm.

Discussion

This study, for the first time shows that, in response to TCR ligation, MHC class II carrying exosomes are secreted in the immune synapse and transferred from DC to T cells. The data in this study indicate that exosomes are used as a vehicle for directional intercellular transfer of MHC class II to cognate T cells but not to non-activated bystander T cells: (i) MHC class II carrying membranes were released into the medium in response to cognate T cells and these had the same sedimentation coefficient, equilibrium buoyant density and morphology as constitutively secreted exosomes (Fig. 2 and Fig. 4). (ii) In the presence of T cells, the number of MHC class II molecules per exosome hardly increased while the total of released MHC class II increased 9-fold, indicating increased exosome secretion (Fig. 4). (iii) About half of MHC class II remained associated to T cells whereas the other half was recovered in the co-culture media, indicating efficient transfer (Fig. 7). Exosomes in the culture medium may either have been released from the synaptic cleft after cell dissociation or have been delivered to the medium

as a consequence of docking of MVB distant from the IS. (iv) MHC class II in the synapse and at the T cell plasma membrane was associated with 100 nm vesicles (Fig. 8). (v) Activated T cells but not resting T cells readily recruited MHC class II carrying exosomes from co-culture-medium (Fig. 7).

Although exosomes have previously been proposed to play a role in transfer of MHC class II from APC to T cells (20, 30, 37) their contribution has never been documented and no information is available on the mechanism and regulation of exosome transfer from DC to T cells. However, Patel and colleagues described the presence of MHC class II containing exosome-like vesicles at the interface of a MHC class II-positive rat T cell line and a second T cell of the same specificity (23). Recently, Wetzel and colleagues described transfer of MHC class II to T cells from the immune synapse using artificial fibroblast APC (25). In their study, involvement of exosomes was thought unlikely because transfer could not be mimicked by adding conditioned exosome containing medium from the fibroblasts. We here show, however, that only resting T cells fail to efficiently recruit DC derived exosomes from the medium in contrast to the efficient recruitment by activated T cells. As an alternative mechanism, shed DC plasma membrane fragments have been proposed as vehicles for transfer to T cells. Indeed, using IEM analysis we found DC plasma membrane fragments attached to T cells, as determined by size and morphology. Plasma membrane fragments were rare, however, and we demonstrate that they were torn from the DC plasma membrane as a consequence of sample preparation rather than by active shedding. Transfer via membrane bridges (26, 27) is also unlikely in our system because MHC class II on T cells was exclusively seen on membranes adhering to T cells and not on the T cell plasma membrane itself (Fig. 7). Noteworthy, this also indicates that exosomes do not fuse efficiently with the T cell plasma membrane.

CD4+ T cells form temporal functional synapses with both mature and immature DC but become activated only upon ligation of their TCR to cognate peptide-MHC class II complexes (38, 39). We found that with their activation T cells increase their capacity to bind DC exosomes. Like cognate DC/ T cell interactions, exosome adhesion to T cells may involve TCR/ MHC class II interactions. However, the number of TCR present on the T cell surface is decreased rather than increased during T cell activation (40), suggesting the involvement of other proteins in binding of DC exosomes to activated T cells. For example the integrin LFA-1, which plays a dominant role in IS formation, alters its conformation as a consequence of T cell activation and as a result binds ligands with increased affinity (41). On exosomes, the LFA-1 ligand ICAM-1 has already been demonstrated to be crucial for efficient naïve T cell priming (11). In addition,

antibodies directed against either LFA-1 or ICAM-1 inhibited transfer of MHC from artificial drosophila APC to T cells (37).
In addition to LFA-1 the co-stimulatory receptor CD28 may also assist binding of DC exosomes to T cells. It has been reported that transfer of MHC from DC to activated wild type T cells may also occur independently of antigen, while transfer to activated CD28-/- T cells was strictly antigen dependent (21, 37), suggesting a role for CD80/CD86-CD28 interactions in exosome transfer.

We obtained no evidence for internalization of DC derived exosomes by T cells. This was unexpected since T-APC interactions can induce rapid endocytosis of TCR (42) and others have reported endocytosis of transferred MHC (21). Based on our IEM (Fig. 5) and CLSM experiments (Fig. 7), however, we noted that transferred exosomes can erroneously be considered to be localized intracellularly. By immuno-double labeling MHC class II prior to and after cell permeabilization we found that most, if not all transferred MHC class II containing membranes were associated with the T cell surface rather than with intracellular compartments (Fig. 8) This observation argues against the idea that exosomes may down-regulate TCR from the cell surface, therewith shutting down TCR signaling. On the contrary, association of exosomes with the plasma membrane might allow continuation of TCR signaling, even after dissociation of T cells from DC.

Determining which cells are targets for DC exosomes is an important first step in unraveling the physiological function of exosomes. Exosomes isolated from cultured DC have been shown in vitro to associate with other DC. T cell activation by isolated exosomes required the presence of acceptor DC (11, 13, 43-45) and exosomes from mature DC were much more potent than exosomes from immature DC (11). Based on these observations, it was proposed that *in vivo* exosomes may transfer peptide-MHC to recipient APC rather than directly present peptide-MHC complexes to naïve T cells. In this scenario, the physiological role of DC exosomes would be to spread antigen specific signals to other DC, therewith amplifying immune responses. Our current observations suggest, however, that T cells may be the physiological target of DC derived exosomes. Even in the absence of acceptor APC, exosomes by themselves can, although less efficiently, exert a stimulatory effect on T cells (11, 37). These observations, together with our findings, suggest that exosomes are secreted in the immune synapse and transferred efficiently only to activated T cells and that recipient APC may not be required for exosome signaling to T cells at physiological conditions. *In vivo*, exosomes may function to maintain signaling to the T cell even after the T cell-DC interaction is lost. This would also explains the observation that transferred MHC remains in close contact with TCR and that TCR signaling is maintained at these locations (25). The latter

can be explained best when MHC class II and the TCR are trans-positioned on distinct membranes.

Acquisition of peptide-MHC class II complexes by T cells may also play a role in immune regulation. MHC class II-peptide complexes may endow T cells with means to signal to other T cells. Exosomes from DC contain co-stimulatory molecules and hence may provide T cells to operate as APC that could enhance immune responses. Alternatively, co-stimulation could be provided by the T cells themselves, analogous to the model proposed for signaling of DC-associated exosomes to T cells (11, 13, 43). In this way, DC-derived exosomes could support T cell-T cell signaling and depending on the quality of co-stimulation, facilitate either immunity or tolerance. T cells from mammalian species other than mice express MHC class II themselves upon their activation and present peptide MHC class II complexes to other T cells, leading to the induction of cytotoxicity, anergy or apoptosis (reviewed by (46)). Mouse T cells do not express MHC class II (34) but can obtain it from DC *in vitro* (this study) and *in vivo* (24). The latter study showed that initial T/T presentation may induce T cell proliferation and IL-2 production, similar to the effects of rat T cells which endogenously express MHC class II (47). However, subsequent re-stimulation by professional APC of thus activated T cells induced apoptosis and hyporesponsiveness. All these findings point to a tolerogenic effect of MHC molecules on T cells and thus of transferred exosomes.

Intriguingly, the release and transfer of exosomes in the immune synapse is reminiscent of what has been described for the so called “viral synapse”, where HIV is transferred from DC to CD4+ T cells ((48); reviewed by (49)). Indeed, budding of enveloped viruses such as HIV, simian immuno-eficiency virus (SIV) and hepatitis C virus (HCV) can occur either at MVB and for budding these viruses exploit the ESCRT machinery, which is also necessary for exosome formation (reviewed by (50)). Recently, Wiley and colleagues reported that HIV is released from DC in association with exosomes. Thus exocytosed HIV-1 particles from DCs were 10-fold more infectious as compared to cell-free virus particles (51). Together with our results these findings strongly suggest that HIV (and possibly other viruses exploiting the ESCRT machinery) use a pre-existing pathway of exosomal secretion and targeting for efficient transfer from DC to T cells.

In conclusion, we here demonstrate for the first time that transfer of MHC class II from dendritic cells to T cells occurs via exosomes and that cognate interactions between these cells trigger this transfer. Moreover, we here identified the T cell plasma membrane as a direct target, suggesting a role for DC exosomes in

modulating T cell responses. Signaling pathways involved in exosome release and the immunological relevance of this pathway are yet unknown and focus of current research.

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Chapter 5

Ubiquitination of MHC class II is required for its targeting to exosomes in response to cognate T cell interaction

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Abstract

In a previous study, we demonstrated that dendritic cells regulate their cell surface expression of MHC class II by ubiquitination of MHC class II-β. Both internalization and sorting at multi vesicular bodies requires ubiquitination of MHC class II. When multi vesicular bodies fuse with the plasma membrane their luminal vesicles are released as exosomes. We here demonstrate that ubiquitination of MHC class II is also required for its targeting to exosomes in response to cognate T cell interaction, providing molecular evidence that secreted MHC class II is associated with exosomes. We further demonstrate that ubiquitination of MHC class II is reduced during DC maturation, consistent with elevated MHC class II surface expression. However, when DC matured as a consequence of cognate interaction with T cells, secretion of exosome-associated MHC class II was increased, despite the redistribution of MHC class II from multi vesicular bodies to the plasma membrane. Together, these data indicate that although both the subcellular distribution of MHC class II and its sorting at MVB/incorporation into exosomes are mediated by ubiquitination of MHC class II-β, these processes are differentially regulated.

Introduction

Dendritic cells (DC) are main players in the initiation of adaptive immune responses. DC are professional antigen presenting cells (APC) and thus have the ability to process endocytosed proteins into peptides for loading onto major histocompatibility complex class II (MHC class II) (reviewed in (1, 2)). Surface exposed peptide-MHC class II complexes may be presented to CD4+ T cells that are activated upon recognition of peptide-MHC complexes by T cell receptors (TCR). Danger signals (e.g. microbial factors, such as lipopolysaccharides (LPS), or endogenous inflammatory signals such as IFN-γ), activate DC to enter a differentiation process, also referred to as maturation. As a consequence of DC maturation, peptide loading and surface expression of MHC class II are enhanced. In addition, co-stimulatory molecules that are also needed for T cell activation are upregulated and DC migrate to the lymphoid organs where they may activate cognate CD4+ T cells.
MHC class II is a heterodimer composed of an α- and a β-chain. During synthesis at the endoplasmic reticulum (ER), MHC class II heterodimers associate with invariant chain (Ii), which major functions are to prevent premature peptide loading onto MHC class II (3) and to direct MHC class II to the endocytic compartment through sorting signals in its cytoplasmic domain (4). In endosomes, Ii is degraded in a stepwise fashion after which peptide loading of MHC class II may occur (reviewed in (5)). In resting DC the majority of MHC class II is retained intracellularly on the luminal vesicles (LV) of Multi Vesicular Bodies (MVB). In general, proteins that are sorted to LV have three possible fates. First, they may be degraded upon fusion of the MVB with a lysosome (6). Second, the LV may be used for storage of membrane proteins (7). Third, MVB can directly fuse with the plasma membrane, thereby releasing their vesicular content as exosomes (8). In resting immature DC, MHC class II has a relative short half-life, probably as a consequence of its sorting at MVB and subsequent transfer to lysosomes. Part of MHC class II (~ 0.1%) is, however, released as exosomes. Upon DC maturation, newly synthesized MHC class II is recruited to the plasma membrane. In addition, the LV of pre-existing MVB may fuse back with their delimiting membrane, thus making stored MHC class II available for transport to the cell surface (7). Furthermore, exosome secretion is reduced during DC maturation in response to LPS (9), consistent with a decrease in the number of MVB. DC also mature when engaged in a cognate interaction with CD4+ T cells through the ligation of DC CD40 by CD40L expressed on the activated T cell (10), and as a consequence elevate their MHC class II surface expression. However, we previously reported that, in contrast to LPS, T cell-induced DC maturation is accompanied by a 5-10

fold increase in exosome secretion (11). This indicates that maturation induced relocalization of MHC class II and the secretion of MHC class II via exosomes may be differentially regulated.
In general, ubiquitination of membrane proteins is crucial for their targeting to MVB (reviewed by (12, 13)). At the plasma membrane, ubiquitinated proteins can be recognized by ubiquitin interacting domain (UIM) containing proteins (e.g. Epsin, Eps15) that mediate their internalization via clathrin-coated pits (12, 13). After uptake, such proteins are targeted to the sorting endosome. Here the ubiquitin tag may be removed by de-ubiquitinating enzymes, resulting in recycling of the membrane protein to the plasma membrane. Alternatively, proteins that remain ubiquitinated may be sorted at MVB into LV by the Endosomal Sorting Complex Required for Transport (ESCRT) (14-16).
Previous data by our group showed that the MHC class II β-chain is poly-ubiquitinated (3-5 ubiquity moieties) at the single lysine residue within its cytoplasmic domain (17). We demonstrated that ubiquitination of the MHC class II β-chain is required both for efficient internalization from the plasma membrane and for incorporation at MVB into LV. LPS-induced surface expression of MHC class II was accompanied by a decrease in β-chain ubiquitination, further indicating that cell surface expression of MHC class II is regulated by its ubiquitination.
The observation that MHC class II ubiquitination is a prerequisite for sorting at MVB, predicts that its incorporation into exosomes may also rely on ubiquitination of the β-chain. Many other plasma membrane receptors are ubiquitinated in response to ligand binding, therewith regulating their internalization, and in some cases, MVB targeting (see (18, 19)). Analogously, we hypothesized that MHC class II may be ubiquitinated upon binding to its ligand, the T cell Receptor (TCR). Here we show that although ubiquitination of the MHC class II β-chain is required for its targeting to exosomes, triggering of exosome secretion by cognate T cell ligation is not accompanied by an increased ubiquitination of total cellular MHC class II. Rather, ubiquitination of MHC class II in DC is reduced, in response to cognate T cell interaction, resulting in elevated surface expression. At the same time, secretion of exosomes carrying MHC class II was up-regulated and those exosomes contained slightly more MHC class II molecules per exosome (11). Taken together, we do not find evidence in support of the idea that TCR ligation may trigger ubiquitination and exosomal targeting of MHC class II. Our results do indicate that ubiquitin mediated trafficking of MHC class II is highly regulated in maturing DC, and suggest that exosomes may derive from a specialized subset of MVB.

Materials & Methods

Cells

D1 is an immature CD8-splenic DC line from C57Bl/6 mice (20). D1 cells were cultured on plastic non-coated bacterial dishes (Greiner), in IMDM (Biowhitaker) containing 10% heat inactivated FCS (Sigma), 100IU/ml penicillin/100µM streptomycin (Gibco), 2mM Ultraglutamine (Biowhitaker), 50 µM 2-mercaptoethanol (Sigma), and 35% conditioned medium from R1 cells (20). FCS was depleted from bovine exosomes by ultracentrifugation for 60 min at 100,000g prior to use.
KO4Cl is a CD4+ T cell clone specific for p53 that was generated in p53 knockout mice (11) and cultured in IMDM (Biowhitaker) supplemented with 10% FCS, 100 IU/ml penicillin/ 100µM Streptomycin, 2 mM Ultraglutamine and 30 µM 2-mercaptoethanol (Sigma) and restimulated every 2-3 weeks with irradiated B6 spleen cells in the presence of p53 (see below) peptides and absence of exogenous IL-2. After 4-5 days of re-stimulation, the T cells were isolated using a Ficoll fradient and grown in the presence of 15 IU IL-2/ ml (Roche). P53 derived peptides corresponded to amino acids 62-91 and 78-107 of murine p53. Peptides were generated as described in (Lauwen et al., Manuscript in preparation) and were used in combination, at a concentration of 1µg/ml for each peptide.
For DC/T cell co-cultures, when indicated, DC cells were first pre-loaded with 1µg/ml of each p53 peptide for two hours prior to adding T cells. 10^7 T cells were added in pre-warmed fresh medium to approximately 5.10^6 DC. The co-cultures where maintained at 37°C, 5% CO_2 for the time indicated.
L (tk-) cells (ATCC: CCL 1.3) are murine fibroblasts. L cell expressing CD40L and non-transfected control L-cells were kindly provided by Dr. C. van Kooten, LUMC, Leiden, The Netherlands). CD40L expressing L-cells, had been transfected with full length rat CD40L cDNA in the pME18 expression vector containing the neomycin resistance gene and clones stably expressing CD40L were isolated by selection on G418. L-cells were cultured in IMDM (Biowhitaker) containing 5% heat-inactivated exosome depleted FCS, 100IU penicillin, 100µg streptomycin (both from Invitrogen).

Antibodies and Reagents

Rabbit polyclonal antibody directed against the cytoplasmic domain of the MHC II β-chain was obtained from Dr. Barois (University of Oslo, Norway). Horseradish peroxidase (HRP) conjugated mouse Mab anti-ubiquitin (P4D1) was purchased

from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). HRP–conjugated secondary antibodies were from Pierce Biotechnology Inc (Rockford IL, USA). Monoclonal IgG2b rat anti–mouse MHC II, A^b (M5/114) (21) was kindly provided by Dr. Amigorena (Curie Institute, Paris, France) and produced as hybridoma culture supernatant. Ascites containing monoclonal mouse anti mouse-MHC class II, A^k (10.2.16) was kindly provided by Dr Savina (Curie Institute, Paris, France). Rat monoclonal anti-mouse MHC II, A^b (M5.114-APC), and isotype control were from Southern Biotech (Birmingham, AL, USA).Carboxyfluorescein diacetate, succinimidyl ester (CFDA, SE) was from Invitrogen. Tissue culture quality lipopolysaccharide from E. coli, (LPS) was obtained from Sigma-Aldrich (St Louis, MO, USA) and used at 10μg/ml. Staphylococcal Enterotoxin A (SEA) was obtained from Sigma-Aldrich and was used at 250ng/ml.

Immunopurification of ubiquitinated MHC class II-β

DC were lysed for 10 minutes at 4°C in freshly prepared lysisbuffer containing 1 % Triton X100, 1 mM EDTA, 150 mM NaCl, 20 mM Tris/HCl pH 8, 10 mM N-ethylmaleimide (Sigma Aldrich, Zwijndrecht, The Netherlands), complete protease inhibitor mix (Roche Molecular Biochemicals, Almere, The Netherlands). Nuclei were removed by centrifugation for 1 min at 10.000 g. Protein G-agarose beads (Sigma Aldrich, Zwijndrecht, The Netherands) were pre-coupled overnight to M5/114 in lysis buffer. Beads were added to the cell lysates and gently mixed for 1,5 hours at 4°C. Subsequently the beads were washed and immunoprecipitated MHC II was eluted in SDS sample buffer (2% SDS, 62.5 mM Tris–HCl, pH 6.8, 10% Glycerol) either for 5 min at 100ºC or for 30 min at room temperature and stored at -20°C or eluted and stored overnight at room temperature.

SDS-PAGE and Western blotting

For SDS-PAGE and Western blotting, proteins were separated on 10% or 12.5% polyacrylamide gels and transferred to Immobilon-P membrane (Millipore, Bedford, USA). Membranes were blocked and probed with antibodies in PBS containing 5% (w/v) non-fat dry milk (Protifar plus; Nutricia, Zoetermeer, The Netherlands) or 0.5% gelatine from cold water fish skin (Sigma-Aldrich, St. Louis, USA) and 0.1% (w/v) Tween 20. Primary antibodies were probed with horseradish-peroxidase-conjugated goat anti-mouse antibodies, purchased from Jackson ImmunoResearch Laboratories Inc. (Soham, UK) and detected on film using Supersignal west pico chemiluminescent substrate from Pierce Biotechnology (Rockford, USA).

Exosome isolation

As a first isolation step, exosomes were collected from DC or DC/T cell co-culture medium by differential centrifugation, principally as described previously (8). In short, cells were removed by centrifugation for 10 minutes at 200xg (2x). Supernatants were collected and centrifuged subsequently two times for 10 minutes at 500 g, 30 min at 10,000xg and 60 min at 70,000xg using an SW40 rotor (Beckman Instruments, Inc., Fullerton, CA). Exosomes were pelleted at the final centrifugation step and collected directly in Laemli sample buffer for Western blot analysis.

Flow cytometry

Cells were removed from the culture dish in PBS containing 2mM EDTA on ice. Subsequently, the cells were fixed in 2% paraformaldehyde (PFA) in PBS for 1 hour. Fixed cells were washed with PBS and free aldehyde groups were quenched with 50mM NH_4Cl in PBS. Cells were labelled with fluorescent antibodies in PBS, 2% BSA, 0.02% Sodium azide and analyzed using a FACS Calibur and CellQuest Software. For microscopic analysis, T cells were CFSE labelled prior to the start of the co-culture: T cells were washed once in PBS at 37°C, labelled in PBS containing 0.5 μM CFSE for 15 minutes at 37°C, washed extensively in IMDM containing 10% FCS and added to the DC culture.

Immuno-fluorescence microscopy

Cells were grown on glass coverslips and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 1 hour. Fixed cells were washed with PBS and free aldehyde groups were quenched with 50nM NH_4Cl in PBS. Cells were permeabilized and immuno-labelled in PBS containing 2% BSA and 0.1 % Saponin (Riedel-de Haën), according to standard procedures. Cells were imaged using a Bio-Rad Radiance 2100MP confocal and
multi-photon system (Bio-Rad, Hertfordshire, UK).

Constructs and retroviral transduction of DC

A^k-β^{WT} and A^k-β^{K225A} cDNA in a pRc/CMV vector were kindly provided by Dr. T. Laufer (Harvard School of Public Health, Boston) (Laufer et al., 1997). cDNAs were amplified by PCR with the primers

TCGCGGCCGCACCATGGCTCTGCAGATCCCCAGC/
GGTCGTCCTGAGGACGTCACTCTTAAGCTAG. Products were EcoR1/Not1 digested and cloned into pQCXIX and pQIXN MoMuLV retroviral Vectors (Clontech, Mountain View, USA) and sequences were verified. Ecotropic retroviral particles were produced by 293T Phoenix-Eco cells after co-transfection with the pQ vectors and pCl-Eco (kindly provided by Dr. H.Rozemuller, Utrecht Medical Centre, Utrecht). Harvested viruses were filtered, mixed with polybrene (Sigma, Zwijndrecht, The Netherlands) to a final concentration of 4 μg/ml and used immediately to infect D1 (17). Spin occulation (1000xg for 90 minutes) was used to infect the cells twice, with a 3 hour interval, on a single day. Transduced D1 were selected using G418 and maintained as stable cell cultures.

Results

We previously demonstrated that in response to a cognate interaction with T cells DC increase their secretion of exosome associated MHC class II (11). Both the number of exosomes and to a limited extent also the amount of MHC class II per exosome was increased. In a separate study we found that sorting of MHC class II at MVB relies on its ubiquitination (17). Together, these results suggested that cognate T cell interactions might target MHC class II to LV of MVB, the precursors of exosomes, by increasing MHC class II ubiquitination. To test whether the increase in secretion of exosome-associated MHC class II in response to T cells is accompanied by enhanced ubiquitination of its β-chain, we immuno-isolated MHC class II from DC that were incubated for 4 hours either in the presence or absence of T cells, or in the presence of both T cells and their cognate peptide. MHC class II was immuno-precipitated and probed for ubiquitin by Western blotting. Opposite to what we expected, ubiquitination of MHC class II-β was decreased in response to cognate T cell interaction (Fig 1A). This apparent discrepancy becomes even more eminent when we take into account that DC mature when they are co-cultured with T cells in the presence of cognate peptide ((11) and Fig. 2). In contrast to DC maturation by T cells, LPS-induced maturation resulted in a concomitant decrease of both exosome secretion and ubiquitination of the β-chain (Fig. 1b and (11, 17). Thus, even though secretion of MHC class II carrying exosomes was stimulated by T cells, reduced amounts of total MHC class II appeared to be sorted at MVB. When DC maturation was induced through CD40 ligation in a co-culture of DC with fibroblasts expressing CD40 ligand, ubiquitination of the MHC class II β-chain was also decreased (Fig. 1C), further demonstrating that decreased ubiquitination of MHC class II is generally associated with the recruitment of MHC class II to the cell surface in maturing DC.

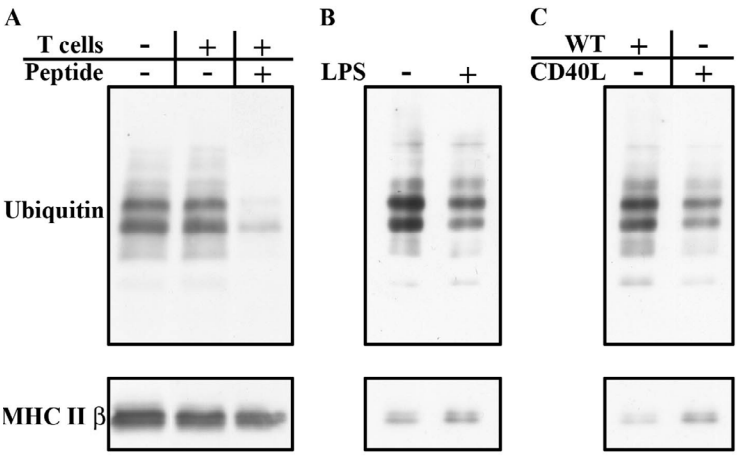

Fig. 1: DC maturation correlates with decreased ubiquitination of MHC class II β. DC were cultured for 4 hours in the presence or absence of A: T cells and p53 peptides, B: LPS, C: WT L cells or L cells expressing CD40L as indicated. Cells were lysed and MHC class II was immuno-isolated and analyzed for MHC class II-β and ubiquitinated MHC class II-β by Western Blotting. Non-ubiquitinated MHC class II-β (bottom panel) was highly abundant compared to ubiquitinated MHC class II-β (upper panel). Regardless of the type of maturation stimulus ubiquitination of the MHC class II β-chain was decreased.

One explanation for the increased secretion of exosome-associated MHC class II and the concomitant decrease of ubiquitination in response to DC activation by T cells is that MVB fusion with the plasma membrane rather than sorting of MHC class II to LV is upregulated. This does, however, not explain the 1.5 fold increase in MHC class II per exosome in response to T cells (11). We argued that perhaps a subset of MHC class II may be preferentially targeted to MVB at those circumstances. For many other plasma membrane receptors, such as receptor tyrosin kinases, ligand binding triggers the ubiquitination of their cytoplasmic domain, resulting in their endocytosis and subsequent delivery to LV of MVB (see (13, 18)). In analogy, we hypothesized that incorporation of MHC class II into exosomes might be triggered by TCR ligation. Since only a limited fraction of MHC class II is loaded with exogenously added peptides (22, 23), only few MHC class II molecules associate to TCR. In contrast, superantigens, like staphylococcal enterotoxin A (SEA), couple non-polymorphic regions of MHC class II to TCR, irrespective of peptide loading (24, 25). By using SEA instead of p53 peptides in the DC/ T cell co-culture we expected to increase the pool of TCR ligated MHC class II.

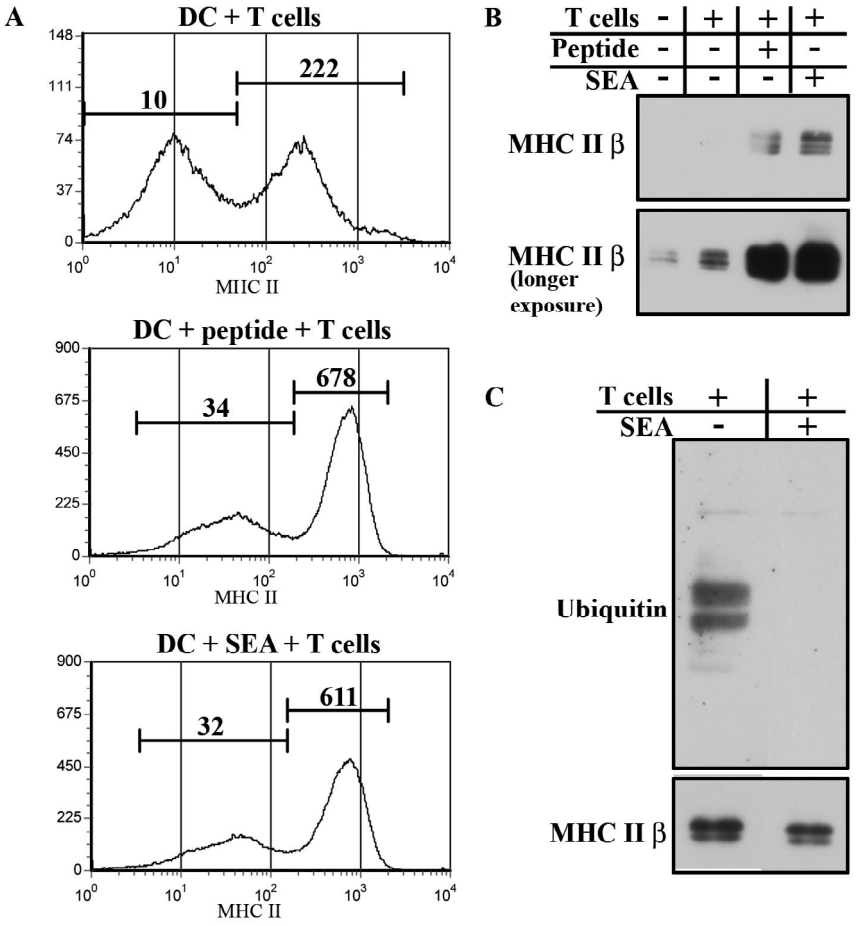

Fig. 2: Both SEA and cognate peptide mediated MHC class II/TCR ligation induce exosome secretion and decrease ubiquitination of MHC class II-β.
A: Control DC or DC loaded with either p53 peptides or SEA, were co-cultured with CD4+ T cells for 24 hours, labeled for MHC class II and analyzed by FACS. The left and right populations represent T cells and DC respectively. The numbers above the peak indicate the mean fluorescence intensity of each population. B: Control DC or DC pre-loaded with p53 peptides or SEA were culture alone or in the presence of T cells for 24 hours as indicated. Exosomes were isolated from the culture medium by differential centrifugation and analyzed by Western blotting for MHC class II-β. The two panels display different exposures of the same blot. C: control DC or DC pre-loaded with SEA were cultured in the presence of T cells for 4 hours. Cells were lysed and MHC class II was immuno-isolated and analyzed for MHC class II and ubiquitinated MHC class II-β by Western Blotting as in figure 1. A-C: Note that SEA- and p53 peptide-loaded DC responded similar to T cells regarding up-regulation of cell surface MHC class II, transfer of MHC class II to T cells, up-regulation of MHC class II secretion via exosomes and decreased ubiquitinated of MHC class II-β.

We first tested whether SEA was capable of establishing DC- T cell interactions, by analyzing cell surface expression of MHC class II using FACS (Fig. 2A). DC-T cell co-cultures were incubated in the presence or absence of SEA or p53 peptides. SEA and p53 peptides elevated the surface expression of MHC class II on DC to the same extent (Fig. 2), indicating DC maturation through ligation of MHC class II to TCR. SEA alone did not have any effect on DC maturation (data not shown and (26, 27)). Interestingly, T cells recruited MHC class II from DC both in the presence of p53 peptides or SEA. This is indicative for transfer of exosomes and consistent with earlier observation (11). Next, we determined the amount of exosomes secreted into the culture medium. Consistent with earlier observations (11), secretion of exosome-associated MHC class II was greatly enhanced by the concomitant presence of T cells and p53 (Fig. 2B). T cells in combination with SEA had similar stimulatory effects. In addition, ubiquitination of MHC class II was reduced in the presence of T cells and SEA, indicating that peptide-independent SEA-mediated ligation of MHC class II to the TCR did not induce massive sorting of MHC class II for targeting to exosomes.

Next, to directly test whether ubiquitination is required for sorting of MHC class II to exosomes, we studied trafficking of mutated MHC class II in which the lysine residue that is targeted by ubiquitin was substituted by an alanine residue. D1 cells were retrovirally transduced to express either a wild-type β-chain of the MHC class II haplotype A^k (A^k-β^{wt}) or a A^k-β^{K225A} substitution mutant (17). Like endogenous A^b-β, A^k-β forms stable heterodimeric complexes with A^b-α and the surface expression of A^k-β/A^b-α dimers is as efficient as that of endogenous A^b-β/A^b-α dimers (28). A^b-β and A^k-β were detected independently using haplotype specific antibodies. When expressed in immature DC, A^k-β^{WT} localized predominantly at intracellular compartments together with endogenously expressed A^b-β (Fig. 3). In contrast, A^k-β^{K225A} was highly abundant on the plasma membrane (Fig. 3). The distinct localization of A^k-β^{K225A} in immature DC illustrates its defect in internalization and MVB sorting as a consequence of lack of ubiquitination (17). The high surface expression of A^k-β^{K225A} was not due to DC maturation, as demonstrated by a low surface expression of the co-stimulatory molecule CD86 ((17) and Fig. 3A). Endogenous I-A^b as well as Ak-β^{WT} and CD86 were expressed at the plasma membrane when DC were triggered to mature with LPS (Fig 3A) or by cognate CD4+ T cells in the presence of p53 peptides (Fig. 3B). Thus, DC expressing A^k-β^{K225A} behaved normally in response to maturation stimuli.
We attempted to monitor whether T cells that were co-cultured with A^k expressing DC acquired MHC class II at their cell surface. This is, however, difficult to determine by fluorescent microscopy of co-cultures as MHC class II at the T cell surface may equally well derive from entangling DC plasma membrane extensions

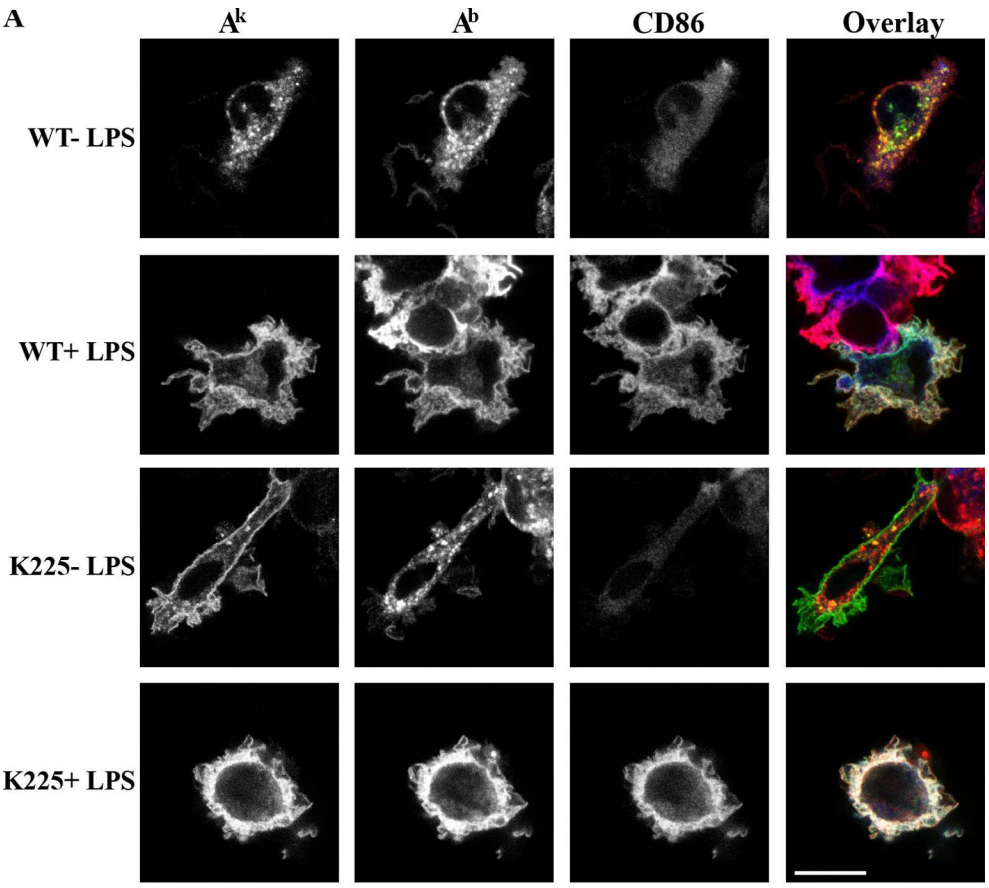

Fig. 3: Ubiquitination of MHC class II-β is required for intracellular retention of MHC class II. DC transduced with either A^k-β^{WT} or A^k-β^{K225A} were incubated for 24 hours in the presence or absence of LPS (A) or in the presence of CFSE labeled CD4+ T cells with or without p53 peptides (B). Fixed cells were labeled either for A^k (cy3) and CD86 (APC) (A) or for A^k (cy3) and A^b (cy5) (B). Bar 10 µm
Note that A^k-β^{K225A} is predominantly localized to the plasma membrane in both immature and mature DC whereas A^k-β^{WT}, CD86 and endogenous A^b are present at the plasma membrane only when DC matured by LPS or cognate T cell interaction.

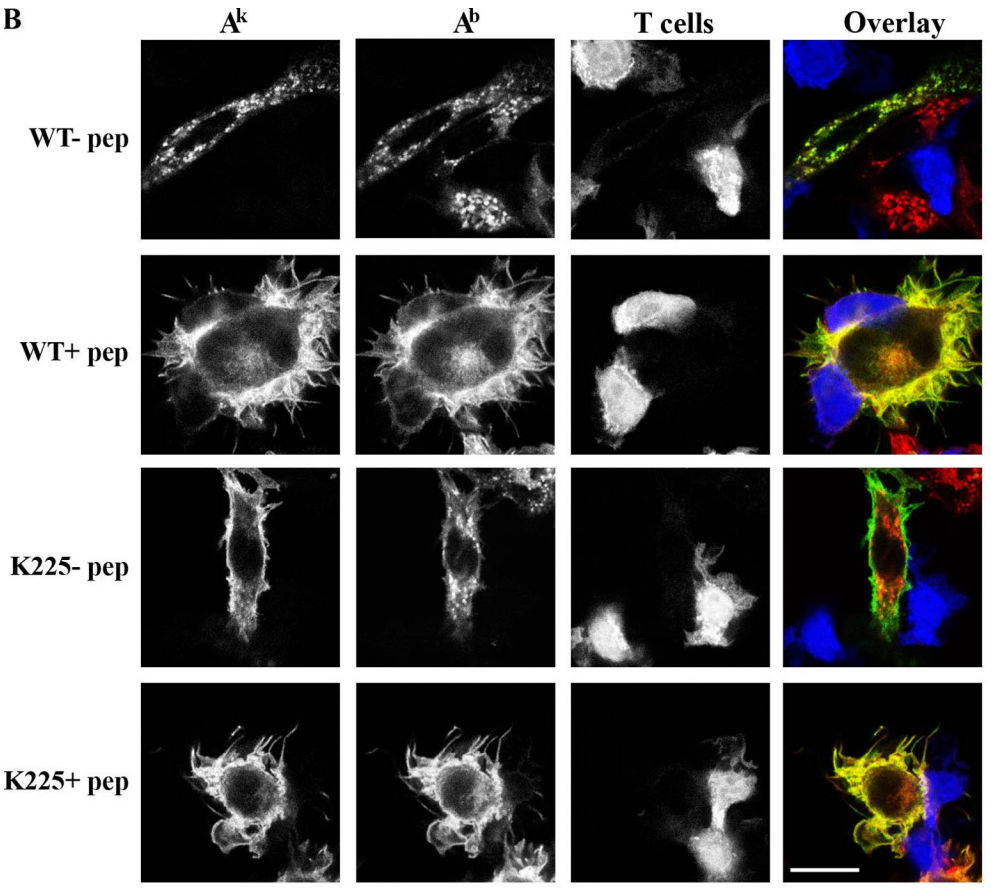

Fig. 3B

(Fig. 3). Previously, we demonstrated transfer of endogenous MHC class II after separating T cells from DC by FACS (11). We could not use this approach here because A^k was expressed by a subset of DC only and expression levels of A^k –β^{wt} and A^k-β^{K225A} were distinct. However, T cell-induced secretion of exosomes by DC also resulted in release of a substantial amount of free exosomes in the culture media (11). As an alternative, we therefore determined the amount of MHC class II associated with freely suspended exosomes in co-culture media. Exosomes were isolated from the culture media by differential centrifugation and probed by Western blotting for A^b and A^k. Consistent with previous observations ((11) and Fig 2B) secretion of exosome-associated endogenous A^b by both A^k-β^{WT} and A^k-β^{K225A} expressing DC was induced by the concomitant presence of p53 peptide and

cognate T cells. Secretion of A^k-β^{WT} was induced similarly, again suggesting that incorporation of MHC-II in exosomes was not induced by an interaction with cognate TCR. Such a conclusion can, however, not be drawn definitely as we have not formally proven that chimeric A^k-β /A^b-α, unlike endogenous A^b- α /A^b-β, cannot associate with this TCR. In contrast to A^b-β and A^k-β^{WT}, secretion of A^k-β^{K225A} was not induced by T cells and p53 peptides (Fig. 4).

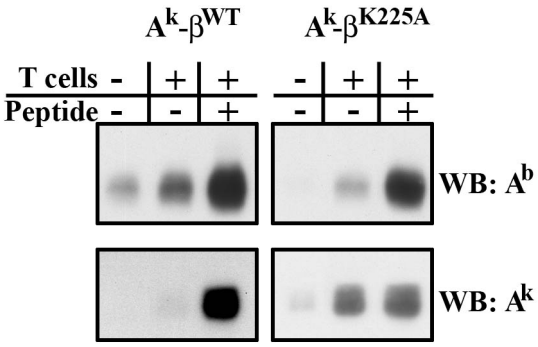

Fig. 4: Ubiquitination of MHC class II-β is required for targeting to exosomes. DC that had been transduced with either A^k-β^{WT} or A^k-β^{K225A} were incubated for 24 hours in the presence or absence of p53 peptides and/or T cells as indicated. Exosomes were isolated from the culture medium by differential centrifugation and analyzed by Western blotting as indicated. Only A^b and A^k-β^{WT} but not A^k-β^{K225A} was secreted via exosomes in response to cognate T cell interaction.

Importantly, this demonstrates that ubiquitination of MHC class II is required for its release in association with exosomes. In the absence of p53 peptides, T cells showed a slight increase in A^k-β^{K225A} release as compared to DC alone. This might be due to contaminating shed plasma membrane fragments, which abundantly expressed A^k-β^{K225A} and were collected together with exosomes from the culture medium by differential centrifugation.
Together, our data do not support the idea that cognate interactions of MHC class II/peptide complexes with TCR result in selective incorporation of such complexes into exosomes. However, we cannot yet exclude this possibility.
Finally, our results demonstrate that ubiquitination of MHC class II is required for incorporation into exosomes and for the first time provide molecular evidence that MHC class II that is released by DC in response to T cell interaction is associated with exosomes rather than with shed plasma membrane.

Discussion

Here for the first time we show that the targeting of MHC class II to exosomes in response to cognate DC- T cell interaction depends on ubiquitination of the MHC class II β-chain. This finding is in agreement with our previous discovery that ubiquitination is required for the targeting of MHC class II to the LV of MVB (17). We further demonstrate that during DC maturation, ubiquitination of MHC class II is decreased, consistent with an elevated MHC class II surface expression. When, DC matured in response to a cognate interaction between DC and T cells, secretion of exosome-associated MHC class II was increased, despite the redistribution of MHC class II from multi vesicular bodies to the plasma membrane.

Excretion of the vesicular MVB content has been demonstrated already in 1985 for maturing reticulocytes using electron microscopy by visualizing fusion profiles of the MVB limiting membrane with the plasma membrane (29, 30). In addition, Raposo and colleagues demonstrated regurgitation of endocytosed BSA-gold together with LV in MVB-plasma membrane fusion profiles (8), demonstrating that the compartment from which exosomes originate is indeed late endosomal in nature. In following years, several reports used EM to visualize MVB fusion profiles in other exosome producing cells, including DC (31) and mast cells (32). Also the abundant presence of MBV resident proteins, including MHC class II and tetraspanins (CD63, CD81 and CD82) rather than plasma membrane constituents (33) strongly indicates that exosomes originate from MVB.
The data we present here for the first time provide direct molecular evidence that the machinery for MVB sorting is linked to exosome biogenesis. Ubiquitination of MHC class II, in agreement with its role in MVB sorting, is also needed for the exosomal targeting of MHC class II. Transduced $A^k\beta^{K225A}$ which lacks a ubiquitination site, failed to target to MVB (17) and exosomes, while $A^k\beta^{WT}$ and endogenous $A^b\beta$ could be sorted to both. A small amount of $A^k\beta^{K225A}$ was found in the exosomal pellet from co-cultures both in the absence and presence of peptides. This might be due to contaminating shed plasma membrane fragments, which abundantly expressed A^k-β^{K225A} and were collected together with exosomes from the culture medium by differential centrifugation. Indeed others have shown that exosomes isolated by differential centrifugation, although quite pure already, may still contain small amounts of plasma membrane fragments that are released by apoptotic cells (34). In any case the amount of secreted $A^k\beta^{K225A}$ was not increased further by the presence of peptides, while exosomes secretion was.
The need for ubiquitination in the targeting of proteins to exosomes provides a helpful tool to discriminate between the contribution of exosomes and plasma

membrane derived membranes in intercellular membrane transfer. Most proteins studied so far, although often highly enriched in MVB/exosomes, were never completely absent from the plasma membrane. We have shown before that exosomes provide a main mechanism for transfer of MHC class II (and likely many other molecules) from DC to T cells during cognate interactions (35). However, our results also indicated that membrane fragments that were sheared from DC as a consequence of experimental procedures may contribute to protein transfer. By preventing ubiquitination of MHC class II we may be able to determine more precisely the contribution of exosomes and plasma membrane to MHC class II transfer. First attempts to study this failed because we have not yet succeeded in generating a homogenous population of DC expressing $A^k\beta^{wt}$ or $A^k\beta^{K225A}$, which is a prerequisite for quantitative analysis of protein transfer by FACS.

We hypothesized that TCR ligation of MHC class II may result in its ubiquitination and subsequent release via exosomes. In the present study, however, we found no evidence that TCR ligation induces MHC class II ubiquitination or incorporation into exosomes.
Ubiquitination of MHC class II and exosomal targeting of MHC class II were not further increased when peptides were replaced by SEA. SEA binds MHC class II outside the peptide binding groove and can crosslink MHC class II to TCR irrespective of its specificity (24, 25), and may thus elevate the amount of MHC class II ligated to TCR. We do not know, however, whether SEA indeed increased the amount of TCR ligated to MHC class II compared to exogenously added p53 peptides.
Our experiments with DC expressing both A^b and A^k suggested that T cell induced incorporation into exosomes may not be restricted to the MHC class II haplotype recognized by the TCR, as both A^b and A^k were efficiently targeted to exosomes upon cognate T cell interaction. The T cell clone we used is generated in a $A^{b+}A^{k-}$ mouse. It is thus unlikely that it recognized p53 peptide in the context of a A^k-β /A^b-α chimera, although we cannot exclude this possibility. Together, these results do not indicate that the targeting of MHC class II to exosomes is directly triggered by TCR ligation but additional experiments are required to get a more definite answer on this matter.

Intriguingly, during DC mature in response to cognate T cell interactions, ubiquitination of MHC class II decreased, therewith elevating its cell surface expression. However, at these conditions, the number of MHC class II molecules per exosome as well as the number of secreted exosomes were increased (35). These observations suggest that DC maintain a subset of MVB, to which MHC class II is very efficiently sorted by the ubiquitin system. These remaining MVB

may be a different from MVB that are used to store MHC class II intracellularly in immature DC. Whether such DC really exist requires further investigation. Furthermore, it is yet not clear how a process that requires a decrease in ubiquitination (cell surface retention of MHC class II) and a process that rather requires an increase in ubiquitination (targeting of MHC class II to exosomes) can occur at the same time. Possibly ubiquitination of MHC class II on MVB versus the plasma membrane requires E3 ubiquitin ligases that are differentially regulated. The transmembrane E3 unbiquitin ligase MARCH VIII was recently shown to be able to ubiquitinate MHC class II and drive its internalization (36). Interestingly, ubiquitination by MARCH ligases may also regulate the cell surface expression other proteins upregulated during DC maturation including MHC class I, CD86 and ICAM-1 (37).These proteins are also found on DC exosomes (38), emphasizing the link between ubiquitination and exosomal targeting. Besides MARCH VIII, the related MARCH I protein may also ubiquitinate MHC class II (36) and differential recruitment of diverse E3 ligases could thus allow separate regulation mechanisms for exosomal targeting and cell surface retention of MHC class II. These ligases may have a preference for MHC class II at a specific cellular location (39) or rely on adaptor proteins. The latter has been described for the related viral E3 ligase K3 (40). The substrate specificity of K3 for MHC class I is determined by sequences in the peptide loading complex proteins TAP-1 and TAP-2 rather than by sequences in MHC class I itself (40). Association of MHC class II with other proteins, such as tetraspanins or HLA-DM, may therefore also influence its ubiquitination and targeting.

In conclusion, our data for the first time provide molecular evidence for the exosomal origin of MHC class II that is secreted upon cognate DC- T cell interaction. Whether a subset of MHC class II molecules is targeted to exosomes remains elusive but our results suggest that during cognate T cell interaction DC may use ubiquitination to differentially regulate MHC class II cell surface expression and exosomal targeting.

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Chapter 6

Summarizing Discussion

Many cell types, including dendritic cells (DC) and B cells secrete small vesicles called exosomes. Exosomes from immune cells are thought to have immuno-regulatory functions but their precise role remains unresolved. The aim of the studies presented in this thesis was to get more insight into the factors that determine exosome formation, composition and secretion as well as to learn more about their physiological relevance.
Exosomes are equivalent to Luminal Vesicles (LV) of Multi Vesicular Bodies (MVB), which are secreted when MVB fuse with the plasma membrane. Studying exosomes may therefore also help to unravel mechanisms that are important for MVB biogenesis.

Incorporation of membrane proteins into the LV of MVB requires, at least for some model proteins, ubiquitination of their cytoplasmic domain. These ubiquitin tags are recognized by a sorting machinery, of which some components are also ubiquitinated. The ubiquitin tags and the sorting machinery are both removed from the cargo before the LV pinch off into the MVB lumen. Exosomes are therefore not expected to contain ubiquitinated proteins. In chapter 2 we demonstrate, however, that exosomes from B cells and DC contain poly-ubiquitinated proteins, many of which are not integrated into the membrane and are relatively enriched as compared to total cell lysates. These results suggest that a subset of poly-ubiquitinated cytoplasmic proteins is incorporated into the MVB. The potential cell biological relevance of this observation is discussed. Furthermore, these data indicate that ubiquitinated proteins can serve as markers for exosomes.

In chapter 3 we present a detailed analysis of the proteome of B cell exosomes using highly sensitive and accurate mass spectrometry. In total 681 different proteins were identified. These include proteins previously detected in exosomes as well as many novel constituents. The latter group also includes many proteins important for protein sorting MVB and endosome dynamics, indicating their role in exosome biogenesis. Furthermore we found that in exosomes, major histocompatibility complex class II (MHC class II) is associated with large protein networks, which include at least 24 other proteins. Although these protein networks may partition into detergent resistant membranes (DRM), their stability does not appear to require abundant association of lipids. These protein networks may reflect a role for protein interactions in exosome biogenesis at MVB. Alternatively, on exosomes, protein clusters may enhance the function of proteins that are important for binding to and signaling in target cells.

In chapter 4 we show that DC secrete exosomes into the immune synapse upon engagement with cognate CD4+ T cells. Furthermore we show that MHC class II is

transferred from the DC to T cells via exosomes. Exosomes are directionally targeted to T cells both by secretion in the immune synapse and trough specific binding to activated but not to resting T cells, indicating that only those T cells that are activated as a result of cognate DC/T cell interaction may acquire DC exosomes. Transferred exosomes were not internalized but rather remained associated to the T cell surface. These results resolve a long-standing question of how MHC II and other membrane constituents are transferred from antigen presenting cells to T cells. Acquisition of DC derived proteins by T cells may have several implications: i) Exosomes remaining on the T cell surface may allow continuation of signaling at the T cell after its dissociation from DC. ii) T cells may acquire MHC class II complexes to downregulate and/ or affinity mature T cell responses. By presenting DC derived antigen-MHC class II complexes to other responding T cells the latter may become anergic or die. iii) Exosomes also transfer, together with cognate peptide-MHC II, antigen-MHC I complexes which may be presented by the CD4+ T cells to CD8+ T cells. In this way CD4+ T cells may help activation of CD8+ T cells without the requirement of a simultaneous association of both T cell types with a single DC.

In chapter 5 we directly studied the molecular mechanism for MHC class II targeting to exosomes and demonstrated that this process requires ubiquitination of its β-chain. In addition, we show that, regardless of the stimulus, DC maturation is accompanied by a decrease in ubiquitination of total cellular MHC class II, consistent with the idea that MHC class II surface expression is stabilized by shutting down ubiquitin-mediated intracellular retention. We demonstrate that during cognate DC/T cell interaction DC increase the sorting of MHC class II to exosomes and at the same time elevate cell surface MHC class II, indicating that ubiquitin mediated trafficking of MHC class II is highly regulated and that exosomes may derive from a specialized subset of MVB.

In conclusion, the studies presented in this thesis provided us with new information on factors that may be important for sorting of proteins into exosomes/MVB and targeting and functioning of exosomes from antigen presenting cells. Based on these studies we hypothesize that exosome biogenesis at MVB may be facilitated by association of cargo proteins with large protein networks and their partitioning into DRM. Such complexes may provide a scaffold for association of cargo with proteins involved in MVB sorting. While sorting into the MVB pathway of some proteins may occur directly as a consequence of ligand binding at the plasma membrane, sorting of other proteins , including MHC class II, may not be regulated by direct ligand binding. For such proteins targeting to MVB/exosomes could be a consequence of their association to other proteins. Although mechanisms are not

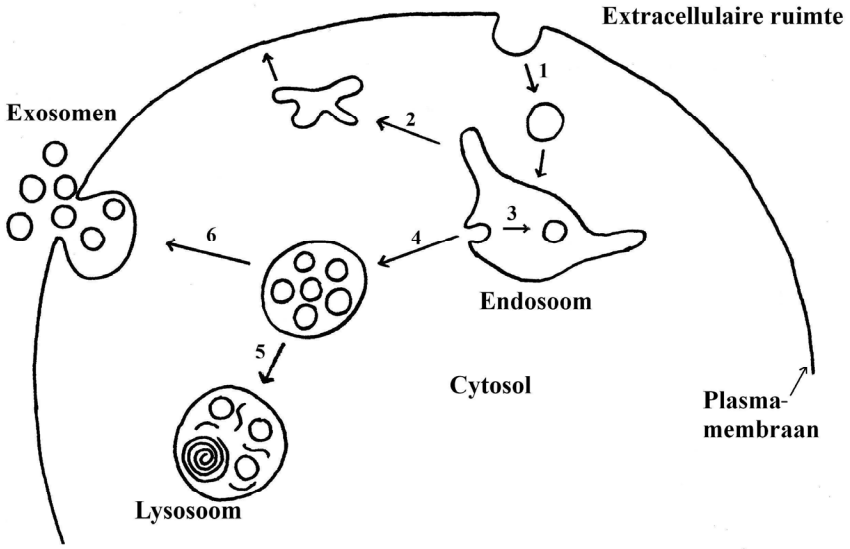
completely clear, exosome content and numbers change in response to interactions with other cells, pathogens or soluble ligands. Therefore, exosomes secreted in response to cell-cell contact may carry a specific set of proteins that will allow acceptor cells to display information on the history of encounters that determined their current state.

Nederlandse samenvatting voor niet-ingewijden

"Somewhere, something incredible is waiting to be known."
-Carl Sagan-

Het lichaam is opgebouwd uit een groot aantal celsoorten, ieder met specifieke functies ten bate van het organisme. Cellen zijn op hun beurt weer opgebouwd uit verschillende kleinere onderdelen, de zogenaamde "organellen", met gespecialiseerde functies. Zo is het lysosoom het afvalvat van de cel en de kern de opslagplaats voor het erfelijke materiaal (DNA). In dit proefschrift worden met name cellen van het afweersysteem ofwel immuunsysteem bestudeerd, dat schadelijke indringers zoals virussen en bacteriën bestrijdt. Ook beschermt het immuunsysteem het lichaam tegen cellen die ongecontroleerd delen (tumoren).
Het immuunsysteem bestaat uit verschillende typen cellen die nauw met elkaar samenwerken. Indringers worden allereerst herkend door zogenaamde "antigeen presenterende cellen (APC)". Deze cellen nemen continu "hapjes" van hun omgeving en "proeven" of er een bedreiging in de buurt is. Vervolgens kunnen APC op hun oppervlak stukjes van eiwitten (antigenen) van de indringer laten zien (presenteren) aan andere cellen. Voor deze presentatie van antigenen maakt de cel gebruik van een eiwitcomplex, "MHC klasse II", dat dient als een soort vlaggenstok waaraan het eiwitstukje wordt opgehangen. Bepaalde witte bloedcellen, T cellen, herkennen de eiwitstukjes van de indringer en doordat de APC ook alarmsignalen geeft (d.m.v. co-stimulatie- en secretie-eiwitten) weet de T cel dat deze antigenen lichaamsvreemd zijn en zal hij een verdedigingsaanval starten. Wanneer er echter geen indringer aanwezig is worden er ook lichaamseigen eiwitstukjes aan de T cellen gepresenteerd, maar dan zonder alarmsignaal, met als gevolg dat de T cellen deze eiwitten tolereren en niet tot een aanval overgaan. Het op deze manier presenteren van "eigen" eiwitten is nodig om het lichaam er continu aan te herinneren welke eiwitten van het lichaam zelf afkomstig zijn en dus geen bedreiging vormen.
Eerder onderzoek heeft aangetoond dat APC net als vele andere celtypen in het lichaam kleine blaasjes uitscheiden, "exosomen" genaamd. Exosomen die door APC worden uitgescheiden kunnen, net als de cellen zelf, antigeen presenteren aan T cellen. Het is nog niet precies duidelijk wat de fysiologische rol van exosomen is en met het onderzoek dat in dit proefschrift wordt gepresenteerd hebben we geprobeerd hier meer inzicht in te krijgen.
Op dit moment wordt door andere onderzoeksgroepen in de kliniek getest of exosomen die geïsoleerd worden uit in het laboratorium gekweekte APC gebruikt kunnen worden in een therapie tegen tumoren. Exosomen die tumorspecifieke eiwitten presenteren worden in het bloed van de patiënt gebracht in een poging T cellen ertoe aan te zetten de tumor te vernietigen. Echter, presentatie van een antigen kan zowel een immuunreactie als tolerantie tot gevolg hebben, afhankelijk van de aanwezigheid van co-stimulatie-eiwitten. Wanneer de context van antigenpresentatie niet juist is kan de T cel een verkeerde tactiek kiezen en de indringer of tumor tolereren in plaats van te lijf te gaan, met alle gevolgen van

dien. Het is dus uitermate belangrijk om precies te kunnen controleren welke eiwitten op exosomen terechtkomen.
De cel beschikt over een uitgebreide infrastructuur (het zgn. cytoskelet), dat is te beschouwen als een soort wegennet waarover organellen en en transportblaasjes die eiwitten tussen organellen transporteren worden vervoerd. De cel bepaalt welke eiwitten via transportblaasjes worden getransporteerd aan de hand van een intrinsieke code waarin staat waar dat eiwit thuis hoort (bijvoorbeeld op het plasmamembraan, het buitenmembraan van de cel). Ook kunnen eiwitten vaak nadat ze gemaakt zijn alsnog een soort oormerk krijgen dat ervoor zorgt dat de locatie van het eiwit kan worden veranderd. Eén type oormerk is ubiquitine, dat aan te transporteren eiwitten kan worden "geplakt". Een geubiquitineerd eiwit kan vervolgens worden herkend door een complex van sorteringseiwitten dat ervoor zorgt dat dit eiwit zijn intracellulaire bestemming bereikt.



Figuur 1: schematisch overzicht van het endocytose systeem van de cel. Eiwitten kunnen worden opgenomen uit de extracellulaire ruimte (de ruimte buiten de cel) d.m.v. endocytose, een proces waarbij de cel hapjes neemt van zijn omgeving (1). Eiwitten komen dan in het endosoom terecht van waaruit ze of terug naar de het plasmamembraan kunnen worden getransporteerd (2), of worden opgenomen in blaasjes die in het endosoom worden gevormd (3). Het endosoom gaat in de tijd steeds meer blaasjes bevatten (4). De blaasjes in endosomen worden of afgebroken wanneer endosomen versmelten met lysosomen (5), of worden uitgescheiden als exosomen in de extracellulaire ruimte wanneer het endosoom met het plasmamembraan versmelt.

Via eiwitten op het plasmamembraan, zoals MHC klasse II, communiceert de cel met zijn omgeving. Wanneer een eiwit niet op het plasmamembraan gewenst is kan de cel dit eiwit internaliseren (ofwel endocyteren) en naar compartimenten in de cel, de endosomen, transporteren (fig. 1). Het hierboven genoemde eiwit ubiquitine speelt een rol bij endocytose van MHC klasse II.
Het endosoom kan op haar beurt ook weer kleine interne membraanblaasjes vormen (fig. 1). Eiwitten worden in deze interne blaasjes opgenomen wanneer er eerst ubiquitine aan wordt geplakt. Het sorteren van eiwitten naar interne blaasjes kan ervoor zorgen dat ze worden afgebroken. Afbraak van de interne blaasjes vindt namelijk plaats wanneer endosomen versmelten met lysosomen, de afvalvaten van de cel. Endosomen met interne blaasjes kunnen echter ook fuseren met de plasmamembraan. Als dit gebeurt komen de interne blaasjes van endosomen in het milieu buiten de cel terecht en worden nu "exosomen" genoemd. Het is nog niet volledig duidelijk hoe de cel bepaalt welke eiwitten in exosomen worden opgenomen en onder welke condities exosomen door APC worden uitgescheiden. Omdat immuunreacties mogelijk in belangrijke mate door deze processen worden gereguleerd is in het in dit proefschrift beschreven onderzoek gepoogd daarin meer duidelijkheid te verschaffen.
In **hoofdstuk 1** van dit proefschrift wordt een overzicht gegeven van de reeds bestaande kennis over de vorming en functie van exosomen, met name door APC geproduceerde exosomen.
In **hoofdstuk 2** laten we zien dat exosomen die worden uitgescheiden door verschillende soorten APC, geubiquitineerde eiwitten bevatten en dat het grootste gedeelte van deze eiwitten geen membraaneiwitten zijn. Hieruit leiden we af dat ubiquitine misschien ook belangrijk is om eiwitten uit het cytosol (fig. 1), die oorspronkelijk niet in organellen zijn ingebouwd, alsnog naar de interne blaasjes van het endosoom te transporteren.
Hoofdstuk 3 beschrijft een uitgebreide studie naar de eiwitten die zich op exosomen bevinden. Onze resultaten laten zien dat MHC klasse II op exosomen een interactie aangaat met verschillende andere eiwitten. Deze eiwitinteracties kunnen belangrijk zijn voor de incorporatie van MHC klasse II in exosomen, en zouden een rol kunnen spelen bij antigeenpresentatie aan T cellen.
In **hoofdstuk 4** laten we zien dat uitscheiding van MHC bevattende exosomen door APC wordt gestimuleerd door de interactie van APC met T cellen . Dit is een aanwijzing dat exosomen belangrijk zijn voor de communicatie tussen deze cellen. Ook laten we zien dat de T cellen, die zelf geen MHC klasse II maken, door middel van exosomen MHC klasse II kunnen opnemen. Deze eiwitoverdracht kan belangrijk zijn voor het aansturen van immuunreacties.
Tot slot laten we in **hoofdstuk 5** zien dat het uitscheiden van MHC klasse II via exosomen, als gevolg van de interactie van APC met T cellen, inderdaad

afhankelijk is van ubiquitinering van MHC klasse II. Eerder onderzoek door onze onderzoeksgroep had al aangetoond dat ubiquitinering belangrijk is voor endocytose van MHC klasse II . Hierop voortbouwend laten we zien dat wanneer APC verschillende soorten gevaar opmerken dit altijd gepaard gaat met een afname van de ubiquitinering van MHC klasse II, met gevolg dat er minder MHC klasse II wordt geëndocyteerd. Op deze manier komt er meer MHC klasse II op het plasmamembraan terechtkomt en dit bevordert antigeenpresentatie aan T cellen.

List of publications

- **Exosomes contain ubiquitinated proteins**
  Sonja I. Buschow, Jolanda M.P. Liefhebber, Richard Wubbolts, Willem Stoorvogel
  Blood Cells Mol Dis 35:398 (2005).
- **Dendritic cells secrete and target MHC class II carrying exosomes to cognate T cells**
  Sonja I. Buschow, Maaike S. Pols, Esther N.M. Nolte-'t Hoen, Marjolein Lauwen, Ferry Ossendorp, Rienk Offringa,
  Cornelis J.M. Melief, Marca H.M. Wauben, Richard Wubbolts, and Willem Stoorvogel
  Submitted
- ***Dendritic cells regulate exposure of MHC class II at their plasma membrane by oligo-ubiquitination.***
  Guillaume van Niel, Richard Wubbolts, Toine A.G. ten Broeke, Sonja I. Buschow, Ferry A. Ossendorp, Cornelis J Melief,
  Graca Raposo, Bas W. van Balkom, and Willem Stoorvogel
  Submitted
- ***Role of Cargo proteins in GGA recruitment***
  Jennifer Hirst, Matthew N.J. Seaman, Sonja I. Buschow and Margaret S. Robinson,
  Submitted
- **A Comprehensive analysis of the proteome of B cell exosomes and MHC class II-associated proteins: implications for exosome biogenesis and function**
  Sonja I. Buschow, Bas W.M. van Balkom, Marian Aalberts, Martijn W.H. Pinkse, Albert J.R. Heck, and Willem Stoorvogel
  Manuscript in preparation
- **Ubiquitination of MHC class II is required for its targeting to exosomes in response to cognate T cell interaction**
  Sonja I. Buschow, Richard W. Wubbolts, Maaike S. Pols, Anko M. de Graaff, Marca H.M. Wauben, and Willem Stoorvogel
  Manuscript in preparation

Curriculum Vitae

Sonja Buschow werd op 29 augustus geboren te Geldrop. In 1994 voltooide zij haar HAVO opleiding aan het Hertog Jan college te Valkenswaard. Na het behalen van haar propedeuse aan de Hogere Laboratorium opleiding te Utrecht begon zij in 1996 aan de opleiding "Medische Biologie"aan de Universiteit Utrecht. Haar hoofdvak stage liep zij op de afdeling Celbiologie op het UMC Utrecht, onder begeleiding van Dr. W. Stoorvogel.
Haar tweede stage verrichte zij onder begeleiding van Dr. A. Motley en Dr. M.S. Robinson op het lab voor klinische biochemie aan de Universiteit van Cambridge, Engeland.
In maart 2001 begon zij aan het onderzoek leidende tot dit proefschrift onder begeleiding van Prof. W. Stoorvogel, allereerst bij de afdeling celbiologie op het UMC Utrecht en later, vanaf januari 2004, bij de afdeling Celbiologie & Biochemie aan de Faculteit der Diergeneeskunde van de Universiteit Utrecht.
Vanaf oktober 2006 is zij werkzaam als postdoc op de afdeling tumor immunologie in de onderzoeksgroep van Prof. C.Figdor aan de Radboud Universiteit Nijmegen.

Dankwoord

"I not only use all the brains I have, but all that I can borrow."
-Woodrow Wilson-

Het is dus toch waar wat iedereen zei. Het komt echt ooit af en alles komt echt goed. Daar heb ik namelijk wel eens aan getwijfeld de afgelopen maanden……..
Het was een top tijd op twee super labs in een fijne stad met een hele hoop geweldige mensen.
Zowel op de het celbiologie lab op het UMC, als dat bij diergeneeskunde maar ook thuis, in de kroeg of elders hebben velen me afgelopen jaren een fijne tijd bezorgd en me geholpen met proeven, schrijven, me voorzien van de nodige uurtjes discussie, me opgepept als het even tegenzat of me juist de ruimte gegeven te stuiteren als het goed ging. Ik wil iedereen hier graag voor bedanken en enkelen van jullie in het bijzonder.

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