Thymic exosomes

Effects on selection and maturation of thymocytes

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Cover illustration by Christina Lundqvist. Human thymic tissue, stained with TSG101 (green) present in exosomes, and AIRE (pink) expressed by mTECs.

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ABSTRACT

T cell tolerance is primarily shaped in the thymus, through direct and indirect presentation of self-antigens to developing T cells. Medullary thymic epithelial cells (mTECs), producing and expressing self-antigens, together with dendritic cells (DCs) are the key antigen presenting cells in the thymus. In addition to direct presentation of self-antigens by mTECs, antigens are transferred to DCs followed by presentation to developing thymocytes. The underlying mechanism of this antigen transfer is not understood. Extracellular vesicles (EVs), and more specifically exosomes, are known to carry antigens and genomic material with a biological function to target cells. This thesis report thymic exosomes as mediators of antigen transfer important for T cell maturation, negative selection and Treg development. Furthermore, we show that thymic exosomes carry co-stimulatory molecules and MHC II. In the first paper, we report that exosomes derived from primary human thymic epithelial cell cultures carry self-antigens associated with autoimmune diseases. The second paper demonstrates how exosomes from mouse thymic tissue induce the final maturation of thymocytes, independently of antigen presenting cells (APCs), before they egress the thymus as T cells, *in vitro*. In order to study the impact of thymic exosomes on central tolerance *in vivo*, we used the transgenic mouse model insHEL-3A9 TCR, which is well described in studies of central tolerance. We report that thymic exosomes from HEL-mice carry the dominant HEL-peptide in complex with MHC II on their surface. Injection of thymic exosomes from HEL-mice into 3A9 TCR mice resulted in a reduction of HEL-specific thymocytes and expansion of peripheral Tregs, suggesting that thymic exosomes are important for tolerance induction. In conclusion, this thesis reports that thymic exosomes carry self-antigens and are mediators for the induction of central and peripheral tolerance.

Keywords: Thymus, T cells, Treg, Extracellular vesicles, exosomes, central tolerance, negative selection

SAMMANFATTNING PÅ SVENSKA

Vårt immunsystem måste utbildas i att agera mot patogener, så som virus och bakterier, men samtidigt vara tolerant gentemot kroppsegna strukturer, så kallade självantigen. Thymus är det organ som utbildar kroppens T-celler, endast några procent av alla blivande T-celler som kommer in i thymus blir fullt fungerande och en del av vårt immunsystem. T celler som reagerar mot kroppens egna strukturer selekteras bort, förutom en del av dessa, som utbildas till regulatoriska T-celler vilka skyddar kroppen mot autoreaktivitet. Om denna process inte fungerar kan T-cellerna börja angripa kroppens egna celler eller vävnader vilket kan leda till autoimmun sjukdom.

Utbildning och selektion av T-celler i thymus är en komplex. Självantigen genereras av epitelceller som tillsammans med dendritiska celler presenterar dem för T-cellerna så att självreaktiva T-celler kan selekteras bort eller utvecklas till regulatoriska T-celler. Det är idag väl beskrivet att självantigen flyttas från epitelcellerna som genererar dem till dendritiska celler, vilka är effektiva på att visa upp dem för T-cellerna. Det är mindre studerat hur självantigenen överförs mellan cellerna.

Avhandlingen syftar till att studera extracellulära vesiklars (exosomers) förmåga att transportera självantigen mellan cellerna i thymus. Exosomer är nanovesiklar som kan skickas ut från de flesta av kroppens celler och är ofta beskrivna som budbärare mellan celler. De är uppbyggda av ett lipidmembran och innehåller proteiner samt genetiskt material som ofta avspeglar cellen som skickat ut dem.

I den första studien odlade vi epitelceller från human thymus och visade att de kan skicka ut exosomer innehållande självantigen associerade med autoimmuna sjukdomar.

Den andra studien är en *in vitro* studie av musthymus, där syftet var att studera thymusexosomers effekt på utmognad av T-celler. T-celler från thymus odlades tillsammans med thymusexosomer, och med eller utan dendritiska celler. Studien visade att thymusexosomerna stimulerade utmognad av T-celler till funktionella T-celler, oberoende av närvaron av dendritiska celler.

I den tredje studien använde vi en välkänd transgen musmodell för att studera selektionen av självreaktiva T-celler *in vivo*. Vi visade att injektion av thymusexosomer från en musthymus, som bär ett specifikt antigen på sin yta, kan minska förekomsten av T-celler som är specifika för antigenet i en annan mustyp. Det talar för att thymus-exosomer är viktiga för selektion av

självreaktiva T-celler. Studien visade också att thymusexosomer har betydelse för utbildningen av regulatoriska T-celler, viktiga för att upprätthålla en tolerans i perifera organ under livet.

Sammanfattningsvis är exosomer från thymus av betydelse för att etablera och bibehålla kontroll av självreaktiva T-celler.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Skogberg, G, **Lundberg V**, Berglund M, Gudmundsdottir J, Telemo E, Lindgren S, Ekwall O. Human thymic epithelial primary cells produce exosomes carrying tissue-restricted antigens. Immunology and Cell Biol 2015; 93(8): 727-734.

II. **Lundberg V**, Berglund M, Skogberg G, Lindgren S, Lundqvist C, Gudmundsdottir J, Thörn K.
Telemo E, Ekwall O. Thymic exosomes promote the final maturation of thymocytes
Scientific Report 2016; 6: 36479.

III. **Lundberg V**, Berglund M, Lindgren S, Thörn K, Hennings V, Lemarquis A, Lingman Framme J, Lundqvist C, Telemo E, Ekwall O. Thymus derived exosomes transfer tissue restricted antigen and affect induction of antigen specific central tolerance in the insHEL-3A9 TCR transgenic mouse model. Manuscript.

PUBLICATIONS NOT INCLUDED IN THE THESIS

Framme JL, Lundqvist C, Lundell AC, van Schouwenburg PA, Lemarquis AL, Thörn K, Lindgren S, Gudmundsdottir J, Lundberg V, Degerman S, Zetterström RH, Borte S, Hammarström L, Telemo E, Hultdin M, van der Burg M, Fasth A, Oskarsdottir S, Ekwall O. Long-Term Follow-Up of Newborns with 22q11 Deletion Syndrome and Low TRECs.
Journal of Clinical Immunology 2022 Jan 26

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CONTENT

ABBREVIATIONS

AIRE/Aire	the human/mouse autoimmune regulator gene
AIRE/Aire	the human/mouse autoimmune regulator protein
APC	antigen presenting cell
APS1	autoimmune polyendocrine syndrome type 1
Atg	Autophagy related
BM	bone marrow
CCL19	c-c chemokine ligand 19
CCL21	c-c chemokine ligand 21
CCL25	c-c chemokine ligand 25
CCR4	c-c chemokine receptor 4
CCR7	c-c chemokine receptor type 7
CCR9	c-c chemokine receptor type 9
CMJ	corticomedullary junction
cTEC	cortical thymic epithelial cell
DC	dendritic cell
DN, DP, SP	double negative, double positive, single positive
DOCK2	dedicator of cytokinesis protein 2
EBV	Epstein-Barr virus
ESCRT	endosomal sorting complexes required for transport
ETP	early T-cell progenitor

EV	extracellular vesicle
Fezf2	Fez family zinc finger 2
FOXP3/foxp3	the human/mouse forkhead box p3 gene
FOXP3/foxp3	the human/mouse forkhead box p3 protein
GDIR1	Rho GDP-dissociation inhibitor 1
HEL	hen egg lysozyme
HSA	heat stable antigen
IL7/IL7R	interleukin7/interleukin7 receptor
INS	insulin
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
K5/K8	keratin 5 / keratin 8
Klf2	Kruppel-like factor 2
LAMP1	lysosomal-associated membrane protein 1
LTßR	lymphotoxin-ß receptor
MBP	myelin basic protein
MFG-E8	milk fat globule-EGF factor 8 protein
MHC	major histocompatibility complex
miRNA	microRNA
mTEC	medullary thymic epithelial cell
MBP	myelin basic protein
MS	multiple sclerosis
MSC	mesenchymal stem cells

MVB	multivesicular body
nTreg/Treg	natural regulatory T cells/regulatory T cell
OVA	ovalbumin
PAK2	p21 protein-activated kinase 2
PGE	promiscuous gene expression
Prdm1	PR domain zinc finger protein
RA	rheumatoid arthritis
$S1P_1$	sphingosine-1-phosphate receptor 1
SLE	Systemic lupus erythematosus
SPGL1	Sphingosine-1-phosphatase lyase
TCR	T cell receptor
Tconv	conventional T cells
TGF-ß	Transforming growth factor beta
TRA	tissue restricted antigen

1 INTRODUCTION

The immune system needs to be educated to react against foreign pathogens and still maintain a tolerance against self-antigens. If this delicate balance is shifted towards a loss of tolerance against self-structures, autoimmune diseases can develop.

The concept of immunological tolerance was first reported in Nature 1953 by Medwar et al, where they discussed an establishment of tolerance in animals when exposed to antigens in fetal life, as well as adapted tolerance later on [1]. This publication came to interest Jacques Miller, whom after studies on thymectomized mice in the neonatal period made the controversial and novel statement that the thymus has an immunological function. Thymectomy of neonatal mice resulted in lymphocytopenia, severe infections and made them unable to reject foreign skin grafts [2]. He suggested a role for the thymus in establishing tolerance to self-tissues, published in the Lancet 1961 [3, 4]. These observations of the thymic function gave rise to the discovery of T and B cells [2, 5].

Congenital athymia in humans is a very rare and severe condition with a lack of functional T cells. The condition is most often associated with a complete DiGeorge syndrome, caused by a microdeletion of chromosome 22q11, even though other genetic defects also can cause athymia [6]. Children undergoing heart surgery early in life are often thymectomized because of technical difficulties otherwise. Thymectomy early in life is associated with increased frequencies of diseases caused by a dysregulated immune system, which reveals an important function of human thymus also after birth [7].

Much knowledge has been gained regarding the thymus and T cell development since it was first described. However, the cell-cell communication important in educating the T cells are not fully understood. This thesis aims at discussing thymic exosomes, nano-sized extracellular vesicles (EVs) as communicators of importance for the selection and maturation of developing T cells. The first study was based on human thymic tissue, and for paper II and III mouse models were used.

1.1 Thymic central tolerance

Thymus

The thymus is a highly productive primary lymphoid organ where thymocytes undergo maturation, proliferation and selection to establish tolerance against self-antigens but still offer the possibility of immunity against pathogens. If too many self-reactive T cells escape the thymus, or if the development of regulatory T cells (Treg) is dysfunctional, autoimmune diseases can occur [8].

The thymus is elegantly structured with a unique microenvironment that enables the development of functional and self-tolerant T cells. It is classically divided into two main areas, histologically defined as the cortex and the medulla, which are separated by the corticomedullary junction (CMJ), rich in blood vessels. The cortex consists mainly of immature thymocytes and cortical epithelial cells (cTEC), as well as macrophages that are responsible for the removal of apoptotic bodies from dying thymocytes. The medulla contains mostly mature thymocytes and medullary epithelial cells (mTECs) as well as other antigen presenting cells such as dendritic cells (DCs) and B cells, which are also present in the CMJ [9]. Other cell types located in the thymus are fibroblasts, which may have a role in cell signaling and are of importance for the thymic structure and organization [10], eosinophils of different maturation stages [11], as well as neutrophils [12] mast cells [13] and tuft cells [14].

The journey of thymocytes through the thymus

Hematopoietic early T cell progenitors (ETPs) from the bone marrow enter the thymus via blood vessels in the CMJ and migrate to the subcapsular zone of the cortex [15]. The identity and characteristics of the progenitors that develop into the thymocyte lineage is still debated. However, it has been suggested that ETPs originate from an IL7R expressing lineage, whereas myeloid cells are not, which makes sense since thymocytes are dependent on IL-7 for their survival [9, 16]. This hypothesis is in contrary to the earlier understanding, where ETPs and myeloid progenitors were thought to share the same pathway [15, 17].

As ETPs enter the cortex they are defined as double negative (DN) thymocytes, which lack expression of both CD4 and CD8, with the important task to produce a functional T cell receptor (TCR) from the genetic elements that codes for the receptor. At their third stage as DN thymocytes, they start to rearrange the β-chain of the TCR. If successful they will express a pre-TCR complex, which consists of the β-chain and a pre α-chain. Signaling through the pre-TCR inhibits further β-chain recombination, induces major proliferation and the expression of CD4 and CD8. At this stage the thymocytes

are denoted as double positive (DP). The thymus can generate as much as 50 x 10^6 $CD4^+CD8^+$DP thymocytes a day [8]. The DP thymocytes further use 3-4 days for rearrangement of their TCR α-chain to achieve a fully functional αβ-TCR [18].

Cortical thymic epithelial cells (cTECs) display self-peptides bound to major histocompatibility complexes (MHC) I and II that are presented to the DP thymocytes. According to the ruling affinity-based model, DP thymocytes need to interact through their TCR with MHC-peptide complexes on cTECs with an intermediate affinity to survive and differentiate into single positive (SP) cells. If they bind with low affinity or fail to bind to the MHC-peptide complex, they will die by neglect and undergo apoptosis. This is referred to as positive selection and will eliminate thymocytes with a dysfunctional TCR not able to recognize MHC-peptide complexes [9, 15, 19]. DP thymocytes that bind to MHC-peptide complexes with high affinity are subject to negative selection and are eliminated through apoptosis, leading to a removal of potentially self-reactive thymocytes. The end result of the interaction between DP thymocytes and cTECs is that cells with an intermediate affinity for the MHC-peptide complexes presented by cTECs will survive and further differentiate into SPCD4 or SPCD8 cells depending on interaction with MHC II or MHC I respectively. [20].

When DP thymocytes differentiate into SP thymocytes they start to express CCR7 which leads to the migration of SP cells from the cortex to the medulla. The thymic medulla is an environment rich in self-peptides, also referred to as tissue restricted antigens (TRAs). TRAs are mainly expressed by medullary thymic epithelial cells (mTECs), and to a large degree regulated by the transcription factor autoimmune regulator (AIRE) [21, 22]. SP thymocytes expressing a TCR that binds with high affinity to an MHC-self peptide complex, often described as autoreactive T cells, are negatively selected by receiving a strong TCR signal which leads to clonal deletion through apoptosis. However, autoreactive SPCD4 thymocytes can also be selected for diversion into the Treg lineage, which will be discussed further. Negative selection is thus occurring both in the cortex and in the medulla, which probably are complementary processes. This reflects the importance of the elimination of self-reactive thymocytes throughout the T cell maturation process [8, 23, 24].

The negative selection of autoreactive T cells is dependent on presentation of TRAs on MHC molecules. mTECs themselves and also DCs have been shown to present TRAs on MHC molecules, originally produced by mTECs. This suggests a transfer of TRAs from mTECs to DCs or between individual mTECs, and for which the mechanisms are still obscure. One hypothesis is that

the transfer of TRAs is mediated by extracellular vesicles (EVs), which will be discussed throughout the thesis.

1.2 Medullary epithelial cells

Thymic epithelial cells, cTECs and mTECs, originate from common progenitors [25, 26]. FOXN1 is a transcription factor crucial for the regulation of TEC development into the cTEC and mTEC lineages. Biallelic mutations of *FOXN1* are associated with athymia, which is treated with thymus transplantation in order to avoid fatal dysfunction of the immune system [27, 28]. The transcription factor PR domain zinc finger protein 1 (Prdm1), mostly studied in B -cells [29], has also been proposed to influence mTEC function and regulation of central tolerance [30]. Variants in *PRDM1* are associated with systemic lupus erythematosus (SLE) [31], and mice lacking expression of Prdm1 in TECs develop an autoimmune phenotype [30].

The development of TECs into the mTEC lineage is dependent on activation of the NF-kB signaling pathway downstream the RANK receptor. RANKL is produced by positively selected thymocytes and critical for AIRE expression in mTECs [32, 33]. The mTECs have a unique capability for promiscuous expression of self-antigens that are normally expressed only in the periphery. These self-antigens are referred to as tissue restricted antigens (TRAs). TRAs are expressed in mTECs for the sole purpose of enabling tolerance to self. AIRE was the first transcription factor reported to regulate TRA expression in mTECs [34]. Biallelic mutations in the *AIRE* gene cause the autoimmune polyendocrine syndrome (APS1) in humans, and today more than 100 disease causing mutations in *AIRE* have been reported [35, 36]. In addition, later publications have reported a reduced AIRE expression related to estrogen levels, which is interesting since autoimmune diseases are more common in females than in males [37, 38]. In addition, autoimmune diseases are associated with improvement during pregnancy, and pregnant humans have shown to have a maintained thymic output of conventional T cells (Tconv) and Tregs during pregnancy [39].

Since the discovery of *AIRE*, the transcription factor Fezf2, regulated by lymphotoxin-ß receptor (LTßR) signaling pathways, has been identified as another transcription factor essential for regulating the expression of TRAs, and independent on AIRE. Mice deficient in *Fezf2* developed autoimmune manifestations [40], and studies on mice deficient in the LTßR-signaling pathway have shown a defect in communication between mTECs and thymocytes leading to autoimmunity [41].

One of the classical examples of a TRA is insulin, well studied both in human and mice and regulated by AIRE [42]. Expression of insulin is restricted to pancreatic β-cells in the periphery, but are also expressed in mTECs under the influence of AIRE. Transgenic mouse models expressing model antigens, such as HEL or OVA, regulated by the insulin promotor are well described systems for studies of negative selection since they are expressing the model antigens in mTECs leading to an AIRE dependent tolerance induction in the thymus. The use of these animal models has contributed to the understanding of negative selection, Treg development and the importance of AIRE, and has resulted in significant knowledge about central tolerance mechanisms [43, 44].

In paper III a transgenic mice model with mice expressing HEL under the rat insulin promotor (insHEL) was used to study antigen transfer by thymic exosomes, as well as the impact of thymic exosomes on thymocyte selection and Treg development (Paper III, in manuscript).

1.3 Maturation of SPCD4 thymocytes

Negative selection of SP thymocytes has been well studied, but less is known about the mechanisms regulating the late state maturation of SP thymocytes. After entering the medulla, thymocytes were first thought to be present there for around 14 days before egressing the thymus [45]. However, later studies have shown that newly developed SP thymocytes spend an average period of 4-5 days in the medulla [46]. Different definitions of maturation stages have been proposed, mainly based on the expression of various surface markers, which will be discussed below and are illustrated in figure 1.

SPCD4 thymocytes are often divided into four different maturation stages (SP1-SP4), based on cytokine production, proliferation and survival capacity, (fig 1) [47, 48].

$S1P_1$ (Sphingosine-1-phosphate receptor) is a G-protein-coupled receptor and a key mediator for mature thymocytes to egress into the blood stream. Hematopoietic cells deficient in $S1P_1$ are unable to leave the thymus, resulting in an elevated proportion of SPCD4 and SPCD8 thymocytes within the thymus, and consequently a lack of peripheral T cells. $S1P_1$ is upregulated on SP thymocytes before leaving the thymus [49, 50].

Kruppel-like factor 2 (Klf2) is a transcription factor which regulates the expression of CD62L and $S1P_1$, both upregulated and necessary for mature T cells to leave the thymus. Mice deficient in *Klf2* have a reduced expression of $S1P_1$ and CD62L. The KLf2-$S1P_1$-axis is upregulated gradually in SP2 and SP3 cells. CD62L and $S1P_1$ expression is enhanced in the last maturation step [51-53]. The expression of CD69 and $S1P_1$ are linked, CD69 is decreased during elevated expression of $S1P_1$ [54, 55]. Qa2 is a non-classical MHC molecule, upregulated in the SP4 thymocyte stage, when they are ready to egress from the thymus. Qa2 is often used as a maturation marker in mice thymocytes, but is not present in human cells [46, 53].

The maturation stages of SP thymocytes have also been suggested to be divided based on the expression of the chemokine receptors CCR4, CCR7 and CCR9. The ligand for CCR9 is CCL25, which is expressed by cTECs and newly differentiated cortical SP thymocytes express CCR9. The chemokine receptor CCR7 is up-regulated on SP thymocytes in the cortex after positive selection, which enable them to migrate towards the medulla where mTECs are producing its ligands CCL19 and CCL21 [56]. An illustration of the importance of the migration of SP cells from the cortex to the medulla is that mice deficient in CCL21 fail to establish central tolerance and as a

consequence develop autoimmunity [57]. As the expression of CCR7 increase, CCR9 and CD69 are downregulated [58-61]. Comparative microarray analyses of SP1-SP4 cells are strengthening this observation by showing that CCR7 levels are still low in newly generated SP1 thymocytes, suggesting that CD4 SP thymocytes migrate to the medulla at stage SP2 [53].

In addition, CCR4 is upregulated in the early positively selected thymocytes, before they start to express CCR7, and is also important for the thymocytes to be able to enter the thymic medulla [62]. Ligands for both CCR4 and CCR7 are expressed by AIRE expressing mTECs [56]. In conclusion, newly positively selected SPCD4 cells express CCR4 and CCR9, whereas more mature SP cells have an $CCR4^{-}CCR7^{+}CCR9^{-}$ chemokine receptor phenotype [15, 63].

To investigate if thymocyte maturation is dependent on an intact medulla, Cowan et al performed a study on $RelB^{-/-}$ mouse that lack an organized structure of the medulla. They showed that mature conventional SPCD4 thymocyte development is not affected when the mTEC compartment is altered, which indicates that thymocyte maturation is not only dependent on mTECs. However, the development of $Foxp3^{+}$ Tregs was shown to be dependent on an organized medulla in this model [64].

Taken together, SPCD4 thymocytes follow a strict maturation pathway, not dependent on a completely organized medulla. Thymocyte selection and egress have been well studied but the mechanisms behind the different stages are still to a high degree unexplored, and hampered by inconsistent data. One factor which could contribute to the selection and maturation process are EVs, that may serve as communicator between cells even in a disorganized medulla, discussed in paper II.

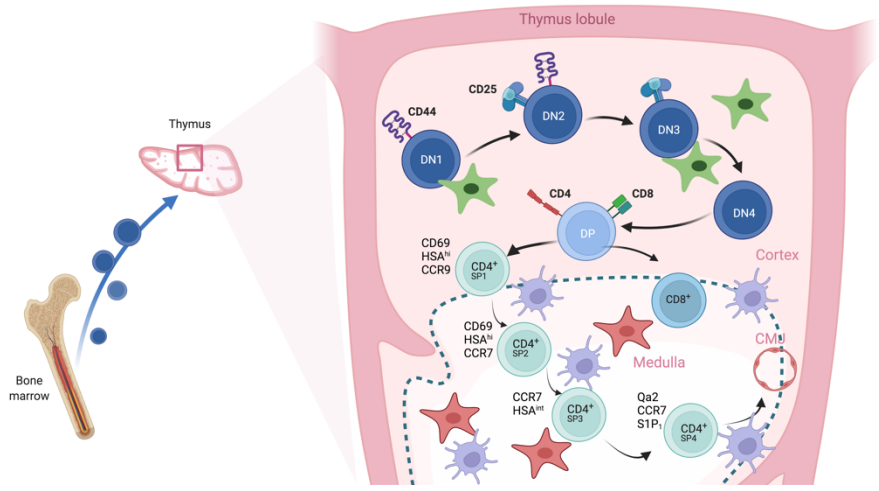

Figure 1. Thymocyte development. Thymocyte precursors enter the thymus as DN thymocytes and undergo several differentiation stages to become DP thymocytes. DP thymocytes are positively selected into SP thymocytes in the cortex and migrate to the medulla. SP thymocytes are divided into different maturation stages SP1-SP4 expressing different maturation markers, as shown in the figure. *Created with Biorender.com*

1.4 Regulatory T cells

Tregs are crucial to maintain an immunological self-tolerance and to prevent autoimmune diseases. Self-reactive T cells can escape the negative selection, egress the thymus to the periphery, and cause T cell mediated autoimmune manifestations [65-67]. In 1969 Nishizuka and Sakakura reported that mice thymectomized during day 2-4 of age developed autoimmune manifestations [67, 68]. The group of Sakaguchi described in 1996 how neonatal thymectomy of mice at day 3, but not at day 7 after birth gave rise to autoimmune diseases, and in adult mice the deletion of $CD25^+$ T cells in the periphery gave rise to a lethal autoimmune state [69]. It was later realised that the removal of $CD25^+$ T cells also deleted the Foxp3 expressing T cells in these mice.

In 1982 Powell et al reported the first case report of IPEX, an X-linked syndrome within a family where 17 males had died within the first year of life with multiple autoimmune manifestation [70]. IPEX was later revealed to be caused by loss of function mutations in the *FOXP3* gene, and the so called scurfy mouse with an almost identical phenotype was shown to carry the same missense mutation, and thus used as a model for the IPEX syndrome in humans. A dysfunctional *Foxp3* gene leads to a complete lack of functional Tregs [71, 72]. Foxp3 expression is required for the thymic development of $CD4^+CD25^+$ Treg, and transfer of $CD4^+CD25^+Foxp3^+$ Treg to neonatal *Foxp3* deficient mice rescue them from disease [73].

1.4.1 Treg development

Natural regulatory T cells (nTreg) are developed in the thymus and originates from SPCD4 thymocytes. The mechanisms controlling Treg formation are still not fully understood. One hypothesis is based on the dependence of a high affinity TCR signal for Treg development, another the abundance of endogenous antigens being presented, and both theories have been confirmed in different experimental studies [67, 74-76]. The current understanding is that both the TCR-affinity and the quantity of antigens presented to the thymocyte are decisive in the induction of Tregs [77].

TCR affinity depends on the strength of the TCR- signal when TCR binds to its corresponding MHC-peptide complex. As described earlier, SPCD4 thymocytes with a high TCR affinity for self-peptides are deleted, and the ones with low affinity egress the thymus as Tconv. Thymocytes bearing a TCR that has an intermediate affinity for MHC-self peptide complex can develop into Tregs expressing Foxp3 [77-79]. This was demonstrated by Lee et al, who

showed in a transgenic OVA model, that the generation of thymic Treg was dependent on higher TCR affinity than Tconv, but below the affinity threshold for clonal deletion. This result suggested that the resulting Treg have a broad range of self-reactivity [78].

Another factor regulating the Treg development is the density of which a self-antigen is presented on MHC II. The level of co-stimulatory molecules present on the APC will also have an impact on the intensity of the TCR-signaling, and the SPCD4 development into the Treg lineage [77, 80].

Furthermore, it has been shown that the expression of TRAs by mTECs is necessary for the development of functional Tregs, able to suppress immune reaction to self-peptides in the periphery [81]. The development of a self-recognising TCR repertoire in the thymus is critical for the suppressive function of Treg cells in the periphery [82-84]

AIRE is a crucial transcription factor for both negative selection and the development of a functional Treg repertoire, and is mainly expressed in medullary epithelial cells of the thymus. AIRE drives the promiscuous expression of TRAs, important both for negative selection of self-reactive T cells and the generation of $Foxp3^+$ Tregs. Most of the functional Tregs are developed during the perinatal period [85]. Thymocytes that normally differentiate into the Treg lineage escapes in the *Aire* deficient mice to become Tconv, which results in autoimmune manifestations [86]. Furthermore, antigen transfer of Aire-dependent TRAs from mTECs to DCs is crucial for clonal deletion as well as induction of Tregs [87].

In conclusion, TCR signaling as a result of self-antigen presentation is necessary for Treg development. To make this process effective in the thymus, intercellular antigen transfer from mTECs to DCs is important [87]. However, co-stimulatory molecules are also needed. Roman et al, elegantly showed that professional APCs and TECs were independently capable of developing Tregs, but that Treg selection always requires a TCR signal and CD28 co-stimulation which is mediated by CD80 and CD86. [88] More knowledge has been gained about essential cytokines in this process such as IL-2, and TGFβ [89]. As an example, Lio et al demonstrated a two-step model of which TCR stimulation generates a $CD25^{hi}$ subset of $SPCD4^+$ cells which then required IL-2 stimulation to initiate the expression of FoxP3 to become $CD4^+CD25^+Foxp3^+$ Treg [90].

1.5 Antigen presentation

The thymic medulla provides a unique microenvironment with self-antigens mostly expressed by the mTECs under the influence of AIRE [22, 91]. Self-reactive thymocytes undergo clonal deletion, partly dependent on the presentation of TRA - MHC II complexes by mTECs [24]. To study the impact of mTECs as antigen presenting cells, Klein et al developed a mouse model with diminished MHC II expression on mTECs. A decrease of MHC II on mTECs resulted in an increase of the SPCD4 population as well as a mild infiltration of T cells in peripheral organs, illustrating the importance of an autonomous APC function of mTECs to avoid autoimmune manifestations [92]. However, a single TRA is only expressed by a small population of mTECs (1-3%) at a given timepoint which would imply a rather ineffective presentation outcome. DCs are effective APCs that are abundant in the thymic CMJ and thymic medulla but absent in the cortex [93, 94]. They are reported to have a key-function in presenting self-antigens to developing thymocytes in the medulla, which is important both for negative selection and Treg induction [8, 21, 87, 95]. Establishment of CD4 T cell tolerance could also be accomplished by self-antigens entering the thymus from the blood stream and picked up by thymic DCs or self-antigens carried by DCs from the periphery to the thymus. This mechanism is suggested as a complementary mechanism for self-antigen presentation [95, 96]. In addition, recent publications have reported that AIRE expressing B cells in the thymic medulla are also able to present antigens and participate in the negative selection process [97].

In summary, mTECs and DCs are the major APCs in the thymic medulla, necessary for induction of central tolerance with deletion of self-reactive T cells and induction of Tregs.

1.5.1 Antigen transfer within the medulla

The mechanisms behind antigen transfer from mTECs to DCs and other potential APCs in the thymic medulla are not well established, even though it is well documented that the negative selection is dependent on an indirect antigen transfer from mTECs to DCs [8]. Intercellular transfer of antigens within the thymic stromal compartments was first reported by the group of Kyewski in 1994. Fifteen years later the group elegantly illustrated an unidirectional transfer of self-antigens from mTECs to DCs, by showing that TECs had to express the self-antigen before it could be presented by the DC [98].

Early publications reported a more efficient antigen presentation ability of TRAs by DCs when compared to mTECs [99, 100]. This resulted in more research and publications about thymic intercellular transfer of antigens, and indirect presentation by DCs [8]. These studies concluded that intercellular transfer of TRAs to DCs is important in the deletion of autoreactive thymocytes [101]. Membrane material including MHC II molecules have also been reported to be transported between epithelial cells as well as from epithelial cells to DCs [102]. Specific self-antigens have also shown to be dependent on indirect presentation through intercellular transfer, reported by Hubert et al. They used a transgenic mouse model in which *Aire* deficient mice were crossed into mice expressing OVA in membrane-bound or secreted form, regulated by the insulin promotor, which is under the influence of Aire. By using this mouse model they could conclude that $CD4^+$ T cell deletion is mediated by Aire, and most importantly dependent on specific self-antigen transfer from mTECs to DCs [103]. In addition, another study on a specific self-antigen showed that tolerance to the myelin basic protein (MBP), associated with multiple sclerosis (MS), is dependent on BM-derived APCs. [104]. Furthermore, both Aire-dependent and independent induction of Treg-development requires BM-derived APCs, also supporting the theory of an antigen transfer from mTECs to DCs [105].

In conclusion, several studies have reported mTECs and DCs as APCs in the thymus, and an intercellular antigen transfer between mTECs and DCs. However, Perry et al was the first to compare the role of mTECs and BM APCs in negative selection and Treg development, respectively. They concluded that neither mTECs nor BM derived APCs had a redundant role. Furthermore, they reported that Aire-dependent tolerance is induced via antigen transfer from mTEC to CD8α+DCs and concluded that CD8α+DC are the primary APC recipient of mTEC derived antigens [87]. The same group later reported that CD36, a scavenger receptor expressed on CD8α DCs, facilitates the transfer of antigens derived from the cell surface but not the ones derived from the cytoplasm of mTECs, and that CD36 promotes Treg development. Interestingly, CD36 can be expressed on EVs [106].

1.6 Extracellular vesicles, exosomes

1.6.1 Extracellular vesicles, history and nomenclature

Extracellular vesicles, (EVs) are membrane derived vesicles released from most cell types. Cell-derived vesicles were first discovered in 1967, described as “platelet dust”. They were isolated by several steps of high-speed centrifugation and finally ultracentrifugation to a sedimented fraction, and characterized using electron microscopy [107, 108]. During the 1980s, two independent groups described how the reticulocyte transferrin receptor was removed from the maturing red blood cells into the extracellular space carried by EVs. The EVs were during a time considered to be a waste product, but rose in interest by researchers in the 1990s after revealing that they can have an immunological function [109, 110].

As for today, a great number of studies have been done on EVs, and the interest and publications are increasing rapidly, where exosomes are the most studied and documented in the EV family. The nomenclature of subgroups of EVs are still debated because of difficulties in distinguishing between them as they have different characteristics, but still overlap in size, morphology and constituents. EVs could be divided into two main subgroups, exosomes with the size range of 50-150nm and microvesicles, including oncosomes, shedding vesicles, apoptotic bodies and others, with the size range of 50-500nm. However, of importance is that exosomes and other microvesicles also differ in their biogenesis and how they are released into the extracellular space, even though they share some contents [111]. Exosomes are endogenous nanoparticles, secreted by most cell-types and escape elimination of macrophages and neutrophils, in contrary to apoptotic bodies [112].

1.6.2 EVs and exosomes biogenesis

Subtypes of EVs differ in their biogenesis, size range immunological functions as well as the mechanism for secretion into the extracellular space. Microvesicles (MVs) are released either through budding of the plasma membrane or as apoptotic bodies from cells undergoing apoptosis through blebbing [113]. However, the production of exosomes start with the formation of nanovesicles achieved by the inward budding of the late endosomal membrane during the maturation process, which then becomes a multivesicular body (MVB). MVBs can either fuse with a lysozyme to recycle its content or fuse with the plasma membrane [114-116]. When the MVBs fuse with the plasma membrane their nanovesicle content is released as exosomes. The constituents of the exosomes depends on the constituents of the endosome, and reflects the biogenesis during the endosome maturation [116].

The formation of MVBs requires the endosomal sorting complex (ESCRT), critical for the transport and the secretion of exosomes [114, 117]. Tetraspanins are cellsurface associated membrane proteins, found in the endosomal compartments, and expressed on secreted exosomes reflecting their biogenesis and therefore used as exosomal markers [118]. The tetraspanins CD9, CD63 and CD81 are often used as exosome markers [114, 118, 119]. Milk fat globule-EGF factor 8 (MFG-E8) is a membrane glycoprotein expressed on exosomes, suggested to be related with exosome secretion and uptake by other cells, and also used as an exosome marker [120]. The endosomal sorting and formation of vesicles, as well as the secretion of exosomes are also dependent on Rab proteins [116, 121] Furthermore, exosomes frequently contain miRNA and functional mRNA, which can be shuttled between cells and influence genes and protein expression in recipient cells [122-124].

1.6.3 Exosomes as antigen carriers

The first immunological study of exosomes was published by Raposo et al, reporting an antigen specific and MHC II class restricted T cell response induced by exosomes derived from EBV-transformed B-cells. The data was confirmed both in murine models and in humans [109]. Most immunological studies of exosomes today are in the in the field of cancer research. The first report of a reduction of tumor growth by exosomes pulsed with tumor antigen were reported by Zitvogel et al in 1998 [110]. In summary, these studies concluded that exosomes can induce a protective immune response and suggested an importance for exosomes as mediators of intracellular communication. These results further lead to an abundance of studies regarding biological functions of exosomes, and set the base to use exosomes as anti-cancer therapy, where the first phase I study was performed in 1999 [125, 126].

As mentioned above exosomes have been shown to induce a T cell response. DC derived exosomes activates T cells in an antigen specific manner, which is true both for CD4 and CD8 T cells. In a study on TCR-transgenic mice, Thery et al reported that exosomes activate antigen specific naïve CD4+ T cells *in vivo*, and further demonstrated that peptide-MHC II complexes are transferred via exosomes between two different DC populations *in vitro* [127]. Further, Admyre et al, showed that exosomes secreted from monocyte-derived DCs loaded with peptides from EBV, CMV and influenza virus directly stimulated human peripheral CD8 T cells. The stimulation was mediated via MHC I and was dependent on the dose of exosomes [128]. Exosomes derived from intestinal epithelial cells mediates antigen specific tolerance to dietary antigen, shown to be MHC II dependent [129].

In summary, exosomes are reported be capable to carry antigens, which can be presented to T cells either directly or indirectly via uptake by DCs, and lead to either priming or tolerogenic responses by the immune system.

1.6.4 Exosomes, fusion and uptake by recipient cells

For exosomes to induce a change in a cell´s physiology it has to interact with the recipient cell and somehow incorporate their cargo. Exosomes can either remain at the plasma membrane, which was illustrated by electron microscopy where follicular DCs had exosomes expressing MHC II attached to their surface [130], or become integrated with the cell membrane of the target cell. They can stay attached to the cell surface by binding to integrins and initiate intracellular signaling pathways. Recipient cells could also take up exosomes by an endocytotic mechanisms which will lead to degradation of the vesicles [111] [125]. The release of miRNA and mRNA into the cytoplasm of recipient cell can either be achieved by fusion of exosome with the plasma membrane or by endocytosis of the exosome [124, 131]. To further investigate the involvement of different mechanism in the uptake of EVs by DCs, research groups have blocked both molecules on the exosomes as well as on recipient DCs. These studies have demonstrated an importance for phosphatidylserine, the tetraspanins CD9 and CD81, CD54 and lactadherin on EVs as well as LFA-1 and DEC205 on the target cells [132].

1.6.5 The role of exosomes in antigen spreading in the thymus

Antigen transfer between mTECs and BM derived APCs for antigen presentation on MHC II to developing thymocytes has been well studied, even though the mechanisms responsible for the transfer is yet unknown [87, 101, 103, 106, 133, 134]. EVs are part of the unique thymic microenvironment and represent potential vehicles for antigen spreading and communication between cells. Thymic EVs have a broad range of content, which includes MHC molecules that may stimulate TCRs in a similar way as direct cell-cell contacts [135]. Human thymic exosomes carry a broad content of molecules, in part mirroring the content of the cell from which they are secreted. Even though exosomes isolated from human thymic tissues probably are derived from different cell types in the thymus, TRAs were readily detected in the thymic exosome fraction which indicates that mTECs are the source of at least a part of the thymic exosomes [136]. This notion was confirmed by our group using cultured human primary TECs, which secreted exosomes that contained TRAs, including several TRAs with an association to autoimmune diseases [137] Some shared TRAs could be detected in exosomes from both from thymic tissues and cultured TECs [138].

A recent interesting observation is that late stage mTECs, which lose their expression of MHC II while still expressing TRAs, upregulate proteins related to the biogenesis of exosomes [139]. Thymic exosomes derived from these late stage mTECs could hypothetically have a function to extend the spreading of TRAs to other APCs when the late stage mTECs decrease their MHC expression. In addition, only a small proportion of all mTECs express an antigen at a given timepoint, and DCs have shown to have a key function in the presentation of TRA to developing thymocytes, even though it is not known how the TRAs reach the DCs.

MHC II molecules on professional APCs are primarily loaded with exogenous antigens that are processed in the cell [140]. TECs express MHC II but are ineffective at loading exogenous antigens on to MHC II [141]. Autophagy in TECs, both cortical and medullary is constitutively active [142], and is therefore suggested to be a key mechanism of intra-cellular loading of endogenous antigens onto MHC II [43, 92, 133, 143]. Exosomes and autophagy share common pathways. Exosomes are formed when early endosomes mature into late endosomes and autophagy is a pathway of intracellular self-digestion and recycling mediated through the lysosome pathway [144]. Klein et al grafted thymus from mice with dysfunctional autophagy, deficient in the autophagy related 5 (Atg5) protein, into athymic nude mice, which resulted in autoimmune manifestations in the recipients. They suggested autophagy as a key mechanism of central tolerance, and a mechanism for loading of endogenous TRAs onto MHC II for presentation by mTECs [145]. Interestingly, exosome production is also inhibited in *Atg5* knockout mice [146]. In addition, autophagy related protein 7, also involved in autophagy, is found in thymic exosomes [136, 138]. In conclusion, autophagy has been reported to have a role in autonomous loading of antigens on MHC II on mTECs.

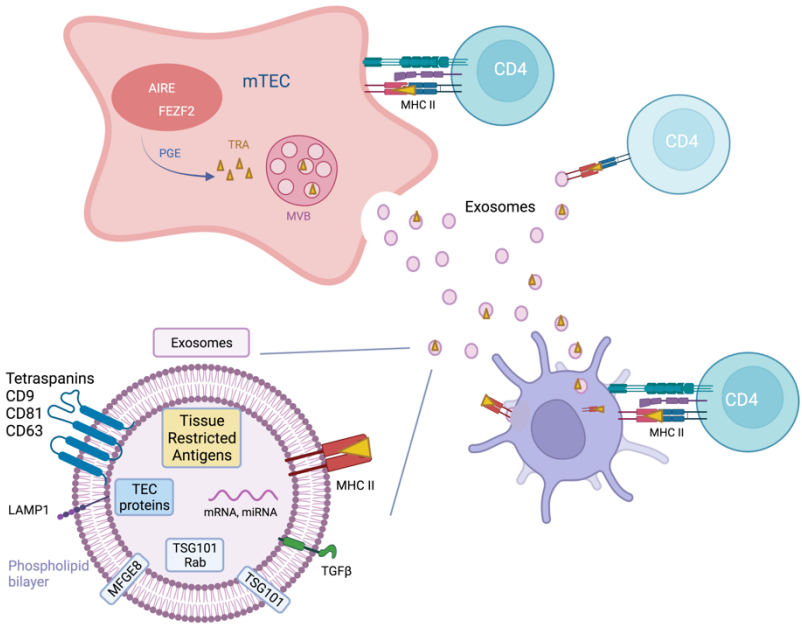

Figure 2. Exosomal transfer of TRAs from mTECs. Thymic exosomes are derived from mTECs and carry TRAs. They can either have a direct effect on developing thymocytes through MHC II presentation of the TRAs or via indirect presentation by DCs. mTECs can also present the TRA themselves on MHC II. Schematic illustration of a thymic exosome which present a MHC II-TRA complex. *Created with Biorender.com*

1.6.6 EVs as treatment

EVs, and especially exosomes with their lipid surface and broad content has drawn attention to researcher for potential uses in the medical field. Exosomes often reflect the cell they are derived from regarding their content, and are therefore suggested to have potential roles as biomarkers for different diseases [123]. The pharmaceutical field are also studying exosomes as a mean for delivering therapeutic contents. Since Zitvogel et al reported exosomes as of potential use in the treatment of cancer [110], multiple studies have been performed in this field. Exosome-based vaccines as a novel strategy in cancer treatment have been developed. During the Covid-19 pandemic, researchers shifted the focus of exosome based vaccines from cancer treatment to the development of exosome based Covid-19 vaccines, and clinical trials in this area are ongoing [147].

An involution of thymic tissue, resulting in a decreased output of naïve T cells is normally age related, but it could also be caused by different medical conditions. Treatment with glucocorticoids, various immunotherapies, and transplantation related conditioning treatment, as well as chronic infections could all damage the thymus [148]. Under these circumstances, exosomes could have a potential role as a treatment to rejuvenate the thymus.

Wnt4 is important in thymic organogenesis and to prevent thymic stroma to change into adipose tissue which could happen in the conditions described above. Banfai et al, isolated exosomes from TECs overexpressing wnt4, and demonstrated that these exosomes could contribute to maintain the thymic stromal structure and prevent the development towards adipose tissue. Based on these results they suggested that exosomes derived from TECs overexpressing wnt4 could have a potential role as a treatment to rejuvenate the thymus [149]. This could, for example, be implied in clinical conditions when thymic tissue is damaged and the output of naïve T cells has to be improved. Furthermore, EVs derived from mesenchymal stem cells (MSC), has been shown to restore reduced thymus function in neonatal mice caused by hyperoxia, which premature infants often are subjected to when treated with O_2. By treatment with EVs derived from MSC the disrupted output of Tregs and T effector cells was restored [150].

Exosomes from numerous cell types such as MSC, intestinal epithelial cells and Tregs have been reported to have a tolerogenic functions [129, 151, 152]. There are several hypotheses as to why some exosomes may have a tolerogenic function, and their expression of immunoregulatory factors is often discussed as well as their ability to transfer antigens and genomic material.

Exosomes have further been shown to restore disturbed self-tolerance and induce T cell mediated tolerance in autoimmune diseases such as MS, RA and Type 1 diabetes [153].

2 AIM

This thesis aims to investigate the role of thymic exosomes, as a transfer of TRAs and their impact on thymocyte selection, Treg formation and maturation of thymocytes

Paper I

Human thymic tissue produces exosomes. We wanted to investigate if human thymic epithelial cells, which express tissue restricted antigens (TRAs), produce exosomes carrying TRAs. Human primary thymic epithelial cells were cultured, and exosomes derived from the cells were isolated and further characterized.

Paper II

Based on the findings in paper I, we wanted to study the influence of thymic exosomes on late-stage maturation of thymocytes. $CD4^+$ thymocytes and thymic dendritic cells (DCs) were co-cultured *in vitro*. We also studied the uptake of thymic exosomes by SPCD4 thymocytes and thymic DCs.

Paper III

The aim of paper III was to study the influence of thymic exosomes on negative selection of self-reactive T cells and induction of Treg *in vivo*. This was achieved through a transgenic mouse model. We also traced thymic exosomes injected *in vivo*.

3 METHODS

Methods used throughout this thesis are well established methods for isolation and characterization of EVs as well as functional immunoassays, cell culture and analyses. The challenging parts will be discussed below.

Paper I, Cell culture of human primary TEC

Human TECs are known to be challenging to culture and at the same time preserve their specific characteristics, eg AIRE expression and expression of self-antigens. In the project a TEC culturing protocol published by Röpke was used [154], and cells were cultured in a 2D structure. However, later publications have shown 3D culture system to be more favorable for maintaining the TEC phenotype when studying cellular interactions [33]. However, the intention of our study was to reveal if TECs produced exosomes containing TRAs, and for this purpose the 2D system was sufficient.

Paper II and III, Isolation of thymic exosomes from thymic tissue

The intention and main purpose of this thesis was to isolate and study an as pure fraction as possible of exosomes. In the current studies exosomes from mouse thymic tissue and human TEC cell cultures were isolated using a standard protocol based on ultracentrifugation [155], which had been used previously in the research group and resulted in the isolation of well characterized exosomes [136]. However, the thymus is an organ rich of apoptotic bodies with a wide size range [113], often over 1000 nm, which should be removed by the extra filtration step of the supernatant through a 200 nm mesh, that was added to the updated exosome isolation protocol used in the studies [137, 155, 156].

For paper II and III, mouse thymic tissue was pressed against a nylon mesh, and a cell free supernatant was collected for further exosome isolation, figure 3a. A challenge when harvesting exosomes from tissue is to avoid particles from broken cells and to distinguish them from intracellular vesicles. As described in MISEV2018, an absolute purified EV isolation of exosomes cannot be achieved with methods of today, even though several methods have been proposed to get purer fractions of different EVs [157]. Tissues could be treated with enzymes to isolate exosomes [158], however it is not known how this would affect the quality of the thymic exosomes and e.g. their immunoregulatory ability *in vivo*. It is even suggested in the MISEV 2018 that less pure EVs could be accepted in some therapeutic situations [157]. This is well in context with the purpose of this thesis, to study the biological functions of exosomes in establishing central tolerance rather than to study EV characteristics in detail.

Paper III Transgenic mice, exosome-dose and timepoints for read out
Characterizations and *in vitro* studies can be performed by using human thymus. However, transgenic mouse models are crucial to study central tolerance *in vivo*. A large proportion of $CD4^+$ T cells in 3A9 TCR mice carry a high-affinity TCR which recognize the dominant HEL-peptide in complex with MHC II, referred to as HEL-specific T cells. The HEL-mice express HEL regulated by the insulin promotor. Thymic exosomes carrying the dominant-HEL peptide were injected into TCR mice using two different protocols, figure 3b. The amounts of exosomes injected was 100µg, but it is not known what proportion of these exosomes that carry the HEL-peptide and how many of the injected exosomes that actually reach the thymus. Another challenge of the study design is the different timepoints, at what age to administer the exosomes, and in how many doses to maintain an peripheral effect. However, we choose to divide the study into short and long term experiment which resulted in different outcomes, with the HEL-specific effects on $CD4^+$ cells seen only in the short term experiment.

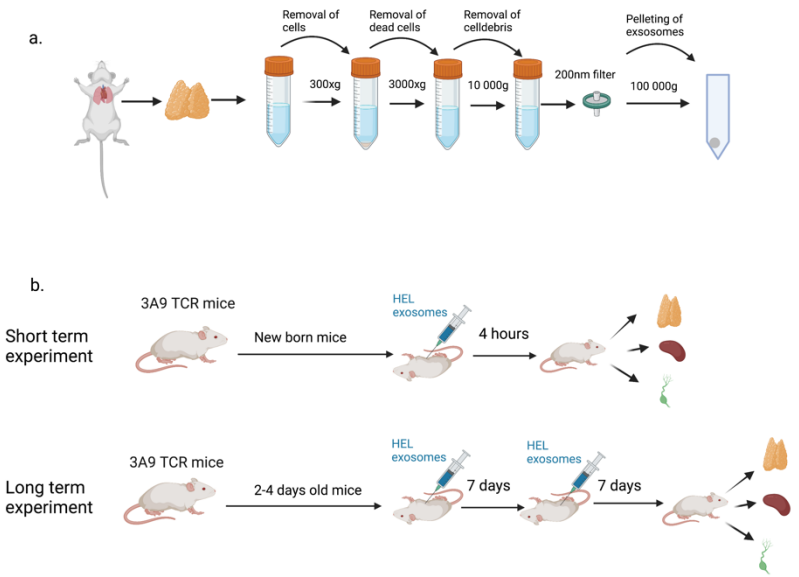

Figure 3a) Schematic illustration of isolation of thymic exosomes. 3b) i.p. injection of thymic exosomes. For the short term experiment thymic exosomes were injected i.p. in new born mice which were sacrificed after 4 hours. In the long term experiment, thymic exosomes were injected at the age of 2-4 days, and repeated after 7 days. Mice were sacrificed after an additional 7 days. For both protocols, thymic tissue, lymph nodes and spleens were collected for further analyses. *Created with Biorender.com*

4 RESULTS AND DISCUSSION

The three papers included in the study are a continuation of earlier studies in the research group. Human thymic exosomes were first characterized by Skogberg et al, which also reported them to carry TRAs [136]. Paper I was designed to characterize exosomes derived from human primary TEC cultures. In Paper II we wanted to study direct and/or indirect effects of murine thymic exosomes via DCs on thymocyte maturation and Treg development, which was done *in vitro*. In order to investigate the influence of thymic exosomes on negative selection and Treg induction *in vivo* we used a transgenic mouse model in paper III.

4.1 PAPER I

4.1.1 Thymic exosomes derived from human TECs

A number of reports have described antigen transfer from mTECs to APCs in the thymus [98, 101-103, 159], but it was not until 2008 that thymic exosomes-like particles in mice were characterized and their immunological effects were studied [160]. In 2013 the first report of human thymic exosomes was published by our group, in which exosomes were characterized in terms of size, morphology, surface markers as well as analyzed regarding their content of proteins and miRNA. Interestingly, thymic exosomes from whole human thymic tissue carried TRAs, and had markers indicating an epithelial origin [136].

These findings lead to an interest in trying to isolate pure human thymic epithelial exosomes. Human thymic tissue was cut in to pieces, enzymatically treated, washed, and then put into cell culture. Cells were cultured for six days in a serum-free medium to avoid contamination, a protocol published by Röpke [154, 161]. RANKL promotes AIRE expression and is therefore needed when culturing TECs to retain their AIRE expression [33]. The cultured TECs showed a similar morphology as previously described [161]. Interestingly, Hassall´s corpuscle-like structures started to appeared after one week of cell culture. Cells were further characterized for their expression of TEC cell surface markers, EpCAM, Keratin 5 and 8, and for their mRNA expression of FOXN1, AIRE, K5, and K8. TRA-mRNA was found for known AIRE-dependent TRAs such as myelin basic protein (MBP) and insulin (INS). In summary, the cultured human TECs were able to express AIRE and TRAs.

Exosomes were isolated from the cell cultures with a size range of 50 to 200 nm and a peak in size at 136nm. The exosomes were characterized using commonly used exosomal surface markers, and stained positive for TSG101, HLA-DR, CD9, and CD81. Furthermore, using MS/MS analysis 1220 cellular proteins and 773 proteins of putative exosomal origin such as TSG101, CD82, CD63, MFG-E8 and FLOTILIN-1 were identified in the thymic exosomes [137]. A main purpose of this thesis was to investigate if thymic exosomes contains TRAs, defined as proteins expressed in at most five different tissues of the body [162]. Interestingly, among the TRAs carried by the exosomes were: Desmoglein 1 and 3, Titin, Collagen 17, Vinculin, Galectin 7, Tissue transglutaminase 2 and MBP, all self-antigens associated with autoimmune diseases. In summary, paper I shows that primary cultures of human primary TECs, produces exosomes with a protein content of TRAs associated with autoimmune diseases [137].

4.2 PAPER II

4.2.1 Thymic exosomes induce maturation of SPCD4 thymocytes

Positively selected thymocytes undergo several maturation steps in the medulla before they egress from the thymus [24]. Mechanisms underlying the thymocyte maturation are not as well studied as the mechanisms involving the negative selection. However, TECs as well as DCs are known to be necessary for both thymocyte maturation and selection [163]. As described in paper I, TECs secrete exosomes, which may serve as communicators between thymic stromal cells and DCs and/or developing thymocytes. The aim for paper II was to study the effects of thymic exosomes on the late-stage maturation of SPCD4 thymocytes. Thymic exosomes were isolated from thymic tissue derived from C57BL/6 or Balb/c mice. The exosomes showed a size range of 50-200 nm, and flow cytometry confirmed the expression of the well described exosome markers CD9, TSG101, MFG-E8, MHC II and Lamp-1 as well as TGFβ. Interestingly, they also expressed EpCAM, which could indicate an epithelial origin. The mouse thymic exosomes contained 1556 proteins when analyzed using MS/MS, including the following exosome markers: Lamp1, Annexin I, II and V as well as Ras-related proteins (Rab) which have a crucial role in exosome biogenesis [113, 156].

In accordance with earlier studies, TRAs were detected in the proteomic analysis of the exosomes. Interestingly, proteins important for thymocyte maturation and for thymocytes to exit the thymus were also detected and this included the following proteins: Sphingosine-1-phosphatase lyase (SPGL1), Dedicator of cytokinesis protein 2 (DOCK2), Rho GDP-dissociation inhibitor 1 (GDIR1) and p21 protein-activated kinase 2 (PAK2) [164-167].

To investigate the influence of thymic exosomes on thymocyte maturation, $CD4^+CD25^-$ thymocytes, DCs and exosomes were isolated from mouse thymic tissue. $CD4^+CD25^-$ thymocytes were co-cultured with thymic exososomes with or without $CDllc^+$ DCs for three days and analyzed for their expression of $S1P_1$, Qa2 and CCR7 to assess late-stage maturation. IL-7, otherwise produced by TECs and necessary for thymocyte development and survival, was added to the cell cultures [156, 168]. Cells with a mature $CD4^+S1P1^+Qa2^+$ phenotype were significantly enriched in the presence of thymic exosomes. Interestingly, this effect was independent of DCs and therefore suggests a direct effect of thymic exosomes on the thymocytes. The direct effect could be a result of

exosomes being internalized by thymocytes, which was confirmed by ImageStream and discussed below [156].

To determine if the effects of thymic exosomes on thymocyte maturation were dependent on expression of surface proteins or transfer of RNA, exosomes were pre-treated with proteinase K or RNase [124, 169, 170]. Pre-treatment with RNase reduced the RNA content in thymic exosomes, which resulted in a loss of effects of the thymic exosomes on thymocyte maturation. Proteinase K treatment reduced the surface expression of exosomal markers but did not reduce the expression of MHC II. Thymic exosomes pretreated with proteinase K were unable to induce maturation of SP$CD4^+$ thymocytes.

Thymic exosomes express MHC II, and to determine if the exosomal effect on maturation was MHC II-restricted a cross strain experiment was performed *in vitro,* which concluded that the observed exosome effect was independent of MHC II. Other groups have reported an immunoregulatory effect of exosomes independent of MHC II restriction [156, 171].

In conclusion, our results indicate a stimulatory effect of thymic exosomes on the induction of the final maturation of $CD4^+CD25^-$ thymocytes, dependent on exosomal surface proteins and RNA but independent of MHC II .

4.2.2 Exosomes impair Treg development *in vitro*

Thymic exosomes were added to $CD4^+CD25^-$ thymocyte cell cultures to study the impact on thymocyte maturation and Treg development, with or without the presence of DCs, as described above. Thymic exosomes impaired the Treg development independently of DCs [156]. Although earlier results have been conflicting, it has been reported that exosomes can suppress the Treg formation *in vitro* [172]. However, Wang et al reported an induction of Tregs by thymic exosome-like particles, even though this was only seen in peripheral organs [160].

4.2.3 Tracing thymic exosomes *in vitro*

In several studies exosomes have been labeled with fluorescent lipophilic dyes to enable to trace them *in vivo* and *in vitro*. Exosomes injected intravenously are reduced by half after two minutes in the circulation and have shown to be internalized by different cell types [173, 174]. There are a few publications reporting the homing of thymic EVs to thymic tissue. However, the first study in which tracing of thymic EVs was reported was by Wang et al who injected thymic exosome like particles (ELPs) labeled with IRDye 800CW iv. They reported that a majority of thymic ELPs were located in the lung and liver three hours after being injected iv. The study aimed at investigating the role of thymic ELPs in peripheral organs, and not in the thymus [160]. Furthermore, exosomes derived from a TEC cell-line and injected iv has been shown to be homing to the thymus, and are detected in the thymus after 24 hours. Interestingly, exosomes injected in young mice (2-month-old) where mostly found in the medulla, while in older mice (21-month-old) they were found in the cortex [149].

In paper II, we report that thymic exosomes co-localize with DCs already after 15 minutes, and that as much as 49% of the DCs showed co-localization with thymic exosomes after 4 hours *in vitro*. Interestingly, co-localization of thymic exosomes and $CD4^+$ cells was also detected [156]. Other groups have reported a reduced uptake of exosomes after pre-treatment with proteinase K [169, 170]. To investigate if the uptake of thymic exosomes was dependent on the expression of surface proteins, we pre-treated thymic exosomes with proteinase K which completely inhibited the co-localization with $CD4^+$ cells, while no inhibition was noted for exosome internalization or co-localization with thymic DCs [156].

4.3 PAPER III

4.3.1 Thymic exosomes transfer tissue-restricted antigen and regulate antigen specific tolerance

In the third paper, we show that thymic exosomes mediate antigen transfer and affect negative selection *in vivo*.

In order to study thymic exosomes *in vivo*, insHEL-TCR, a well described mouse model for studying central tolerance was used [175-177]. A significant part of the $CD4^+$ T cells in 3A9 TCR transgenic mice express a TCR with high affinity for peptide 46-61 of hen egg lysosome (HEL) complexed with MHC II molecule I-A^k [178]. HEL transgenic mice express HEL protein regulated by the rat insulin promotor (insHEL), which leads to an expression mainly in the pancreatic beta cells and the thymus, more specifically in the mTECs under the influence of Aire [179, 180]. A majority of the high-affinity HEL-specific T cells are deleted through negative selection in the thymus when 3A9 TCR transgenic mice are crossed with insHEL mice (insHEL-TCR) [43, 181]. The deletion of 3A9 TCR thymocytes is dependent on Aire expression by mTECs in this model [43]. The transgenic insHEL mouse model has been used to show that indirect antigen transfer is important for deletion of the self-reactive thymocytes [44].

In paper III, thymic EVs were isolated by ultracentrifugation of thymic tissue from HEL mice, with the purpose to isolate mainly exosomes, which were characterized to have a size range of 50-200nm, when analyzed with the ZetaView NTA methodology. The well described exosomal markers, CD9, MFG-E8 and LAMP-1 were expressed on the surface and their presence in the thymic exosomes was also confirmed by proteomic analysis. The C4H3 monoclonal antibody specific for HEL-(46-61)-I-A^k was used to confirm the presence of HEL peptide-MHC II complexes on the surface of thymic exosomes from HEL mice. That the exosomes contained HEL was further confirmed by proteomics, where the only HEL peptides present were the dominant aa 46-61, and the adjoining aa 34-45. In conclusion, thymic exosomes from HEL mice contain HEL peptides, which strengthens the theory that exosomes are involved in thymic antigen transfer. In the model, HEL is expressed under the insulin promotor and controlled by Aire in mTECs, indicating that exosomes carrying HEL are derived from mTECs.

To study the impact of exosomes on negative selection, thymic exosomes were isolated from HEL mice and 100μg was injected intraperitoneally (i.p.) into

newborn 3A9 TCR mice. Wild-type (wt) thymic exosomes were used as a control. Thymic tissue was collected after 4 hours and cells were analyzed using the 1G12 anti-TCR clonotype specific antibody, detecting $CD4^+$ T cells bearing a high affinity TCR for HEL, referred to as HEL-specific $CD4^+$ cells ($CD4^+1G12^+$). Injection of thymic exosomes from HEL mice significantly reduced total- and mature HEL-specific thymocytes in 3A9 TCR mice. This protocol is referred to as "short term experiment" in figure 3b.

In another protocol, thymic exosomes from HEL mice (100μg) were injected into newborn 3A9 TCR mice followed by an additional injection after one week. The mice were sacrificed at two weeks of age. This is referred to as "long term experiment" in figure 3b. An increased frequency of Treg was detected in lymph nodes and spleen, as well as an increased frequency of HEL-specific Treg in the thymus. However, there were no detectable long-term effects when looking at the whole population of HEL-specific $CD4^+$ T cells, even if there was an increased frequency of peripheral $CD4^+$ T cells expressing $CD25^+$ and $CD69^+$. This indicates that thymic exosomes derived from HEL mice induce a peripheral activation of $CD4^+$ T cells in 3A9 mice (paper III). However, the reduction of the frequency of HEL-specific thymocytes was only detected in the short-term experiment. In the long-term experiment, mice were sacrificed one week after the second dose of HEL-exosomes, and thymocytes stay in the medulla during 4-5 days for negative selection. This could explain why we were unable to detect effects on selection of HEL-specific thymocytes in the long-term experiment.

Studies on immunoregulatory functions of exosomes have mainly been done with exosomes isolated from primary cell cultures, and cell-lines, which can result in a diversity and a content of exosomes far from physiological conditions *in vivo*. However, by using the insHEL-3A9TCR transgenic mouse model, we report thymic exosomes to take part in the central tolerance induction, without any manipulation of cells by adding stimulatory factors in cell cultures before injecting the exosomes *in vivo.*

In summary, an indirect antigen transfer in the thymus could be mediated by thymic exosomes that are able to carry antigens expressed in mTECs under the influence of Aire. This is the first report showing the effects of thymic exosomes on negative selection and induction of Treg *in vivo*.

4.3.2 Exosomes and regulatory T cells

The development of FoxP3$^+$ Tregs is dependent on an organized thymic medulla, and the presence of mTECs as well as the transfer of Aire-dependent TRAs to DCs. CDllc$^+$ DCs have been reported to be the DCs mainly involved in Treg development when compared with other bone marrow derived APCs [64, 87]. Antigen transfer from mTECs to DCs could be mediated by thymic exosomes, which, as shown by our group, are able to carry TRAs [138]. Furthermore, TGF-β is crucial for induction of Treg in the periphery, by inducing the expression of Foxp3 in CD4$^+$CD25$^+$ T cells. Development of thymic Treg is dependent on TCR signaling but also on the presence of TGF-β, which is expressed on thymic exosomes, true for both humans and mice [156, 182]. Thymic-exosome like particles are reported to induce Foxp3$^+$Tregs in peripheral organs, which was suggested to be an effect of their high content of TGF-β [160] Exosomes are also described to influence tolerance in the periphery. EVs from intestinal epithelial cells, have high levels of TGF-β1and prevents IBD development in mice by the inducing Tregs [152]. In summary, exosomes are able to express co-stimulatory molecules which could be of importance in the development of Treg precursors [183].

4.3.3 Thymic exosomes induce Tregs *in vivo*

As described above, thymic exosomes from HEL-mice carry the HEL-peptide regulated by the insulin promotor, influenced by Aire. Thymic Tregs are developed before 7 days of life in mice [67, 68], and to study the influence of thymic exosomes on Treg development, thymic exosomes from HEL-mice were injected i.p. into new born 3A9 TCR mice before the age of 4 days. This was followed by an additional injection after one week, and the mice were sacrificed one week after the last injection (figure 3b). Interestingly, a significant increase in the frequency of the Treg population was detected in peripheral organs when compared to mice injected with wt thymic exosomes. In addition, thymic exosomes from HEL-mice significantly increased the frequency of HEL-specific Tregs in the thymus, even though the cell numbers analyzed were low, which should be taken in consideration when drawing conclusions (paper III). This data shows an induction of Tregs, dependent on indirect antigen transfer of TRA, mediated by thymic exosomes *in vivo* [105, 106].

In conclusion, thymic exosomes induced Treg development *in vivo* (paper III), which was in contrary to the observations *in vitro* [156]. This illustrates the difficulties of comparing *in vitro* studies to physiological conditions *in vivo*. It

is therefore important to be able to test observations seen *in vitro* in an *in vivo* model.

4.3.4 Tracing thymic exosomes *in vivo*

In paper II, uptake and co-localization of thymic exosomes and SPCD4 thymocytes or thymic DCs was studied *in vitro*. To study homing of thymic exosomes *in vivo*, thymic exosomes were labeled with VybrantDiD and injected i.p. in 3 week old mice. Thymic exosomes were present in the thymus 24 hours after injection, mostly detected in the CMJ, but also in the cortex and the medulla (figure 3, paper III). A co-localization of thymic exosomes with mTEC and thymic DCs was seen both by confocal microscopy and ImageStream analysis. Flow cytometry was performed to analyze the frequency of exosome co-localization with mTECs and DCs at 24 hours, 48 hours and 5 days. The frequency of thymic DCs that stained positive for exosomes was 40%, and as much as 80% of the mTECs stained positive for exosomes, with a maximum peak at 48 hours. The co-localization of exosomes with mTECs and thymic DCs did not vary much during the different timepoints. In addition, spleens were used as a control, and thymic exosomes were mainly located in the marginal zone and 70% of splenic DCs showed a co-localization with exosomes at 48 hours (Paper III). In conclusion, thymic exosomes home to the thymus and are co-localized with thymocytes, mTECs and DCs.

Exosomes have previously been reported to be homing to the thymus. BMDC derived exosomes labeled with PKH26 dye and injected into rats were detected after 3 days mainly in the CMJ and the cortex. Exosomes were also shown to be internalized by keratin positive cortical epithelial cells [184].

5 CONCLUSIONS

In **Paper I**, we cultured human primary epithelial cells and showed that they expressed self-antigens and secreted exosomes carrying the self-antigens, some associated with autoimmune diseases. The exosomes also expressed immunoregulatory molecules.

In **Paper II,** we demonstrate using an *in vitro* model, that thymic exosomes induce the final maturation of thymocytes in which they express molecules necessary to egress the thymus and migrate to the periphery.

Paper III shows that thymic exosomes are able to transfer self-antigens and mediate central tolerance *in vivo*.

We suggest that exosomes are important in the thymic microenvironment, as a communicator in the transfer of antigens from thymic epithelial cells to other APCs, as well as being directly involved in the process of thymocyte maturation.

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