Characteristics and functions of thymic exosomes in human and mouse

Gabriel Skogberg

Department of Rheumatology and Inflammation Research
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg,
Sweden

UNIVERSITY OF GOTHENBURG

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Till alla **barn födda med hjärtfel**; denna avhandling var av er och för er!

More than meets the eye – George Arthur Bloom

ABSTRACT

Thymocytes develop in the thymus to become a functional pool of T-cells in the periphery. To achieve this, the thymocytes go through several steps of maturation and selection within the thymus. The goal is a cell population able to recognize foreign structures, in order to help in the defense against invading pathogens/infectious agents, which at the same time tolerate self-structures and thereby avoid autoimmunity. Exosomes are nano-sized vesicles released into the extracellular space by cells. They carry components such as proteins, micro-RNA and mRNA between cells and are able to participate in inter-cellular communication. We have isolated and characterized exosomes from human thymus and we demonstrate that exosomes are abundant in thymic tissue and that they share features with other exosomes but have their own characteristic niche. Tissue-restricted antigens (of which many are under the control of the autoimmune regulator gene) are antigens expressed by highly specialized medullary thymic epithelial cells. The role of these antigens is to mirror self-antigens found in the periphery, in order to deplete self-reactive thymocytes in the negative selection process. Tissue-restricted antigens were found in exosomes from human thymic tissue and also in exosomes from primary cultures of human thymic epithelial cells. Also, several known autoantigens e.g. myelin basic protein and transglutaminase 2 were found in these exosomes. When studying the effects of exosomes on thymocyte maturation, with an *in vitro* model, we showed that thymic exosomes stimulated the final steps of single-positive CD4+ thymocyte maturation. Finally, since Down syndrome patients share autoimmunity and autoantibodies with patients carrying mutations in the *autoimmune regulator*, and this gene is located on chromosome 21, we investigated the expression of the autoimmune regulator in thymus from Down syndrome patients. The expression was up regulated and also accompanied by other thymic abnormalities such as accumulation of CD11c+ cells in the medulla and an altered protein composition of thymic exosomes.

Keywords: Exosome, thymus, tissue-restricted antigen, Aire, Down syndrome

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List of Original Papers

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

I. **Gabriel Skogberg**, Judith Gudmundsdottir, Sjoerd van der Post, Kerstin Sandström, Sören Bruhn, Mikael Benson, Lucia Mincheva-Nilsson, Vladimir Baranov, Esbjörn Telemo, Olov Ekwall. Characterization of Human Thymic Exosomes. *PLoS ONE 8(7): e67554. doi:10.1371/journal.pone.0067554*

II. **Gabriel Skogberg**, Vanja Lundberg, Martin Berglund, Judith Gudmundsdottir, Esbjörn Telemo, Susanne Lindgren and Olov Ekwall. Human thymic epithelial primary cells produce exosomes carrying tissue restricted antigens. *In manuscript.*

III. Vanja Lundberg*, Martin Berglund*, **Gabriel Skogberg**, Susanne Lindgren, Judith Gudmundsdottir, Esbjörn Telemo and Olov Ekwall. Thymic exosomes promote the maturation of developing thymocytes. *In manuscript.* *Equal contribution.

IV. **Gabriel Skogberg**, Vanja Lundberg, Susanne Lindgren, Judith Gudmundsdottir, Kerstin Sandström, Olle Kämpe, Göran Annerén, Jan Gustafsson, Jan Sunnegårdh, Sjoerd van der Post, Esbjörn Telemo, Martin Berglund and Olov Ekwall. Altered Expression of Autoimmune Regulator in Infant Down Syndrome Thymus, a Possible Contributor to an Autoimmune Phenotype. *The Journal of Immunology, 2014, 193: 2187–2195.*

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
CCR7	c-c chemokine receptor type 7
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5
CHRNA1	the α-subunit of the nicotinic acetylcholine receptor
cTEC	cortical thymic epithelial cell
DC	dendritic cell
DN, DP, SP	double negative, double positive, single positive
DSCR	down syndrome critical region
EV	extracellular vesicle
HC	hassall´s corpuscle
HLA	human leukocyte antigen
ICAM-1	intracellular adhesion molecule 1
IHC	immunohistochemistry
IL	interleukin
INS	insulin
IRBP	interphotoreceptor retinoid-binding protein
ITGB2	integrin beta-2
LAMP1	lysosomal-associated membrane protein 1

LCL	B-lymphoblastoid cell line
MFGE8	milk fat globule-EGF factor 8 protein
MHC	major histocompatibility complex
miRNA	microRNA
MS	mass spectrometry
mTEC	medullary thymic epithelial cell
MUC1	mucin1
MVB	multi vesicular body
NFAT	nuclear factor of activated T-cells
nTreg	natural regulatory T-cell
PGE	promiscuous gene expression
SOD1	intracellular Cu,Zn superoxide dismutase
$S1P_1$	sphingosine-1-phosphate receptor 1
TCR	T-cell receptor
TRA	tissue-restricted antigen
TNT	tunneling nano tube
TG	thyroglobulin
TPO	thyroid peroxidase
TREC	T-cell receptor excision circle
Treg	regulatory T-cell
TSLP	thymic stromal lymphopoietin
XCL1	chemokine (C motif) ligand 1

Aims

Paper I: Previous work described the isolation and characterization of exosome-like particles in mouse thymus. No work was however performed using human thymic tissue so, in this paper we wanted to study if exosomes were present in human thymic tissue and if so, characterize these vesicles.

Paper II: Based on the findings in Paper I we wanted to investigate whether thymic epithelial cells are able to produce exosomes. To do this, we cultured primary human thymic epithelial cells and used the cultures as source for exosomal isolation and characterization.

Paper III: In this paper we wanted to study the effects of thymic exosomes. We used an *in vitro* system including mouse thymic exosomes and CD4+ thymocytes. We were also interested in possible contribution from an indirect exosomal route, so co-cultures were included in which thymic dendritic cells (DCs) were present.

Paper IV: Patients with Down syndrome and patients with mutations in the chromosome-21-located gene *autoimmune regulator* (*AIRE*) share both autoimmunity and autoantibodies. In Paper IV we wanted to investigate whether the increased *AIRE* gene dose was accompanied by an altered expression of AIRE and AIRE regulated tissue-restricted antigens (TRAs) in thymic tissue from Down syndrome patients. We also investigated DS thymic tissue for differences in cell composition and exosome characteristics.

Central tolerance – an introduction

Thymus - As a primary lymphoid organ, the thymus is responsible for selection, maturation and output of a functional T-cell pool into the periphery. Mature T-cells must, as key adaptive immune cells, be able to recognize foreign structures and at the same time be blind to self-structures to avoid autoimmunity. To perform this selectional-educational task, the thymus consists of a highly specialized core stromal network made up mainly by epithelial cells. Two major microenvironments constitute the thymus: the cortex and the medulla in which thymocytes are positively and negatively selected, respectively. An organ-specific feature of the thymic medullary stroma is the expression of otherwise tissue-restricted antigens: TRAs (also known as tissue specific antigen – TSA, or peripheral tissue-restricted antigen - PTA). The presence of such antigen repertoire minimizes educational gaps in the negative selection/clonal deletion of thymocytes and the occurrence of autoimmune events in the periphery is impaired. Breakdown of thymic function and central tolerance will ultimately lead to autoimmune disease.

Thymocytes - Developing thymocytes interact with the stroma in different thymic microenvironments to complete their maturation and selection before being exported to the periphery (See Figure 1 for schematic illustration). Briefly, thymocyte precursors migrate from the bone marrow via the blood and enter the thymus in the area of cortico-medullary junction. At this stage, they migrate toward the subcapsular zone of the cortex and also rearrange their T-cell receptor (TCR) β-locus to express pre-TCRs. The thymocytes have so far been double negative (DN), lacking expression of the co-receptors CD4 and CD8, but now acquire these molecules to become double positive (DP). They also rearrange their TCR α-locus to express TCRs, which interact with cortical thymic epithelial cells (cTECs) in the process of positive selection. If the positive selection is successful, i.e. if the TCRs are able to bind to major histocompatibility complexes (MHC) of class I or II with a moderate affinity, the thymocytes mature into CD8 or CD4 single positive (SP) cells. However, if no interaction, the thymocytes go into apoptosis due to neglect since a cell carrying a TCR that is unable to interact with either peptide/MHC I or peptide/MHC II is useless to the immune system. Alternatively, the interaction with peptide/MHC complex is too

strong which leads to negative selection. The thymocytes that survive the positive selection migrate towards and into the medulla where they interact with medullary TECs (mTECs) in a self-peptide rich microenvironment for another round of negative selection. Only the thymocytes with non-activating interactions to self-peptide/MHC complexes will be exported to the periphery.

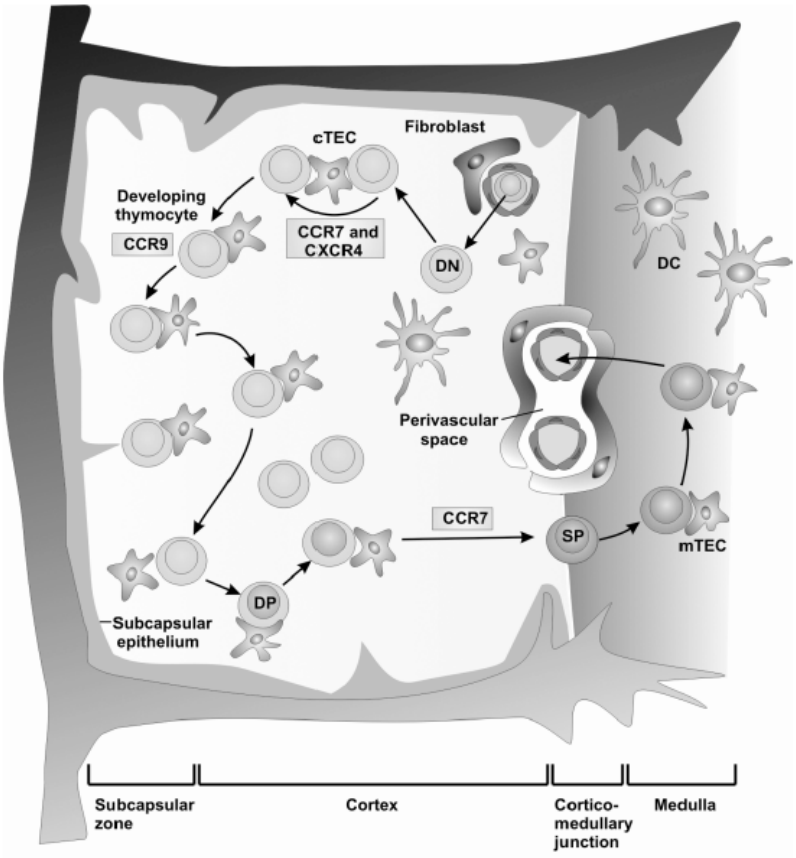

Figure 1. *Schematic overview of thymocyte migration, development and selection in the thymus*. After thymic entry, thymocytes go through several states, highlighted in this cartoon are DN, DP and SP-phases and thymic exit. cTECs mediate both negative and positive selection and are located in the cortex. mTECs mediate negative selection and present a battery of self-peptides. DCs mediate indirect presentation of stroma-expressed antigens.

C-C chemokine receptor type 7 (CCR7) is important for thymocyte migration into the medulla. CCR7: C-C chemokine receptor type 7. CXCR4: C-X-C chemokine receptor type 4. Adopted from (1).

Exosomes, mediators of communication

Hallmarks in exosome research - Exosomes are small vesicles released by cells into the extracellular space (see Figure 2 for a schematic exosome). They are a subgroup of extracellular vesicles (EVs) formed by inward budding of membranes in late endosomes, which create multi-vesicular bodies (MVBs) that contain exosomes (2, 3). The recycling of the transferrin receptor back to the cell membrane from an endocytic route in developing erythrocytes was, in hindsight, the first description of exosomes (4) according to the "endocytic origin definition" – see next section for vesicle terminology. In 1996, the first description of exosomes with antigen presenting capability was reported by Raposo et al (5). This study showed that both human and murine B-cell derived exosomes could induce an antigen-specific MHC II-restricted T-cell response. This article confirmed the mechanism of vesicle formation in late endosomes and the release of these vesicles at the plasma membrane that was first demonstrated in reticulocytes (4, 6). Much attention to exosomes was drawn by a study of Zitvogel and co-workers in which they could show that tumor peptide-pulsed DCs release exosomes which carry MHC I and II as well as co-stimulatory molecules, and that these exosomes primed cytotoxic T-cells and suppressed growth of established tumors (7). Further in the context of antigen presentation, exosome-like structures named tolerosomes, are released from small intestinal epithelial cells and have been shown to induce antigen specific tolerance (8). This study is interesting since the biological context is similar to that of thymic exosomes with an intimate interaction between epithelial cells and T cells and with focus on tolerance induction (fed antigens and self-antigens respectively). Since exosomes are abundant in, and fairly easy to purify from, fluids such as saliva (9), peripheral blood (10), bronco alveolar lavage fluid (11) and urine (12, 13) one area of extensive research is exploring the use of exosomes as diagnostic biomarkers (14). One particularly striking finding, which in recent years has heavily influenced exosome research, was the identification of microRNA (miRNA) and mRNA in exosomes (15). This study also showed transfer of

exosomal mRNAs between cells, leading to translation of proteins in recipient cells. In the context of T-cells it is particularly interesting to note that exosomes produced by regulatory T-cells (Tregs) contribute to a tolerogenic environment. Smyth and co-workers isolated exosomes from CD4+CD25+Foxp3+ cells and suggested that the observed suppression of T-cell response induced by the exosomes was mediated via CD73 molecules found in the exosomal surface membrane (16). CD73 has earlier been shown to contribute to Treg suppressing activity by converting adenosine monophosphate into anti-inflammatory adenosine (17). Exosomes from various cancer cell lines have also been shown to exert effect by using the mechanism of adenosine production via CD73 (18). More recent work regarding Treg exosomes suggests that their suppressing mode of action is exerted via miRNAs (19).

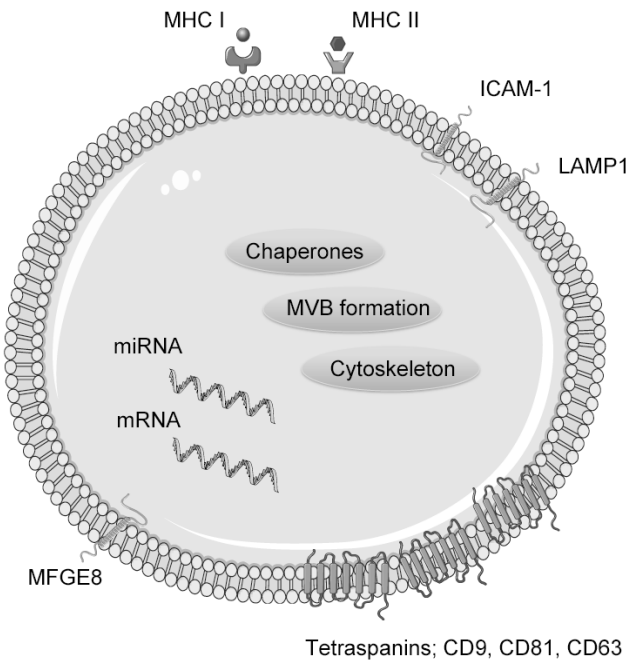

Figure 2. *Schematic illustration of components typically found in exosomes.* The lipid bilayer of exosomes resembles that of cellular membranes with a high content of lipid raft domains and sphingomyelins (20, 21). Proteins are found both in the lumen and in the enclosing membrane of exosomes. Nucleic acids are also often identified within exosomes. Intercellular

adhesion molecule 1 (ICAM-1) has for example been shown to be enriched on exosomes released by mature DCs (22). Thymic exosomes share typical exosomal proteins such as CD9 and CD81 but seem to lack others, such as CD63. LAMP1: Lysosomal-associated membrane protein 1, MFGE8: Milk fat globule-EGF factor 8 protein. The figure was produced using Servier Medical Art with inspiration from (23) and (24).

Vesicle terminology – Today there is considerable confusion regarding terminology in the field of exosomes. The field has for long been aided by the use of the term exosomes, but lately it has been partly hampered by it. The distinction between exosomes and other EVs, such as microvesicles and ectosomes, has been a recurring point of discussion and illustrates the need of an exosomal definition (24). It started with the hijacking of the term exosomes that originally referred to pieces of DNA (25) and has continued with a change in vesicle definition. The first exosome definition suggests that the term exosomes refers to vesicles with a plasma membrane origin (26) while the second definition states that an exosome is a vesicle with an endocytic pathway origin formed by inward membrane budding in a late endosome resulting in a MVB (2, 27). This is the dominating definition in theory, but even so, since the use of a differential centrifugation protocol to isolate exosomes is considered standard this means that the practical definition is often based upon isolation protocol (28, 29). Adding complexity, numerous other vesicle names have been proposed based on the source or function of the vesicles.

Exosomal isolation – All exosomal isolations in the current work are made using stepwise ultracentrifugation based upon the protocol introduced by Thery and co-workers (28). A step of filtration through a 200 nm mesh was added to the original protocol (see Figure 3 for procedure). In Papers II and III the last step of exosomal wash was excluded. This approach has since introduced been considered standard approach within the field. Additional approaches have been suggested as alternative methods for exosomal isolation, but the ultracentrifuge approach is still widely used. Recently however, a commercially available, gradient based method of isolation has demonstrated promising purity (30). Another novel approach to exosomal isolation is the use of small peptides that depending on the membrane curvature and lipid composition of the vesicles in the sample bind with high

affinity to certain vesicles while not to others (31, 32). Also in other specific methodological areas efforts are focused on developing approaches adapted for exosomes, one example being the accurate and specific measurement of exosome concentration (33).

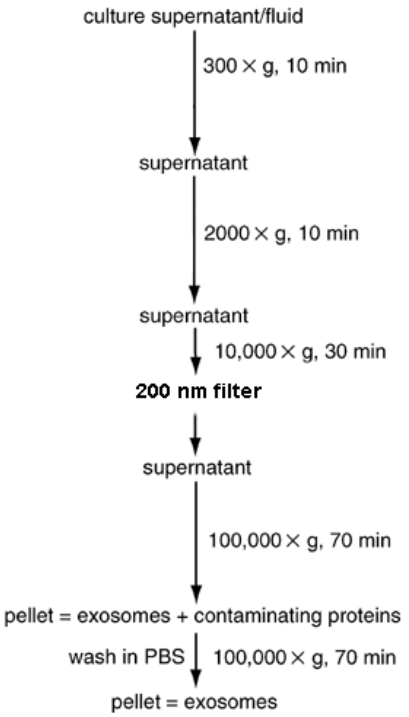

Figure 3. *Methodological approach for isolation of exosomes using ultracentrifugation*. The method is based upon protocol in (28) with addition of a filter step (200 nm) after the 10 000 x g centrifugation.

Cultured TECs – In Paper II we specifically wanted to answer the question if TECs are able to produce exosomes. Previously, other epithelial cell types have been reported to be competent producers of exosomes such as human intestinal epithelial cells (IEC) whose exosomes carry the IEC marker A33 (34). Human tracheobronchial ciliated epithelium releases exosomes that can inhibit influenza infection of the epithelia via exosomal α-2,6-sialic acid residues (35). Exosomes have also been isolated from cultured normal human bronchial epithelial cells (36). To avoid the introduction of contaminating exosomes, we used a serum-free approach presented by

Röpke and co-workers (37, 38), see Figure 4. Other culturing-conditions have been described by Fernandez and co-workers who used 10% FBS, and the resulting cloned TEC line displayed a cTEC-like phenotype (39). Berrih and co-workers also developed a similar culture protocol that use 10% FBS (40). Although suitable for the current exosomal question, which demands as pure TECs as possible, a 2D culture approach has its limitations. Mouse thymic stromal cell monolayer cultures fail in supporting thymopoiesis due to lack of 3D-architechture leading to expressional loss of Delta-like 1 and Delta-like 4 in contrast to fetal thymic organ cultures (41) (importance of Delta-like 4 has been confirmed in vivo (42)). Another more recent approach aims at mimicking the three-dimensional structure of the thymic epithelial network and thus succeeds in maintaining other key features of mTECs such as Aire-expression (43). The authors in REF43 emphasize the need of adding RANK-ligand for promoting Aire-expression, which made us include RANK-ligand in the culturing protocol in Paper II. The cultured cells in our work resemble TECs from many points of view: typical epithelial morphology (Figure 5), formation of structures which resemble Hassall´s Corpuscle (HCs) (Figure 5), they are to varying degree EPCAM+, Keratin 5+, Keratin 8+, HLA-DR+ and express *FOXN1*, *KRT5*, *KRT8* and *AIRE*-mRNA (immunofluorescent stainings and RT-PCR respectively). Also, the cultured TECs did produce exosomes that carried typical TEC-proteins such as Keratin 5, Keratin 14, HLA-molecules and immunoproteasome subunits. For TRA and autoantigen-content see "Human TRAs and autoantigens in the literature and Paper II" below.

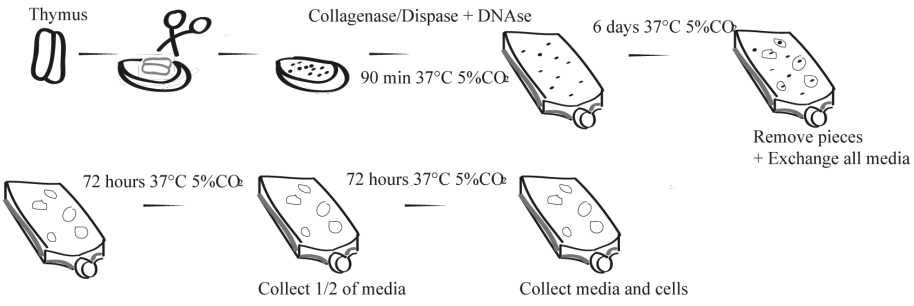

Figure 4. *Scheme of thymic culture approach; tissue piece removal and exosome-supernatant collection.* Briefly**,** the tissue was washed, trimmed, cut into millimeter-sized pieces, and then incubated with enzymes. The pieces were then added to complete media and cultured for 6 days. Pieces

and media were discarded and replaced with fresh complete media. Cells were cultured for another 72 hours and half of the media was collected and replaced. Finally, after another 72 hours of culture, all media was collected for exosomal isolation and cells were trypsinized and collected for analysis.

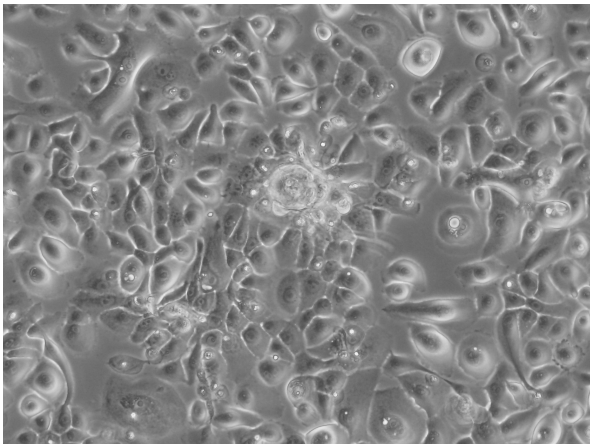

Figure 5. *Cultured human epithelial TECs seen in light microscope.* As described in Paper II the cultured cells have a typical epithelial morphologic appearance. Note the presence of the HC-like structure just above the central point of the image.

Exosomal morphology – Traditionally, exosomes are considered to be in the size range of 40-100 nm (3). The size is however varying depending on the method of measuring it and depending on the sample origin, which has led to a questioning of the narrow exosomal size range (44). When visualized in electron microscopy these vesicles often appear cup-shaped, which is considered to be an artifact from sample fixation prior to analysis with transmission electron microscopy. The cup shape was evident in exosomes analyzed with transmission electron microscopy (Paper I and Figure 6). The vesicles isolated in the current work range roughly from 30-250 nm in size for human exosomes (see Paper I and II), while the mouse exosomes were below 100 nm in size (Paper III). Exosomal size was determined using the Brownian motion-based instrument NanoSight LM10. Also, exosomes have been shown to differ in density from other vesicles such as apoptotic bodies. This difference depends on different protein composition. Apoptotic bodies for example contain larger amounts of heavy

nucleosomes than exosomes and these vesicle groups float in different density levels (exosomes: 1.15-1.18 g/ml, apoptotic bodies: 1.24-1.28 g/ml) when examined using a sucrose gradient (45). In Paper I the isolated exosomes have a density of approximately 1.15-1.21 g/ml.

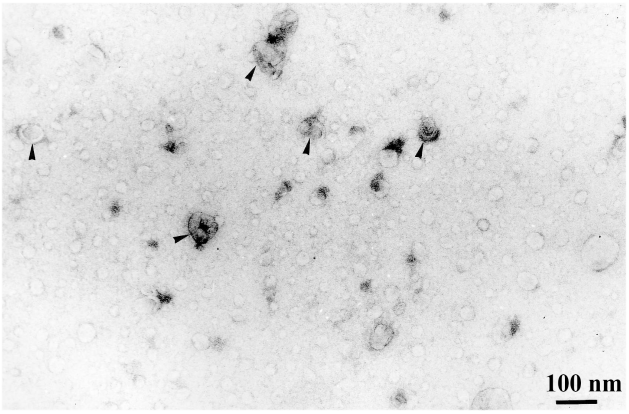

Figure 6. *Human thymic exosomes visualized in transmission electron microscopy (Paper I).* Arrowheads point toward exosomes with the typical cup-shaped morphology and a size range of 50–100 nm. Also note the presence of smaller vesicles.

Typical exosomal proteins – No protein, peptide, lipid, nucleic acid or other structure has been found to be common for all exosomes (finding an unique exosomal element would yet again alter the exosomal definition if applied). Researchers use a combinatorial approach to characterize exosomes. When it comes to proteins exosomes frequently contain in their membrane and/or lumen: CD9, CD81, CD63, moesin, annexins, rabs and tubilins to mention a short list (23, 46). By coupling exosomes to 4 μm beads, vesicle surface markers can be analyzed using standard flow cytometry. In Paper I, II, III and IV the exosomes were analyzed with this approach and shown to carry typical exosomal markers, e.g. CD9, CD81, TSG101 and MHC II. Notable is that the human thymic exosomes and human TEC exosomes are low in CD63 (Paper I, Paper II). This is a protein-profile that could be thought of as atypical when compared with other exosomes described in the literature. However, this lack of CD63 could have functional implications for thymic exosomes since it has been reported that

knockdown of the tetraspanin CD63 in a B-lymphoblastoid cell line (LCL) using short hairpin RNA resulted in increased CD4+ T-cell recognition as evaluated by INFγ-production. The increase in T-cell response could not be explained by changes in antigen processing or MHC II-expression (47). It turned out that equal amounts of exosomes from $CD63^{low}$ LCL cells and control LCL cells stimulated the T-cells to comparable degrees, but the $CD63^{low}$ LCL cells produced more exosomes. This could in the end enhance the total T-cell-stimulatory capacity of the $CD63^{low}$ LCL cells. Of further potential importance is the presence of ICAM-1 on thymic exosomes (Paper I), since ICAM-1 has been suggested to enable direct T-cell stimulation by exosomes (48). Another interesting protein present on thymic exosomes is MFGE8, which has been described to bind to αvβ3 and αvβ5 expressed by human DCs and macrophages, and has been proposed to be involved in the homing of exosomes to DCs (49). Admyre and co-workers reported that MHC I alone was critical for the stimulation of CD8+ T-cells by monocyte-derived DC exosomes (50). This notion is interesting since it has been shown that the activation of a T-cell requires over 60 copies of a relevant peptide/MHC complex (51). Apparently, exosomes are, at least under favorable conditions, able to provide a sufficient amount of peptide/MHC complexes for T-cell stimulation. With this being noted, the mouse thymic exosomes reported in Paper III did however not exert an effect on CD4+ thymocyte maturation via MHC II since in cross-strain experiments (e.g. thymic exosomes isolated from BALB/c mice and cultured with C57BL/6 thymocytes) no differences were seen between exosomes from different strains. Also, blocking of MHC II on the exosomes had no effect. In addition, membrane bound TGFβ was identified on the exosomes in Paper I and Paper II (not stained for in flow cytometry experiment in Paper II). In Paper I this prompted us to speculate about a possible role for thymic exosomes in thymic Treg formation. This since it has been suggested before in mouse thymus, where thymic exosome-like particles induced Treg formation (52), and for tumor-derived microvesicles that could induce Tregs from peripheral T-cells and up-regulate Treg suppressor function (53). However, in Paper III we see that thymic DCs are able to induce CD4+CD25+FoxP3+ thymocyte formation *in vitro*, but addition of thymic exosomes suppresses this formation both in presence and absence of thymic DCs.

AIRE and autoimmune polyendocrine syndrome type 1 (APS1)

Using positional cloning approaches the gene mutated in APS1 (also known as Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy - APECED) was identified by two groups in parallel in 1997 and given the name *AIRE* (54). Currently, over 60 APS1-associated mutations have been reported in the *AIRE* gene (55). The identification of *AIRE* has been important not only for knowledge regarding this syndrome but it also paved way for deeper understanding concerning thymic function at the cellular and molecular level. APS1 is regarded as a monogenetic autosomal recessive disease and is diagnosed by the presence of two out of three of the following major clinical manifestations: primary hypoparathyroidism, primary adrenocortical insufficiency and chronic mucocutaneous candidiasis (56). Besides this, the clinical manifestations in the APS1 patient group vary considerably in both severity and number of disease components (57). Even siblings carrying the same *AIRE* mutations are reported to vary in their clinical phenotype (58). While these siblings carried the same *HLA* (Human Leukocyte Antigen) genotype other studies have highlighted the importance of *HLA*-genotype for the APS1 phenotype (59). The presence of numerous autoantibodies, many of them exclusively found in APS1, is a hallmark of APS1. Among others, APS1 patients frequently have autoantibodies against the parathyroid autoantigen NALP5 (60), the adrenal antigen 21-hydroxylase (61), the intestinal antigen tryptophan hydroxylase (62) and the Th17-associated cytokines IL-17A, IL-17F and IL-22, which are thought to hinder the immune system with development of candidiasis as a consequence (63, 64).

Function of AIRE – As mentioned above, mutation in *AIRE* was identified as the underlying cause of APS1. Two years later AIRE was shown to be expressed in epithelial cells located in thymic medulla (65). It was known from before that the thymic medullary epithelium is important in regulating immune tolerance (66). Further advance in understanding mTEC function came from Derbinski and co-workers when they showed that this cell type is responsible for promiscuous gene expression (PGE) in order to mimic the peripheral self (67). That is, a large number of peripheral proteins, probably thousands (67), are expressed in mTECs with the sole task to be presented

for thymocytes in the process of negative selection. Later, Anderson and colleagues showed that it specifically was Aire in mouse mTECs that was responsible for PGE (68). Following this finding, Liston and co-workers could show that Aire deficiency leads to failure of thymic tolerance mechanisms and that Aire indeed was responsible for the deletion of autoreactive CD4+ islet-specific T-cells (69). How Aire functions on a molecular level has been an area of intense studies. Aire was early considered as a transcription factor based on the presence of known transcriptional control elements such as PHD-type zinc-finger domains, a nuclear targeting signal (54), a SAND domain (70) and by its nuclear body formation (65, 71). More recently, identification of proteins to which Aire binds in co-immunoprecipitation experiments revealed partners of four classes: nuclear transport, chromatin binding, transcription and pre-mRNA processing (72). In other work, Aire was shown to localize to all RNA polymerase-II rich transcription start sites, but only affected a subset of them. Since absence of Aire resulted in overabundance of short transcripts the suggested mechanism of action for Aire was to release stalled RNA polymerases for the induction of PGE (73). Also, mRNA processing factors have been indicated to cooperate with Aire to release stalled polymerases (74). The mTECs that express Aire are typically postmitotic and express MHC II, CD80, CD40 and PD-L1 (75). In addition to PGE, Aire has later also been coupled to other functions in mTECs namely the transfer of antigens from mTECs to thymic DCs (76), production of chemokines (77), maturation (75, 78, 79) and differentiation (80). Hence, an overexpression of *AIRE*/AIRE, which is reported in Paper IV (quantitative RT-PCR/IHC), probably leads to a complex set of changes within the mTEC population resulting in a net biological outcome that is hard to predict. Probably, the simplistic view that overexpression of AIRE leads to generally higher levels of TRAs and in the end a more comprehensive clonal deletion is probably not correct since more frequent autoimmunity within the DS groups is documented (81-83). Altering the expression of TRAs probably not only affects the selection and repertoire of conventional CD4 and CD8 thymocytes, but also of developing Tregs. In Paper IV, we observed elevated expression levels of *INSULIN* (*INS*) and the α-subunit of the nicotinic acetylcholine receptor (*CHRNA1*). This is not however, to be extrapolated to all TRAs. As seen, even though H/K-ATPaseβ (*ATP4b*) is known to be controlled by *AIRE* (84), its expression is not up-regulated in DS thymic

tissue. The reason could be either that other effects of trisomy 21 than the increased AIRE gene-dose mask its AIRE-dependency or that other effects of AIRE indirect result in a dampened total expression. Further, knockout of *Aire* leads to altered expression of individual miRNAs (85). By studying TECs from dicer- deficient mice, a global role for miRNAs has been postulated in which they play a crucial role for thymopoiesis (86). Absence of miR-29a in thymic epithelium has been shown to result in elevated expression of the IFN-α receptor, which resulted in a rapid loss of thymic cellularity analogous to induction of thymic involution (87). Also, during mTEC differentiation, a subpopulation of miRNAs is up-regulated and correlates to the expression of Aire. Microarray analysis in Paper I demonstrated that, as most exosomes, thymic exosomes are rich in miRNAs and that the miRNA-profile is more like that of Raji B-cells than Jurkat T-cells which indicates that the majority of thymic exosomes in this paper do not originate from thymocytes.

Thymus logistics – a role for indirect presentation of antigens?

Overall, thymocytes vastly outnumber TECs, with estimates of proportion as low as 0.25% of the total numbers of cells being CD45- in mouse thymus (88). The traditional view holds that a vast majority of them die by apoptosis due to expressing a TCR with an affinity too low for sufficient interaction with peptide/MHC complex (death by neglect) (89). This view has however been complemented in recent work where it was found that roughly six times more cells undergo negative selection than complete positive selection (90). Moreover, negative selection of cortical DP thymocytes was 2.5 times higher than the number of negatively selected medullary SP thymocytes. Combination of experimental data with mathematical modeling has estimated that 65.8% of pre-DP thymocytes undergo death by neglect (91). Further 91.7% of post-DP thymocytes undergo death via negative selection while 4.7% develop into CD4 SP and 3.6% into CD8 SP. In the medulla, negative selection removes 8.6% of CD4 SP and 32.1% of CD8 SP thymocytes (91). The negative selection in the medulla includes the testing of peptide/MHC-TCR affinity also in the context of dilute TRAs. Both differences in cell population sizes and expression of any given TRA by only a subpopulation of mTECs (92) have fueled the discussion concerning

how negative selection on TRAs can be efficiently and reliably performed. While TRA genes seem to be expressed in the order of 2-15% of the mTEC population at the gene level in the mouse thymus (92), TRA proteins, exemplified with carcinoembryonic antigen-related cell adhesion molecule 5 (*CEACAM5*) and mucin 1 (*MUC1*), seem to be expressed by lower numbers of mTECs in the human thymus (1-3%) at a given time (93).

Oscillating TRA expression? - A transient TRA expression has been proposed to aid the negative selection process by making a larger antigen pool available for the thymocytes. This proposition was based upon results from experiments showing lacZ-expression when the connexin 57 promotor drove Cre-recombinase and this transgenic line was crossed with a ROSA26/lacZ reporter strain in comparison to when lacZ was directly driven by the connexin 57 promotor. In the first setup lacZ was detected in every cell that ever had activated its connexin 57 promoter (resulting in 1,2% positive purified mTECs) while lacZ driven directly by the connexin 57 promotor (resulted in <0.1% positive purified mTECs) represented the cross section expression (94). Similarly, Pinto and co-workers have suggested a model in which mTECs sequentially express different groups of TRAs depending on level of AIRE-expression (95) (also discussed in "TRAs?" below). That is, with increased levels of AIRE, mTECs would shift through distinct gene pools and hence, the need for thymocyte scanning for antigen would be reduced. These two studies are interesting, but a global model of oscillating TRA expression in mTECs has still not been proven.

Thymocyte agility - Since thymocytes have been demonstrated to be an agile cell type, their movement is often used as an argument for direct presentation of antigens from TECs to be enough for a robust central tolerance development. The idea is that this agility enhances the medulla scanning potential of each thymocyte. However these speculations originate from two-photon microscopy studies, in which besides the demonstration of thymocyte agility, observations were made of frequent and transient interactions between the rapidly moving thymocytes and thymic DCs (96). Also, in the presence of a negative selecting ligand, the thymocytes moved more slowly and made slightly prolonged contacts with DCs. Le Borgne and coworkers themselves further speculate that cross-presentation of mTEC antigens to DCs could somewhat broaden the distribution of TRAs. If

combining agile thymocytes with an oscillating TRA-expression in mTECs the question arises, if an oscillating mode would yield an efficient tolerance-inducing milieu anyhow since the thymocyte might migrate in suboptimal areas in terms of where their antigen is expressed.

Direct antigen presentation in the thymus - Still, considering the studies mentioned below regarding indirect presentation, the dogma remains that TECs themselves are responsible for a vast majority of the antigen presentation within the thymus (97). Currently however, different arguments are raised for or against the need of antigen spreading mechanisms within the thymus. In indirect presentation, TECs, and more specifically mTECs, partly function as producers and reservoirs for antigens and are partly relieved of antigen presentation by other thymic antigen presenting cells (APCs). Even though it is firmly established that mTECs are able to negatively select thymocytes through direct interaction, this does not *per se* exclude antigen presentation contribution from indirect routes such as exosomes. Further, studies of to what extent direct and indirect antigen presentation in the thymus work could be hampered by redundancy of these presentation routes. Examples follow below in which indirect presentation seems to contribute and a schematic view of direct versus indirect antigen presentation is seen in Figure 7.

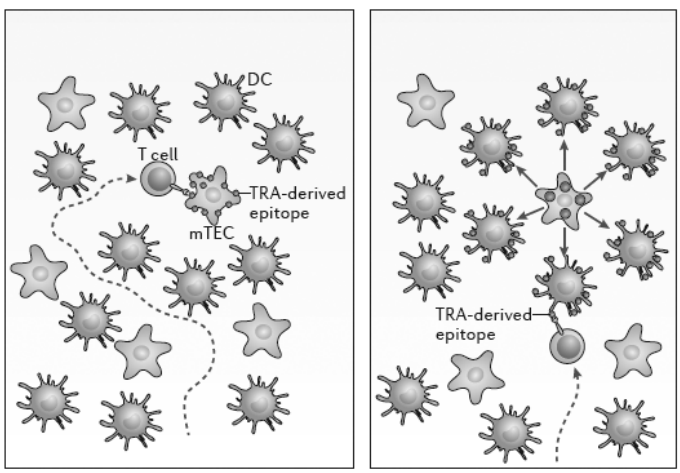

Figure 7. *Models of antigen presentation to developing thymocytes.* Left: direct presentation. In direct presentation TRAs are presented to thymocytes

by mTECs who also expressed the TRA. Right: Indirect presentation (or antigen handover). In indirect presentation mTEC-produced TRAs are transferred to DCs that in turn present them for thymocytes. Exosomes could be one route of antigen handover from mTECs to DCs. Figure adopted from (98).

Indirect antigen presentation in the thymus - Twenty years ago Kyewski and co-workers observed intercellular antigen-transfer from the thymic stromal compartment to DCs. They proposed that this mechanism "may enhance the efficacy of tolerance induction by spreading self antigens" (99). Koble and coworkers carried out a study that strengthens DC-presentation of TEC antigens. In this work, thymic but not peripheral DCs present TEC-derived OVA to OVA-specific T-cells and were constitutively provided with mTEC-derived proteins (100). Further, the transfer of material in this study was unidirectional towards DCs and the discussed mechanisms were primarily exosomes and apoptotic bodies. Transfer of material from TECs to DCs has also been demonstrated in work by Hubert and co-workers in which OT-II restricted thymocytes were deleted by a soluble form of OVA that required presentation by bone marrow derived cells (76). In reaggregated thymic organ cultures both the thymic epithelium, as well as conventional DCs (as opposed to plasmacytoid DCs), have been shown capable of eliminating autoreactive CD4 thymocytes and to support natural Treg (nTreg) development on their own (101). Further, non-redundant contribution of DCs and mTECs was suggested from data based on simultaneous hematopoietic MHC class II deficiency and reduced MHC II expression on mTECs since this combination had an additive worsening effect on negative selection compared to either of them alone (102). Likewise, an essential role of DCs for a tolerogenic CD4 population has been demonstrated in DC-depleted mice in which increased frequencies of CD4 SP thymocytes and CD4 T-cell infiltration into peripheral tissues were observed (103). Using tetramer staining and transfer of bone marrow with ablated expression of MHC II, Taniguchi and co-workers found an abolished negative selection of T-cells specific for the Aire-controlled self-antigen retinoid-binding protein (104). They concluded that intracellular transfer of the interphotoreceptor retinoid-binding protein (IRBP) peptide epitope of amino acids 277-290 from Aire-expressing mTECs to bone marrow-derived APCs is important for negative selection of the investigated peptide. IRBP

peptide of amino acids 771-790 did not undergo thymic negative selection at all due to complete lack of presentation. Finally, Aschennbrenner and colleagues have observed that DCs capture mTEC-derived antigens and mediate deletional tolerance (also thymocyte-TEC interactions mediated tolerance in this study) (105). Possible TRA-distributing routes in the process of indirect presentation are presented below.

Tunneling nano tubes (TNTs) - It has been shown that filipodia-like processes formed by cells are able transfer material between cells (106). These structures, called TNTs, have also been shown to be formed by the MTE4.14 TEC cell line (107). The MTE4.14 TNTs transferred both membrane material and functional MHC II molecules between epithelial cells *in vitro*. *In vivo* this transfer was suggested to take place from epithelial cells to both other epithelial cells, but also DCs. Recently, an article was published in which exosomes were suggested to be of importance in TNT formation (108).

Apoptotic bodies – It is plausible that TECs who undergo apoptosis could spread antigens via apoptotic bodies. This is further interesting since Aire is suggested to exert pro-apoptotic functions in mTECs (75). This route of spreading antigens might however not be able to distribute functional intact MHC molecules since apoptotic material undergo breakdown in lysosomal compartments. Still, apoptotic bodies could be taken up by DCs or other APCs, for example CD4+CD11b+ macrophages (109), prior to presentation for thymocytes.

Thymic exosomes – The first observation of exosomes in thymic tissue was made by Wang and co-workers in mice (52). The main finding in their work was that the exosomes (or exosome like particles, which the vesicles are designated in this article) induced thymic CD4+CD25- cells into CD4+CD25+Foxp3+ cells *in vitro*. This is in contrast to the *in vitro* co-culture results presented in Paper III where thymic DCs induce CD4+CD25+Foxp3+ formation, and this formation is suppressed by thymic exosomes in a dose dependent manner. Wang et al did not include control-cultures where APCs (in their case T-cell-depleted irradiated splenic cells) were excluded. Hence, the induction seen could result from the APCs rather than the exosomes as the case is in our study. Prior to the work presented in Paper I, II and IV there is no report concerning human thymic exosomes.

Presence of exosomes in human thymic tissue was first presented in Paper I, and TEC-produced exosomes were described in Paper II. The thymic exosomes from DS patients in Paper IV differed from control exosomes in an altered proteomic profile. It is plausible that exosomes released by TECs contribute in antigen presentation for thymocytes, both indirectly via DCs and directly by themselves (Figure 8).

Thymocyte vesicles - Thymocytes themselves have been shown to produce microvesicles (110). These vesicles were pelleted at 12 200 x g, and probably contain high contamination of apoptotic bodies. At the same time this centrifugal force is too low to pellet exosomes (28). Indeed there were a high proportion of proteins found on both apoptotic bodies (2000 x g pellet) and microvesicles (47.2% of totally identified proteins were present in both apoptotic bodies and microvesicles).

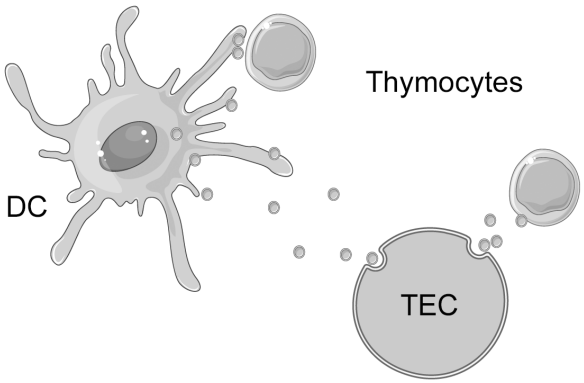

Figure 8. *Schematic illustration of exosomal transfer from TECs.* To the left exosomes transfer from a TEC to a DC which in turn presents exosomes processed/unprocessed for thymocytes. To he right exosomes transfer from TECs directly to a thymocyte. The figure is produced using Servier Medical Art.

Thymic TRAs

TRAs in thymic exosomes – To examine TRAs in the proteomic content of thymic exosomes (Paper I) we used tandem mass spectrometry (MS) together with expression protein profiles based upon expression data in the human protein atlas (HPA) (111). The four identified TRAs: 2´,3´-cyclic nucleotide 3´phosphodiesterase, reticulon 3, tropomyosin 3 and the GNAS protein have been identified using a definition with protein-expression allowed in maximum five tissues in the HPA database. This is a moderate definition in that, typically, a protein is expressed in a larger number of tissues in the HPA compared to for example the BioGPS database (112) and it is noted that other proteins classically thought of as TRAs actually are expressed in more than five tissues in the HPA database. Examples from a study by Gotter et al (113) are secreted phosphoprotein 1 (9 of 82 tissue cell types), adenylate kinase 1 (32 of 80 cell types) and metalloproteinase (symbol:*ADAM23* 46 of 82 cell types). Also, the four TRAs identified in Paper I are present in both proteomic samples analyzed in this paper, hence TRAs present in only one sample fall out of this analysis. With this in mind, these four identified TRAs are possible candidates to participate in the selection/maturation processes within the human thymus. With the exception of one study that address thymic expression of 2´,3´-cyclic nucleotide 3´phosphodiesterase (114) the four TRAs identified in Paper I seem unaddressed in thymic research. Interestingly, 2´,3´-cyclic nucleotide 3´phosphodiesterase are recognized by IgG autoantibodies in multiple sclerosis patients (115). In addition, tropomyosin 3 has been suggested to be a candidate antigen in endometriosis (116). Also, 2',3'-cyclic nucleotide 3' phosphodiesterase was present within the TEC-exosomes in Paper II (reticulon 3 and tropomyosin 3 were found in the cultured cells but not in the exosomes while GNAS protein was not found). This strengthens the findings in Paper I to be of epithelial origin. All four proteins could be interesting candidates to address in future studies. More about Paper II TRAs in the following section.

Human TRAs and autoantigens in the literature and Paper II – To examine the proteomic content of TEC exosomes in Paper II we shifted into using the BioGPS database to evaluate TRAs within the MS-datasets (112), yet again allowing expression in a maximum of five tissues. This database

has been repeatedly used to evaluate TRA-expression by other groups (117, 118). This results in a higher number of TRAs than if using the HPA database, and the data is also more comparable to the existing literature. Sospedra and co-workers conducted a pioneering study that examined multiple TRA transcripts in human thymic tissue. Among others, they identified *INS*, glutamate decarboxylase (*GAD1*), thyroglobulin (*TG)* and myelin basic protein (*MBP)* (119). In our current work, Paper II describes the finding of several disease-associated autoantigens in the proteome of exosomes released from cultured human TECs. Among them is MBP, which is an autoantigen in multiple sclerosis (120), that has been found to be expressed in fetal human thymus in the long golli-gene variant (121) and MBP epitopes have also been shown expressed in human TECs (122). Collagen type II is an autoantigen associated with rheumatoid arthritis and besides being present in the TEC exosome proteome (123), mRNA-expression has been shown both in human cTECs and mTECs (113). TITIN, described as an autoantigen in myasthenia gravis, was identified in TEC exosomes while the acetylcholine receptor was not found (124). The Graves´ disease associated thyrotropin receptor is another interesting example of an autoantigen shown to be present in human thymic tissue both at mRNA and protein level (125). Wong and co-workers uncovered the presence of the α3 chain of collagen type IV in human thymus, which is an antigen in Goodpasture´s disease. Interestingly it localized around and in HCs (126). Desmoglein 3 (DSG3), present in TEC exosomes, is another example of an autoantigen found to localize to HCs (127). DSG3 is a known autoantigen in pemphigus vulgaris, as is DSG1 which is also present in TEC exosomes (128). Heat shock protein 60 (HSPD1), connected with various autoimmune diseases, is yet another autoantigen that has been found in human thymus with staining seen in HCs (129) and now also identified at protein level in TEC exosomes (Paper II). Further, the autoantigen in coeliac disease, transglutaminase 2 (TGM2) (130, 131) was found in TEC exosomes (Paper II).

TRAs? - Possibly the most used example of a TRA, and one of the first described to be expressed in thymic tissue, is insulin. It has been thoroughly studied in the context of thymic expression both in mouse and human (132-135). The reason for the continued use of insulin as an example when describing a TRA is probably not only due to its important function in

glucose metabolism and involvement in diabetes, but also its strict expression pattern. It is well known that insulin is expressed solely by pancreatic β-cells. This is also true when searching in databases such as BioGPS (112) (INS-expression only in "pancreatic islets" and "pancreas") and the HPA database (111) (INS-expression in 1 of 82 analyzed normal tissue cell types "Islets of Langerhans"). For other TRAs however, a sole TRA-function in thymus it is not as intuitive as for insulin. In a recent study Pinto and co-workers chose to study the expression of three TRAs (one TRA criteria was "expression at low frequency, as typical for pGE") in sorted human mTECs: MUC1, CEACAM5 and sodium/glucose cotransporter (SGLT1) (95). They found that while both MUC1 and CEACAM5 could be detected in all samples SGLT1 was "more variable precluding recovery from several samples" (95). Further they concluded that AIRE-expression at mRNA and protein level were highest in the SGLT1+ subset and lowest for the MUC1+ subset. Expression of these three TRAs in the HPA database are the following: SGLT1 6/82 tissue cell types, CEACAM5 14/82 tissue cell types and MUC1 24/82 tissue cell types. That is, the more ubiquitous proteins CEACAM5 and MUC1 are despite lower expression of AIRE always found in their samples, while SGLT1 seems to need a higher expression of AIRE to be expressed in human mTECs. Firstly, this is interesting in the context of defining what a TRA is. SGLT1 clearly seems to more profoundly match the thought of what a TRA is than CEACAM5 and MUC1 do, and also there seem to be different levels of AIRE dependency between different TRAs as discussed in (136). Secondly, it also strengthens the discussion in Paper IV; that altered level of AIRE alters the proteomic and TRA profile of TECs in DS thymus. Notable is further that, as mentioned above (see first section in chapter "Thymus logistics – a role for indirect presentation of antigens?"), the expression frequencies of CEACAM5 and MUC1 in human mTECs are 1-3% of mTECs. Hence, the expression frequencies of TRAs more dependent on AIRE might be lower than in 1-3% of mTECs.

Incomplete PGE – good enough?

Even though the phenomenon of PGE is extensive in terms of numbers of expressed TRA-genes it does not constitute a complete TRA coverage. This leaves gaps in the central tolerance induction that potentially could lead to

autoimmunity in the periphery. These gaps in central tolerance are acceptable since also peripheral tolerance mechanisms contribute to avoid autoimmunity. It is also possible that an incomplete tolerance is accepted during normal circumstances if it is kept below pathogenic level. Though, it is known that healthy individuals harbor self-reactive T-cell clones (137). MBP is a specific example where not only patients but also healthy controls could mount antigen-specific T-cells response (138, 139). In the paragraphs below peripheral tolerogenic mechanisms that add redundancy when central tolerance fails are briefly discussed.

Peripheral tolerance: ignorance, deletion, anergy and Tregs – As noted previously, there is a lower limit of the number of peptide/MHC complexes needed in order to activate a T-cell (51, 140). Hence, a self-antigen that fails to reach these levels is ignored. Immune privilege is a complex process partly due ignorance based on physiological features (such as limited lymphatic drainage of the brain) (141). In a groundbreaking study, immature DC-mediated (no bacterial stimulation or CD40 stimulation present) T-cell deletion and unresponsiveness were shown by using hybrid antibodies. This construct consists of HEL-peptide conjugated to a monoclonal DEC-205 antibody that directs HEL-peptides to DCs specifically. A majority of the responder 3A9 T-cells disappeared after 7 days and the remaining cells were unresponsive (clonal anergy) to further stimulation with HEL (142). Since then, a prevailing idea of peripheral tolerance holds that cross presentation of peripheral antigens by DCs is critical and that the local milieu in which the DC sampled its antigens determines the T-cell response in terms of immunity or tolerance (143). A final, well-established, mechanism of peripheral tolerance is the presence of Tregs that can be identified with the use of Foxp3 (144, 145). The Treg population is heterogenic and while nTregs (or thymic Tregs -tTregs) are formed in the thymus, others (collectively known as induced Tregs) form in the periphery such as T-regulatory 1 cells that form from naïve CD4+ T-cells in the presence of IL-10 (146).

Peripheral bystander supression - Modigliani and colleagues proposed a ”model of natural tolerance” (147) in which nTregs with affinity for antigen A suppress an autoreactive T-cell with affinity for antigen B. In this model both types of cells recognize their respective antigens presented from the

same APC which brings the autoreactive T-cell in close proximity to the nTreg and its suppressive milieu. Kyewski and Derbinski specified this model (148) using α-H^+/K^+-ATPase, which in humans is a known mTEC expressed TRA, and β-H^+/K^+-ATPase which is not expressed in the thymus (113). In this study, tolerance spreads, via suppression, from a central tolerance covered TRA to an antigen that is not covered by central tolerance (131).

Peripheral expression of Aire and TRAs – Lee and co-workers introduced lymph node stromal cells expressing ER-TR7 (expressed on cells in thymic capsule, septa and around blood vessels (149)) and the vascular cell adhesion molecule as a population of cells with capacity to present endogenously expressed TRAs to T-cells (150). The same group later identified these cells as lymph node fibroblastic reticular cells (FRCs), and at the same time investigated both gp38-CD31- cells, hitherto uncharacterized, and found these to express Aire and proteolipid protein (151). Using a gene reporter system, yet another interesting cell population in lymph nodes and spleen was identified as Aire-expressing cells; the so called extrathymic *Aire*-expressing cells (eTACs). These stromal cells were shown to interact with and to delete autoreactive T-cells and to express a set of TRAs. Even though both eTACs and mTECs depend on Aire for the expression of TRAs, only a small overlap of Aire-regulated genes was observed. This surprisingly small overlap indicates the presence of another level of regulation of TRA-expression (152). In later work, Gardner and co-workers concluded the eTAC-population to be MHC $II^{high}CD80^{lo}CD86^{lo}EpCAM^{hi}CD45^{lo}$ and with a bone-marrow origin (153). Cohen and co-workers have reported that peripheral TRA-tolerance can be mediated directly by gp38+CD31+ lymph-node lymphatic endothelial cells (LECs) without cross-presentation to DCs (154). Due to lack of TRA Aire-dependency for certain antigens in LECs and FRCs the authors emphasize the probability of other transcriptional regulators involved in TRA control. The expression of peripheral Aire has been questioned in other work (155).

Late stage thymocyte development

Studies of positive and negative selection have resulted in a large body of knowledge concerning that topic, but late stage thymocyte maturation is an

area that is less explored (especially studies regarding maturation of CD8 SP thymocytes are rare). As thymocytes mature they go through several shifts in expression cell surface receptors. Ccr7 is up-regulated on immature DP cortical thymocytes upon TCR engagement and is crucial for thymocyte migration from cortex to medulla. This was shown by CD4 and CD8 SP thymocyte arrestment in the cortex in Ccr7 knockout animals as opposed to accumulation into medulla (156). As for conventional αβ T-cells, also Tregs depend on Ccr7 for medullary entry, and this is possibly also the case for invariant natural killer T-cells (157). Sphingosine-1-phosphate receptor 1 ($S1P_1$), which is responsible for thymocyte egress from thymus, is a membrane marker of thymocyte maturation and starts being expressed at the DP stage and increases in expression sequentially in SP CD4+ thymocytes with increased levels of L-selectin (CD62l) (158). Qa-2 is yet another marker suitable for evaluation of thymocyte maturation since it is first expressed on CD4 SP cells at stage 4 (159). CD69 is known to be expressed in a transitional state after positive selection but prior to a more mature state including expression of Qa-2. Further, TCR engagement is required for CD69 expression (160). Expression of CCR9 peaks at the DP stage and is thereafter lowered in expression (161). For a schematic summary of late thymocyte maturation, see Figure 9.

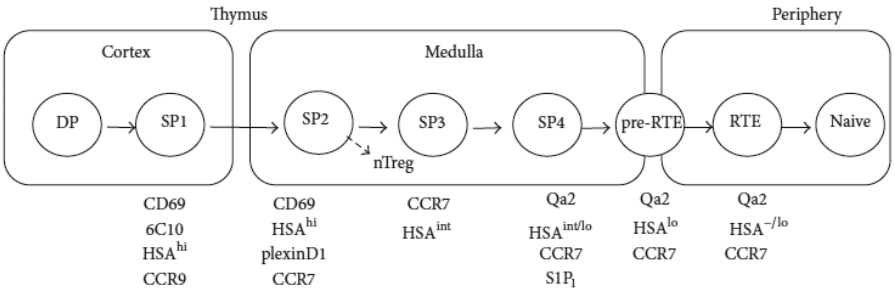

Figure 9. *Schematic illustration of late stage thymocyte maturation.* HSA – heat stable antigen, RTE – Recent thymic emigrant. Adopted from Xu and co-workers (162).

Effect of thymic exosomes on late thymocyte maturation - In Paper III we wanted to investigate exosomal effects on the maturation of thymocytes. An *in vivo* approach was hampered both by the challenge of distributing

exosomes to the thymus (intravenous injection did not work in our hands with a low portion of exosomes ending up in thymus, and intrathymic injection is not an established protocol in our lab) and by the presence of endogenous exosomes, so we turned to an *in vitro* approach. As thymocytes are very prone to go into apoptosis *in vitro* (and *in vivo*), culturing them in optimal conditions to evaluate surface marker expression and survival is a delicate task (evaluation of negative selection is also hampered by this). Co-cultures were therefore supplemented with IL7 and IL2 to enhance thymocyte survival and to make evaluation of Treg formation plausible (163). By evaluating maturation markers with flow cytometry we could report that thymic exosomes influence the maturation of thymocytes. The frequencies of $S1P_1$+Qa2+ and Ccr7+Qa2+ cells were increased following exosomal stimulation, and at the same time the percentage of Ccr9- cells were increased. These effects on thymocyte maturation behaved in a dose-dependent manner. Further, the observed effects were mostly direct and also independent of MHC II. The latter, since the effects were similar in cross-strain experiments where, independently of mouse-strain origin, the thymic exosomes induced maturation of thymocytes with respect to CD4+$S1p_1$+Qa2+, CD4+Ccr7+Ccr9- and CD4+Ccr7+Qa2+ formation. Also using a blocking antibody against MHC II did not alter the exosomal-induced effects. In Paper III we used the ImageStream technique, which is a combined flow cytometer and fluorescence microscope, to evaluate exosomal uptake. ImageStream has previously been used for this purpose (164, 165). The thymic exosomes were efficiently taken up by both CD11c+ thymic cells and by CD4+ thymocytes. Finally, as discussed in "Typical exosomal proteins" and "Thymic exosomes" above, the thymic exosomes in Paper III suppressed DC-dependent formation of CD4+Foxp3+ cells even though they expressed surface bound TGFβ.

Down Syndrome (DS)

DS is a genetic condition in which a person has three copies of chromosome 21 instead of two (166). DS is most often caused by nondisjunction during oogenesis in 93.2% of cases followed by spermatogenesis errors in 4.1% of cases and post-zygotic errors in 2.7% of cases (167). The main known risk factor for DS is maternal age (168). For the period of 1993-2004 the incidence of DS in Sweden was 13.07 newborn DS per 10 000 births (169).

DS is accompanied by increased frequencies of autoimmune disorders such as hypothyroidism (81), hyperthyroidism (170) and insulin-dependent diabetes mellitus (83, 171, 172). While the cause of these increased autoimmune frequencies is unknown, it is interesting to note that the DS group shares autoantibodies with the APS-1 patient group (173). Further, since the *AIRE* gene is located on chromosome 21 we wanted to investigate expression of *AIRE* and other thymic features in DS thymus.

Thymic differences in DS – Perhaps the most striking feature of DS thymuses is the small size at birth (174). This is also the case already at fetal stage (175, 176). Enlarged HCs (177), thymocyte depletion and cortico-medullary demarcation (178) are other know features of DS thymus. Further, both compartments of single positive cells, CD4+ and CD8+, have been found altered with lowered percentages indicating an affected thymocyte maturation (179). In Paper IV we saw an increased expression of *AIRE* in thymic tissue using quantitative RT-PCR and also an elevated number of AIRE+ cells in the medulla, visualized using immunohistochemistry (IHC). As mentioned above (see "Function of Aire") the expression of *INS* and *CHRNA1* mRNAs were elevated in DS tissue, while for *ATP4b* expression no change was seen. Further, no differences were seen in the mRNA expression of *TG*, thyroid peroxidase (*TPO*) and *GAD1*. Also, using IHC, an elevated number of CD11c+ cells were seen in DS medulla, and a skewed proteomic profile of exosomes was the result of MS-analysis (including a pool of TRAs that were not represented in control exosomes, Paper IV-Table I). It has been demonstrated that chemokine (C motif) ligand 1 (Xcl1) attracts DCs into the thymic medulla in mice (180). However, the accumulation of CD11c+ cells in DS medulla is probably not dependent on altered levels of *XCL1* since no difference is seen in expression of this chemokine between DS and control thymic tissue. Since thymocyte-DC crosstalk is important for cDC numbers in mouse thymus (181), it is possible that the altered sub-populations of thymocytes in DS thymus are directly responsible for the elevated numbers of CD11c+ cells seen in DS medulla in Paper IV. The elevated expression of *AIRE* observed in Paper IV together with the large HCs that are typical for DS thymus could be an important contribution towards a functional understanding of AIRE regarding its role in mTEC maturation. Peterson and co-workers have earlier suggested a central but not dispensable role of Aire in mTEC differentiation/HC

development since, in Aire KO mice, the reduced number of HCs can be induced back to almost normal numbers by stimulation with RANK-ligand and CD40-ligand (78). Also interesting regarding HC-size (see Figure 10 for DS medulla morphology with staining for involucrin) is the theory regarding HC function that states that HCs produce thymic stromal lymphopoietin (TSLP) which, induce activation of thymic myeloid DCs who up regulate CD80 and CD86 and are now in turn able to induce formation of CD4+CD25+ Tregs in the human thymus (182). Later work has also shown that human thymic pDCs, induced by TSLP, can generate FOXP3+ Tregs that differ in chemokine and cytokine production compared with mDC-TSLP induced Tregs (183). From this line of events, and the observation of DS medulla accumulation of CD11c+ cells, a logical assumption is that DS thymus harbors more Tregs than controls. However in Paper IV we saw no difference in the number of FOXP3+ cells in DS medulla compared with controls. Possibly, this indicates a functionally affected population of DCs in DS thymus. In the end, DS could be viewed as a syndrome with immunodeficiency and autoimmunity, possibly both, partly as consequences of abnormal development of the thymus. The combination of lymphocyte deficiency and autoimmunity has been investigated previously (184). Further, it has actually been proposed that DS should be considered a primary immunodeficiency rather than a syndromatic deficiency (185). Kisand and colleagues have argued for the combination of Aire loss together with lymphopenia induced proliferation of autoreactive T-cell clones in the periphery as basis of Aire-/- autoimmunity (186). This combination could speculatively also be valid in DS (AIRE-malfunction and peripheral lymphopenia).

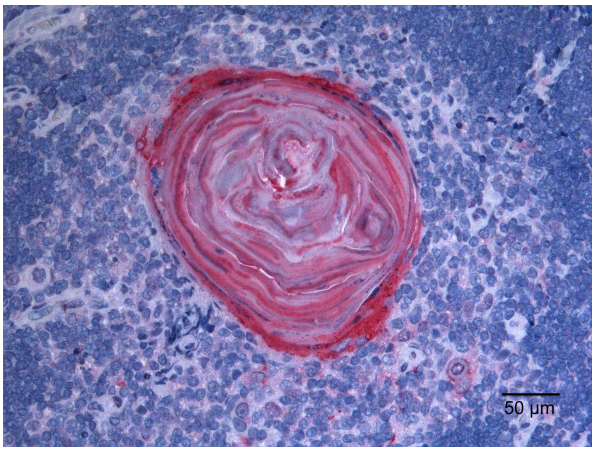

Figure 10. *Morphology of DS thymic medulla.* Notice how the medullary space is occupied by the huge amorphous HC. Tissue is stained with anti-involucrin antibody in IHC.

Interferon regulatory factor 8, AIRE and *CHRNA1* – Considering *CHRNA1*, it is somewhat surprising that the thymic expression, when examined by Wakkach and co-workers, did not differ between the myasthenia gravis group and controls. This indicates that a lack of central tolerance for this protein is of limited importance for the onset of disease (187). However, expression levels and localization of nicotinic acetylcholine receptor subunits have been considered controversial and in a later study, Bruno and co-workers revisited this topic and found highly variable expression between individuals (188). Even though AIRE controls the expression of many TRAs genes, and among them *CHRNA1* (84), the exact number is unknown; in addition it is probably not so that all AIRE-controlled TRAs are equally controlled by AIRE. As noted previously (see "TRAs?"), it is tempting to speculate about other TRA-controlling factors within mTECs and different degrees of AIRE-dependency. In fact, interferon regulatory factor 8 has been shown to, together with AIRE, control the expression of *CHRNA1* in human TECs (189). Interestingly, compatible overall Spearman R values between *CHRNA1* and *AIRE* are presented by Giraud and co-workers and in Paper IV (R_{Giraud}=0.71, $R_{Paper\ IV}$=0.77) as results from analysis with quantitative RT-PCR (see Figure 11). This strengthens the probability that the pressed pieces of thymic tissue used

in Paper IV for mRNA analysis are reflective of the expression within the mTEC population.

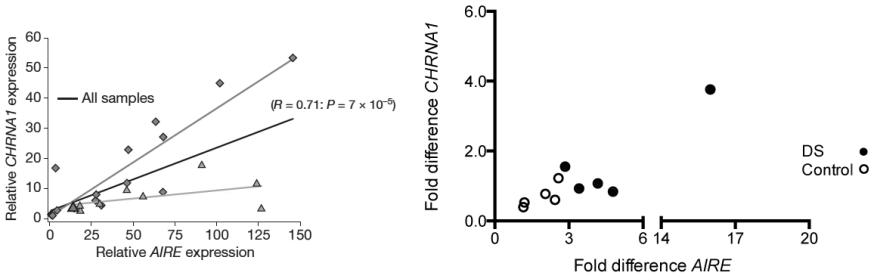

Figure 11. *Comparison of CHRNA1 expression versus AIRE expression* (*Paper IV and REF189*). Left: *CHRNA1* expression versus *AIRE* expression in purified human mTECs, adopted from (189). Right: *CHRNA1* expression versus *AIRE* expression in pressed thymic pieces in Paper IV.

Peripheral immune cells in DS – It has long been a dogma that DS patients suffer from moderate lymphopenia both in the T-cell, B-cell and NK-cell compartments. Using cordocentesis this was confirmed already at fetal stage (190). Using dried blood spot samples, an elegantly performed study showed reduced copy numbers of T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles in DS patients. The aim of this study was actually to investigate if DS could impact the performance of neonatal screening for severe combined immunodeficiency and X-linked agammaglobulinemia (191). One advantage of these two aforementioned studies is that they rely on data gathered from two groups where none of the fetuses/infants yet have undergone thymectomy. This could be of importance since the frequent congenital heart defects seen in DS (192) probably lead to more frequent thymectomies in this group which could potentially bias the analysis of peripheral T-cells. Further, higher numbers of NK-cells in DS patients have been reported, which has been coupled to premature thymic involution (193). This has been questioned in later work in which use of a broader panel in flow cytometry resulted in lowered absolute numbers of NK-cells with increasing age (194). The authors concluded that the relative increase earlier reported in the NK-cell population was rather due to decreased numbers of T-cells and B-cells and also by incomplete information regarding changes in subpopulations of T-cells. Another study

has demonstrated elevated plasma levels of IL7 and IL15, as well as a reduction in the number of TREC+ lymphocytes in DS peripheral blood (195).

"Chromosome 21 immune genes" - The revealing of the human chromosome 21 DNA sequence was of obvious interest to researchers in the DS field (196). Among other important genes, some genes located on this chromosome are of particular interest regarding immune function. Below follows a discussion of some highlighted genes. See figure 12 for a chromosome 21 schematic drawing including highlighted immune genes. Although it is tempting to couple individual genes on chromosome 21 to specific features of DS it is important not to exaggerate the effects of a single triplet gene on DS phenotype, which is probably to high degree a result of the synergistic effect from many triplicated genes. Extrapolations of observations from mice to human must also be made with caution since inter-species differences surely are considerable on this level of complexity.

Regulator of calcineurin 1 *(RCAN1)* – *RCAN1*, also known as Down syndrome critical region 1 (*DSCR1*), was shown both to be an inhibitor of calcineurin-mediated signaling and to be overexpressed in DS-fetal brain in the same study (197). The inhibiting effect of RCAN1 was via binding to the catalytic subunit of calcineurin and thus inhibiting it from dephosphorylating the nuclear factor of activated T-cells (NFAT), resulting in an inhibited NFAT nuclear accumulation and its dependent transcription. *RCAN1* has further been suggested to cause DS-like immune dysfunction based on thymic dysfunction (198). In this study, a mouse model overexpressing *RCAN1* was showed to display defects in thymic T-cell development and reduced T-cells numbers in peripheral immune organs. Notable was a loss of thymic CD4+ and CD8+ thymocytes, resembling DS thymocyte depletion. Transferring wild type bone marrow into *RCAN1*-transgenic recipients could restore these populations. This transfer did however not improve the observed impaired T-cell function, which prompted the authors to speculate in an additional non-hematopoietic role for *RCAN1*. Finally, *RCAN1* has been shown to stimulate expression of intracellular Cu,Zn superoxide dismutase (*SOD1*, also located on chromosome 21) and increase activity of this enzyme (199). *SOD1* in turn has been investigated as a possible contributor to DS congenital heart defects (200) and also speculated to be

involved in the onset and progression of Alzheimer's disease pathogenesis in DS (201).

Integrin beta-2 (*ITGB2)* – The *ITGB2* gene codes for β2 integrin (also known as CD18) which is the β-subunit of lymphocyte function-associated antigen 1 (LFA-1), macrophage-1 antigen, integrin αXβ2 and integrin αDβ2 (202). Increased adhesion in a lymphoblastoid DS cell line has been reported. This increase was abrogated with the use of antibodies against CD11a and CD18 (203). Further, while no difference was seen in the LFA-1 expression in DS monocytes and granulocytes isolated from peripheral blood, Barrena and co-workers observed an increased proportion of lymphocytes with a high expression of LFA-1 (204).

Runt related transcription factor 1 (*RUNX1*) – *Runx1* is expressed in thymocytes and overexpression of this transcription factor in mouse thymocytes (Runx1-tg;Lck-Cre-tg) impairs the development of thymocytes from the DN to DP stage (205). *RUNX1* has not been investigated in DS thymocytes but is an interesting candidate with possible effect on DS thymocyte development. It has been speculated that GATA1 mutations in DS – related acute megakaryoblastic leukemia (DS-AMKL) result in inhibited GATA1 binding (through its zinc-finger domains) to RUNX1. However, when investigated the interaction between GATA1 and RUNX1 was retained in DS-AMKL (206).

Down syndrome cell adhesion molecule (DSCAM) – *DSCAM* was discovered as a putative member of the immunoglobulin superfamily that mapped into the *DSCR* (207). While little is know regarding DSCAM function in human and mouse its homolog has been shown to bind to bacteria and participate in bacteria phagocytosis in insects (208). In DS individuals, this adhesion molecule has been reported important in retinal ganglion cells and thus suggested to be involved in DS visual impairment (209).

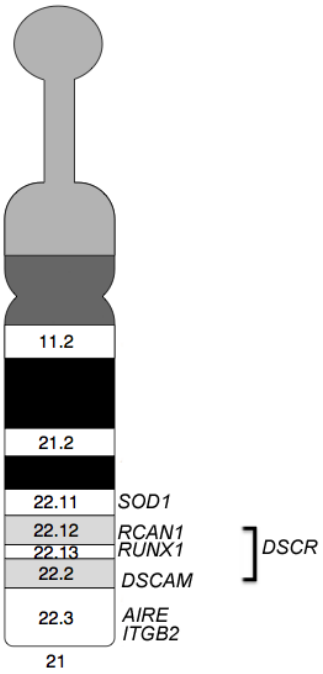

Figure 12. *Schematic illustration of chromosome 21, including highlighted immune genes*. Note that, as opposed to *RCAN1*, *RUNX1* and *DSCAM*, *AIRE* is not included in the *DSCR*. Figure is adopted and modified from (210).

Summary of main findings

Paper I describes the presence and characteristics of vesicles isolated from human thymic explants. These vesicles matched exosomal characteristics. In short, the thymic exosomes had a size between 20 and 150 nm, typical exosomal density, carried tetraspanins, antigen presentation molecules, TRAs, mRNAs and miRNAs.

Paper II shows that cultured human primary TECs produce exosomes and that these exosomes were rich in TRAs. Also, several autoantigens were identified in these exosomes, for example MBP, desmoglein 3 and transglutaminase 2.

Paper III illustrates that exosomes isolated from mouse thymic tissue were taken up by thymocytes and DCs *in vitro*. In a dose-dependent fashion, the exosomes promoted *in vitro* maturation of CD4+ SP thymocytes by inducing up-regulation of $S1P_1$, Qa2 and CCR7 whereas CCR9 was down regulated. In addition, the exosomes suppressed the formation of Foxp3+ thymocytes.

Paper IV investigates *AIRE*/AIRE-expression in DS patients, and concludes an elevated expression in thymic tissue in DS compared with controls. Further, the elevated expression of AIRE was accompanied by an elevated expression of *INS* and *CHRNA1*. In addition to enlarged HCs (and larger involucrin+ area), CD11c+ cells accumulated in DS thymic medullas. Finally, the proteomic profile of exosomes from DS thymic tissue was skewed compared with controls and showed TRAs that were restricted to DS exosomes.

Populärvetenskaplig sammanfattning

I thymus (brässen) utbildas och utvecklas T-celler som är en viktig celltyp för immunsystemet och dess försvarsförmåga mot invaderande patogener såsom bakterier och virus. För att utvecklas korrekt i thymusen interagerar T-cellerna med thymusens egna celler, i första hand dess epitelceller. Dessa epitelceller producerar och presenterar kroppsegna strukturer för T-cellerna och testar därigenom hur starkt T-cells receptorn binder till den presenterade strukturen. Det är denna receptor på T-cellerna som senare ska känna igen strukturer på patogener för att kunna bekämpa dem. Om T-cellerna binder för svagt eller för starkt när de testas så kommer de att elimineras i thymus. Detta för att T-celler som binder för svagt kan ej känna igen presenterade patogen-strukturer emedan T-celler som binder för starkt riskerar att även binda till och därmed attackera kroppsegna strukturer och orsaka skada. Det är detta som händer vid autoimmuna sjukdomar som diabetes typ 1 och multipel skleros. Denna avhandling har undersökt förekomsten av exosomer i thymusvävnad från människa och mus. Exosomer är små vesiklar som släpps ut av celler och deltar i kommunikation celler emellan. I thymus skulle exosomer kunna bidra till kommunikationen genom att presentera epitelproducerade kroppsegna strukturer för T-cellerna. En sådan typ av indirekt presentation i thymus har diskuterats eftersom T-cellerna är många gånger fler än epitelcellerna och därför ger upphov till frågetecken kring hur logistiken för presentationen av kroppsegna strukturer fungerar. Här skulle alltså exosomer kunna fungera som en spridningsmekansim för dessa strukturer. Denna spridning skulle kunna vara direkt från epitelet till T-cellerna eller från epitelet via så kallade dendritiska celler (denna celltyp är expert på att presentera strukturer för immunceller) som i sin tur presenterar strukturerna för T-cellerna. Vi visar i avhandlingen att exosomer kan isoleras både från human- och musthymus och att dessa bär på kroppsegna strukturer. Även odlade epitelceller från thymus visade sig producera exosomer och dessa innehöll ett stort antal kroppsegna strukturer och även strukturer som man sedan tidigare vet är inblandade i autoimmuna sjukdomar. Dessutom undersökte vi effekten av exosomerna på T-celler genom att odla T-celler ihop med exosomer och dendritiska celler. Exosomerna visade sig bidra till utmognaden av T-cellerna. Till sist ville vi även undersöka thymusvävnad från patienter med Downs syndrom eftersom de har en extra kopia av den för thymus viktiga genen autoimmune regulator

(denna gen styr uttrycket av många kroppsegna strukturer i tymusepitelet). Genen visade sig överuttryckt liksom proteinet. Vi hittade även andra skillnader i thymus vid Downs syndrom, såsom ackumulation av dendritiska celler i thymus-märgen och en ändrad protein-komposition av exosomerna.

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