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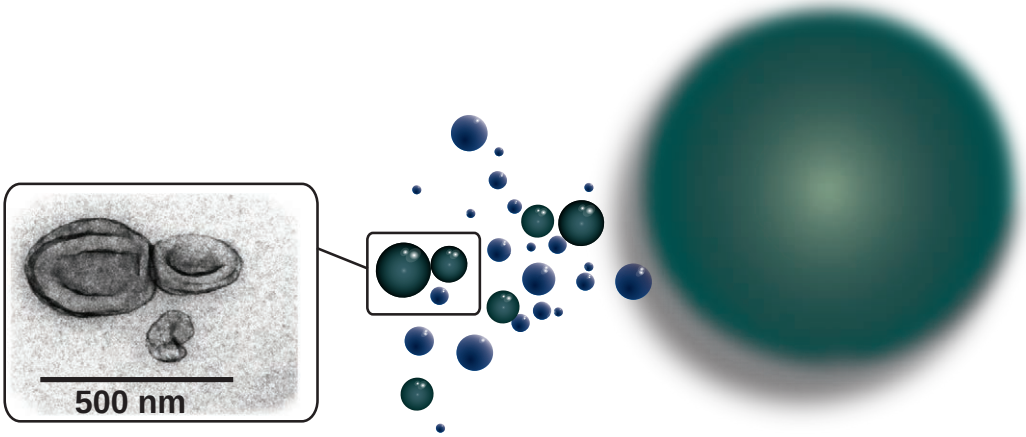

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Extracellular vesicles - mediators of immune modulation in the lung and as therapeutic vehicles
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EXTRACELLULAR VESICLES – MEDIATORS OF IMMUNE MODULATION IN THE LUNG AND AS THERAPEUTIC VEHICLES

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For my family.

ABSTRACT

Extracellular vesicles (EVs) are released from all cell types, and carry a wide setup of proteins, nucleic acids, lipids and other cargo. The overall aim of this thesis is to explore EV-based immune therapy, but also to find clues on mechanisms of the inflammatory disease sarcoidosis, and of lung cancer. Exosomes from dendritic cells (DCs) pulsed with antigen can induce antigen-specific responses *in vitro* and *in vivo*. **Study I** is an investigation comparing exosomes and microvesicles (MVs), which may complement exosomes therapeutically. We found surprisingly similar phenotypes of the two EV subtypes, including size distribution and immune-stimulatory molecule expression. However, when tested *in vivo*, only exosomes induced a significant antigen-specific $CD8^+$ T cell response. Antigenic re-stimulations *ex vivo* did, however, suggested that also MVs had such capacity, and both vesicle types induced antigen-specific IgG production. We further targeted the inflammatory disease pulmonary sarcoidosis in **study II** and **study III** with the aim to increase understanding of disease mechanisms, but also to search for disease biomarker candidates, and possible new treatment regimens. Broad proteomic characterizations of exosomes from patients revealed high abundance of pro-inflammatory molecules including leukotriene (LT)-forming enzymes. Large portions of the complement system were elevated, and we flagged vitamin D-binding protein as a possible biomarker for sarcoidosis. Functional tests of patient exosomes further suggested that they can engage monocytes and favor release of pro-inflammatory cytokines. The effects were partly dependent on LTs, and we could reduce cytokine production using the commercially available LT receptor antagonist Montelukast. **Study IV** on exosomes in lung cancer is focused on how exosomes may contribute to tumor progression via LTs. Exosomes from pleural effusions of patients favored generation of tumorigenic LTD_4, as well as tumor cell migration, which could be reduced using Montelukast. In summary, this thesis highlights the importance of investigating all EV subtypes in both basic and applied research. Further, the ability of EVs to contribute to inflammatory processes in the lungs underscores the potential of EVs in understanding disease mechanisms and finding diagnostic and prognostic disease markers. Finally, all three lung studies II-IV point to the possibility of interfering with LTs in inflammatory conditions, with possible applications also in cancer therapy.

POPULAR SCIENTIFIC SUMMARY

Synthetical nanoparticles are developed and used in state-of-the-art applications, and offer exciting new technical possibilities. Like many other innovations, nature has its own sophisticated equivalence. Every cell type of your body is capable of releasing nanoparticles fully loaded with cargo that can be shipped to other cells. These biological nanoparticles, or *extracellular vesicles* (EVs), can be down to 30 nanometers in diameter. This is extremely small considering that the beard of an average man has been anecdotally reported to grow 5-10 nanometers per second! Interestingly, these little entities travel around in you, and based on thousands of published studies, they seem to contribute to many of your biological functions including keeping the immune system in check.

Research on EVs isolated from blood, urine, or saliva provides clues about how diseases affect you via EVs, and explores the potential to diagnose diseases based on EVs. Another idea is to use EVs as vaccine or treatment, as they can stimulate your own immune system to fight a virus or tumor.

Exosomes are intensely investigated EVs, and in this thesis we tested also another EV type for its ability to stimulate an immune response. We found that both EV types could stimulate immune cells in mice, which suggests that treatments based on EVs should include both EV types. We also investigated exosomes from patients with the lung disease sarcoidosis. These exosomes were enriched in molecules which can induce inflammation, and when testing them on blood cells from healthy volunteers, they indeed induced inflammatory effects. Lastly, we also tested exosomes from lung cancer patients, and found signs that they can help tumor cells to survive and possibly also to spread the cancer. Several of these effects depended on molecules involved in inflammation. We could reduce several of these inflammatory effects using an asthma drug which reduces inflammation, suggesting that this drug may protect from cancer progression, and perhaps can be used in inflammatory diseases.

In summary, we show that several EV types should be tested further for their possible use in new treatments, and that they may contribute to inflammations and even cancer in the lungs.

LIST OF SCIENTIFIC PAPERS

I. **Wahlund CJE**, Güclüler G, Hiltbrunner S, Veerman RE, Näslund TI, Gabrielsson S

Exosomes from antigen-pulsed dendritic cells induce stronger antigen-specific immune responses than microvesicles *in vivo*

Sci Rep 2017 Dec 6;7(1):17095

II. Martinez-Bravo MJ*, **Wahlund CJE***, Qazi KR, Moulder R, Lukic A, Rådmark O, Lahesmaa R, Grunewald J, Eklund A, Gabrielsson S

Pulmonary sarcoidosis is associated with exosomal Vitamin D-binding protein and inflammatory molecules

J Allergy Clin Immunol. 2017 Apr;139(4):1186-1194

* Shared first authorship

III. **Wahlund CJE**, Güclüler G, Lepzien R, Smed-Sörensen A, Kullberg S, Eklund A, Grunewald J, Gabrielsson S

Sarcoidosis exosomes stimulate monocytes to produce proinflammatory cytokines and CCL2, which can be inhibited by Montelukast

Manuscript

IV. Lukic L, **Wahlund CJE**, Gomez C, Brodin D, Samuelsson B, Wheelock CE, Gabrielsson S, Radmark O

Exosomes from lung cancer pleura exudates form LTD_4, promoting cell migration and survival via CysLT1

Manuscript

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. **Wahlund CJE**, Eklund A, Grunewald J, Gabrielsson S.

Pulmonary Extracellular Vesicles as Mediators of Local and Systemic Inflammation.

Front Cell Dev Biol. 2017 Apr 26;5:39

II. Schmidt A, Zhang XM, Joshi RN, Iqbal S, **Wahlund CJE**, Gabrielsson S, Harris RA, Tegnér J.

Human macrophages induce CD4(+)Foxp3(+) regulatory T cells via binding and re-release of TGF-β

Immunol Cell Biol. 2016 Sep;94(8):747-62

LIST OF ABBREVIATIONS

AM	Alveolar macrophage
APC	Antigen-presenting cell
BALF	Bronchoalveolar lavage fluid
BCR	B cell receptor
BHL	Bilateral hilar lymphadenopathy
CAF	Cancer-associated fibroblast
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CME	Clathrin-mediated endocytosis
COX	Cyclooxygenase
cTec	Cortical thymic epithelial cell
CTL	Cytotoxic T lymphocyte
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot assay
EN	Erythema nodosum
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
FDC	Follicular dendritic cell
FLT3L	FMS-like tyrosine kinase 3 ligand
GC	Germinal center
GGT	Gamma-glutamyl transferase
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILV	Intraluminal vesicle
IPF	Idiopathic pulmonary fibrosis
LFA	Lymphocyte function-associated antigen
LOX	Lipoxygenase
LS	Löfgren´s syndrome
LT	Leukotriene

LTRA	Leukotriene receptor antagonist
MHC	Major histocompability complex
moDC	Monocyte-derived dendritic cell
mTec	Medullary thymic epithelial cell
MV	Microvesicle
MVB	Multivesicular body
MZ	Marginal zone
NSAID	Non-steroidal anti-inflammatory drug
NSCLC	Non-small cell lung cancer
NTA	Nanoparticle tracking analysis
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood monouclear cell
pDC	Plasmacytoid dendritic cell
PDL-1	Programmed death ligand 1
PG	Prostaglandin
PRR	Pattern recognition receptor
PS	Phosphatidylserine
SCLC	Small-cell lung cancer
TEM	Transmission electron microscopy
TGF	Tumor growth factor
Th	T helper
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
VDBP	Vitamin D-binding protein

CONTENTS

1 THE IMMUNE SYSTEM

The human immune system has evolved over millions of years to recognize and neutralize threats with as little effort and collateral damage as possible. The result is a system of enormous complexity. This is a brief overview of the most central concepts including lymphocytes and the mononuclear phagocyte system. The central aspects of immunity addressed in the studies of this thesis include induction of an antigen-specific immune response or inflammation. Immune stimulatory mechanisms are therefore mainly described below.

B CELLS

B cells comprise follicular B (FOB) cells residing in follicles of peripheral lymphoid organs, marginal zone B (MZB) cells of the spleen capturing blood-borne antigens, and B1 cells which confer mucosal immunity by monitoring pleural and peritoneal cavities. The latter two respond rapidly, and independently of T cells whereas FOB cells undergo somatic mutation and affinity maturation in germinal center (GC) reactions [1]. B cells are generated in the bone marrow, where their B cell receptor (BCR) is assembled from a heavy and a light chain, composed of a unique combination of three gene segments (V, D and J). This results in a cell surface-bound immunoglobulin (Ig) with high specificity for one antigenic epitope. At this stage, in the bone marrow, if a BCR recognizes any antigen it is highly likely self-structures. Any such BCR will therefore be treated as autoreactive, and the B cell will undergo receptor editing, basically switching the BCR specificity. Failure to do so will induce apoptosis or anergy in the B cell. This central tolerance induction reduces the number of mature autoreactive B cells to approximately 10% for adults, still a surprisingly high number, which however rarely causes problems as autoreactive B cells in the periphery are anergic [2].The BCR can increase its affinity greatly, and be produced in great quantities when needed. For this to occur, a GC reaction has to take place which is located to peripheral lymphoid organs. B cells are attracted to follicles by follicular dendritic cells (FDCs) which release CXCL13 to attract T and B cells [3]. The FDCs there display antigen to B cells, which in turn recognize the antigen and migrate towards the T cell zone, where antigen-specific T cells meet their B cell counterpart. The B cell has now internalized the antigen, processed it and here presents it to the $CD4^+$ T follicular helper (TFH) cell, which via its T cell receptor

(TCR) recognizes the MHC-antigen complex on the B cell. In return the T cell ligates its CD40L to the B cell CD40, which is necessary for the B cell to further differentiate either into plasma cells secreting high affinity antibodies, or into memory B cells [4].

In parallel with this, some B cells exit the GC early as plasma blasts to generate low affinity antibodies. These act as a first line of defense, until the long-lived plasma cells are finally produced, releasing high-affinity antibodies. These long-lived PCs home to the bone marrow via unknown signaling, and reside there dependent on partly unknown mediators which may include IL-6 and TNF [5].

Memory B cells are generated via GCs, but can also be formed independently of T cell help via mechanisms far less investigated, but generally the T cell-dependent (GC-induced) memory B cells generate more robust secondary responses. The memory B cells express surface-oriented Ig, and depending on the Ig isotype they elicit different secondary responses. IgG^+ memory B cells more readily differentiate into plasma cells, whereas IgM^+ B cells are more prone to re-enter GC reactions. They also show great differences in how long they maintain immunological memory, i.e. the capacity to induce a robust secondary response [6].

T CELLS

T cells are selected in the thymus for their ability to recognize foreign, but not self, peptides presented in self-MHC. T cell progenitors are attracted to the thymus, where they stepwise become $CD4^+CD8^+$ and are positively selected by cortical thymic epithelial cells (cTECs) based on their ability to recognize self-MHC with low affinity. Thereafter, the T cells differentiate to single positive cells ($CD4^+$ or $CD8^+$), and encounter medullary thymic epithelial cells (mTECs) which display self-antigens, and any T cells recognizing self will be deleted in a negative selection process [7]. Elegantly, however, a $CD4^+$T cell recognizing self-antigen in the thymus can also follow another path and differentiate into a regulatory T cell (Treg) with the capacity to modulate tolerance [8]. In pursuit of an antigen, the T cells circulate between the blood and lymphoid organs to find an antigen-presenting cell (APC) presenting the antigen peptide they recognize. Most commonly, the antigen encounter takes place in peripheral lymphoid organs such as a lymph node which represents a crossing point for APCs and lymphocytes [9].

Necessary for antigen recognition is the T cell receptor (TCR), composed of an alpha and a beta chain, which in conjunction with CD3, can engage the MHC-

peptide complex expressed by an APC. Consequently, the T cell and the APC form an immunological synapse, where the TCR-MHC interaction is accompanied by association of adhesion molecules including CD54 binding to lymphocyte function-associated antigen 1 (LFA-1). Further, the T cell is equipped with the immune stimulatory receptor CD28, as well as the inhibitory CTLA-4 which both can be engaged by the costimulatory CD80/CD86 on the APC. As CD28 interaction with CD80/CD86 is a necessity for T cell activation, the competition for CD80/CD86 provides a tool to steer the T cell response towards immunogenic or tolerogenic responses [10].

Activated $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTL) capable of recognizing MHC I with associated peptide, and recognize and kill cells expressing foreign peptides derived from pathogens or tumors. $CD4^+$ T cells on the other hand rather support other immune cells including $CD8^+$ T cells, B cells, and NK cells to tailor the immune response according to the infection or threat. $CD4^+$ T cells can differentiate into many subtypes including T helper (T_h)1, T_h2 and T_h17 cells depending on the stimulatory environment, including T_h-skewing cytokines and costimulation [11].

T_h1 cells are classically described as promoting an immune response to intracellular pathogens including viruses, with IFNγ being the hallmark cytokine. T_h2 cells on the other hand, release IL-4, IL-5 and IL-13, are vital in fighting parasitic infections but also play a major role in allergies. T_h2 cells are also closely associated with ILC2s, which react to epithelial cell-released IL-25 and IL-33 by producing Th2 cytokines [12].

T_h17 cells reside in the intestines during steady state, where the local microbiota contributes to T_h17 cell development. Here the T_h17 cells have an immune regulatory role and contribute to maintaining the integrity of the mucosal barrier. However, T_h17 cells can contribute significantly to inflammatory disorders, a pathogenic role which seems to be shaped by environmental factors including dietary components and microbiotal imbalance [13].

MONONUCLEAR PHAGOCYTES

Monocytes, DCs and macrophages are closely associated to one another to the degree that they are referred to as the mononuclear phagocytes [14]. All three are

capable of phagocytosing pathogens, to process them for destruction and antigen presentation to other immune cells. **Monocytes** can differentiate into monocyte-derived DCs and macrophages, and were previously considered precursors of tissue macrophages, whereas more recent appreciations are that tissue macrophages at steady state are generally derived from precursor cells seeded in the tissues already at embryonic stages [15]. During inflammation, however, there is a substantial increase in migration of monocytes into inflamed tissues where they can contribute to an expanding pool of macrophages and DCs to participate in resolving the inflammation [15]. Monocytes in the blood can be categorized in classical, intermediate and non-classical based on their expressions of CD14 and CD16. Generally, the classical monocytes ($CD14^+CD16^-$) which are the most abundant of the three, are considered to have the most potent migratory abilities and respond to infection and inflammation by migrating to the affected tissue. The intermediate ($CD14^+CD16^+$) and the non-classical ($CD14^-CD16^+$) are less prone to emigrate from circulation, and are dedicated to patrolling the endothelial linings of the blood vessels. Of note, the majority of monocyte migration studies are based on mouse experiments. There is phenotypic and functional overlap with human monocyte counterparts, but there are clearly gaps in the understanding of human monocyte behavior *in vivo*. Monocytes are equipped with chemokine receptors including CCR2, upon which they rely for their exit from the bone marrow into systemic circulation when stromal cells of the bone marrow releases CCL2. Monocytes constitutively migrate to tissues in steady state, but during immunological challenge, the trafficking is dramatically increased. Monocytes are attracted to the site of inflammation where they undergo substantial transcriptional changes and generate pro-inflammatory cytokines such as TNF, and can differentiate to DCs or macrophages with inflammatory capacities [16]. Monocytes are also capable of presenting antigen, and contribute to mounting adaptive immune responses. When activated, they upregulate MHC, costimulatory molecules, and as they are the most commonly occurring APC, their contribution to antigen presentation may have been underestimated [17].

Macrophages are present throughout the body, in most organs and tissues. Their phenotype is shaped by the local milieu of cytokines and growth factors. The proportions of tissue resident macrophages and monocyte-derived macrophages depend on the state of inflammation, and which tissue is affected. Macrophages display a vast number of functions. To name a few examples, both in lymph nodes

and within the marginal zone (MZ) of the spleen are macrophages which cross-talk with B cells to keep them correctly positioned, and display captured antigen to the B cells. In the lungs are alveolar macrophages contributing to remove excess amounts of surfactant, and in the liver are macrophages (Kupffer cells) which not only monitor for pathogens, but also aid in degrading erythrocytes to maintain homeostasis [18].

DCs are described as the most professional APCs of the immune system, with high expressions of MHC II and costimulatory molecules. To heighten their ability to act as immunological sentinels, DCs are also competent cross-presenters, displaying external antigenic peptides in MHC I. The ability to present both endogenous and exogenous antigens, combined with a high capacity to activate antigen-specific lymphocytes is the key to DC supremacy as professional APCs. Categorizing DCs is a challenging task. Mouse and human DCs do not fully overlap phenotypically and functionally, and *in vitro* studies of DCs are greatly dependent on the culturing conditions including the combination of growth factors present. In a simplified manner, however, human DCs can be subdivided into plasmacytoid DCs (pDCs), and classical DCs (cDCs). The cDCs can be further divided into cDC1 ($CD141^+$) and cDC2 ($CD1c^+$).The pDCs respond vigorously to viral infection, by sensing viral components mainly via TLR7 and TLR9, and releasing large amounts of interferons as well as cytokines including TNF and IL-6. The cDCs are generally located in tissues, and their main roles are to maintain immunological tolerance in steady state, and to prime adaptive immune responses against pathogens. $CD141^+$ cDCs are mainly described to cross-present antigens to $CD8^+$ T cells, and promote T_h1 responses, whereas $CD1c^+$ cDCs more potently primes $CD4^+$ T cells and are able to favor T_h1, T_h2 or T_h17 responses [19].

To prevent the risk of DCs priming an immune response to self-antigen, the elegant solution is that costimulatory molecule expression on the DCs is required for T cell activation. Signals inducing DC-expressed costimulators include TLR ligands, pro-inflammatory cytokines and the binding of CD40 to CD40 ligand. In absence of such signals, costimulatory molecules are not sufficiently expressed to activate the T cells, and instead of immunogenic activation the T cells are tolerized. Beyond their ability to initiate a specific response, DCs thus play important homeostatic roles in immunity [20].

DC-based immune therapy of cancer represents a personalized medicine with great potential, with the aim of raising an immune response against the tumor. As of 2018,

over 200 clinical trials using DC-based vaccines have been conducted, but the optimal settings to generate the most efficient vaccines are yet to be established [21]. The central concept of DC-vaccines is that antigen-loaded DCs are generated and expanded *ex vivo*, re-infused into the patient where they hopefully encounter T and B cells and prime an immune response against the tumor. The phenotype of cultured DCs is directly dependent on the culturing conditions and the source of the DC precursor cells, whether they are progenitors or monocytes. Further, many different antigen loading strategies can be applied, resulting in DCs of greatly varying capacity to prime an anti-tumor response in the patients [22, 23]. Melanoma is a highly immunogenic cancer type, which is positive when designing DC-based vaccines where the aim is to obtain as efficient immune response as possible. Several early DC-vaccine studies were aimed at treating melanoma, generally with successful inductions of tumor antigen-specific CTLs, but of varying clinical efficacy [24-26]. Since then, many cancer types have been included in trials including liver and prostate cancer, leukemia and myeloma [21]. DC-based cancer vaccines show great promise, but many challenges are yet to be solved, and so far only one vaccine has been approved by the US food and drug administration, targeting prostate cancer [27].

On a final note, **innate lymphoid cells** (ILCs) are tissue-resident lymphoid-like cells derived from lymphoid progenitors, but lacking the highly specific antigen receptors of B and T cells. ILCs share T cell properties in terms of transcription factor expressions and cytokine production, and are categorized in relationship to their T_h cell counterparts. ILC1s produce IFNγ, the main T_h1 cytokine, whereas ILC2s secrete T_h2-skewing cytokines including IL-5, IL-9 and IL-13, and ILC3s match the T_h17 cell cytokine profile including IL-17 and IL-22. Emerging evidence of ILCs in immunity suggest they exert a role as sentinels for threats including infection and tissue damage, mainly via cytokine receptor expression. Thus, they act as second messengers, by responding to a cytokine change induced by other cells, and in turn rapidly release their own cytokines. This fine-tuning of immune responses likely contributes to steady state homeostasis, but also tumor surveillance and to counter infections. ILC dysregulation has been associated with primarily asthma, where ILC2 activities further amplify an already exaggerated T_h2 cytokine response [28, 29].

2 EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) comprise several categories of vesicles released from probably all cell types, mediating intercellular communication in steady state and disease. Most frequently studied are the exosomes, for which the scientific interest has risen to substantial proportions. Other EVs include microvesicles (MVs) and apoptotic bodies, which are less investigated. Anecdotally, exosomes are reported in nearly 7500 publications, apoptotic bodies and MVs in approximately 3000 publications each, to be compared e.g. to “innate lymphoid cells” reported in 1600 publications (PubMed database, August 2018). Apoptotic bodies are generally released during apoptosis, and are vehicles for disassembling the dying cell with as little immunological effect as possible, and are therefore less suited to function in immunogenic settings. There is however a generally increasing understanding that the overlap in characteristics and functionality between the EV subtypes is greater than previously appreciated, and broad investigations beyond just exosomes are warranted. EV nomenclature is still debated, and MVs are sometimes termed ”microparticles, ”ectosomes” or ”shedding vesicles”. Other EV descriptions are based on the cellular origin or function of the EVs e.g. prostasomes released from the prostate or tolerosomes which induce immunological tolerance. Attempts have been made for a uniform nomenclature, resulting in the agreement to disagree. However, the term EVs should refer to all released EVs [30-36], and is used throughout this thesis.

EV BIOGENESIS AND COMPOSITION

Exosomes are formed when early endosomes mature to late endosomes, and undergo an inward budding process which produces intraluminal vesicles (ILV) inside the endosomes, which when filled with ILVs are named multivesicular bodies (MVB). MVBs have two distinct destinies; fusing with a lysosome to degrade and recycle its interior cargo, or fusing with the cell membrane to expel its contents of ILVs to the extracellular environment. The fusion with the cell membrane releases the ILVs, which are generally 50-100 nm in size, and are thereafter defined as exosomes. MVs, on the other hand, are instead formed through pinching of the cell membrane, which is an ATP-dependent process involving rearrangements of actin cytoskeletal components [37]. MV formation is accompanied by an increase in

intracellular Ca^{2+}, resulting in shedding of vesicles being 100-350 nm diameter [32, 35, 38]. In the original finding of MVs released side-by-side with exosomes, however sizes of MV up to 1000nm in diameter [39] were noted.

Extensive proteomic studies conducted on EVs of various origin has revealed thousands of proteins in EVs, which together with the results from some lipidomic studies have been added to a common and public database (”Vesiclepedia”) [40]. The proteins seemingly most conserved between exosomes of different origins are the tetraspanins CD9, CD63 and CD81 [41], as well as components of endosomal pathways including ESCRT, TSG101 and Alix [34]. In exosome formation, several subtypes of MVBs have been found, and endosomal sorting complexes required for transport (ESCRT) believed to be vital for exosome formation can be silenced with only partial loss of exosome production [42]. Complete independence of ESCRTs for the formation of some exosomes was shown in 2008 when a central concept of exosome formation as either ESCRT-dependent or -independent was coined [43]. In an attempt to dissect exosome formation mechanisms, Ostrowski *et al* [44] silenced several Rab proteins - members of small GTPases implicated in exosome formation. They found that Rab 27a and 27b were vital to exosome biogenesis in their experimental settings, but that silencing both proteins was not enough to completely abrogate vesicle release. The two Rab proteins were also found to contribute to formation of phenotypically different MVBs, suggesting a complex system of vesicle biogenesis with redundant and overlapping mechanisms for formation of subtypes of EVs. The same research group later blocked the same Rab proteins in tumor cells, and this time found that Rab 27a blockade affected tumor growth, presumably via decreased exosomal release, whereas 27b blockade had no effect on exosome release or tumor growth [45]. Rab 27 silencing also results in release of vesicles with reduced CD63, TSG101 and HSP70, whereas CD9 levels remain unchanged [46]. These studies point to a complexity of the EV systems, with consequences for the research as there are seemingly no EV-exclusive pathways which can be modified for investigative purposes.

As mentioned, it is of increasing interest to distinguish between the EV subtypes to evaluate their respective biological activities, but also their potential in applied settings. Considering their MVB origin, exosomes should reasonably have membrane and cargo composition overlapping with that of late endosomal compartments, whereas MVs should carry mainly plasma membrane components.

It has however become increasingly clear that the differences between vesicle subtypes are more diffuse than previously imagined. The ESCRT components involved in exosome formation and some of their interacting molecules e.g. TSG101 and ALIX are involved also in the release of MV [35], so distinguishing between exosomes and MVs based on these markers may not be suitable. Tetraspanins have long been considered “exosome markers”, but are also present in cytoplasmic membranes, and consequently MV membranes [41]. More recent proteomic evaluations of multiple EV subtypes showed that CD9, CD63 and CD81, as well as several other markers previously considered exosome-defining were found also in EVs corresponding to MVs [47].

Another possibility would be to segregate EVs based on lipid profiles. Lipid compositions of exosomal membranes generally display enrichment of cholesterol, phosphatidylserine (PS), and sphingomyelin compared to the cytoplasmic membrane [31]. Up to two thirds of small EV components are lipids, based on calculations assuming a 5 nm membrane thickness [48]. Despite this, surprisingly few broad lipidomic investigations of EVs which could be used to discriminate EV subtypes have been conducted. One explanation may be the complexity of lipid metabolism, a recent lipidomic analysis of exosomes and MVs detected nearly 2000 lipid species in the vesicles [49]. Two lipid species enriched in MVs compared to exosomes were ceramide and sphingomyelin, but further lipidomic investigations are warranted, preferably based on vesicles from several cell types and species.

Lastly, RNA contents of EVs may also define their subtype and, possibly, physiological activity. The finding of functional RNA in EVs [50] spiked interest as it opened up a whole new world of intercellular communication in need of investigation. There are controversies in EV-RNA research, however. It has been discussed whether EV-associated RNA is circulating in quantities high enough to be of biological significance. Chevillet *et al* reported that carefully conducted calculations estimate that far less than one miRNA molecule was present per exosome [51]. Further, it is unclear whether RNA is tethered to the exosomes or actually inside them [52]. This could clearly question whether RNA is indeed packed in EVs or merely attached to them during isolation, and if so how this would be of biological significance. However, the RNA profiles seem to differ between EV subtypes [53], the same for miRNA species [54], and ongoing EV-RNA research is likely to contribute to further understanding of EV subtypes within the near future [55].

How specific cargo is incorporated into EVs is not fully understood, but the mechanisms involved greatly overlap with those of endosomal recycling and intracellular protein sorting. For both exosomes and MVs, cargo is clustered in the cytosolic membrane (for MVs) or endosomal membranes (for exosomes) with support from tetraspanins. ESCRT components regularly sort proteins tagged by ubiquitination into MVBs, for subsequent degradation. As mentioned above, an alternative fate of the MVB is to release its contents including ILVs, which then are defined as exosomes. By ubiquitinating proteins, they may thus be sorted into MVBs of which some will be incorporated in released exosomes [56]. However, ubiquitination is not a pre-requisite for sorting into exosomes, as shown by Buschow *et al* who showed that MHC II sorting into exosomes was independent of ubiquitination, but depended on CD9 [57]. Further suggestions of tetraspanin-associated cargo sorting into exosomes include CD81-enriched micro domains where proteins are clustered [58], and CD63-mediated sorting into endosomes [59]. This tetraspanin-associated cargo loading of exosomes is most strongly associated with ESCRT-independent loading [60]. MHC II loading into EVs is expected, as MHC II peptide loading occurs in late endosomes [61], coinciding with the site of exosome biogenesis. MHC I, on the other hand, is loaded with antigen peptides in the endoplasmic reticulum [61] separate from endosomes, so it is interesting that exosomes also can express MHC I. ARF6 is a protein involved in intracellular trafficking of MHC I and other molecules, and has been shown to regulate incorporation of both MHC I and integrins in exosomes [62]. Fully understanding how cargo is incorporated into EVs will open for applications aimed at modulating these processes.

CELLULAR UPTAKE OF EVS

For EVs to convey an intercellular message, the cargo of interest must be incorporated and delivered to the right compartment. *In vivo*, exosomes have been found to have short half-life in circulation. The levels of intravenously injected exosomes were reduced to half after approximately 2 minutes [63], which has been confirmed with exosomes from several different cell sources [64]. This may be limiting for certain drug delivery applications where long circulatory half-life often is warranted. On the other hand, it may also be an advantage if the EVs are taken up by the desired target cell. How to engineer EVs to reach a certain destination is complex, but equipping them with surface molecules affecting their uptake is a main

approach. To mention some of the molecules on the surface of EVs involved in their cell engagement and following uptake, tetraspanins as well as integrins and lectins are prime candidates. The optimal set of molecules on EV surfaces for their efficient targeting will most likely depend on both which specific exosome population is used, as well as properties of the recipient cell type. By blocking molecules on either EVs or recipient DCs, Morelli *et al* [65] elegantly showed that molecules important for uptake included phosphatidylserine (PS), tetraspanins CD9 and CD81, CD54, and lactadherin on the EVs. On the DC surface, the αv/β3 integrin, and CD54 was most important. A similarly conducted experiment later confirmed a role for CD54 also on EVs, then binding to LFA-1 on DCs, as well as a role of the lectin DEC-205 on DCs for EV uptake [66]. This was supported by the finding that LFA-1 expression on T cells was vital for capture of DC-released exosomes [67]. Exosomes from breast milk with expressions of mucin-1 (MUC-1) have been shown to partly be dependent on the pathogen-recognizing molecule DC-SIGN for their uptake by DCs [68], further pointing to surface molecule dependency of EV uptake. B cell-derived exosomes were taken up by macrophages of the spleen in a report showing interaction with CD169 on the macrophages binding to sialic acids on the exosomes [63]. As mentioned above, PS is strongly enriched on exosomes [34], and has for long been known as an important component in recognition of apoptotic cells by macrophages [69]. Further, EV uptake is reduced if any of the PS-recognizing molecules TIM4 [70], or Annexin V [67, 71] are blocked, speaking for a role of PS in EV binding to target cells.

After association to the target cells, EVs can be internalized via several possible pathways. Substantial evidence point to EV uptake as an active process, including dramatically reduced uptake in cold incubations, as well as uptake by fixed cells, and by cells with disrupted endocytic pathways [72]. There is evidence of EVs fusing with the recipient cell membranes [73, 74], or being phagocytosed [70, 75], the latter possibly via PS expression on the EVs [71]. Clathrin-mediated endocytosis (CME) is dependent on the binding of ligands to receptors which are anchored by clathrin in the recipient cell [76]. This represents the most specific uptake mechanism. CME-dependent uptake of exosomes have been found [77, 78], which argues for a possibility of EVs to engage highly specific targets. Taken together, EVs are able to enter cells in many different ways, so therapeutic strategies based on EV uptake, or blocking of detrimental EV-communication, must be carefully designed.

EVS IN IMMUNITY AND CANCER

Published findings have shown that EVs are likely released from all cell types and transport thousands of proteins, lipids and other molecules. So to define their activities is a task as comprehensive as describing what proteins do. However, focusing on the role of EVs in the immune system, they have been ascribed both stimulatory and inhibitory effects.

On a brief note, an example of beneficial immune inhibitory EVs is pregnancy-associated EV release contributing to immune tolerance of fetal tissue. As reviewed in [79], placental exosomes seem have immune inhibitory functions and downregulate activities of both T cells and NK cells. Interestingly, however, MVs from the same source can engage monocytes and B cells to produce a large number of pro-inflammatory cytokines including TNF, IFNγ, IL-1β and IL-12 [79].

Focusing on pathogenic roles of immune inhibitory EVs, however, overwhelming evidence show that tumor-derived EVs are important players in cancer. The multitude of publications on cancer EVs do not present a uniform picture of their phenotype and behavior, but instead point to the complex nature of cancer itself, with as many phenotypic variants as can be imagined. Programmed death ligand 1 (PDL-1) binds PD-1 on immune cells and inhibits their activation. In a study of head and neck squamous cell carcinoma (HNSCC), plasma exosomes from 40 patients showed a PD-L1 expression correlating strongly to disease activity and the presence of lymph node metastasis [80]. Although less clear-cut, glioblastoma EVs have also been found to express PD-L1 and prevent T cell activation [81]. Further, tumor EVs have been found to induce Treg activity and survival [82], and promote immunosuppressive activities of myeloid-derived suppressor cells [83, 84].

Beyond immune inhibitory effects, tumor EVs have been implicated in metastasis mechanisms by promoting cell migration and a tumor-supportive milieu. For example, melanoma exosomes can travel to draining lymph nodes to settle there and both attract melanoma cells and support the metastatic formation [85]. Further, a major finding revealed a role of exosomes in directing metastatic migration, preparing a metastatic niche, as well as maintaining a chronic low-level inflammation, which is associated with tumor progression [78]. Exosomes from tumors metastasizing to the lungs, liver or the brain preferentially engaged cells of the destination tissue, and could even alter the metastatic destination of other

tumors. The different metastatic destinations were associated with differential expressions of integrins on the exosomes [86]. In line with the observed ability of exosomes to maintain a low degree of inflammation, Nabet *et al f*ound that breast cancer cells stimulated fibroblasts to release exosomes carrying RNA species recognized as danger-associated molecular patterns (DAMPS) [87]. Other harmful cancer EV strategies involve chemo resistance induction and contribution to a pathogenic tumor microenvironment (TME). In breast cancer, EVs were shown to transport the full mitochondrial genome, and in a tumor model of therapy-resistant breast cancer, the delivery of this cargo favored an exit from a dormant state of tumor cells [88]. There are plenty of findings supporting the idea that EVs favor chemo resistance in tumor cells [89-93]. Further, cancer associated fibroblasts (CAF) are stromal cells contributing to tumorigenesis and metastasis by releasing cytokines and growth factors, and favoring angiogenesis [94]. Exosomes from tumors promote differentiation and tumor-favoring activities of CAF [95, 96], supporting the TME. Other EV strategies contributing to a pathological TME induce modulating vascularization [97-99], or even to reduce nutrient uptake of non-tumor cells [100].

EV-BASED IMMUNE THERAPY

How can we utilize EVs to improve human health? Aside of implementing the knowledge gained on basic physiological functions for better understanding of human health, the main areas of EV-based medicine are diagnostics and immune therapy. To briefly touch upon EV-based diagnostics, the concept is to isolate vesicles, preferably from a body fluid accessible with as little invasivity as possible, and characterize their cargo, most likely proteins or nucleic acids. RNA transported by EVs show different compositional profile compared to healthy subjects in many cancer types investigated so far including glioma, breast cancer, colorectal cancer, ovarian cancer and melanoma, as reviewed by Jia *et al* [101]. The first exosome-based diagnostic tool for cancer has been approved by the US food and drug administration (FDA), tested in clinical cohorts of prostate cancer patients and is based on a disease-specific pattern of exosomal RNA (www.exosomedx.com).

Focusing on EV-based therapy, there have been suggestions to interfere with the function of harmful EVs in disease, by inhibiting formation, release or uptake of EVs [102]. Yet another way could be to reduce the circulatory burden of exosomes, as

suggested by Marleau et al, which could be based on techniques already in use to reduce viral loads in blood [103]. Interestingly, tetraspanins are involved in all steps of cancer metastasis, and tetraspanin-blocking antibodies have shown some promise in cancer settings [104]. Antibodies targeting CD9 reduced tumor cell proliferation and tumorigenicity *in vivo* in a colon carcinoma setting [105], and human gastric cancer cells implanted in mice showed reduced proliferation and angiogenesis [106]. When targeting either CD9 or CD63 with antibodies in a metastatic breast cancer model in mice, the metastases to lungs and liver were significantly reduced [107]. EVs are strongly enriched in tetraspanins, but whether the therapeutic antibodies did target EVs has not been elucidated, and as tetraspanins are involved in many cellular processes the risk of off-target effects are substantial. Further, as several of the studies mentioned above are based on human cancer cells implanted in mice, the therapeutic antibodies used are less likely to have side-effects, so in a more realistic setting there is greater risk of unacceptable toxicity.

A more plausible therapeutic implementation of EV knowledge is drug delivery, or priming antigen-specific immune responses. It should be mentioned that EVs from hematopoietic or mesenchymal stem cells have been proven efficient in inducing tissue repair after myocardial infarctions, and tissue damage in kidneys, muscle tissue and even pancreatic cells [102]. This thesis is however focused on immunogenic EVs for antigen-specific response induction. For cancer vaccination or therapy, the most likely approach is using tumor antigen-carrying EVs released from APCs [102]. As mentioned above, DCs have been thoroughly tested in nearly 200 clinical trials with varying success. As tumor cells can induce anergy or apoptosis in therapeutic DCs [22, 23], DC-derived EVs represent an attractive alternative approach, either alone or in combination with DCs [108].

DC-EVs are capable of priming $CD8^+$T cells *in vitro* and *in vivo*, and attracted great attention when they were found to reduce tumor burden *in vivo* by inducing an antigen-specific T cell-dependent immune response [109]. In dissecting the mechanisms behind this, Thery *et al* found that exosomes activate antigen-specific $CD4^+$ T cells *in vivo*, which was confirmed *in vitro* but only in the presence of DCs [110]. This suggested that EVs need APCs for optimal immunogenicity, but Hwang *et al* showed an ability of EVs to directly prime naive $CD8^+$ T cells *in vitro* [111]. Montecalvo *et al* found in an engraftment model that donor tissue exosomes did not

directly activate T cells but were shuttled via host engraftment-infiltrating DCs to splenic DCs, which in turn induced an immune response [112]. Taken together, it is highly likely that although EVs may prime T (or B) cells directly, the presence of APCs strongly increases the chance of a successful immune response. Further, the maturation status of the DCs is of importance for the EV immunogenicity. Exosomes from mature DCs are 50-100 times more efficient compared to immature DC-exosomes in activating naive T cells [113]. Nolte-´t Hoen *et al* found that DC-exosomes released during DC-T cell interaction transferred MHC II to the T cells, and again that cellular LFA-1 played a central role [67]. Our group demonstrated that B cell-derived exosomes from allergy patients presented allergen, induced T cell proliferation and favored a T_h2-like cytokine release [114]. Further, DC-exosomes are far more potent in inducing an antigen-specific immune response if they carry both T-, and B cell antigen epitopes, and successful induction of $CD8^+$ T cell responses have been found to be dependent on B cells [115, 116].

In cancer research, phase I clinical trials using patient cell-derived exosomes to induce anti-tumor responses have been completed, but with limited clinical improvement of patient health status [117, 118]. One possible explanation to the low efficacy of the exosomes is that they were derived from immature DCs, but also a phase II trial based on mature DC-exosomes showed very modest clinical success [119]. The above mentioned B cell dependency could be one reason for this. On the positive side, the trials showed little toxicity induced by EVs, which encourages continued optimization of EV-based therapy. To just briefly mention other areas of EV-based immune therapy, exosomes have been used successfully in vaccine models to confer protection against e.g. Mycobacteria [120], avian parasites [121] and Leishmanial species [122].

TECHNICAL ASPECTS OF EV RESEARCH

In 1987, Johnstone *et al* [123] used differential ultracentrifugation (UC) to isolate exosomes, and centrifugation is still the most frequently used technique with adjusted protocols [124]. Other options of isolation include size exclusion chromatography (SEC) and ultrafiltration (UF), all three techniques having advantages and limitations [125]. UC is straight-forward and easy to use with all sample-contacting equipment easy to sterilize, but initial costs of ultracentrifuges and rotors are very high. SEC is most likely to isolate vesicles without disrupting

membranes or altering their biological activities [126]. Caveats of the techniques include filtration-associated risk of shearing vesicles into smaller fragments and clogging filters. UC is also associated with exceptionally high forces leading to collapsed and fused vesicles, and SEC requires substantial setup, tuning and training, and vesicle isolation is solely based on their size [125]. The choice of isolation method will affect downstream analyses of the vesicles, and in ideal several methods should be used in parallel, which however often is not feasible.

Characterization of the vesicles should be conducted using several methods such as electron microscopy, ELISA, Western Blot, and conventional flow cytometry in which the EVs have to be bound to beads due to their small size [124]. There is increasing usage of direct flow cytometry, independent of beads, to analyze EVs. The technique is based on tuning the settings of the flow cytometer, and detecting vesicles based on fluorescence rather than forward/side scatter properties, however with the substantial limitation of a resolution around 100 nm [127]. Transmission electron microscopy (TEM) has been frequently used, and is still one of very few imaging techniques with resolution high enough to visualize single exosomes. Exosomes are commonly noted as having a cup-shaped appearance when imaged by TEM [124], and a fully rounded shape when cryo electron microscopy is used [128], a consequence of TEM sample preparation where the specimen is dried.

A method initially developed for industrial nanoparticles, nanoparticle tracking analysis (NTA), has been used to measure concentrations and size distributions of exosomes. Early versions of the instruments showed large variations in the results depending on user and the settings applied [129], with the conclusion that NTA must be used with carefully evaluated settings and very well standardized protocols. By comparing NTA with other methods for size estimation of EVs, Van der pol *et al* found that ”the minimum detectable vesicle sizes were 70-90 nm for NTA” [130], indicating that large portions of all exosomes are outside of the dynamic range of the instrument. Further, evaluations of NTA are often conducted using beads, which are inherently homogenous in sizes and properties compared to biological vesicles. As exosomes span a size range from at least 40-100 nm [34], but up to 30-150 nm [46] the smallest and the largest exosomes differ greatly in properties affecting how much light they disperse in NTA analysis [130], leading to large potential errors in light scattering-based analysis.

When studying proteins, inhibiting their release can reveal many clues on their functions. As EVs are far more complexly composed entities, no methods are available to robustly inhibit their production or release. GW4869 is a compound reducing exosome release by inhibiting sphingomyelinases [43], and this has been used repeatedly but as it interferes with lipid metabolism it most likely has broad off target effects.

As mentioned above, studies of EV uptake have shown that they are taken up by several mechanisms including receptor-mediated endocytosis and membrane fusion [72]. However, many of the studies are based on staining EVs with lipophilic dyes such as PKH67, R18 or DiL, followed by fluorescence microscopy-based detection of EVs, a method with resolution insufficient to detect single EVs [72]. This, taken together with the fact that lipophilic dyes themselves may cause aggregation, and possibly alter the EV uptake, or leak into the cytoplasmic membranes, speaks for that results must be interpreted with caution. However, they may provide important clues to EV uptake mechanisms. Touching upon these difficulties in isolation, characterization and nomenclature of EVs, attempts have been made to encourage broad investigations and very clear descriptions of how experiments were conducted in each publication [131].

3 THE LUNGS

LUNG IMMUNE COMPONENTS

A consequence of inhaling large volumes of air is exposure of the airways to antigens and irritants of the surroundings. Biological and chemical dangers such as airborne pathogens, pollen, cigarette smoke, and particulate dust are all constantly challenging the airways. The nasal cavity, the pharynx and the lungs are fitted with sophisticated defensive systems to counteract infection, and to resolve any insult to the barriers shielding us from environmental hazards. Epithelial cells collaborate with innate immune cells including granulocytes, DCs, and ILCs, via effector molecules including cytokines, chemokines and lipid mediators to confer immunological protection in the lungs.

Epithelial cells compose tight epithelial barriers, the first line of defense against aggressors, equipped with cilia and mucus-lined surfaces which capture, immobilize and shuttle threats upwards and out of the airways [132]. Beyond a role as physical barrier, airway epithelium is actively involved in upholding immunity e.g. by monitoring for dangers via pattern recognition receptors (PRRs) including toll-like receptors (TLRs) [133] and RIG1 [134]. Upon recognition of pathogens or injury, epithelial cells contribute to immune activation in part by producing and secreting antimicrobial products including lysozyme, aggressive oxidant species [132], and antimicrobial peptides including cathelicidin (LL37) and defensins [132, 135]. Epithelial-derived GM-CSF, IL-25 and IL-33 further potently activates DCs, ILCs and basophils and inflict airway inflammation [136].

Mast cells, which can be activated by epithelial signals including IL-33, are important players in pulmonary inflammation, capable of releasing potent pro-inflammatory cytokines, chemokines and pre-formed granules [137]. Mast cells act both as sentinels monitoring airways for threat, with a broad repertoire of PRRs, and FCε receptors binding IgE antibodies which upon antigen recognition leads to Fc receptor crosslinking and consequential release of inflammatory granule contents including histamine, TNF and antimicrobial peptides [137]. Mast cells are also an important source of eicosanoids including LTs, which can be released already a few minutes after activation [137], and mast cell released LTB_4 can further enhance the inflammation by promoting chemo attraction of mast cell progenitors [138].

ILCs (generally described in chapter 1) are lymphoid-like cells lacking antigen receptors and instead respond to soluble factors by releasing cytokines modulating inflammation [139]. ILC group 2 (ILC2) produce Th2 promoting cytokines including IL-4, IL-5 and IL-13 and increased ILC2 activity has been associated to airway disorder including asthma [139]. Again speaking for a role of epithelial cells in modulating airway inflammation, epithelial cell-derived IL-25 and IL-33 can contribute to ILC2 activation, and as IL-33 can activate mast cells it is not surprising that also ILC2s appear to co-operate with mast cells. ILC2s and mast cells have been found in proximity of one another in the airways [140], and mast cell-released prostaglandin D2 (PGD2) potently enhances ILC2 activation mediated by IL-25 and IL-33 [140]. So, epithelial cells have the potential to activate both mast cells and ILCs, and mast cells add positively to the ILC2 effect via PGD2, which further can upregulate the ILC2 receptors for IL-25 and IL-33 [141] leading to a self-amplifying spiral of pro-inflammatory events.

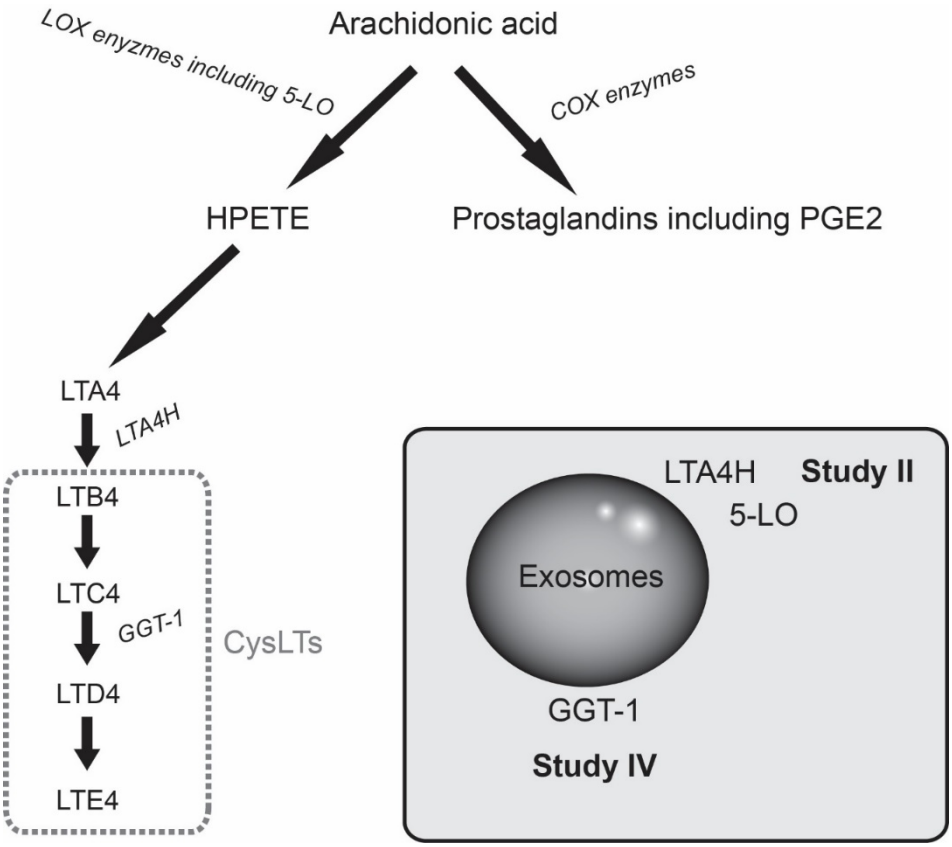
DCs, in conjunction with other innate immune cells of the airways including macrophages, and granulocytes, are all components of a robust system to maintain immune integrity [142]. Broadly, DCs in the lungs capture and clear pathogens, with following tolerogenic or immunogenic response induction [143]. DC populations in the lungs are a complicated matter. Based on mainly mouse studies, there are at least three defined pulmonary DC subsets, monocyte-derived DC (moDC), plasmacytoid DCs (pDCs), and conventional DCs (cDC) which can be divided in $CD103^+$ DCs and $CD11b^+$DCs [142]. The two latter DCs are also considered migratory, as they are most capable of capturing antigen and transporting it to local lymph nodes to prime immune responses. The airways consist of the conducting (upper) airways in which relatively few DCs are present, and with low capacity of sampling the airway lumen [144], whereas DCs present beneath the epithelial cells of the alveoli are richly branching their dendrites into the airway lumen for antigen sampling [145]. Infections initiated in the airways stimulate DC activity, influenza e.g. triggers antigen-experienced DCs to migrate to draining lymph nodes [146] in a CCR7-dependent manner [147] to prime CD8+ T cells to counter the viral infection. Also B cells are primed, and antibodies class-switched to IgA are generated to reside in the airway mucosa where they can bind and immobilize antigens including respiratory viruses, and act together with tissue resident memory T cells which are highly abundant in the mucosa and confer protection against pathogens [148]. Based on which (migratory) DC subset is performing this activity, effector or memory

$CD8^+$T cells are primed [149] to shape the most efficient response to each pathogen and circumstance. Connecting back to epithelial cells, the local environment including expression of the DC differentiation-inducing FMS-like tyrosine kinase 3 ligand (FLT3L) affects DC phenotype, and the lungs are supplied with FLT3L likely by epithelial cells [150], pointing to a complex interplay affecting DC phenotype, and again involvement of epithelial cells in shaping pulmonary immune components.

Macrophages are capable of presenting antigen, however alveolar macrophages (AMs) are generally inefficient at doing so as they normally have low levels of costimulatory molecules [151] and even can inactivate antigen-specific $CD4^+$ T cells [152]. AMs develop from embryonically seeded precursors rather than monocytes [153], and during steady state they are they are kept in check by epithelial cells via both direct contact to inhibitory receptors. Epithelial cell-expressed CD200 binds macrophage-expressed CD200R to inhibit pro-inflammatory responses [154]. Further, epithelial cells release IL-10 which binds the IL-10R on AMs. Moreover, integrins on the epithelial cell surfaces adhere to TGFβ, which in turn binds TGFβ receptors on AMs, resulting in an anti-inflammatory state of the AMs, with downstream further promotion of FoxP3 expression in T regulatory cells [155]. However, during infection or insult, damage to the epithelial cells reduces their IL-10 production, and disturbs their ability to regulate AMs via TGFβ and CD200 (mentioned above), resulting in activation of the AMs [156]. The AMs turn into potent factories of pro-inflammatory molecules including interferons, IL-12, IL-23 and IL-1β which sets off a series of events leading to activation and attraction of lymphoid cells, neutrophils and monocytes with a following inflammatory cascade [156].

Beyond cellular protectors of the airways**, eicosanoids** are signal mediating lipids with substantial impact on pulmonary immunity. Together, the eicosanoids are several hundred species with a very large number of biological activities in both steady state and disease, most studied are their effects on inflammation [157]. They are all derived from arachidonic acid, which can be processed by cyclooxygenases (COX enzymes) to form prostanoids including prostaglandins (PGs), by lipoxygenases (LOX enzymes) to form LTs, or by cytochrome P450 enzymes generating hydroxy-or epoxy-eicosatetranoids [158]. Some of the eicosanoids most studied in immunological contexts are prostaglandin E2 (PGE_2), a product of the COX pathway, and the cysteinyl-LTs (CysLTs) which are products of 5-lipoxygenase (5-LO) enzymatic conversion. The enzymes catalyzing each step throughout

eicosanoid metabolism are differentially expressed in different cell types, so to most efficiently generate eicosanoids e.g. during inflammation, cells harboring different intermediates and/or eicosanoid forming enzymes work in concert to complete the biogenesis, a behavior termed transcellular eicosanoid biosynthesis [157].



A schematic, simplified, overview of the eicosanoids most relevant to this thesis. Exosomes associated to LTA_4H and 5-LO are reported in **study II**, whereas exosomes harboring GGT-1 are investigated in **study IV**.

PGs are often strongly pro-inflammatory, and reducing their biogenesis by inhibiting COX enzymes using non-steroidal anti-inflammatory drugs (NSAID) such as aspirin is a common strategy to reduce systemic inflammations and fever [157, 158]. However, PGs are not inflammatory under all circumstances. PGE_2 has four different receptors (EP_1-EP_4), and depending on the expressions of these, PGE_2 can even act in an anti-inflammatory fashion in the lungs and to dilate bronchi [159]. Notably in a cancer setting, PGE_2 can even prevent apoptosis in epithelial tumor cells [160]. CysLTs, on the other hand, are strongly associated with inflammation. Concerning pulmonary disorders they promote bronchoconstriction, as summarized by the LT-discovering scientist Samuelsson [161], as well as migration of leukocytes including neutrophils [162]. In asthma, the levels of CysLTs are highly increased, and amongst

detrimental activities in allergies are CysLT-induced increase in vascular permeability, attraction and activation of type 2 ILCs as well as elevated airway resistance [163]. LTs are targets for therapy in asthma using LT receptor antagonists (LTRA) including Montelukast [157, 163].

SARCOIDOSIS

Sarcoidosis is an inflammatory disorder of unknown cause. It has the potential to affect any organ of the body, and is associated with granuloma formation which can lead to irreversible damage. Although it has been studied since it was first described in 1869, it is not known what causes sarcoidosis or why some patients are affected by an acute form of disease (with better prognosis), whereas other have a slow onset disease progression associated with chronicity. Factors complicating research on sarcoidosis include a wide heterogeneity of patients and cohorts, complex diagnostic procedures and pathological traits overlapping with other diseases. There is a great need to understand the disease better, to facilitate diagnostic and prognostic procedures and to find new treatment regimens.

Sarcoidosis results in anything from asymptomatic disease and complete recovery to neurological dysfunction and death [164]. Although any organ can be affected, there is a clear predominance for symptoms from the lungs (in more than 90% of cases) [165]. This thesis is based on investigations of pulmonary sarcoidosis patients, whose symptoms most commonly include shortness of breath (dyspnea) and dry cough [166]. Generally, 35-40% of sarcoidosis patients presents with the acute form of disease [167], known as Löfgren's syndrome (LS). This after Sven Löfgren, who studied patients with signs of swollen hilar lymph nodes (bilateral hilar lymphadenopathy, BHL) and subcutaneous inflammations (erythema nodosum (EN)), and eventually associated these events with a sarcoidosis patient phenotype [168]. The majority of LS patients display with arthritis of the ankle joints, BHL, and EN [169], the latter to a larger extent displayed by female patients [170]. LS patients generally have a better prognosis, in particular those with the HLA-DRB1*03 haplotype, which is strongly associated with a complete remission within two years [171]. The proportions of patients developing chronic disease also varies with studies, but around 25% is commonly reported [172], which is a substantial proportion and associates with long-term medication and severe consequences.

Epidemiology of sarcoidosis

Epidemiological numbers vary greatly between studies, and large cohort studies of sarcoidosis are few. Sweden is one of the countries with highest sarcoidosis prevalence [166], and a large study recently conducted by Arkema *et al* reported a prevalence of 160 per 100´000 inhabitants [173]. This is strikingly higher than in the US, where a study on 29´000 US patients found a prevalence of 60 patients per 100´000 adults [174]. The study further showed that majority of patients were 55 or older at time of diagnosis, with a higher prevalence in females and almost threefold higher prevalence in African Americans compared to Caucasians [174]. This has been supported by other reports, showing up to eightfold higher prevalence in African Americans compared to Caucasian Americans [166], and a large cohort study reported mortality rates up to twelve times higher for the African Americans [175].

Sarcoidosis diagnostic and therapeutic approaches

Examination includes lung function test, blood sampling, bronchoscopy with bronchoalveolar lavage (BAL), and chest radiography to rule out e.g. tuberculosis and define sarcoidosis [176]. The ratio of BAL $CD4^+$ to $CD8^+$ T cells is typically elevated (<3.5) in sarcoidosis patients as a result of $CD4^+$ T cell accumulation and expansion in BAL. Chest radiography is conducted to certify diagnosis and to stage the disease from 0-IV based on degree of pulmonary involvement from none (0) to full fibrotic development (IV). Combined with measures to exclude other disease such as infections including tuberculosis, and the identification of sarcoid granulomas in tissue or lymph node biopsies, a diagnosis is confirmed [176]. Searches for biomarkers have so far not resulted in any clinically applied tool. Elevated serum levels of angiotensin converting enzyme (ACE), and general cytokine profiles associated with inflammation have been observed [176], but not proven sufficient to diagnose sarcoidosis. There are reports suggesting possible sarcoidosis biomarkers, which include the acute phase protein Serum Amyloid A [177], and Chitotriosidase, which is produced by activated macrophages and has been associated with sarcoidosis [178, 179].

Treatment is generally aimed at dampening the symptoms, and initial stage disease is usually observed without medical intervention. In later stages, treatments are aimed at reducing the risk of fibrotic development, and decreasing inflammation and

pain by using corticosteroids, anti-inflammatory agents including NSAID, and cytotoxic agents, mainly Methotrexate [166]. In a large US study of more than 9000 sarcoidosis patients, 23% were pharmacologically treated, the most common medication was glucocorticoids with 56% of the patients treated [174]. Corticosteroids are however associated with side effects and toxicity [172], and efforts should be made to carefully evaluate its necessity. A recent cohort study aimed at reducing corticosteroid usage showed that the antimetabolite drugs Methotrexate or Azathioprine could be used to reduce corticosteroid dosage [180]. An option for steroid refractory disease is anti-TNF therapy. Infliximab has shown improvements in lung function in sarcoidosis patients [181, 182], and Adalimumab reduced cough and shortness of breath [183]. As a last resort, the most severely ill sarcoidosis patients receive lung transplantation, which however is associated with a median survival time of less than six years post transplantation [184].

Sarcoidosis genetics

Familial studies show a higher risk for sarcoidosis in families with at least one patient [185, 186]. A recent study of 23´880 sarcoidosis patients in Sweden reported a 3.7-fold increased risk for sarcoidosis in families with at least one case [187]. A twin study showed that for monozygotic siblings to a sarcoidosis patient, the risk of disease was increased 80 times [188]. This clearly indicates a genetic component contributing to sarcoidosis, one such predisposition seems to include HLA genes. HLA-DRB1*03 has long been associated with sarcoidosis [189], in particular with acute onset and good prognosis [189], which has been firmly established [171]. Also HLA-DQB1*0201 has been associated with a good prognosis [190], although this haplotype is closely linked to HLA-DRB1*03. DRB1*04, on the other hand, even seems to confer protection against sarcoidosis [189, 191], and HLA-DRB1*01 protects against sarcoidosis [192], or against chronicity within non-LS patients [167]. Further, Berlin *et al* found that HLA-DRB1*15 was more frequent amongst patients with chronic disease [189], and it has been reported that the combined HLA-DRB1*1501-HLADQB1*0602 predisposes for a worse outcome [193].

Altogether, there is strong evidence of a genetic predisposition for sarcoidosis based on familial studies, as well as beneficial or detrimental associations to HLA haplotype. Further, as HLA molecules present antigens to $CD4^+$T cells, it is not surprising that particular subsets of $CD4^+$ T cells have been found expanded in sarcoidosis.

Immunology of sarcoidosis

A generally accepted hypothesis for how sarcoidosis is induced is that an environmental trigger such as a pathogen or a toxic irritant sets off a series of immunological events in those who are genetically predisposed, leading to formation of granulomas and dissemination of disease [165, 194]. $CD4^+$ T cells are expanded in the BAL fluid to the degree that it is included for the diagnostic procedures [195]. The CD4/CD8 ratio is however not conclusive evidence of sarcoidosis as the ratio can vary greatly [196] and a recent study of sarcoidosis publications conclude that the ratio itself should only be used in conjunction with other parameters to diagnose sarcoidosis [197]. The role for T cells is however central in sarcoidosis, which is typically considered a T_h1 skewed disorder [165] with increased expression of inflammatory cytokines including IFNγ, IL-1β, IL-6 and TNF [198-200], and generally reduced levels of T_h2 cytokines [165].

The central hallmark of sarcoidosis is the granuloma formation, which occurs in affected tissues and is the result of a series of immunological events. Granulomas consist of a core of epithelioid, and multinucleated giant cells, surrounded by mainly $CD4^+$ T cells [201]. TNF is central to granuloma formation [202], and alveolar macrophages from sarcoidosis patients release TNF spontaneously [203], and vigorously [204] and they are suggested to even be the main source of TNF in sarcoidosis patients [205]. Genetic alterations of TNF is associated with increased risk of developing sarcoidosis [206], further speaking for a central role of TNF. Other key cytokines involved in sarcoidosis include IL-12 and IL-18. The two cytokines in combination have synergistic effects leading to favor T_h1 skewing by strongly increasing production of IFNγ [207, 208], which is central in sarcoid inflammation. And, clearly, in sarcoidosis, both IL-12 and IL-18 are elevated in BAL fluid [209, 210], and have been proven to favor IFNγ production in the patients [211]. Also the epithelial lining fluid of sarcoidosis patients contains elevated IL-18 levels, matching an increased expression of the IL-18 receptor on $CD4^+$ T cells in patients, possibly amplifying IL-2 generation from these $CD4^+$ T cells [212].

What initiates sarcoid inflammation and granuloma formation is not known, and putative antigens and triggers have been searched for. Dust exposure and living and working near hospital environments have been reported to associate with sarcoidosis [198, 213]. Mold, nanoparticles and mycobacteria in working and living

environments have also been connected to sarcoidosis, but surprisingly smoking has been suggested to correlate with a lower risk of sarcoidosis [214]. It was noted that rescue workers of the World Trade Center catastrophe in 2011 presented with higher incidence of sarcoidosis-like disease [215]. This has however been questioned, referring to insufficient proof of causality [216]. For almost 100 years, mycobacteria have been pointed out as a pathogen possibly implicated in sarcoidosis [217]. Mycobacterial DNA has been found in 50% of lung samples from sarcoidosis patients [217], and the mycobacterial enzyme Mycobacterium tuberculosis catalase-peroxidase (mKatG) has been found in sarcoidosis patients but not in healthy controls [218]. The role of Mycobacteria was further implicated in sarcoidosis by the finding of mKatG-reactive T cells in patients [219]. Mycobacterial involvement has however not been found in all patient groups and cohorts, and evidence of a disease-driving role by mycobacteria is lacking [217].

Stronger support of disease-driving antigens have however been found based on investigations of T cell receptors. As mentioned above, HLA-DRB1*03 is associated with sarcoidosis, although the acute form with good prognosis. When isolating lung cells from HLA-DRB1*03-positive sarcoidosis patients and eluting peptides from their HLA, Wahlström *et al* found peptides matching self-antigens including vimentin. Further, $CD4^+$ T cells with a specific alpha chain of the TCR have been found expanded in patients with HLA-DRB1*03 [220]. Also beta chain variants of the TCR has been shown to be expanded in sarcoidosis patients [221], and even a combination of alpha and beta chains; Vα 2.3, Vβ 22 [222]. This strongly implies reactivity towards a specific antigen or antigens. And, most striking, in a model fitting, peptides of the protein vimentin was found to fit perfectly in HLA-DRB1*03 as well as matching the Vα 2.3, Vβ 22 TCR [222].

LUNG CANCER

Lung cancer is one of the deadliest cancers, and its prevalence is increasing rapidly. Beyond the necessity to understand lung cancer mechanism to improve detection and treatment regimens from an ethical perspective, there are also enormous societal costs associated with lung cancer.

Epidemiology

In the US, lung cancer has increased dramatically the past century and cancer-related death due to lung cancer was before 1940 rare, but has increased by

multitudes compared to other cancer forms [223]. Globally, the risk of dying by cancer is largest for lung cancer, and lung cancer has been predicted to increase in women due to changed smoking habits [224]. The five year survival rate is around 18% for the US population [225], one reason for very high death rates is the late discovery of most cases, at the point where substantial pathological alterations throughout the airways are irreversible [225].

Immunology and pathology

Lung cancer is divided in subtypes depending on the cellular origin. Non-small cell lung cancer (NSCLC) is the most common type, representing around 85% of all patients, with the remaining 15% being small cell lung cancer (SCLC) [226]. NSCLC is further subdivided in adenocarcinoma, and squamous or large cell cancer, and adenocarcinoma is the by far most common diagnose amongst lung cancer patients [224, 226].

Tumor growth is the result of disturbed cellular homeostasis leading to the classical hallmarks of cancer which essentially defines immortal and invasive cells growing out of control [227]. The alterations contributing to tumorigenesis are generally induced by mutations. For lung cancer it is strongly related with inhaled irritants, mainly cigarette smoke, but also exposure to other toxic substances such as industrial dust including asbestos fibers, or inhalation of radon gas [228]. For lung cancer to metastasize, tumor cells must detach by degrading the surrounding extracellular matrix, migrate directly into a neighboring tissue or extravasate into blood or lymph circulation, and last settle and form a metastasis [229].

Genetical aberrations in lung cancer

Frequently occurring oncogenic mutations in lung cancer involve proteins with large impact on cell growth and differentiation including the epidermal growth factor receptor (EGFR), the anaplastic lymphoma kinase (ALK) and KRAS (from Kirsten Rat Sarcoma Virus) [226]. The alterations are induced during years of smoking, but giving up smoking after many years is no guarantee to safeguard against lung cancer. As many smoking-induced alterations represent permanent damage, smoking correlates with a lifelong increased risk of lung cancer, and 50% of diagnosed cases are ex-smokers [225].

Diagnosis and treatment

The majority of cancer-related deaths are due to metastatic spread, and lung cancer is often diagnosed at a point where substantial metastatic spread has already occurred [226]. Diagnostic procedures include biopsies of the lungs and affected tissues combined with radiology (most frequently computer tomography (CT)) to confirm and define the tumor characteristics and stage of disease, which includes the degree of spread to lymph nodes [226].

Early treatments were entirely based on chemotherapy, with a median survival of 8 months after diagnosis compared to 4-5 months for untreated lung cancer [230]. Improved drug development and usage has increased survival to 12 months [231]. However, first line of treatment depends on the stage of the disease. Stages I and II of (non-small cell) lung cancer are the most uniform stages (later stages are more heterogeneous and treatments must be individualized), and are approached with surgery to remove tumor mass, whereas stage II to III patients usually undergo combined surgery and chemotherapy. Radiotherapy has been reported to actually associate with a worse outcome compared to surgery, although comparisons may be unfair as there is a bias in which patients are selected for the two treatment options. However, more recent development of radiotherapy, as well as its use as a post-operative combination therapy supports its role in at least some patients [232]. Immune therapy provides new outsights for treatment, and patients with high expressions of the checkpoint blocking (immune inhibitory) PD-L1 are actually suggested to receive the PD-1 blocking antibody treatment Pembrolizumab as first line of treatment [233].

4 PULMONARY EVS

With their great diversity in phenotype, cargo and mediated effects it is highly likely that EVs play roles in intercellular communication also between cells of the airways.

The airways can be sampled for luminal contents using bronchoalveolar lavage (BAL), and the first findings of human airway EVs revealed BAL fluid (BALF) exosomes with surface expressions of MHC I and II, costimulatory CD86 and CD54 [234]. CD54 is a key molecule in immune crosstalk, as it binds LFA-1, which is expressed by most leukocytes [235] and mediates adhesion. This cocktail of immune stimulatory molecules speaks for that BALF EVs interact with immune cells and contribute to disease and/or immune homeostasis. We recently postulated the hypothesis that pulmonary EVs even may be a general vehicle initiating or aggravating inflammation also in non-pulmonary disorders [236].

CELLULAR ORIGIN OF LUNG EVS

An important question in dissecting the roles of EVs in the lungs is what cells they originate from. Mapping the EV cell sources is difficult, and further complicating is the fact that the condition of the releasing cell affects the EV amounts and phenotypes being released. It is thus highly likely that during steady state the released EVs differ from those released during inflammation or disease. It is tempting to assume that the most numerous cells of the airways are the main source of EVs. It is however not necessarily the most common lung EVs that are of greatest interest, certainly not so in disease where even a minority of EVs may be responsible for pathogenic effects.

However, considering **epithelial cells**, they line the entire airways, and it is highly likely that epithelial EVs thus are present in the respiratory system. Epithelial cells are readily exposed to inhaled antigens and irritants, and it is becoming increasingly clear that epithelial cells are important players in pulmonary immunity [237], possibly also releasing EVs upon stimuli. Epithelial cells of the bronchi co-express CD63 and CD81, which was considered evidence that they are a central source of EVs in the lungs [238]. Several findings point to a role of epithelial cell-derived exosomes modulating lung immunity by interacting with mononuclear cells. Gazdhar *et al* cultured monocytes in media conditioned by bronchial epithelial cells, which favored

expansion of inflammatory monocytes positive for CD141, CD123 and DC-SIGN [239]. Further, they found monocytes of the same unique phenotype expanded in sarcoidosis patient BAL fluid. Connecting the dots, it is tempting to assume that the monocyte-engaging media conditioned by the epithelial cells is indeed enriched in EVs. In an attempt to dissect further the role of epithelial exosomes, the exosome inhibitor GW4869 [43] (mentioned earlier) was used in an asthma model which alleviated symptoms of asthma, presumably by decreasing leukocyte infiltration [238], however with the cautionary note that GW4869 most likely has unwanted effects beyond reduced exosomal release. In support of a role for epithelial cell-derived EVs as contributors to inflammation, experiments exposing epithelial cells to cigarette smoke extract (CSE) has repeatedly been shown to result in release of pro-inflammatory EVs. *In vitro* modeling of chronic obstructive pulmonary disease (COPD) showed that epithelial cells released large amounts of exosomes upon CSE-stimulation, and that these exosomes contributed to IL-8 production by shuttling the protein cysteine-rich angiogenic protein 61 (CCN1), which is associated to chronic inflammation in both COPD, RA and cancer [240]. CSE-stimulated bronchial epithelial cells release EVs with seemingly pro-fibrotic features including promoting differentiation of fibroblasts of the lungs into myofibroblasts [241].

The most numerous immune cells in BALF are **macrophages**. Alveolar macrophages (AMs) are central in pulmonary immunity, contributing with surveillance and phagocytic capacity. In COPD they are strongly expanded [242], and in Sarcoidosis there is a general increase in all leukocytes in the BAL fluid with macrophage counts rising several fold [202]. Findings have supported a role for AM-derived EVs in inflammation in pulmonary compartments, e.g. by reversing IFNγ-mediated induction of signal transducer and activator of transcription (STAT) [243]. They were also found to be taken up by alveolar epithelial cells, speaking for an intercellular communication within the lungs mediated by EVs which also could transport suppressor of cytokine signaling (SOCS) proteins [243]. Mycobacteria are intracellular bacteria infecting alveolar macrophages, which in turn release EVs capable of modulating inflammation [244] and even priming $CD4^+$ as well as $CD8^+$ T cells [245].

Although far less frequent than macrophages, **granulocytes** are present throughout the airways, and are a possible source of pulmonary EVs. Eosinophils and neutrophils respond to noxious stimuli via pattern recognition receptors, and can

release potent pro-inflammatory granules [246, 247]. They both release EVs, potentially with cargo modulating inflammation during both steady state and disease. Neutrophils are strongly associated with pulmonary disorders including asthma and allergy and can release exosomes with antibacterial peptides including myeloperoxidase [248]. Interesting from an asthma perspective, where LTs are mediating inflammatory effects, neutrophil EVs are capable of transporting arachidonic acid, the starting substrate for LT synthesis, to platelets and contribute to innate immunity [249]. Again this EV –based crosstalk is more complex than just contributing to disease, as it was shown that neutrophilic EVs engaging platelets actually reduced the mortality in a model of lung inflammation [249]. For asthma patients, cultured eosinophils release more exosomes compared to healthy controls, and both healthy and patient eosinophils activated by IFNγ increase EV release [250]. The granulocytes perhaps most associated to airway disease are mast cells. Mast cells are central in airway immunity, equipped with PRRs and surface-bound IgE readily acting upon antigen encounter. They too release EVs, and in an attempt to map the proteomes of mast cell EVs, Veerappan *et al* compared exosomes isolated from tracheal aspirations, and from a human mast cell line and found great similarities [251] indicating that mast cell EVs indeed are present in human airways. Mast cell EVs have further been found to engage both DCs [252], B and T cells [253], and endothelial cells [254] to induce maturation, proliferation and activation respectively.

Beyond the innate cells mentioned above, it is possible that also **DCs** contribute to the pool of airway EVs, although DCs are in clear minority compared to most other cells of the airways. Considering that sarcoidosis BALF exosomes are enriched in MHC II [255], it is however possible that many of them are APC-derived and that e.g. DCs play immune modulatory roles in the lungs via EVs.

T cells are expanded in sarcoidosis, as discussed above, and may be another source of pulmonary EVs. T cells release exosome-sized ILVs with cytolytic granules [256, 257], and upon activation they release exosomes with incorporated portions of the TCR [258]. T cell exosomes may also act as deadly vehicles, as they can incorporate membrane-bound Fas ligand [259, 260] and thereby regulate immunity by inducing apoptosis in recipient cells. In an *in vivo* tumor model, T cell exosomes induced elevated expression of a metalloproteinase implicated in tumor migration, and favored lung tumor invasivity [261]. Activated T cells seem to release exosomes

stimulating bystander T cells [262], including promoting $CD8^+$T cell-release of IFNγ and granzyme B [263].

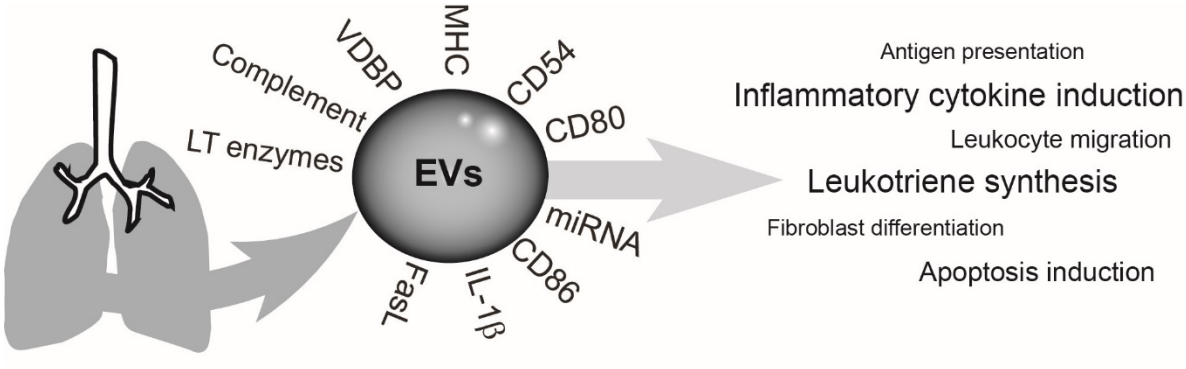
In conclusion, many cell types present throughout the airways are capable of releasing EVs with cargo and functions that may contribute to pathological development or aggravation in the respiratory system.

EVS IN LUNG DISEASE

The Gabrielsson group showed that exosomes with MHC II and costimulatory molecules are present in bronchoalveolar lavage fluid (BALF) [234], which directed attention to pulmonary EVs. In asthma patients, BALF exosomes have a dysregulated miRNA profile [264], carry LT-forming enzymes, and promote LT formation and IL-8 generation in bronchial epithelial cells [265]. Asthma patient eosinophils release more exosomes compared to healthy controls [250], and Kulshreshtha *et al* reported that IL-13 stimulated epithelial cells released exosomes capable of increasing macrophage proliferation and chemotaxis [238].

Fibrotic diseases including Idiopathic pulmonary fibrosis (IPF) and sarcoidosis have also been associated with potentially pathological EVs. IPF patients have serum exosomes enriched for micro RNA (miR)-21-5p, which is mostly of biomarker interest [266], but may also reveal clues about the disease. IPF involves imbalance in WNT-protein signaling, and recently, primary lung fibroblasts from IPF patients were found to release EVs enriched in WNT-5A, and contribute to fibroblast proliferation and TGFβ formation, suggesting EV contribution to IPF [267]. In pulmonary sarcoidosis, exosomes isolated from BALF are elevated in numbers and enriched in MCH class I and II compared to healthy controls [255]. Patient exosomes further stimulated PBMCs to produce more IL-13 and IFNγ, as well as bronchial epithelial cells to release more IL-8 compared to healthy exosome stimulations [255]. However, which cells within the PBMC compartment that exosomes engage was not studied, leading to the design of study III (below) in this thesis. Also in COPD, implications of EV involvement have been found, including transport of CCN1 (as mentioned above) by exosomes from cigarette smoke-exposed epithelial cells [240], and a suggested COPD fibrosis-promoting feature of exosomes via miR-210 [241].

In summary, many interesting findings point to a role of exosomes and other EVs in lung diseases, but clinical studies on pulmonary EVs are still very few, and this thesis contributes with three studies (II-IV) on clinical samples.



Schematic figure of pulmonary EV cargo and effects. Based on the findings of us and others, it is clear that EVs from the lungs, but also other compartments, can incorporate both nucleic acids and proteins with a potential to engage the immune system in numerous ways. VDBP = Vitamin D Binding Protein (study II).

5 METHODS

Most methods used in the projects of this thesis are commonly used immunoassays, for detailed description see the materials and methods section of each study. EV research does, however, involve techniques less commonly used as well as adaptations of methods, which may require explanation. For these, a section follows below.

Overview of methods used

Method	**Study**			
	I	**II**	**III**	**IV**
Lipidomics				x
Cytometric bead array			x	
Western blot	x	x		x
Migration assay				x
Patient sample		x	x	x
Ultracentrifugation	x	x	x	x
ELISA	x	x		x
ELISpot	x			
Flow cytometry	x	x	x	x
Animal studies	x			
Nanoparticle tracking	x	x		x
Cell culture	x		x	x
Electron microscopy	x	x		x
Sucrose gradients		x		x

EV Flow cytometry

Flow cytometry is designed to detect cells, which are magnitudes larger than EVs, and is therefore not suitable for direct acquisition of single EVs. There are alternative approaches to solve this issue, including using modified instruments to enhance

resolution and setting primary gates according to fluorescence rather than forward/side scatter properties [127]. We and others apply a technique based on using beads large enough to be detected in the flow cytometer FSC/SSC panel. The beads can be coated with antibodies directed against exosome epitopes of choice, most commonly we target CD9, CD63 or MHC II. The antibody-coated beads are incubated with the EV samples to capture and bind EVs to the beads. The beads are washed and the captured EVs can be stained using a conventional flow cytometric protocol. The advantage of this adapted flow cytometry is that it requires relatively little material (compared to e.g. western blot analysis). It is also robust in the sense that the beads are very easily detected in the flow cytometer, and a fluorescent signal is only achieved if both a capturing and a detection antibody have bound their targets, which reduces false positive signals. The main disadvantage of the method is that only a subgroup of EVs are captured, and the choice of capturing antibody will strongly affect the results.

Nanoparticle Tracking Analysis (NTA)

This technique is based on a converted microscope combined with a laser and a high resolution camera. The laser is passed through the sample in a chamber, and live video sequences are recorded and analyzed using a designated software. The software detects the refracted laser light of each particle in the sample, and tracks its movements. All nano-sized entities are constantly vibrating (Brownian motions), and these vibrations are proportional to particle size. The NTA software can therefore calculate the particle size distribution of the sample. The software also reports absolute particle concentration, which we however interpret carefully. The instrument is calibrated and tested using monodisperse suspensions of synthetical nanoparticles. Aquiring polydisperse, biological samples is associated with a far greater complexity including a very large difference in capacity to refract light (refractive index) between the smallest and the largest particles. The resulting absolute concentrations must therefore be very carefully evaluated, and all technical parameters must be strictly controlled not to induce any bias. In study I and II, where particle concentration analyses were made using NTA, the results are therefore always discussed in relation to another sample, for example between patients and healthy volunteer exosomes.

6 RESULTS AND DISCUSSION

The four projects included in this thesis are built on previous findings of the Gabrielsson group and others, indicating immunogenic potential of exosomes. Previous findings have contributed to improved engineering of exosomes for immune therapy, the discovery of exosomes in human BALF, and exosomal capacities to transport and generate LTs. Study I on exosomes and MVs *in vivo* was designed to further broaden investigations on EVs and shift focus from only exosomes to include several EV subtypes. Study II and III are continuations of the discovery of BALF exosomes. These clinical collaborations focus on sarcoidosis patient exosomes, and in study III also MVs, with the aim to contribute to understanding the disease better but also to search for possible biomarkers. Study II presents a substantial proteomic characterization of the patient exosomes and a highlights a possible biomarker. Many of the proteins upregulated in patients were immune modulatory with potential effects on monocytes including chemotaxis. Considering that sarcoidosis is an inflammatory disorder, project III was designed to functionally evaluate whether the patient EVs can engage and stimulate monocytes to modulate cytokine production. Project IV aims at shedding light on the role of exosomes in a lung cancer setting, with main focus on their LT-associated effects on tumor cell migration and survival.

STUDY I – EXOSOMES AND MICROVESICLES IN VIVO

Concept and main findings

An exciting application where EVs may contribute to human health is immune therapy. As exosomes can transport antigen and induce antigen-specific immune responses *in vivo* [268], they have the potential to reach clinical use. Exosomes have been intensely investigated since the first reports of their immunogenic capacity, whereas MVs have attracted far less attention in these settings. Study I was designed to address the abilities of also MVs to generate antigen-specific immune responses *in vivo*. Bone marrow-derived DCs (BMDCs) can be pulsed with ovalbumin (OVA), which together with MHC and costimulatory molecules is incorporated into BMDC exosomes with OVA-specific immunogenic capacity [109, 116, 269, 270]. In the current study we expanded the setting to also include MVs

isolated from the same BMDC cell cultures, to compare exosomes and MVs side-by-side.

Characterizations of the two EV types showed great phenotypic overlap. Similar surface marker expressions were found for exosomes and MVs including MHC I and II, CD54, CD80, CD86, and CD40. The vesicle size distributions were also similar, MVs being slightly larger, and approximately equal numbers of exosomes and MVs seemed to be released per cell. EM imaging further showed that exosomes and MVs resemble each other morphologically. We thus hypothesized that both EV subtypes have immune-modulatory capacities. To evaluate this *in vivo*, both vesicle types were injected into C57Bl/6 mice with a boosting dose after seven days and immunological analysis conducted 14 days after the initial immunization. Mice immunized with exosomes showed significantly increased proportions of OVA pentamer$^+$ CD8$^+$ T cells, as well as OVA-specific IgG production, which is supported by previous findings [115, 116, 269]. MVs on the other hand showed no induction of CD8+T cell responses, and only a slight production of OVA-specific IgG. Further, the proportions of GCB cells were elevated by exosomes only, and surprisingly the effect was independent of the OVA antigen being present on the vesicles. In IFNγ ELISpot assays, splenocytes from the immunized mice were re-stimulated with the whole OVA antigen or either of its MHC I or MHC II immune-dominant peptides. When re-stimulating with the MHC I peptide, splenocytes from both exosome-treated and MV-treated mice displayed a significant, and equally high, IFNγ response. Further, we hypothesized that MVs and exosomes combined may have synergistic effects, so mice were also immunized with a combination of the two EVs. However, this did not show effects stronger than what the exosomes were inducing. To investigate how exosomes have this immunogenic advantage over MVs, we analyzed the contents of OVA in the vesicles. Substantially higher levels of OVA were found on the surface of the exosomes by ELISA. By western blot analysis, OVA was found strongly enriched in exosomes, but OVA was barely detectable in in the MVs.

Discussion

Exosomes have a history, and promising future, of investigations concerning their immunogenicity and possible use in therapy. MVs are most frequently bypassed in studies. Accumulating evidence is however indicating that MVs may be more similar

to exosomes than previously realized, and on a speculative basis possibly even more stimulatory given the right conditions. Our findings that MVs and exosomes seem to be fairly similar in size contradict previous appreciations of MVs as much larger than exosomes [32]. The size of the vesicles may affect how they traffic circulatory systems, and where and how they are taken up, so equal sizes speaks for similar abilities from this viewpoint. On a technical note, NTA which was used to determine the EV sizes is not suitable for greatly polydispersed samples, and if MVs have a very large size span, analyses of these would be more error prone. However, by EM imaging we also found quite similar sizes, which is further supported by a recent publication [271]. There it was reported that the diameters of DC-derived exosomes were 150 nm and MVs 168 nm, to be compared to our findings of 153 nm and 170 nm respectively. The overlap also in surface marker expressions further strengthens an image of exosomes and MVs as rather close siblings. The expressions of syntenin and actinin was, however, inversely expressed in the two vesicle pellets, indicating that they are indeed different vesicle types [47], and that MVs are not just exosome-contaminated pellets.

Interestingly, exosomes, but not MVs, induced OVA-specific $CD8^+$ T cells as detected by pentamer-based staining, whereas both EVs induced splenocytes to react similarly to MHC I peptide re-stimulation. As the re-stimulation is conducted for 22 hours, it is possible that the two EV types induce $CD8^+$ T cell responses with different kinetics. It is thus possible that longer time is needed for MVs to induce their strongest effect. Further, although less potent than exosomes, the ability of MVs to induce an OVA-specific response is interesting when considering that we found very little OVA associated to the MVs by ELISA and no OVA at all by western blot. Is then less antigen needed when associated to MVs, suggesting a strong immunogenic potential?

Another intriguing question is how OVA can be taken up by MVs. DCs are capable of antigen uptake via several pathways including phagocytosis, macropinocytosis and receptor-mediated endocytosis [272]. As all three pathways converge into the MVBs [272] where exosomes are formed, it is possible that DC exosomes incorporate exogenous antigen. Further, beyond cargo derived from endosomes, exosomes can also incorporate molecules from the cytosol as a result of recruitment of cytoplasmic components to membrane micro-domains [56]. MVs can be packed with cargo from the cytosol in the same manner [56], but clearly not from endosomal compartments.

One possibility is, that OVA bound to the surface of the DC would be incorporated into forming MVs. As mentioned above, DCs have several options for antigen uptake and OVA has been shown to be taken up by micropinocytosis, but mainly by receptor-mediated endocytosis dependent on the mannose receptor [273, 274]. In our system where OVA is added in enormous amounts, it is possible that phagocytic mechanisms of the DCs are saturated. On a speculative basis, this could lead to OVA being present both in the cytosol (from where it may be incorporated into MVs), as well as stuck to the surface of the DCs bound to receptors such as the mannose receptor. Budding MVs would thus be able to depart from the cell with OVA attached to them.

On a final note, we found great overlap in phenotype but distinct functional activities of exosomes and MVs. Recently, Tkach *et al* reported that DC-derived MVs and exosomes had very similar stimulatory effects on T cells *in vitro* [271]. As the two studies have nearly identical EV isolation protocols, it is highly interesting to relate the findings. Tkach *et al* found that immature DCs released both exosomes and MVs favoring release of Th1-skewing cytokines from primary $CD4^+$ T cells, and after maturing the DCs with IFNγ, both exosomes and MVs induced $CD4^+$ T cell proliferation equally efficient [271]. Although *in vitro* evaluations cannot always be extrapolated to the *in vivo* situtation, the study supports our findings of an immunogenic potential of MVs.

STUDY II – CHARACTERIZATIONS OF SARCOIDOSIS EXOSOMES

Concept and main findings

A main focus of this thesis is pulmonary sarcoidosis, and how EVs may play a role in the course of disease, or be exploited for biomarker search. In study II we investigated exosomes isolated from the BALF of sarcoidosis patients, to characterize their proteomic content and thereby draw a road map for further investigation and biomarker search. Patient and healthy control BALF exosomes were analyzed by proteomic characterizations, flow cytometry, ELISA and Western Blot.

Flow cytometry was initially used to verify that the exosomes in the current study overlap characteristically with what has been published previously. We therefore

used beads coated with anti-MHC II as capturing antibodies, as previously published [255] and found CD9 and CD63 which verified a vesicular character as well as MHC II and CD54, supporting an immune-modulatory role of the exosomes.

Next, the proteomic analysis detected more than 690 proteins in the BALF exosomes, which provides an excellent outsight for further investigation of proteins implicated in sarcoidosis, and possibly EV-associated mechanisms in lung diseases in general. A brief technical note is that the proteomic technique ITRAQ (isobaric tag for relative and absolute quantification) is in the current setting conducted in a semi-quantitative manner, so protein levels are not reported in absolute amounts. To relate the expressions in patients and healthy individuals, all results were therefore normalized to internal controls, which were composed of a mix of patient and healthy exosomes. The expression of each protein can thus be presented as a proportion relative to the internal control, and the relative abundance of each protein is presented.

Many proteins upregulated in patient exosomes were associated with inflammation, including immunoglobulins, many components of the complement system, and the LT-forming enzyme LTA4H. Of the complement components, C3 was elevated two-fold on sarcoidosis exosomes, and western blotting validated an enrichment on patient exosomes. Vitamin D metabolism is frequently disturbed in sarcoidosis patients [166], and we found a clear increase in the Vitamin D binding protein (VDBP) in patient exosomes. Indeed, others who investigated the full BALF proteome of sarcoidosis patients have highlighted Vitamin D metabolism, and multiple components of the complement system [275, 276], and even VDPB [276] as strongly elevated in sarcoidosis. We validated the VDBP findings by ELISA, and found that VDBP was significantly higher on BALF exosomes from sarcoidosis patients compared to healthy control exosomes, but also compared to exosomes from six patients with inflammatory lung disorders other than sarcoidosis. To further evaluate the potential of exosomal VDBP as biomarker, we also tested exosomes isolated from patient serum. Again, we found a significant increase in exosomal VDBP for patients compared to healthy. Moreover, when also analyzing whole plasma we did not see this difference, which speaks for an enrichment of VDBP in exosomes specifically.

Discussion

EVs are laborious to investigate, and patient material including BALF adds further heterogeneity and complexity. Broad characterizations are therefore highly warranted. Our findings in study II of nearly 700 proteins in the BALF exosomes is promising for future EV experiments in sarcoidosis, possibly also for other pulmonary investigations. Although the patient cohort used in the proteomic characterization was quite small (n=15 patients + 5 healthy), the list of proteins can be used both to study differences between patient/healthy, but also regardless of differences as a list of protein candidates for further pursuit of biomarkers.

Perhaps most striking, virtually all complement components were increased, whereas complement regulatory CD55 was decreased. Both alveolar type II epithelial cells and bronchial epithelial cells can produce and release complement proteins, and increased complement activity has been reported in lung disorders including asthma, IPF, and COPD [277]. Further, previous findings of LT-components in exosomes [265, 278] were again supported in the current study, now in sarcoidosis patient BALF with a different pattern in patients compared to controls. LTs are fundamentally associated with inflammatory lung disease including asthma [279], and our current findings imply a role for exosomal LTs in lung disorders.

The findings of increased exosomal VDBP according to both proteomics, and in validation experiments was interesting. Sarcoidosis patients frequently show dysregulated Vitamin D metabolism, possibly as a consequence of cells within the granulomas which are hyperactive in metabolizing Vitamin D [280, 281]. Further, beyond transporting Vitamin D to tissues, VDBP can activate macrophages [282] and in concert with complement components stimulate chemotaxis of monocytes [283] and neutrophils [284]. A speculation is that exosomes carrying VDBP, as well as other chemotactic proteins, exit pulmonary compartments and reach circulation where they engage immune cells to favor migration.

From a biomarker point-of-view, although there was an overlap in the levels of exosomal VDBP between patients and healthy, one has to consider that this was a proof-of-principle that disease-associated markers may be enriched on plasma exosomes in sarcoidosis (and other pulmonary disorders for that matter). Refined and optimized settings could very well produce diagnostic aid based on EVs, especially if several molecules are studied in combination.

Sarcoidosis can affect multiple organs, and it is not clear if the disease spreads from one organ to another or if it simultaneously develops in several organs. Transplanted individuals receiving organs from donors with sarcoidosis have been found to develop sarcoidosis [285-289], which speaks for an ability to disseminate. EVs are capable of passing the blood-brain-barrier as well as other borders of cellular integrity. Further, they have inherent abilities to transport antigen and prime antigen-specific immune responses, but also to shuttle thousands of proteins and to induce pro-inflammatory effects in both innate and adaptive immunity. Is it possible that EVs could transfer sarcoidosis-inducing effects between organs? Sarcoidosis is an inflammatory disorder, and study II, together with previous reports, indicates that exosomes can promote an inflammatory milieu, possibly contributing to sarcoid progression.

STUDY III – FUNCTIONAL TESTS OF SARCOIDOSIS EVS

Concept and main findings

Previous findings indicated that sarcoidosis BALF exosomes have pro-inflammatory effects including induction of IFNγ in autologous PBMCs [255]. Study II further highlighted the cargo of sarcoidosis exosomes as enriched in pro-inflammatory molecules affecting both innate and adaptive immunity [290]. Study III is a continuation of this track, with the aim to functionally evaluate exosomal inflammatory capacities. Although sarcoidosis is caused by unclear mechanisms, inflammation and granuloma formation are central aspects. In inflammation, innate immune cells including neutrophils and monocytes are rapidly recruited to the site of inflammation and contribute to inflammatory events [16, 291]. Our findings in study II included increased exosomal complement components and VDBP, as well as proteins with chemotactic properties on T cells and monocytes (in supplementary material, [290]). We therefore hypothesized that BALF exosomes contribute to sarcoid inflammation, in part by engaging innate immune cells and induce cytokine release, which would accelerate inflammatory events. When stimulating allogeneic PBMCs for six hours with patient exosomes, and analyzing the cells by intracellular flow cytometry we found that IFNγ was not increased in $CD4^+$ or $CD8^+$ T cells or natural killer (NK) cells (the latter potently releasing IFNγ during first hours of infection [292]). There was a significant increase of IFNγ in monocytes, however of

low magnitude and not with dose-dependency. IFNγ release in the cell cultures was also evaluated, but no detectable levels were seen after six or 22 hours. However, IL-1β$^+$ monocytes were significantly, and strongly, increased in a dose-dependent manner by patient exosomes (but not by healthy control exosomes). To include several EV subtypes, and to delineate whether the monocyte effect is dependent on bystander PBMCs or is a direct effect on the monocytes, we stimulated either whole PBMCs or enriched monocytes with exosomes or MVs. Patient exosomes induced significant IL-1β induction on monocytes both within the PBMC population and on enriched monocytes, which speaks for a direct activation of the monocytes induced by the patient exosomes. MVs had trends of similar effects as the exosomes, but not of significant difference to PBS stimulations. We further analyzed the release of more innate early cytokines in the supernatants and found IL-6, IL-1β and TNF induced by patient, but not healthy, exosomes at six hours and for some also after 22 hours. Further, CCL2 was strongly induced by the exosomes from a number of patients. As study II showed an increase in LT-forming enzymes in patient exosomes, we added the LT receptor antagonist Montelukast to investigate whether any of these effects were LT-dependent. The high levels of CCL2 induced by some patients were significantly reduced by Montelukast. Reactive oxygen species (ROS) were also induced in both PBMCs and monocytes by patient exosomes compared to healthy exosome stimulations, which adds to an image of pro-inflammatory EVs in sarcoidosis.

Discussion

We could not detect responses supporting an IFNγ-inducing role of sarcoidosis EVs, which was seen in a previous study from our group. It is however possible that the discrepancies in these findings are due to the different setup of the two studies. We here used an allogeneic system with healthy donor PBMC recipient cells, whereas the previous publication was based on autologous patient recipient cells. Exosomes can prime lymphocytes directly, but in allogeneic system there is no self MHC recognition and therefore no direct priming of T cells can occur. Looking into other early innate cytokines, however, the induction of IL-1β both intracellularly in monocytes and released by both PBMCs, and monocytes, was clearly promoted by patient exosomes. As also IL-6, TNF, and CCL2 were released significantly more by cells stimulated with patient exosomes compared to healthy, this suggests a pro-inflammatory nature of the sarcoidosis exosomes. The induced cytokines are all

implicated in sarcoidosis [293], strengthening the image of EVs contributing to sarcoid inflammation.

The finding that also cultures enriched for monocytes could be induced by sarcoidosis exosomes to release the cytokines, was highly interesting, and suggests that these cells are a major responder cell to exosomes in the lung. Monocytes have also been implicated in sarcoidosis, with expanded intermediate monocytes [294, 295], which is a population capable of producing large amounts of TNF [296]. Strikingly, sarcoidosis patients with expanded intermediate monocytes have been shown to have the best response to anti-TNF therapy [297]. It is thus possible that exosomes contribute to sarcoid inflammation by engaging monocytes to release pro-inflammatory cytokines. Monocytes express the CysLT1 receptor [298] and respond to LT-mediated signaling, one effect being production of CCL2 [299]. The Montelukast-induced reduction of the highest CCL2 response by exosomes provides outsight for clinical applications.

STUDY IV – LUNG CANCER PLEURAL EFFUSION EXOSOMES

Main findings

Here we studied exosomes isolated from pleural effusions of patients with lung cancer to investigate their capacities to promote tumor cell survival and migration by modulating LT metabolism. LTs are implicated in cancer, and a lower expression of CysLT1R is associated with better prognosis in colorectal [300] and breast cancer [301]. One of the most tumorigenic LTs is LTD_4, which binds CysLTR1 with high affinity [302]. We here found that cultured tumor cells from the PEs of lung cancer patients released low levels of LTD_4. However, exosomes from the same PEs were enriched in the enzyme GGT-1, which converts LTC_4 to LTD_4. We verified a capacity of the exosomes to promote this conversion by adding LTC_4, and noted a rapid conversion to LTD_4. Further, the amounts of LTC_4 available for conversion to LTD_4 *in situ* may therefore directly limit the tumorigenic effects of PE exosomes. We therefore investigated cellular sources of LTC_4 in the PEs and found that monocytic cells had a greatly increased ability to generate LTs. They had an almost 100-fold higher capacity than the mixed cancer cell population in the PEs, strongly suggesting that monocytic cells are a plentiful source of LTC_4 within the TME.

Exosomes have been thoroughly proven to support the TME and favor metastastic migration, and to even prepare a metastatic niche [86]. Exosomes from seven lung cancer patients were therefore further tested for their capacity to promote migration of the lung epithelial cancer-cell line A549, as well as of cells isolated from one of the patients. The exosomes clearly accelerated wound healing in a migration assay, indicating a potential to favor migration and metastasis of tumor cells in lung cancer. The tumor cells isolated from the PE were also sensitive to the CysLT receptor antagonist Montelukast, and they could partly be rescued by PE exosomes.

Discussion

Study IV expands the scope of this thesis to also touch upon cancer-promoting features of exosomes. As mentioned, GGT-1 converts LTC_4 to LTD_4, and LTD_4 levels have been correlated with cancer in the liver [303], as well as the stomach and the lungs [304]. Further, GGT-1 has been found in prostate cancer exosomes and even been suggested as a biomarker [305]. Our findings of GGT-1 in the PE exosomes are thus supported by previous reports, but also strengthens the hypothesis that exosomes contribute to tumorigenic LT formation. The PE exosomal capacity to actually also generate LTD_4 provided proof that the GGT-1 was indeed intact and functional. Moreover, as exosomes promoted cancer cell migration which was reduced by Montelukast, it is even clearer that the cancer exosomes use LT pathways to contribute to pathogenesis.

Previous findings have reported that Montelukast interferes with survival or progression of lung cancer cells [306], and prostate cancer cells [307], and it was found to inhibit tumor growth in a mouse model of colon cancer [308]. We here found that Montelukast induced apoptosis in patient tumor cells, and perhaps most interestingly that the effect was decreased by PE exosomes. Taken together, PE exosomes are enriched in GGT-1 which favors generation of tumorigenic LTD_4, and they favor migration and survival of cancer cells in a LT-dependent manner.

7 CONCLUDING REMARKS

EVs have a potential to revolutionize biological research and human medicine. Clinical trials based on EVs have already been conducted, and the number of EV publications is increasing exponentially. Previously considered randomly released waste disposal vehicles, EVs are today evaluated for their characteristics, functions, and potential applications in most areas of medical research. This thesis hopefully contributes to the field.

Study I on MVs and exosomes showed only modest efficacies of the MVs, but even so, it is of great importance to consider MVs when designing immune therapies, searching for biomarkers, or mapping EV functions. It is highly likely that settings optimized for generation of efficient exosomes and MVs are not the same. Thus, based on study I, re-evaluating all aspects of cell culturing and antigen-loading may very well reveal greater potential of the MVs. Should EV subtypes favor different immune responses, one could argue that combined EV populations provide broader responses. Or could it be that different EV subtypes are suitable depending on the character of the tumor or pathogen against which an immune response is warranted, which require different immune responses?

Sarcoidosis is caused by unclear factors, and diagnostic as well as prognostic evaluations are difficult and consume time and resources. The proteomic characterization of sarcoidosis exosomes in **study II** with its validations of selected findings provided a long list of protein candidates for biomarker hunting, but also for providing clues and perhaps drug targets in the mysterious disorder. **Study II and III** can hopefully contribute to increased understanding of sarcoidosis, but also how inflammation may be spread both within pulmonary compartments, but also to distant sites. **Study IV** touches upon cancer, and the role of exosomes, and supports findings in study II and III on LT association to lung EVs.

To what degree EVs play a role in sarcoidosis and lung cancer remains to be fully elucidated, but we have found clear indications that pulmonary EVs contribute to inflammation. Inflammation is central to many pathologies including sarcoidosis, but also to cancer, which is strongly associated with a low level of chronic inflammation.

EVs are capable of inducing inflammation via many different mechanisms. Exosomes [210] but also MVs [211, 212] can transport IL-1β, and have even been suggested to be one of the main exocytic transport mechanisms for IL-1β [309]. Inflammations in pulmonary compartments often lead to increased permeability of endothelial barriers [218]. Further, in multiple sclerosis (MS) there are inflammatory EVs in the affected areas [207-209], which plausibly contributes to degraded endothelial integrity [209], and increased leakage across the blood brain barrier [208]. There is also plenty of evidence showing that EVs can cross healthy blood brain barriers [203-206]. In conclusion, it is possible that EVs deliver their cargo and induce effects beyond barriers, either by crossing them or by promoting their degradation.

On a final note, it is highly interesting to connect the inflammatory capacities of EVs to their cargo of LT components. Exosome-modulated LT pathways may contribute to pathology in multiple lung disorders, presumably also to inflammation in other contexts. This underlines the clinical potential for LT receptor antagonists, which are safe, well-tolerated, and readily available.

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