Department of Medicine
Clinical Allergy Research Unit &
Centre for Allergy Research
Karolinska Institutet, Stockholm, Sweden

EXOSOMES IN IMMUNE REGULATION AND ALLERGY

Charlotte Admyre

Karolinska
Institutet

Stockholm 2007

All previously published papers were reproduced with permission from the publisher.

© Charlotte Admyre, 2007
ISBN 978-91-7357-157-9

Published and printed by

www.reproprint.se
Gårdsvägen 4, 169 70 Solna

ABSTRACT

Exosomes are nano-sized vesicles of endosomal origin which are secreted from several different cell types. Depending on their cellular source they have been suggested to have different functions, like having a role in antigen delivery and T cell activation (antigen presenting cell-derived exosomes) or a role in tolerance induction (epithelial cell-derived exosomes). In addition, exosomes have shown potential to be used in immunotherapy both against infections and cancer. This thesis aimed at elucidating the presence of exosomes *in vivo*, develop methods to asses their function as immune regulators and to investigate if they may have a role in inflammatory responses such as allergies.

A lot of investigations have been published on exosomes derived from *in vitro* culture supernatants but very few studies have been performed showing the presence of exosomes *in vivo*. Since the lung contain many antigen presenting cells (APCs) and is a site of antigen entry we hypothesized that bronchoalveolar lavage fluid (BALF) might contain exosomes. By flow cytometry analysis and immune electron microscopy we describe the novel finding of exosomes in BALF. These exosomes expressed the antigen presenting molecules MHC class I and II, the co-stimulatory molecule CD86 and the tetraspanin protein CD63, suggesting them to be of APC origin, and to have a role in the immune defense of the lung.

Exosomes from APCs have been proposed to have a role in T cell activation. However, there are contradictory data on how this activation is achieved; i.e. if exosomes can directly activate T cells or if they exert their effect via APCs. We show that dendritic cell (DC)-derived exosomes loaded with viral peptides can activate human autologous peripheral $CD8^+$ T cells to produce IFN-γ and TNF-α without the addition of APCs by using the sensitive enzyme-linked immunospot (ELISPOT) assay. This stimulation was more efficient when using exosomes from mature DCs and was dependent of exosomal MHC class I. These data suggest that DC-derived exosomes may have a role in T cell activation during an immune response and show that the ELISPOT assay is a suitable method to use for evaluating exosome induced immune responses.

Breast milk is a complex liquid with immune competent cells and soluble proteins that provide immunity to the infant and affect the maturation of the infant's immune system. We further demonstrate the presence of exosomes *in vivo* with the finding that exosomes expressing molecules such as MHC, CD63, CD81, CD86, MUC-1 and heat shock proteins are present in human breast milk. These exosomes inhibited anti-CD3 induced cytokine production from peripheral blood mononuclear cells (PBMC). Furthermore, PBMC incubated with milk-derived exosomes showed a higher number of $Foxp3^+CD4^+CD25^+$ T regulatory cells. Our results show that human breast milk contains exosomes with the capacity to influence immune responses, and this suggests that they might influence the development of the infant immune system.

Since both we and others have shown that exosomes from APCs can have a function in T cell activation, we wanted to further investigate if APC-derived exosomes could have a role in activation of allergen specific T cells. We demonstrate that B cell-derived exosomes loaded with peptides from the major birch pollen allergen Bet v 1 can activate Bet v 1 specific T cells to both proliferate and produce the Th2-like cytokines IL-5 and IL-13 in a dose-dependent manner. This finding suggests that APC-derived exosomes may have a role in activation of T-cells during allergic immune responses.

In conclusion, the work presented in this thesis has increased our knowledge of the presence, phenotype and function of exosomes found *in vivo*. Furthermore, it sheds light on the function of APC-derived exosomes in T cell stimulations with the implication of a possible role in allergic responses.

Thesis summary in Swedish – Svensk sammanfattning av avhandlingen

Betydelsen av exosomer vid immunreglering och allergi

Exosomer är små blåsor, 30-100 nm i diameter, som bildas inuti celler och som sedan frisätts till den omgivande miljön. Exosomer produceras av många olika typer av celler t ex av immunologiska celler såsom antigenpresenterande dendritiska celler (DC) och B celler, men även av epitelceller och blodplättar. Beroende på vilken typ av cell exosomerna har frisatts ifrån har man funnit att de kan ha olika funktioner. Exosomer från DC och B celler har visat sig kunna inducera ett immunologiskt svar mot främmande ämnen (antigen). Detta sker genom att de fungerar som transportörer av antigen mellan celler eller genom att de presenterar antigen på sin yta för T celler som då aktiveras, vilket leder till ett specifikt immunsvar mot antigenet. Exosomer från epitelceller verkar istället vara involverade i att inducera tolerans mot antigen.

Det övergripande målet med min forskning har varit att öka kunskapen om exosomer och deras betydelse i vårt immunförsvar. Detta gjordes genom att karakterisera exosomer i kliniska prover, genom att studera exosomers förmåga att aktivera T celler, samt genom att undersöka om exosomer kan ha en betydelse vid inflammatoriska reaktioner som allergier.

Våra lungor utgör en viktig barriär mot den yttre miljön och utsätts konstant för olika typer av partiklar i luften som vi inandas. På grund av detta är det viktigt med ett kraftfullt immunförsvar i lungorna som därför är rika på immunologiska celler. Ett sätt att studera celler och lösliga immunologiska faktorer i luftvägarna är att skölja dem med en saltlösning. Genom att analysera sköljvätskan, som kallas bronkoalveolärt lavage (BAL), får man en bild av den immunologiska situationen i luftvägarna. I min första studie kunde vi som första forskargrupp påvisa exosomer i BAL, och dessutom visa att dessa exosomer uttrycker molekyler som till stora delar är gemensamma med exosomer från B celler och DC. Detta tyder på att exosomer kan ha betydelse i lungans immunförsvar.

Som nämnts ovan har man visat att exosomer från DC kan aktivera T celler. Hur detta sker är dock omtvistat, då vissa studier tyder på en direkt interaktion mellan exosomer och T celler, medan andra studier indikerar att exosomerna har sin effekt först efter interaktion med DC. I min andra studie kunde vi visa att exosomer från DC direkt kan stimulera T celler utan närvaro av DC. Detta understryker exosomers betydelse i aktivering av T celler. Den utvecklade metoden för att detektera exosomers aktivering av T celler kan även användas för att utvärdera funktionen av exosomer som utvecklas för kliniska applikationer.

Bröstmjölk är komplext sammansatt och innehåller bland annat immunologiska celler och lösliga protein som både ger immunologiskt skydd för det nyfödda barnet samt kan påverka utvecklingen av barnets egna immunsystem. Min tredje studie visar att exosomer också finns i bröstmjölk. Dessa exosomer uttrycker både molekyler som finns på antigen-presenterand celler och på bröstepitelceller, vilket indikerar att exosomer i bröstmjölk härstammar från flera olika celltyper. Exosomer i bröstmjölk hämmar aktivering av T celler. Detta tyder på att de kan influera immunologiska reaktioner och skulle kunna påverka immunsystemet hos det ammade barnet.

Allergier är inflammatoriska sjukdomar som ofta leder till kroniska besvär. I dessa sjukdomar överreagerar immunförsvaret på vanligt förekommande ämnen i vår miljö, så kallade allergen. Aktivering av T celler som specifikt känner igen allergen har en central roll vid allergiska reaktioner. I min fjärde studie har vi funnit att exosomer från antigen-presenterande celler kan presentera allergen från björkpollen på sin yta och aktivera björkpollen specifika T celler. Det är därför rimligt att tro att exosomer är involverade i aktiveringen av T celler under en allergisk reaktion. För att exakt klargöra denna interaktion krävs dock fler funktionella studier.

Denna avhandling har ökat kunskapen om exosomers förekomst, egenskaper och funktion ur ett immunologiskt perspektiv och kan förhoppningsvis inspirera till fortsatta studier inom detta område.

LIST OF PUBLICATIONS

I. Charlotte Admyre, Johan Grunewald, Johan Thyberg, Sofia Gripenbäck, Göran Tornling, Anders Eklund, Annika Scheynius and Susanne Gabrielsson. Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid.
Eur Respir J. 2003, 22(4): 578-583.

II. Charlotte Admyre, Sara M. Johansson, Staffan Paulie and Susanne Gabrielsson. Direct exosome stimulation of human peripheral T-cells detected by ELISPOT.
Eur J Immunol. 2006, 36(7): 1772-1781.

III. Charlotte Admyre, Sara M. Johansson, Khaleda Rahman Qazi, Jan-Jonas Filén, Riitta Lahesmaa, Mikael Norman, Etienne P.A. Neve, Annika Scheynius and Susanne Gabrielsson. Exosomes with immune modulatory features are present in human breast milk.
Under revision for the Journal of Immunology

IV. Charlotte Admyre, Barbara Bohle, Sara M. Johansson, Rudolf Valenta, Annika Scheynius and Susanne Gabrielsson. B-cell derived exosomes can present allergen and stimulate allergen specific T-cell proliferation and Th2-like cytokine production.
Submitted

CONTENTS

1 Introduction 1
1.1 The immune system 1
1.2 Antigen presenting cells 1
1.2.1 Dendritic cells 2
1.2.2 B lymphocytes 2
1.2.3 Antigen processing and presentation 3
1.3 T lymphocytes 3
1.3.1 $CD8^+$ T cells 4
1.3.2 T helper cells 4
1.3.3 T regulatory cells 4
1.4 Exosomes 5
1.4.1 Exosome formation 5
1.4.2 Exosome composition 7
1.4.3 Exosome function 8
1.4.4 Exosomes in inflammation 10
1.4.5 Exosomes derived from tumor cells 10
1.4.6 Exosomes in pregnancy 11
1.4.7 Exosomes and infectious agents 12
1.4.8 Exosomes in immunotherapy 12
1.5 Breast milk 13
1.6 Allergy 14
1.6.1 The allergic immune response 14
1.6.2 Bronchoalveolar lavage (BAL) 15
1.6.3 Breast feeding and allergy 15
2 Aims of the thesis 16
3 Methodology 17
4 Results and discussion 18
4.1 Exosomes are present in human bronchoalveolar lavage fluid (Paper I) 18
4.2 DC-derived exosomes can activate peripheral human $CD8^+$ T cells (Paper II) 20
4.3 Exosomes with immune modulatory features are present in human breast milk (Paper III) 22
4.4 B cell-derived exosomes can present allergen and stimulate allergen specific T cells (Paper IV) 25
5 Conclusions 28
6 Future perspectives 29
7 Acknowledgements 31
8 References 33

LIST OF ABBREVIATIONS

APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCR	B cell receptor
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
EM	Electron microscopy
ER	Endoplasmatic reticulum
ESCRT	Endosomal sorting complex required for transport
Foxp3	Forkhead box protein 3
GM-CSF	Granulocyte-macrophage stimulatory factor
HLA	Human leukocyte antigen
Hsp	Heat shock protein
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAMP	Lysosome-associated membrane proteins
LPS	Lipopolysaccharide
mDC	Myeloid dendritic cell
MFG	Milk fat globule
MFG-E8	Milk fat globule elongation factor 8
MHC	Major histocompatibility complex
MIC	MHC class I chain related protein
MIIC	MHC class II enriched compartment
MS	Mass spectrometry
MVB	Multivesicular body
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cells
PHA	Phytohemagglutinin
PLAP	Placental type alkaline phosphatase
PRR	Pattern recognition receptor
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Tumor growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tr1	T regulatory type 1
Tregs	T regulatory cells

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system has developed to protect us from infectious agents, and this is achieved by a combination of innate and adaptive immunity. The non-specific innate immune system is an evolutionarily ancient form of host defense found in most multicellular organisms and serves as a first line of defense against invading microbes. The innate immune system is triggered upon pathogen recognition by a set of pattern recognition receptors (PRRs), one example being the family of Toll-like receptors (TLRs), recognizing conserved molecular patterns shared by large groups of microorganisms. These patterns are called pathogen-associated molecular patterns (PAMPs) [1] with one example being bacterial lipopolysaccharide (LPS) found in the cell wall of gram negative bacteria [1]. One important role of the innate immune system is the barrier function of the epithelial surfaces preventing entry of microbes into our body. In addition, the surface epithelia constitutively produce anti-microbial peptides [2]. If an infectious agent crosses the epithelial barrier and is further recognized by the innate immune system it leads to elimination of the invading pathogen through various mechanisms, one being phagocytosis by macrophages and neutrophils [3]. Another important mechanism is activation of the complement system, which is important in opsonizing pathogen for promoting the uptake by phagocytic cells, and in the formation of the membrane-attack complex resulting in lysis of the pathogen. Phagocytic cells can also release cytokines, which in turn can induce the mobilization of antigen-presenting cells (APCs) which are important for the induction of the adaptive immune system [1].

Adaptive immunity is mediated by B cells and T cells through their highly specific receptors. B cells have cell-surface immunoglobulin (Ig) molecules as receptors and upon activation they secrete the immunoglobulin as soluble antibody that provides defense against pathogens in the extracellular spaces of the body [1]. T-cells have receptors that recognize peptide fragments of pathogens presented on the surface of APCs on special molecules called major histocompatibility complexes (MHCs). Depending on which type of T cells that are activated the effect could be killing of infected cells (cytotoxic $CD8^+$ T cells), activation of macrophages and B cells ($CD4^+$ T helper cells) or inhibition of an immune response (T regulatory cells) [1]. The adaptive immune system is specific and develops during our life-time. In addition, it gives rise to immunological memory that protects us from re-infection with the same pathogen.

1.2 ANTIGEN PRESENTING CELLS

Antigen presenting cells express PRRs and are among the first cells to respond to invading pathogens. Triggering of PRRs leads to increased expression of co-stimulatory molecules on APCs which allow them to stimulate T cell responses [1]. There are three types of professional APCs: macrophages, B cells and dendritic cells (DCs) [1].

1.2.1 Dendritic cells

DCs are the main form of APC with an exceptional capacity to initiate both primary and secondary immune responses [4] providing a link between the innate and adaptive immune system. They develop from stem cells in the bone marrow giving rise to circulating progenitors in the blood [5]. These DC progenitors home to a variety of different tissues where they reside as immature DC with high capacity to take up antigens [6]. In their immature state DC express low levels of costimulatory molecules and surface MHC. After capturing of antigen and being triggered by proinflammatory signals, the DC mature and migrate to lymphoid organs. The mature DC express higher levels of surface MHC and costimulatory molecules and in the lymphoid organs they can present antigen on their MHC to naïve T cells [6]. Ag-specific T cell activation requires the engagement of the T cell receptor (TCR)/CD3 complex with the antigenic peptide and the MHC molecule (signal 1), and further also the engagement of costimulatory receptors on the T cell, like CD28, with costimulatory ligands on the DC like CD80 and CD86 (also called B7-1 and B7-2, respectively) [7]. By providing different signals to the $CD4^+$ T cell the DC can influence the differentiation of the cells into either T helper (Th) 1, Th2 or T regulatory cells depending on the expression of costimulatory molecules and the type of cytokines that they produce [7].

There exist several different types of DCs. In human blood two major subsets have been characterized based on their expression of the integrin CD11c [8]. One of these two subsets belongs to the myeloid lineage and is called myeloid DC (mDCs). The other subset belong to the lymphoid lineage and is called plasmacytoid DCs (pDCs) [9]. These two subsets have different morphology and have both distinct and shared molecular expression and functions. The mDCs are characterized by their irregular shape and expression of myeloid markers like CD11c, CD13 and CD33 and their low expression of the IL-3R α-chain CD123. mDCs can be generated from monocytes *in vitro* by culturing with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [10] and produce high levels of IL-12 in response to LPS. PDCs have a morphology that resembles plasma cells and have low expression of myeloid markers like CD11c [11] but high expression of CD123. PDCs produce low amounts of IL-12 in response to LPS but instead produce high levels of interferon (IFN)-α in response to viruses [11]. In mice additional subsets have also been identified such as the $CD8^+$ or $CD8^-$ splenic DCs [12].

1.2.2 B lymphocytes

The major role of B cells is to produce antibodies specific for invading pathogens. These antibodies protect the extracellular spaces causing destruction of extracellular microbes and prevent the spread of intracellular infections. The activation of B cells and their differentiation to antibody-secreting plasma cells is triggered by antigen binding to the B cell receptor (BCR) and usually requires T cell help through CD40-CD40L interaction and through stimulation by cytokines produced by the T cell [1]. An immature B cell express IgM as well as IgD on their surface, but activated B cells subsequently undergo isotype switching where the B cells switch to secrete antibodies

of a different isotype like IgG, IgA or IgE. The different isotypes have different effector functions which are mediated by the Fc part of the antibody [1].

In addition to being antibody-secreting cells B cells can also function as antigen presenting cells. When the BCRs are cross-linked by their specific antigen it leads to presentation of antigen peptides on MHC class II molecules on the surface of the B cell together with low expression of co-stimulatory molecules like CD86. The TCR of T cells specific for the antigen peptide bind to the peptide/MHC complex on the B cell and to CD86 by its receptor CD28. These signals induce expression of low levels of CD40L on the T cell which bind CD40 on the B cell leading to higher expression of co-stimulatory molecules on the B cell. After continued signaling between the cells the end result is complete activation of both cells [13].

1.2.3 Antigen processing and presentation

After encountering antigen APCs can internalize it in three different ways: by phagocytosis, fluid-phase pinocytosis or receptor mediated endocytosis [14]. Examples of receptors involved in receptor-mediated endocytosis are the mannose-receptor [15], Fc receptors and for B-cells the BCR. After being captured and internalized the antigen is proteolytically processed into peptide fragments and loaded onto MHC class II molecules for presentation to $CD4^+$ T cells [1]. The loading of MHC class II with antigen peptides occur in special compartments called MHC class II enriched compartments (MIICs).

MHC class I molecules are instead usually loaded with peptide in the endoplasmatic reticulum (ER) and most of the peptides are derived from cytosolic proteins. The degradation of cytosolic proteins for loading on MHC class I is a tightly regulated process to prevent nonspecific destruction of essential self-proteins. Peptides presented on MHC class I molecules are recognized by $CD8^+$ T cells. In addition to these two pathways exogenous antigens can be processed and loaded onto MHC class I, which is called cross-presentation. One way by which this can occur is by loading exogenous antigens on recycling MHC class I molecules present in the endosomal compartments [16].

1.3 T LYMPHOCYTES

T cells are positively selected during their development in the thymus by interaction with self-peptides bound to MHC class II for CD4 T cells and self-peptides bound to MHC class I for CD8 T cells. The T cells are also subjected to negative selection by which self-reactive T cells are removed from the lymphocyte repertoire [1]. After the positive selected T cells leave the thymus they re-circulate through lymphoid organs and are dependent on repeating contact with DCs for their survival [3]. After activation by APCs presenting the specific antigen the T cells develop to effector cells with different effector functions depending on the type of T cell.

1.3.1 CD8$^+$ T cells

CD8$^+$ T cells (cytotoxic T cells) are selected in the thymus to recognize and respond to foreign peptides presented by MHC class I on APCs. When activated they can kill their target cells by releasing cytotoxic effector molecules like perforin and granzymes, which form pores in the target cell plasma membrane destroying the membrane integrity leading to cell death. They can also induce apoptosis via binding of FasL to Fas on the target cell [1]. Cytotoxic T cells can in addition release cytokines like IFN-γ and TNF-α, which can contribute to host defense by for example activating macrophages and increase the expression of MHC class I [1].

1.3.2 T helper cells

CD4$^+$ T cells have traditionally been divided into two distinct lineages based on their cytokine production profile [17, 18]. Th1 cells, which evolved to enhance eradication of intracellular pathogens, are characterized by their production of IFN-γ, which is a potent activator of cell-mediated immunity. Th2 cells, which instead evolved to enhance elimination of parasitic infections, are characterized by production of IL-4, IL-5 and IL-13, which are potent activators of B-cell immunoglobulin production (mainly of the IgE type) and eosinophil recruitment [1]. Recent studies have further led to the characterization of a new type of Th cell called the Th17 cell, which produces the cytokine IL-17 [19]. These cells seems to be highly proinflammatory and involved in many autoimmune disorders [20, 21].

Th1 differentiation is initiated by signaling through the TCR and signal transducer and activator of transcription (STAT)1 associated receptors like the IL-27 receptor together with STAT4 associated receptors like the receptor for IL-12 [22]. STAT1 signaling upregulates the transcription factor T-bet [23, 24], which potentiate the expression of IFN-γ and upregulates the IL-12 receptor while suppressing Th2 associated factors. Th2 differentiation is initiated by TCR signaling together with IL-4 receptor signaling via STAT6. This leads to activation of the transcription factor GATA-3, which drives epigenetic changes in the Th2 cytokine cluster while suppressing factors crucial for the Th1 pathway like expression of the IL-12 receptor.
The differentiation of Th17 cells requires blockage of IL-4 and IFN-γ and is mediated by tumor growth factor (TGF)-β1 together with IL-6 [25, 26]. After their differentiation IL-23 is essential for Th17 cell expansion and survival [27]. (Fig. 1)

1.3.3 T regulatory cells

T regulatory cells (Tregs) can be divided into two types, the natural occurring Tregs and the adaptive Tregs [28]. Natural occurring Tregs develop in the thymus and go to the periphery with a functional suppressive phenotype where they make up around 5-10% of peripheral CD4$^+$ T cells [29]. These naturally occurring Tregs have high expression of the IL-2Rα chain (CD25) and express the transcription factor forkhead box protein 3 (Foxp3) [30, 31]. Naturally occurring Tregs seem to mediate their suppression in a contact dependent way. They need TCR interaction to become

suppressive but once activated they suppress T cells independently of antigen specificity [29].
Adaptive Tregs develop from naïve $CD4^+$ T cells in the periphery as a result of specific immune stimulations. They can be divided into T regulatory type 1(Tr1) and Th3 cells [32]. Tr1 cells secrete high levels of IL-10 and low levels of TGF-β and IL-5. Th3 cells secrete TGF-β together with IL-4 and IL-10 and seem to mediate its suppression via a TGF-β dependent mechanism. Stimulation of naïve T cells can under certain conditions also lead to the generation of $Foxp3^+$ Tregs that seem indistinguishable from the natural occurring Tregs [29] (Fig. 1).

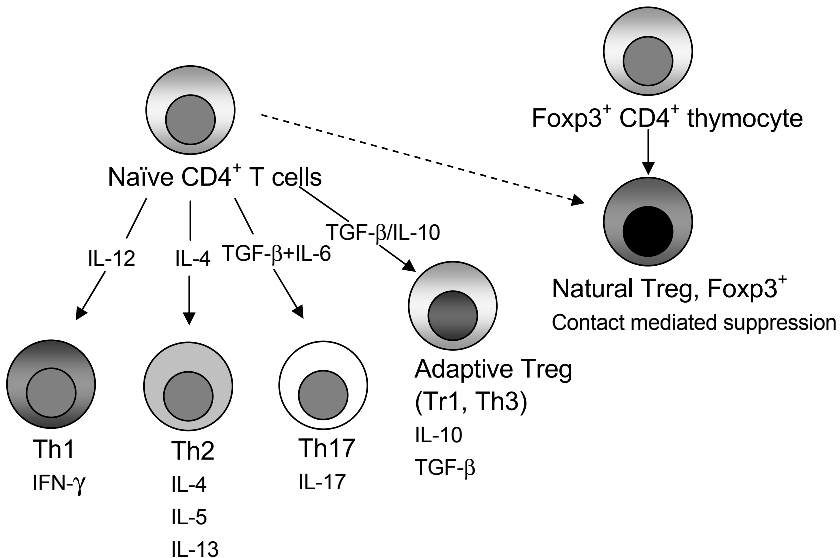

Fig 1. Development of different $CD4^+$ T cell subsets. (Modified from [29, 33])

1.4 EXOSOMES

1.4.1 Exosome formation

Exosomes are small, 30-100 nm, membrane vesicles of endocytic origin that are secreted by a variety of cell types like B-cells [34], T-cells [35], mast cells [36], DCs [37], platelets [38], neurons [39] and epithelial cells [40]. They were first described as microvesicles containing 5´-nucleotidase activity secreted by neoplastic cell lines [41]. A few years later another group reported secretion of small vesicles of endocytic origin by cultured reticulocytes. Using electron microscopy (EM) they observed these vesicles in late endosomes, and the fusion of these late endosomes with the cell membrane resulted in the release of the vesicles extracellularly [42, 43]. In addition to cultured cells, exosomes have today further been isolated from a number of body fluids such as plasma [44], urine [45], synovial fluid [46], malignant effusions [47], epididymal fluid

[48] and from seminal plasma, in which the vesicles are derived from prostate cells and called prostasomes [49]. Moreover, in this thesis we demonstrate the presence of exosomes in bronchoalveolar lavage (Paper I) and breast milk (Paper II).

Exosomes are believed to originate from the intraluminal vesicles of late endosomal compartments called multivesicular bodies (MVBs). These intraluminal vesicles are formed by inward budding of the limiting endosomal membrane and contain cytosol from the cell. MVBs are involved in transporting proteins for degradation in lysosomes. Alternatively, the MVBs can fuse with the plasma membrane leading to the release of the intraluminal vesicles extracellularly which are then called exosomes [34, 43]. Proteins and lipids are sorted at the limiting membrane of endosomes during the formation of the intraluminal vesicles and as a consequence the released exosomes will contain molecules reflecting their origin from late endosomes [50]. The mechanisms leading to exosome release are unknown. However, the transmembrane protein TSAP6 has been suggested to be involved in regulating exosome production [51]. Furthermore, Rab11, a member of the small GTPase family, together with calcium were shown to be important for the docking and fusion of MVBs with the plasma membrane [52-54]. A machinery responsible for sorting proteins in intraluminal vesicles has recently been identified called ESCRT (Endosomal Sorting Complex Required for Transport) [55]. This complex is believed to recognize mono-ubiquitinated transmembrane proteins and induce their inclusion into membrane domains that generate the intraluminal vesicles of MVBs. Lipid rafts has also been suggested to be involved in protein sorting into intraluminal vesicles [56] and typical raft components has been identified on exosomes such as glycolipids, Src tyrosine kinases and cholesterol [56, 57]. How MVBs discriminate between proteins that are destined for exosomal secretion or lysosomal degradation remains to be determined.

In APC MHC class II are accumulated in MVBs which are called MIICs. These MIICs are the major site for peptide loading, and subsequently exosomes from APC bear peptide-loaded MHC on their surface (Fig. 2).

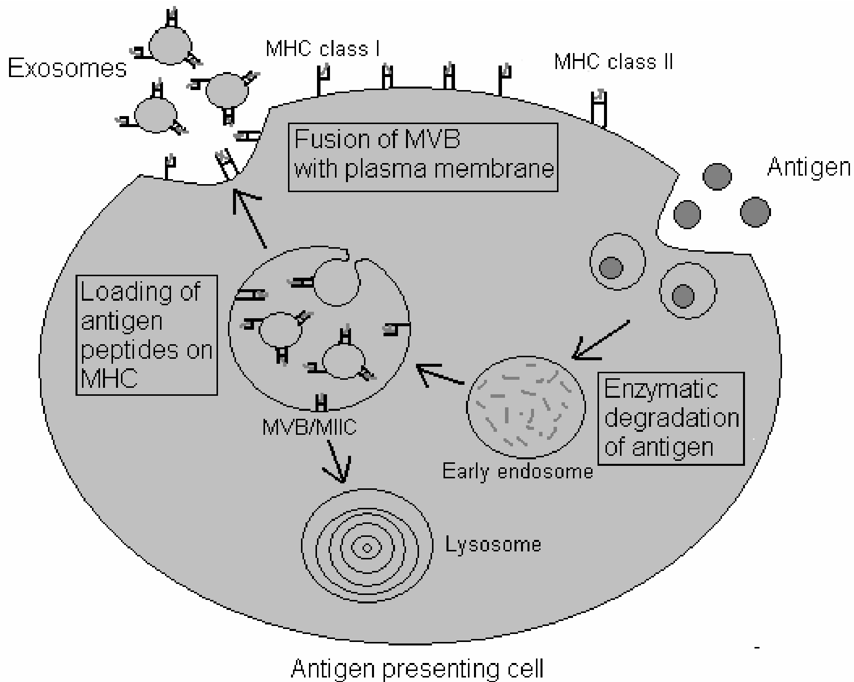

Fig 2. Schematic picture of the formation of exosomes in antigen presenting cells. Antigen is taken up by the antigen presenting cell into early endosomes were the antigen are degraded to peptides by proteases. In multiple vesicular bodies (MVB) or MHC class II enriched compartments (MIIC) the antigen-derived peptides are loaded onto MHC class I and II molecules. The MVB contains intraluminal vesicles formed by inward budding of their limiting membrane. For the peptide-loaded MHC to reach the surface of the cell the MVB fuse with the plasma membrane. This also leads to the release of their intraluminal vesicles which are now called exosomes (modified from [58]).

1.4.2 Exosome composition

The molecular composition of exosomes reflects the cell type from which they are secreted and their endosomal origin. For example exosomes from APCs express co-stimulatory molecules like CD54 (also called ICAM-1), CD80 and CD86 [59-61], exosomes from intestinal epithelial cells (IECs) express the IEC specific marker A33 [40], exosomes from T cells bear CD3 [35], exosomes from reticulocytes contain the transferrin receptor [62], and subunits of the glutamate receptor are found on exosomes from neurons [39]. In addition to cell-specific molecules exosomes also contain common components. They are enriched in a family of proteins called tetraspanin proteins which are cell-surface proteins that span the membrane four times [61]. Tetraspanin proteins are found on the surface of many cell types but also in endosomal compartments. They have been suggested to be involved in cell adhesion, activation,

proliferation and antigen presentation. They form complexes with other molecules, for example MHC class II, and are thought to keep the proteins in an optimal conformation [63]. Examples of tetraspanin proteins found on exosomes are among others CD9, CD63 and CD81. Exosomes have also been demonstrated to contain heat shock proteins (Hsps) like Hsp70, Hsc70, Hsc73 and Hsp90 [64, 65]. Heat shock proteins are a family of proteins which act as chaperones to facilitate the folding of protein intracellularly. Hsps can also be secreted and have extracellular functions such as being immuno-regulating. Hsps can be both constitutively expressed and be induced by cellular stress. Heat stressed cells have been shown to increase the expression of Hsps on their released exosomes [64]. Moreover, exosomes contain cytoskeleton proteins like actin and Moesin, ESCRT proteins like Tsg101 and alix and proteins involved in transport and fusion like Rab and annexins [66, 67]. Furthermore, exosomes express CD55 and CD59 which have been shown to protect them from complement lysis [68] suggesting them to be stable *in vivo* (Fig. 3).

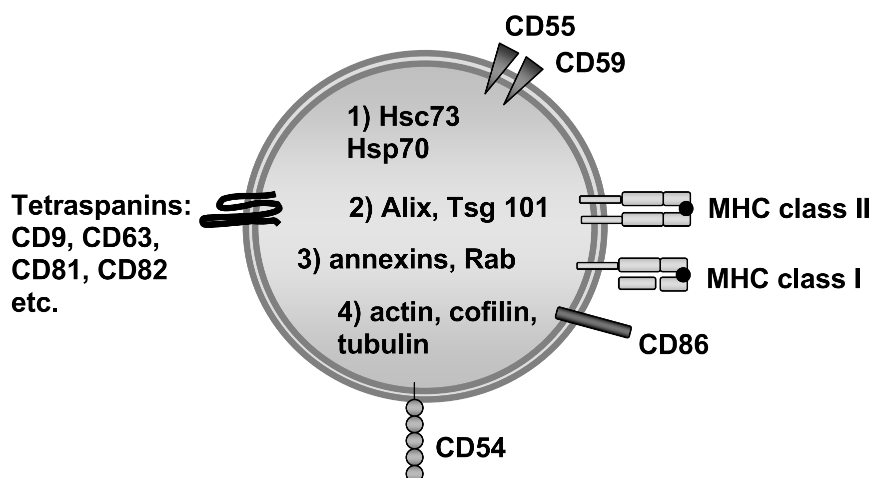

Fig 3. A simplified schematic presentation of the molecular composition of exosomes. Exosomes bear molecules for antigen presentation like MHC class I and II and co-stimulatory molecules like CD86, adhesion molecules like CD54 and tetraspanin proteins, and regulators of complement activation like CD55 and CD59. Exosomes contain cytosol from the cells including 1) heat shock proteins, 2) ESCRT proteins, 3) membrane transport and fusion proteins, and 4) cytoskeleton associated molecules (modified from [69, 70]).

1.4.3 Exosome function

The function of exosomes depends to large extent from which cell type they originate and their protein expression. When exosomes were initially discovered from reticulocytes, they were shown to be a way of removing unnecessary proteins such as the transferrin receptor during the maturation of reticulocytes into erythrocytes [71]. Later this was also shown for the integrin $\alpha 4\beta 1$ which was down regulated from the red

blood cell surface and instead found on the surface of released exosomes, and in addition this made the exosomes able to bind to fibrinonectin [72].

Exosomes from APCs have been demonstrated to be involved in T-cell stimulation both *in vitro* [34, 73-75] and *in vivo* [76, 77]. How this stimulation occurs is debated, with some studies showing that exosomes can stimulate T cells directly without the presence of APC [34, 75] while other studies demonstrate that exosomes need APC to exert their effect [73, 77]. These differences may be due to among other things the phenotype of the exosomes and of the responder cells, the affinity for the antigen or the doses of the exosomes used. CD54 and MHC class II was shown to be required for exosomes to prime naïve T cells [76]. Moreover, another group demonstrated that exosomes need to express CD54 and B7 together with MHC class I/peptide complexes to be strongly immunogenic [75]. Several studies have demonstrated that exosomes from mature DCs are more potent in inducing antigen-specific T-cell activation than exosomes from immature DCs [74, 76], possibly due to their increased expression of these molecules. In addition, it has been demonstrated that exosomes from monocyte-derived DCs (MDDCs) can support the survival of naïve T cells via activation of the transcription factor NF-κB which was induced by interaction of human leukocyte antigen (HLA-DR) and TCR [78].

Exosomes have also been suggested to have a role as transporters of molecules between cells. Several studies show that exosomes can transfer MHC/antigen complexes between DCs making the recipient DCs able to efficiently activate antigen specific T cells [79, 80]. It was shown that exosomes can be internalized by DCs and sorted into endocytic compartments for processing and loading of exosome-derived peptides in MHC class II molecules for presentation to $CD4^+$ T cells. The targeting of exosomes to DCs were further discovered to be mediated via exosomal expression of milk fat globule elongation factor 8 (MFG-E8), CD11a, CD54, phosphatidylserin and the tetraspanins CD9 and CD81 [81]. Another example of exosomes as transporters is that *in situ* analysis of follicular DCs show attachment of exosomes like vesicles expressing MHC class II on the surface of the follicular DCs [82]. Since follicular DCs do not express MHC class II themselves these exosomes are probably derived from other cell types and may have a role in tuning the immune response [82]. In addition, a recent study by Zakharova *et al* showed that exosomes released from PHA activated $CD4^+$ T cells can enhance cholesterol accumulation in cultured human monocytes by internalization via the phosphatidylserine receptor, suggesting that T cell-derived exosomes could have atherogenic properties [83]. Furthermore, exosomes from B cells were demonstrated to bind to extracellular matrix components like collagen and fibronectin and may be an indication that exosomes are able to deliver signals at distances beyond that of direct cell-cell contact [84].

Mast cell-derived exosomes have also been shown to have immunological effects. When incubating mast cell-derived exosomes with splenocytes, the exosomes induced blast formation, proliferation and production of IL-2 and IFN-γ [85]. In addition, mast cell-derived exosomes were demonstrated to induce maturation of DCs showing up-regulation of MHC class II, CD40, CD80 and CD86 [86], which may be a way to potentiate an immune response.

In contrast to being immunostimulatory, exosomes from IECs have been shown to be tolerogenic and have therefore been called tolerosomes [87]. Tolerosomes isolated from ovalbumin (OVA) pulsed IEC lines and from serum after OVA antigen feeding were capable of inducing antigen specific tolerance in naïve recipient rats [87]. The tolerance induction was dependent on MHC class II expression of the IECs and is only functioning in syngeneic recipients [88]. This may be an important mechanism for oral tolerance induction. Contradictory results have, however, been reported by van Niel *et al* who demonstrated that IEC derived exosomes induced more of an immunogenic rather than tolerogenic response [89].

1.4.4 Exosomes in inflammation

The role of exosomes in different inflammatory disorders has not been extensively studied. However, one very interesting finding by Zhang *et al* was that synovial fibroblasts produce exosomes, and that these exosomes obtained from patients with rheumatoid arthritis contained a membrane form of tumor necrosis factor (TNF)-α which was cytotoxic to the TNF-α sensitive cell line L929 [90]. In addition, the exosomes from rheumatoid arthritis patients were taken up by activated T cell making these T cells resistant to apoptosis [90]. This suggests that synovial fibroblasts may communicate with infiltrating T cells through the release of exosomes in the joint making the infiltrating T cells resistant to activated-induced cell death.

Another example of that exosomes may have a role in inflammation is the finding that exosomes released from platelets obtained from patients with sepsis had higher NADPH oxidase activity compared with healthy controls, measured by the generation of reactive oxygen species and the apoptosis inducing activity. This might be one way with which platelet-derived exosomes contribute to vascular cell apoptosis and may constitute a new pathway involved in the pathophysiology of sepsis [91].

1.4.5 Exosomes derived from tumor cells

The majority of the studies performed on exosomes have been in the context of tumor biology and cancer therapy.

Wolfers *et al* showed in 2001 that both human and mouse tumor cell lines produce exosomes which are 60-90 nm in size, that are positive for MHC class I, lysosome-associated membrane protein (LAMP) 1 and Hsc 70 and negative for the ER marker calnexin [92]. Later it was further demonstrated that tumor-derived exosomes positive for MHC class I, Hsps, CD81 and tumor antigens like MART1 could be found in malignant effusions from cancer patients [47]. In both studies it was also shown that tumor-derived exosomes could deliver tumor antigen for loading on DCs allowing activation of specific cytotoxic T cells [47, 92]. When injected *in vivo* into mice tumor derived-exosomes were further demonstrated to protect against tumor establishment [92].

Since tumor-derived exosomes express tumor antigen they have been of interest for their potential use in anti-tumor immunotherapy, however tumor-derived exosomes have in several studies also been demonstrated to have immunosuppressant effects and be part of tumor immune evasion. Tumor-derived exosomes have been found to be able to inhibit $CD8^+$ T cell cytotoxic killing [93] and to induce apoptosis of $CD8^+$ T cells via FasL-Fas interaction [94]. Tumor-derived exosomes can also have an effect on NK cells by inhibiting their cytotoxic activity through the reduction of perforin release [95]. Tumor-derived exosomes have in addition been suggested to have a role in tumor angiogenesis by the expression of the tetraspanin protein D6.1A/CO-029 [96]. This tetraspanin protein has been associated with poor prognosis in patients with gastrointestinal cancer and has been shown to be a strong inducer of angiogenesis [96]. The release of this molecule on exosomes may initiate angiogenesis that reaches organs distant from that of the tumor site.

1.4.6 Exosomes in pregnancy

During a pregnancy the semi-allogenic fetus is tolerated by the mother's immune system. This is achieved by several suggested mechanisms one being the expression of FasL of fetal trophoblasts which may induce apoptosis of maternal immune cells [97]. Abrahams *et al* showed that trophoblast cells isolated during the first trimester lacked plasma membrane associated FasL, but expressed a cytoplasmic form of FasL in association with the secretory lysosomal pathway. This intracellular FasL was secreted by the trophoblast cells through the release of microvesicles [98]. In a study by Mincheva-Nilsson *et al* they confirmed these findings and further showed that the majority of intracellular FasL was concentrated in cytoplasmic granules on microvesicles with the size of 60-100 nm [99]. These microvesicles were later found to also be positive for MHC class I chain-related proteins A and B (MIC) [100]. Soluble MIC can be immunosuppressive by competing with membrane-bound MIC, found on the surface of stressed cells, for binding to the receptor NKG2D, which is expressed on the surface of NK cells and cytotoxic T cells, blocking immune effector functions [101]. The level of soluble MIC was elevated in the blood of pregnant women compared with non-pregnant controls and soluble MIC was able to down regulate the NKG2D receptor on peripheral blood mononuclear cells (PBMC) and inhibit their cytotoxic activity [100]. Secretion of exosomes expressing FasL and MIC could be one mechanism by which the fetus evades immune recognition.

Placenta-derived exosomes has been characterized in sera from pregnant women by the expression of the placenta specific enzyme placental-type alkaline phosphatase (PLAP) [102]. The levels of placental derived exosomes were shown to be elevated in sera from mothers delivering at term compared to preterm and were shown to express higher levels of FasL and HLA-DR. Exosomes from term-delivering mothers were further able to inhibit phytohemagglutinin (PHA) induced IL-2 production by T cells which were not seen for preterm exosomes [102], suggesting that exosomes could be a major regulating factor for pregnancy maintenance.

1.4.7 Exosomes and infectious agents

The Trojan exosome hypothesis was first proposed by Could *et al* which suggested that retroviruses use the exosome release pathway for viron biogenesis and that they may use an exosome uptake pathway as an alternative way of infecting cells [103]. It has been demonstrated that macrophage-derived HIV have a host-derived protein phenotype that matches that of macrophage-derived exosomes with expression of the tetraspanin proteins CD63 and CD81, MHC class II and the lysosomal protein Lamp-1 [104]. Immuno EM studies of HIV infected macrophages showed co-localization of tetraspanin proteins and HIV proteins in multivesicular bodies [105]. It was later demonstrated that exocytosed HIV-particles in DC supernatants were associated with vesicles that were around 100 nm in size and expressed HLA-DR and tetraspanin proteins, suggesting to be exosomes. These exosome-associated HIV particles could further infect $CD4^+$ T cells and were shown to be more infectious than cell-free virus particles [106]. This could suggest new strategies of interfering with virus production for antiretroviral therapy. In addition to retroviruses, other studies have also found association of infectious prion proteins with secreted exosomes indicating that prions may also use exosomes as vehicles for infection [107, 108].

1.4.8 Exosomes in immunotherapy

1.4.8.1 Exosomes in immunotherapy against cancer

The first anti-tumor effect of exosomes was demonstrated by Zitvogel *et al* where tumor peptide pulsed DCs-derived exosomes suppressed growth of established tumors when injected into mice [37]. Since then a lot of studies have been made regarding the potential use of exosomes in immunotherapy against cancer. Different strategies have been applied, using either exosomes from tumor cells or exosomes from DCs loaded with tumor peptides, to induce anti-tumor immune responses [92, 109-111]. Tumor derived exosomes represents a natural source of tumor antigens but as mentioned above there are also studies showing immuno-inhibitory effects of tumor-derived exosomes [93-95]. In a study comparing DC-derived exosomes with tumor-derived exosomes it was shown that DC-derived exosomes induced a more efficient anti-tumor immunity than tumor-derived exosomes and may therefore represent a more effective type of vaccine [112]. In several studies one has tried to improve exosome-based tumor vaccines. For example, heat shocked lymphoma cells were demonstrated to release exosomes with higher levels of Hsps and immunogenic molecules like MHC class II, CD40 and CD86. It was further shown that these exosomes were more potent in inducing T cell responses and anti-tumor immunity compared to control exosomes in a lymphoma mouse model [113]. In addition, IL-2 genetically modified tumor cells were demonstrated to release exosomes containing IL-2 which were more efficient in inhibiting tumor growth [114]. Combining exosomes with adjuvants like CpG has also shown promising effects [110]. The first phase 1 clinical trials using autologous DC-derived exosomes loaded with tumor peptides for vaccination of melanoma and lung cancer patients have now been published. These studies showed low toxicity of the exosome treatment [115, 116] and a phase II clinical trial has know been designed [117].

1.4.8.2 Exosomes in other types of immunotherapies

Exosomes have not only shown potential use in cancer treatment but also in other disorders like prophylactic therapy against pathogens. Aline *et al* demonstrated that *Toxoplasma gondii* pulsed DC-derived exosomes can induce an efficient Th1 immune response specific for *T. gondii*, which provides good protection against both acute and chronic toxoplasmosis [118]. Furthermore, Peche *et al* demonstrated that exosomes derived from donor DCs given before heart transplantation can induce prolonged allograft survival with a decrease of graft-infiltrating leukocytes [119]. In two studies by Kim *et al* it was further shown that exosomes may also be used for suppressing inflammation. They found that exosomes produced by DCs treated with IL-10 or that were genetically modified to express FasL could suppress delayed-type hypersensitivity and reduce the severity of collagen-induced arthritis in a mouse model [120, 121]. The exosome display technology, in which proteins by fusion with the Lactadherin C1C2 domain can be targeted to exosomes, makes it possible to manipulate the protein expression of exosomes and tailor make them for different applications [122]. This may be a useful tool when developing exosome-based therapies.

1.5 BREAST MILK

Breast milk contains many components that provide immunological information and may promote the development of neonatal immune competence and protect the neonate from infections. These components include antibodies, mainly IgA which constitutes over 90% of all immunoglobulins in milk [123], as well as cytokines and chemokines like IL-4 [124], IL-6 [125], IL-8 [126], IL-10 [127], IL-12 [128], IL-18 [129], IFN-γ [124, 125] and RANTES [126], and immunological cells including APC and effector/memory T cells [123]. It has been demonstrated that human milk leukocytes can adhere to the gut epithelium, cross the gut mucosa and go through the circulation to the spleen and the liver, indicating that milk cells are able to influence not only the local immune system of the gut but also the systemic immune response of the neonate [130]. In addition, breast milk contain nonspecific protective factors like Lactoferrin and Lysozyme which have been shown to inhibit bacterial growth [131, 132], oligosaccharides and lipids that can inhibit binding of certain pathogens [133, 134] and complement factors [135]. Fat is a major nutrient of milk and over 90% of the total lipid content of milk is found in milk fat globules (MFG). MFG are fat droplets, 95% being triacylglycerols, surrounded by cellular membrane and are secreted from mammary epithelial cells [136]. The MFG membrane contains membrane proteins such as the mucin MUC-1, Lactadherin (also called MFG-E8) CD36 [136] and HLA-DR [137], several which have been shown to have anti-infectious functions [138]. Milk does not only contain immunostimulatory but also immunosuppressive factors. In mice, immunization of mothers can down regulate specific immune responses in the offspring via the milk but not the placenta [139] suggesting an additional suppressive factor in milk specific for the antigen. It has previously further been demonstrated that colostrum proteins in high concentration can have an inhibitory effect on T cell growth [140]. In addition, colostrum contain the anti-inflammatory cytokine TGF-β which was shown to play a role in the immunosuppressive effect of colostrum on stimulated cord blood mononuclear cells [141]. Breast milk has also been shown to have anti-tumor effects

through the protein-lipid complex HAMLET (human alpha-lactalbumin made lethal to tumor cells), which selectively enters tumor cells, accumulates in their nucleus and induces apoptosis-like cell death [142].

Human colostrum is the first milk produced after birth and the secretion gradually changes to mature milk. As compared with the composition of mature milk, colostrum has a higher protein content, lower fat content, and is rich in immunoglobulins and other important immune factors and mediators [143-145].

1.6 ALLERGY

Allergic diseases are chronic inflammatory disorders where the immune system is reacting to innocuous antigens, allergens, in the environment. IgE-mediated allergic diseases affect more than 25% of the children in industrialized countries [146]. Examples of clinical manifestations of allergies are allergic rhinitis, allergic asthma, food allergy and atopic eczema, which act locally within the target organ, and anaphylaxis which is systemic. The reasons why some develop allergies and others do not are not fully known, but it seems that numerous different factors, both genetic and environmental, are of importance. Several susceptibility genes have been reported [147, 148], and different life styles may also be of influence [149, 150]. In addition, “the hygiene hypothesis” proposes that exposure to pathogens early in life may protect from allergic diseases [151].

1.6.1 The allergic immune response

Sensitization to an allergen involves presentation of allergen and priming of allergen-specific T cells by APCs inducing a Th2 type of response with the production of cytokines like IL-4 and IL-13, which are able to class switch the antibody production of B cells to IgE. The IgE can then bind to the IgE high-affinity receptor (FcεRI) present on the surface of mast cells and basophils. When IgE/FcεRI complexes are cross linked by allergen the cells degranulate leading to the release of mediators that drives the immediate response to the allergen. Examples of mediators are histamine, which causes increase in local blood flow and vessel permeability, enzymes like tryptase and mast-cell chymase that activated metalloproteinases which causes tissue destruction, and cytokines like IL-4 and IL-13 which further promote a Th2 response. IgE can also bind to FcεRI on DCs and monocytes, as well as to the low-affinity receptor for IgE, FcεRII, at the surface of B cells. This facilitates the uptake of allergen by these APCs and in this way increases the presentation of allergen-derived peptides to T cells which drives the late phase of the allergic reaction. This late phase reaction is characterized by recruitment of effector cells like Th2 cells, eosinophils and basophils to the site of inflammation (Fig. 4).

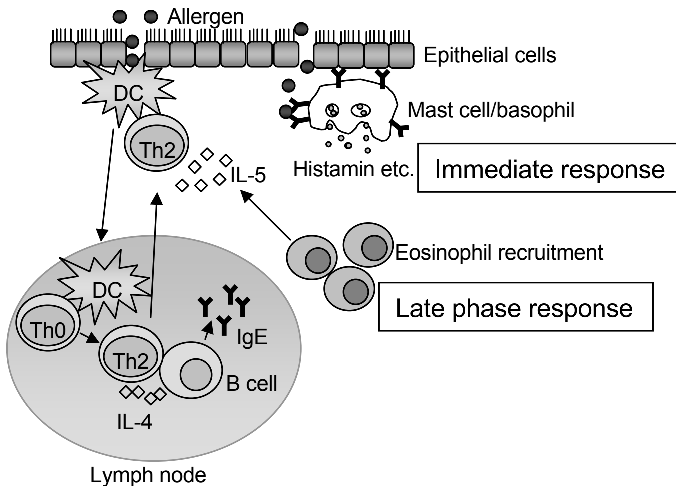

Fig 4. Illustration of an allergic immune response. (Modified from [152])

1.6.2 Bronchoalveolar lavage (BAL)

A great deal of information about the pathophysiology of asthma and other airway diseases and its treatment have been obtained through the use of bronchoalveolar lavage (BAL). BAL is a technique for sampling the epithelial lining fluid of the respiratory tract. Saline is instilled into the airways and cells and soluble components like cytokines can be analysed in the returned fluid, which will give valuable information about the inflammatory status.

1.6.3 Breast feeding and allergy

There are conflicting results regarding the effect of breast feeding on allergy development, with some studies showing that breast feeding increase the risk of sensitization [153, 154], while several studies show a protective effect of breast feeding against allergy development [155-157]. There are also studies showing that the effect of breast feeding depends on the atopic heredity [158]. Most evidence is though pointing towards a protective effect of breast feeding on allergic disease [159] and that breast feeding should be recommended also for its overall beneficial effects. Some difference in the composition of breast milk from allergic and non-allergic mothers have been reported such as the concentrations of the cytokine IL-4 and the chemokines IL-8 and RANTES which were higher in milk from allergic mothers compared to non-allergic mothers [126, 160], and the immunosuppressive cytokine TGF-β_2 which was lower in breast milk from mothers with allergic disease [161]. If these difference has any impact on the child's immune system remains to be elucidated.

2 AIMS OF THE THESIS

The overall aims of this thesis were to assess the presence of exosomes *in vivo*, develop methods to elucidate their function as immune regulators and to investigate if they may have a role in inflammatory diseases such as allergies. The more specific aims were to investigate:

I: Whether exosomes are present in bronchoalveolar lavage fluid (BALF) and if so to establish if these exosomes bear MHC and co-stimulatory molecules.

II: If DC-derived exosomes can stimulate antigen specific peripheral $CD8^+$ T cells without the presence of DCs using the enzymed-linked immunospot (ELISPOT) assay.

III: Whether human breast milk contains exosomes and if so to assess if exosomes in milk can have immune modulatory features.

IV: The role of antigen presenting cell-derived exosomes in allergen presentation and stimulation of allergen specific T cells.

3 METHODOLOGY

Methods used for paper I-IV are described in detail in the respective "Materials and methods" sections. The following methods were used in this thesis:

- Bronchoscopy with BAL (paper I)
- Cytometric bead array for cytokine analysis (paper III and IV)
- Cytospins with May-Grünwald Giemsa staining (paper I)
- Electron microscopy (paper I-III)
- ELISPOT (paper II and III)
- Enzyme-linked immunosorbent assay (ELISA) (paper IV)
- Flow cytometry analysis (paper I-IV)
- Generation of MDDCs (Paper I and II)
- ImmunoCAP (paper IV)
- *In vitro* stimulation of T cells (paper II-IV)
- Mass spectrometry analysis (paper III)
- Peptide synthesis and biotin-labeling (paper IV)
- Preparation of exosomes with ultracentrifugations and anit-MHC class II beads (paper I-IV)
- Proliferation analysis using [^{3}H]-thymidine incorporation (paper IV)
- Separation of PBMC (Paper I-IV)
- Skin prick test (paper IV)
- Statistical analysis (paper III and IV)
- Sucrose gradient fractionation (paper III)
- Western blot analysis (paper III)

4 RESULTS AND DISCUSSION

4.1 EXOSOMES ARE PRESENT IN HUMAN BRONCHOALVEOLAR LAVAGE FLUID (PAPER I)

When we performed this study a lot of investigations had been published on exosomes derived from *in vitro* culture supernatants but very few studies were done showing the presence of exosomes *in vivo*. Denzer *et al* gave the first indications of exosomes *in vivo*, when they by EM analysis showed that follicular DCs isolated from tonsils have MHC class II positive exosomes attached to their surface [82]. In addition, Andre *et al* later demonstrated that tumor-derived exosomes could be isolated from malignant effusions [47]. Since the lung contain many APC and is a site for antigen entry we hypothesized that BALF might contain exosomes. Therefore BALF from healthy volunteers was subjected to exosome isolation methods using differential centrifugations as previously described [34]. As a reference we in parallel isolated exosomes from MDDC culture supernatants. Due to the small size of exosomes, only 30-100 nm, we needed to adhere them to larger particles for further analysis using flow cytometry and EM. For this purpose we used 4.5 μm magnetic beads coated with anti-MHC class II antibodies, which allowed attachment of the exosomes to the surface of the beads [60] (Fig. 5). We were interested in APC derived exosomes why we used beads coated with anti-MHC class II.

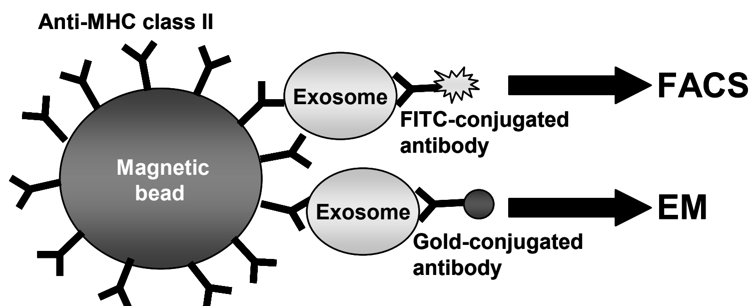

Fig 5. Isolation of exosomes on anti-MHC class II beads.
FACS: flow cytometer, EM: electron microscopy

EM analysis of the pelleted material from the BALF revealed the presence of vesicles attached to the magnetic beads, which had similar shape and size, 30-100 nm, as the DC-derived exosomes. In addition, using immune EM we could illustrate that these vesicles were positive for HLA-DR and the tetraspanin protein CD63 (Fig. 6). Further analysis using flow cytometry confirmed the presence of HLA-DR and CD63 on the vesicles and additionally revealed the expression of MHC class I, CD54 and CD86 molecules, which was also found on the DC-derived exosomes. The morphology and protein expression were in accordance with previous studies on exosomes [34, 60, 61], indicating that these vesicles were true exosomes.

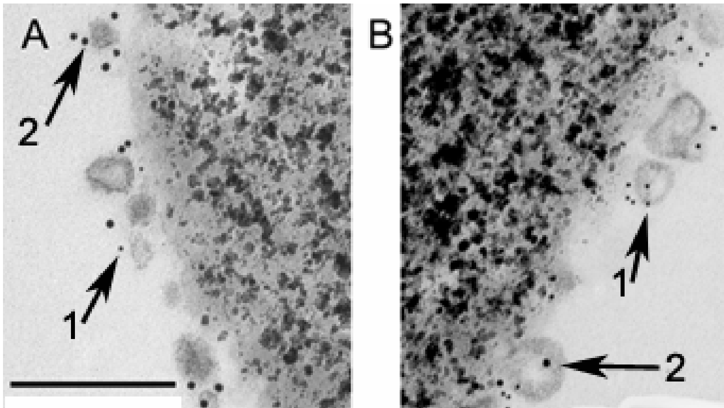

Fig 6. EM picture of exosomes in BALF. Exosomes isolated from BALF (A) or MDDC culture supernatant (B) were coated on anti-MHC class II beads, stained with anti-HLA-DR (arrow 1) and anti-CD63 (arrow 2) monoclonal antibodies and analyzed with immune EM. Scale bar = 200 nm.

Both flow cytometry analysis and immune EM analysis showed that exosomes from BALF have higher expression of CD63 but lower expression of HLA-DR compared to DC-derived exosomes suggesting that they may be derived from other cell types. Most probably the exosomes found in BALF are derived from a mix of different cell types. Since macrophages were the most abundant cell found in the BAL one would expect that most of the exosomes were of macrophage origin. However, we here counted the BAL cells by May-Grünwald Giemsa and with this method it is not possible to distinguish between macrophages and dendritic cells, which are counted as one cell type [162]. Furthermore, the dendritic nature of DCs, with long protrusions around other cells, makes them less disposed to detach and be part of BALF. In this study we analysed exosomes bound to anti-MHC class II coated beads and therefore did not examine the possible existence of MHC class II negative exosomes in BALF. However, MHC class II has also been detected on exosomes from both epithelial cells [40], mast cells [85], B cells [60, 61] and T cells [35] and these exosomes should be able to bind to the beads. The exosomes in BALF were negative for CD3 suggesting them not to be of T cell origin, since T cell-derived exosomes previously have been shown to be positive for this molecule [35].

To get an idea about the amount of exosomes in the preparations we measured the protein content. When coating exosomes to the anti-MHC class II beads we needed about 10 times more protein to saturate the beads when using BALF-derived exosomes compared to DC-derived exosomes. This may be due to that other proteins in the BALF were co-purified with the exosomes during the preparation, for example surfactant proteins. Another possibility is that MHC class II negative exosomes are also present in the preparation and these will not bind to the beads. In general, we saw higher molecular expression in the flow cytometer with DC-derived exosomes compared to BALF-derived exosomes. This is possibly due to that we had very restricted amounts of

BALF-exosomes and were not able to saturate the beads, giving rise to lower signals, or this could reflect a true difference.

The role of exosomes *in vivo* in the lung remains to be investigated. Due to the restricted amount of BALF-exosomes we were not able to perform any functional experiments in this study. One can however speculate that exosomes expressing antigen presenting molecules such as MHC class I and II and co-stimulatory molecules in the lung might have a role in T cell stimulation after airway antigen exposure. Exosomes have previously been shown to be able to stimulate T cells both directly [34, 75] or by transporting antigen to DCs [73, 77]. This could be a way of transporting antigen to other parts of the body. Exosomal expression of MHC, CD54 and CD86 were found to essential for exosome induced T cell activation [75, 76], molecules which were all found on the exosomes in BALF. Exosomes in the lung could possibly also be involved in promoting tolerance. Airway pDCs have been suggested to be more tolerance inducing compared to mDCs [163] and it could be that their exosomes are also more tolerogenic, however, this has never been investigated. It would be interesting to study the role of exosomes during an airway inflammation like sarkoidosis or asthma. Our preliminary data show presence of exosomes in BALF from both sarkoidosis and asthmatic allergic patients, but their exact function need to be further explored.

In conclusion, our data show for the first time that exosomes are present in the lung and that these exosomes express MHC and co-stimulatory molecules, suggesting them to have a role in the immune defense of the lung.

4.2 DC-DERIVED EXOSOMES CAN ACTIVATE PERIPHERAL HUMAN CD8$^+$ T CELLS (PAPER II)

Exosomes have been suggested to have a role in T cell activation. Raposo *et al* first reported that B cell-derived exosomes can stimulate antigen specific T cells [34]. Several studies have later confirmed this, but there are contradictory data regarding the mechanism behind this activation. Some studies show that exosomes can stimulate T cells by their own [34, 75] while others have reported that exosomes exert their effect by transferring antigen to DCs [73, 77]. Previous studies have used T cell lines [73, 80], T cell hybridomas [74, 80] or T cell clones [34, 75, 77, 79] when investigating the effect of exosomes on T cells. These cells have been cultured *in vitro* and might have differed from their original state. Therefore, we investigated the capacity of DC-derived exosomes to stimulate peripheral T cells *ex vivo*. To do this we needed a sensitive method, why we took advantage of the ELISPOT assay, by which you can visualize cytokine production at the single cell level. Exosomes prepared from MDDC culture supernatants and loaded with a viral peptide mix containing 23 viral MHC class I specific peptides from the three viruses Epstein-Barr virus, cytomegalovirus and influenza virus could stimulate autologous CD8$^+$ T cells to produce IFN-γ and TNF-α in a dose-dependent manner without addition of DCs. The purity of the cell separations was always high, around 99% T cells. In addition, no correlation between the purity of the T cells and the degree of activation could be seen, indicating that possible contaminating APC were not responsible for the stimulation seen. Exosomes not loaded

with the peptide mix could not activate the T cells showing that the stimulation was antigen-specific. The stimulation was further demonstrated to be dependent on exosomal MHC class I, since blocking of MHC class I on the exosomes with antibodies reduced the cytokine release from the T cells. This is in agreement with previous data where both MHC class I, CD86 and CD54 were needed to be expressed by the exosomes for them to be immunogenic [75]. Previous studies on mouse DC-derived exosomes have illustrated that increased T cell activation is induced when using exosomes from mature compared to immature DC [74, 76]. In line with these studies, we wanted to investigate if we in our system could confirm these data using human cells. Furthermore, we wanted to explore how sensitive our assay is. Therefore, exosomes from immature and LPS-matured MDDCs loaded with the viral peptide mix were compared for their capacity to induce cytokine production of autologous peripheral $CD8^+$ T cells. Exosomes from mature MDDCs gave a 4-13 fold increase in the number of IFN-γ producing T cells compared with immature exosomes, showing for the first time in humans that exosomes from mature MDDC are more efficient than exosomes from immature MDDC in stimulating T cells. When comparing the phenotype of exosomes from immature and mature MDDCs, we found that exosomes from mature MDDCs hade higher levels of MHC class I and II, the co-stimulatory molecules CD40, CD80 and CD86 and the adhesion molecule CD54, while no difference were seen for the tetraspanin proteins CD63 and CD81. The presence of higher levels of MHC and co-stimulatory molecules is probably one explanation to the increased stimulation, since a higher density of these molecules would give a more efficient activation. Higher levels of antigen presenting and co-stimulatory molecules and higher T cell stimulatory capacity are well documented for mature DC compared to immature DC, showing that the exosomes are mirroring the “mother” cells.

The contradictory results between different studies concerning the capacity of exosomes to stimulate T cells may be due to differences in the phenotype of the exosomes used, differences in the phenotype of the responder cells, what type of antigen that was used or what detection method that was applied. We here could detect activation of peripheral $CD8^+$ T cells which contain a mix of cells with different specificities. This was likely due to that we used the sensitive ELISPOT method instead of ELISA or proliferation assays like [H3]-thymidine incorporation. This shows that the ELISPOT method is very suitable to use for studying the effect of exosomes on T cell responses, and that this assay may constitute a useful tool when evaluating the stimulatory or immuno-modulatory capacity of exosomes for therapeutic purposes.

The mechanism how exosomes interact with T cells and which molecules that are of importance needs further investigation. We have performed pilot confocal microscopy studies using PKH67-labelled DC-derived exosomes showing binding of the exosomes to the surface of the $CD8^+$ T cells (Fig. 7), giving further proof of direct interaction between exosomes and T cells. Exosomes have previously been reported to be able to adhere to follicular DCs [82] and other DCs [81] indicating them to be part of intercellular communication.

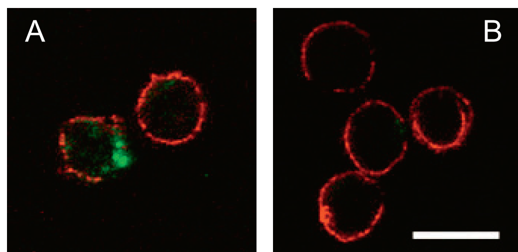

Fig 7. Exosomes bind to CD8$^+$ T-cells. CD8$^+$ T cells stained with anti-CD3 Alexa Fluor 546 (red) were incubated with LPS-matured MDDC-derived exosomes labeled with the green fluorescent dye PKH67 (A) or with only free PKH67 (B). Binding of exosomes to T-cells were studied with confocal laser-scanning microscopy (TCS SP2; Leica Microsystems). The inserted scale bar represents 10 µm.

Taken together, our results demonstrate that DC-derived exosomes are able to activate peripheral human CD8$^+$ T cells in a dose-dependent and antigen-specific manner. The stimulation was shown to be dependent on exosomal MHC class I and could be further increased by using exosomes from mature DCs. This suggests that DC-derived exosomes may have a role in T cell activation during an immune response. In addition, we here show that the ELISPOT assay is a sensitive and suitable method to use for evaluating exosome induced immune responses.

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

Breast milk is a complex liquid containing a diverse set of components including antibodies, cytokines, immune competent cells and MFG [123], which provides protection against neonatal infections and are important for the development of the neonatal immune system. Since we previously have shown that exosomes could be found in BALF and that later studies had demonstrated that exosomes are present in other body fluids like plasma [44] and urine [45], and since several studies have established that exosomes can have a role in immune responses, we wanted to investigate if exosomes were also present in human breast milk and if so if these exosomes have immuno-modulatory features. We therefore subjected human colostrum, which was collected within 4 days after delivery, and mature milk, which was collected between 1 and 6 months after delivery, to exosome preparation procedures by differential ultracentrifugations [34]. The pelleted material was further analysed morphologically with immune EM, for density using sucrose gradient fractionation and phenotypically with flow cytometry, Western blot and mass spectrometry (MS). Immune EM revealed vesicles with shape and size, around 50 nm in diameter, according to previous reports on exosomes [69]. In addition, these vesicles had CD63 and HLA-DR on their surface. The presence of CD63 and HLA-DR was further confirmed by flow cytometry by which we also could detect low levels of MHC class I and the co-stimulatory molecule CD86, and high levels of the tetraspanin

proteins CD63 and CD81 as well as MUC-1. MUC-1 is a glycoprotein expressed on the surface of glandular and ductal epithelia of various organs such as mammary glands, lung, pancreas and the gastrointestinal tract [164], and has also been found on MFG in breast milk [165]. MUC-1 has been suggested to have a role in the protection of epithelium by binding pathogens but also in cell adhesion. MUC-1 has for example been shown to be able to bind to the adhesion molecule CD54 [166]. It has further been demonstrated that MUC-1 is over expressed in several cancers and suggested to play a role in the metastasis process [164]. To verify that our vesicles were not MFG we isolated MFG from breast milk as previously described [167] and analysed them with flow cytometry. As expected the MFG were positive for HLA-DR and MUC-1 but negative for all the other markers found on our vesicles, indicating that our vesicles were not MFG. Further phenotypic analysis of our isolated vesicles by Western blot illustrated that they according to the characteristics of exosomes in addition were positive for the heat shock protein Hsc70 and negative for the ER specific protein calnexin, indicating that the exosomes were not of ER origin. As a last verification that our vesicles were true exosomes, sucrose gradient fractionation revealed the vesicles to have a density between 1.10 and 1.18 g/ml, which corresponds to the density previously shown for exosomes [34]. MS analysis of the exosomes confirmed the finding of several previously reported exosome-associated molecules such as MHC class II, CD81 and heat shock proteins, and further confirmed our flow cytometry data of the finding of MUC-1 in association with the exosomes. Furthermore, we detected Lactadherin, also called MFG-E8, in the exosome preparation. This molecule have previously been suggested to be involved in clearance of apoptotic cells [168] and has previously been described on DC-derived exosomes [169]. MFG-E8 has in addition been found to be released in association with vesicle-like structures from cultured mammary epithelial cell lines *in vitro* [170]. Moreover, several proteins involved in vesicle budding and endocytic membrane fusion were detected in the MS analysis, supporting that the vesicles found are of endocytic origin.

The exosomes in milk were negative for the molecules CD40, CD54 and CD80, which are present on exosomes from DCs and B cells [59-61], suggesting that the exosomes might come from other cells. The presence of CD86 however proposes an APC origin, while MUC-1 implies that they might be derived from breast epithelium. Most likely, the exosomes in breast milk are derived from a mix of different cellular sources and possibly exosomes from other parts of the body could home to the breast milk via the blood, since plasma previously have been shown to contain exosomes [44]. We could here demonstrate that the expression of HLA-DR were higher on exosomes in colostrum compared to mature milk. This might be due to that the exosomes are derived from different cellular sources during the first days of lactation or from cells in another activation status, and suggests that the exosome composition of milk might change over time.

The finding of exosomes in breast milk made us ask questions regarding if these exosomes could have any immuno modulatory features. To test this we incubated mature breast milk-derived exosomes with PBMC, after which the PBMC were stimulated with anti-CD3. In this way we would detect both if the milk exosomes would potentiate or suppress T cell activation. Since exosomes from the mother would

be semi-autologous to the child we set up this assay using both autologous and allogeneic PBMC. Unfortunately we had not the possibility to collect any cord blood cells in this study. There was a significant reduction in IL-2 and IFN-γ production from both anti-CD3 stimulated autologous and allogeneic PBMC when incubated with 500 μg/ml of milk exosomes. The suppression was not due to increased cell death, since Annexin V/propidium iodide staining showed similar levels of viable cells in PBMC incubated with or without milk derived-exosomes. The suppressive effect could be partially blocked when reducing the levels of exosomes in the preparation by using anti-MHC class II and anti-CD81 beads, showing that exosomes are contributing to the suppression. The partial blocking effect seen might be due to that not all exosomes were removed by the beads or that there are other factors co-purified with the milk exosomes that also have suppressive effects. Examples of these other factors could be immunosuppressive cytokines like IL-10 and TGF-β. TGF-β has previously been shown to be present in colostrum and have immunosuppressive effects [141]. However, neither IL-10 nor TGF-β was detected in the MS analysis of the exosome preparations. Another possibility would be if the exosome preparations also contained soluble MUC-1. Soluble MUC-1 secreted by epithelial cancer cells has previously been shown to be able to inhibit T-cell proliferation [171]. It has also been demonstrated that a high concentration of colostral milk protein can have an inhibitory effect on mitogen induced T cell proliferation, while a low concentration of colostral milk protein can enhance T cell proliferation. The inhibitory effect was not seen for late milk proteins, and the inhibitory activity was suggested to be associated with glycoprotein [140]. The functional test in our study was due to lack of material only performed with mature milk-derived exosomes and it is possible that exosomes from colostrum might have different effects. Taylor *et al* have previously demonstrated an inhibitory effect of pregnancy associated exosomes on PHA induced IL-2 production in Jurkat T cells in the same concentration range as we used here [102]. In addition, exosomes released from IECs, tolerosomes, have been shown to be able to induce tolerance [87]. These tolerosomes express the IEC specific marker A33 [172]. Our preliminary data show that the milk derived-exosomes are negative for A33, indicating that exosomes have not been homing to the milk from the intestine epithelium.

Tregs are present in the normal immune system for maintaining immunological self-tolerance and immune homeostasis by immunosuppressive mechanisms. We therefore were interested to investigate if the immunosuppressive effect seen for milk-derived exosomes were in some way associated with Tregs. Interestingly, the number of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells was significantly increased in PBMC incubated with milk-derived exosomes compared to PBMC not incubated with milk-derived exosomes. How exosomes could influence Tregs remains to be investigated but it suggests that Tregs might have a role in the suppression seen, however further studies on this are needed.

If exosomes in breast milk could have a true effect on the neonate's immune system remains to be elucidated. The quantification of exosomes was here based on protein measurements and 500 μg of protein/ml of culture medium might seem like a lot of protein. However, the amount of exosomes used per stimulation corresponds to exosome preparations from only approximately 2 ml of milk, indicating that the

neonate would easily ingest the amount of exosomes used here during breast feeding. In addition, exosomes are very stable structures and our preliminary data show that they are stable to at least pH 4, suggesting that they would tolerate the gastric environment of the neonate which has a pH of above 5 [173]. It is however, not known if the exosomes would tolerate the digestive enzymes of the gastrointestinal tract. The fact that human milk leukocytes can survive passage through the gastrointestinal tract, cross the gut epithelium and enter the circulation [130], suggests that this might also be true for exosomes. One could speculate that the suppressive effect of exosomes might have a role in tolerance development to food and environmental antigens in the neonate, and be a protective factor for the development of allergies.

To summarize, we here present the novel finding that exosomes are present in both colostrum and mature human breast milk. We further show that these exosomes express MHC class I and II, CD86, tetraspanin proteins, heat shock proteins and MUC-1, and that these exosomes can suppress anti-CD3 stimulated cytokine production from PBMC. This inhibition might be associated with the increased level of Tregs seen in PBMC after incubation with milk-derived exosomes. This suggests that exosomes in breast milk might be able to influence the immune system of the neonate although this needs further investigation.

4.4 B CELL-DERIVED EXOSOMES CAN PRESENT ALLERGEN AND STIMULATE ALLERGEN SPECIFIC T CELLS (PAPER IV)

Both we (paper II) and others [34, 74, 75] have previously demonstrated that APC-derived exosomes can have a role in T cell activation. In allergic diseases activation of allergen-specific T cells releasing Th2 like cytokines such as IL-4, IL-5 and IL-13 play a central role. We therefore further wanted to investigate if APC-derived exosomes could have a role in activation of allergen specific T cells. Exosomes were prepared from EBV-transformed B cells from birch pollen allergic individuals. Phenotypic analyses of the exosomes showed expression of MHC class I and II, the co-stimulatory molecules CD40, CD80 and CD86, and the adhesion molecule CD54, which are in accordance with previous studies on exosomes from healthy individuals [60, 61]. We could here in addition demonstrate the presence of the B cell-specific marker CD19 on these exosomes. The B cell-derived exosomes were further loaded with peptides derived from the major birch pollen allergen Bet v 1, Bet v 1 4-18 corresponding to the amino acid residues 4-18 in the N-terminal region and Bet v 1 142-156 corresponding to amino acid residues 142-156 in the C-terminal part of Bet v 1. Both peptides have previously been shown to contain relevant T cell epitopes and Bet v 1 142-156 was demonstrated to represent the immunodominant T-cell epitope of Bet v 1 [174]. By using biotinylated Bet v 1-derived peptides we could detect the peptides in association with the exosomes using ELISA, showing that the peptides were present on the surface of the exosomes probably in association with MHC class II. The Bet v 1-loaded exosomes could further activate Bet v 1 specific T cell clones to proliferate and to produce the Th2 like cytokines IL-5 and IL-13 in a dose-dependent manner. No or very low levels of IL-4, IFN-γ and TNF-α were detected. Stimulation with exosomes not loaded with Bet v 1-derived peptides did induce neither proliferation nor cytokine

production from the T cells, showing that the activation was antigen-specific. As a positive control the Bet v 1-specific T cell clones were stimulated with Bet v 1-derived peptide loaded B cells, which gave rise to similar responses with high levels of the Th2 cytokines IL-4, IL-5 and IL-13 and lower levels of the Th1 cytokines IFN-γ and TNF-α, showing, as expected, that the T cells were mostly of the Th2 type. As pointed out previously it is still under debate how exosomes stimulate T cells, whether it is done by direct interaction of exosomes with the T cells or if the exosomes mediate its effect via APCs. In this study we could detect T cell activation of exosomes without adding APCs, which are in line with that the exosomes expressed high levels of MHC and co-stimulatory molecules.

The role of exosomes in an allergic response *in vivo* needs further investigations. Our preliminary data show that exosomes are present in the lung of asthmatic patients, indicating that exosomes are present in the target organ during allergic inflammation. Publications from other groups show that exosomes are present also in other compartments of the body [45, 46] and can travel via the blood [44] suggesting that exosomes could also have effects in other parts of the body. Hypothetically, allergen-loaded exosomes could be released from allergen-specific B cells upon allergen encounter, which could contribute to the activation of allergen-specific T cells. A previous study has demonstrated that B cell receptor triggering can lead to increased release of exosomes from the B cell [175]. One could also speculate that exosomes from DCs could have a role in stimulating allergen specific T cells since both our previous studies (Paper II) and studies from other groups have shown that DC-derived exosomes can activate T cells [74-77]. This could possibly be achieved by direct interaction of the exosomes with the T cells or by transferring allergen between DCs. It has been demonstrated that B cell-derived exosomes express functional integrins by which the exosomes can bind to extracellular matrix components and to cell surface adhesion molecules [84] suggesting that exosomes might be able to deliver signals beyond that of direct cell to cell contact. The presence of CD55 and CD59 on the exosomes further suggests them to be stable *in vivo* [68]. Other cells that are of importance in an allergic immune response have also been shown to release exosomes like mast cells [36, 85]. Mast cell-derived exosomes have been shown to be immunostimulatory in that they can induce proliferation and cytokine production of cultured splenocytes [85]. Furthermore, they have been demonstrated to be able to induce maturation of DCs [86]. Degranulation of mast cells after allergen IgE cross-linking could possibly lead to the release of exosomes with the capacity to promote an allergic immune response by for example delivering maturation signals to DCs (Fig. 8).

If exosomes are of importance in an allergic response, they may constitute a new therapeutic target. Alternatively, exosomes could be used to manipulate the immune response to induce more of a Treg response instead of the disease promoting Th2 response. Previous studies have demonstrated that exosomes from DCs which were stimulated with IL-10 alternatively genetically modified to express FasL were able to suppress delayed type hypersensitivity and collagen-induced arthritis in a mouse model [120, 121]. One advantage of using exosomes in immunotherapy could be that exosomes are unable to change their phenotype after they have been released, making them stable and insensitive to *in vivo* stimuli, compared to cells.

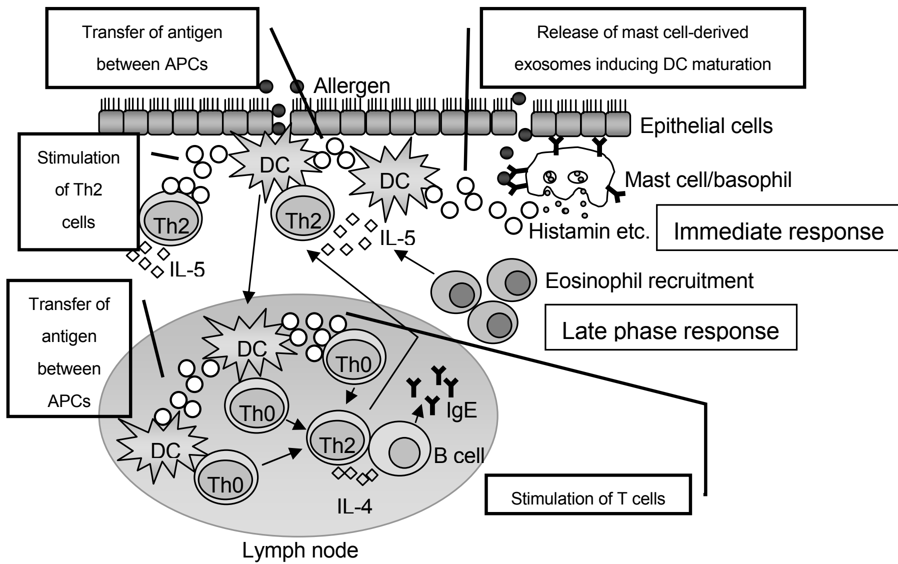

Fig 8. Hypothetical scheme over possible interaction of exosomes with cells during an allergic response.

Taken together, we here demonstrate for the first time that B cell-derived exosomes are able to present allergen-derived peptides and activate allergen-specific T cells to proliferate and produce Th2 like cytokines. Further *in vivo* experiments will reveal if allergen presenting exosomes released from APCs could have a role in T cell activation during an allergic immune response, and if exosomes could constitute a new therapeutic target, alternatively be used as a tool in allergy treatment.

5 CONCLUSIONS

I: In this study we present the novel finding that exosomes are present in BALF from healthy individuals, and that they are similar to DC-derived exosomes in that they express MHC class I and II, CD54, CD63 as well as CD86. Since they contain co-stimulatory molecules it is likely that they are derived from APCs. These results demonstrate that exosomes are present in the lung and suggest that they might have a role in antigen delivery or immune regulation during airway antigen exposure.

II: In this study we show, for the first time, that DC-derived exosomes can directly stimulate human *ex vivo* peripheral $CD8^+$ T-cells to produce IFN-γ and TNF-α in an antigen-specific manner. The stimulatory capacity of the exosomes was enhanced when derived from LPS-matured DCs, compared to immature DCs, and was shown to be dependent on exosomal MHC class I. Our data suggest a mechanism for exosome interaction with T-cells independently of DCs. The results further demonstrate that ELISPOT is a suitable method for detecting exosome-induced peripheral T-cell responses. This method may provide a useful tool for monitoring exosomal immune responses when developing exosomes as therapeutic agents, and when investigating the immune response from differently generated or transformed exosomes.

III: Here we found nano vesicles in human breast milk from healthy mothers and they had all characteristics of exosomes including shape and a size of around 50 nm, a density between 1.10 and 1.18 g/ml, and the expression of exosome-associated molecules like MHC, Hsps, and tetraspanin proteins. Functional analysis revealed that the exosome preparation inhibited anti-CD3 induced IL-2 and IFN-γ production from both allogeneic and autologous PBMC. This effect could be reduced by removing vesicles with anti-MHC class II and anti-CD81 beads. In PBMC incubated with milk exosome preparations an increased number of $Foxp3^+CD4^+CD25^+$ T regulatory cells were observed, which might have a role in the inhibition seen. These results illustrate that human breast milk contains exosomes and that these exosomes have the capacity to affect immune responses.

IV: In this study we demonstrate that B cell-derived exosomes can present Bet v 1-derived peptides and activate Bet v 1 specific T cells in a dose-dependent manner. The peptide-loaded exosomes stimulated the T cells to both proliferate and to produce Th2 like cytokines such as IL-5 and IL-13. These results illustrates for the first time that exosomes can present allergen and activate allergen specific T cells, suggesting that exosomes could be an immuno-stimulatory factor in an allergic immune response.

In summary, this thesis supports that exosomes play several roles in immune regulation and defense. They are likely to have a role in both T cell activation and suppression depending on their cellular source, and could possibly be part of allergic responses.

6 FUTURE PERSPECTIVES

My studies have provided answer to some of the questions raised but have also led to the generation of new questions. I will here briefly outline what I think would be interesting to explore in the future.

Our finding that exosomes are present in BALF raises the questions from where these exosomes are derived and what their functions are. It would be interesting to perform a more thorough phenotypic analysis of the exosomes and the cells in BALF, and maybe compare the phenotype of exosomes in BALF with exosomes derived from different types of cells in culture, to see if this could give more knowledge about their origin. As pointed out previously some exosomal molecules reflect the cell type from which they are secreted at least *in vitro*, such as the presence of co-stimulatory molecules on APC-derived exosomes [59-61], expression of CD3 on T cell-derived exosomes [35], expression of A33 on IEC-derived exosomes[40] and the presence of the transferrin receptor on exosomes from reticulocytes [62]. However, more studies on the phenotypic differences of exosomes from different cell types are needed to be able to distinguish their source *in vivo*. In our study we isolated exosomes on anti-MHC class II beads for phenotypic analysis and one could argue that this might select for only MHC class II highly expressing exosomes. It would therefore be interesting to isolate exosomes by selection for other molecules for example a tetraspanin protein like CD63 or CD81, to see if other exosomes are also present in the BALF. In addition, we are now testing the possibility to analyze exosomes directly in the flow cytometer. This would give the advantage of analyzing exosomes without prior selection methods and would give the possibility to investigate different populations of exosomes derived from different cell types simultaneously. In this way one might also be able to sort out different types of exosomes in regards to their phenotype and further analyze their different functions. Our preliminary data show that exosomes are present in the BALF from asthmatic patients. It would be interesting to further investigate if the phenotype and amount of exosomes released during airway inflammation differs from a non-inflammatory state by comparing exosomes in BALF between healthy and allergic individuals. Furthermore, since we have demonstrated that APC-derived exosomes can stimulate allergen specific T cells it would be interesting to analyze the effect of BALF-derived exosomes in allergen stimulation of T cells both *in vitro* and *in vivo*. We are currently looking into the effect of BALF-derived exosomes on airway inflammation by transferring BALF-derived exosomes from allergic or healthy mice to mice during airway allergy development. This will hopefully give more insight into the role of exosomes in an allergic response and show if exosomes produced during and out of an allergic reaction have different functions. It would further be interesting to explore if modified exosomes could be used as tools in treatment of allergic diseases. Previous studies have demonstrated that exosomes derived from DC stimulated with IL-10 or that were genetically modified to express FasL could inhibit delayed type hypersensitivity and collagen-induced arthritis [120, 121]. It would be exciting to study the effect of these exosomes in a mouse model of allergic asthma to see if also allergic inflammation and airway hyperreactivity can be suppressed.

We have demonstrated that exosomes can activate both $CD4^+$ and $CD8^+$ T cells, and we have preliminary data that visualizes the direct interaction of exosomes with T cells. The preliminary data further show that the interaction of exosomes with T cells occurs to a higher extent if the exosomes are loaded with antigen compare to unloaded exosomes. Previous studies have illustrated that CD86 and CD54 are important factors for exosome induced T cell activation [75, 76]. It would be interesting to further investigate this interaction to see what additional molecules that are involved and to analyze the kinetics of the interaction by following exosomes co-incubated with T cells over time *in vitro* or *in vivo* by injecting fluorochrome labeled antigen-loaded exosomes into sensitized mice. The distribution of radioactively labeled DC-derived exosomes injected into mice have previously been investigate and the exosomes were found mainly in the spleen, the intestine and the cervical lymph nodes [118]. Additional studies on with which cells the exosomes interact when injected *in vivo* and if this differs depending on the phenotype of the exosomes would be interesting to perform.

Our findings show that the effect of exosomes depends on their origin, since we illustrate that DC-derived exosomes can activate T cells while exosomes in breast milk seem to suppress T cell stimulation. It would be interesting to further investigate how this suppression is mediated and what molecules that are involved. Blocking experiments using antibodies against different candidate molecules may give some clues. MUC-1 would be one interesting molecules to investigate since it has previously been demonstrated to inhibit T cell proliferation [171]. Further investigation of if and how the increased number of Tregs seen is responsible for the inhibition are needed. One way to prove their effect could be to deplete the Tregs from the PBMC incubated with milk to see if the suppressive effect is lost. In addition, it would be very exciting to investigate how the exosomes are inducing the Tregs. In this study we have used PBMC and it would be interesting to see if the suppressive effect is the same if using cord blood mononuclear cells, which would more resemble the interaction of exosomes with cells of the neonate. Moreover, one would like to investigate if milk-derived exosomes could have an effect on tolerance development in the neonate. Giving milk-derived exosomes to neonate mice and follow their tolerance development to a certain antigen may give useful information. In addition, it would be intriguing to see if milk-exosomes could have any impact on allergy development by giving allergen-loaded milk-exosomes to neonate mice before allergen sensitization. A recent study using a mouse model showed that breast milk from OVA sensitized mice contains factors that are sufficient to increase susceptibility of the offspring to development of allergic airway disease [176]. It would therefore be interesting to compare the phenotype and suppressive effect of milk-derived exosomes from healthy and allergic mothers *in vitro* and from healthy and allergic mice *in vivo*.

7 ACKNOWLEDGEMENTS

I would like to thank all present and former colleagues who have helped me to accomplish this thesis and all my friends and family for always being there for me during these years. In particular I would like to acknowledge:

Susanne Gabrielsson, my fantastic supervisor, who has been both an excellent supervisor and a friend during these years. Thank you for your endless support and enthusiasm, for being a true inspiration and for always believing in me. Thanks for all nice discussions and laughter both at work and outside work, and for making my PhD education such a great time!

My co-supervisor, **Annika Scheynius**, for always being supportive and encouraging, for always believing in me and for excellent guidance into the scientific world.

My co-supervisor, **Rudolf Valenta**, for sharing your outstanding scientific knowledge and for a nice collaboration.

Everyone at the Centre for Allergy Research: the former and present directors, **Bengt Björkstén**, **Lars Gustafsson**, **Sven-Erik Dahlén** and **Gunilla Hedlin** for making it possible for me to do my PhD-studies within the Cfa network, the former and present study administrator, **Gunilla Jacobsson Ekman** and **Anne Renström** and coordinator **Marianne Heibert-Arnlind** for excellent support to the PhD-education, and all past and present **Cfa PhD-students** for fun times and nice discussions about allergy research.

My co-authors: **Sara Johansson**, **Khaleda Rahman Qazi**, **Staffan Paulie**, **Johan Grunewald**, **Anders Eklund**, **Johan Thyberg**, **Sofia Gripenbäck**, **Göran Tornling**, **Jan-Jonas Filén**, **Riita Lahesmaa**, **Mikael Norman**, **Etienne Neve**, **Barbara Bohle** and **Margarete Focke-Tejkl** for fruitful and encouraging discussions, for help with experimental work and for useful input in manuscript preparations. Thanks for nice collaborations!

The head of the department of Medicine, Professor **Anders Hamsten**, for providing excellent research conditions.

All members of the "Exosome group": **Sara**, for making exosome research so much more fun (I was so glad when you decided to come back!), for sharing ups and downs in the "nano-world" and in the "real world" and for fun traveling times! **Mukti,** for always being so positive and helping out with experimental work. Thanks for all good scientific advice and for the nice dinner you prepared, it was really yummy! **Helen,** for always being so kind and helpful and for teaching me a lot about nanoparticles.

All former and present members in Annika Scheynius group: **Sara Johansson**, **Theresa Neimert Andersson**, **Monica Vilhelmsson**, **Fredrik Wermeling**, **Helen Vallhov**, **Maaike Joerink**, **Mikael Karlsson**, **Sara Lind**, **Christine Selander**, **Erika**

Rindsjö, **Catharina Johansson**, **Khaleda Rahman Qazi**, **Anna Andersson**, **Andor Pivarcsi**, **Britt Berggren, Maria Tengvall Linder, Anne Rasikari**, **Anita Waara**, **Eva Buentke**, **Ulrika Holmlund**, **Arezou Zargari**, **Eva Sverremark Ekström**, **Gunilla Jacobsson Ekman**, **Omid Rasool** and **Hojjat Eshaghi** for scientific support and a nice working atmosphere.

Anne Rasikari and **Gerd Franzon Lundquist** for excellent secretarial help.

All friends and colleagues at L2:04 for nice lunches, "fika", parties and scientific discussions.

All my former and present room mates: **Theresa**, **Monica**, **Helen**, **Fredrik**, **Maaike**, **Arezou**, **Sara** and **Mukti** for nice discussions about all sorts of things and for a lot of laughter. **Theresa** "The Beast" and **Fredrik** "Flameboy", for challenging me in "DN:s nutidstest" (I am still in the lead! 7 out of 7, yes!) and sharing a lot of chili nuts and soft corn. **Theresa**, for being my "Friskis och Svettis" partner number one (Intensiv-Robban rules)! For fun traveling times (inkluderat 120 cm säng och fästmassa för löständer) and for having the same childish sense of humor as me! **Monica**, for a great time in Davos! For being the best guide possible and for introducing me to my first cheese fondue experience, yummy! I miss you at the lab!

All my friends outside the lab, especially my favorite girls and dearest friends **Anna**, **Kattis**, **Maria** and **Åsa**. We have shared a lot and have had tons of fun during the years. Thanks for being the best friends ever! Puss på er! Thanks also for having such great boyfriends (**Magnus** and **Andreas**)!

My old study friends Jessica, **Johanna** and **Katarina**, for our nice dinners which I hope we will continue to have for a long time, and thanks for all fun times when doing "tjejklassikern" (except Lidingöloppet = not fun!), and **Alex** and **Stefan** for nice "after work" and career discussions.

My family, pappa **Jan** and mamma **Yvonne,** for your love and support during the years and for always being there no matter what. My sister **Cecilia,** for all fun times including traveling, swimming at Eriksdalsbadet and diving (advance course, still waiting….) and for always being so generous and helpful.

Finally, I would like to thank **Agne**, for being in my life and for making me happy. Thanks for always being supportive and encouraging and for complementing me in such a good way, calming me down when I am worried. I hope you know how much you mean to me.

TACK!

8 REFERENCES

1. Janeway, C.A., Travers, T., Walport, M., and Shlomchik, M. (2004) *Immunobiology - the immune system in health and disease, 6th edition*. Garland Science Publishing.
2. Medzhitov, R., Janeway, C., Jr. (2000) Innate immune recognition: mechanisms and pathways. *Immunol Rev* **173**, 89-97.
3. Janeway, C.A., Jr. (2001) How the immune system protects the host from infection. *Microbes Infect* **3**, 1167-71.
4. Steinman, R.M. (2003) The control of immunity and tolerance by dendritic cell. *Pathol Biol (Paris)* **51**, 59-60.
5. Flores-Romo, L. (2001) In vivo maturation and migration of dendritic cells. *Immunology* **102**, 255-62.
6. Banchereau, J., Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature* **392**, 245-52.
7. Schuurhuis, D.H., Fu, N., Ossendorp, F., Melief, C.J. (2006) Ins and outs of dendritic cells. *Int Arch Allergy Immunol* **140**, 53-72.
8. O'Doherty, U., Peng, M., Gezelter, S., Swiggard, W.J., Betjes, M., Bhardwaj, N., Steinman, R.M. (1994) Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* **82**, 487-93.
9. Robinson, S.P., Patterson, S., English, N., Davies, D., Knight, S.C., Reid, C.D. (1999) Human peripheral blood contains two distinct lineages of dendritic cells. *Eur J Immunol* **29**, 2769-78.
10. Romani, N., Gruner, S., Brang, D., Kampgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P.O., Steinman, R.M., Schuler, G. (1994) Proliferating dendritic cell progenitors in human blood. *J Exp Med* **180**, 83-93.
11. Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., Colonna, M. (1999) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* **5**, 919-23.
12. Shortman, K. (2000) Burnet oration: dendritic cells: multiple subtypes, multiple origins, multiple functions. *Immunol Cell Biol* **78**, 161-5.
13. Rodriguez-Pinto, D. (2005) B cells as antigen presenting cells. *Cell Immunol* **238**, 67-75.
14. Thery, C., Amigorena, S. (2001) The cell biology of antigen presentation in dendritic cells. *Curr Opin Immunol* **13**, 45-51.
15. Sallusto, F., Cella, M., Danieli, C., Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* **182**, 389-400.
16. Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J., Harding, C.V. (1993) Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361**, 359-62.
17. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-57.
18. Romagnani, S. (1994) Lymphokine production by human T cells in disease states. *Annu Rev Immunol* **12**, 227-57.
19. Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* **6**, 1133-41.
20. Bettelli, E., Oukka, M., Kuchroo, V.K. (2007) T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* **8**, 345-50.

21. Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., Cua, D.J. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* **201**, 233-40.
22. Lucas, S., Ghilardi, N., Li, J., de Sauvage, F.J. (2003) IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms. *Proc Natl Acad Sci U S A* **100**, 15047-52.
23. Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G., Glimcher, L.H. (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655-69.
24. Afkarian, M., Sedy, J.R., Yang, J., Jacobson, N.G., Cereb, N., Yang, S.Y., Murphy, T.L., Murphy, K.M. (2002) T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* **3**, 549-57.
25. Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M., Stockinger, B. (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* **24**, 179-89.
26. Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., Weaver, C.T. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* **6**, 1123-32.
27. Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J., Gurney, A.L. (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* **278**, 1910-4.
28. Bluestone, J.A., Abbas, A.K. (2003) Natural versus adaptive regulatory T cells. *Nat Rev Immunol* **3**, 253-7.
29. Wing, K., Fehervari, Z., Sakaguchi, S. (2006) Emerging possibilities in the development and function of regulatory T cells. *Int Immunol* **18**, 991-1000.
30. Hori, S., Nomura, T., Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057-61.
31. Fontenot, J.D., Gavin, M.A., Rudensky, A.Y. (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* **4**, 330-6.
32. Lohr, J., Knoechel, B., Abbas, A.K. (2006) Regulatory T cells in the periphery. *Immunol Rev* **212**, 149-62.
33. Weaver, C.T., Harrington, L.E., Mangan, P.R., Gavrieli, M., Murphy, K.M. (2006) Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* **24**, 677-88.
34. Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., Harding, C.V., Melief, C.J., Geuze, H.J. (1996) B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* **183**, 1161-72.
35. Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G., Hivroz, C. (2002) TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol* **168**, 3235-41.
36. Raposo, G., Tenza, D., Mecheri, S., Peronet, R., Bonnerot, C., Desaymard, C. (1997) Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol Biol Cell* **8**, 2631-45.
37. Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., Amigorena, S. (1998) Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* **4**, 594-600.
38. Heijnen, H.F., Schiel, A.E., Fijnheer, R., Geuze, H.J., Sixma, J.J. (1999) Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* **94**, 3791-9.
39. Faure, J., Lachenal, G., Court, M., Hirrlinger, J., Chatellard-Causse, C., Blot, B., Grange, J., Schoehn, G., Goldberg, Y., Boyer, V., Kirchhoff, F., Raposo, G., Garin, J., Sadoul, R. (2006) Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* **31**, 642-8.

40. van Niel, G., Raposo, G., Candalh, C., Boussac, M., Hershberg, R., Cerf-Bensussan, N., Heyman, M. (2001) Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* **121**, 337-49.
41. Trams, E.G., Lauter, C.J., Salem, N., Jr., Heine, U. (1981) Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta* **645**, 63-70.
42. Harding, C., Heuser, J., Stahl, P. (1984) Endocytosis and intracellular processing of transferrin and colloidal gold-transferrin in rat reticulocytes: demonstration of a pathway for receptor shedding. *Eur J Cell Biol* **35**, 256-63.
43. Pan, B.T., Teng, K., Wu, C., Adam, M., Johnstone, R.M. (1985) Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J Cell Biol* **101**, 942-8.
44. Caby, M.P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G., Bonnerot, C. (2005) Exosomal-like vesicles are present in human blood plasma. *Int Immunol* **17**, 879-87.
45. Pisitkun, T., Shen, R.F., Knepper, M.A. (2004) Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* **101**, 13368-73.
46. Skriner, K., Adolph, K., Jungblut, P.R., Burmester, G.R. (2006) Association of citrullinated proteins with synovial exosomes. *Arthritis Rheum* **54**, 3809-14.
47. Andre, F., Schartz, N.E., Movassagh, M., Flament, C., Pautier, P., Morice, P., Pomel, C., Lhomme, C., Escudier, B., Le Chevalier, T., Tursz, T., Amigorena, S., Raposo, G., Angevin, E., Zitvogel, L. (2002) Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* **360**, 295-305.
48. Gatti, J.L., Metayer, S., Belghazi, M., Dacheux, F., Dacheux, J.L. (2005) Identification, proteomic profiling, and origin of ram epididymal fluid exosome-like vesicles. *Biol Reprod* **72**, 1452-65.
49. Ronquist, G., Brody, I. (1985) The prostasome: its secretion and function in man. *Biochim Biophys Acta* **822**, 203-18.
50. Kleijmeer, M.J., Escola, J.M., UytdeHaag, F.G., Jakobson, E., Griffith, J.M., Osterhaus, A.D., Stoorvogel, W., Melief, C.J., Rabouille, C., Geuze, H.J. (2001) Antigen loading of MHC class I molecules in the endocytic tract. *Traffic* **2**, 124-37.
51. Amzallag, N., Passer, B.J., Allanic, D., Segura, E., Thery, C., Goud, B., Amson, R., Telerman, A. (2004) TSAP6 facilitates the secretion of translationally controlled tumor protein/histamine-releasing factor via a nonclassical pathway. *J Biol Chem* **279**, 46104-12.
52. Savina, A., Fader, C.M., Damiani, M.T., Colombo, M.I. (2005) Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. *Traffic* **6**, 131-43.
53. Savina, A., Furlan, M., Vidal, M., Colombo, M.I. (2003) Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J Biol Chem* **278**, 20083-90.
54. Savina, A., Vidal, M., Colombo, M.I. (2002) The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci* **115**, 2505-15.
55. de Gassart, A., Geminard, C., Hoekstra, D., Vidal, M. (2004) Exosome secretion: the art of reutilizing nonrecycled proteins? *Traffic* **5**, 896-903.
56. de Gassart, A., Geminard, C., Fevrier, B., Raposo, G., Vidal, M. (2003) Lipid raft-associated protein sorting in exosomes. *Blood* **102**, 4336-44.
57. Mobius, W., van Donselaar, E., Ohno-Iwashita, Y., Shimada, Y., Heijnen, H.F., Slot, J.W., Geuze, H.J. (2003) Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. *Traffic* **4**, 222-31.
58. Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., Geuze, H.J. (2000) Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci* **113 Pt 19**, 3365-74.
59. Lamparski, H.G., Metha-Damani, A., Yao, J.Y., Patel, S., Hsu, D.H., Ruegg, C., Le Pecq, J.B. (2002) Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods* **270**, 211-26.

60. Clayton, A., Court, J., Navabi, H., Adams, M., Mason, M.D., Hobot, J.A., Newman, G.R., Jasani, B. (2001) Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *J Immunol Methods* **247**, 163-74.
61. Escola, J.M., Kleijmeer, M.J., Stoorvogel, W., Griffith, J.M., Yoshie, O., Geuze, H.J. (1998) Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* **273**, 20121-7.
62. Johnstone, R.M., Adam, M., Hammond, J.R., Orr, L., Turbide, C. (1987) Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* **262**, 9412-20.
63. Hemler, M.E. (2005) Tetraspanin functions and associated microdomains. *Nat Rev Mol Cell Biol* **6**, 801-11.
64. Clayton, A., Turkes, A., Navabi, H., Mason, M.D., Tabi, Z. (2005) Induction of heat shock proteins in B-cell exosomes. *J Cell Sci* **118**, 3631-8.
65. Thery, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Raposo, G., Amigorena, S. (1999) Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J Cell Biol* **147**, 599-610.
66. Wubbolts, R., Leckie, R.S., Veenhuizen, P.T., Schwarzmann, G., Mobius, W., Hoernschemeyer, J., Slot, J.W., Geuze, H.J., Stoorvogel, W. (2003) Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem* **278**, 10963-72.
67. Thery, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., Raposo, G., Garin, J., Amigorena, S. (2001) Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol* **166**, 7309-18.
68. Clayton, A., Harris, C.L., Court, J., Mason, M.D., Morgan, B.P. (2003) Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59. *Eur J Immunol* **33**, 522-31.
69. Thery, C., Zitvogel, L., Amigorena, S. (2002) Exosomes: composition, biogenesis and function. *Nat Rev Immunol* **2**, 569-79.
70. Fevrier, B., Raposo, G. (2004) Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol* **16**, 415-21.
71. Pan, B.T., Johnstone, R.M. (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* **33**, 967-78.
72. Rieu, S., Geminard, C., Rabesandratana, H., Sainte-Marie, J., Vidal, M. (2000) Exosomes released during reticulocyte maturation bind to fibronectin via integrin alpha4beta1. *Eur J Biochem* **267**, 583-90.
73. Vincent-Schneider, H., Stumptner-Cuvelette, P., Lankar, D., Pain, S., Raposo, G., Benaroch, P., Bonnerot, C. (2002) Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. *Int Immunol* **14**, 713-22.
74. Utsugi-Kobukai, S., Fujimaki, H., Hotta, C., Nakazawa, M., Minami, M. (2003) MHC class I-mediated exogenous antigen presentation by exosomes secreted from immature and mature bone marrow derived dendritic cells. *Immunol Lett* **89**, 125-31.
75. Hwang, I., Shen, X., Sprent, J. (2003) Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules. *Proc Natl Acad Sci U S A* **100**, 6670-5.
76. Segura, E., Nicco, C., Lombard, B., Veron, P., Raposo, G., Batteux, F., Amigorena, S., Thery, C. (2005) ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* **106**, 216-23.
77. Thery, C., Duban, L., Segura, E., Veron, P., Lantz, O., Amigorena, S. (2002) Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol* **3**, 1156-62.

78. Matsumoto, K., Morisaki, T., Kuroki, H., Kubo, M., Onishi, H., Nakamura, K., Nakahara, C., Kuga, H., Baba, E., Nakamura, M., Hirata, K., Tanaka, M., Katano, M. (2004) Exosomes secreted from monocyte-derived dendritic cells support in vitro naive CD4+ T cell survival through NF-(kappa)B activation. *Cell Immunol* **231**, 20-9.
79. Andre, F., Chaput, N., Schartz, N.E., Flament, C., Aubert, N., Bernard, J., Lemonnier, F., Raposo, G., Escudier, B., Hsu, D.H., Tursz, T., Amigorena, S., Angevin, E., Zitvogel, L. (2004) Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J Immunol* **172**, 2126-36.
80. Hsu, D.H., Paz, P., Villaflor, G., Rivas, A., Mehta-Damani, A., Angevin, E., Zitvogel, L., Le Pecq, J.B. (2003) Exosomes as a tumor vaccine: enhancing potency through direct loading of antigenic peptides. *J Immunother* **26**, 440-50.
81. Morelli, A.E., Larregina, A.T., Shufesky, W.J., Sullivan, M.L., Stolz, D.B., Papworth, G.D., Zahorchak, A.F., Logar, A.J., Wang, Z., Watkins, S.C., Falo, L.D., Jr., Thomson, A.W. (2004) Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* **104**, 3257-66.
82. Denzer, K., van Eijk, M., Kleijmeer, M.J., Jakobson, E., de Groot, C., Geuze, H.J. (2000) Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. *J Immunol* **165**, 1259-65.
83. Zakharova, L., Svetlova, M., Fomina, A.F. (2007) T cell exosomes induce cholesterol accumulation in human monocytes via phosphatidylserine receptor. *J Cell Physiol*.
84. Clayton, A., Turkes, A., Dewitt, S., Steadman, R., Mason, M.D., Hallett, M.B. (2004) Adhesion and signaling by B cell-derived exosomes: the role of integrins. *Faseb J* **18**, 977-9.
85. Skokos, D., Le Panse, S., Villa, I., Rousselle, J.C., Peronet, R., David, B., Namane, A., Mecheri, S. (2001) Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J Immunol* **166**, 868-76.
86. Skokos, D., Botros, H.G., Demeure, C., Morin, J., Peronet, R., Birkenmeier, G., Boudaly, S., Mecheri, S. (2003) Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. *J Immunol* **170**, 3037-45.
87. Karlsson, M., Lundin, S., Dahlgren, U., Kahu, H., Pettersson, I., Telemo, E. (2001) "Tolerosomes" are produced by intestinal epithelial cells. *Eur J Immunol* **31**, 2892-900.
88. Ostman, S., Taube, M., Telemo, E. (2005) Tolerosome-induced oral tolerance is MHC dependent. *Immunology* **116**, 464-76.
89. Van Niel, G., Mallegol, J., Bevilacqua, C., Candalh, C., Brugiere, S., Tomaskovic-Crook, E., Heath, J.K., Cerf-Bensussan, N., Heyman, M. (2003) Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut* **52**, 1690-7.
90. Zhang, H.G., Liu, C., Su, K., Yu, S., Zhang, L., Zhang, S., Wang, J., Cao, X., Grizzle, W., Kimberly, R.P. (2006) A membrane form of TNF-alpha presented by exosomes delays T cell activation-induced cell death. *J Immunol* **176**, 7385-93.
91. Janiszewski, M., Do Carmo, A.O., Pedro, M.A., Silva, E., Knobel, E., Laurindo, F.R. (2004) Platelet-derived exosomes of septic individuals possess proapoptotic NAD(P)H oxidase activity: A novel vascular redox pathway. *Crit Care Med* **32**, 818-25.
92. Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Thery, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., Tursz, T., Angevin, E., Amigorena, S., Zitvogel, L. (2001) Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* **7**, 297-303.
93. Clayton, A., Tabi, Z. (2005) Exosomes and the MICA-NKG2D system in cancer. *Blood Cells Mol Dis* **34**, 206-13.

94. Abusamra, A.J., Zhong, Z., Zheng, X., Li, M., Ichim, T.E., Chin, J.L., Min, W.P. (2005) Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells Mol Dis* **35**, 169-73.
95. Liu, C., Yu, S., Zinn, K., Wang, J., Zhang, L., Jia, Y., Kappes, J.C., Barnes, S., Kimberly, R.P., Grizzle, W.E., Zhang, H.G. (2006) Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. *J Immunol* **176**, 1375-85.
96. Gesierich, S., Berezovskiy, I., Ryschich, E., Zoller, M. (2006) Systemic induction of the angiogenesis switch by the tetraspanin D6.1A/CO-029. *Cancer Res* **66**, 7083-94.
97. Trowsdale, J., Betz, A.G. (2006) Mother's little helpers: mechanisms of maternal-fetal tolerance. *Nat Immunol* **7**, 241-6.
98. Abrahams, V.M., Straszewski-Chavez, S.L., Guller, S., Mor, G. (2004) First trimester trophoblast cells secrete Fas ligand which induces immune cell apoptosis. *Mol Hum Reprod* **10**, 55-63.
99. Frangsmyr, L., Baranov, V., Nagaeva, O., Stendahl, U., Kjellberg, L., Mincheva-Nilsson, L. (2005) Cytoplasmic microvesicular form of Fas ligand in human early placenta: switching the tissue immune privilege hypothesis from cellular to vesicular level. *Mol Hum Reprod* **11**, 35-41.
100. Mincheva-Nilsson, L., Nagaeva, O., Chen, T., Stendahl, U., Antsiferova, J., Mogren, I., Hernestal, J., Baranov, V. (2006) Placenta-derived soluble MHC class I chain-related molecules down-regulate NKG2D receptor on peripheral blood mononuclear cells during human pregnancy: a possible novel immune escape mechanism for fetal survival. *J Immunol* **176**, 3585-92.
101. Collins, R.W. (2004) Human MHC class I chain related (MIC) genes: their biological function and relevance to disease and transplantation. *Eur J Immunogenet* **31**, 105-14.
102. Taylor, D.D., Akyol, S., Gercel-Taylor, C. (2006) Pregnancy-associated exosomes and their modulation of T cell signaling. *J Immunol* **176**, 1534-42.
103. Gould, S.J., Booth, A.M., Hildreth, J.E. (2003) The Trojan exosome hypothesis. *Proc Natl Acad Sci U S A* **100**, 10592-7.
104. Nguyen, D.G., Booth, A., Gould, S.J., Hildreth, J.E. (2003) Evidence that HIV budding in primary macrophages occurs through the exosome release pathway. *J Biol Chem* **278**, 52347-54.
105. Kramer, B., Pelchen-Matthews, A., Deneka, M., Garcia, E., Piguet, V., Marsh, M. (2005) HIV interaction with endosomes in macrophages and dendritic cells. *Blood Cells Mol Dis* **35**, 136-42.
106. Wiley, R.D., Gummuluru, S. (2006) Immature dendritic cell-derived exosomes can mediate HIV-1 trans infection. *Proc Natl Acad Sci U S A* **103**, 738-43.
107. Robertson, C., Booth, S.A., Beniac, D.R., Coulthart, M.B., Booth, T.F., McNicol, A. (2006) Cellular prion protein is released on exosomes from activated platelets. *Blood* **107**, 3907-11.
108. Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., Laude, H., Raposo, G. (2004) Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* **101**, 9683-8.
109. Altieri, S.L., Khan, A.N., Tomasi, T.B. (2004) Exosomes from plasmacytoma cells as a tumor vaccine. *J Immunother* **27**, 282-8.
110. Chaput, N., Schartz, N.E., Andre, F., Taieb, J., Novault, S., Bonnaventure, P., Aubert, N., Bernard, J., Lemonnier, F., Merad, M., Adema, G., Adams, M., Ferrantini, M., Carpentier, A.F., Escudier, B., Tursz, T., Angevin, E., Zitvogel, L. (2004) Exosomes as potent cell-free peptide-based vaccine. II. Exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. *J Immunol* **172**, 2137-46.
111. Hao, S., Ye, Z., Yang, J., Bai, O., Xiang, J. (2006) Intradermal vaccination of dendritic cell-derived exosomes is superior to a subcutaneous one in the induction of antitumor immunity. *Cancer Biother Radiopharm* **21**, 146-54.
112. Hao, S., Bai, O., Yuan, J., Qureshi, M., Xiang, J. (2006) Dendritic cell-derived exosomes stimulate stronger CD8+ CTL responses and antitumor immunity than tumor cell-derived exosomes. *Cell Mol Immunol* **3**, 205-11.

113. Chen, W., Wang, J., Shao, C., Liu, S., Yu, Y., Wang, Q., Cao, X. (2006) Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells. *Eur J Immunol* **36**, 1598-607.
114. Yang, Y., Xiu, F., Cai, Z., Wang, J., Wang, Q., Fu, Y., Cao, X. (2007) Increased induction of antitumor response by exosomes derived from interleukin-2 gene-modified tumor cells. *J Cancer Res Clin Oncol*.
115. Escudier, B., Dorval, T., Chaput, N., Andre, F., Caby, M.P., Novault, S., Flament, C., Leboulaire, C., Borg, C., Amigorena, S., Boccaccio, C., Bonnerot, C., Dhellin, O., Movassagh, M., Piperno, S., Robert, C., Serra, V., Valente, N., Le Pecq, J.B., Spatz, A., Lantz, O., Tursz, T., Angevin, E., Zitvogel, L. (2005) Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J Transl Med* **3**, 10.
116. Morse, M.A., Garst, J., Osada, T., Khan, S., Hobeika, A., Clay, T.M., Valente, N., Shreeniwas, R., Sutton, M.A., Delcayre, A., Hsu, D.H., Le Pecq, J.B., Lyerly, H.K. (2005) A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med* **3**, 9.
117. Mignot, G., Roux, S., Thery, C., Segura, E., Zitvogel, L. (2006) Prospects for exosomes in immunotherapy of cancer. *J Cell Mol Med* **10**, 376-88.
118. Aline, F., Bout, D., Amigorena, S., Roingeard, P., Dimier-Poisson, I. (2004) Toxoplasma gondii antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against T. gondii infection. *Infect Immun* **72**, 4127-37.
119. Peche, H., Heslan, M., Usal, C., Amigorena, S., Cuturi, M.C. (2003) Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection. *Transplantation* **76**, 1503-10.
120. Kim, S.H., Bianco, N., Menon, R., Lechman, E.R., Shufesky, W.J., Morelli, A.E., Robbins, P.D. (2006) Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. *Mol Ther* **13**, 289-300.
121. Kim, S.H., Lechman, E.R., Bianco, N., Menon, R., Keravala, A., Nash, J., Mi, Z., Watkins, S.C., Gambotto, A., Robbins, P.D. (2005) Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol* **174**, 6440-8.
122. Delcayre, A., Estelles, A., Sperinde, J., Roulon, T., Paz, P., Aguilar, B., Villanueva, J., Khine, S., Le Pecq, J.B. (2005) Exosome Display technology: applications to the development of new diagnostics and therapeutics. *Blood Cells Mol Dis* **35**, 158-68.
123. Xanthou, M. (1998) Immune protection of human milk. *Biol Neonate* **74**, 121-33.
124. Eglinton, B.A., Roberton, D.M., Cummins, A.G. (1994) Phenotype of T cells, their soluble receptor levels, and cytokine profile of human breast milk. *Immunol Cell Biol* **72**, 306-13.
125. Bocci, V., von Bremen, K., Corradeschi, F., Franchi, F., Luzzi, E., Paulesu, L. (1993) Presence of interferon-gamma and interleukin-6 in colostrum of normal women. *Lymphokine Cytokine Res* **12**, 21-4.
126. Bottcher, M.F., Jenmalm, M.C., Bjorksten, B., Garofalo, R.P. (2000) Chemoattractant factors in breast milk from allergic and nonallergic mothers. *Pediatr Res* **47**, 592-7.
127. Garofalo, R., Chheda, S., Mei, F., Palkowetz, K.H., Rudloff, H.E., Schmalstieg, F.C., Rassin, D.K., Goldman, A.S. (1995) Interleukin-10 in human milk. *Pediatr Res* **37**, 444-9.
128. Bryan, D.L., Hawkes, J.S., Gibson, R.A. (1999) Interleukin-12 in human milk. *Pediatr Res* **45**, 858-9.
129. Takahata, Y., Takada, H., Nomura, A., Ohshima, K., Nakayama, H., Tsuda, T., Nakano, H., Hara, T. (2001) Interleukin-18 in human milk. *Pediatr Res* **50**, 268-72.

130. Jain, L., Vidyasagar, D., Xanthou, M., Ghai, V., Shimada, S., Blend, M. (1989) In vivo distribution of human milk leucocytes after ingestion by newborn baboons. *Arch Dis Child* **64**, 930-3.
131. Brock, J.H. (1980) Lactoferrin in human milk: its role in iron absorption and protection against enteric infection in the newborn infant. *Arch Dis Child* **55**, 417-21.
132. Chipman, D.M., Sharon, N. (1969) Mechanism of lysozyme action. *Science* **165**, 454-65.
133. Newburg, D.S., Pickering, L.K., McCluer, R.H., Cleary, T.G. (1990) Fucosylated oligosaccharides of human milk protect suckling mice from heat-stabile enterotoxin of Escherichia coli. *J Infect Dis* **162**, 1075-80.
134. Crouch, A.A., Seow, W.K., Whitman, L.M., Thong, Y.H. (1991) Effect of human milk and infant milk formulae on adherence of Giardia intestinalis. *Trans R Soc Trop Med Hyg* **85**, 617-9.
135. Nakajima, S., Baba, A.S., Tamura, N. (1977) Complement system in human colostrum: presence of nine complement components and factors of alternative pathway in human colostrum. *Int Arch Allergy Appl Immunol* **54**, 428-33.
136. Heid, H.W., Keenan, T.W. (2005) Intracellular origin and secretion of milk fat globules. *Eur J Cell Biol* **84**, 245-58.
137. Wiman, K., Curman, B., Tragardh, L., Peterson, P.A. (1979) Demonstration of HLA-DR-like antigens on milk fat globule membranes. *Eur J Immunol* **9**, 190-5.
138. Peterson, J.A., Patton, S., Hamosh, M. (1998) Glycoproteins of the human milk fat globule in the protection of the breast-fed infant against infections. *Biol Neonate* **74**, 143-62.
139. Bednar-Tantscher, E., Mudde, G.C., Rot, A. (2001) Maternal antigen stimulation downregulates via mother's milk the specific immune responses in young mice. *Int Arch Allergy Immunol* **126**, 300-8.
140. Mincheva-Nilsson, L., Hammarstrom, M.L., Juto, P., Hammarstrom, S. (1990) Human milk contains proteins that stimulate and suppress T lymphocyte proliferation. *Clin Exp Immunol* **79**, 463-9.
141. Bottcher, M.F., Fredriksson, J., Hellquist, A., Jenmalm, M.C. (2003) Effects of breast milk from allergic and non-allergic mothers on mitogen- and allergen-induced cytokine production. *Pediatr Allergy Immunol* **14**, 27-34.
142. Svanborg, C., Agerstam, H., Aronson, A., Bjerkvig, R., Duringer, C., Fischer, W., Gustafsson, L., Hallgren, O., Leijonhuvud, I., Linse, S., Mossberg, A.K., Nilsson, H., Pettersson, J., Svensson, M. (2003) HAMLET kills tumor cells by an apoptosis-like mechanism--cellular, molecular, and therapeutic aspects. *Adv Cancer Res* **88**, 1-29.
143. Ho, F.C., Wong, R.L., Lawton, J.W. (1979) Human colostral and breast milk cells. A light and electron microscopic study. *Acta Paediatr Scand* **68**, 389-96.
144. Hamosh, M., Bitman, J., Wood, L., Hamosh, P., Mehta, N.R. (1985) Lipids in milk and the first steps in their digestion. *Pediatrics* **75**, 146-50.
145. Sanchez-Pozo, A., Lopez, J., Pita, M.L., Izquierdo, A., Guerrero, E., Sanchez-Medina, F., Martinez Valverde, A., Gil, A. (1986) Changes in the protein fractions of human milk during lactation. *Ann Nutr Metab* **30**, 15-20.
146. Asher, M.I., Montefort, S., Bjorksten, B., Lai, C.K., Strachan, D.P., Weiland, S.K., Williams, H. (2006) Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* **368**, 733-43.
147. Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J., O'Regan, G.M., Watson, R.M., Cecil, J.E., Bale, S.J., Compton, J.G., DiGiovanna, J.J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C.S., El Houate, B., McElreavey, K., Halkjaer, L.B., Bisgaard, H., Mukhopadhyay, S., McLean, W.H. (2006) Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* **38**, 441-6.

148. Laitinen, T., Polvi, A., Rydman, P., Vendelin, J., Pulkkinen, V., Salmikangas, P., Makela, S., Rehn, M., Pirskanen, A., Rautanen, A., Zucchelli, M., Gullsten, H., Leino, M., Alenius, H., Petays, T., Haahtela, T., Laitinen, A., Laprise, C., Hudson, T.J., Laitinen, L.A., Kere, J. (2004) Characterization of a common susceptibility locus for asthma-related traits. *Science* **304**, 300-4.
149. Alfven, T., Braun-Fahrlander, C., Brunekreef, B., von Mutius, E., Riedler, J., Scheynius, A., van Hage, M., Wickman, M., Benz, M.R., Budde, J., Michels, K.B., Schram, D., Ublagger, E., Waser, M., Pershagen, G. (2006) Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle--the PARSIFAL study. *Allergy* **61**, 414-21.
150. Alm, J.S., Swartz, J., Lilja, G., Scheynius, A., Pershagen, G. (1999) Atopy in children of families with an anthroposophic lifestyle. *Lancet* **353**, 1485-8.
151. Weiss, S.T. (2002) Eat dirt--the hygiene hypothesis and allergic diseases. *N Engl J Med* **347**, 930-1.
152. Lambrecht, B.N. (2005) Dendritic cells and the regulation of the allergic immune response. *Allergy* **60**, 271-82.
153. Wetzig, H., Schulz, R., Diez, U., Herbarth, O., Viehweg, B., Borte, M. (2000) Associations between duration of breast-feeding, sensitization to hens' eggs and eczema infantum in one and two year old children at high risk of atopy. *Int J Hyg Environ Health* **203**, 17-21.
154. Sears, M.R., Greene, J.M., Willan, A.R., Taylor, D.R., Flannery, E.M., Cowan, J.O., Herbison, G.P., Poulton, R. (2002) Long-term relation between breastfeeding and development of atopy and asthma in children and young adults: a longitudinal study. *Lancet* **360**, 901-7.
155. Kull, I., Bohme, M., Wahlgren, C.F., Nordvall, L., Pershagen, G., Wickman, M. (2005) Breast-feeding reduces the risk for childhood eczema. *J Allergy Clin Immunol* **116**, 657-61.
156. Kull, I., Almqvist, C., Lilja, G., Pershagen, G., Wickman, M. (2004) Breast-feeding reduces the risk of asthma during the first 4 years of life. *J Allergy Clin Immunol* **114**, 755-60.
157. Oddy, W.H., Holt, P.G., Sly, P.D., Read, A.W., Landau, L.I., Stanley, F.J., Kendall, G.E., Burton, P.R. (1999) Association between breast feeding and asthma in 6 year old children: findings of a prospective birth cohort study. *Bmj* **319**, 815-9.
158. Siltanen, M., Kajosaari, M., Poussa, T., Saarinen, K.M., Savilahti, E. (2003) A dual long-term effect of breastfeeding on atopy in relation to heredity in children at 4 years of age. *Allergy* **58**, 524-30.
159. van Odijk, J., Kull, I., Borres, M.P., Brandtzaeg, P., Edberg, U., Hanson, L.A., Host, A., Kuitunen, M., Olsen, S.F., Skerfving, S., Sundell, J., Wille, S. (2003) Breastfeeding and allergic disease: a multidisciplinary review of the literature (1966-2001) on the mode of early feeding in infancy and its impact on later atopic manifestations. *Allergy* **58**, 833-43.
160. Bottcher, M.F., Jenmalm, M.C., Garofalo, R.P., Bjorksten, B. (2000) Cytokines in breast milk from allergic and nonallergic mothers. *Pediatr Res* **47**, 157-62.
161. Laiho, K., Lampi, A.M., Hamalainen, M., Moilanen, E., Piironen, V., Arvola, T., Syrjanen, S., Isolauri, E. (2003) Breast milk fatty acids, eicosanoids, and cytokines in mothers with and without allergic disease. *Pediatr Res* **53**, 642-7.
162. van Rijt, L.S., Kuipers, H., Vos, N., Hijdra, D., Hoogsteden, H.C., Lambrecht, B.N. (2004) A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J Immunol Methods* **288**, 111-21.
163. Hammad, H., Lambrecht, B.N. (2006) Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation. *J Allergy Clin Immunol* **118**, 331-6.
164. Baldus, S.E., Engelmann, K., Hanisch, F.G. (2004) MUC1 and the MUCs: a family of human mucins with impact in cancer biology. *Crit Rev Clin Lab Sci* **41**, 189-231.
165. Patton, S., Gendler, S.J., Spicer, A.P. (1995) The epithelial mucin, MUC1, of milk, mammary gland and other tissues. *Biochim Biophys Acta* **1241**, 407-23.

166. Regimbald, L.H., Pilarski, L.M., Longenecker, B.M., Reddish, M.A., Zimmermann, G., Hugh, J.C. (1996) The breast mucin MUCI as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. *Cancer Res* **56**, 4244-9.
167. Mather, I.H., Jack, L.J., Madara, P.J., Johnson, V.G. (2001) The distribution of MUC1, an apical membrane glycoprotein, in mammary epithelial cells at the resolution of the electron microscope: implications for the mechanism of milk secretion. *Cell Tissue Res* **304**, 91-101.
168. Miksa, M., Wu, R., Dong, W., Das, P., Yang, D., Wang, P. (2006) Dendritic cell-derived exosomes containing milk fat globule epidermal growth factor-factor VIII attenuate proinflammatory responses in sepsis. *Shock* **25**, 586-93.
169. Veron, P., Segura, E., Sugano, G., Amigorena, S., Thery, C. (2005) Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells. *Blood Cells Mol Dis* **35**, 81-8.
170. Oshima, K., Aoki, N., Kato, T., Kitajima, K., Matsuda, T. (2002) Secretion of a peripheral membrane protein, MFG-E8, as a complex with membrane vesicles. *Eur J Biochem* **269**, 1209-18.
171. Chan, A.K., Lockhart, D.C., von Bernstorff, W., Spanjaard, R.A., Joo, H.G., Eberlein, T.J., Goedegebuure, P.S. (1999) Soluble MUC1 secreted by human epithelial cancer cells mediates immune suppression by blocking T-cell activation. *Int J Cancer* **82**, 721-6.
172. Mallegol, J., van Niel, G., Heyman, M. (2005) Phenotypic and functional characterization of intestinal epithelial exosomes. *Blood Cells Mol Dis* **35**, 11-6.
173. Alcorn, J., McNamara, P.J. (2003) Pharmacokinetics in the newborn. *Adv Drug Deliv Rev* **55**, 667-86.
174. Jahn-Schmid, B., Radakovics, A., Luttkopf, D., Scheurer, S., Vieths, S., Ebner, C., Bohle, B. (2005) Bet v 1142-156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. *J Allergy Clin Immunol* **116**, 213-9.
175. Rialland, P., Lankar, D., Raposo, G., Bonnerot, C., Hubert, P. (2006) BCR-bound antigen is targeted to exosomes in human follicular lymphoma B-cells. *Biol Cell* **98**, 491-501.
176. Leme, A.S., Hubeau, C., Xiang, Y., Goldman, A., Hamada, K., Suzaki, Y., Kobzik, L. (2006) Role of breast milk in a mouse model of maternal transmission of asthma susceptibility. *J Immunol* **176**, 762-9.