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UNIVERSITY OF TARTU
Press

Department of Biomedicine, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

This dissertation has been accepted for the commencement of the degree of Doctor of Philosophy in Medicine on August 29, 2018 by the Council of the Faculty of Medicine, University of Tartu, Estonia.

Supervisors: Martti Laan, MD, PhD, Senior Research Fellow in Molecular Immunology, Lecturer in Human Biology
Department of Biomedicine, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

Pärt Peterson, PhD, Head of Department of Biomedicine, Professor in Molecular Immunology, Lead Research Fellow in Molecular Immunology
Department of Biomedicine, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

Kai Kisand, MD, dr. med., Senior Research Fellow in Immunology
Department of Biomedicine, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

Reviewers: Raivo Uibo, MD, dr. med, Academician, Head of Department of Immunology, Professor of Immunology
Department of Immunology, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

Kersti Lilleväli, PhD, Senior Research Fellow of Physiology
Department of Physiology, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

Opponent: Graham Anderson, BSc, PhD, Professor of Experimental Immunology
Institute of Immunology and Immunotherapy, University of Birmingham, UK

Commencement: November 20, 2018

Publication of this dissertation is granted by University of Tartu. This study was supported by the Estonian Research Council and the European Union through the European Regional Development Fund.

ISSN 1024-395X
ISBN 978-9949-77-849-2 (print)
ISBN 978-9949-77-848-5 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

I. **Bichele R**, Kisand K, Peterson P, Laan M. TNF superfamily members play distinct roles in shaping the thymic stromal microenvironment. Mol Immunol. 2016 Apr;72:92–102.

II. Haljasorg U, **Bichele R**, Saare M, Guha M, Maslovskaja J, Kõnd K, Remm A, Pihlap M, Tomson L, Kisand K, Laan M, Peterson P. A highly conserved NF-κB-responsive enhancer is critical for thymic expression of Aire in mice. Eur J Immunol. 2015 Dec;45(12):3246–56.

III. Wang X, Laan M, **Bichele R**, Kisand K, Scott HS, Peterson P.Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens. Front Immunol. 2012 Mar;3:19.

IV. **Bichele R**, Kärner J, Truusalu K, Smidt I, Mändar R, Conti HR, Gaffen SL, Peterson P, Laan M, Kisand K. IL-22 neutralizing autoantibodies impair fungal clearance in murine oropharyngeal candidiasis model. Eur J Immunol. 2018 Mar;48(3):464–470.

Contributions of Rudolf Bichele to original publications:

Study I	Performed the experiments and the data analysis, prepared the figures and wrote the paper.
Study II	Performed fetal thymic organ culture experiments and the corresponding RT-PCR analysis, participated in corresponding figure preparation and data analysis.
Study III	Performed thymus tissue stimulation experiments, RT-PCR analysis and the corresponding data analysis.
Study IV	Performed the animal experiments and RT-PCR analysis, participated in the figure preparation, data analysis and writing the paper.

ABBREVIATIONS

2'dG	2'-deoxyguanosine
AIRE/*Aire*	Autoimmune regulator (gene designation in human/mouse)
AIRE	Autoimmune regulator (protein designation in human and mouse)
αLTβR	lymphotoxin beta receptor agonist antibody
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS-1	autoimmune polyendocrine syndrome type I
CD	cluster of differentiation
Ccl	CC-chemokine (gene designation in mouse)
CCL	CC-chemokine (protein designation in human/mouse)
Ccr	CC-chemokine receptor (gene designation in mouse)
CCR	CC-chemokine receptor (protein designation in human/ mouse)
CFU	colony forming unit
Cld (*Cld3*, *Cld4*)	claudin (claudin 3, claudin 4) (gene designation in mouse)
CMC	chronic mucocutaneous candidiasis
CNS1	conserved noncoding sequence 1
Csn	casein (gene designation in mouse)
Crp	C-reactive protein (gene designation in mouse)
cTEC	cortical thymic epithelial cell
Cxcl	CXC chemokine (gene designation in mouse)
CXCL	CXC chemokine (protein designation in human/mouse)
Cxcr	CXC chemokine receptor (gene designation in mouse)
CXCR	CXC chemokine receptor (protein designation in human/ mouse)
DC	dendritic cell
DLL4	Delta-like 4
DMEM	Dulbecco's Modified Eagle Medium
DN	double-negative (for CD4 and CD8)
DSG	desmoglein
E16.5	embryonic day 16.5
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
Fezf2	FEZ family zinc finger 2 (gene designation in mouse)
FOXN1/*Foxn1*	forkhead box N1 (gene designation in human/mouse)
FOXN1	forkhead box N1 (protein designation in human/mouse)
Foxp3	forkhead box P3 (gene designation in mouse)
FTOC	fetal thymic organ culture
Gad67	glutamate decarboxylase 67 (gene designation in mouse)
HC	Hassall's corpuscle
HRP	horseradish peroxidase
IFN	interferon
IKK	IκB kinase
IκB	inhibitors of NF-κB
IL	interleukin

Ins2	insulin 2 (gene designation in mouse)
IP	intraperitoneal
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked
Ivl	involucrin (gene designation in mouse)
IVL	involucrin (protein designation in mouse)
Krt	keratin (gene designation in mouse)
KRT	keratin (protein designation in mouse)
KO	knockout
LEKTI	lympho-epithelial Kazal-type related inhibitor
LTα/LTβ	lymphotoxin α/β
LTβR	lymphotoxin β receptor
MHC	major histocompatibility complex
mTEC	medullary thymic epithelial cell
$mTEC^{hi}$	mTEC with high expression of MHC class II
$mTEC^{lo}$	mTEC with low expression of MHC class II
NF-κB	nuclear factor kappa-B
NIK	NF-kB-inducing kinase
NOD	non-obese diabetic
OPC	oropharyngeal candidiasis
OPG	osteoprotegerin
Psmb11	proteasome subunit beta 11 (gene designation in mouse)
RAG	recombination-activating genes
RANK	receptor activator of nuclear factor kappa-B
RANKL	ligand of receptor activator of nuclear factor kappa-B
RT-PCR	real time polymerase chain reaction
Spt1	salivary protein 1 (gene designation in mouse)
TAK1	transforming growth factor β (TGFβ)-activated kinase 1
TCR	T-cell receptor
TEC	thymic epithelial cell
TFBS	transcription factor binding site(s)
TGFβ	transforming growth factor β
Th17	T helper 17 cells
TNF	tumor necrosis factor
TNFα	tumor necrosis factor α
TNFSF	tumor necrosis factor superfamily
Tnfrsf11b	TNF Receptor Superfamily Member 11b (gene designation in mouse)
TPA	*Tetragonolobus purpureas* agglutinin
TRAF	TNF-receptor-associated factor
Treg	regulatory T-cell
TSA	tissue specific antigen
TSLP	thymic stromal lymphopoietin
UEA-1	*Ulex europaeus* agglutinin 1
WT	wild-type
YPD	yeast extract/peptone/dextrose

1. INTRODUCTION

The thymus was acknowledged to be a separate, distinct organ already by the ancient Greeks, who believed it was there, that the soul of a man could reside. As time went by, no legitimate explanation for its existence emerged, at least not until the past half century of human history, which has actually brought scientific meaning to this lump of tissue resting on the pericardium. Found in all jawed vertebrates, the thymus has finally got the credit it deserves as an organ with an indispensable role in shaping the acquired immune system, which protects the host from foreign pathogens and immune attacks towards self alike.

This is achieved through a complex mechanism of cellular crosstalk, at the center of which are thymic epithelial cells (TEC), which in essence teach developing T-lymphocytes to differentiate between self and non-self. TECs do that by presenting immature T-cells with a plethora of peptides derived from various potential antigens expressed in different tissues, making these cells completely unique in the context of the body, as they must be capable of expressing a large number of otherwise tissue specific antigens (TSA). This so-called promiscuous gene expression is especially characteristic of TECs found in the thymic medulla, aptly named medullary thymic epithelial cells (mTECs), where it is largely under the control of Autoimmune Regulator (AIRE), a transcription regulator influencing the expression of thousands of TSAs.

AIRE is vital for the development of a properly functional immune tolerance, as its deficiency in humans leads to a complex autoimmune syndrome called autoimmune-polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED). APECED is characterized by autoimmune attacks against multiple tissues, primarily endocrine organs, as well as the presence of neutralizing auto-antibodies against multiple inflammatory cytokines.

Since AIRE has such a key role in shaping the immune system, many studies have delved into the underlying mechanisms that regulate its expression in mTECs as well as the broader role AIRE and its deficiency plays in the immune system. For that purpose, various genetically modified mouse models have been established and the data derived from them have been indispensable for our current understanding of how AIRE functions and how it is controlled. Nevertheless, many questions regarding this important gene and the cells primarily associated with it remain unanswered.

This thesis explores the various molecular pathways that regulate mTEC maturation with a focus on signals that control *Aire* expression in the murine thymus as well as examines the natural maturation process of mTECs and the role *Aire* plays in regulating mTEC maturation. In addition to that, this thesis explores the pathogenic potential of autoantibodies against cytokines produced by T helper 17 cells (Th17), which develop in response to AIRE deficiency and their possible role in precipitating susceptibility to *C. albicans* infections.

2. LITERATURE REVIEW

2.1. Thymic epithelium in immune tolerance

The thymus is a primary lymphoid organ which provides the unique micro-environment needed for the development of T lymphocytes. Thymic tissue is comprised of a number of lymphoid and non-lymphoid cell types, which create a complex three-dimensional structure, consisting largely of two compartments – the outer cortex and inner medulla, both of which play distinct roles in T cell maturation (Figure 1).

As T cell precursors from the bone marrow enter the thymus at the cortico-medullary junction (Lind et al., 2001), they first travel to the cortex where they undergo T-cell receptor (TCR) gene recombination and are committed to either the CD4+ or the CD8+ T cell lineage (Anderson and Takahama, 2012). Due to the largely random way TCR clones are generated, however, there is firstly a need to eliminate those cells that have undergone an unsuccessful TCR recombination, a process called positive selection. Secondly, the developing population of thymocytes bearing functional TCR variants needs to be checked for potentially autoreactive cells through negative selection (Takaba and Takayanagi, 2017).

Both positive and negative selection processes rely largely on thymic epithelial cells (TECs), which are further divided into subsets of cortical (cTECs) and medullary cells (mTECs) (Takahama et al., 2017), as well as dendritic cells. These cells, along with thymic fibroblasts, form a three-dimensional scaffold that supports the different stages of thymocyte differentiation (Breed et al., 2017).

Positive selection of thymocytes takes place in the thymic cortex and is mediated predominantly by cTECs (Takada and Takahama, 2015; Takahama et al., 2012). For this purpose, cTECs display an array of self-peptide–MHC complexes on their surface and in order to survive, thymocytes need do express TCRs with weak or intermediate avidity towards these complexes. A situation where thymocytes fail to recognize self-peptide–MHC complexes leads to so called death by neglect, a form of programmed cell death (Klein et al., 2009). Negative selection, meanwhile, takes place both in the cortex and the medulla, albeit the medullary compartment does provide a much wider array of self-peptide–MHC, which is why negative selection is generally regarded as medulla-dependent (Klein et al., 2014).

mTECs play a critical role in the negative selection of developing thymocytes due to their unique ability to express a wide array of otherwise tissue specific genes in a process called promiscuous gene expression (Brennecke et al., 2015; Gäbler et al., 2007; Sansom et al., 2014). This ability of mTECs to express thousands of tissue specific antigens (TSAs) in essence projects an immunological self-shadow within the thymus which for the most part guarantees that thymocytes with high avidity towards self-antigens are clonally deleted before entering the periphery (Klein et al., 2014).

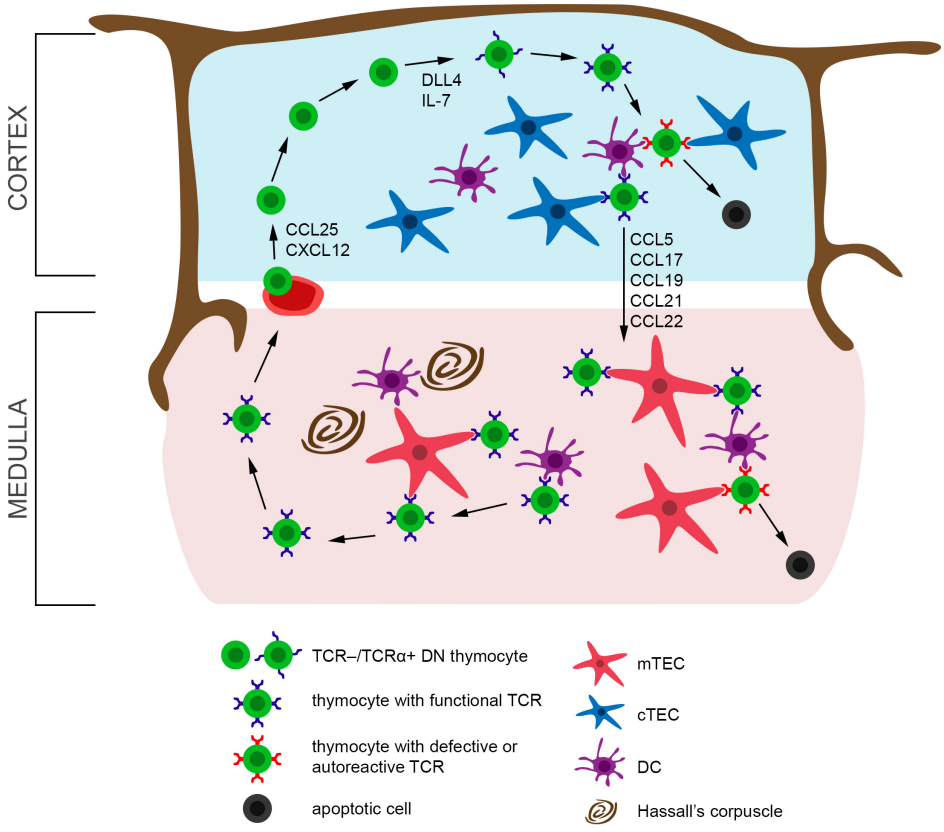

Figure 1. Intrathymic differentiation of immature T-cell progenitors. Bone marrow derived lymphoid progenitor cells enter the thymus at the corticomedullary junction and migrate into the cortex under the influence of chemokines, predominantly CCL25 and CXCL12. Through multiple maturation stages driven largely by Notch-DLL4 and IL-7 signalling, they undergo V(D)J recombination of their TCR locus and start expressing newly recombined TCR. Up until this point thymocytes do not yet express costimulatory molecules CD4 or CD8 and are thus labelled as double-negative (DN) for these markers. Once thymocytes acquire the expression of TCR, they concurrently start expressing both CD4 and CD8. Positive selection follows, where thymocytes are forced to recognize either MHC I or MHC II bound peptides on the surface of cTECs or dendritic cells (DC), appointing them to either the CD4 or CD8 lineage or face death by neglect. Once positively selected, thymocytes are subjected to negative selection, starting in the cortex and continuing in the medulla, where potentially autoreactive cells are clonally deleted or converted into regulatory T-cells. Having successfully undergone negative selection, mature naïve T-cells exit the thymus and travel to the periphery to take on their designated functions.

Thymocyte selection is additionally facilitated by thymic dendritic cells (DC). While they are not involved in promiscuous gene expression themselves, they can still provide thymocytes with the necessary survival signals throughout their development (Oh and Shin, 2015; Perry et al., 2014). The main mechanism through which DCs are hypothesized to facilitate negative selection involves cross-presentation of TEC-derived TSAs. While the mechanism of antigen uptake by thymic DCs is not yet fully understood, it has recently been shown that the acquisition and presentation of cell surface antigens by $CD8\alpha^+$ thymic DCs is mediated by the scavenger receptor CD36 (Perry et al., 2018). This antigen transfer increases the likelihood of thymocytes to encounter certain TSAs that would otherwise be expressed by only a handful of mTECs. (Dresch et al., 2012; Hubert et al., 2011; Klein et al., 2009)

Since mTECs are the main primary source of various TSAs in the thymus, a lot of effort has been put into understanding the mechanisms that allow this unique cell subset to express such a variety of genes. One of the biggest breakthroughs in this field came with the discovery that a large portion of the TSAs that are expressed in mTECs are under transcriptional regulation of the Autoimmune Regulator (*Aire*) gene (Anderson et al., 2002). *Aire* plays a key role in enforcing self-tolerance by inducing the expression of thousands of TSAs as well as being an important factor for mTEC maturation and thymic chemokine expression (reviewed in Laan and Peterson, 2013). AIRE deficiency in humans results in a complex autoimmune syndrome called APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy), also known as APS-1 (autoimmune polyendocrine syndrome type I) (Finnish-German APECED Consortium, 1997; Nagamine et al., 1997). *Aire*-deficient mice also exhibit an autoimmune phenotype, albeit to a significantly lesser extent (discussed in greater detail later) (Anderson et al., 2002; Hubert et al., 2009; Kuroda et al., 2005; Ramsey et al., 2002).

The AIRE-dependent expression of TSAs by mTECs has been a subject of debate and research for some time. mTECs express *Aire* only in a limited time frame during their maturation process, after which they down-regulate the expression of *Aire* as well as molecules related to antigen presentation and continue on to a so-called post-*Aire* phase (Nishikawa et al., 2010; Yano et al., 2008), discussed in more detail below. Previous findings indicate that not every mTEC expressing *Aire* actually expresses the whole array of TSAs. Instead, every TSA is expressed, on average, by about 1–3% of $Aire^+$ mTECs (Derbinski et al., 2008). It has also been shown that *Aire* is not the only factor responsible for the expression of TSAs in mTECs, as more recent research has demonstrated that the transcription factor *Fezf2* is responsible for the expression of a set of TSAs, which are not transcriptionally regulated by *Aire* (Takaba et al., 2015). However, the way this stochastic TSA expression pattern ultimately results in a relatively true to life representation of self-antigens within the thymus leading to successful tolerance remains enigmatic.

In addition to its role as a transcription regulator, AIRE appears to be an important factor in the development of a T lymphocyte subset called regulatory

T-cells (Treg) (Malchow et al., 2013; Perry et al., 2014; Yang et al., 2015). The generation of Tregs, characterized by their expression of FOXP3 (Fontenot et al., 2003; Hori et al., 2003), in the thymus appears to be an alternative to clonal deletion during negative selection. Instead of being sent into apoptosis, some thymocytes recognizing self-antigens with high avidity differentiate into regulatory cells, which later go on to prevent spontaneous autoimmunity (reviewed in Kitagawa and Sakaguchi, 2017). In fact, one hypothesis states that the primary function of the thymic medulla is to facilitate the generation of a functional Treg compartment, whereas conventional CD4+ and CD8+ T-cells can arise without a direct input of mTECs, being more dependent on thymic DCs (Cowan et al., 2013; Herbin et al., 2016; Perry et al., 2014). These studies suggest that the mechanisms through which mTECs maintain self-tolerance could be more complex than originally thought.

While TECs no doubt play a vital role in thymocyte selection, they are also responsible for creating the thymic chemokine and cytokine gradients needed for T cell precursor ingress, intrathymic migration, proliferation and eventual egress. It has been well established that thymic seeding by precursors and their subsequent migration requires the expression of chemokine receptors CCR9, CXCR4 and CCR7 on the surface of T cell precursors and the expression of their respective ligands CCL25, CXCL12, CCL19 and CCL21 by thymic stromal cells during embryogenesis (Ara et al., 2003; Benz et al., 2004; Bleul and Boehm, 2000; Calderón and Boehm, 2011; Ueno et al., 2002) as well as postnatally (Benz et al., 2004; Misslitz et al., 2004; Plotkin et al., 2003; Ueno et al., 2004). However, there is currently no clear consensus whether the initial thymic seeding during embryogenesis and subsequent thymocyte migration processes are regulated by identical signalling pathways. The correct regulation of thymocyte migration is well illustrated though by the fact that defects in the thymocyte migratory process brought about by CCR7 deficiency in mice results in multi-organ autoimmunity (Davalos-Misslitz et al., 2007), which surprisingly shares many similarities with *Aire*-deficiency.

Additionally, proliferation and early differentiation of thymocytes are regulated by cytokines such as IL-7 as well as Notch ligands (e.g. DLL4) in the thymic cortex (Balciunaite et al., 2005; Fry and Mackall, 2005; Radtke et al., 1999). Postnatally, thymic fibroblasts and cTECs have been shown to be the main cell populations that express CCL25 and CXCL12 while mTECs are responsible for the expression of CCL19 and CCL21. This suggests that CCR9 and CXCR4 signalling is required for the initial entry and positive selection of thymic lymphoid precursors while CCR7 and its ligands attract the positively selected thymocytes to the medulla where they undergo negative selection (Laan et al., 2016).

2.2. Thymic epithelial cell differentiation

TECs are a heterogeneous cell population, which, during embryonic development, are derived from a common thymic progenitor and undergo several stages of maturation characterized by the expression of specific genes and surface markers at certain points (Figure 2). TEC maturation is a sequential process during which cTECs and mTECs up- and down-regulate various genes depending on their maturation stage and this gene expression pattern defines the maturation stages of TECs with different functional capabilities.

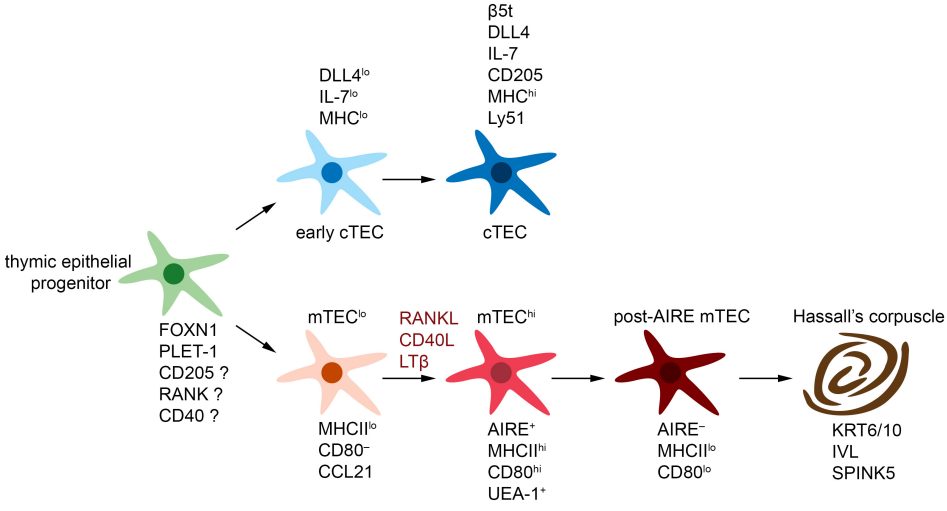

Figure 2. Differentiation of thymic epithelial cells. Thymic epithelial progenitor cells have been demonstrated to express both cTEC- and mTEC-related markers and exhibit a certain level of plasticity up until a certain, currently unclear developmental stage (Hamazaki et al., 2016). As of yet unknown signals promote the differentiation of these cells towards the cTEC lineage, which eventually start expressing several factors critical for the early developmental stages of thymocytes such as DLL4 and IL-7 as well as genes necessary for antigen processing and presentation. Differentiation towards the mTEC lineage is dependent on positively selected thymocytes and various TNFSF signals they provide, predominantly RANKL, CD40L and LTβ. mTECs differentiation begins with the so-called mTEC^{lo} stage, in which immature mTECs are not yet potent antigen presenting cells, but already express some chemokines (e.g. CCL21), which facilitate intrathymic thymocyte migration. After acquiring the expression of AIRE, mTECs are at their maximum negative selection potential, expressing thousands of TSAs and high levels of antigen presenting and costimulatory molecules. AIRE expression and promiscuous gene expression are transient and in the later stages of development, mTECs lose their antigen expression and presenting capabilities and eventually degenerate into Hassall's corpuscles expressing various markers characteristic of keratinocyte end-stage maturation.

2.2.1. Thymic epithelial progenitors

Although long debated, it is now widely accepted that the thymic epithelium is derived from a single germ layer, the endoderm, with multiple studies supporting the notion that endodermal epithelial cells alone are sufficient for the generation of functional thymic structures in mice (Blackburn et al., 2002; Gordon et al., 2004; Rodewald et al., 2001; Rossi et al., 2006). However, this raises the question of how do these progenitor cells give rise to two functionally distinct TEC subsets.

A range of bipotent (giving rise to both cTECs and mTECs) as well as unipotent (only giving rise to either the cTEC or mTEC lineage) progenitor cells present in the thymus at various stages of development have been described (reviewed in Hamazaki et al., 2016). It has been shown that in murine thymic organogenesis at around E11-E12 days of development a population of early thymic progenitor cells sequentially acquires the expression of CD205, a marker for mature cTECs in the postnatal thymus, and later on the surface expression of mTEC regulators CD40 and receptor activator of nuclear factor kappa-B (RANK) (Baik et al., 2013). These cells can give rise to fully functional cortical and medullary compartments when transplanted into immunocompetent mice, which shows that the development of mTECs and cTECs does not follow a simple binary differentiation mechanism with the two cell types deriving from a single lineage negative progenitor cell. Instead, bipotent thymic epithelial progenitors exhibit the hallmarks of both cTECs and mTECs, suggesting a certain degree of plasticity at this stage (Alves et al., 2014; Mayer et al., 2016). It is important to note that bipotent progenitors persist also in the postnatal thymus (Bleul et al., 2006), with recent studies characterizing two distinct cell populations derived from postnatal thymic tissue capable of regenerating both mTECs and cTECs in mice (Ucar et al., 2014; Wong et al., 2014).

Although there is currently no clear consensus on the phenotype of thymic progenitor cells, one common trait for the differentiation of all TECs from early progenitors is its complete dependence on the transcription factor FOXN1. This is illustrated by the fact that mice lacking a functional *Foxn1* gene fail to develop any TECs and thymopoiesis in these mice does not occur (Blackburn and Manley, 2004; Nehls et al., 1996; Rodewald, 2008). Subsequent differentiation of TECs along the cTEC and mTEC lineages is discussed in more detail below.

2.2.2. cTECs and positive selection

Since T cell progenitors entering the thymus begin their differentiation in the cortex, cortical epithelial cells need to provide the necessary conditions for the early stages of T cell development. The induction of T cells to undergo V(D)J recombination in the TCRα and TCRβ genomic loci and to start expressing costimulatory molecules CD4 and CD8 are achieved by the high expression of

Notch1 ligand DLL4 as well as IL-7 by cTECs (Alves et al., 2009; Hara et al., 2012; Hozumi et al., 2008; Koch et al., 2008). Additionally cTECs express chemokines CCL25, CXCL12 and a chemokine binding protein CCRL1, which are needed for the colonization of primordial thymic tissue and later on regulate the ingress and initial migration of thymocytes (Ara et al., 2003; Benz et al., 2004; Calderón and Boehm, 2011; Plotkin et al., 2003; Rode and Boehm, 2012).

A rather unique aspect of cTECs is the so-called thymoproteasome, a specific form of the proteasome, which produces MHC class I-associated peptides in the cytoplasm of cTECs. What sets the thymoproteasome apart from other proteasomes is the β5 subunit, β5t (encoded by the gene *Psmb11*), which is expressed only in cTECs, providing a unique repertoire of MHC I associated peptides (Murata et al., 2007; Sasaki et al., 2015). These peptides are enriched for low-affinity TCR ligands, thereby facilitating optimal positive selection of $CD8^+$ thymocytes (Sasaki et al., 2015; Xing et al., 2013), as positive selection requires thymocytes to receive weak stimulation through the TCR, which is interpreted as a survival signal.

Apart from DLL4, IL-7 and β5t, cTECs are typically identified and isolated based on their expression of CD205, Ly51, MHC II and Keratin 8 (reviewed in Ohigashi et al., 2016). While these markers are not expressed uniformly on all cTECs and there exists a level of heterogeneity, especially in the expression of MHC class II (Yang et al., 2006), they are nonetheless commonly used to identify the population of cortical epithelial cells.

2.2.3. mTECs and negative selection

Medullary thymic epithelial cells play a critical role in creating immune tolerance to self partly by creating the necessary cytokine microenvironment that facilitates the migration of thymocytes from the cortex into the medulla following positive selection, but more importantly through their unique ability to express thousands of TSAs in order to screen positively selected thymocytes against possible reactivity towards self. mTECs develop from the same bipotent progenitors as cTECs (discussed previously), although there also appear to be unipotent mTEC progenitor cells within the postnatal thymus, which guarantee the constant replenishment of the mTEC pool due to the rapid turnover of mTECs (Gray et al., 2007; Ohigashi et al., 2015).

After undergoing successful positive selection, thymocytes up-regulate the chemokine receptor CCR7 and migrate into the thymic medulla under the influence of chemokines CCL19 and CCL21, which are primarily produced by mTECs (Laan et al., 2016; Misslitz et al., 2004; Ueno et al., 2004, 2002). There, thymocytes undergo negative selection based on their TCR avidity towards self-antigens. At this stage, thymocytes showing higher affinity towards self-antigens are deleted by apoptosis (Palmer, 2003), or in certain cases deviate towards the Treg lineage, albeit the mechanisms behind this process remain somewhat unclear (Bains et al., 2013).

Not all mTECs are equally efficient at presenting antigens to thymocytes, as the most effective antigen presentation primarily occurs only during a restricted stage of mTEC maturation. The different stages of mTEC maturation are primarily characterized by the differential expression of claudins 3 and 4 (*Cld3* and *Cld4*), keratin 5, MHC class II, CD80, *Aire* and reactivity to *Ulex europaeus* agglutinin 1 (UEA-1). During ontogeny, a subpopulation of the $CD205^{+}$ bipotent progenitors start expressing RANK, a key regulator of mTEC development, which is thought to direct them towards the mTEC lineage (Baik et al., 2013). These early mTEC progenitors acquire the expression of *Cld3* and *Cld4*, setting them further apart from the bipotent thymic progenitors displaying a more cTEC-like phenotype (Ohigashi et al., 2015).

At this stage, immature mTECs express low levels of MHC class II and no CD80 (MHC II^{low} $CD80^{-}$) and are therefore not effective antigen presenting cells. This so-called $mTEC^{lo}$ compartment is heterogeneous, as it appears to consist of a non-cycling pool of immature progenitors that sustain the turnover of those mTECs that express TSAs and take part in negative selection (Gäbler et al., 2007) but also contains cells that functionally influence thymocyte development by producing CCL21 (Lkhagvasuren et al., 2013). In mice as well as in humans, an effective way to differentiate this cell population from the similarly MHC II^{low} end-stage mTECs appears to be *Tetragonolobus purpureas* agglutinin (TPA) staining, which selectively marks out the later stages of mTEC development (Michel et al., 2017).

RANK signalling promotes the transition of mTECs from the $mTEC^{lo}$ pool to a subsequent stage known as $mTEC^{hi}$, cells that highly express MHC class II as well as costimulatory molecule CD80 (Rossi et al., 2007). It is at this stage where they also start to express a range of TSAs and present them to developing thymocytes. This initial self-antigen repertoire is limited, however, and it is only after mTECs acquire the expression of *Aire* when they start expressing a full range of TSAs, including *Aire*-dependent antigens such as insulin 2 (*Ins2*) caseins α and γ (*Csna* and *Csng*), salivary protein 1 (*Spt1*) and many others in a process called promiscuous or ectopic gene expression (Anderson et al., 2002; Derbinski et al., 2005; Sansom et al., 2014). Not all TSAs expressed in mTECs are regulated by *Aire*, however, and are therefore aptly divided into *Aire*-dependent and *Aire*-independent antigens. *Aire*-independent TSAs include for example C-reactive protein (*Crp*) and glutamate decarboxylase 67 (*Gad67*) (Yano et al., 2008) and appear to be partly regulated by other transcription factors such as *Fezf2* (Takaba et al., 2015). Nevertheless, *Aire*-dependent expression of TSAs is regarded as one of the key processes that leads to the negative selection of self-reactive thymocytes.

The main function of *Aire* in mTECs is primarily regarded to be that of a transcription activator for TSAs, although it has been shown that *Aire* is also required for the overall maturation of mTECs by as of yet unknown means. When comparing the thymic staining patterns for UEA-1 and Keratins 5, 8 and 14, there appear to be detectable changes in the thymic morphology when comparing *Aire* knock-out mice with their wild-type counterparts (Dooley et al.,

2008; Gillard et al., 2007) and the ultrastructure of mTECs is radically changed (Milićević et al., 2010). *Aire* deficient mice also exhibit disrupted thymic production of CCR4 and CCR7 ligands (Laan et al., 2009), thus supporting the notion that *Aire* regulates thymic functions at multiple levels and is critical for proper negative selection of thymocytes.

2.2.4. Hassall's corpuscles and end-stage mTEC maturation

For a long time it was uncertain, whether *Aire* expression marks the terminal differentiation stage for mTECs, but in light of recent discoveries it appears that mTECs continue to develop further into the so-called post-*Aire* maturation stage. They down-regulate the expression of *Aire*, MHC II and CD80, thus losing their unique ability to directly present TSAs (Nishikawa et al., 2010; Yano et al., 2008).

Another indication of the role of post-*Aire* mTECs in the thymus are Hassall's corpuscles (HCs), enigmatic structures within the thymic medulla believed to be formed by terminally differentiated mTECs. HCs are characterized by their positive staining for terminal differentiation markers that are usually associated with epidermal cells such as keratin 6 (KRT6), KRT10 and involucrin (IVL) (Hale and Markert, 2004) and are absent in *Aire*-deficient mice (Yano et al., 2008). It is therefore likely, that these thymic structures represent a post-*Aire* mTEC population.

For a long time, the potential function of HCs remained uncertain and they were considered to simply be a "thymic mTEC graveyard". However, studies have revealed that HCs could actually be involved in the generation of Tregs through their expression of thymic stromal lymphopoietin (TSLP), which in turn facilitates thymic DC-dependent proliferation and differentiation of $FOXP3^+$ Tregs in the human thymus (Watanabe et al., 2005). Seeing how *Aire*-deficiency in mice leads to a drastic decrease in HC numbers as well as defects in Treg development (discussed previously), these facts strengthen the hypothesis linking *Aire* and Treg generation. Nevertheless, many questions remain unanswered about the post-*Aire* stages of mTEC development and their direct connection with HCs has yet to be established.

2.2.5. TNF superfamily in TEC differentiation

The human tumor necrosis factor superfamily (TNFSF) consists of 29 receptors and 19 ligands which have varying functions within the body. The binding of ligands to TNF receptors may trigger a number of processes inside a cell from differentiation and cell division to apoptosis, but without exception, all TNF superfamily members exhibit pro-inflammatory activity (Aggarwal et al., 2012).

It has been well established that the development of a fully functional thymus is dependent on a number of TNFSF receptors, ligands and downstream

signalling pathways (Akiyama et al., 2008; Boehm et al., 2003; Dunn et al., 1997; Gray et al., 2006; Hikosaka et al., 2008; Rossi et al., 2007; Venanzi et al., 2007). Out of the main signals driving mTEC homeostasis, receptors RANK, CD40 and the lymphotoxin beta receptor (LTβR) as well as their respective ligands have predominantly been studied. Various knock-out mouse models of these genes have been constructed, all having thymic defects of varying degrees (Table 1).

Table 1. Thymic phenotypes of transgenic mice deficient for TNFSF receptors/ligands (modified from Irla et al., 2010).

Mouse genotype	Resulting defects	Thymic phenotype
RANK$^{-/-}$ or RANKL$^{-/-}$	Defective RANK-RANKL signalling	Thymic architecture mostly normal, UEA-1^{+} Aire^{+} mTECs absent in embryonic thymus, postnatally develop decreased numbers of UEA-1^{+} mTEC, Aire^{+} mTECs strongly reduced (Akiyama et al., 2008; Rossi et al., 2007)
OPG$^{-/-}$	OPG deficiency, defective inhibition of RANK-RANKL signalling	Strongly increased medulla/cortex ratio and number of UEA-1^{+} mTECs (Hikosaka et al., 2008)
CD40$^{-/-}$ or CD40L$^{-/-}$	Defective CD40-CD40L signalling	No defects in embryonic thymus (Akiyama et al., 2008), postnatally normal thymic architecture, mTEC^{hi}/mTEC^{lo} ratio skewed towards mTEC^{hi}, number of mTECs normal (Dunn et al., 1997; Gray et al., 2006; Hikosaka et al., 2008)
RANKL$^{-/-}$ CD40$^{-/-}$	Defective RANK-RANKL and CD40-CD40L signalling	Thymus architecture strongly disorganized, UEA-1^{+} and Aire^{+} mTECs almost completely absent (Akiyama et al., 2008)
LTβR$^{-/-}$	Defective LT signalling	mTEC organization disrupted, medulla/cortex ratio unchanged, number of UEA-1^{+} mTECs reduced (Boehm et al., 2003), AIRE protein level in mTECs unchanged (Venanzi et al., 2007)
LTα$^{-/-}$	Defective LT signalling	Normal thymic architecture, number of Aire^{+} mTEC^{hi} cells unchanged, (Rossi et al., 2007; Venanzi et al., 2007)
LTβ$^{-/-}$	Defective LT signalling	Disrupted organization of UEA-1^{+} mTECs, numbers not reduced (Boehm et al., 2003)
LTβ$^{-/-}$ LIGHT$^{-/-}$	Defective LT signalling	Disrupted organization of UEA-1^{+} mTEC, numbers not reduced (Boehm et al., 2003)

The most evident effect on the thymic structure is observed in the case of RANK and RANKL deficiency, as neither RANK nor RANKL KO mice possess any mature mTECs in the embryonic thymus and have severely reduced numbers of mature mTECs in their post-natal life, causing severe defects in thymocyte selection (Akiyama et al., 2008; Hikosaka et al., 2008; Rossi et al., 2007). A deficiency in osteoprotegerin (OPG), a soluble decoy receptor for RANKL and a negative regulator of RANK signalling, conversely results in thymic medullary hypertrophy and an increased number of UEA-1^+ mature mTECs (Hikosaka et al., 2008). In addition to studies conducted on genetically modified animals, administration of RANKL following bone marrow transplantation has also been shown to induce TEC proliferation and thymic regeneration in mouse thymi following the loss of cellularity induced by irradiation treatment (Lopes et al., 2017), suggesting a clear role in regulating TEC homeostasis for this pathway.

As RANK- and RANKL-deficient animals nevertheless develop a small number of mature Aire$^+$ mTECs (Akiyama et al., 2008; Rossi et al., 2007), it suggests that there exist other signals, which help to compensate for the lack of RANK signalling. This is demonstrated by the effectively complete lack of mature mTECs even in the adult thymi of mice lacking both functional RANK and CD40 signalling (Akiyama et al., 2008; Mouri et al., 2011). Mice deficient for CD40 or CD40L alone show a relatively mild thymic phenotype compared to RANK/RANKL deficient animals (Akiyama et al., 2008; Gray et al., 2006) and signalling through the CD40-CD40L pathway has been suggested to acquire more importance in the post-natal thymus (Dunn et al., 1997).

Additionally, signalling mediated by the LTβ receptor has been shown to play a role in thymic chemokine expression (Lkhagvasuren et al., 2013; Seach et al., 2010) and defects in LTβR signalling result in a reduced number of mature mTECs (Boehm et al., 2003) as well as defective thymic medullary architecture (Irla et al., 2013). RANKL-LTβR double knockout mice exhibit a similar phenotype to the aforementioned RANK-CD40 double knockout animals and there appears to be a degree of synergy between LTβR and RANK signalling, as $LT\alpha_1$-$LT\beta_2$ has been shown to up-regulate the expression of RANK on mTECs (Mouri et al., 2011). Nevertheless, it must be stressed that while TEC-specific LTβR-deficiency results in thymic architectural defects, it does not have a negative impact on T-cell tolerance. Instead, LTβR-signalling appears to affect the availability of the thymic DC pool and through that project its control over negative selection (Cosway et al., 2017).

The LTβ receptor is also bound by TNFSF member LIGHT (Schneider et al., 2004), albeit its absence does not result in a significant effect on lymphoid organ development (Scheu et al., 2002). The LTα and LTβ proteins also share a structural similarity with TNFα but apart from TNFα KO mice exhibiting a normal thymic stromal architecture (Grech et al., 2000) there exists little information on the effects TNFα might have on thymic functions.

Taken together, existing data suggest that the TNFSF signalling pathways in the thymus are intertwined into a more complex regulatory network. Never-

theless, the majority of our information regarding this topic comes from various KO mouse models, which often result in developmental blocks in mTECs. Thus, the direct transcriptional effects of these signals on the thymic stroma remain largely unknown. The aforementioned members of the TNF receptor family eventually work through the activation of nuclear factor kappa-B (NF-κB) via a complex intracellular signal transduction network, as further discussed below.

2.2.6. NF-κB signalling in TEC differentiation

A growing number of studies implicate the critically important role of NF-κB signalling in the formation of a correctly organized thymic medullary structure and the generation of functional mature mTECs (Akiyama et al., 2005; Boehm et al., 2003; Franzoso et al., 1998; Gray et al., 2006; Kajiura et al., 2004; Kinoshita et al., 2006; Lomada et al., 2007; Riemann et al., 2017; Zhang et al., 2006; Zhu et al., 2006) (Table 2).

As discussed previously, a number of TNFSF receptors needed for thymic medulla and mTEC generation act through the activation of NF-κB, which is achieved through TNF-receptor-associated factors (TRAF6, TRAF2 and TRAF5). There are two main pathways that activate NF-κB transcription factors, known as the canonical (classical) and alternative (non-canonical) pathways (Bonizzi and Karin, 2004) and both have been shown to affect mTEC maturation and medulla formation (Akiyama et al., 2005; Boehm et al., 2003; Kajiura et al., 2004; Kinoshita et al., 2006; Lomada et al., 2007; Weih et al., 1995; Zhang et al., 2006).

The canonical pathway relies on the activation of the IκB kinase (IKK) complex (consisting of IKKα, IKKβ and IKKγ) by transforming growth factor β (TGFβ)-activated kinase 1 (TAK1), which leads to the proteasome mediated degradation of inhibitors of NF-κB (IκB). This in turn releases the NF-κB transcription factors, mainly the p50/RelA heterodimer, into the cytoplasm, allowing it to translocate into the nucleus and activate gene expression.

In the alternative pathway, on the other hand, the IKK complex (consisting of IKKα dimer) is activated by NF-kB-inducing kinase (NIK). Instead of a p50/RelA dimer inhibited by IκB, NIK acts on a latent cytoplasmic complex consisting of p100/RelB and induces the proteolytic degradation of the C terminal domain of the p100 subunit, resulting in the release of a p52/RelB heterodimer, which can modulate transcription after translocating into the nucleus.

Table 2. Thymic phenotypes of transgenic mice deficient for NF-κB signalling pathway components downstream on TNFSF signalling (modified from Irla et al., 2010).

Mouse genotype	Resulting defects	Thymic phenotype
TRAF6$^{-/-}$	Defective canonical NF-κB pathway	Disrupted thymic architecture, UEA-1^{+} mTECs absent, *Aire* expression strongly reduced (Akiyama et al., 2005)
RelA$^{TEC-/-}$	TEC specific defective canonical NF-κB pathway	Relatively normal thymic architecture, Aire^{+} UEA-1^{+} mTECs reduced (Riemann et al., 2017)
NIK$^{-/-}$	Defective alternative NF-κB pathway	Thymic architecture strongly disrupted, UEA-1^{+} and Aire^{+} mTECs strongly reduced, *Aire* expression reduced (Boehm et al., 2003; Kajiura et al., 2004; Kinoshita et al., 2006)
IKKα$^{-/-}$	Defective alternative NF-κB pathway	Thymic architecture strongly disrupted, UEA-1^{+} and Aire^{+} mTECs absent (Kinoshita et al., 2006; Lomada et al., 2007)
RelB$^{-/-}$	Defective alternative NF-κB pathway	Thymic architecture disrupted, mTEC numbers reduced (Gray et al., 2006)
RelB$^{TEC-/-}$	TEC specific defective alternative NF-κB pathway	Relatively normal thymic architecture, Aire^{+} UEA-1^{+} mTECs absent (Riemann et al., 2017)
NF-κB2$^{-/-}$ (p100/p52$^{-/-}$)	Defective alternative NF-κB pathway	Relatively normal thymic architecture, UEA-1^{+} mTEC numbers reduced, conflicting evidence on the effect on AIRE expression (Zhang et al., 2006; Zhu et al., 2006)
NF-κB2$^{-/-}$ NF-κB1$^{-/-}$ (p100/p52$^{-/-}$ p50$^{-/-}$)	Defective canonical and alternative NF-κB pathways	Thymic architecture strongly disrupted, UEA-1^{+} mTECs absent (Franzoso et al., 1998)

The importance of both the canonical and alternative pathways has been demonstrated using various mouse models, which are deficient for components of NF-κB signalling pathways. Mice deficient for TRAF6, a key upstream mediator of the canonical NF-κB pathway, exhibit severe thymic abnormalities, lacking mature UEA-1^{+} and Aire^{+} mTECs and having a disorganized arrangement of mTECs in the thymus (Akiyama et al., 2005). The importance of the alternative NF-κB pathway is highlighted by gene-targeted mouse models lacking NIK (Kajiura et al., 2004; Kinoshita et al., 2006), IKKα (Kinoshita et al., 2006; Lomada et al., 2007) and RelB (Akiyama et al., 2005; Weih et al., 1995), which all exhibit a loss of UEA-1^{+} and Aire^{+} mTECs and defective thymic organization of the remaining mTEC population. RelB in particular has been identified as a key transcription factor needed for proper mTEC development (Jin and Zhu, 2018; Riemann et al., 2017) and it has been shown

to be required for the emergence of RANK-expressing mTEC progenitors from mTEC stem cells (Baik et al., 2016).

Although the importance of TNFSF receptors and NF-κB signalling on the overall maturation of mTECs is well established, the direct role of specific TNFSF signals, predominantly RANKL, and NF-κB signalling pathways on the expression of *Aire* and therefore the generation of mTECs capable of promiscuous gene expression remains unknown.

2.3. APECED and *Aire*-deficient mouse models

As discussed previously, AIRE functions primarily in the thymic epithelium by facilitating the clonal deletion of potentially autoreactive thymocytes. That is supported by the fact that situations where AIRE-dependent thymic selection is impaired typically result in a breakdown in immune tolerance.

2.3.1. APECED

In humans, AIRE deficiency leads to a severe autoimmune disease called APECED, a rare, mostly recessive autosomal disorder with an incidence in the general population of <1:100,000 (Kahaly, 2009). However, in certain historically isolated populations that have undergone genetic bottlenecks and founder effect, it has a significantly increased prevalence, the best known examples of which are Iranian Jews (1:9,000), Sardinians (1:14,000) and Finns (1:25,000) (Perheentupa, 2006; Rosatelli et al., 1998; Zlotogora and Shapiro, 1992).

The clinical manifestations of the disease are multiple and complex – classical APECED symptoms consist of chronic mucocutaneous candidiasis (CMC), hypoparathyroidism and adrenocortical failure, a set of diseases characterised as the "APECED triad," at least two of which are usually required for a diagnosis (Husebye et al., 2009). The full classical triad, however, is present only in roughly two thirds of all patients and additionally, many develop more uncommon diseases associated with APECED such as vitiligo, alopecia, autoimmune hepatitis, type I diabetes, keratoconjuctivitis, periodical fevers and gastrointernal tract dysfunctions (Ahonen et al., 1990; Kisand and Peterson, 2015; Perheentupa, 2006). In fact, APECED patients on average have three to five of the aforementioned conditions over the course of their lifetime. These diseases, in addition to autoantibody analysis and the sequencing of the *AIRE* gene are used to give the final APECED diagnosis (Husebye et al., 2009; Meloni et al., 2008; Perheentupa, 2006). Following diagnosis, the subsequent management of APECED usually follows a case-by-case scenario due to a very wide array of possible manifestations and is focused on managing the individual conditions (Husebye et al., 2009; Kisand and Peterson, 2015).

2.3.2. Autoantibodies in APECED

APECED patients have been shown to develop a plethora of different autoantibodies. The first group of these are organ-specific autoantibodies, primarily targeting various endocrine organs and often being closely connected with the corresponding diseases. The main endocrine organs that are affected are the parathyroid glands (77–96% of all cases), the adrenal cortex (63–92%), the ovaries (60%) and pancreatic islets (up to 30%) (Kisand and Peterson, 2015; Meloni et al., 2012; Perheentupa, 2006; Wolff et al., 2007).

A second group of autoantibodies that are a rather unique and peculiar feature in APECED patients, are neutralizing cytokine-specific autoantibodies, which typically have a high titer in patients' sera. This is especially noteworthy, as there is currently no evidence suggesting that AIRE directly regulates the expression of inflammatory cytokines in the thymic epithelium. Most prevalent are autoantibodies against type I interferons – 100% of APECED patients have antibodies recognizing and neutralizing IFN-ω and about 95% have autoantibodies against IFN-α, with reactivity against IFN-β (22%) and IFN-λ (14%) being less common (Meager et al., 2006; Meloni et al., 2008). Interestingly, these IFN-neutralizing antibodies do not appear to bring about visible susceptibility towards viral infections nor do they affect the numbers of IFN-producing dendritic cells despite inhibiting the effect of interferons *in vitro* and suppressing IFN-stimulated gene expression. It is likely that the mostly para- and autocrine way IFN signalling works is largely unaffected by these antibodies and possible deficiencies may be compensated by other interferon types (Kisand et al., 2008; Meager et al., 2006). However, the occurrence of neutralizing autoantibodies against type I IFNs is strikingly in inverse correlation with type I diabetes in APECED patients (Meyer et al., 2016), suggesting these antibodies might act as a disease-limiting factor. In recent years, other instances of complex autoimmune syndromes have been described where patients develop autoantibodies against type I IFNs, such as in the case of mutations of the recombination-activating genes (RAG) (Walter et al., 2015) as well as patients suffering from immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) (Rosenberg et al., 2018). Nevertheless, these autoantibodies have not been associated with any particular aspects of disease in either of those cases.

In addition to anti-IFN antibodies, a majority of APECED patients also have autoantibodies against cytokines produced by T helper 17 (Th17) cells, primarily IL-22 (91%), IL-17F (75%) and IL-17A (41%) (Kisand et al., 2010; Puel et al., 2010). Autoantibodies targeting Th17 cytokines have also been associated with clinical symptoms, as they correlate with chronic mucocutaneous candidiasis in patients (Kisand et al., 2010). However, there is still lacking direct evidence of the pathogenic potential of autoantibodies against IL-17A/F or IL-22.

2.3.3. Aire deficient mouse models

Due to the rarity of APECED and obvious limitations of acquiring and studying human patient material, an *Aire*-deficient mouse model has been extensively used to understand the complexities of AIRE deficiency. The AIRE proteins in humans and mice are 71% similar, containing the same domains (Blechschmidt et al., 1999; Wang et al., 1999) and have a similar overall expression pattern (Eldershaw et al., 2011). A lot of what is known about thymic selection (discussed previously) has been made possible through the use of various *Aire*-deficient mouse models, which replicate the most common *AIRE* mutations in humans, resulting in a truncated protein or deletions in vital protein domains (Hubert et al., 2009; Jiang et al., 2005).

Originally on the C57BL/6 background, *Aire*-deficiency has been crossed into multiple other strains such as BALB/c, NOD and SJL, revealing that these mouse strains exhibit different susceptibilities to autoimmune diseases (Jiang et al., 2005). Different organs are affected depending on the genetic background and the severity of the phenotype varies greatly.

The original C57BL/6 *Aire*-deficient mice exhibit the mildest phenotype – while they do have autoimmune infiltrations in multiple tissues, they remain functional despite developing keratoconjuctivitis and uveitis (DeVoss et al., 2010; Hässler et al., 2006; Hubert et al., 2009; Jiang et al., 2005; Yeh et al., 2009). On the other hand, gastritis is common in BALB/c *Aire*-deficient mice while NOD.Aire^{-} and SJL.Aire^{-} animals develop pancreatitis and thyroiditis respectively. These autoimmune reactions are also correlated with corresponding organ-specific autoantibodies in different mouse strains (Jiang et al., 2005). Mortality of these animals equally varies, from unaltered life expectancy of *Aire*-deficient mice on C57BL/6 background to up to 90% in NOD.Aire^{-} animals, who are prone to weight loss and subsequent death between 5 to 15 weeks of age (Jiang et al., 2005). Overall, while the symptoms seen in *Aire*-deficient mice partially match the ones seen in APECED patients, they are generally less pronounced and not consistent over different strains of mice (Table 3).

One aspect that clearly sets *Aire*-deficient mice apart from their human counterparts is the lack of the classical APECED triad. They also completely lack type I IFN-specific autoantibodies, which are so indicative of APECED patients (Hubert et al., 2009). However, aged *Aire*-deficient BALB/c mice have been shown to develop autoantibodies capable of recognizing and neutralizing IL-17A, which is a common feature shared between mice and humans (Kärner et al., 2012). This, along with their low mortality compared to NOD.Aire^{-} mice, makes BALB/c.Aire^{-} mice one of best available animal models to study *Aire* deficiency.

Table 3. Phenotype observed in BALB/c, C57BL/6, NOD and SJL *Aire*-deficient mice and the corresponding disease/autoantibodies found in APECED patients, where applicable. Modified from (Kisand et al., 2014).

	Aire $^{-/-}$ mouse	**APECED**
Diseases / autoimmune infiltrations		
	Thyroid gland infiltrations*	Addison's disease
	Infertility	Ovarian failure Testicular failure
	Liver infiltrations	Autoimmune hepatitis
	Pancreas infiltrations*	Pancreatitis
	Lung infiltrations	Interstitial lung disease
	Gastritis	
	Uveoretinitis	
	Dacryoadenitis	
	Salivary gland infiltrations	
	Prostate infiltrations	
	*on NOD or SJL background only	
Autoantibodies		
	IL-17A (IL-17F)	IL-22, IL-17F, IL-17A
	BPIFB9	BPIFB1
	OBP1a	
	SVS2	
	IRBP	
	alpha-fodrin	
	TRP-1	
	Mucin 6	

2.3.4. Candidiasis in APECED

Chronic mucocutaneous candidiasis is one of the classical diseases associated with AIRE deficiency in humans and usually one of the first signs of APECED. The onset of CMC, primarily caused by the fungal species *Candida albicans*, typically occurs within the first few months or years of life and is one of the most common diagnostic markers for APECED (Ahonen et al., 1990; Kisand and Peterson, 2015; Perheentupa, 2006).

The current notion is that susceptibility to candidiasis in APECED patients is caused by the neutralizing autoantibodies against IL-22 and IL-17A/F, which in turn impairs the host defence against fungal infections. The reasoning is based on observations that autoantibodies against these cytokines correlate with CMC in patients (Kisand et al., 2010) and Th17 cytokines are known to be an essential part of providing protection against *Candida* infections in humans as well as in mice (Conti et al., 2009; Engelhardt and Grimbacher, 2012; Kisand and Peterson, 2013; Lilic, 2012; Sparber and LeibundGut-Landmann, 2015).

Relations between CMC and AIRE deficiency have been difficult to study though, as *Aire*-deficient mouse strains appear to be resistant towards spontaneous *Candida* infections (Kisand et al., 2014). Mice and humans share little in terms of phenotype when it comes to anti-cytokine autoantibodies, the only clear similarity being antibodies against IL-17A (discussed previously).

While it has been shown that neutralizing IL-17A or IL-17A and IL-17F using monoclonal antibodies in mice leads to impaired fungal clearance (Whibley et al., 2016), little is known about the pathogenic potential of the naturally occurring autoantibodies in aged *Aire*-deficient mice. Additionally, experimental evidence so far is lacking in regards to anti-IL-22 autoantibodies and their role in CMC susceptibility, despite being the most prevalent autoantibodies against Th17 cytokines in APECED patients.

3. AIMS OF THE STUDY

The general purpose of this study was to characterize in greater detail the various roles thymic expression of *Aire* plays in shaping central immune tolerance and host defence as well as the regulatory molecular mechanisms controlling *Aire* expression.

The specific aims of this study were as follows:

- to study the effects of various TNFSF molecular pathways on the global gene expression in mTECs and more specifically to assess the extra- and intracellular pathways regulating *Aire* expression in the thymus.
- to study the role of *Aire* in the latter stages of mTEC development and characterize the nature and function of post-*Aire* mTECs and Hassall's corpuscles in the murine thymus.
- to assess, whether *Aire* deficiency and the concurrent autoantibody background in aged Aire KO mice makes them susceptible to oropharyngeal candidiasis as well as understand the role IL-22 neutralizing antibodies play in providing protection against *Candida albicans* infections.

4. MATERIALS AND METHODS

4.1. Mice (Studies I, II, III and IV)

Wild-type (WT) and *Aire* KO C57BL/6 and BALB/c mice used in all studies were bred and maintained at the animal facilities of the Institute of Molecular and Cellular Biology (University of Tartu, Estonia) and/or the Institute of Biomedicine and Translational Medicine (University of Tartu, Estonia).

In study III, the Aire KO mice on the C57BL/6 background (obtained from Dr. Hamish Scott) were generated at the Walter and Eliza Hall Institute for Medical Research to have a disruption in exon 8 of the *Aire* gene which brings the *lacZ* reporter gene under the control of the endogenous *Aire* promoter, creating an AIRE-*lacZ* fusion (Hubert et al., 2009). From these mice, Aire KO mice on the BALB/c background were generated by backcrossing for at least ten generations.

Conserved noncoding sequence 1 (CNS1) deficient mice (CNS1 KO) used in study II were generated at the Laboratory Animal Centre of Tartu University by targeted disruption of the CNS1 region upstream of the *Aire* gene as described in study II and maintained at the animal facilities of the Institute of Biomedicine and Translational Medicine (University of Tartu, Estonia).

In experiments requiring embryonic tissues, day 0.5 of pregnancy was determined as the day a vaginal plug was detected after overnight mating.

For performing oropharyngeal candidiasis (OPC) susceptibility experiments on *Aire*-deficient mice in study IV, Aire KO mice and their WT littermates on the BALB/c background were aged for at least 1.5 years. Male and female mice were both equally allocated into study gruoups. All animal experiments were conducted in accordance with the European Communities Directive (86/609/EEC) and were approved by the ethical committee of animal experiments at the Ministry of Agriculture, Estonia (approved 29.08.2013 no 13).

4.2. Fetal thymic organ culture (FTOC) generation (Studies I and II)

T-cell maturation as well as normal thymic gene expression rely heavily on the complex 3D thymic architecture. Fetal murine thymic tissue has long been used to imitate the *in vivo* conditions as closely as possible while still maintaining a relatively high level of contol over the experimental system. The ability to selectively deplete hematopoietic cells allows us to study the effects of isolated signals on the thymic stroma with greatly reduced background noise otherwise coming from thymocytes, making the FTOC system preferable over various *in vitro* alternatives.

Pregnant WT C57BL/6 or CNS1 mice were sacrificed by cervical dislocation prior to the removal of embryos and FTOCs were established from

E16.5 day mouse embryos. Thymic lobes were cultured for 6 days on Isopore™ membrane filters (pore size 0.8 μm; Merck Millipore) placed on Artiwrap sponges (Medipost Ltd.) in DMEM supplemented with 4.5 g/L glucose, L-glutamine and sodium pyruvate, 10% (v/v) FBS, 0.1 mg/mL streptomycin, 100U/mL penicillin, and 0.25 μg/mL amphotericin B (at different points in time supplied by PAA, Naxo, Corning, PAN Biotech). To deplete hematopoietic cells, 2'-deoxyguanosine (2'dG, Sigma-Aldrich) was added to the media at a final concentration of 1.35 mM.

4.3. Stimulation of thymic tissue (Studies I, II and III)

In study I, FTOCs were stimulated with different TNFSF members for 48 hours following 6 days of 2'dG-treatment at the following concentrations: 500 ng/ml RANKL (eBioscience), 100 ng/ml CD40L, 50 ng/ml TNFα 500 ng/ml LIGHT with 2.5 μg/ml poly-His (all R&D systems), or 2 μg/ml lymphotoxin beta receptor agonist antibody (αLTβR) (eBioscience). In study II, following 2'dG treatment for 6 days, one lobe from each thymus was cultured for 24 hours on DMEM alone, the other on DMEM with 500 ng/ml RANKL (eBioscience). To test the effect of NF-κB inhibitors on *Aire* expression, FTOCs were prepared using the aforementioned method from C57BL/6 mice. After 6 days, 2'dG-treated FTOCs were treated with inhibitors for IKKβ (TPCA-1, Tocris Bioscience) or NIK (isoquinoline – 1, 3(2H, 4H) – dione, Santa Cruz Biotechnology) at indicated concentrations with or without 500 ng/mL RANKL. In study III, thymi from 4–6 week old WT or Aire KO mice were removed and thymic tissue was incubated at the air-liquid interface for 24h or 48h on Isopore Membrane Filters (Millipore) in DMEM supplemented with 10% FCS and antibiotics and treated with LIGHT (50 ng/ml + polyHis 2.5μg/ml), RANKL (15 ng/ml), or CD40L (10 ng/ml, all from R&D). In all experiments, a minimum of three biological replicates were used for every stimulation as well as control, where one replicate consisted of two pooled thymic lobes.

4.4. Oropharyngeal candidiasis mouse model (Study IV)

Induction of OPC was performed as described previously (Conti et al., 2009; Kamai et al., 2001). All OPC experiments were conducted on mice on the BALB/c background. The *C. albicans* laboratory strain SC5314 (ATCC) was grown in yeast extract/peptone/dextrose (YPD) broth at 30 °C for 14–16 h prior to infection. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally (IP) and inoculated sublingually with a 0.0025 g cotton ball saturated in *C. albicans* suspension for 90 min under anaesthesia. When necessary, boost anaestesia was applied consisting of ketamine at 50 mg/kg IP. Mice were caged individually after infection.

To test the effect of IL-22 neutralization, 200 μg of the APECED patient-derived IL-22 IgG antibody 30G1 (Meyer et al., 2016) or human IgG control (Sigma-Aldrich) was injected on days −1, 1, 3 and 5. Eight female mice (2 month old) were allocated to each antibody-treated group. 30G1 treated group also included 4 male and 5 female mice over 1.5 year of age and IgG treated group 3 male and 4 female mice over 1.5 years of age. As a positive control for fungal infectivity, 2–3 mice per experiment were immunosuppressed with 225 mg/kg cortisone acetate (Sigma-Aldrich) injected subcutaneously starting from day 1 prior to infection and continued every other day. Since acute OPC results in loss of bodyweight due to discomfort and pain associated with eating, the bodyweight of mice was monitored throughout the course of the experiments as well as the signs of pain and distress that required euthanization.

On day 6 (or on day 3–4 for immunosuppressed group) mice were sacrificed and the tongues were evenly split into two. One half was snap-frozen for gene expression analysis and the other half homogenized with GentleMACS (Miltenyi Biotec) in PBS and plated in serial dilutions on YPD agar with antibiotics to quantify the fungal loads.

4.5. Flow cytometry analysis and FACS sorting of cells (Studies I and III)

In study I, to assess the effect of TNFSF signalling on mTEC maturation in organ cultures, 2'dG treated FTOCs were prepared and stimulated as described above. To obtain a single-cell suspension, FTOCs were washed twice in Ca^{2+}/Mg^{2+} free PBS (Lonza) and disaggregated in 600 μL of 0.25% trypsin solution in 0.2% EDTA [1 in 10 dilution of 10× trypsin with 0.2% EDTA (both Sigma-Aldrich)] at 37°C for 10 min. Cells were then pipetted to aid disaggregation and the suspension was neutralized with an equal volume of DMEM containing 10% (v/v) FBS and spun down at 300 × g for 10 min at 4 °C. Disaggregated FTOCs were analysed using LSRFortessa flow cytometer (BD Biosciences) and FCS Express 5 (De Novo Software). The antibodies used were CD45 (30-F11) PerCP-Cy5.5; EpCAM (G8.8) Pe-Cy7; Ly51 (BP1) PE (all from eBioscience); CD80 (16–10A1) BV421 (Biolegend).

In study III, to isolate different mTEC populations, fifteen thymi from six to eight week old WT, heterozygous *Aire-lacZ* ($Aire^{+/-}$) or Aire KO C57BL/6 mice were dissected and collected into ice-cold PBS. GentleMACS C-Tubes (Miltenyi Biotec) were used for mechanical disruption followed by enzymatic digestion in 0.5 mg/ml dispase/collagenase (Roche) and 5μg/ml DNase I (AppliChem) in PBS at 37 °C for 3×30 min with gentle agitation. After each incubation period, the released cells were counted and, starting from the last fraction, the cells were pooled to gain 200×10^6 cells. In this population, a negative selection was performed with CD45 microbeads and AutoMACS (Miltenyi Biotec) to obtain the $CD45^-$ cells. To get the populations of diverse

mTECs, the $CD45^-$ thymic stromal cells were stained with LacZ (X-gal, ImaGene Red C12RG β-galactosidase substrate, Invitrogen), anti-EpCAM-FITC monoclonal Ab (generated in-house from G8.8 hybridoma cell line, which was obtained from Developmental Studies Hybridoma Bank, University of Iowa, USA), and anti-MHCII-APC monoclonal Ab (Miltenyi Biotec), followed by FACS sorting with a FACS Vantage (BD). According to EpCAM, LacZ, and MHCII expression, the mTEC fractions were further divided into the $EpCAM^+LacZ^-MHCII^{lo}$, $EpCAM^+LacZ^-MHCII^{hi}$, and $EpCAM^+LacZ^+$ mTECs.

4.6. Immunofluorescence and immunohistochemistry analysis (Study III)

Thymic tissue was either fixed in 10% neutral buffered formalin and processed into paraffin blocks using standard protocols or snap-frozen in TissueTek (Vogel, Germany) and 5µm frozen sections were made and stored at −80 °C for later use. For immunohistochemistry, the immunoperoxidase assays were performed using standard techniques on 4% formaldehyde-fixed frozen sections or chamber slides. For histochemical β-galactosidase staining, frozen sections were fixed in ice-cold 4% formaldehyde for 10 min and stained for lacZ overnight, after which co-immunostaining with different markers was carried out. For more detailed protocols of stainings, see Study III. The images were captured using an Olympus IX70 inverted microscope equipped with WLSM PlanApo 20× or 40× water immersion objective and Olympus DP70 CCD camera (Olympus Corp., Tokyo, Japan). For all stainings, a minimum of three experiments were carried out. For all immunohistochemical or immunofluorescent stainings one stained section on a glass was paralleled with one negative control section on the same glass, which went through exactly the same procedures but instead of the primary antibody was incubated with phosphate buffer solution (PBS) only. On the glasses used for analysis, the negative controls showed virtually no signal.

The following primary antibodies were used: anti-LEKTI rabbit polyclonal antibody, anti-desmoglein-1 goat polyclonal, anti-desmoglein 3 goat polyclonal antibody (all from Santa Cruz); anti-CK6 rabbit polyclonal, anti- CK10 rabbit polyclonal, anti-involucrin rabbit polyclonal, anti-LAMP-1 rat monoclonal (all from Abcam) and anti-CD11c–Cy5.5 recombinant human IgG1 (Miltenyi Biotec). The following horseradish peroxidase (HRP) labelled secondary antibodies were used for immunohistochemistry: anti-rabbit swine polyclonal, anti-rat goat polyclonal (both from Santa Cruz Biotechnology). For immunofluorescence the following secondary antibodies were used: anti-goat donkey polyclonal antibody conjugated with Alexa Fluor 594, anti-rabbit chicken polyclonal antibody conjugated with Alexa Fluor 488 (both from Life Technologies).

4.7. 2'dG-FTOC microarray analysis (Study I)

Four or five FTOCs were pooled, and RNA was extracted using an RNeasy Micro kit (Qiagen). Prior to microarray analysis, RNA quality was assessed using a NanoDrop ND-1000 (Thermo Scientific) and a 2100 Bioanalyzer (Agilent Technologies). Next, cRNA was synthesized using a TargetAmp™ Nano Labeling Kit (Epicentre) from 300 ng of total RNA according to the manufacturer's instructions, and a genome-wide gene expression analysis was carried out using MouseRef-8 v2.0 Expression BeadChip® arrays (Illumina) with two replicates per treatment. Array data were analysed using Genome-Studio software (Illumina). Rank invariant normalization was applied and sample groups were compared to untreated control samples. If a single gene was represented by multiple probes on the array, the mean fold change of all probes was used for further analysis. Genes were considered to be differentially up-regulated compared to the untreated control if their fold changes in treated samples were ≥ 2 and the array detection p-value was ≤ 0.01. Functional profiling was performed using g:Profiler software (http://biit.cs.ut.ee/gprofiler/). Best per parent group filtering with a maximum size of 1000 genes for a functional category and Benjamini-Hochberg FDR significance thresholds were used. Hits were sorted by p-values.

4.8. Transcription factor binding site enrichment analysis (Study I)

To identify over-represented transcription factor binding sites (TFBS), the lists of differentially expressed genes were analysed with oPOSSUM 3.0 software (http://opossum.cisreg.ca/oPOSSUM3/) using the following settings: conservation cut-off: 0.40; matrix score threshold: 85%; amount of upstream/downstream sequence: 2000/0. To determine statistically significant TF binding sites, we took into account the calculated Z-scores and Fisher scores. The Z-score compares the rate of occurrence of a TFBS in the target set of genes to the expected rate estimated from a pre-computed background set. Because this analysis can be skewed by the presence of multiple TF binding sites in a single promoter region, we plotted these values against corresponding Fisher scores. These were calculated as one-tailed Fisher exact probability, which compares the proportion of co-expressed genes containing a particular TFBS to the proportion of a background set containing the site and is therefore unaffected by the aforementioned bias. Values considered statistically significant for Z-scores were calculated as the sample set mean + 2×standard deviation (default for oPOSSUM 3.0) and $-\ln(0.01) = 4.61$ for Fisher scores where 0.01 represents a p value.

4.9. Quantitative RT-PCR analysis (Studies I, II, III and IV)

In order to extract RNA, thymic and tongue tissues were homogenized in TRIzol (Thermo Scientific) either manually using a pestle (in case of FTOCs) or with GentleMACS (Miltenyi Biotec) using M tubes (Miltenyi Biotec). Total RNA was then purified using the RNeasy Micro kit (Qiagen) for FTOCs and sorted cells or isopropanol precipitation for other thymic and tongue tissue and was converted to cDNA using the SuperScript III kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was carried out using the ViiA7 Real-Time PCR System (Applied Biosystems). The amplification program included an initial denaturation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min for 45 cycles. SYBR® Green (Thermo Scientific) fluorescence was measured after each extension step and the specificity of amplification was evaluated by melting curve analysis. Every sample was run in three parallel reactions. Relative gene expression levels were calculated using the comparative Ct (ΔΔ*C*t) method, where the relative expression is calculated as $2^{-\Delta\Delta Ct}$, and where *C*t represents the threshold cycle. Cytokeratin 8 was used as a housekeeping gene for normalization of thymic epithelium-specific genes, in other instances beta-microglobulin was used as a housekeeping gene. Primers used for qRT-PCR analysis are listed in Table 4.

Table 4. Primers used in RT-PCR gene expression analysis.

Primer name	Primer sequence 5'→3'	Gene
B2M F	TGAGACTGATACATACGCCTGCA	*B2m*
B2M R	GATGCTTGATCACATGTCTCGATC	*B2m*
CCL5 F	GTGCCCACGTCAAGGAGTAT	*Ccl5*
CCL5 R	CCCACTTCTTCTCTGGGTTG	*Ccl5*
CCL19atg F	CTGCCTCAGATTATCTGCCAT	*Ccl19*
CCL19atg R	TCATTAGCACCCCCCAGAGT	*Ccl19*
CCL21 F	CCCTGGACCCAAGGCAGT	*Ccl21*
CCL21 R	AGGCTTAGAGTGCTTCCGGG	*Ccl21*
Crp F	CAGACTTTTCCGCACCTTGGCTTT	*Crp*
Crp R	AGTGGGTGGTGCTGAAGTACGATT	*Crp*
CSNb F	GGCACAGGTTGTTCAGGCTT	*Csn2*
CSNb R	AAGGAAGGGTGCTACTTGCTG	*Csn2*
CXCL9-F	GAGTTCGAGGAACCCTAGTG	*Cxcl9*
CXCL9-R	AAACTGTTTGAGGTCTTTGAGG	*Cxcl9*
GAD1 F	ATAGAAAGGGCCAATTCAGTC	*Gad1*
GAD1 R	TGCATCCTTGGAGTATACCC	*Gad1*
IL1B F	CAACCAACAAGTGATATTCTCCATG	*Il1b*

Primer name	Primer sequence 5'→3'	Gene
IL1B R	GATCCACACTCTCCAGCTGCA	*Il1b*
IL2Ra F	TCACTAAACTGGACTTGCCAT	*Il2ra*
IL2Ra R	TTGGGCCCTCTCTCCCATTA	*Il2ra*
IL18R1 F	TAGGCGCATAGCGGAAAGAG	*Il18ra*
IL18R1 R	GGGTGCAGGCACAAAAACAT	*Il18ra*
KRT2-8 F	AGGAGCTCATTCCGTAGCTG	*Krt8*
KRT2-8 R	TCTGGGATGCAGAACATGAG	*Krt8*
KRT6b F	CAAACTCACATCTCAGACAC	*Krt6*
KRT6b R	GCAGCTCCTCATATTTAGTC	*Krt6*
KRT10 F	GTTCAATCAGAAGAGCAAGGA	*Krt10*
KRT10 R	GTAGTTCAATCTCCAGACCC	*Krt10*
mCD80 F	GTCCATCAAAGCTGACTTCTC	*Cd80*
mCD80 F	ATGCCAGGTAATTCTCTTCCA	*Cd80*
mCxcl1 F	ACCGAAGTCATAGCCACACTC	*Cxcl1*
mCxcl1 R	CTCCGTTACTTGGGGACACC	*Cxcl1*
mCxcl2 F	TGAACAAAGGCAAGGCTAACT	*Cxcl2*
mCxcl2 R	TCAGGTACGATCCAGGCTTC	*Cxcl2*
mCXCL2 F	GCCCAGACAGAAGTCATAGCC	*Cxcl2*
mCXCL2 R	CTTTGGTTCTTCCGTTGAGGG	*Cxcl2*
mCxcl5 F	CCTACGGTGGAAGTCATAGC	*Cxcl5*
mCxcl5 R	GCTTTCTTTTTGTCACTGCCCA	*Cxcl5*
mDefb3 F	TCTCCACCTGCAGCTTTTAGC	*Defb3*
mDefb3 R	CAATCTGACGAGTGTTGCCA	*Defb3*
mH2-Aa F	CTCAGAAATAGCAAGTCAGTC	*H2-Aa*
mH2-Aa R	AATCTCAGGTTCCCAGTG	*H2-Aa*
mIL10 F	CATGGCCCAGAAATCAAGGA	*Il10*
mIL10 R	GGAGAAATCGATGACAGCGC	*Il10*
mIL17A F	TTCATCTGTGTCTCTGATGCT	*Il17a*
mIL17A R	GTTGACCTTCACATTCTGGAG	*Il17a*
mIL22 F	GAGTCAGTGCTAAGGATCAG	*Il22*
mIL22 R	CTGAGTTTGGTCAGGAAAGG	*Il22*
mIns2 F	CAAGTGGCACAACTGGAC	*Ins2*
mIns2 R	CAGCACTGATCTACAATG	*Ins2*
mINV F	GTGAGTTTGTTTGGTCTACAG	*Ivl*
mINV R	GAAAGCCCTTCTCTTGAATCTC	*Ivl*
mLcn2 F	CCACGGACTACAACCAGTTC	*Lcn2*
mLcn2 R	CAGCTCCTTGGTTCTTCCATAC	*Lcn2*
mOPG F	CATCCAAGACATTGACCTCTG	*Tnfrsf11b*

Primer name	Primer sequence 5'→3'	Gene
mOPG R	TCTCAATCTCTTCTGGGCTG	*Tnfrsf11b*
mS100a8_n2 F	AATCACCATGCCCTCTACAA	*S100a8*
mS100a8_n2 R	CACCCACTTTTATCACCAT	*S100a8*
mTff3_exp_F	TACGTTGGCCTGTCTCCAAG	*Tff3*
mTff3_exp_sh2_R	CAGGGCACATTTGGGATACT	*Tff3*
Mup1F	TCTGTGACGTATGATGGATTCAA	*Mup1*
Mup1R	TCTGGTTCTCGGCCATAGAG	*Mup1*
Mup3 F	AGCTGATGGAGCTCTATGGCCGA	*Mup3*
Mup3 R	CGAGGCAGCGATTGACATTGGTTAGG	*Mup3*
QmAIRE13 14/14 F	TCCTCAATGAGCACTCATTTGAC	*Aire*
QmAIRE13 14/14 R	CCACCTGTCATCAGGAAGAG	*Aire*
QmAIRE3/4 5 F	CCTCAAAGAGCGTCTCCAG	*Aire*
QmAIRE3/4 5 R	TGGTCTGAATTCCGTTTCCA	*Aire*
SPINK5 F	CGAAGGCTAAGGATGAATGTG	*Spink5*
SPINK5 R	GCAGTAGTTCTTTACACATGA	*Spink5*
Spt1_F	AACTTCTGGAACTGCTGATTCTG	*Spt1*
Spt1_R	GAGGCCTCATTAGCAGTGTTG	*Spt1*
Tff1 F	CCCGGGAGAGGATAAATTGT	*Tff1*
Tff1 R	GCCAGTTCTCTCAGGATGGA	*Tff1*

4.10. Statistics

Statistical significance for RT-PCR and ELISA analysis was determined by applying an unpaired two-tailed t-test. For fungal burden enumeration analysis comparing multiple groups, Dunn's multiple comparison test was used. Data were analysed using GraphPad Prism software. Gene expression microarray data were analysed using GenomeStudio software (Illumina) and array detection p-value was used to assess statistical significance of differentially expressed genes.

5. RESULTS

5.1. RANKL, TNFα and αLTβR induce NF-κB responsive genes in the thymic stroma (Study I)

In order to study the direct transcriptional effect different TNFSF signals have in shaping the thymic stromal environment, we stimulated FTOCs depleted of hematopoietic cells with RANKL, CD40L, TNFα, LIGHT and an agonist antibody for the LTβR for 48h. The subsequent microarray analysis revealed that, out of these ligands, RANKL, TNFα and αLTβR were the primary inducers of gene expression in the embryonic thymus, whereas LIGHT and CD40L had a very modest effect on overall gene expression (Figure 3, A). The genes up-regulated by RANKL, TNFα and αLTβR partially overlapped, but there were also clearly distinct sets of genes up-regulated by specific TNFSF signals (Figure 3, B, C).

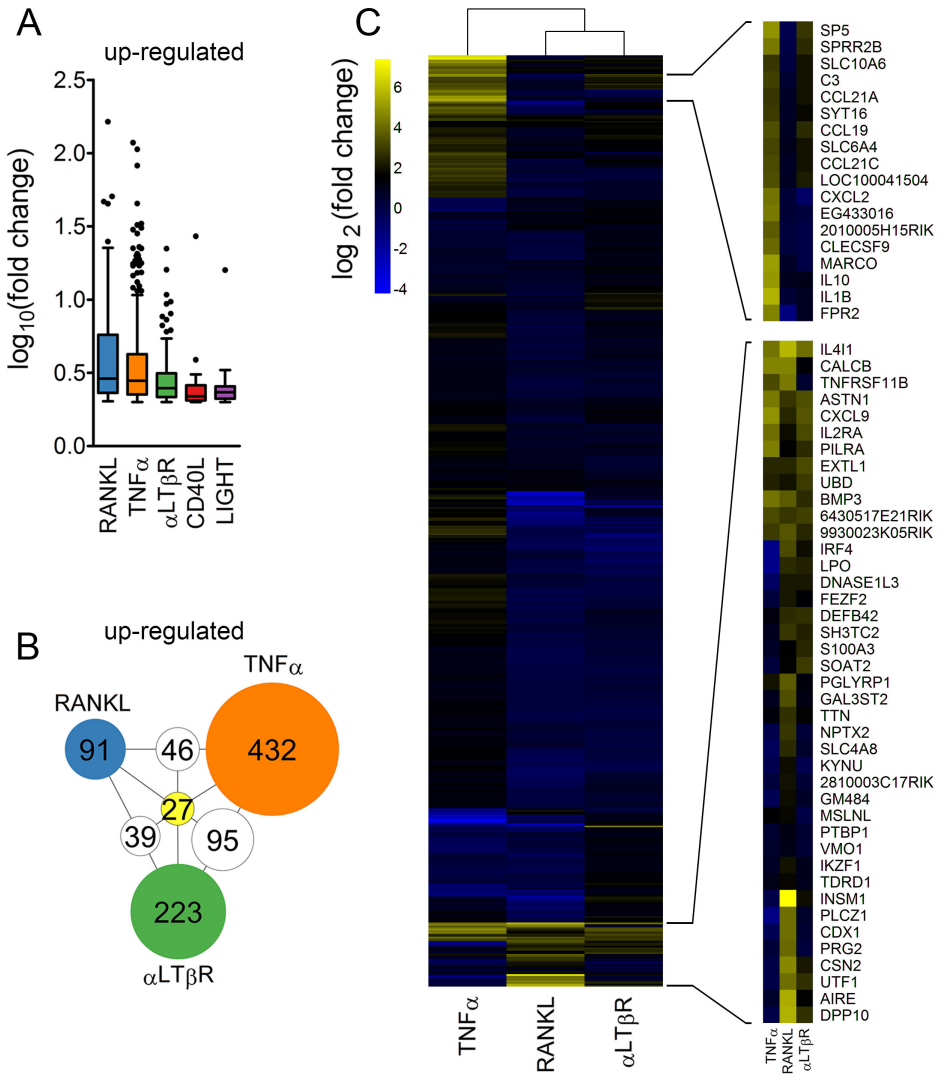

Figure 3. RANKL, TNFα and αLTβR induce gene expression in the thymic stroma. (A) The distribution of gene expression fold change for differentially up-regulated genes showing the median, 1st and 3rd quartiles and whiskers spanning a 1.5 interquartile range. Genes with a fold change falling outside of a given range are represented as separate dots. (B) The overlap of genes differentially expressed following RANKL, TNFα and αLTβR treatment. The numbers of genes induced by stimulations are shown as scaled circles positioned in the outer corners of the triangles. The number of genes co-occurring in given sets is shown in between. (C) A heat map showing genes up-regulated by RANKL, TNFα and αLTβR, with two sections expanded to show gene clusters regulated specifically by one or by multiple TNFSF members. Log2 expression values for each gene are colour coded from blue (lower) to yellow (higher).

When analysing the promoter regions (up to 2000 bp upstream of the transcription start site) of gene sets up-regulated by RANKL, TNFα and αLTβR for common motifs of transcription factor binding sites we could see that, rather predictably, the genes up-regulated by these signals are enriched for various subunits of the NF-κB family (Figure 4). Promoters of genes induced by RANKL were primarily enriched for NF-κB1 (p50) binding sites, whereas TNFα and αLTβR gene sets additionally showed enrichment for RelA (p65) and c-Rel.

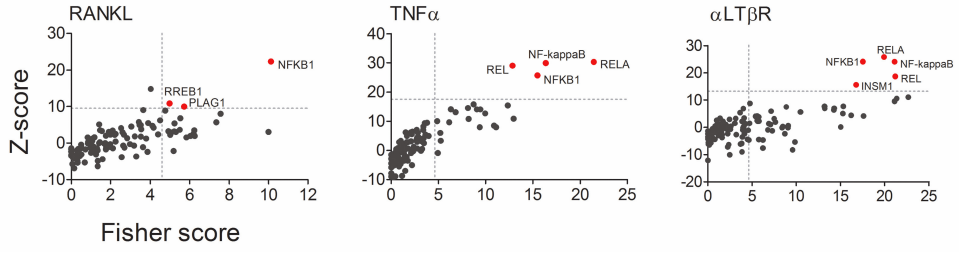

Figure 4. RANKL, TNFα and αLTβR induce NF-κB responsive genes. Analysis of over-represented transcription factor binding sites in the promoter areas (2000 bp upstream of the transcription start site) of genes induced by RANKL, TNFα and αLTβR. Cut-off levels for statistically significant transcription factors are represented by grey dotted lines. Z-score statistical significance: mean + 2 × SD, Fisher score statistical significance: $-\ln(0.01) \approx 4.61$. Transcription factors that have significantly enriched binding sites in the gene sets are visible in the upper right section as red dots.

5.2. TNFα and LTβR induce thymic chemokine and cytokine expression (Study I)

To identify and assess the molecular pathways primarily regulated by RANKL, TNFα and αLTβR, we subjected the up-regulated gene sets to gene profiling software analysis and saw that the primary molecular pathways affected were associated with cytokine-cytokine receptor interactions. In particular, TNFα and αLTβR were the primary inducers of cytokine and chemokine expression, while the effect of RANKL on chemokine expression was rather modest. Among the chemokines induced by TNFα and/or αLTβR were several chemokines that have been shown to play an important role in various steps of thymocyte development (Figure 5). CXCL2 mobilizes hematopoietic progenitor cells from the bone marrow into circulation (Pelus et al., 2002), CXCL9 functions as a homing chemokine to attract mature CXCR3+ T-cells from the periphery to the thymus (Nobrega et al., 2013), CCL19 and CCL21 facilitate thymocyte migration after undergoing positive selection (Misslitz et al., 2004; Ueno et al., 2004, 2002) and CCL5 modulates thymic emigration of T-cells (Kroetz and Deepe, 2011). In addition to chemokines, multiple genes related to interleukin

signalling were also up-regulated (such as *Il1b*, *Il10* and *Il18r1*), suggesting that intrathymic inflammatory processes may be in part regulated by various TNFSF signals.

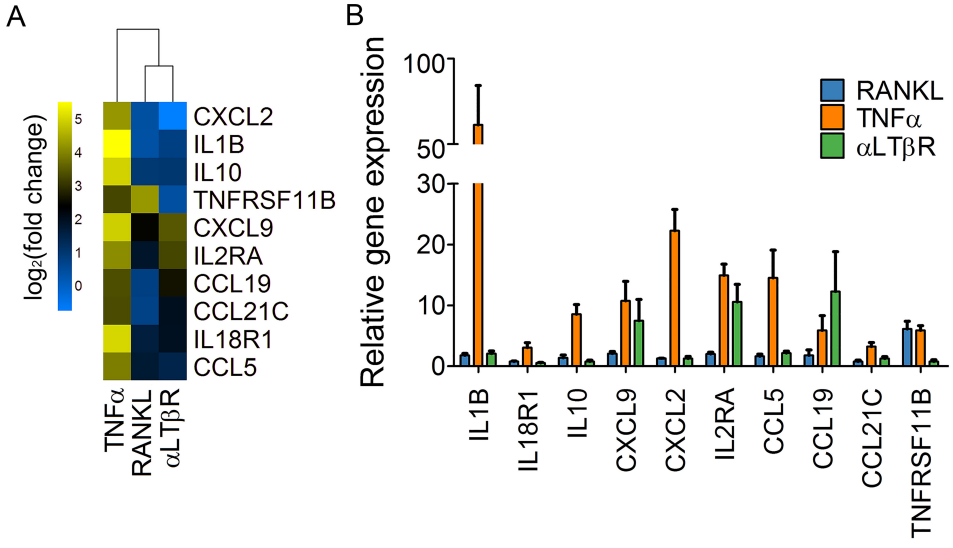

Figure 5. TNFα and αLTβR induce chemokine expression in the thymic stroma. (A) A heat map of the top 10 genes related to cytokine-cytokine receptor interactions, as identified by g:Profiler analysis, and (B) a validation of the expression of these genes by RT-PCR. In (A), log2 expression values for each gene are colour coded from blue (lower) to yellow (higher). The data in (B) are shown as the mean + SEM of at least three replicates, each replicate contains material from 2–3 pooled FTOCs.

5.3. RANK signalling directly and specifically up-regulates Aire expression through the classical NF-κB pathway (Studies I and II)

While there was a significant overlap among the genes induced by RANKL, TNFα and αLTβR, we observed that *Aire* and TSAs were almost exclusively up-regulated following RANKL stimulation. We validated this result using RT-PCR analysis and saw that *Aire* as well as the majority of tested *Aire*-dependent (*Ins2*, *Spt1*, *Mup3*) and *Aire*-independent (*Csn2*, *Tff1*) TSAs were induced only by RANKL and not by other TNFSF ligands, the only exception being *Crp*, an *Aire*-independent TSA, which was also induced by αLtβR (Figure 6).

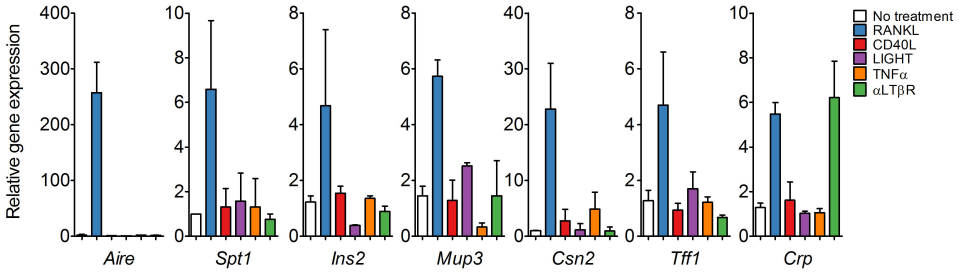

Figure 6. RANKL specifically induces *Aire* and TSA expression in thymic stroma. The relative fold change of *Aire*, as well as *Aire*-dependent (*Spt1*, *Ins2* and *Mup3*) and *Aire*-independent autoantigens (*Csn2*, *Tff1*, *Crp*), after stimulation with TNFSF ligands was measured by RT-PCR. All RT-PCR data are shown as the mean ± SEM of at least three replicates, where each replicate contains material from 2–3 pooled FTOCs.

To assess, whether the changes in *Aire* and TSA expression were a result of RANKL-induced mTEC maturation, we analysed the emergence of $CD80^+$ mTECs following RANKL stimulation at different time points. Following 48h of RANKL treatment, there was only a marginal increase in $CD80^+$ mTECs compared to the substantial mature mTEC population detectable after 96h of RANKL stimulation (Figure 7, A). Additionally, gene expression analysis revealed a clearly detectable increase in *Aire* expression already as early as 6h after RANKL treatment (Figure 7, B), indicating that RANKL-induced *Aire* expression is a direct transcriptional effect, rather than a by-product of gradual mTEC maturation.

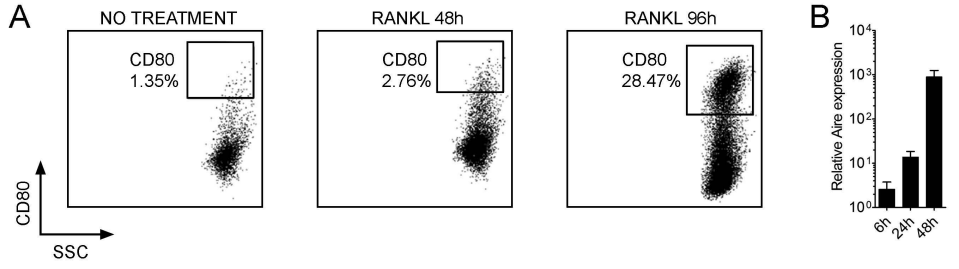

Figure 7. mTEC maturation and *Aire* expression following RANKL stimulation. (A) Percentage of $CD80^+$ mTECs in FTOCs after 48h and 96h of RANKL stimulation was determined by flow cytometry. Plots shown represent data for 8 pooled FTOCs. The no treatment panel shows $CD80^+$ mTEC data at the 96h time point, but is equally representative of 48h. (B) Relative Aire expression at 6 h, 24 h and 48 h after RANKL stimulation measured by RT-PCR. All RT-PCR data are shown as the mean ± SEM of at least three replicates, where each replicate contains material from 2–3 pooled FTOCs.

As RANK-RANKL interactions lead to the intracellular activation of NF-κB, we decided to test, whether the signals for RANKL-induced *Aire* expression are transmitted through the canonical or alternative NF-κB pathway. We treated

2'dG FTOCs with RANKL in the presence of TPCA1, a canonical NF-κB pathway inhibitor with high specificity for IKKβ over IKKα, or a specific inhibitor for NIK, which blocks signalling through the alternative NK-κB pathway. We observed a clear, dose-dependent decrease in RANKL-induced *Aire* expression in FTOCs treated with TPCA1, whereas inhibition of NIK did not appear to have any significant effect on *Aire* expression (Figure 8, A). We therefore concluded that RANKL-induced *Aire* expression is dependent on canonical NF-κB signalling.

Since upstream of the *Aire* gene lies a conserved 90 bp sequence identified as CNS1 (Blechschmidt et al., 1999), which contains two well-conserved 10 bp motifs matching known NF-κB binding sites, we wanted to determine, whether this DNA region could play a part in RANKL-induced *Aire* expression. We stimulated 2'dG FTOCs derived from mice deficient for the NF-κB binding sites in the CNS1 region (CNS1 KO mice) with RANKL and compared the resulting *Aire* induction to WT littermates. Strikingly, RANKL stimulation did not induce *Aire* expression in CNS1 KO FTOCs (Figure 8, B), suggesting this NF-κB binding site containing region plays a crucial role in RANKL-induced *Aire* expression in the thymus.

Together with additional data published in Study II, this supports the notion that *Aire* expression in the thymus is dependent on signalling through the RANK-RANKL interaction, which acts through the canonical NF-κB pathway to directly induce *Aire* expression via the CNS1 region located upstream of the *Aire* gene.

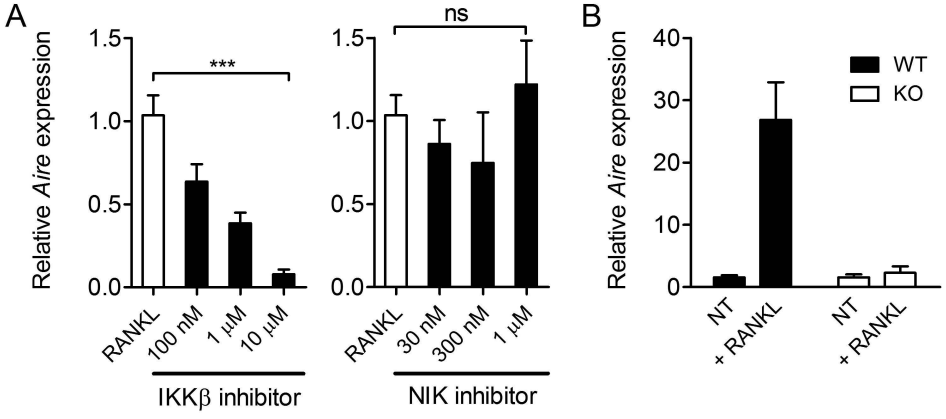

Figure 8. (A) Relative *Aire* mRNA expression in WT 2'dG FTOCs stimulated for 24 h with RANKL or a combination of RANKL and inhibitors with high specificity for IKK-β (left) or NIK (right) at indicated concentrations. Data are shown as the mean + SEM of three to five replicates with material from two pooled thymic lobes making up one sample. ***$p \leq 0.001$, unpaired t-test, two-tailed; ns = not significant. (B) Relative *Aire* mRNA expression in WT and CNS1-KO thymi following FTOC stimulation with RANKL for 24 h. For each embryo, the expression of *Aire* was compared between the untreated and RANKL-treated lobe of the same thymus. Data are shown as the mean + SEM of three replicates. NT, no treatment control.

5.4. *Aire*-deficiency results in defective mTEC late-stage maturation, which is partially rescued by RANKL and CD40L (Study III)

Hassall's corpuscles are believed to contain terminally differentiated mTECs and are known to stain for markers associated with differentiated cells of the epidermis (Bodey et al., 2000; Hale and Markert, 2004; Nuber et al., 1996). In order to study the possible relation between *Aire*-expressing mTECs and HCs, we first compared end-stage mTEC and HC formation in the thymus to keratinocyte maturation in the skin. We looked at KRT6, KRT10, SPINK5 and IVL expression in mouse skin and the thymus and saw a previously described correlation between the expression pattern observed in the skin compared to the thymus – KRT6 and 10 are expressed at earlier stages of keratinocyte development (KRT6 in the stratum basale, stratum spinosum and stratum granulosum, KRT10 in the stratum spinosum, stratum granulosum and stratum corneum). SPINK5 expression was restricted to the stratum granulosum and IVL was expressed in the stratum granulosum as well as the stratum corneum. In the murine thymus, KRT6 and 10 were expressed by a relatively large number on mTEC-like cells as well as the outer layers of HCs (KRT10 also stained the inner layers of HCs). SPINK5 and IVL were, on the other hand, never expressed in isolated thymic cells, being clearly restricted to HCs (Figure 9). We therefore concluded, that epidermal maturation markers can indeed be used to identify and characterize end-stage mTEC populations as well as HCs in the murine thymus and we used these markers in our further study.

In addition to inducing TSA expression, *Aire* is implicated to have a role in the broader mTEC maturation process, as *Aire*-deficient mice are reported to lack IVL positive HCs (Yano et al., 2008). To further characterize and understand the developmental block seen in Aire KO mice, we decided to determine the expression of HC markers in WT, Aire heterozygous and Aire KO mice. Aire KO mice indeed had a clearly reduced expression level of CK6, CK10, LEKTI and IVL in the thymus along with a reduced number of CK6 and IVL positive cells/HCs (Figure 10, A). There were, however, still detectable numbers of IVL positive HCs in the thymi of Aire KO mice, despite their number being greatly reduced.

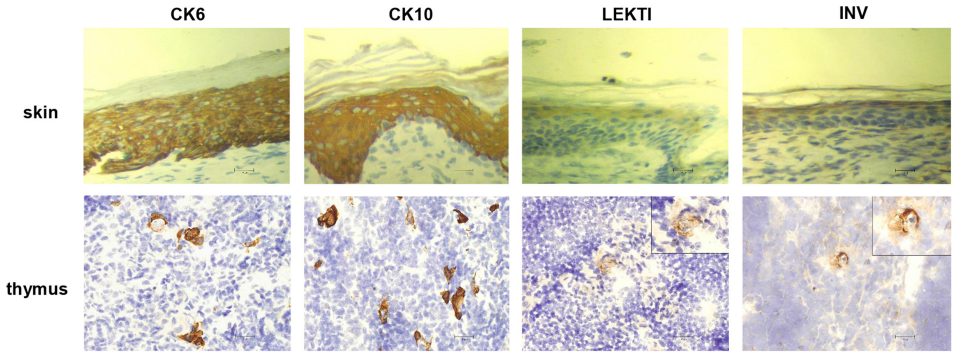

Figure 9. Expression of epidermal differentiation markers KRT, KRT10, SPINK5, and IVL in thymi and skin of WT mice stained with immunohistochemistry. CK6 reacted intensely with the stratum basale, the stratum spinosum and the stratum granulosum of skin epidermis. In the thymus, CK6 stained the outer layers of HCs and some medullary thymic epithelial-like cells. CK10 showed intense reaction with the stratum spinosum layer, the stratum granulosum and the stratum corneum of skin epidermis. In the thymus, CK10 strongly stained the outer layers of HCs in the medulla as well as giving a weaker staining in the central portion of HCs and some medullary thymic epithelial-like cells. LEKTI stained the stratum granulosum of skin epidermis and localized in the outer layers of HCs in the thymus. Involucrin mainly stained the stratum granulosum and also gave minimal staining in the stratum corneum of skin epidermis. In the thymus, involucrin stained the outer layer of HCs. Shown are representative stainings of at least three experiments.

Since the formation of HCs has been associated with TNFSF signalling coming from positively selected thymocytes (White et al., 2010), we decided to assess, whether this was also an *Aire*-dependent process. We stimulated thymic tissue from 4–6 week old Aire KO mice with RANKL, CD40L and LIGHT and saw that RANKL and CD40L increased IVL levels at both the mRNA as well as protein levels (Figure 10, B), suggesting that the induction of HC formation is not directly dependent on *Aire*.

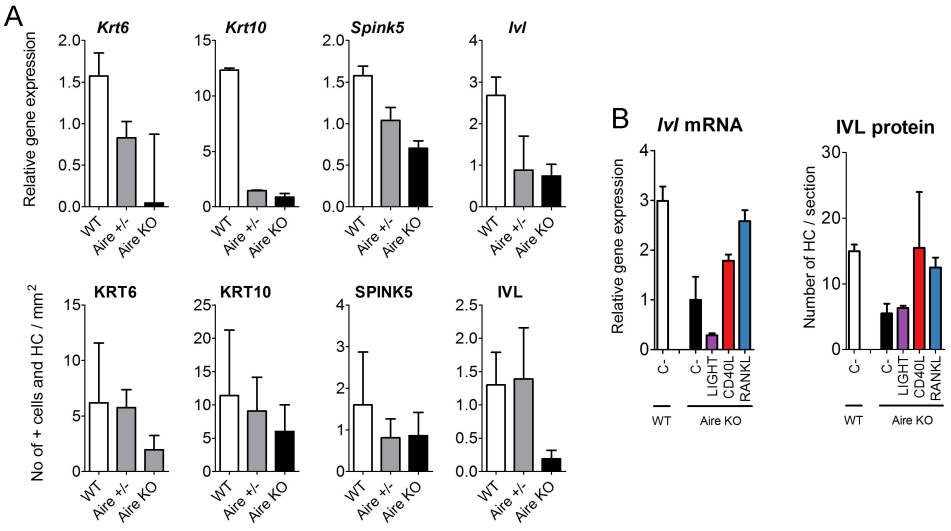

Figure 10. (A) Relative gene and protein expression of epidermal differentiation markers in the thymus of wild-type (WT), heterozygous (Aire +/−) and Aire KO mice. Whole thymi were collected for RNA purification and analysed by RT-PCR or stained for KRT6, KRT10, SPINK5 and IVL. In the Aire KO mouse, there was a statistically significant reduction of all four epidermal differentiation markers at the mRNA level and of KRT6 and IVL at the protein level compared to WT ($p < 0.05$, unpaired two-tailed t-test), differences in KRT10 and SPINK5 did not reach statistical significance. (B) Relative gene and protein expression of involucrin in Aire KO thymus after treatment with LIGHT, RANKL, or CD40L. Thymic tissue from Aire KO or WT mice was treated *ex vivo* for 24 (for mRNA) or 48h (protein) and analysed thereafter for involucrin expression by RT-PCR or immunofluorescence. All values are mean±SEM of triplicate experiments.

5.5. Post-Aire mTECs lose promiscuous gene expression and start expressing pemphigus vulgaris-related TSAs desmoglein 1 and 3 during their keratinization (Study III)

Aire is expressed only in a relatively short window during mTEC maturation, after which not much is known about the so called post-*Aire* mTEC population. To further characterize this population of thymic cells, we used the *Aire*$^{+/-}$ *lacZ* reporter mice, which express *lacZ* from the endogenous *Aire* promoter on the disrupted copy of the gene, thus making it possible to track the lineage of *Aire*-expressing cells for a while after they have stopped expressing *Aire*. To analyse gene expression patterns in different stages of mTEC development, we sorted out the following three cell populations: (1) *lacZ*$^-$ MHCII^{hi} mTECs known to express *Aire*, (2) *lacZ*$^-$MHCII^{lo} mTECs not expressing *Aire* and (3) post-Aire *lacZ*$^+$ mTECs.

As expected, *Aire* and TSA expression was highest in the *Aire*-expressing mTEC^{hi} cell population, which also exhibited the highest expression of MHCII (as measured by the expression of *H2-Aa*) and *Cd80* needed for antigen presentation (Figure 11). However, post-*Aire* cells had lost their expression of *Cd80* and *H2-Aa* along with both *Aire*-dependent and *Aire*-independent TSAs (with the exception of *Tff3*). The only genes showing persistent or even higher expression were related to keratinization (*Krt8*, *Krt10*, *Spink5*).

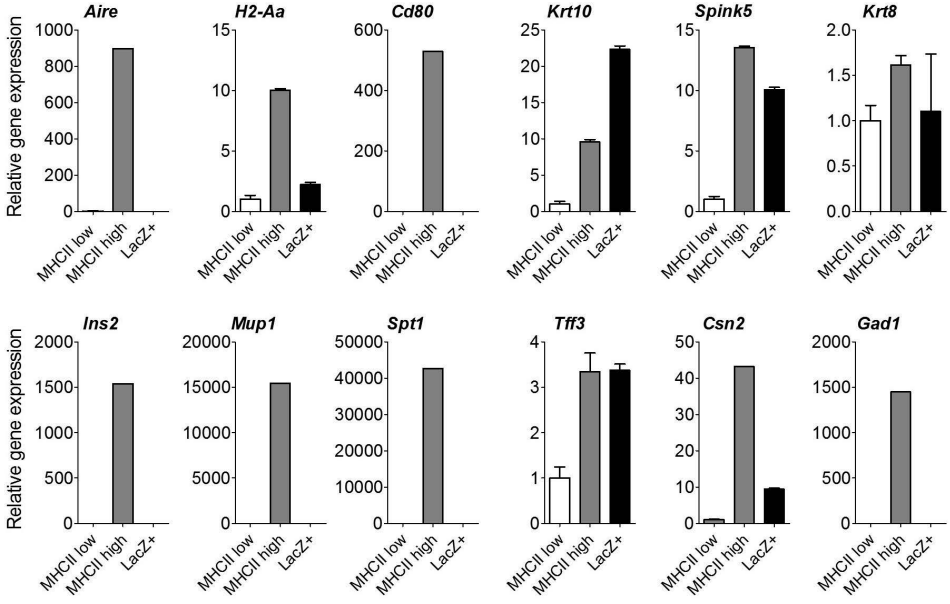

Figure 11. Gene expression of *Aire*, selected genes associated with antigen presentation (*H2-Aa*, *Cd80*), epidermal markers (*Krt8*, *Krt10*, *Spink5*), *Aire*-dependent (*Ins2*, *Mup1*, *Spt1*, *Tff3*) and *Aire*-independent TSAs (*Gad1*, *Csn2*) in the post-*Aire* EpCAM^{+} LacZ^{+}mTECs compared with the EpCAM^{+}LacZ^{-}MHCII^{lo} and EpCAM^{+}LacZ^{-}MHCII^{hi} mTECs as measured by RT-PCR. Data are mean±SEM of triplicate measurement of pooled samples from 15 mice

Seeing how the thymic post-*Aire* cell population specifically expresses genes that are characteristic of keratinocytes and despite the general loss of promiscuous gene expression, some TSA expression capability still remains in these cells, we decided to evaluate, whether epidermis-specific autoantigens could be expressed in this population of mTECs. When we stained thymic sections for pemphigus vulgaris associated TSAs desmoglein 1 (DSG1) and DSG3 (Wada et al., 2011), we could see that these proteins were expressed exclusively by HCs and post-*Aire* cells (Figure 12). Seeing how this post-*Aire* mTEC population appears to lose the ability to independently present antigens to developing thymocytes due to the progressive loss of MHCII and CD80, we analysed whether cross-presentation could be a viable option for these TSAs to be

presented to developing thymocytes. When we co-stained thymic sections for HCs and CD11c/LAMP1, both markers for dendritic cells, we could indeed see that post-*Aire* mTECs were often in close proximity to thymic dendritic cells, which supports the notion that TSAs expressed by terminally differentiated mTECs could be presented to thymocytes via cross-presentation by other antigen-presenting cells.

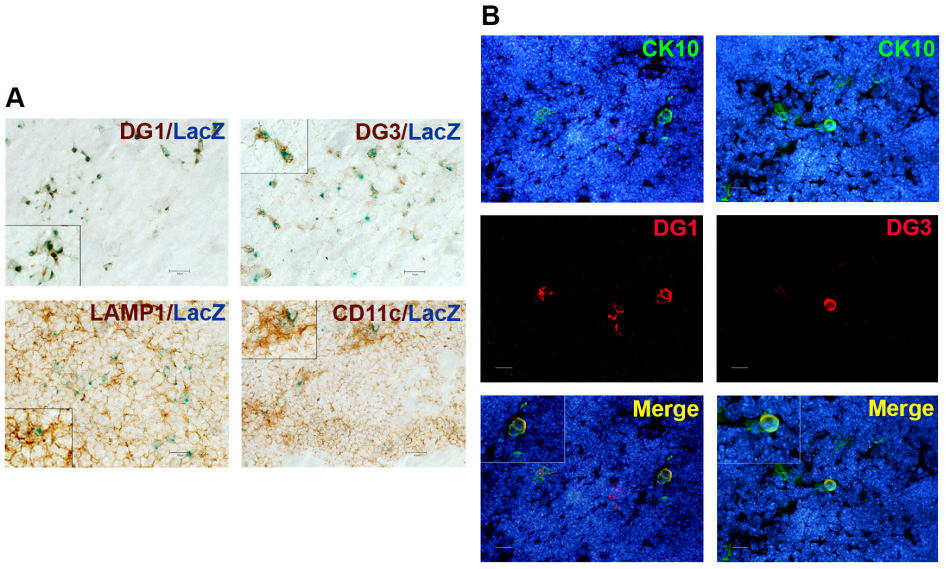

Figure 12. Co-localization of post-Aire mTECs and HCs with epidermal autoantigens and APC markers. (A) Thymi from 4–6 week old Aire+/− mice stained for LacZ, DG-1, DG-3, LAMP-1, and CD11c. (B) Thymi from 5–6 week old Aire+/− mice stained for HCs with CK10 and DG-1, DG-3. DAPI was used for nuclear staining. Figures are representatives of at least three experiments.

5.6. Aged *Aire*-deficient mice do not display increased susceptibility to oral candidiasis (Study IV)

As discussed above, *AIRE* deficiency in humans leads to APECED, typical symptoms of which include infections by *Candida albicans*, which are in correlation with various cytokine-specific autoantibodies in the patients' sera (Kahaly, 2009). Albeit *Aire*-deficient mice appear to be unsusceptible to spontaneous *Candida* infections, it has been shown, that blocking IL-17A in WT mice leads to susceptibility to *Candida* infections. It would thus be reasonable to assess, whether the naturally occurring autoantibody background (predominantly against IL-17A) that develop in aged *Aire*-deficient mice leads to similar symptoms. We therefore infected aged (>1.5 years of age) *Aire*-deficient BALB/c mice with *C. albicans* and assessed the fungal loads in the oral mucosa

of these animals after 6 days, the time it takes for WT mice to fully clear OPC. What we saw was that despite all testing positive for IL-17A neutralizing antibodies (Figure 13, A), the Aire-deficient mice showed no symptoms of weight loss (Figure 13, B) and with the exception of one mouse, had no fungal outgrowth from their tongue tissue (Figure 13, C).

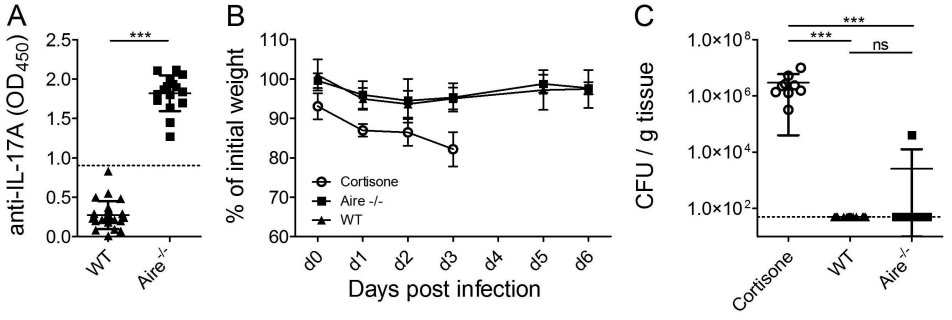

Figure 13. The impact of Aire-deficiency on the susceptibility for OPC. (A) Autoantibodies binding to IL-17A quantified from mouse serum samples using ELISA. Data are representative of tests performed three times in two replicates. (B) Control mice (n = 5) were treated with cortisone at day −1, 1 and 3, and were subjected to OPC at day 0 together with aged *Aire*-deficient mice (n = 16) and their wild-type littermates (n = 22). Weight loss was calculated daily. (C) Tongues were obtained on day 3–4 (cortisone-treated mice) or 6 for fungal burden enumeration. Dotted line indicates limit of detection (50 CFU/g). In B and C, data were pooled from three independent experiments with 13–15 mice per experiment. Mean and SD are depicted in the graphs. Unpaired two-tailed t-test (A, C) and Dunn's multiple comparison test (B) were used. ***$p < 0.001$.

In addition to that we also measured Th17 cytokine related gene expression in the tongues of mice that had been subjected to oral *Candida* infection six days prior and saw no significant differences between WT and *Aire*-deficient mice (Figure 14), suggesting that aged *Aire*-deficient mice have successfully cleared the fungal infection by that point to the same extent as their WT counterparts. Thus, it appears that the naturally occurring autoantibodies against IL-17A are probably not enough to bring about susceptibility to OPC in mice. Since among APECED patients the prevalence of IL-17A neutralizing autoantibodies is significantly lower than that of CMC (Kisand et al., 2010) is safe to assume that the naturally occurring autoantibodies against IL-17A cannot be the only factor driving CMC pathogenesis in the human setting.

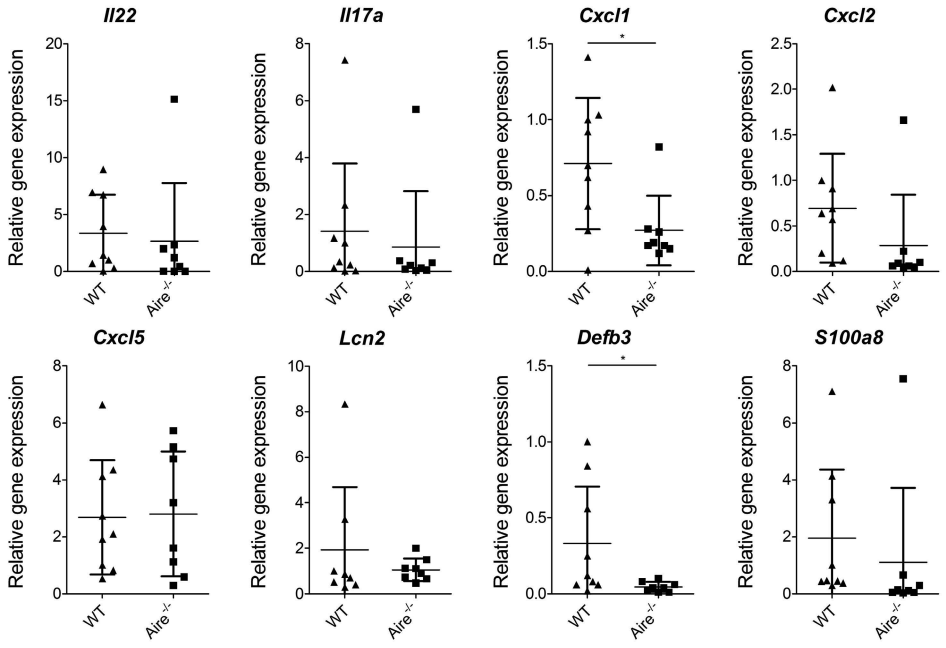

Figure 14. Th17 cytokine related gene expression in tongue tissue at day 6 after oropharyngeal *C. albicans* infection. *Aire*-deficient (BALB/c, n = 8, >1.5 years of age) and wild-type littermates (n = 9) mice were compared for *Il22* and *Il17a*, and their downstream gene expression relative to *B2m* with the comparative Ct (ΔΔCt) method. Data are pooled from four different experiments with 8–18 mice per experiment. Mean and SD are depicted in the graphs. Two-tailed unpaired t-test was used. *$p < 0.05$.

5.7. Blocking IL-22 delays mucosal fungal clearance in mice (Study IV)

In APECED patients, CMC is closely correlated with the presence of autoantibodies against IL-17F and IL-22, but the pathogenic potential of these antibodies is not yet known. Since *Aire*-deficient mice do not develop antibodies against either of these cytokines (Kärner et al., 2012) and IL-17F neutralizing antibodies derived from APECED patients proved to be exclusively human-specific, we used an APECED patient derived monoclonal antibody (30G1) that cross-reacts with murine IL-22 (Meyer et al., 2016) in order to study the effect blocking IL-22 may have on clearing oral mucosal *Candida* in mice.

We treated WT BALB/c mice with 30G1 or control IgG one day prior to *Candida* infection and following that on days 1, 3 and 5 post infection and at the end of the experiment, IL-22 binding activity could readily be detected in the sera of 30G1 treated mice (Figure 15, A) at levels comparable to those seen in APECED patients. Nevertheless, the body weight fluctuations were indistinguishable from control antibody treated animals and no visible signs of infection

could be seen on the oral mucosa on day 6. However, when analysing the presence of *Candida* in the tongue tissue, 30G1 treated mice showed an elevated fungal load (53% of 30G1 treated mice had *Candida* levels above the lower limit of detection).

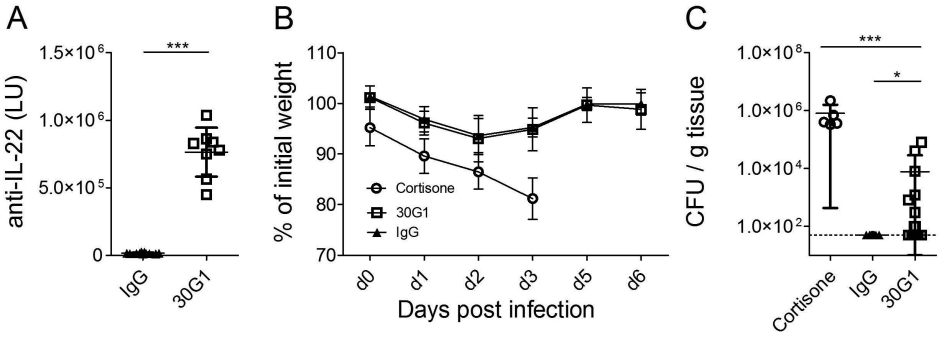

Figure 15. (A) Intra-peritoneal injection of 30G1 at days −1, 1, 3 and 5 resulted in measurable anti-IL-22 activity in mouse serum samples at day 6 as measured using luciferase based immunoprecipitation systems. (B) Weight loss after *C. albicans* infection was recorded in cortisone (n = 5), 30G1 (n = 17) or IgG (n = 15) treated mice. (C) Tongues were obtained on day 3–4 (cortisone-treated mice) or 6 for fungal burden enumeration. Dotted line indicates LOD (50 CFU/g). Data are pooled from three independent experiments with 8–18 mice per experiment. Mean and SD are depicted in the graphs. *p < 0.05, ***p < 0.001 determined by unpaired two-tailed t-test (A) or Dunn's multiple comparison test (C).

Additionally to the elevated fungal loads, anti-IL-22 treated mice had elevated expression of *Il17a*, *Il22*, *Cxcl1*, *Cxcl2*, *Cxcl5*, *Defb3*, *Lcn2*, and *S100a8* in their tongue tissue (Figure 16), clearly indicating a prolonged effort to keep the infection in check due to higher activation of Th17 response related genes.

All in all, these results indicate that patient-derived antibodies, which neutralize IL-22 *in vivo*, are capable of increasing the susceptibility to OPC, despite not being able to directly lead to an infectious pathological condition in mice.

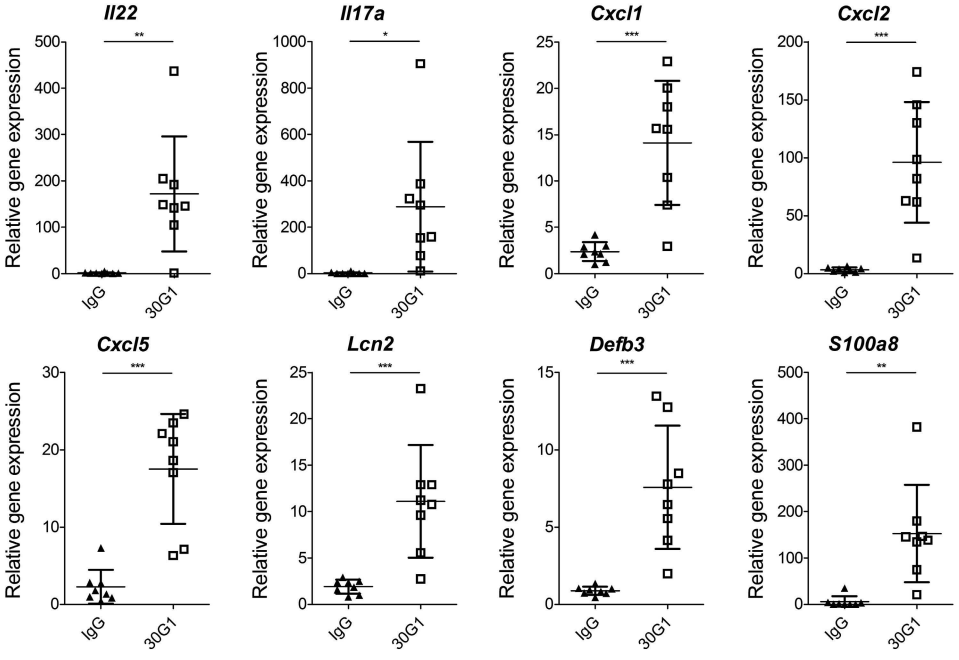

Figure 16. Th17 cytokine related gene expression in tongue tissue at day 6 after oropharyngeal *C. albicans* infection in mice treated with anti-IL-22 antibody (30G1) or IgG control. 30G1 (n = 8) and IgG (n = 8) treated mice were compared for *Il22* and *Il17a*, and their downstream gene expression relative to *B2m* with the comparative Ct (ΔΔCt) method. Data are pooled from four different experiments with 8–18 mice per experiment. Mean and SD are depicted in the graphs. Two-tailed unpaired t-test was used. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

6. DISCUSSION

6.1. RANK and LTβR signalling are key regulators of gene expression in the embryonic thymus

The importance of TNFSF signalling in the thymus has been demonstrated in a number of scientific works, which have studied mice deficient for various receptors and ligands of this signalling network. Nevertheless, a comprehensive analysis on the transcriptional effects these signals have on the thymic stroma was rather lacking. We therefore set out to study the specific roles various TNFSF receptor-ligand interactions have in the embryonic thymic tissue of mice.

What we could see was that RANKL, αLTβR as well as TNFα all worked as potent inducers of gene expression in thymic organ cultures. Additionally, we tested the effects of CD40L and LIGHT, but these ligands brought about only minor changes in the gene expression pattern. This is, for the most part, in relatively good correlation with the respective thymic phenotypes of mice deficient for components of these receptor-ligand pairs. The most surprising is perhaps the discrepancy between the very potent gene expression induction potential of TNFα and the apparently normal thymic architecture observed in TNFα KO mice (Grech et al., 2000). This leads one to believe that while potentially potent, TNFα signalling plays little, if any persistent role in shaping the thymic microenvironment. Alternatively, the deficiency in TNFα signalling could be compensated by LTα, which can bind the same TNF receptors in the form of a homotrimer (Calmon-Hamaty et al., 2011), thus suggesting that it would be potentially interesting to study the thymic phenotype of mice deficient for both TNFα and LTα.

It should also be pointed out that the likely reason behind the very weak transcriptional effect observed following CD40L treatment stems from the use of embryonic tissue. Even though we can detect CD40 expression in FTOCs by RT-PCR, the expression is considerably lower compared to postnatal thymic tissue. Nevertheless, the same can be said of RANK as well as TNFR1/TNFR2, which both seem to successfully facilitate signal transduction. CD40-CD40L signalling has been shown to gain more importance postnatally (Dunn et al., 1997), so it is possible that at this developmental stage functional receptor complexes along with the complete intracellular signal transduction network are not yet fully established.

The fact that there is a considerable overlap of genes induced by different ligands might help to explain the much more severe phenotype that is seen in mice deficient for multiple TNFSF signalling pathways compared to their single KO counterparts, such as the RANKL-LTβR and RANKL-CD40 double KO mice (Akiyama et al., 2008; Mouri et al., 2011). The deficiency of one of the central signals can partly be compensated by another, which speaks for a partial redundancy in TNFSF signalling in the thymus. This is also supported by the results of co-stimulation experiments with multiple TNFSF ligands (performed

in Study I), which show a clearly more pronounced effect on gene expression when treating FTOCs with αLTβR and RANKL cooperatively.

Given our gene expression analysis results and considering the phenotypic severity of animals deficient for these signalling pathways, LTβR and RANK are most likely the two central TNFSF signals that have the most profound effect on the early development and formation of the thymic microenvironment.

6.2. *Aire* expression in the embryonic thymus is dependent on RANKL

When discussing mTEC functionality, one cannot overlook *Aire*, as it is one of the key genes characteristic of this cell type. What we could clearly see was that only RANK signalling was capable of inducing *Aire* up-regulation. RANK signalling is a well-known factor driving the formation of *Aire*-expressing mTECs (Akiyama et al., 2008; Hikosaka et al., 2008; Rossi et al., 2007), but whether this is down to the natural maturation process of mTECs, or a direct transcriptional activation, had not been properly explored.

Based on the results of our group as well as other studies discussed in detail above, it appears that the effect of RANKL on mTECs is two-fold. One important function of RANKL is to drive the mTEC maturation process to induce the formation of $CD80^{hi}$ mTECs. mTEC precursors can be defined by them acquiring the expression of RANK, which then allows them to differentiate further along the mTEC lineage (Akiyama et al., 2016). Nevertheless, the process of mature $mTEC^{hi}$ cells differentiating from the immature $mTEC^{lo}$ populations takes a considerable amount of time. At 48h of RANKL stimulation, only a marginal difference can be observed in the emergence of $mTEC^{hi}$ cells in 2'dG treated FTOCs and it takes another 1–2 days for there to be a properly distinct population of these mature mTECs. Interestingly, we did not detect the induction of terminal differentiation markers such as *Krt10* and *Ivl* after 48h of RANKL stimulation in embryonic thymi, unlike in adult mouse thymic tissue where changes in mRNA of *Ivl* can already be detected after 24h of RANKL treatment (Study III), suggesting that the transcriptional activation of terminal differentiation markers might be differently regulated in the pre- and postnatal thymus.

The second function of RANKL appears to be the direct transcriptional activation of *Aire*. Compared to mTEC maturation in organ cultures, the induction of *Aire* mRNA expression is a much faster process, which can be detected within the first 6 hours of RANKL stimulation in thymocyte-depleted embryonic thymi. This clearly suggests that the induction of *Aire* and mTEC maturation into $mTEC^{hi}$ cells are two separately regulated events or at least that the sequence in which these events take place is not suggestive of *Aire* expression being strictly dependent on CD80 expression by mTECs.

Despite being the only signal capable of inducing *Aire* expression in the embryonic thymus, evidence suggests that postnatally, other TNFSF ligands such as CD40L are also capable of inducing *Aire* expression, albeit to a lesser extent. This is supported by the fact that in RANK/RANKL-deficient mice, some *Aire*-positive cells can still be detected after birth (Akiyama et al., 2008; Rossi et al., 2007), but in RANKL-CD40 double KO animals, there are effectively no AIRE-positive mTECs even in the adult thymus (Akiyama et al., 2008). Additionally, *Aire* expression has been described in thymic B cells, where it is induced by CD40 signalling through interactions with autoreactive $CD4^+$ thymocytes (Yamano et al., 2015). Thus, there appears to be more flexibility in *Aire* regulation postnatally compared to the early stages of thymic development.

One key aspect of mature, functional mTECs is their ability to express a uniquely large amount of different otherwise tissue specific antigens for presentation to developing thymocytes during negative selection. A large number of those TSAs is considered to be dependent on *Aire* (Anderson et al., 2002), whereas some are expressed in an *Aire*-independent manner and are, at least in part, regulated by other "master regulators" such as *Fezf2* (Takaba et al., 2015). We could clearly see that *Aire*-dependent TSAs were induced only by RANKL, fully correlating with the fact that RANKL was the only TNFSF signal capable of inducing *Aire* expression. *Aire*-independent TSAs were also predominantly induced by RANK signalling, albeit αLTβR was also capable of inducing some *Aire*-independent TSA expression (as well as *Fezf2*), which again suggests there is a partial redundancy between RANK and LTβR signalling in the thymus. Nevertheless, it has been shown that while LTβR influences the development and organization of the thymic medulla, it is RANK signalling in particular, which acts as a master regulator of mTEC development, including the emergence of $Fezf2^+$ mTECs (Cosway et al., 2017).

6.3. Thymic chemokines are not under direct control of *Aire* and RANK signalling

Normal thymic functions are strongly dependent on the expression of multiple thymic chemokines (Lancaster et al., 2018), which have also been shown to be substantially dependent on *Aire*. In *Aire*-deficient mice, the level of several CCR4 and CCR7 ligands is clearly disrupted (Laan et al., 2009) but interestingly, despite being a strong inducer of *Aire* expression, RANKL appears to have a very modest direct effect on thymic chemokine expression. We detected only minor changes in the expression of key thymic chemokines following RANKL stimulation. Instead, LTβR signalling (and in our system especially TNFα) were much more potent inducers of chemokine expression. LTα, a ligand which binds the same receptors as TNFα, has also recently been shown to regulate the expression of thymic chemokines controlling the entry of APCs

into the thymus (Lopes et al., 2018), suggesting that TNFR-mediated signalling plays an important role in regulating the thymic microenvironment. Among the top ten most strongly induced cytokines and chemokines were CXCL9 and CCL19, up-regulated by both αLTβR and TNFα and being important factors for thymocyte homing (Nobrega et al., 2013) and intrathymic migration respectively (Misslitz et al., 2004; Ueno et al., 2004, 2002). This is in line with previous research, as CCL19 has been reported to be down-regulated in mice deficient for LTβ signalling (Lucas et al., 2016; Seach et al., 2010). Seeing how there appears to be a rather weak induction of chemokine expression by RANKL, the thymic chemokine expression dependence on *Aire* could therefore be more suggestive of it being related to different developmental stages of mTECs, as *Aire* deficiency leads to a broader disruption in mTEC maturation in general (discussed below).

6.4. RANKL induces *Aire* expression through the canonical NF-κB pathway

The transduction of signals received through TNF superfamily receptors continues through a complex intracellular signalling network, one branch of which ends in the activation of different NF-κB subunits through either the canonical or alternative pathways and subsequent transcriptional activation of target genes (reviewed in Aggarwal et al., 2012).

When it comes to studies regarding which of these two NF-κB pathways is directly responsible for regulating *Aire* expression in the thymus, there appears to be evidence both ways. KO mouse models of various components of the canonical and alternative NF-κB pathways suggest that both are needed for proper mTEC maturation, as just about all of them result in disrupted thymic architecture and/or a reduction in the number of mTECs to a varying degree (reviewed in Irla et al., 2010). A recent study has confirmed this by showing that there is a mechanism whereby the canonical and alternative NF-κB pathways regulate the development of mTECs through molecular crosstalk, where RelA and c-Rel, both activated by the canonical pathway, go on to induce RelB, which in turn is vital for the development of mature mTECs (Riemann et al., 2017). Nevertheless, the direct effect of either pathway on *Aire* expression is not as straightforward.

Since mTECs express *Aire* only during a specific stage of their life cycle, it may very well be that the early block in the mTEC maturation process brought about by defective NF-κB signalling does not allow the emergence of mTECs capable of expressing *Aire* in the first place. If this were the case, deficiencies in NF-κB pathway components resulting in, among other things, disrupted *Aire* expression might not be suggestive of a direct cause and effect but rather a more indirect mechanism arising from defective mTEC maturation. Therefore,

exploring other possibilities to determine the direct links between different NF-κB pathways and *Aire* expression should be prioritized.

Seeing how, in our experiments, we can clearly observe RANKL-dependent direct transcriptional activation of *Aire* expression, we decided to assess the contribution of different NF-κB pathways to this effect. What we could see was a very clear reduction in RANKL-induced *Aire* expression in the presence of inhibitors for the canonical NF-κB pathway by blocking IKKβ, whereas direct inhibition of the alternative pathway through blocking NIK did not lead to a visible decrease in *Aire* expression. In addition to that, in Study II we characterized a conserved cis-regulatory element, CNS1, upstream of the *Aire* gene which contains two NF-κB binding sites and appears to have a central role in regulating *Aire* expression, as CNS1-deficient mice almost exactly mimic the phenotype seen in their *Aire*-deficient counterparts and RANKL stimulation of CNS1 KO mouse thymi completely failed to induce *Aire* expression. Additional experiments showed that it is predominantly components of the canonical NF-κB pathway (RelA, c-Rel and p50) that bind to and facilitate transcription from this region. RelB and NF-κB2, both alternative NF-κB pathway components, had a much weaker, albeit observable, effect. An analogous study, on the other hand, concluded that NF-κB2 and RelB are factors which activate transcription through this element (LaFlam et al., 2015). However, since no testing of the transcriptional activation potential of canonical NF-κB pathway components was carried out in that study, it is difficult to compare the two studies. Nevertheless, we hypothesize, that *Aire* expression in mTECs is directly induced by RANK-RANKL signalling, acting through the canonical NF-κB pathway, which activates transcription through the CNS1 regulatory sequence upstream of the *Aire* gene.

6.5. Hassall's corpuscles are a natural final step in mTEC maturation

Despite being derived from different germ layers (the endoderm and ectoderm respectively), thymic epithelial cells and keratinocytes follow a surprisingly similar path of maturation when it comes to their expression of certain genes. Various terminal differentiation markers (such as KRT6, KRT10, SPINK5 and IVL) correlate well with different developmental stages of these cells and can thus be used to track the progressive end-stage maturation of keratinocytes and mTECs alike.

Thymic epithelial cells are known to have a relatively quick turnover period of 2–3 weeks (Gäbler et al., 2007). During their maturation process they actively proliferate (Michel et al., 2018), after which their fate is not completely understood. One idea, that has been floated, is that terminally differentiated mTECs end up in thymic structures known as Hassall's corpuscles, which is what we also investigated in our study. Assuming a similar differentiation

pattern of keratinocytes and mTECs, we would expect both to start out as single cells and follow a path towards a stratified structure. This can in fact be observed in the cornified epithelium as well as in the thymus, since judging by the expression pattern of the aforementioned maturation markers, mTECs appear to follow a similar path as they start out as single cells and during their end-stage maturation localize to the outer layer of HCs and eventually to the inner part of HCs. The eventual clearance of these terminally differentiated mTECs appears to be facilitated by antigen presenting cells such as thymic dendritic cells (Nishikawa et al., 2010), something we also observe in our study.

By using different mouse models that allow fate mapping of mTECs, which have at some point expressed *Aire*, we were able to determine that HCs are predominantly composed of these post-*Aire* cells. By analysing different maturation stages of mTECs we could observe that after they lose *Aire* expression, mTECs also subsequently lose their expression of most TSAs (both *Aire*-dependent and -independent) as well as their ability to present these antigens due to down-regulated expression of MHC II and CD80. It is worth mentioning though, that more work needs to be done to fully characterize the function of HCs and understand the changes mTECs undergo in their final differentiation stages. Since HCs consist of anuclear cells, it is hard, if not impossible to accurately determine the expression of genes via conventional methods such as PCR. Instead, further studies should focus on the proteomic analysis of different stages of mTECs all the way up to HCs in order to better understand the underlying processes.

One aspect that still remains somewhat unclear is the exact role *Aire* plays in steering the mTEC maturation process. The majority of mTECs express AIRE during their differentiation (Kawano et al., 2015) and the lack of AIRE clearly hinders the normal developmental process of mTECs, as indicated by the abnormally increased numbers of $CD80^{hi}$ mTECs seen in *Aire*-deficient mice (Nishikawa et al., 2014) as well as substantially reduced level of end-stage mTEC maturation markers and a reduced number of HCs in these animals. On the other hand, some HCs are still present even in *Aire*-deficient thymi and treating this thymic tissue with RANKL and CD40L seems to induce terminal differentiation marker expression, apparently bypassing the need for *Aire* expression. Therefore *Aire* seems to play an important, but somewhat dispensable role in mTEC maturation and HC development.

6.6. The pathologic potential of AIRE-deficiency induced anti-IL-22 autoantibodies

Generally speaking, one of the central roles of *Aire* is to facilitate the expression of peripheral tissue antigens in the thymus and through that provide one of the first lines of defence against autoimmune reactions against self. This is clearly evident in APECED patients, who, due to the lack of functional AIRE develop a

complex autoimmune phenotype, including the production of autoantibodies against multiple tissue restricted antigens. A very peculiar aspect of APECED are autoantibodies against inflammatory cytokines such as type I interferons or Th17-related cytokines, which do not appear to be under direct transcriptional control of AIRE, although RANK signalling which is directly upstream of AIRE expression is capable of inducing some interferon stimulated genes in the thymus (Ohshima et al., 2011). It is still enigmatic, what causes APECED patients to develop autoantibodies against these cytokines. Nevertheless, in humans, it is a phenotype very clearly associated with AIRE-deficiency, where antibodies against Th17 cytokines clearly correlate with candidiasis, one of the classical symptoms of APECED (Kisand et al., 2010; Puel et al., 2010).

However, cytokine-neutralizing autoantibodies and their clinical significance have been difficult to study in mice, as they do not develop the same selection of autoantibodies as human patients. The studies examining *Aire*-deficiency and candidiasis in mice have so far been carried out using either young mice who have not yet developed anti-cytokine antibodies or by applying models resembling a systemic *Candida albicans* infection (Ahlgren et al., 2011; Hubert et al., 2009), neither of which properly addresses the possible role of auto-antibodies in disease development. It has been demonstrated, that treating WT mice with high amounts of monoclonal antibodies against IL-17A (or IL-17A + IL-17F) impairs their immunity to oral candidiasis (Whibley et al., 2016), but the pathogenic potential of naturally occurring anti-IL-17A antibodies found in *Aire*-deficient mice as well as anti-IL-22 antibodies commonly found in APECED patients had yet to be determined.

In our work presented here we have provided evidence that the anti-IL-17A antibodies that develop in aged *Aire*-deficient mice do not represent a susceptibility factor for candidiasis. This demonstrates that there is a clear discrepancy between the naturally occurring anti-cytokine antibodies when compared to an artificial system of non-patient derived monoclonal antibody treatment in mice. It should be noted, however, that a slight increase in the incidence of *C. albicans* infections has been observed in human patients being treated for psoriasis and psoriatic arthritis with anti-IL-17A therapy (Saunte et al., 2017), suggesting that a pathological link in APECED patients harbouring anti-IL-17A/F antibodies is also possible. Antibodies against IL-17A and IL-17F are, however, not the most prevalent anti-Th17 cytokine specific antibodies in human patients. Instead, antibodies neutralizing IL-22 are considerably more prevalent, being present in upwards of 90% of APECED patients (Kisand et al., 2010; Puel et al., 2010). *Aire*-deficient mice, on the other hand, do not develop these antibodies, leaving no choice but to use somewhat artificial means to study the potential clinical significance of IL-22 neutralization.

Mice deficient for IL-22 have been demonstrated to be susceptible to OPC, clearly suggesting IL-22 plays an important role in fungal clearance (Conti et al., 2009). In our study we demonstrate, that antibodies against IL-22 derived from APECED patients, which also cross-react with and neutralize murine IL-22, lead to delayed clearance of fungi after infection with *C. albicans*. Mice

treated with 30G1, a human-derived anti-IL-22 antibody, exhibited impaired fungal clearance from their oral mucosa and an elevated level of Th-17-related genes in their tongues compared to their control group, demonstrating that high-affinity neutralizing anti-IL-22 antibodies in possess a potential to impair fungal clearance.

It should be noted, that neither in our study nor the previously conducted experiments with IL-17 neutralization (Whibley et al., 2016), was antibody treatment able to precipitate the full clinical manifestation of a *Candida albicans* infection. This suggests that, while clearly a susceptibility factor, these antibodies are unlikely to be the only element contributing to *Candida albicans* infections in human patients.

6.7. Closing remarks

This thesis explores different aspects of establishing immune tolerance, focused around one central element – the *Aire* gene. We describe the key transcriptional networks associated with thymic epithelial cell functions necessary for the establishment of the thymic microenvironment and explore in detail the activation principles of *Aire* in the thymic epithelium. We demonstrate that signalling through RANK and downstream activation of the canonical NF-κB pathway are central to inducing *Aire* expression in the murine thymus and that *Aire* plays an important role in coordinating the mTEC maturation process, which ends in the formation of Hassall's corpuscles from mTECs that have lost their expression of *Aire* and most TSAs. Additionally, we explore the pathogenic properties of IL-22 neutralizing antibodies arising in APECED patients deficient for AIRE and show that these antibodies could act as a susceptibility factor for developing candidiasis, a characteristic APECED-related disease.

Taken together, these studies expand our knowledge of the key mechanisms that regulate thymic processes central to the formation of immune tolerance.

7. CONCLUSIONS

Based on the studies presented in this thesis, the following conclusions can be drawn:

- TNFSF signals play distinct roles in shaping the thymic microenvironment. Most importantly, RANKL is the only signal capable of inducing *Aire* expression in the embryonic thymus, where it functions via the canonical NF-κB pathway through a cis-regulatory region CNS1. This signalling pathway thus plays a key function in facilitating the development of mTECs capable of promiscuous gene expression.
- *Aire* is a necessary, but partially dispensable regulator of mTEC maturation. After mTECs lose the expression of *Aire*, they continue to develop into Hassall's corpuscles. In addition to several maturation markers characteristic of the skin epidermis, HCs contain a reduced selection of TSAs such as pemphigus vulgaris-associated TSAs desmoglein 1 and desmoglein 3. HCs could thus play a role in establishing central tolerance through cross-presentation in association with thymic dendritic cells.
- *Aire*-deficient mice do not exhibit a heightened susceptibility towards *C. albicans* infections despite developing autoantibodies against IL-17A as they age. However, autoantibodies against Th17 cytokine IL-22 that develop in AIRE-deficient human patients can function as a susceptibility factor for candidiasis. Nevertheless, multiple susceptibility factors are most likely needed to develop the fully precipitated disease in APECED patients.

8. SUMMARY IN ESTONIAN

TNF perekond ja AIRE tüümuse diferentseerumise ja *Candida albicans*-vastase kaitse ristteedel

Tüümust (ehk harknääret) on teadvustatud eraldiseisva organina juba vanade kreeklaste ajast, kes uskusid, et just seal paikneb inimese hing. Pika aja vältel ei osatud sellele rinnaku taga, südame peal paiknevale organile välja pakkuda ühtegi tõsiseltvõetavat rolli, kuni lõpuks umbes poole sajandi eest omistati talle reaalne teaduslikku kinnitust leidnud funktsioon. Tänapäeval mõistame me, et tüümus, mis on leitav kõikides selgroogsetes organismides, on asendamatu tähtsusega organismi omandatud immuunsuse kujunemisel, mis hoiab ühtviisi ära nii nakkushaigusi kui ka immuunrünnakuid organismi enda kudede vastu.

Mõlemad funktsioonid saavutatakse läbi keeruka rakkudevahelise suhtluse, mille keskmes on tüümuse epiteelirakud (TEC –*thymic epithelial cells*). Tegu on rakkudega, mis piltlikult öeldes õpetavad arenevatele T-lümfotsüütidele ehk tümotsüütidele tegema vahet kehaomastel ja võõrastel valkudel. TECid suudavad seda, kuna nad on võimelised ekspresseerima tuhandeid geene, mis muidu avalduvad vaid üksikutes spetsiifilistes kudedes ning esitavad sellisel viisil saadud antigeene arenevatele tümotsüütidele. Taoline nn avatud geenieksressiooni muster on organismi seisukohast unikaalne, kuna ükski teine rakutüüp ei ole võimeline ekspresseerima taolisel kombel tuhandeid erinevaid koespetsiifilisi geene. Eriti omane on selline geenieksressiooni muster TECidele, mis asuvad tüümuse säsis, nn medulaarsed ehk säsi epiteeli rakud (mTEC – *medullary thymic epithelial cells*). mTECides on paljude koespetsiifiliste geenide ekspressioon kontrollitud ühe transkriptsiooni regulaatori poolt, milleks on Autoimmuunregulaator – AIRE geeni pealt sünteesitud samanimeline valk.

AIRE on hädavajalik normaalselt talitleva immuunsüsteemi arenemiseks, mida ilmestab hästi fakt, et selle puudulikkuse korral areneb inimestel välja raskekujuline ja kompleksne autoimmuunsündroom APECED – Autoimmuunne polüendokrinopaatia-kandidoos-ektodermaalne düstroofia. APECEDi iseloomustavad autoimmuunrünnakud mitmete organismi kudede (ennekõike sisenõrenäärmete) vastu ning mitmete põletikuliste tsütokiinide vastased neutraliseerivad autoantikehad, mis võivad olla seotud patsientidel esineva seeninfentsiooniga. APECEDi enamlevinud sümptomiseks ongi varajases lapsepõlves algav krooniline naha ja limaskestade kandidoos, millele järgnevad neerupealiste ja kõrvalkilpnäärmete puudulikkus.

Kuna AIRE roll immuunsüsteemis on sedavõrd oluline, on paljud uuringud süüvinud mehhanismidesse, mille kaudu käib selle keskse tähtsusega geeni regulatsioon mTECides ning püüdnud selgitada laiemalt AIRE rolli immuunsüsteemis. Selleks otstarbeks on loodud mitmeid geneetiliselt modifitseeritud hiireliine, mille peal tehtud uuringud on andnud asendamatut informatsiooni, paljastamaks AIRE funktsiooni ja regulatsiooni. Vaatamata sellele on siiski veel palju vastamata küsimusi, mis on seotud antud geeniga ja rakutüübiga, kus see peamiselt avaldub.

Käesolev väitekiri keskendub molekulaarsetele signaalradadele, mis reguleerivad erinevaid mTECide küpsemise aspekte ning ennekõike faktoritele, mis mõjutavad *Aire* ekspressiooni hiire tüümuses. Antud töös lahatakse ka mTECide loomulikku küpsemist ning *Aire* rolli selle protsessi koordineerimises. Sellele lisaks uuritakse AIRE puudulikkusest tingitud T-abistajarakkude alatüübi Th17 poolt toodetud tsütokiinide vastaste autoantikehade võimalikku patogeenset rolli *C. albicans* nakkuse väljakujunemises.

Uurimistöö eesmärgid:

- Uurida erinevate tuumori nekroosifaktori perekonna (TNFSF – *tumor necrosis factor superfamily*) signaalradade toimet geenikspressioonile tüümuse epiteelis keskendudes ennekõike rakuvälistele ja rakusisestele signaalradadele, mille kaudu on reguleeritud *Aire* ekspressioon tüümuses.
- Uurida, millist funktsiooni omab *Aire* mTECide hilises küpsemises ning Hassall'i kehade moodustumises hiire tüümuses, sealhulgas kirjeldades mTECide populatsiooni, mis enam ei ekspresseeri *Aire*-t.
- Hinnata, kas *Aire*-puudulikkus ja sellega kaasnevad tsütokiinide vastased autoantikehad vanades *Aire*-puudulikes hiirtes on piisav põhjus, et kutsuda nendes loomades esile eelsoodumust kandidoosile ning uurida, millist rolli mängivad interleukiin (IL)-22 neutraliseerivad autoantikehad kaitses *Candida albicans* infektsiooni vastu.

Materjal ja meetodid:

Et uurida erinevate TNFSF signaalide toimet geenikspressioonile tüümuse epiteelis, kasutati embrüonaalse tüümuse organkultuuri mudelsüsteemi. Tüümuse sagarad eraldati metsiktüüpi C57BL/6 või CNS1-puudulike hiirte embrüotest E16,5 tiinuspäeval, hematopoeetilised rakud kõrvaldati 2'–deoksüguanosiini töötlusega ning seejärel kasvatati tüümuse organkultuuri erinevate TNFSF ligandide juuresolekul 48h. Geenikspressiooni analüüsiks eraldati organkultuurist RNA ja rakendati mikrokiipanalüüsi, mille alusel tuvastati statistiliselt usaldusväärselt muutunud ekspressiooniga geenid. Osade geenide puhul valideeriti tulemus kvantitatiivse polümeraasi ahelreaktsiooniga. Et tuvastada erinevate TNFSF signaalide poolt mõjutatud geenide promootorpiirkonnas asuvaid transkriptsioonifaktorite seondumisjärjestusi, kasutati oPOSSUM 3.0 tarkvara. TNFSF signaalide toimet tüümuse epiteeli küpsemisele organkultuuris hinnati voolutsütomeetrilise analüüsiga.

Kirjeldamaks *Aire* rolli mTECide hilises küpsemises ning Hassall'i kehade moodustumises, uuriti võrdlevalt metsiktüüpi ja *Aire*-puudulike hiirte tüümuse kudet BALB/c ning C57BL/6 taustal. Erinevas küpsusastmes mTECide ja Hassall'i kehade tuvastamiseks koelõikudel kasutati immuunohistokeemiat ja immuunofluorestsents-analüüsi. Erinevas küpsusastmes mTEC-ide geenikspressiooni uurimiseks eraldati rakupopulatsioonid esmalt voolutsütomeetriliselt, rakkudest puhastati RNA ja sellest sünteesiti cDNA, kust määrati kindlate geenide ekspressioonitase kvantitatiivse polümeraasi ahelreaktsiooniga.

Uurimaks, kas *Aire*-puudulikkus põhjustab eelsoodumust *C. albicans* nakkusele, kasutati 1,5–2 aasta vanuseid *Aire*-puudulikke BALB/c hiiri ning nende metsiktüüpi pesakonnakaaslasi. IL-22 vastaste neutraliseerivate antikehade toimet *C. albicans* nakkusele hinnati metsiktüüpi 1,5–2 aastat vanades BALB/c hiirtes. Hiirte suukaudne nakatamine *C. albicans* kultuuriga viidi läbi anesteesia all, milleks kasutati ketamiini ja ksülasiini. Kuus päeva pärast nakatamist hiired hukati tservikaalse dislokatsiooniga. Keel eemaldati ja seda kasutati geeniekspressiooni analüüsiks ning hindamaks *C. albicans*'i esinemist suuõõnes. Samuti võeti vereproov seerumis leiduvate antikehade analüüsiks. Kõik hiirtega tehtud tööd olid kooskõlas Euroopa Liidu direktiiviga 86/609/EEC.

Uurimistöö peamised tulemused ja järeldused:

Käesolev töö lubab järeldada, et erinevad TNFSF signaalrajad omavad kindlaid unikaalseid rolle tüümuse keskkonna kujundamisel. Peamise leiuna võib välja tuua, et embrüonaalses tüümuses on NF-κB retseptori aktivaatori (RANKL – *receptor activator of NF-κB*) signaal ainuke, mis suudab indutseerida *Aire* ekspressiooni. Seega on RANK embrüonaalses tüümuses asendamatu, kuna selle kaudu kutsutakse mTECides esile avatud geeniekspressioon. Samuti demonstreerib käesolev töö, et RANK signaali ülekanne toimib läbi kanoonilise NF-κB signaalraja ja *Aire* geeni ees paikneva cis-regulatoorse elemendi CNS1.

Uurides *Aire* rolli mTEC'ide küpsemise reguleerimises selgus, et *Aire* on vajalik, kuid mitte täielikult asendamatu mTECide küpsemise regulaator. *Aire* ekspresseerub vaid suhteliselt lühikeses ajalises aknas mTECide küpsemise käigus ning pärast seda, kui mTECid on kaotanud *Aire* ekspressiooni, muunduvad nad Hassall'i kehadeks. *Aire*-puudulikel hiirtel on märkimisväärselt langenud Hassall'i kehade hulk tüümuses, mis viitab omakorda *Aire* rollile mTECide hilise küpsemise suunamises. Hassall'i kehade kirjeldamisel selgus, et need struktuurid ekspresseerivad muuhulgas erinevaid keratinotsüütide-spetsiifilisi antigeene, näiteks *pemphigus vulgaris*-seoselised antigeenid *desmoglein1* ja *desmoglein 3*. See viitab, et Hassall'i kehad võivad omada olulist rolli immuuntolerantsuse kujunemisel läbi antigeenide ristesitamise koostöös tüümuse dendriitrakkudega.

Kuna inimestel kaasneb AIRE-puudulikkusega reeglina kandidoos, uuriti käesolevas töös lähemalt *Aire*-puudulikke hiiri kui potentsiaalset mudelit sellele konditsioonile. Kuna APECED patsientides on kandidoosi seostatud tsütokiinide vastaste autoantikehadega, uuriti lähemalt ka IL-22 vastaste antikehade potentsiaalset rolli kandidoosi kujunemisel hiiremudelis. Selgus, et vaatamata sellele, et *Aire*-puudulikes hiirtes tekivad vananedes autoantikehad IL-17 vastu, ei oma need loomad kõrgenenud eelsoodumust *Candida albicans* nakkusele. Seevastu IL-22 vastased autoantikehad, mis tekivad AIRE-puudulikkusega patsientides, mõjuvad loommudelis eelsoodumusfaktorina kandidoosile. Antud tulemused kinnitavad interleukiinide vastaste autoantikehade tähtsust kandidoosi väljakujunemisel. Vaatamata sellele viitavad antud tulemused, et APECED patsientides on tõenäoliselt vaja mitmete eelsoodumusfaktorite koosesinemist kandidoosi täielikuks väljakujunemiseks.

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ACKNOWLEDGEMENTS

The studies this thesis is based on were performed at the Molecular Pathology research group, Institute of Biomedicine and Translational Medicine at the University of Tartu. I would like to express my deep gratitude to the people who have helped me out along the way, in particular:

- my supervisors Martti Laan, Pärt Peterson and Kai Kisand who have guided me a great deal on this academic journey;

- my colleagues, past and present, who have always been helpful and created a friendly environment to work in;

- my co-authors for their valuable contributions to our shared work;

- Raivo Uibo and Kersti Lilleväli, who gave their constructive review of this thesis;

- and last but not least, my friends and family who have had to endure endless ranting and moaning in regards to this whole ordeal and despite all this have been there for me on many an occasion. You have done well in keeping me (relatively) sane throughout this time. Cheers mates!

PUBLICATIONS

CURRICULUM VITAE

Name: Rudolf Bichele
Date of birth: July 5th 1989, Tartu, Estonia
Citizenship: Estonian
Address: Molecular Pathology Study Group,
Institute of Biomedicine and Translational Medicine,
University of Tartu, Ravila 19, 50411, Tartu
Phone: +372 737 4202
E-mail: rudolf.bichele@ut.ee

Education:

1996–2005	Miina Härma Gymnasium
2005–2008	Hugo Treffner Gymnasium
2008–2011	University of Tartu, Faculty of Science and Technology, Genetic engineering, BSc
2011–2013	University of Tartu, Molecular and Cell Biology, MSc
2013–2018	University of Tartu, Faculty of Medicine, PhD student

Institution and position:

2013–2017	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Molecular pathology study group, laboratory specialist
2017–…	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Molecular pathology study group, Junior Research Fellow in Biomedicine and Translational Medicine
2017–…	University of Tartu, Faculty of Medicine, Institute of Biomedicine and Translational Medicine, Molecular pathology study group, Assistant in Human Biology

Publications:

Bichele R, Kärner J, Truusalu K, Smidt I, Mändar R, Conti HR, Gaffen SL, Peterson P, Laan M, Kisand K. IL-22 neutralizing autoantibodies impair fungal clearance in murine oropharyngeal candidiasis model. Eur J Immunol. 2018 Mar;48(3):464–470.

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Wang X, Laan M, **Bichele R**, Kisand K, Scott HS, Peterson P.Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens. Front Immunol. 2012 Mar;3:19.

ELULOOKIRJELDUS

Nimi: Rudolf Bichele
Sünniaeg: 5. juuli 1989, Tartu, Eesti
Kodakondsus: eesti
Aadress: Molekulaarpatoloogia uurimisrühm,
Bio- ja siirdemeditsiini instituut, Tartu Ülikool,
Ravila 19, 50411, Tartu
Telefon: +372 737 4202
E-mail: rudolf.bichele@ut.ee

Hariduskäik:

1996–2005	Miina Härma Gümnaasium
2005–2008	Hugo Treffneri Gümnaasium
2008–2011	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Geenitehnoloogia eriala, BSc
2011–2013	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Molekulaarbioloogia ja rakubioloogia eriala, MSc
2013–2018	Tartu Ülikool, Arstiteaduskond/Meditsiiniteaduste valdkond, doktorant

Teenistuskäik:

2013–2017	Tartu Ülikool, Arstiteaduskond/Meditsiiniteaduste valdkond, Bio- ja siirdemeditsiini instituut, molekulaarpatoloogia uurimisrühm, laborispetsialist
2017–…	Tartu Ülikool, Meditsiiniteaduste valdkond, Bio- ja siirdemeditsiini instituut, molekulaarpatoloogia uurimisrühm, nooremteadur
2017–…	Tartu Ülikool, Meditsiiniteaduste valdkond, Bio- ja siirdemeditsiini instituut, molekulaarpatoloogia uurimisrühm, inimese bioloogia assistent

Publikatsioonid:

Bichele R, Kärner J, Truusalu K, Smidt I, Mändar R, Conti HR, Gaffen SL, Peterson P, Laan M, Kisand K. IL-22 neutralizing autoantibodies impair fungal clearance in murine oropharyngeal candidiasis model. Eur J Immunol. 2018 Mar;48(3):464–470.

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