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IMMUNE MODULATION BY EXOSOMES

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SUMMARY

Exosomes are small membrane vesicles ranging in size from 40-90 nm secreted by a number of different cells such as dendritic cells, B- and T cells, mast cells and intestinal epithelial cells. Exosomes have also been isolated from several body fluids such as bronchoalveolar lavage (BAL), urine, serum and breast milk. Exosomes are believed to function as communicators between cells, carrying a message of both activation and suppression of the immune response. They are also believed to be utilised as a transport for retroviruses and to be responsible for the intracellular membrane exchange and the spread of prions. The different functions of exosomes are most likely dependent on their origin since the composition and expression of surface markers of the exosomes vary with the type of cell they are secreted from. Recent studies indicate that exosome formation is a highly regulated process but the mechanisms behind exosome biogenesis are still far from being completely understood. There is no doubt though that these small vesicles play an important part in the spread of immunological information and immune regulation. This review aims to adress some of the many functions of exosomes with emphasis on the role of intestinal epithelial cell (IEC) derived exosomes in tolerance induction.

EXOSOME BIOLOGY

Exosomes are small, 40-90 nm membrane vesicles of endocytic origin that are secreted by a variety of cells in culture. They were described for the first time in 1981 as microvesicules containing 5´-nucleotidase activity secreted by neoplastic cell lines (*Trams* et al., 1981). A few years later two independent groups reported secretion of small vesicles of endocytic origin by cultured reticulocytes. Using electron microscopy they observed these small vesicles in the late endosomes which by fusion with the cell membrane released the vesicles extracellularly. The supposed function of the exosome in this study was to remove the transferrin receptor from the cell surface (*Harding* et al., 1983; *Pan* et al., 1985). A decade later, in 1996, exosomes were for the first time shown to have an immunological function. Antigen pulsed B cells secreted exosomes originating from multivesicular bodies which activated antigen specific T cells (*Raposo* et al., 1996). Today we know that exosomes can be secreted by a variety of cells in culture and can be isolated *in vivo* from

body fluids such as serum (*Caby* et al., 2005; *Janiszewski* et al., 2004), bronchoaveolar lavage (*Admyre* et al., 2003) and urine (*Pisitkun* et al., 2004). So far intestinal epithelial cells (*Karlsson* et al., 2001; *Van Niel* et al., 2001), T- and B-lymphocytes, dendritic cells, macrophages, reticulocytes, mast cells and platelets have been shown to produce exosomes (*Thery* et al., 2002). Exosomes are believed to function as communicators between cells, carrying an antigen specific message resulting in either activation or suppression of the immune response. The significance of the message that the exosomes carry seem to depend on what cell they originate from and the state of the cell during exosome formation.

The process of exosome formation starts with invagination of the cell membrane and formation of endosomes. Invagination and inward budding of the membrane of late endosome then forms the exosome. Upon fusion with the cell membrane these multivesicular endosomes release exosomes extracellularly (Figure 1). So far two different mechanisms has been suggested which supports the idea that exosome formation and release is a highly regulated process. The first one is the identification of the endosomal sorting complex required for transport (ESCRT) in association with exosomes (*Williams* and *Urbe*, 2007), the second was just recently identified as ceramide-triggered budding (*Trajkovic* et al., 2008). The ESCRT sorts ubiquinated proteins for transport in the endosomal network, however not all proteins found in exosomes are ubiquitinated. So far no "exosome-specific" marker has been identified hence they are characterised on morphological and biochemical criteria. Exosomes are commonly defined as small membrane bound vesicles originating from the cell surface and processed/modified intracellularly resulting in a multi-vesicular compartment, which is emptied to the extra cellular space, thus releasing the exosomes (Figure 1). Due to their size exosomes can only be visualized in electron microscope. Figure 2 shows exosomes isolated from serum stained with ICAM-1 (*Ostman* et al., 2005).

Due to the formation pathway of exosomes, the molecules found on their surface are typically of endocytic/lysosomal origin e.g., CD9, CD63 and CD81 (*Escola* et al., 1998). These molecules belong to a family of proteins called tetraspanins, which have been suggested to be involved in cell adhesion, activation, proliferation and antigen presentation. Exosomes from antigen presenting cells express MHC class I and II together with co-stimulatory molecules like CD54, CD80 and CD86, which explains their capacity to activate T cells (*Escola* et al., 1998; *Lamparski* et al*., 2002)*. The enrichment of these co-stimulatory molecules on the exosome seems to depend on the maturation state of the antigen presenting cell (APC), indicating that they would also stimulate the immune system in different ways (*Segura* et al., 2005). Exosomes from intestinal epithelial cells (IECs) also express MHC class II along with e.g., CD63, CD81 and A33 which is a marker specific for IECs (*Karlsson* et al., 2001; *Van Niel* et al., 2001). IEC exosomes can be immunostimulatory, both as suppressors and activators of the immune system (*Karlsson* et al., 2001; *Van Niel* et al., 2003). Mast cells secret exosomes expressing MHC class II, CD86, LFA-1 and ICAM-1 which mediates Mast cell-dependent B and T cell activation (*Skokos* et al., 2001). Recently it has also been reported that mast cell derived exosomes contain mRNA which can be transferred between cells (*Valadi* et al., 2007). Depending on what cell they originate

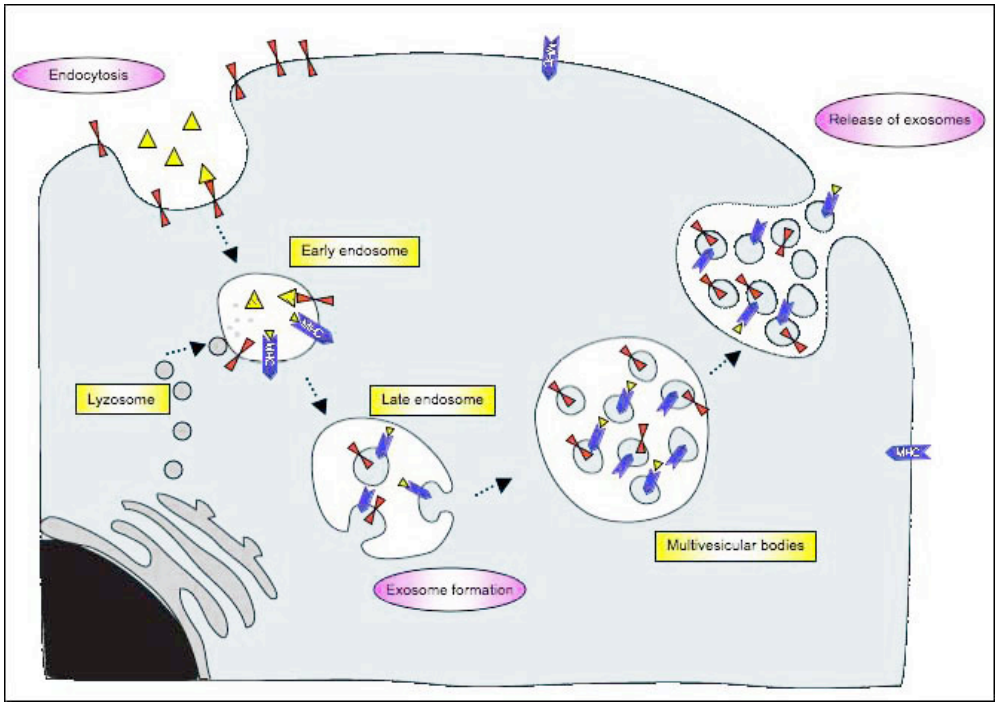

Figure 1: The process of exosome formation starts with invagination of the cell membrane and formation of endosomes, invagination and budding of the membrane of late endosome then forms exosomes. Upon fusion with the cell membrane these multivesicular endosomes release exosomes extracellularly.

from, exosomes express different surface markers and have different lipid composition (*Laulagnier* et al., 2004), hence they are likely to have different functions.

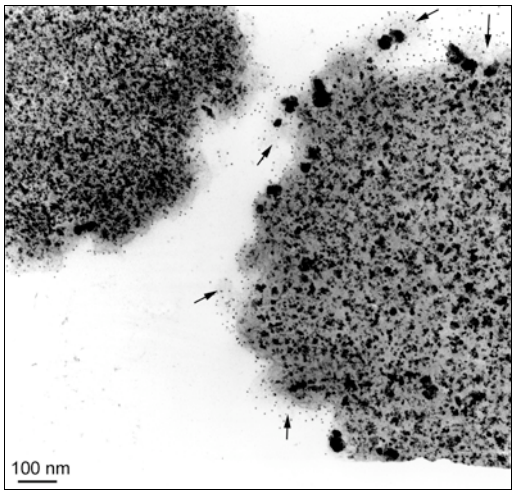

Figure 2: Electron microscope picture of exoxomes.

EXOSOME FUNCTION

Exosomes have been studied most extensively in cancer therapy due to their T-cell stimulatory capacity. One of the pathogenic problems with cancer is the unresponsiveness of the immune system and one way to overcome this is to give the patient autologous APC's primed with tumour antigen. However it has been shown that also the exosomes, derived from these primed DC's, most effectively trigger a tumour specific T cells response. The advantage exosomes have over the whole cell in this case is their size; they spread through the system more efficiently then an activated dendritic cell, but still carry the same message. They can also be collected at one occasion and stored frozen for multiple dosing. Studies show that exosomes loaded with tumour antigens can stimulate CD4+ and CD8+ T cells. For example, exosomes isolated from *in vitro* cultured dendritic cells, pre-pulsed with tumour antigen, induced rejection of established tumours (*Amigorena* 1998; *Hsu* et al., 2003; *Zitvogel* et al., 1998). Depending on the maturation state of the secreting dendritic cell, the exosomes released from the latter appear to trigger the immune response in different ways. It has been shown that ICAM-1, which is more abundant on exosomes from mature DCs, is crucial for naïve T cell priming by exosomes (*Segura* et al., 2005). Another recent study shows that exosomes from IL-10 treated DCs are capable to suppress inflammation and collagen-induced arthritis (*Kim* et al., 2005). In conclusion, the current literature shows that the outcome of an immune response to DC derived exosomes depend on the state of the originating DC. Moreover, along with the type of markers on the surface, the amount of exosomes secreted from dendritic cells at different maturation state seems to differ (*Segura* et al., 2005).

As in the case of DC derived exosomes, the exosomes secreted from IEC can both suppress as well as activate the immune system. We have shown that IEC exosomes can transfer antigen specific tolerance (*Karlsson* et al., 2001) while another group showed that IEC exosomes prime for an aggressive immune response rather than tolerance (*Van Niel* et al., 2003). The conclusion of these experiments is that IECs are capable of producing exosomes that can initiate an immune response, but the outcome of such response may differ in different experimental settings.

We have also shown that exosomes isolated from serum shortly after an antigen feed have the capacity to transfer tolerance to recipients and protect against allergic airway sensitization (*Almqvist* et al., 2008). Moreover, it has recently been shown that exosomes from B cells, isolated and cultured from human PBMC, can present allergen peptides and activate allergen specific T cells to proliferate and produce Th2 cytokines (*Admyre* et al., 2007). Taken together these findings suggests that exosomes from different sources may play a role in the development of asthma and allergy in at least two ways; either as a failure to induce effective tolerance or as enhancers of an already established allergic response. This makes exosomes highly interesting as therapeutic targets in anti-allergy treatment.

Exosomes are also believed to be utilised as a transport for retroviruses (*Pelchen-Matthews* et al., 2004). Gould et al suggests 'The Trojan exosome hypothesis' which states that retroviruses use the pre-existing nonviral exosome biogenesis pathway for the formation of infectious particles (*Gould*

et al., 2003). The exosome-like vesicles would contain virus particles undetectable to the host's own immune system. It has been shown that macrophages infected with HIV release HIV particles displaying, to a certain extent, similar molecules as exosomes (*Nguyen* et al., 2003). In addition HIV virons assemble in the MVBs of macrophages, which is the site where exosomes are formed (*Kramer* et al., 2005). Another study has shown that HIV infected immature DC release exosomes that can transfer HIV to CD4+ T cells. They also show that the exosome-associated HIV was 10 fold more infectious then free virus particles (*Wiley* et al., 2006). Using exosomes as a transport is an excellent strategy to escape the host defence. The virus is protected inside a membrane bound vesicle that is readily taken up and processed by a number of different cells unaware of its infectious content, just like a true 'Trojan Horse'.

Furthermore there is evidence suggesting that exosomes are contributing to the intracellular membrane exchange and the spread of prions (*Fevrier* et al., 2004; *Vella* et al., 2007). These studies show that infectious prion proteins, abnormally folded prion proteins (PrPs), scrapie (PrPsc) are associated with exosomes. Furthermore the exosomes had the capacity to transfer the PrPsc to uninfected cells and transform normal PrPs into scrapie PrPsc. Exosomes enriched in PrP have also been isolated from sheep cerebral spinal fluid and is suggested by the authors to be a way of detecting abnormal forms of the prion (*Vella* et al., 2008).

IEC EXOSOMES AND TOLERANCE

Oral administration of an antigen gives rise to a generation of $CD4^+$ T cells that down regulate the immune response and induce tolerance to the antigen (*Chen* et al., 1994; *Groux* et al., 1997; *Karlsson* et al., 1999). The mechanisms behind oral tolerance and the induction of these regulatory CD4+ T cells remain largely unknown. We have shown that one possible route for tolerance is via exosomes produced by intestinal epithelial cells (tolerosomes) (*Karlsson* et al., 2000, 2001, 2002). The initial step in this process is active sampling by the small intestinal epithelial cells of the luminal content at the mucosal surface. The antigen is processed and peptides are loaded on MHC class II molecules, which are constitutively present in IECs (*Lin* et al., 2005). Exosomes, carrying MHC class II-peptide complexes, are formed and released at the basolateral side of the IEC (*Karlsson* 2001). We believe that these exosomes are transported across the endothelium, enters the circulation and quite rapidly reaches the liver. This is supported by the fact that we can isolate exosomes from serum expressing the A33 molecule (*Ostman* et al., 2005). The liver is important for oral tolerance induction since it is the major draining site of the intestinal circulation. It contains APC's that effectively clears particulate matter of similar size as the exosomes (≈40nm) from the blood (*Matsuno* et al., 1996; *Willekens* et al., 2005). In addition it has previously been reported that portal drainage through the liver is a prerequisite for establishing this form of tolerance (*Callery* et al., 1989; *Yang* et al., 1994). The environment in the liver is naturally very tolerogenic with high levels of TGF-β and IL-10 (*Knolle* and *Gerken*, 2000), and the message sent as a consequence of the processing and presentation of antigen-loaded exosomes by liver APC's is therefore

likely to be one of tolerance induction. The presentation by APC's to naïve T cells, resulting in an increased population of induced antigen-specific regulatory T cells, is likely to occur in the liver or in the liver draining lymph nodes, due to migrating APC's. This theory is supported by a previous study, which shows that after oral administration of an antigen there is an induction of regulatory T cells in the liver draining lymph node (*Hultkrantz* et al., 2005). The same study shows that the celiac lymph nodes draining the liver rapidly become engaged in the response to fed antigens and that the T-cells become activated within 6h and later develop into a distinct antigen specific T-cell population with a regulatory phenotype and a suppressive function (*Hultkrantz* et al., 2005). These results have been confirmed in a recent study which also shows that regulatory, Foxp3 expressing, T cells are induced in the liver draining lymph nodes after feeding an antigen orally (*Siewert* et al., 2008). This strengthens the idea of a central role for the celiac lymph node (CLN) in tolerance induced by feeding an antigen, and suggests that CLN function as a "boot camp for regulatory T cells". The possible fate of exosomes/tolerosomes and their involvement in tolerance induction is overviewed in Figure 3.

As previously mentioned we have shown that exosomes can be isolated from serum 1h after an antigen feed and transfer antigen specific tolerance when injected into naïve recipients (*Almqvist* et al., 2008; *Karlsson* et al., 2001). The recipient animals were protected against both Th1 and Th2 dominated responses. Moreover we have shown that exosome-mediated tolerance is MHC class II dependent and requires an intact immune system in the fed donor (*Ostman* et al., 2005). Germ-free mice lack MHC II-expression in the small intestinal epithelium, which results in the formation of non-informative exosomes that without MHC class II lack antigen presenting capacity, and failure to induce regulatory T cells after oral antigen administration (*Rask* et al., 2005). A full flora generally provides the required stimuli for the maturation of the intestinal immune system and the intestinal epithelial cells, but it is not known which individual bacteria or bacterial products that delivers the necessary signals. A collaborating group investigated whether neonatal mucosal exposure to SEA could influence the capacity to develop oral tolerance and reduce sensitisation and allergy. Their results show that SEA pretreated mice are more efficiently tolerised by OVA feeding. This suggests that strong T cell activation in infancy promotes the development of oral tolerance (*Lönnqvist* et al., unpublished data). We have examined the role of mucosal exposure to *S. aureus* enterotoxin A regarding the capacity of tolerogenic processing by the intestinal epithelium in adult mice. Our results indicates that the *S. aureus* enterotoxin A potentiates the development of oral tolerance, and we show for the first time that this effect can be transferred to naive recipient mice by the adoptive transfer of serum. Our results suggest that the exosome fraction produced by SEA-exposed epithelium more efficiently modulates the immune system into a tolerogenic response to a fed antigen (*Hultkrantz et al*., unpublished data). In conclusion, bacterial stimuli are important both for the tolerogenic processing and the development of oral tolerance.

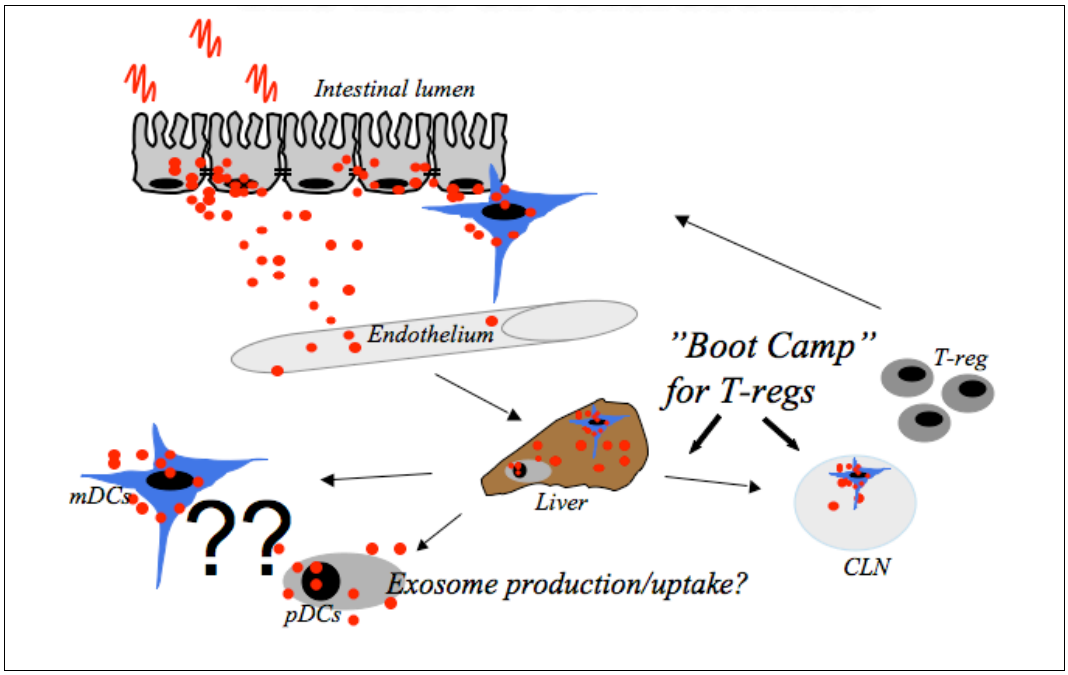

Figure 3: The fate of tolerosomes. The Intestinal epithelial cells release exosomes at the basolateral side. The exosomes are transported across the endothelium and travels with the blood to the liver were they are taken up and processed by local APCs. The APCs present the exosome-message in a tolerogenic milieu resulting in elevted numbers of regulatory T cells.

CONCLUDING REMARKS

It is clear that the functions of exosomes are many and their complete biological role is yet to be understood. What can be concluded so far is that exosomes are important players for passing on immunological information between cells and thus take active part in immune regulation. They are released by many different cell types including professional APC's and the epithelial cells lining the major mucosal interface to the environment, and found in various body fluids. Exosomes can be produced and manipulated *in vitro* and safely administered to patients. This makes them highly interesting as therapeutic targets e.g. as vaccine vehicles in cancer. Their tolerogenic capacity could further be exploited in autoimmune and allergic diseases.

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