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TO MY SATISFACTION

ABSTRACT

Nano-vesicles (30-100 nm) with an endosome-derived limiting membrane are called exosomes. These are released from the cell when the endosome fuses with the outer cell membrane. Exosomes from antigen presenting cells (APC) carry MHC class I and class II as well as integrins, tetraspanins and co-stimulatory molecules. They can either stimulate T cell responses or induce tolerance. Exosomes are presently being evaluated as therapeutic tools but still little is known about their biological roles. The overall aim of this thesis was to study exosomes as immune-regulators *in vitro* and to investigate how exosomes found *in vivo* may exert immune regulatory effects. To achieve this, new methods were developed.

It is debated whether exosomes can stimulate T cells directly or if APC are needed. Exosomes produced by human monocyte-derived dendritic cells (MDDC) were analyzed for their $CD8^+$ T cell stimulatory capacity. This was explored in a virus peptide specific, highly sensitive enzyme-linked immunospot (ELISPOT)-assay measuring IFN-γ or TNF-α release. The stimulation occurred without APCs and was dependent on exosome-dose and MHC class I. Exosomes from lipopolysaccharide (LPS)-matured MDDC were more efficient stimulators than exosomes from immature MDDC. Thus, the method proved to be a sensitive way to measure exosome T cell stimulatory capacity.

Further, MDDCs generated in two different ways and their released exosomes were compared. Exosomes from IL-4/IL-3 generated MDDCs showed significantly higher levels of HLA-ABC, HLA-DR, CD11c, CD63 and CD81 than exosomes from conventional MDDCs. Both kinds of exosomes could stimulate IFN-γ release from $CD8^+$ T cells using the virus peptide-specific ELISPOT, however, no difference in stimulatory capacity was detected. On the cellular level, the IL-4/IL-3 generated MDDC showed a slightly more efficient T cell stimulatory capacity as compared to conventionally generated MDDC. Thus, exosomes inherited some features from their parent cells, but not all, and both types were able to stimulate virus specific T cells and could potentially be used in therapy.

Next we investigated whether human breast milk contains exosomes. By immuno-capture and flow cytometry, electron microscopy, sucrose density gradient centrifugation, Western blot, and mass-spectrometry, we identified exosomes. Interestingly, these exosomes inhibited anti-CD3 induced cytokine production, which correlated with an induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells. For the first time the presence of exosomes in human breast milk has been shown and they have a potential to influence immune responses.

To assess if exosomes from different sources have preferences for specific cell types in peripheral blood mononuclear cells (PBMC), we compared the association patterns of exosomes isolated from MDDC, B cells and human breast milk. Flow cytometry and confocal laser scanning microscopy showed that both MDDC- and milk-exosomes preferred monocytes, which mainly ingested the exosomes, whereas B cell exosomes targeted $CD19^+$ B cells and remained surface associated. Multispectral imaging flow cytometry supported our findings and made possible the objective analysis of a high number of cells in each sample. This novel technique can be useful for the development of different exosomes for therapies and for exploring biological roles of exosomes.

In conclusion, the work presented in this thesis has increased our understanding of the presence of human exosomes *in vivo,* and addressed how different exosomes may exert distinct immunomodulatory effects.

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I. Charlotte Admyre, Sara M Johansson, Staffan Paulie, Susanne Gabrielsson. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur J Immunol.* 2006, Jul;36(7):1772-81.

II. Sara M Johansson, Charlotte Admyre, Annika Scheynius, Susanne Gabrielsson. Different types of *in vitro* generated human monocyte-derived dendritic cells release exosomes with distinct phenotypes. *Immunology* 2008, Apr;123(4):491-9.

III. Charlotte Admyre, Sara M Johansson, Khaleda Rahman Qazi, Jan-Jonas Filén, Riitta Lahesmaa, Mikael Norman, Etienne P.A. Neve, Annika Scheynius, Susanne Gabrielsson. Exosomes with immune modulatory features are present in human breast milk. *J Immunol.* 2007, Aug 1;179(3):1969-78.

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Morales-Tirado V, Johannson S, Hanson E, Zhang J, Siminovitch KA, and Fowell DJ. Cutting edge: selective requirement for the Wiskott-Aldrich syndrome protein in cytokine, but not chemokine, secretion by $CD4^+$ T cells. *J Immunol,* 2004*,* 173:726-30.

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CONTENTS

LIST OF ABBREVIATIONS

Ab	antibody
APC	antigen-presenting cell
BAL(F)	bronchoalveolar lavage (fluid)
BCR	B cell receptor
CBA	cytometric bead array
CD	clusters of differentiation
CEF	CMV, EBV, Flu (virus peptide mix)
CMV	cytomegalovirus
CTL	cytotoxic lymphocyte ($CD8^+$ T cell)
DC	dendritic cell
DTH	delayed-type hypersensitivity
EBV	Epstein-Barr virus
ELISPOT	enzyme-linked immunospot assay
ER	endoplasmic reticulum
ESCRT	endoplasmic sorting complex required for transport
Flu	influenza virus
Foxp3	fork-head box protein 3
GM-CSF	granulocyte macrophage stimulatory factor
HLA	human leukocyte antigen
Hsc	heat shock cognate
Hsp	heat shock protein
IEC	intestinal epithelial cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
MIIC	MHC class II enriched compartment
mDC / pDC	myeloid dendritic cell / plasmacytoid dendritic cell
MDDC	monocyte derived dendritic cell
MFG-E8	milk-fat globule factor E8
MHC	major histocompatibility complex
MS	mass spectrometry
MUC-1	mucin-1
MVB	multivesicular body
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PHA	phytoheamagglutinin
PLAP	placental type alkaline phosphatase
PRR	pattern recognition receptor
RNA	ribonucleic acid
TCR	T cell receptor
TEM	transmission electron microscopy
TGF	tumor growth factor

TLR	Toll-like receptor
TNF	tumor necrosis factor
Th	T helper cell ($CD4^+$ T cell)
T reg	T regulatory cell
WB	Western blot

1 INTRODUCTION

1.1 THE HUMAN IMMUNE SYSTEM

Our immune defense is an intricate system made up of physical barriers and organ-structures, consisting of different cell types and mediators that collaborate to protect the body from harmful agents. The constant microbial pressure from both infectious and harmless microorganisms has shaped both sides in this constant combat. Invaders can adapt quickly to evade immune recognition, and the immune system has adapted throughout evolution to handle the pressure by trimming its recognition repertoire. The immune system consists of two main parts, the innate and the adaptive[1].

The innate arm is evolutionarily ancient and present in different versions in most multi-cellular organisms. The barrier function of the skin and epithelial surfaces prevents entry of pathogens by physical hindrance or through anti-microbial peptides. Innate players often act fast, and rely largely on germ-line encoded pattern recognition receptors (PRRs) such as Dectin-1 or Toll-like receptors (TLRs)[2] expressed on e.g. phagocytic cells like antigen presenting cells (APC) and neutrophils. PRRs interact with pathogen-associated molecular patterns (PAMPs) which are conserved structures commonly expressed on many microorganisms. This interaction leads to the activation of signaling cascades and release of cytokines and inflammatory mediators that recruit additional cells and restrict spread of infection by tissue swelling. The complement cascade is a part of innate immunity and functions by opsonizing microbial structures for membrane-attack and microbial cell destruction. Triggering the complement cascade can occur in three different ways; the alternative pathway, where spontaneously activated complement components are directly deposited on pathogen surfaces, the mannose-binding lectin pathway, or the classical pathway where opsonization through antibodies from the adaptive immune system play a part.

Adaptive immunity is the other arm of the immune system and owes its name to the occurrence of adaptation of the response towards a pathogen during an individual's lifetime. The key players are the lymphocytes, consisting of T- and B-cells, that carry highly specific antigen receptors on their surfaces. The broad repertoire of receptor specificities is a result of somatic gene-rearrangements in the cells. B cell receptors, are surface bound immunoglobulins (Ig), which can bind a great diversity of antigens in their native form. Activated B cells also release Ig as soluble antibodies. T cell receptors are surface-bound and recognize peptide antigens in association with major histocompatibility complexes (MHC) on APC. Whereas the innate immunity acts immediately when a pathogen enters, the adaptive events take longer time to develop. Recall responses are however quicker, and result from the long-lived memory cells of the adaptive system.

1.2 CELLS OF THE IMMUNE SYSTEM

The immune system is spread throughout the body. Primary lymphoid organs like the bone marrow and thymus are sites of lymphocyte generation and primary education where B- and T-cells learn to discriminate self from non-self. The cells circulate through the blood out to secondary lymphoid organs like the spleen and lymph nodes,

where antigen recognition takes place after interaction with APC. The lymph drains the tissues and transports APC and antigens through afferent vessels to lymph nodes where effector cells are activated and leave the nodes through the efferent vessels, which ultimately empty back into the blood stream. Overall, secondary lymphoid structures vary in size depending on where activity is currently intense, and swell during infection. Different cells are specialized to reside in tissues or to circulate, and resting cells can be activated and recruited by inflammatory signals such as cytokines or chemokines.

1.2.1 Dendritic cells

Dendritic cells (DCs) are the most professional of the antigen presenting cells, with the capacity to initiate primary and secondary immune responses. Whereas the macrophages are engulfing foreign material for destruction, the main function of DCs is to process antigens and pass on the information as a link between innate and adaptive immunity. DCs originate from the bone marrow and reside as sentinels in the tissues where they have high phagocytic capacity, with a phenotype often referred to as immature. Humans have two main sub-types of DCs in peripheral blood called myeloid (m)DC that are $CD11c^+$, and plasmacytoid (p)DC that are $CD11c^-$ (reviewed by Liu *et al* [3]) There is no consensus whether mDC and pDC have a common progenitor or if they originate from different lineages early in the development. DCs are mainly present in tissues but are quite rare in the circulation where mDC constitute approximately 0.6 % and pDC 0.4 % of the peripheral blood mononuclear cells (PBMC)[4]. In order to explore DCs in experimental studies and for therapeutic purposes, different ways of *in vitro* generation have been established to generate high numbers of DC-like cells. $CD34^+$ precursor cells from cord blood or, most commonly, $CD14^+$ monocytes in peripheral blood are the starting materials and by culture with combinations of growth factors (e.g. GM-CSF) and cytokines (e.g. IL-4, IL-3, TNF-α)[5-7] high numbers of DCs can be generated *in vitro* in about 6 days.

1.2.1.1 Antigen uptake, processing and presentation

DCs are situated in the body where antigens are encountered, as in epithelia of mucosal surfaces and in the skin, and sample their surroundings constantly. Antigen uptake, called endocytosis, can be of various kinds; phagocytosis (uptake of particles), macro- or micropinocytosis (uptake of soluble antigens or very small particles), or receptor mediated. For the latter there are different kinds of receptors (e.g. PRR) and for example Fc-receptors, which bind to the shaft of antibodies and incorporate structures recognized by antibodies. Other receptors are c-type lectins, including DC-SIGN and DEC-205 that bind to specific sugar-structures on pathogens[8]. Another group of receptors are named scavenger receptors, and contain e.g. SR-A1-2, MARCO, and CD36.

Specialized intracellular organelles take care of the engulfed material; endosomes harbor soluble antigens whereas particulate antigens localize in phagosomes. These compartments mature gradually by acidification and ultimately fuse with lysosomes containing degradative enzymes like proteases. Some endosomal compartments are specialized in loading of MHC class II molecules (MIIC, specialized multivesicular bodies (MVB)). Exogenous antigens are degraded to fit in the antigen binding cleft of MHC molecules for presentation on the DC surface, whith a typical length of 13-17 amino acids[1,9] for MHC class II presentation. Endogenous and viral antigens (produced in the cytosol), are instead processed by the proteasome in the cytosol, and loaded to the smaller cleft of MHC class I in the endoplasmic reticulum[1].

Upon encountering a danger signal[10], e.g. in the form of a microbial structure binding to a TLR or other PRR, the DC is reprogrammed to migrate from its peripheral site to a lymph node, where it interacts with T cells to activate effector cells. Simultaneously, a number of events occur that changes the phenotypes of the DCs, from its immature state it (i) becomes less phagocytic, (ii) starts producing cytokines and (iii) upregulates co-stimulatory molecules like CD40, CD80 (B7-1), CD86 (B7-2) and the maturation marker CD83[1]. In the absence of a danger signal and in their steady state, DCs take up and present self-antigens that do not trigger maturation, leading to T cell unresponsiveness, due to low levels of co-stimulatory signals[1].

1.2.2 T lymphocytes

T cells originate from the bone marrow but are educated in the thymus (hence named T)[1]. The T cell receptor (TCR) is highly specific which results from gene rearrangement events in the thymus. Here the T cells express a wide repertoire of TCR specificities for both self- and foreign peptides and undergo thymic selection in two steps, first positive and then negative. In the positive selection, thymic stroma cells express MHC molecules with peptides and T cells with TCR that are able to recognize self-MHC are positively selected, and survive. Then, in the negative selection, the T cells with TCR that strongly reacts with MHC:self-peptide complexes are deleted through apoptosis. However, some self-reactive T cells slip through unaffected by the negative selection and leaves the thymus along with the properly educated T cells and re-circulate in the blood through the lymphoid organs. Luckily, there are mechanisms in the periphery to regulate these self-reactive T cells. T cells that leave the thymus are considered naïve, but will develop into effector cells after interaction with an activated APC. Once the T cell finds a "perfect match" together with co-stimulatory signals, it differentiates into an effector cell, of which there are several different types. After a pathogen has been cleared most T cells undergo apoptosis, but some descendants from the effector cells survive as long-lived memory cells that are quickly activated if the individual is re-infected.

1.2.2.1 Cytotoxic T cells

T cells with the main role to kill cells, harboring intracellular pathogens, or cancer cells express the co-receptor CD8 and are named cytotoxic T lymphocytes (CTL). The TCR together with the CD8 binds to MHC class I with bound peptides and upon activation release cytotoxic effector molecules like perforin and granzymes. These mediators damage the target cells by creating pores and destroying the membrane integrity leading to cell death. CTL can also induce apoptosis of target cells by providing signaling through FasL/Fas interactions. To amplify immunity against intracellular pathogens, CTL release the cytokines TNF-α and IFN-γ that can activate macrophages and trigger up-regulation of MHC class I on APC.

1.2.2.2 Regulatory T cells

Since the thymic selection process does not completely exclude auto-reactive T cells and some are released into re-circulation, there is a need to prevent them from being activated. For this purpose there exist regulatory T cells (T regs) which suppress effector functions through release of IL-10 and TGF-β[11]. T reg cells are divided in two main subtypes, naturally occurring and adaptive[12]. Naturally occurring T regs are $CD4^+CD25^+$ and educated in the thymus from which they exit as fully suppressive cells to constitute 5-10 % of the peripheral $CD4^+$ T cell pool. These cells express the transcription factor fork-head box protein 3 *(FOXP3),* whose presence as an intracellular protein, or detection as mRNA, is to date the most used marker of naturally occurring T regs. The distinction between naturally occurring and adaptive T regs is

most clear in the murine system, but can be applied to humans to some extent. Mutation in the *FOXP3* region in humans results in a disease called IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) that causes severe immunodeficiency in an autoimmune way[12]. The adaptive T regs originate from naïve peripheral T cells and come in two flavors; Tr1 and Th3. They acquire their suppressive phenotype in the periphery and produce the suppressive cytokines IL-10 (Tr1 and Th3) and TGF-β (Th3)[11].

1.2.2.3 T helper cells

T helper cells have the CD4 co-receptor in association with the TCR on the cell surface, and this complex binds to the MHC class II:peptide complex. Intracellular signaling triggered by the TCR engagement leads to production of different cytokines and commitment into one of two major T helper subsets, Th1 or Th2[13,14]. The cytokines produced by the two cell types counter-inhibit each other. The main duties of $CD4^+$ T cells are to facilitate eradication of intracellular pathogens (Th1) and to aid B cells in the production of antibodies (Th2). The Th1 subset is characterized by the release of IFN-γ that stimulates activation of natural killer (NK) cells and macrophages to eliminate cells harboring intracellular pathogens (cell-mediated immunity). Th2 cells on the other hand, are considered to have evolved to combat parasitic infections through the release of IL-4, IL-5 and IL-13, which activate B cells to produce immunoglobulins (humoral immunity) and recruits eosinophils by their chemo-attracting properties. In addition to Th1 and Th2, another subtype of helper cells has been discovered called Th17, releasing the cytokines IL-17, IL-12, and IL-23. These cells are involved in protection against bacteria and fungi and play a role in several autoimmune disorders[15].

In a healthy individual the immune system is in homeostasis and, in a simplified view, there is a balance between Th1 and Th2 cells. A shift towards either of these sides is either a response to infection or, when provoked by mistake, is the cause of disease. Th1 domination is related to autoimmune diseases whereas a deviation towards Th2 often associates with allergic diseases[1]. The balance of Th cells is plastic and reversible. There also exist Th cell subtypes that are developmental precursors called Th0 which can be driven in either Th1 or Th2 direction depending on the cytokine milieu[1].

1.2.3 B lymphocytes

B cells originate from the bone marrow, where they also mature and self-reactive versions are eliminated. They re-circulate and populate the spleen and lymph nodes. Their main responsibility is to produce antibodies against antigens that are recognized through the B cell receptor (BCR), which is a surface bound immunoglobulin (Ig) consisting of a conserved shaft binding to the cell membrane (Fc) and a part with two variable regions at the ends. Unlike the TCR, which needs to interact with both MHC plus peptide, the BCR binds antigens in their native form. Initially, the BCR is expressed in the form of IgM or IgD. When a specific antigen is encountered, the B cell starts production and release of antibodies (soluble Ig's) and undertakes class switching to an appropriate isotype depending on the location of the B cells and the nature of the pathogen. The Fc parts of the antibodies mediate the isotype specific effector functions, e.g. IgA has a role in mucosal immune defense and IgG function as neutralizing antibodies. There are in total five isotypes; IgM, IgD, IgA, IgG, and IgE that are located in the genome in the above order. IgG is the mot abundant antibody in the circulation of healthy individuals followed by IgA, IgM and IgE[1].

B cells become activated when two BCR are cross-linked by a specific antigen. The BCR then ingest the antigen into the cell, which process and present the peptides on MHC class II. In this way, the B cell can interact with the $CD4^+$ helper T cells that provide CD40 signaling and cytokines to support further class switching.

1.3 BREAST MILK

Human breast milk has an ideally suited composition of nutrients to provide the newborn infant with the best prerequisites for growth, development and immune protection. The secretion during the first few days after birth, called colostrum, is especially rich in nutrients and is highly concentrated in comparison to the mature breast milk. Since the newborn's immune system is not fully matured, the immune relevant components of the breast milk are important for the survival, especially in developing countries.

Such components include immunological cells, e.g. neutrophils (40–65% of the total leukocyte population), macrophages (35–55%) and lymphocytes (5–10%, of which the majority are T cells)[16]. Studies in baboons have revealed that milk leukocytes can adhere to gut mucosal epithelial cells and transfer to the blood stream to end up in the liver and spleen[17]. Except cells, other membranous structures identified in milk are the milk fat globules (MFG) (0.8-2 μm in diameter) that are fat droplets secreted from mammary epithelial cells that contain about 95 % triacylglycerides[18]. Colostrum is dominated by small MFG but in mature milk they can range up to 4 μm[19]. The membranes of MFG express CD36, lactadherin (MFG-E8), MHC class II, and mucin (MUC)-1 of which several have shown immune suppressive functions[20]. Other immune relevant components of breast milk are oligosaccharides and glycoproteins that inhibit pathogen binding, immunoglobulins[21] (primarily IgA[16]), antimicrobial peptides (e.g. lactoferrin[22] and lysozymes[22]), chemokines, and cytokines identified in a number of studies e.g. IL-4, IL-6, IL-8 (CCL-8), IL-10, IL-12, IL-18, IFN-γ and RANTES[23-31]. The finding of the anti-inflammatory cytokine transforming growth factor (TGF)-β in breast milk, has suggested that breast milk may induce regulatory mechanisms and has been shown to account for some of the immunosuppressive effects seen when cord blood cells were stimulated with colostrum[32]. Recently it was discovered that airborne antigens can be transferred from a murine mother to her pups and induce tolerance in a TGF-β dependent fashion[33].

Several protective effects of breast-feeding have been described and although debated, there are data supporting beneficial and protective effects against infections[34,35] and allergy[33,36,37]. Convincing evidence for a specific component of breast milk that mediates these effects is lacking. However, a multitude of different factors are likely to contribute.

1.4 EXOSOMES

1.4.1 Exosome history

It is known since long that vesicles of different sizes are released naturally from cells. When progenitor cells develop by differentiation some molecules need to be discarded and this is the case for instance for reticulocytes, which shed their transferrin receptors during maturation[38]. Most reports on vesicle shedding have described this as budding from the cell surfaces, however, there exist other intricate ways of release. In one of

these reports the term exosomes was coined by Johnstone in 1987 who isolated exosome-vesicles from culture supernatants of reticulocytes[39]. The term had also been proposed for released micro-vesicles with 5'-nucleotidase activity as early as 1981[40]. In 1996 Raposo adopted the term and modified the definition to describe nano-vesicles isolated from cultures of B cells, which she called exosomes, which were released from the MHC class II loading compartments of these antigen presenting cells[41]. Raposo was also the first one to describe their T cell stimulatory capacity[41]. Since this milestone achievement in exosome history, the exosomes have rendered interest from immunologists in particular, and exosomes are now considered as important players in antigen presentation and immune regulation *in vivo*. Although their biological role remains elusive, they have shown great potential as therapeutic tools (see section 1.5.5). Exosomes are nano-sized membrane vesicles (30-100 nm) released from a wide variety of cell types including B cells[41], DC[42], macrophages[43], mast cells[44], T cells[45], epithelial cells[46], platelets[47], reticulocytes[39], tumor cells[48] and placental trophoblasts[49]. Different clinical isolates and body fluids such as bronchoalveolar lavage (BAL) fluid[50], malignant effusions[51,52], ascites[53], urine[54] and plasma[55] contain exosomes, probably of mixed cell origin.

It should be noted that there exists a relatively loose definition of exosomes and in the literature, the term is used broadly. They should also not be confused with the more recently described ribonuclease complex that has also been named exosome[56]. The sections 1.5.2 to 1.5.5 describes exosomes from an immunological viewpoint.

1.4.2 Exosome formation

Exosomes are formed within the multivesicular bodies/endosomes (MVB) of the cell which function as an intermediate in the degradation pathway of proteins, either internalized from the plasma membrane or from the trans Golgi network[57]. This compartment harbors intraluminal vesicles which are formed by inward budding of the limiting membrane of the MVB. Upon fusion of the MVB with the cell limiting membrane the intraluminal vesicles are released as exosomes[56] (Fig. 1).

Two different mechanisms for exosome formation, have thus far been suggested, which supports the hypothesis that it is a highly regulated process. The first one is marked by the identification of an endosomal sorting complex required for transport (ESCRT)[58], [59], and just recently a second one was identified as a cermaide-triggered budding[60]. The ESCRT sorts ubiquitinated proteins for transport in the endosomal network[58], however, not all proteins found in exosomes are ubiquitinated. It has also been suggested that p53 is involved in the release of exosomes from human tumor cell lines[61], something which might be of importance in tumorigenicity.

In an antigen presenting cell, as for example DC, the MVBs are also the site for MHC class II storage and loading and are thus called MHC class II enriched compartments (MIIC)[62]. Antigen peptide is bound to MHC class II in this compartment and thus when the MIIC fuses with the outer cell membrane to dress the cell surface with MHCII:peptide complexes, there is also a release of exosomes carrying these complexes to the extra-cellular space (Fig. 1). Furthermore, co-stimulatory molecules are also incorporated into APC-derived exosomes.

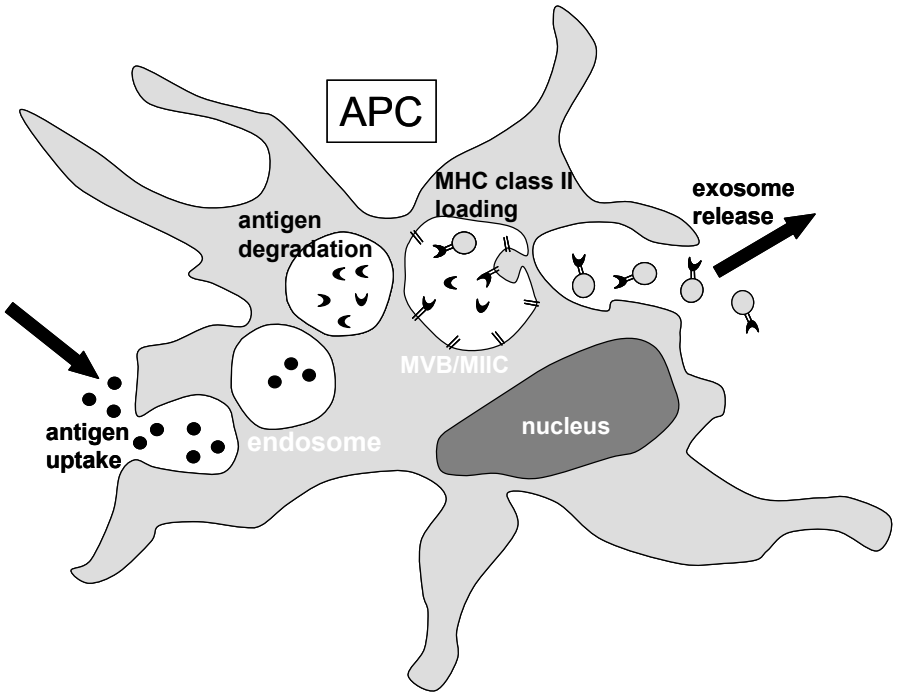

Figure 1. Model for exosome formation and antigen loading to MHC class II in an antigen presenting cell. Antigen presenting cells (APC) take up antigen in to the endosomal pathway, where the antigen is degraded through acid-activated proteases. Degraded antigen is loaded to MHC class II in multivesicular bodies (MVB/MIIC) and upon fusion of this late endosomal compartment with the outer cell membrane, internally accumulated vesicles formed by inward budding are released to the cell exterior as exosomes. Image modified from[42].

1.4.3 Exosome composition

The molecular composition of exosomes is dependent on the cell type of origin and typically enriched in certain molecules (Fig. 2). Tetraspanins are a family of molecules that span the membrane four times, and which suggestively stabilizes other proteins and co-localize in complexes with other proteins, for instance MHC class II molecules. In exosomes the tetraspanins CD9, CD63, CD81 and CD82 are highly enriched, in a similar way as lipid rafts[63], therefore a biochemical relation between these structures has been suggested.

Exosomes from antigen presenting cells are also enriched in protein antigen presenting molecules MHC class I and II (capable of presenting peptides) and have co-stimulatory molecules as for example CD40, CD80 (B7-1) and CD86 (B7-2) on their surface. In addition exosomes carry cell adhesion molecules like CD11c, ICAM-1 (CD54) and DC-SIGN. The exosomes also express lipid antigen presenting molecules CD1a, b, c and d[64]. B cell exosomes have been shown to express CD55 and CD59 which are glycosylphosphatidylinositol (GPI)-anchored regulators of complement attack and may protect the exosomes from complement lysis[65].

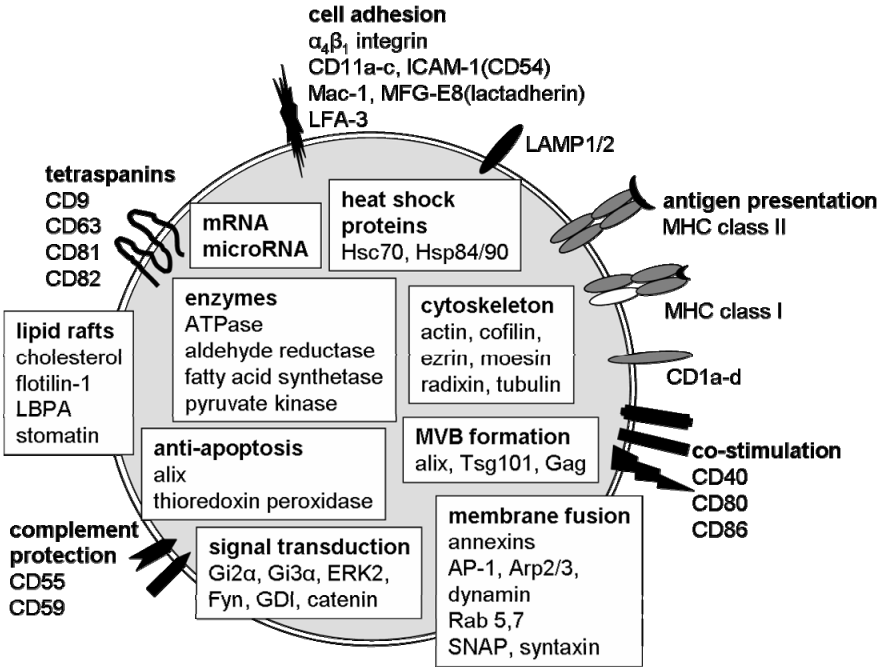

Figure 2. Schematic image of selected molecular components of exosomes. Exosomes show surface expression of molecules for antigen presentation (e.g. MHC and CD1), co-stimulation (e.g. CD80 and CD86), cell adhesion (e.g. integrin, ICAM-1), tetraspanins (e.g. CD63 and CD81), and complement protection (CD55, CD59). Inside are a variety of cytosolic and cytoskeletal components such as RNA, heat shock proteins, enzymes, and signal transduction molecules. AP-1 (activator protein-1), Arp 2/3 (actin-related protein 2/3), ERK2 (extracellular signal-regulated kinase 2), GDI (GTP dissociation inhibitor), Gi2/3α (alpha-subunit of the heterotrimeric G-protein Gi2/3), LBPA (lysobisphosphatidic acid), LFA-3 (lymphocyte function-related antigen-3 or CD58), Mac-1 (M2 integrin of leucocytes or CD11b/CD18), Tsg101 (tumor susceptibility gene 101). Image adapted from[66,67].

All of the molecules described here so far are present on the exosome surface. Heat shock proteins (Hsp) have been identified as part of the exosome proteome in several studies, e.g. Hsp70, Hsc70, Hsc72, Hsc73 and Hsp90[68-72] and has been suggested to reside in the exosomal lumen. Hsp are mostly known for their involvement in supporting proper protein folding while acting as chaperones intracellularly[73] but have also received attention as immune regulating molecules[74]. In addition exosomes have a mixture of cytosolic proteins in their lumen, for instance; actin, tubulin, and cofilin as well as alix, Tsg101, annexins and different Rab-proteins. Most recently, exosomes have also been shown to contain functional RNA (both mRNA and microRNA)[75]. This suggests that exosomes act as a shuttle of nucleic acids and most interestingly, there appears to be a selective sorting of a limited number of regulatory microRNA molecules to exosomes[75] (and unpublished data Ekström *et al*, Gothenburg University, Sweden).

1.4.4 Exosome functions

Exosome composition suggests several potential functions in the immune system. Sometimes, the information provided to the cells by exosomes directly leads to triggering of responses in the cells. Other times, the exosomes have been considered as

a random way of shedding waste molecules. Thanks to their small size exosomes can potentially access many sites in the human body. Indications that they are resistant to complement lysis, suggests that they may be durable and stable over a long time. The exosomes most likely lack the possibility to change their structure once formed, since they do not harbor any protein synthesis of their own. The surface molecules are involved in attachment to cells and structures of the extracellular matrix. Exosomes can function as vehicles to transport information between cells in the form of proteins, like the MHC:peptide complex or through RNA molecules. For the cells to gain access to the internal contents, the exosomes probably need to be ingested by the target cell and degraded. The process of exosome uptake have thus far been described as phagocytosis-like[76], but a lot remains to be explored in this area.

1.4.4.1 Exosomes in inflammation

Increasing attention has recently been devoted to the role of exosomes in inflammation. The exosomes may have both suppressive and stimulating effects on inflammation. In rheumatoid arthritis (RA) patients, synovial fibroblasts have been shown to produce exosomes that express a membrane bound form of tumor necrosis factor (TNF)-α. TNF-α has previously been suggested to have a pathogenic role in RA[77]. These exosomes also induced signaling events in T cells that led to reduced apoptosis and delayed T cell activation-induced cell death. Another study in RA, identified citrullinated proteins in exosomes isolated from synovial fluid, which are known to be autoantigens in this disease[78]. Previously, TNF receptor 1 has been identified on exosomes released from lung epithelial cells, endothelial cells and serum[79].

Moreover, platelet derived exosome-like microparticles showed a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity which contributed to vascular cell apoptosis. This effect was stronger for exosomes from patients with sepsis than for exosomes from healthy individuals[80]. Thus, exosomes may be part of a novel redox-signalling pathway.

Exosomes from DC containing milk fat globule factor (MFG)-E8 (lactadherin) have been tested as a treatment for sepsis in a rat model[81]. MFG-E8 works as a bridging protein between "eat-me" signals like phosphatidylserine (PS) and integrins on phagocytic cells. DC exosomes with MFG-E8 were able to attenuate systemic inflammation and were beneficial against sepsis in the rat model. Microparticles, among which exosomes are included, are also explored in neuroinflammatory disorders[82].

1.4.4.2 Exosomes and infectious agents

Microorganisms tend to exploit the cellular facilities of their host and exosomes were first discussed in this context in 2003 when Gould *et al* introduced "The Trojan exosome hypothesis" for cellular trafficking of retroviruses[83]. In this article, it was suggested that HIV uses the exosome pathway for production of virions in its natural life-cycle. In line with this, HIV infected macrophages release HIV particles displaying host-derived protein phenotypes that match that of exosomes released from macrophages displaying tetraspanins, MHC molecules and lysosomal Lamp-1[43]. HIV virions have also been shown to be assembled in the MVBs of macrophages[84], the site where exosomes are formed, which supports the hypothesis that HIV uses a similar release pathway as exosomes. Later studies have shown that immature DC derived exosomes can transmit HIV to $CD4^+$ T cells and that the exosome-associated HIV was 10 fold more infectious as compared to free virus particles[85]. HIV taken up by DCs were sorted to MVB-like compartments positive for tetraspanins, and upon release, the

HIV particles were associated with HLA-DR$^+$, CD1b$^+$, CD9$^+$ and CD63$^+$ exosomes. Interfering with the exosome pathway thus may be a strategy to include in anti-retroviral therapies.

Infectious prion proteins, abnormally folded PrP scrapie, have been found in exosomes from rat and mouse cell lines[86]. The exosomes had a proteome similar to that described previously for exosomes. The study showed that exosomes transmit the prion protein to uninfected cells and convert normal cellular PrP into the dangerous PrP scrapie conformation. In acquired prion disease, the infection first takes place in lymphoid tissues and then spread to the central nervous system. There has been no mobile cell type identified to account for this spread, however, prion infected neuronal cell culture supernatant is infectious. Exosomes from a neuronal cell line infected with prions could transmit both normal cellular PrP and PrP scrapie to uninfected cells and induced prion disease in recipient mice[87].

Moreover, mycoplasma infected DCs release mycoplasma-contaminated exosomes which could induce both T cell and polyclonal B cell proliferation[88]. Exosomes isolated from the BAL of *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* infected mice contain mycobacterial components and can stimulate a proinflammatory response *in vitro* and *in vivo*[89]. In a study of another intracellular pathogen, *Leishmania donovani*, the proteomic analysis of the culture supernatant from parasite cultures revealed extensive overlap with the exosomal proteome, however, in the cultures the serum source was not pretreated to eliminate bovine exosome contaminations[90].

1.4.4.3 Exosomes and immune tolerance

In contrast to the early dominating view of exosomes as immune stimulators, a constantly growing number of observations focus on the role of exosomes in immune tolerance. Karlsson *et al* first described exosomes with tolerance inducing effects in 2001 and coined the term "tolerosomes"[46]. In their study, they showed that rat intestinal epithelial cells (IEC) take up dietary antigens, process them and release them to the circulation in association with MHC class II on exosome-like structures. The serum or tolerosome isolates from serum, were capable of transferring tolerance to naïve recipient rats. In a follow up study, it was shown that human IEC contain the structural characteristics of a functional antigen-processing compartment for presentation on MHC molecules and prerequisites required for exosome production and release from the baso-lateral side[91]. A follow-up study additionally showed that the tolerance mediated by tolerosomes was MHC dependent and only functional in transfer to syngeneic recipients[92].

In 2003 Pêche *et al* reported the first study of the role of exosomes in transplantation. The authors showed that by giving the recipient animal DC derived exosomes from the donor before transplantation of a heart, the allograft survival was significantly improved[93], whereas no tolerance effect was observed with syngeneic exosomes. The prolonged allograft survival coincided with a decrease in graft infiltrating leukocytes and a decreased IFN-γ mRNA expression. In a recent study, the same author showed that exosomes could have moderate allograft protective effects also when given after allograft transfer[94]. An immune-suppressive drug LF 15-0195 (LF), which inhibits DC maturation, was then used in combination with exosome treatment. This led to a donor-specific allograft tolerance which was also transferrable to naïve allograft recipients. Further, a new study in mice showed that recipient or host-derived exosomes presenting alloantigens could trigger alloreactive CD4$^+$ T cells in a process that depended on host DC MHC class II molecules[95]. However, donor allogeneic exosomes were not released

in sufficient amounts to stimulate alloreactive T cells *in vivo*. Instead, the authors suggested that donor DCs migrate from the allograft to lymphoid organs where an exchange of exosomes between donor and host DCs takes place with subsequent alloreaction as a consequence.

Maintaining tolerance is central in pregnancy and also for this purpose exosomes have been proposed to play a role[96-98]. The foetus and the placenta represents a semi-allograft and trophoblasts are evading the attack of the maternal immune system towards cells expressing paternal antigens by eliminating reactive lymphocytes, possibly through the FasL/Fas system. Trophoblasts have no membrane expression of FasL but it has been demonstrated that these cells release exosomes with FasL expression which may be participating in creating an immune privileged area around the trophoblasts[96]. These data are reinforced by the specification that it is syncytiotrophoblasts in particular but also Hofbauer cells of the placenta that contain the intracellular FasL which is released through exosomes[49]. Taylor *et al* showed that placental exosomes positive for the marker PLAP (placental alkaline phosphatase) could be isolated from the circulation of pregnant women and that pre-term delivering mothers had lower levels of FasL and HLA-DR on their placenta-exosomes[98]. Exosomes from mothers delivering at term showed greater suppression of CD3ξ an JAK3 than exosomes from preterm delivering mothers. Following up these results the authors were also able to show that blocking antibody to FasL partially reversed the suppression of CD3ξ[97]. These studies indicate that exosomes may have a role in maintaining tolerance between the mother and the foetus and might be an important factor for succesful pregnancy.

1.4.4.4 Exosomes in allergy

During the work with this thesis we showed for the first time that B cell derived exosomes can present allergen (Betv1) and stimulate specific T cell proliferation and induction of Th2-like cytokines[99]. This study and data from others[33] made us speculate whether exosomes might have a role in allergy. Mast cells, considered as key players in IgE-mediated allergies, produce exosomes that induce functional maturation of DC[100]. The study by Karlsson *et al* showed that tolerosomes could transfer tolerance to naïve recipient animals and thus exosomes may have a role in the tolerance of dietary antigens[46]. Recently, an additional mouse study from the same group showed that serum or isolated serum exosomes from animals orally tolerized with ovalbumin (OVA), mediated protection from allergic sensitization in naïve recipients[101]. The treated mice were given tolerosomes intraperitoneally and were then sensitized and exposed to OVA intranasally. In mice given tolerosomes, there was a decrease in eosinophils in BAL and lower levels of both total IgE and OVA-specific IgE in serum as compared to control mice, suggesting that tolerosomes might protect from allergy development.

1.4.4.5 Exosomes in malignancies

Tumor cell derived exosomes carrying MHC class I, tetraspanins and Hsps' from both human cell lines and from mice were first described in 2001 and was presented as a source of tumor rejection antigens[102] that could transfer tumor antigens to DCs. Several studies on different tumor sources showed that exosome release was a common feature to several tumor cell types as for example mesothelioma[103], plasmacytoma[104] and melanoma[105]. Tumor exosomes, however, seem to have dual functions with completely opposite effects. Either they can stimulate an immune response against the tumor [102, 48,51], or they mediate immune tolerance towards the tumor e.g. via FasL:Fas interaction[106], TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)[107], TGF-β[108], NK cell suppression[109], expression of

NKG2D-ligands diminishing CTL efficiency[110] or membrane bound TGF-β1 mediated T cell suppression[111]. Exosomes and micro-vesicles released from platelets have also been reported to promote metastases as they act as chemo-attractants on cancer cell lines and induce stimulation of angiogenetic factors[112] maybe mediated through a newly discovered tetraspanin protein D6.1A/CO-029[113]. Tumor exosomes, which started out as a hypothetical therapeutic agent *per se*, are now rather considered to mould the host environment in favor of protecting the tumor[111,114], and thus the exosome pathway can be a target for therapeutic intervention.

1.4.5 Exosome immunotherapy

1.4.5.1 Immunotherapy against cancer

Ever since Zitvogel *et al* in 1998 showed that injection of tumor peptide loaded DC derived exosomes could reverse tumor growth in mice[115], the area of cancer immunotherapy has been the context where exosomes have received the most attention. Several recent reviews describe different strategies to use exosomes, from various sources, as therapeutic agents to combat cancer. Although tumor derived exosomes showed promise at first, more and more concerns are raised around the use of tumor-derived exosomes as therapeutic agents. According to the pioneers in the field, tumor derived exosomes should either be pulsed onto *ex vivo* matured DCs or isolated from heat stressed cells in order to mediate immuno-stimulatory functions *in vivo*[116]. An alternative to tumor derived exosomes is to harvest exosomes from clinical isolates from locations in the tumor vicinity[51,117]. It was recently reported that ascites derived exosomes from colorectal cancer (CRC) patients were tested in a phase I clinical trial[53]. These exosomes were administered either alone or together with granulocyte macrophage stimulating factor (GM-CSF). Although both approaches were well tolerated, no therapeutic responses were seen shortly after treatment, for patients who recieved ascites exosomes alone[53]. Tumor specific cytotoxic T cells could however be detected upon *in vitro* analysis which motivates further studies.

DC derived exosomes are so far the exosome type that has reached furthest in therapeutic development. In a direct comparison between tumor exosomes and DC exosomes, the outcome was in favor of DC exosomes, since DC exosomes were better stimulators of $CD8^+$ cytotoxic T cells as compared to tumor exosomes[118]. Two clinical phase I trials have been conducted which both showed that exosome administration was safe. In GMP protocols monocyte-derived DC (MDDC) or leukapheresis isolated DC have been used to generate exosomes which are harvested and loaded with tumor specific peptides[64]. Patients with non-small cell lung cancer or with metastatic melanoma were treated with tumor peptide loaded DC exosomes and the outcome was promising. The treatment was well tolerated and some patients in the lung cancer study showed long term stability of the disease[119,120]. A phase II clinical trial is planned to follow up the initial positive results[121].

DC have been used for some time in tumor immunotherapy. However, the half-life of transferred cells *in vivo* is probably quite short. In mouse experiments, it was shown that, when administering labelled dendritic cells *in vivo*, they homed to the spleen, but after one week they could no longer be detected. Importantly, their released exosomes were still plentiful after several weeks [122]. Therefore, DC derived exosomes have the potential of being long-lasting immune stimulators *in vivo.*

1.4.5.2 Exosomes in other immunotherapies

In the murine collagen induced arthritis model it was shown that adenovirus-transduced DC expressing IL-4 as well as their released exosomes could reduce the severity of

established arthritis and dampen the inflammatory delayed type hypersensitivity reaction(DTH)[123]. This effect was MHC class II dependent and to some extent mediated through the FasL/Fas mechanism. In this model the same authors have also shown that both IL-10 treated DC derived exosomes as well as FasL transduced DC derived exosomes also can reduce a delayed-type hypersensitivity (DTH) reaction and the severity and even prevent induction of collagen induced arthritis[124,125].

Another therapeutic area where exosomes have been studied is in the protection against microbial infections in the form of prophylactic vaccines. Aline *et al* have demonstrated that *Toxoplasma gondii* pulsed DC derived exosomes induced an efficient and antigen specific Th1 response which gave subsequent protection against toxoplasmosis[126].

It was first believed that exosomes only contained T cell epitopes presented in the MHC groove. However, a few studies suggest that intact antigens, or at least partly degraded antigens, might be present in exosomes. Colino *et al*[127] have done pioneering work on the induction of humoral responses triggered by exosomes. They utilized diphtheria toxoid (DT) pulsed murine bone marrow derived DCs as an exosome source[127]. Through injecting these DT expressing exosomes into naïve mice, they provoked DT-specific Ig responses. In another study exosomes from DC were shown to express a glycoconjugate which cross-reacted with a capsular polysaccharide from S*treptococcus pneumoniae*[128]. These exosomes could raise IgM, IgG_3, and IgG_1 antibodies when injected in complete Freund's adjuvant primed naïve mice. After the injection, the mice were tolerant to an otherwise lethal dose of *S. pneumoniae.* Recently, our group has shown that OVA pulsed DC derived exosomes can function as an adjuvant and modulate the humoral response to a Th1 type in naïve recipients (K. Rahman Qazi *et al*, Karolinska Institutet, Stockholm, Sweden, submitted manuscript).

An increasing number of studies highlight the possibilities for manipulating exosome phenotypes. One of the most advanced ways of tailoring exosomes is a method developed, called exosome display technology, which allows expression of virtually any protein on their surface[129]. This technology exploits the fusion of a protein with the C1C2 domain of lactadherin (MFG-E8) through which the protein can be targeted to exosomes. This may increase the usefulness of exosomes in various therapies.

2 AIMS OF THE THESIS

The overall aim of this thesis was to study exosomes as immune-regulators *in vitro* and to investigate how exosomes found *in vivo* may exert immune regulatory effects. The specific aims of each paper were to:

Paper I. Investigate whether DC derived exosomes can stimulate peripheral $CD8^+$ T cells directly and to develop a sensitive assay to detect this response.

Paper II. Generate monocyte derived dendritic cells in different cytokine milieus and phenotypically and functionally compare their released exosomes.

Paper III. Explore if exosomes exist in human breast milk and study their potential immune modulatory properties.

Paper IV. Investigate if exosomes from different sources selectively target specific cells in human peripheral blood.

3 METHODOLOGY

Methods used in paper I-IV are described in detail in the respective "Materials and methods" sections. Below follows an overview of each method with reference to the papers in which they were used:

Confocal laser scanning microscopy (CLSM) [IV]	Microscopic technique enabling scanning through samples in sections of the z-plane.
Cytometric bead array (CBA) [III]	Capture-antibody on beads-based flow cytometric measurement of cytokines in cell culture supernatants.
Enzyme linked immunospot assay (ELISPOT) [I, II, III]	Antibody and enzyme conjugate based detection of cytokine release on a per cell basis.
Exosome preparation [I, II, III, IV]	Isolation of exosomes by consecutive ultra centrifugations.
Flow cytometry (FACSCalibur and FACSAria) [I, II, III, IV]	Laser based analysis of cell phenotypes in flow using fluorochrome conjugated antibodies.
^{3}[H]-thymidine incorporation [III]	Proliferation assay of *in vitro* stimulated T cells by radioactive isotope incorporation and scintillation measurement.
Mass spectrometry [II]	Protein analysis with Liquid chromatography-electrospray ionization-quadropole time of flight-tandem mass spectrometry (LC-ESI-QTOF-MS/MS).
MDDC generation [I, II, III, IV]	Cell culture of peripheral blood monocytes in the presence of cytokines to differentiated dendritic cells.
Multispectral imaging flow cytometry (ImageStream) [IV]	Flow cytometry combined with fluorescence microscopy for simultaneous phenotypic and morphological analysis on a large cell number.
PBMC separation [I, II, III, IV]	Density separation of mononuclear cells from blood with Ficoll Hypaque.
Refractometer [III]	Measurement of densities in sucrose fractionated exosome samples.
Statistical analysis [II, III]	Wilcoxon's matched pairs test was employed for comparing paired samples.
Sucrose gradient fractionation [III]	Continuous density gradient separation by ultra centrifugation of exosomes.
Transmission electron microscopy (TEM) [I, II, III]	Microscopic technique for morphological analyses of exosomes.
Western blot [II, III]	Gel electrophoretic separation of proteins and transfer to protein binding membranes for enzyme-conjugated antibody detection.

4 RESULTS AND DISCUSSION

The growing interest for exosomes as therapeutic tools and the enigma of their biological roles stresses the need to develop methods for studying exosome composition and functions. In this thesis, large efforts have thus been devoted to method development which also resulted in new functional insights for *in vitro* generated (papers I, II, IV) and directly *ex vivo* obtained (papers III, IV) exosomes.

4.1 DIRECT EXOSOME STIMULATION OF PERIPHERAL HUMAN T CELLS DETECTED BY ELISPOT (PAPER I)

DC exosomes have been shown to stimulate T cells *in vitro* in studies using T cell hybridomas[130,131], clones[41,132-134] or cell lines[130,135]. It is debated whether DC exosomes can directly stimulate T cells[41,131,132] or if other cells are needed as intermediates in the process[130,133,134,136]. With exosomes receiving increased attention as candidates for immunotherapy, we identified a need for a sensitive assay to evaluate T cell stimulatory capacities of exosomes on T cells directly *ex vivo*. We designed a sensitive assay for measuring DC exosome mediated $CD8^+$ T cell stimulation in an MHC class I restricted antigen specific context. We evaluated the production of IFN-γ or TNF-α, cytokines essential for tumor destruction, using a sensitive ELISPOT assay. ELISPOT allows for highly sensitive analysis (as few as 1 responding cell out of 100.000) of cytokine responses on a per cells basis. It has been shown previously that the *in vitro* frequencies reflect the *in vivo* situation to a large extent[137].

$CD14^+$ monocytes were isolated from healthy blood donors and differentiated to immature monocyte-derived dendritic cells (MDDC) by culturing in IL-4 and GM-CSF for 6 days[5]. In some individuals the MDDC were matured by adding lipopolysaccharide (LPS) on day 6, and supernatants were harvested on day 8[138]. Cell-depleted supernatants were stored at -80ºC until exosome preparations. Exosomes were loaded with a viral peptide mix from cytomegalovirus (CMV), Epstein-Barr virus (EBV) and influenza virus (Flu) (CEF), either by acid elution or by co-incubation. The majority of the population reacts against this mix of common viruses[137]. Autologous $CD8^+$ T cells were isolated by magnetic bead separation from the $CD14^-$ fractions and the purity was typically 99 % (ranging from 93-99 %, n=16) as measured by flow cytometry.

The exosomes from both immature and LPS-matured MDDC were coated to anti-HLA class II beads and phenotyped by flow cytometry. The exosomes had similar morphology as to previously reported data, and likewise expressed antigen presenting molecules HLA-DR and HLA-ABC, cell adhesion molecule CD54 (ICAM-1), and tetraspanin molecules CD63 and CD81[72]. In MDDC culture supernatants virtually all exosomes were HLA-DR expressing with co-expression of HLA-ABC, CD63 or CD86, since when coating the remaining material (after anti-HLA class II bead isolation) to latex beads, no signals for either of these molecules could be detected. As seen previously in the murine system, exosomes from mature MDDC had higher expression of both MHC-antigens, CD40, CD54, CD80 and CD86, whereas the tetraspanins remained unaltered compared to immature MDDC exosomes[139]. These molecular patterns were similar on the cells and thus the exosomes phenotypically reflected their cells of origin.

To hinder that the viral peptide mix would bind to MHC class I on the T cells, instead of associating with exosomal MHC class I, and thus prime other T cells directly, the $CD8^+$ T cells were pre-incubated with the MHC class I blocking antibody W6/32. We evaluated the blocking over a time-course of 48 h and could see that the blocking capacity was maintained over time. To measure the IFN-γ release in ELISPOT, two different approaches were used for exosome stimulation of the $CD8^+$ T cells. Either, the MHC class I was blocked on the T cells as described above, and the CEF peptide mix was added together with the exosomes, or the exosomes were pre-loaded with the CEF mix by acid elution and free CEF was removed by a filtering step. Blocking W6/32 antibody added to the exosomes prior to mixing with CEF and the T cells, showed that the stimulation was dependent on MHC class I on the exosomes. The stimulatory response was exosome dose dependent both in the stimulation with free CEF and with pre-loading of the exosomes. In addition to IFN-γ we also report that virus peptide loaded exosomes have the ability to stimulate tumor necrosis factor (TNF)-α release from $CD8^+$ T cells. We were also able to show that the stimulatory capacity was higher when exosomes from mature MDDC exosomes were used in the assay. This is in line with previously reported data from the murine system[139].

The contradictory results in the literature regarding whether exosomes need APC or not for T cell stimulation may depend on the nature of the antigen, the state of the T cells or the nature of the exosomes used. Also, although a high T cell purity was reached, the contamination of few antigen-presenting cells cannot be excluded but is unlikely to have influenced the stimulation significantly. No correlation between purity and the level of activation was detected, suggesting that contaminating APC were not responsible for the activation. It needs to be clearly stated in this context that the stimulations we assessed are not of naïve T cells, but represent a secondary immune response from memory T cells. It may however be necessary to have APCs present in order to trigger naïve T cells, as has been described previously[133,134].

Herein, we show that exosomes can directly stimulate T cells, in a dose dependent and antigen specific manner, without the need of antigen presenting cells as intermediates. The stimulation was dependent on MHC class I on the exosomes. We have presented the successful development of a sensitive immunoassay for evaluation of the T cell stimulatory capacity of exosomes to T cells directly *ex vivo*. This suggests that DC exosomes may have a role in T cell stimulation *in vivo*. The assay can be easily modified to test other antigens or T cell subsets for stimulation and release of different cytokines. The method can be used to efficiently screen for individual responses in the development of exosomes as therapeutic agents in for instance cancer treatments. The objectivity of the analysis is secured by automatic counting which is also time-efficient. Significant work remains to establish which exosome phenotypes that are most efficient and to find out what antigens that are best suited for immunotherapy. Whole DC cancer therapy has shown limited success[122] and the cell-free exosome system provides a novel strategy for triggering immune response against tumor cells[134].

4.2 DIFFERENT TYPES OF *IN VITRO* GENERATED HUMAN MONOCYTE-DERIVED DENDRITIC CELLS RELEASE EXOSOMES WITH DISTINCT PHENOTYPES (PAPER II)

Others had shown that the maturation state of the DCs could be reflected on the exosomal level, in studies performed in the murine system[139]. Tumor cell derived exosomes[48,53,103,121], and DC-derived exosomes[134,140], has recently become a focus of interest in the area of tumor immunotherapy and studies have shown that mouse tumors

could be completely eradicated when animals were administered tumor peptide loaded DC-derived exosomes[115]. There has also recently been several studies in humans where MDDC-derived exosomes loaded with tumor peptides have been used to boost an immune response against cancer[119,120]. These studies used an established protocol by culturing in IL-4 and GM-CSF to generate clinical grade[64] MDDC from which exosomes were isolated. MDDCs generated with IL-4/IL-3 had previously been suggested to have a more Th2-inducing phenotype as compared to conventionally IL-4/GM-CSF generated MDDC[6]. We wanted to see if the $CD8^+$ T cell stimulatory capacities of exosomes could be modified *in vitro* by generating MDDC in different cytokine milieus. Therefore we utlilized the ELISPOT method developed in paper I to assess the $CD8^+$ T cell stimulatory capacities of the different MDDC and their released exosomes.

Immature MDDC were analyzed by flow cytometry. Our results show, in line with Ebner et al[6] that we could differentiate $CD14^+$ monocytes from peripheral blood cells into MDDC using interleukin (IL)-4 in combination with either IL-3 or granulocyte macrophage colony stimulating factor (GM-CSF). Typical immature dendritic cell phenotypes included moderate expression of MHC classes I and II as well as co-stimulatory molecules e.g. CD40, CD63, CD80 (B7-1), CD81, and CD86 (B7-2) and cell adhesion molecules CD11c, CD54 (ICAM-1) and DC-SIGN. MDDC generated in both ways had low CD83 expression indicative of an immature phenotype. Despite an overall morphologic and phenotypic similarity between the two MDDC types there were also some differences. Both CD1a and CD40 were present on a higher percentage of IL-4/GM-CSF generated MDDC as compared to IL-4/IL-3 MDDC. Another interesting difference was seen in the CD80 and CD86 expression patterns. While the median of $CD80^+$ MDDC was 62 % for conventional MDDC, only 26 % of MDDC were $CD80^+$ when generated in IL-4/IL-3. CD86 expression showed the reverse pattern with a higher percent positive MDDC among the IL-4/IL-3 MDDC. Interestingly, there were also differences in the levels of expression of certain molecules as indicated by increased mean fluorescence intensity values (MFI). MHCs were present on close to 100 % of the MDDC but when comparing MFI values for MHC class I and II we found that IL-4/IL-3 MDDC had a higher density of expression of these molecules. Thus, we hypothesized that these cells also had a more efficient T cell stimulatory capacity compared to conventionally generated MDDC (IL-4/GM-CSF). With the highly sensitive ELISPOT assay we could detect a slightly more efficient IFN-γ release from autologous $CD8^+$ T cells stimulated with virus peptide mix loaded IL-4/IL-3 MDDC as compared to conventional MDDC. This difference could not be detected when analyzing T cell proliferation responses by ^{3}[H]-thymidine incorporation assay, something that may be due to the sensitivity of the proliferation assay.

When analyzing exosomes from the two culturing conditions by transmission electron microscopy (TEM) we saw that they had similar morphology. Both for TEM and for phenotypic analysis by flow cytometry we used the anti-HLA class II magnetic beads described previously[50]. In this study however, we wanted to capture the exosomes directly from the MDDC culture supernatants in order to exclude the hypothetical possibility that some exosomes might be formed as an artifactural consequence from the ultra centrifugations or that variations in pelleted exosome recovery affected our attempt to a quantitative comparison. We could see by transmission electron microscopy that the exosomes captured directly were structurally identical to exosomes isolated by ultra centrifugation (paper I). Direct capture was preceded by a set of titration experiments, to verify that the magnetic beads were not saturated with exosomes, thus enabling a semi-quantitative analysis of the two exosome types. Our

results show that there was a higher expression on IL-4/IL-3 MDDC exosomes of CD11c, CD63, CD81 and both MHC classes I and II, as compared to conventionally generated MDDC exosomes whereas other molecules remained unaltered. Enrichment of MHC class II was confirmed by Western blot. Since some but not all markers were enriched we speculate that there was no general increase in exosome numbers.

While $CD8^+$ T cell stimulatory differences could be detected between the MDDC types, using the virus specific ELISPOT assay, there was no consistent difference found between the two different exosome types. However, due to the individual variation of exosome supply, only a limited number of donor exosomes could be tested functionally. In addition, HLA-DR differences were more pronounced between the two exosome types and thus should be addressed in future studies when an MHC class II restricted antigen peptide mix has been developed.

4.3 EXOSOMES WITH IMMUNE MODULATORY FEATURES ARE PRESENT IN HUMAN BREAST MILK (PAPER III)

Human breast milk is a complex liquid that contain immune cells, antibodies, cytokines, and antimicrobial peptides[141] as well as MHC class II containing milk fat globules (MFG)[142]. We speculated that breast milk might contain exosome-like vesicles, which could be a novel way of transferring immunological information from the mother to her infant.

We modified the method used for isolating exosomes from cell culture supernatants to be applicable on breast milk. Healthy mothers donated breast milk. Fat and milk cells (mainly macrophages) were removed by centrifugation and the remaining liquid was consecutively filtered through syringe filters down to a pore size of 0.2 μm. Serial ultra centrifugations were then used to isolate the nano-vesicles and pellets were generally larger as compared to exosome pellets from e.g. DC culture supernatants. The breast milk nano-vesicles had to be dissolved over night in PBS and were washed an extra time. Using several methods, we identified the isolated vesicles as exosomes. First, they were coated to anti-HLA class II beads and subjected to phenotypic analysis in flow cytometry using a panel of mAb's. Hereby, we could identify expression of typical exosome molecules like HLA-DR, CD63, CD81 and CD86 but also the milk related molecule mucin (MUC)-1. MFG have also been shown to express MUC-1 and we therefore prepared MFG to compare with the exosomes in parallel showing that MHC class II and MUC-1 were the only common markers identified. The exosome coated beads were also prepared for transmission electron microscopy analyses which revealed an exosome characteristic morphology of the vesicles of about 30-100 nm in diameter and with intact lipid bi-layers. Immunolabeling showed HLA-DR and CD63 surface expression. Western blot analysis confirmed the presence of HLA-DR and CD81 and also identified the heat shock cognate protein (Hsc)70 in the vesicles. When comparing HLA-DR in colostrum and mature milk in parallel, by Western blot, we could conclude that colostrum had a higher concentration of this molecule than mature milk exosomes. Importantly, the lack of the endoplasmic reticulum marker calnexin supported the notion that the vesicles were of an endosomal origin. Sucrose density gradient centrifugation also revealed that the vesicles had a density of 1.10-1.18 g/ml corresponding to that of previously described exosome densities[116]. Coating anti-HLA class II beads with the sucrose fractions also confirmed that CD63, CD81, and HLA-DR localized to these same fractions. These data led us to define the vesicles isolated, both from colostrum and mature milk, as exosomes.

The sucrose gradient purified colostrum and mature milk exosomes were trypsin digested and analyzed by mass-spectrometry using a liquid chromatography (LC) electrospray ionization (ESI) quadropole time of flight (QTOF) MS/MS. The results showed that several peptides also identified in previous mass-spectrometric experiments of other exosome types were found in milk exosomes. We present in paper III only the identifications confirmed in both individuals tested and noticed that colostrum and mature milk had some proteins in common but also some that were uniquely identified in one or the other. While HLA-DR and several heat-shock proteins were only found in colostrum using this method, annexins and macrophage migration inhibitory factor (MIF) were only present in mature milk exosomes. Our results from Western blot showed that HLA-DR was more enriched in colostrum, and colostrum is generally considered to be more enriched in immune relevant factors, which may contribute to explaining why mass-spectrometry only identified these proteins in colostrum. Likewise, annexins and MIF may be reversely related and more enriched in mature milk than in colostrum exosomes. The typical high abundance milk proteins; e.g. xanthine dehydrogenase, lactadherin (MFG-E8), CD36, casein and fatty acid synthase, were generally identified in both colostrum and mature milk exosome samples. Consequently, these high abundance proteins constituted a proportionally large fraction of the total number of resulting peptides after the trypsin digestion. Some of these proteins may be exosome associated or just coincidently isolated together with the exosomes due to aggregation and their high concentration in breast milk. However, our sucrose gradient preparation prior to analysis should reduce the risk for this. The resolution of the analysis might have been decreased due to these high abundance peptides potentially masking (by out-numbering) more rare peptides in the analyses. This could lead to a decreased sensitivity of the method to detect the whole proteome, leading to an underestimation of its complexity.

We proceeded to evaluate potential functions of the milk exosomes by co-incubating them with PBMC. Preliminary experiments using ELISPOT analysis of IL-2 release showed that the milk exosomes themselves did not initiate T cell stimulation. We thus designed an assay to assess the potential of milk exosomes to regulate T cells by analyzing their IL-2 release upon anti-CD3 stimulation after 4 days of PBMC co-incubation with mature milk exosomes. Strikingly, we could see that the PBMC, which were co-incubated with the milk exosomes, were inhibited to release IL-2 upon anti-CD3 stimulation. This inhibition was seen both in an autologous and an allogenic situation. The infant is semi-allogenic in relation to the mother, and thus a tolerance inducing property of breast milk ought not to be strongly MHC-dependent. The inhibition was not observed when performing the anti-CD3 stimulation on PBMC that had been incubated with control MDDC derived exosomes. Thus, the effect was not caused by sterical hindrance but was rather a property associated with the milk exosomes. In this context we can speculate that MUC-1 may have a role since it has been published that this molecule can inhibit T cell proliferation[143]. We attempted to remove the MHC class II and/or CD81 positive exosomes before co-incubation with PBMC. By incubating the exosome isolates with anti-HLA class II magnetic beads and/or anti-CD81 latex beads prior to co-incubation, we could observe a decreased inhibition of the anti-CD3 stimulated IL-2 release. The inhibition was however not completely abolished which could either be due to that the removal of exosomes was not total or there are additional factors in the milk exosome isolate that contribute to the inhibitory effects. There might also be exosomes with low expression of both MHC class II and CD81 contributing to the effect. We can however conclude that the exosomes are partly responsible for the inhibition. In addition we report that the inhibitory effect was also visible for IFN-γ and TNF-α whereas IL-5 release from

PBMC incubated with milk exosomes was rather increased as compared to without the exosomes. The reasons for this are unclear and needs continued investigation.

To further explore how the co-incubation of milk exosomes with PBMC may influence the cells, we assessed the phenotypes of the PBMC after co-incubation for 4 days with milk exosomes by flow cytometry. We could identify a relative increase in the number of $CD4^+CD25^+Foxp3^+$ T cells in the PBMC that had been with milk in comparison to PBMC incubated in medium alone. This cell type is described as regulatory in the literature, however functional proof in our system is not at hand. The overall level of $CD4^+$ lymphocytes in the PBMC remained unaltered and the viability was the same whether incubated with or without milk exosomes.

We show in this paper, for the first time, that both colostrum and mature breast milk contained exosomes and that the exosome isolates were immune-modulating and potentially induced T regulatory cells. The infant gastric pH is not as acidic[144] (pH >5) as the adults pH(1-3), and thus the milk exosomes may pass undisturbed to become available for uptake by cells of the evolving immune system in the newborn's intestines. Intestinal epithelial cells possess the ability to release exosomes and these have been found in the serum of experimental animals and identified by the gut epithelium specific marker A33[46,92]. However, preliminary studies did not reveal any A33 on our milk exosomes. The immune system of the newborn is constantly educated and breast milk is one very important factor for the development of a potent defense. Increased knowledge of the composition of human breast milk can be useful in the formulation of alternatives.

4.4 EXOSOMES OF DIFFERENT ORIGIN TARGET SPECIFIC CELL POPULATIONS IN HUMAN BLOOD – CELLULAR LOCALIZATION QUANTITATIVELY RESOLVED BY MULTISPECTRAL IMAGING FLOW CYTOMETRY (PAPER IV)

Previous studies by others[139] and us (paper I and II) had shown that exosomes from different cells largely reflect their cell types of origin phenotypically. Whether some of the functional properties of a cell are also inherited by the released exosomes remains to be clarified. Most studies on exosome interactions with different cells have been done in isolated *in vitro* systems and on specific cell types, assessed one at a time. We strived to make an unbiased assessment of what cell types in human peripheral blood that associated with exosomes from different sources. By using PKH67, which is a general membrane dye[145], exosomes from MDDC- or B cell culture supernatants or isolated from human breast milk could be directly stained. By using TEM it was verified that green fluoresceing (flow cytometry) exosomes captured on HLA class II magnetic beads had intact exosome morphology. Association with different cell populations of peripheral blood mononuclear cells (PBMC) from healthy blood donors was analyzed, after 1 h and 4 h of co-incubation, using flow cytometry and confocal laser scanning microscopy (CLSM). Some experiments were also conducted under cold conditions to assess if interactions were active (phagocytic) or not (most likely receptor mediated).

Our results showed that MDDC- and breast milk exosomes shared a similar pattern of cell association and both showed preferences for HLA-DR^+CD14^+ monocytes above HLA-$DR^+CD14^-CD19^+$ B cells. After 1 h of co-incubation 46 % of the HLA-DR^+CD14^+ monocytes were positive for DC exosomes whereas only 17 % of the HLA-$DR^+CD14^-CD19^+$ B cells were associated with DC exosomes. In contrast, B cell

exosomes preferred to interact with the B cells and this interaction was little affected by co-incubation in cold conditions whereas the exosome interaction with HLA-DR^+CD14^+ monocytes was decreased in cold. Interestingly, the B cell exosome-B cell interaction was uniquely unaffected by cold since both DC- and breast-milk exosome interactions with B cells were both decreased by more than 50 % in cold. We therefore suggest that there are B cell exosome features that mediate specific targeting to the B cells and which are probably mediated through specific adhesion molecules. CLSM analyses also revealed that exosomes mainly localize on the surface of $CD19^+$ cells whereas $CD14^+$ cells mainly incorporate the exosomes intracellularly. We also showed that all three types of exosomes primarily interacted with antigen presenting cells and only to a low extent, at most 7.5 %, of the T cells. We could not identify any consistent difference between exosome association with either $CD4^+$ or $CD8^+$ T cells.

In addition to conventional flow cytometry and CLSM we explored a novel technique which combines flow cytometry with simultaneous imaging by fluorescence microscopy (ImageStream). This enabled a robust analysis of a high number of cells (typically 10,000) in each sample. The system used in this study has three lasers which enables collection of data from brightfield plus four different fluorochromes. The system generates an image of each cell that passes in the flow stream which can be inspected using the Ideas Software. In addition to visual inspection a variety of computerized quantitative analyses can be made from the images. In this study a specific algorithm was applied that enables objective analysis of internalization versus surface localization. There are also methods to analyze co-localization of molecules in the cells. Although a limited number of analyses were done, we were able to conclude that exosome interaction with different cell types could be analyzed with the ImageStream and that there was a high level of consistency between our findings in conventional flow cytometry and CLSM with the novel ImageStream. The ImageStream system is based on regular fluorescence microscopy and thus the resolution of the images is less good than in CLSM. It is also a weakness that the possibility to create sections in the z-plane is not at hand, something which would also be difficult due to the limited time that the cell passes the microscope. This can however be compensated for by the use of a nuclear stain which secures the possibility to capture cells interior for analysis. Compared to regular flow cytometry the ImageStream is slower in acquiring data yet the gain in information with the image of each cell increases its attractiveness.

This study gave insights into how exosomes of different origin targets different cell types in human peripheral blood and show a clear selectivity in their binding. We here chose to stain a limited number of cell-populations and this approach can be expanded to include other populations of interest. The ImageStream method shows promise to be useful in studies of exosome biology for the development of exosomes as therapeutic agents.

5 CONCLUSIONS

Paper I. In this study we developed a sensitive assay for assessment of the $CD8^+$ T cell stimulatory potential of exosomes in the presence of a viral peptide mix. Exosomes from monocyte derived dendritic cells (MDDC) could stimulate IFN-γ and TNF-α release in an antigen specific manner and without aid from antigen presenting cells. Stronger stimulation could be seen with exosomes from LPS-matured MDDC as compared to immature MDDC. These findings indicate that the method can be useful for the development of exosomes as therapeutic tools, for evaluation of the functional potential of different exosome phenotypes in the quest for desirable properties to combat tumors or facilitate vaccine development.

Paper II. MDDCs cultured in two different ways acquired different phenotypes which were also reflected by their released exosomes. Both IL-4/IL-3 and IL-4/GM-CSF generated MDDC stimulated virus specific $CD8^+$ T cell IFN-γ release with a slightly higher efficiency by the latter type. Both cell-types released exosomes, which could be loaded with viral peptides and subsequently stimulated a virus specific response from $CD8^+$ T cells in the form of IFN-γ release. No differences in their stimulatory abilities could, however, be established.

Paper III. Herein, we describe for the first time that exosomes are present in human breast milk and that they may have immune suppressive effects. A method for isolation of breast milk exosomes was developed and an extensive characterization was performed to verify the exosomal nature of the vesicles. Exosomes thus may be a novel way for transferring immune information between the mother and her infant and may contribute to the immunological maturation process of the newborn's defense against infections. They could also ultimately protect against or increase susceptibility to allergic manifestations later in life.

Paper IV. In this study we used a novel approach to visualize and quantify exosome associations to peripheral human blood cells. Conventional flow cytometry and confocal laser scanning microscopy revealed that over all, exosomes mainly associated to antigen presenting cells and very little to T cells. DC derived exosomes preferred HLA-DR^+CD14^+ monocytes whereas B cell exosomes specifically target HLA-$DR^+CD14^-CD19^+$ B cells. The later process is most likely receptor mediated. The origin of the exosome thus directs targeting to different immune cells. A multispectral imaging flow cytometer, ImageStream, enabled robust analysis of a high number of cells in each sample, and will be a useful method in future exosome research.

In summary, the work in this thesis has led to the development of new techniques for analyzing exosomes and has shown that exosomes can have different immune regulatory functions depending on their source and ultimately their cell type of origin. Exosomes can function both as T cell stimulators and as immune suppressors. The exosome origin also dictates selective targeting to distinct human peripheral blood cells. This thesis contributed to our understanding of exosomes as immune modulatory tools that may be useful in therapies, and increased the knowledge of the biological roles of exosomes.

6 FUTURE PERSPECTIVES

The results of this thesis have touched upon some issues in exosome biology, but many questions remain and new ones constantly arise. Below follows some of my thoughts on how to proceed with the exploration of exosomes.

Do exosomes really have a role *in vivo*? These small particles appear to be omnipresent and can originate from many cell types. Where should we start to look? The immunological focus of exosome research has stimulated speculations about their usefulness in immunotherapies, but much remains unknown about their physiological relevance. No one has yet identified a disease state where the imbalance of exosomes contributes to disease severity or the onset of disease. With more studies of exosomes in different disease states we might clarify the relevance of exosomes in disease, thus translational and cross-disciplinary research is of high importance.

We have learnt that exosomes can stimulate T cells directly in an antigen specific system, probably this is possible as long as the responder T cells are of a memory cell phenotype. The $CD8^+$ T cells in the assay were pre-selected and in an *in vivo* situation, there may be other target cells involved as well. It is important to avoid limitations caused by semantics such as for instance the concept of "stimulation" which often indicates a positive reaction from effector cells with a boost effect on the defense. However, stimulation of a regulatory phenotype, as for instance suggested for $CD4^+CD25^+Foxp3^+$ T cells induced by the milk exosomes, may ultimately control and inhibit immune responses. A response is a complex network between different cell types and one should look further than the signaling cascades induced within one cell type. Thus, further work *in vitro* using the ImageStream system to visualize exosome interactions with different cell types will be useful. This technique allows an unpredjudiced quantitative analysis of the exosome interactions with different cell types.

Turning to animal studies may reveal the distribution and life-span of exosomes *in vivo.* A lot may also be learnt about exosome distribution and interactions with different cell populations *in vivo* from advances in imaging techniques such as for example positron emission tomography (PET) in combination with radioisotope labeled exosomes. Further examinations of the composition of exosomes from different origins, and interactions with different cell types *in vitro* and *in vivo,* are highly relevant to understand the prospects of exosomes in therapy.

In the case of possible T regulatory cell induction by milk exosomes, functional proof that the Foxp3-expressing cells are really suppressive is desired, something which may be tested *in vitro.* A way to evaluate this could be to deplete the population of these cells using magnetic bead separation and stimulate the remaining cells. In order to dissect the mechanisms behind the inhibitory effects observed in the anti-CD3 stimulation of PBMC co-incubated with milk exosomes, blocking antibodies will be helpful. Strong candidates for blocking experiments are e.g. MUC-1 and MFG-E8. In our preliminary experiments, TGF-β known as a factor able to induce regulatory T cells *in vitro,* has been identified on milk exosomes (P. Torregrosa Paredes, *et al,* Karolinska Institutet, Stockholm, Sweden, unpublished), hence this is also a candidate molecule.

It has been suggested that a weak stimulation of T cells and B cells is necessary to maintain a long-term memory. Antigen retention may be a role for exosomes but a lot

more knowledge about exosome turnover rates in the body is needed, maybe the spleen and liver clears exosomes quickly? Again, animal studies can tell us more about this. Exosomes are suggested to be durable and with their small size and diluted concentrations over large areas, it could be argued that they would interact only weakly with target cells. However, in an immune response e.g. at a site of infection, cells may release exosomes in a polarized manner so that exosome conscentration increases enough to stimulate potently.

The usefulness of exosomes in therapy needs a lot of further examination. The promising data from mouse studies in cancer treatment[115] and as suggested vaccine components[126] have yet to be followed up. Basic knowledge of exosome formation and the specificity of sorting of proteins to this pathway needs competent cell biological evaluation. Unraveling the pathway of exosome formation would introduce possibilities to inhibit the exosome formation in experimental animals.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Exosomer är mycket små membranbubblor, mindre än en tiotusendels millimeter i diameter. De frisätts från flertalet celltyper och cirkulerar genom kroppen via blodet. Cellerna i vårt immunförsvar utgör den celltyp som undersökts mest i exosomsammanhang. De flesta immunceller verkar kunna frisätta exosomer som bildats genom inåtvänd avknoppning till större membranavgränsade rum inuti cellen (endosomer) och frisätts när hela endosomen smälter samman med det yttre cellmembranet.

Ett ständigt informationsutbyte pågår mellan celler, genom direktkontakt eller lösliga faktorer (proteinmolekyler) som t ex cytokiner och kemokiner. Exosomers ringa storlek gör att de nästan kan betraktas som lösliga faktorer eftersom de troligen kan passera överallt. Samtidigt är de på sätt och vis ett slags "mini-celler" som på ytan bär liknande molekyler som sin modercell och de är även anrikade med särskilda antigen-presenterande molekyler kallade MHC. Dock saknar exosomerna ett eget maskineri för proteinbildning och istället har de inuti sig faktorer som följt med från ursprungscellens innandöme. Innehållet i exosomerna tyder på att deras huvuduppgift är att överföra information mellan celler, men det finns ännu inget slutgiltigt svar på vilken roll exosomer spelar i kroppen. Det saknas kunskap om sjukdomar där eventuell brist eller överskott på exosomer skulle påverka förloppet. Framför allt har exosomer undersökts som möjliga terapiverktyg eftersom de visat sig kunna stimulera ett immunsvar som bekämpar tumörer i möss.

I den här avhandlingen har huvudmålet varit att vidga kunskaperna kring exosomers betydelse i immunförsvaret. För att uppnå detta mål har jag behövt utveckla metoder för att studera exosomers immunreglerande förmåga.

Dendritceller (DC) är de celler som är mest specialiserade på att känna av främmande inkräktare och signalera till andra celler att aktivera ett immunsvar. I det första delarbetet utvecklades en metod för att undersöka odlade DC-exosomers förmåga att stimulera T celler isolerade från blodet. Metoden (ELISPOT) mäter förekomsten av cytokinfrisättande celler. Fördelarna med denna metod är att den möjliggör en snabb och känslig analys av en individs immunsvar. En blandning av sönderdelade virus-proteiner (även kallade peptider eller antigen) som de flesta friska blodgivare reagerar mot användes för att ladda exosomerna. Vi kunde visa att exosomer från aktiverade DC stimulerade ett starkare svar i jämförelse med exosomer från icke aktiverade DC. ELISPOT är alltså användbart för att undersöka hur exosomer med olika sammansättning och ursprung förändrar sina funktionella egenskaper.

I delarbete II undersöktes hur exosomers egenskaper kan manipuleras genom olika odlingbetingelser i laboratoriet. Genom att odla DC på två olika sätt förändrades cellernas utseende. DC från kulturer där cytokinet IL-3 använts istället för GM-CSF visade sig vara mer effektiva att stimulera T celler. Även exosomerna ändrade utseende, däremot kunde ingen skillnad påvisas mellan de två olika exosomtypernas stimulerande förmåga.

I nästa delarbete identifierades för första gången exosomer i bröstmjölk. Det nyfödda barnets immunförsvar är inte färdigutvecklat vid födseln utan utbildas under de första levnadsåren genom bland annat interaktioner med barnets omgivande miljö. Eftersom

bröstmjölk visats ha gynnsamma effekter på spädbarnets immunförsvar så väcktes hypotesen att exosomer skulle kunna vara en bidragande komponent som överför immunologisk information från modern till barnet. Isoleringsmetoden för exosomer anpassades till mjölk och genom att använda flera olika tekniker kunde så många exosomkaraktäristiska egenskaper visas att det stod klart att mjölken innehåller exosomer. Blodceller som odlats tillsammans med mjölkexosomer ökade uttrycket av genen Foxp3, samtidigt som de visade sig svårare att stimulera än celler som inte varit i kontakt med mjölkexosomer. Därför spekulerar vi att mjölkexosomer hjälper barnets immunförsvar att veta vad det ska reagera på och inte.

Slutligen ställdes frågan vilka vita blodkroppar som exosomer från olika källor interagerar med. Detta undersöktes genom att färga exosomer från tre olika källor (DC, B celler och bröstmjölk) och analysera vilka typer av vita blodkroppar som de helst interagerar med. Vi kunde visa att exosomer från en viss celltyp föredrar att interagera med celler som har samma ursprung som exosomen. Till exempel sökte sig B cellsexosomer till B celler medan DC exosomer associerade med monocyter, vilka kan utvecklas till DC. I likhet med DC exosomer associerade mjölkexosomer med monocyter vilket indikerar att exosomer i mjölken eventuellt har sitt ursprung i DC eller monocyter. I detta arbete användes ett instrument kallat ImageStream, som är en kombination av två etablerade metoder, flödescytometri och fluorescensmikroskopi. Detta är en ny och mycket lovande teknologi som kommer att vara användbar i framtida exosomstudier.

Den här avhandlingen har bidragit till ökade kunskaper om hur exosomer interagerar med olika celltyper och utvärderat olika exosomers T cellsstimulerande förmåga. Detta arbete innehåller även det första beviset för exosomer i mänsklig bröstmjölk vilka har potential att vara en bidragande faktor till utvecklingen av barnets immunförsvar.

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