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UKU HALJASORG

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Molecular Pathology Research Group, Institute of Biomedicine and Translational Medicine, University of Tartu.

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LIST OF ORIGINAL PUBLICATIONS

I. **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. European Journal of Immunology. 2015; 45(12):3246–56

II. Laan M, **Haljasorg U**, Kisand K, Salumets A, Peterson P. Pregnancy-induced thymic involution is associated with suppression of chemokines essential for T-lymphoid progenitor homing. European Journal of Immunology. 2016;46(8):2008–17

III. **Haljasorg U**, Dooley J, Laan M, Kisand K, Bichele R, Liston A, Peterson P. Irf4 Expression in Thymic Epithelium Is Critical for Thymic Regulatory T Cell Homeostasis. Journal of Immunology. 2017; 198 (5):1952–1960.

Uku Haljasorg's contribution to original publications is as follows:

Study I: Performed flow cytometric and FACS experiments, immunofluorescence and RT-PCR gene expression experiments with the exception of the FTOC-experiment. Participated in the study design, figure preparation and data analyses.

Study II. Performed the experiments and participated in the study design, figure preparation and data analyses.

Study III. Performed all the experiments with the exception of the FTOC-experiment. Analyzed the results, prepared the figures and wrote the paper.

ABBREVIATIONS

Aire	Autoimmune regulator
APC	antigen-presenting cell
APS1	autoimmune polyendocrine syndrome 1
ARTI	age-related thymic involution
BCR	B cell receptor
CARD	caspase-recruitment domain
Ccl	CC-chemokine
Ccr	CC-chemokine receptor
CD	cluster of differentiation
cKO	Tissue-specific conditional knockout
CNS	conserved noncoding sequence
CMJ	cortico-medullary junction
CSR	class-switch recombination
cTEC	cortical thymic epithelial cell
CTL	cytotoxic T cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Cxcl	CXC chemokine
Cxcr	CXC chemokine receptor
DBD	DNA-binding domain
DDR	DNA-damage response
DC	dendritic cell
Dll4	Delta-like 4
DN	double negative
DP	double positive
FACS	fluorescence-activated cell-sorting
Foxn1	forkhead box n1
Foxp3	forkhead box p3
FTOC	fetal thymic organ culture
GM-CSF	granulocyte macrophage colony-stimulating factor
IFN	interferon
IL	interleukin
IPEX	immune dysfunction, polyendocrinopathy, and enteropathy, X-linked
Irf	interferon regulatory factor
Kitl	Kit-ligand
KO	knockout
Krt	keratin
LTβ	lymphotoxin β
Mϕ	macrophage
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 κB
NIK	NF-κB-inducing kinase
NLS	nuclear localization sequence
Nrp1	neuropilin 1
PHD	plant homeodomain zinc finger
PRTI	pregnancy-related thymic involution
pTreg	Treg induced by peripheral tolerance
RANK (RANKL)	(ligand of) receptor activator of NF-κB
SAND	SP100, AIRE1, NucP41/P75 and DEAF1 domain
SP	single positive
Tc	interleukin-secreting $CD8^+$ T cell
TCR	T cell receptor
TLP	thymic lymphoid progenitor
TEC	thymic epithelial cell
TEPC	thymic epithelial progenitor cell
Th	helper T cell
TNFα	tumor necrosis factor α
TNFSF	TNF superfamily
Treg	regulatory T cell
Tri	trimester
TSA	tissue-specific antigen
tTreg	Treg induced by central tolerance
UEA	*Ulex europeaus* agglutinin

1. INTRODUCTION

Thymus has been appreciated for its culinary properties already for hundreds of years. Its role as an organ required for the induction and maintenance of central tolerance however, has been acknowledged for just over half a century. Up until the early 1960s the thymus was thought to be a redundant organ. The fact that the thymus involutes and almost disappears in aged individuals only seemed to justify those convinced of the organ's vestigiality. Although the thymus was known to be filled with lymphocytes, at best it was thought to serve as their graveyard.

While working as a PhD student in a shack near London, Jacques Miller discovered that severe autoimmune disorders develop in neonatally thymectomized mice – an effect not witnessed in mice thymectomized as adults. When his groundbreaking paper was published in 1961, Jacques Miller was met with bewilderment, as only a few leading scientists of the era believed the thymus to have a major function at all, lest to say, to be pivotal for the development of central tolerance.

Today we know that the thymus provides a unique microenvironment for the stepwise maturation of thymocytes that give rise to peripheral T cell populations. The main populations indicated in influencing thymocyte maturation are thymic epithelial cells (TEC) in the cortex (cTEC) and medulla (mTEC). A proportion of mTEC expresses a transcription factor Autoimmune Regulator (Aire) which has been shown to be crucial for the negative selection of autoreactive thymocytes and induction of central tolerance. Mutations in the *Aire* gene cause a rare multiorgan autoimmune disorder named autoimmune polyendocrine syndrome 1 (APS1 or APECED). Although widely accepted as a pivotal protein in central tolerance induction, relatively little is known how the highly specific expression of Aire is regulated on transcriptional level.

Immune tolerance induction is required to avoid immune responses towards self-antigens but it also plays a central role in mammalian reproduction. Pregnancy in mammals requires substantial reorganization of the maternal peripheral immune system to allow for the normal development of the semiallogenic fetus. Aside from the general thymic involution occurring during pregnancy, pregnancy-related effects on the thymic stromal cells have, by and large, remained an unstudied subject in reproductive immunology.

In multiple cell-types of the peripheral immune system the transcriptional changes taking place during their activation require the expression of Interferon regulatory factor 4 (Irf4). These include the cells involved in pathogen clearance as well as cells responsible for peripheral tolerance induction towards self- and foreign antigens. Despite being a well-known factor in peripheral immune homeostasis, it has not been studied in the context of central tolerance.

Current thesis is focused on the transcriptional mechanisms involved in thymic epithelial cell function. We characterize the role of the conserved non-coding sequence 1 (CNS1) in *Aire* induction, study the transcriptional changes taking place in thymic stroma during pregnancy-related thymic involution and dissect the role of Irf4 in TEC function.

2. REVIEW OF THE LITERATURE

2.1. Central and peripheral tolerance of the immune system

A functional immune system is capable of protecting and clearing the host from invading pathogens and maintaining immune tolerance towards autologous and beneficial or benign foreign antigens. Immune tolerance is divided into central and peripheral tolerance depending on the time and site of induction. The two sites of central tolerance induction in mammals are the bone marrow for maturing B cells and the thymus for maturing T cells (Kyewski & Klein 2006). Peripheral tolerance induction occurs mainly in secondary lymph organs and mucosal tissues and concentrates on mature autoreactive B and T lymphocytes that have escaped central tolerance and on lymphocytes targeting antigens derived from the gut or from a developing fetus. As a result of central and peripheral tolerance induction, auto-reactive lymphocytes are either deleted or differentiated into regulatory cells suppressing immune responses to the specific antigen (Plitas & Rudensky 2016, Rosenberg et al. 2016).

2.2. Thymus, its formation and thymic epithelial cells

Thymus is a two-lobed organ positioned in the thoracic cavity anterior to the heart and is indispensable for the development of thymocytes and tolerance induction. In size it is proportionally the largest during infancy but starts to involute at puberty. In aged mammals most of the thymic tissue involutes and is replaced with connective and fatty tissue. In addition to age-related decrease in thymic size and T cell production, temporary thymic involution also takes place during infections and pregnancy after which the thymus restores to its age-befitting size (Ansari & Liu 2017).

Thymus is a primary lymphoid organ that arises from the third pharyngeal pouch at around embryonic day 10 or 11 in mice. In humans the thymic domain is evident by week 6 of gestation (Vaidya et al. 2016). The development of thymic tissue into organized outer cortex and inner medulla depends on the transcription factor Forkhead box N1 (Foxn1) (Takahama et al. 2017). A single base pair mutation in *Foxn1* is responsible for the athymic and hairless phenotype of the nude mouse (Vaidya et al. 2016).

The adult thymus contains thymic epithelial progenitor cells (TEPC) (Bleul et al. 2006) able to give rise to the main populations of TEC. TEPC population is most likely to reside in the thymic cortico-medullary junction (CMJ), however the markers defining this rare population have not been agreed on. Sca1, Dec205 and Plet1 have all been suggested to distinguish TEPC from differentiated TEC (Abramson & Anderson 2017). TEPC further differentiate into cTEC and mTEC that have distinct roles in promoting thymocyte differentiation (Takahama et al. 2017). cTEC are responsible for the early stages of thymocyte maturation up until the positive and negative selection of $CD4^{+}CD8^{+}$ double

positive (DP) cells. They express several molecules indicated in early thymocyte differentiation and maturation, such as β5t (a thymoproteasome subunit), Dll4 (Delta-like 4), Kitl (Kit-ligand), IL7 (Interleukin 7) and Cd83 (Takahama et al. 2017) (discussed in detail in the next chapter). mTEC are responsible for the negative selection of autoreactive thymocytes and the generation of a subpopulation of T cells called regulatory T cells (Treg). Based on major histocompatibility complex class II (MHCII) and CD80 surface expression, mTEC can further be divided into $mTEC^{lo}$ ($CD80^{low}$ $MHCII^{low}$) and the more mature *Ulex europeaus* agglutinin 1 (UEA1) positive $mTEC^{hi}$ ($CD80^{hi}$ $MHCII^{hi}$). The expression of Aire (Autoimmune regulator) in $mTEC^{hi}$ has been shown to be crucial in the transcriptional activation of tissue specific antigen (TSA) required for thymic negative selection and thereby central tolerance induction (Anderson et al. 2002).

cTEC differentiation relies mainly on the expression of Foxn1 and its target genes although arrest in a very early stage of thymocyte development has been shown to result in a disorganized cortex and cTEC arrested in an immature stage (Takahama et al. 2017). mTEC differentiation however, is dependent on external signaling from maturing thymocytes (Ohigashi et al. 2016). Positively selected thymocytes produce various tumor necrosis factor superfamily (TNFSF) members that have been shown to regulate mTEC maturation. Mice deficient in receptor activator of nuclear factor κB (RANK) on TEC or its corresponding ligand (RANKL) produced by $CD4^{+}$ single positive (SP) thymocytes have a significantly decreased $mTEC^{hi}$ population and develop no Aire-expressing cells (Rossi et al. 2007, Akiyama et al. 2008, Hikosaka et al. 2008). CD40L and lymphotoxin β (LTβ) produced by thymocytes have also been indicated in TEC differentiation. Both CD40-KO and LTβR-KO mice show aberrant mTEC maturation profiles, but combining either deficiency with a RANK-deficient background results in a phenotype virtually lacking $mTEC^{hi}$ and Aire-expressing cells (Akiyama et al. 2008, Mouri et al. 2011). $mTEC^{hi}$ cells have been shown to differentiate past the Aire-expressing stage and lose the expression of MHCII, CD80 and both Aire-dependent and independent TSA expression (Yano et al. 2008, Nishikawa et al. 2010, Wang et al. 2012). Eventually these post-Aire $mTEC^{lo}$ differentiate into structures called Hassall's corpuscles that express keratinocyte-specific antigens such as desmogleins (Dsg), keratins (Krt) and involucrin (Ivl).

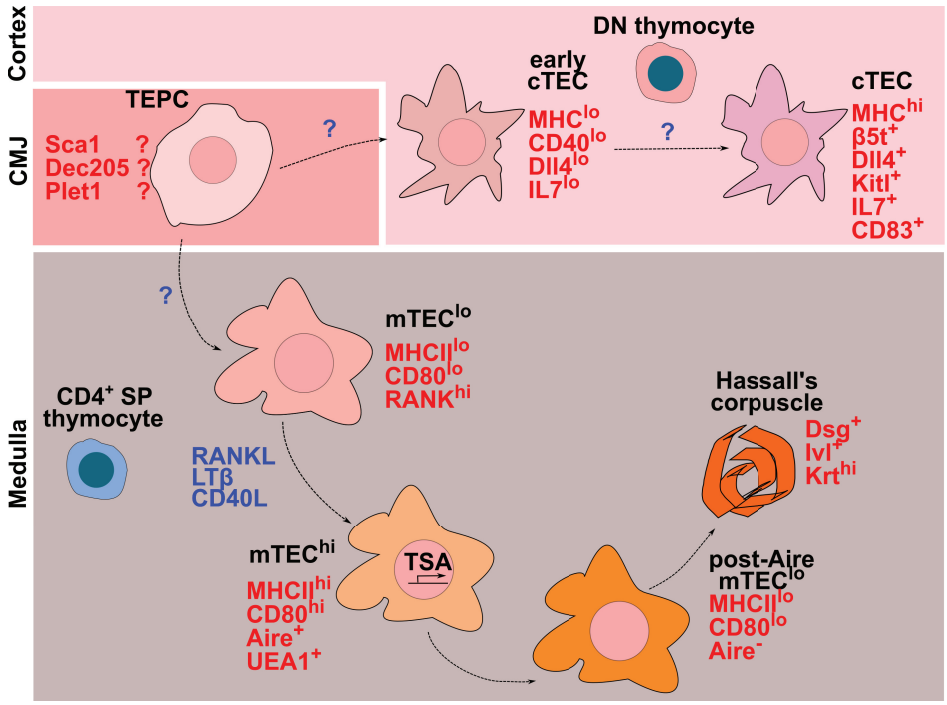

Figure 1. Differentiation of thymic epithelial cells. The differentiation of early cortical thymic epithelial cells (cTEC) from the undefined thymic epithelial progenitor cell (TEPC) depends on unidentified signals (?). This differentiation is likely to take place in the cortico-medullary junction (CMJ), but is presently unknown. Further differentiation depends on unknown signals from early $CD4^-CD8^-$ double-negative (DN) thymocytes. cTEC express multiple proteins facilitating early thymocyte differentiation and positive selection of the $CD4^+CD8^+$ double-positive population. Medullary TEC (mTEC) maturation following the $mTEC^{lo}$ stage requires external signaling from $CD4^+$ single positive ($CD4^+$ SP) thymocytes (written in blue). $mTEC^{hi}$ is the main population responsible for the negative selection and central tolerance induction as they express a vast array of tissue specific antigens (TSA). In later stages $mTEC^{hi}$ lose TSA and Aire expression and eventually accumulate into Hassall's corpuscles expressing keratinocyte TSA. MHC – major histocompatibility complex; β5t – thymoproteasome subunit, Dll4 – delta-like ligand 4, Kitl – Kit- ligand, IL7 – interleukin 7, RANK (L) – (ligand of) receptor activator of nuclear factor κB, LTβ – lymphotoxin beta, Aire – Autoimmune regulator, UEA1 – *Ulex europeaus* agglutinin 1, Dsg – desmogleins, Ivl – involucrin; Krt – keratins including Krt6, Krt10 etc.

2.3. Thymocyte maturation

By far the largest populations of cells in the thymus are thymocytes in different maturation stages (>95%). These stages range from thymic lymphoid progenitors (TLP) to mature naïve single positive (SP) $CD4^+$ T-helper (Th) or regulatory T cells (Tregs); and $CD8^+$ cytotoxic T cells (CTL) (Figure 2). The recruitment of TLP to the embryonic thymus depends on Notch1 and several CC-chemokine receptors (Ccr) such as Ccr7, Ccr9 and CXC-chemokine receptor 4 (Cxcr4), which direct the cells towards high gradients of Ccl19, Ccl21 expressed by mTEC and Ccl25 and Cxcl12 expressed by cTEC and fibroblasts (Calderón & Boehm 2011, Takahama et al. 2017)

TLP enter the thymus at the CMJ as $CD4^-CD8^-$ double negative (DN) cells and relocate to the subcapsular cortex (Takahama 2006). IL7 and Kitl produced by both thymic fibroblasts and cTEC promote thymocyte survival and proliferation at these early stages (Gray et al. 2007b, Takahama et al. 2017). Separate DN stages are identified based on the expression of CD25 and CD44. Following the upregulation of CD25 in late DN1, cells commit to T cell lineage in DN2 stage ($CD25^+CD44^+$). Only thymocytes that succeed in the in-frame rearrangement of the gene encoding the T cell receptor (TCR) beta-chain and express the pre-TCR in DN3 ($CD25^+CD44^-$) enter the proliferating DN4 stage ($CD25^-CD44^-$). These cells give rise to the $CD4^+CD8^+$ DP thymocyte population via a cycling immature $CD8^+$ intermediate SP cell population (Crompton et al. 2007).

During cortical positive and negative selection DP thymocytes migrate towards the medulla and interact with cTEC and dendritic cells (DC) that present ubiquitous and serum-derived self-antigens on their cell surface (Klein et al. 2014). cTEC promote both the $CD4^+$ and $CD8^+$ SP thymocyte lineages by presenting MHC-associated self-peptides to newly formed DP thymocytes. Foxn1-induced thymoproteasome component β5t expressed specifically by cTEC is required for the processing of self-peptides loaded to MHCI thus influencing the $CD8^+$ T cell differentiation (Uddin et al. 2017). cTEC-specific expression of CD83 on the other hand regulates the stability of MHCII molecules on cTEC surfaces and is required for $CD4^+$ thymocyte positive selection (Liu et al. 2016, von Rohrscheidt et al. 2016). A vast majority of TCR clones fail to interact with the self-MHC at all and die by neglect or activate TCR-signaling in response to MHC-associated ubiquitous peptides presented on cTEC and DC which at this stage results in apoptosis. As a result, only a fraction of the produced DP thymocytes become SP and enter the thymic medulla in a Ccr7-dependent manner (Takahama 2006, Klein et al. 2014)

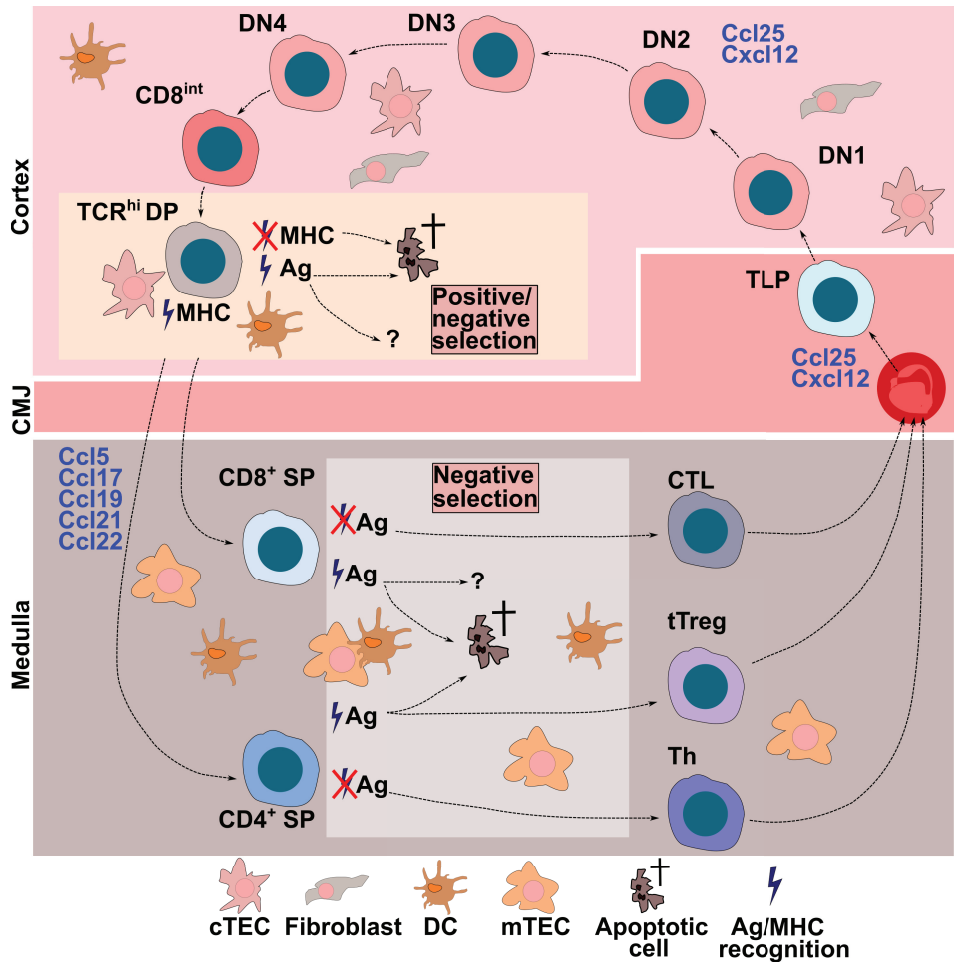

Figure 2. Intrathymic differentiation of T cell progenitors. Thymic lymphoid progenitors enter the thymus in the cortico-medullary junction (CMJ) and migrate towards the subcapsular cortex as $CD4^-CD8^-$ double negative (DN1-4) cells. Migration is dependent on chemokines (written in blue) secreted by cortical thymic epithelial cells (cTEC) and fibroblasts. Following the DN-stages proliferating $CD8^+$ intermediate ($CD8^{int}$) cells mature into $CD4^+CD8^+$ double positive T cell receptor-expressing cells (TCR^{hi}DP). These cells are positively and negatively selected by cTEC and dendritic cells (DC). Positively selected $CD4^+$ or $CD8^+$ single positive ($CD4^+$ SP and $CD8^+$ SP, respectively) migrate towards the medulla and are negatively selected by medullary TEC (mTEC) and DC. Autoreactive cells are either removed, or at least in the $CD4^+$ T cell lineage, central tolerance mechanisms can also differentiate them into Treg (tTreg). Negative selection ideally results in a mature naïve T cell pool not reactive to self and consists of naïve cytotoxic T cells (CTL) and helper T cells (Th) from the $CD8^+$ SP and $CD4^+$ lineages, respectively. MHC – major histocompatibility complex; Ag – antigen.

Thymic medulla is crucial in the selection of immunocompetent thymocytes. Medullary antigen-presenting cells (APC) including mTEC, DC and B cells present self-antigens to $CD4^+$ or $CD8^+$ SP thymocytes in the context of MHCI or MHCII in a process called negative selection (Takahama 2006, Klein et al. 2014, Yamano et al. 2015). The main population responsible for negative selection is the mTEC (Klein et al. 2014). Whereas cTEC are thought to present ubiquitous antigens, $mTEC^{hi}$ harbor a unique ability to express most of the TSA genes and proteins present in the genome (Sansom et al. 2014) Peptides from the encoded TSA are presented in the context of MHC to allow elimination of the possible pathogenicity of autoreactive thymocytes (Takahama 2006). If a thymocyte has a TCR that does not recognize any presented antigen as foreign, it will be considered safe for self and is allowed to exit to the periphery (Takahama 2006). *Nolens volens*, the random rearrangement occurring in the TCR locus results in thymocytes with high-affinity TCRs specific for self-antigens. These cells, should they escape negative selection, can potentially cause autoimmunity in the periphery. Autoreactive thymocytes and peripheral T cells do exist in healthy individuals, but both central and peripheral tolerance maintaining mechanisms exist that render the autoreactive cell anergic, delete it or differentiate it into a Treg (Xing & Hogquist 2012, Plitas & Rudensky 2016).

2.3.1. Regulatory T cells

The path from the initial phenotypic description of a $CD4^+$ T cell population with immunosuppressive capacity to the general acceptance of the existence and importance of Tregs took nearly two decades. In 1982 a paper was published showing that the transfer of sorted splenic $CD4^+CD90^+$ ($Lyt1^+Thy\text{-}1^+$) cells to neonatally thymectomized female mice protected them from the development of oophoritis (Sakaguchi et al. 1982). Years later it was found that the adoptive transfer of a subset of $CD4^+$ T cells expressing high levels of CD25 (IL2Rα) but not $CD25^-$ cells to athymic mice suppresses the development of autoimmune diseases (Sakaguchi et al. 1995). It was only in 2000, when transcription factor forkhead box protein 3 (Foxp3) was found to be specifically expressed in $CD4^+CD25^+$ T cells, that Tregs were truly acknowledged to be a separate T cell subpopulation (Benoist & Mathis 2012). The *FOXP3* gene is located in the X-chromosome and was found to be mutated in IPEX (immune dysfunction, polyendocrinopathy, and enteropathy, X-linked) (Chatila et al. 2000), a rare disorder where male patients are affected by multiorgan autoimmunity (Benoist & Mathis 2012). In mice, both the germline deletion and conditional deletion of *Foxp3* in T cells result in a phenotypically identical T cell dependent severe autoimmunity (Fontenot et al. 2005).

Tregs are MHCII restricted $CD4^+$ T cells that can be divided into two major subpopulations depending on the site of tolerance induction: those induced by central tolerance are of thymic origin (tTreg) and peripherally induced Tregs (pTreg) emerge mostly form mucosal tissues and are induced by peripheral

tolerance (Lin et al. 2013). tTregs develop in thymic medulla from autoreactive αβ T cells that for a long time were thought to be rendered anergic and deleted after TCR-activation during negative selection (Benoist & Mathis 2012). In fact apoptosis or deletion of autoreactive thymocytes has recently been shown to be far less common than previously thought (Malchow et al. 2016) and rather these autoreactive cells differentiate into Treg. The stromal cells mostly indicated in tTreg differentiation are $mTEC^{hi}$ that present a vast library of self-peptides on their surface (Anderson et al. 2002, Klein et al. 2014). Similarly to Th and CTL, tTreg differentiation requires additional signaling from costimulatory molecules such as CD40, CD80 and CD86 on $mTEC^{hi}$ (Williams et al. 2014). However, aside from Aire, little is known about other mTEC-specific factors that regulate tTreg differentiation in the thymus. pTregs differentiate mainly in the gut from peripheral $Foxp3^-$ Th upon exposure to antigenic stimulation under tolerogenic conditions: encounter of a foreign tolerizing antigen or a self-antigen, chronic and/or suboptimal stimulation by agonist peptide etc. (Benoist & Mathis 2012). If an autoreactive Th meets its specific auto-antigen in the periphery it is usually rendered anergic. Recent evidence shows that anergy in peripheral autoreactive Th does not necessarily lead to deletion, but could actually be an important intermediate stage required for pTreg differentiation (Kalekar et al. 2016).

Although not yet elaborated on as to why, central and peripheral mechanisms exist to restrict Treg differentiation. Tregs in the thymus of a 5-week old mouse are a roughly 1:1 mixture of differentiating tTregs and Tregs that have recirculated back to the thymus and the proportion of new tTregs declines with age (Thiault et al. 2015). Although the mechanism is still unclear, recirculating Tregs restrain the development of thymic Treg precursors (Thiault et al. 2015). In T cell-mediated colitis the peripheral induction of Tregs, Ccr9 expression appears to have a negative effect on pTreg differentiation, as Ccr9-KO mice exhibit an enlarged Treg population in the spleen and mesenteric lymph nodes (Evans-Marin et al. 2015).

Although the existence of both tTreg and pTreg is not disputed, the discrimination between the two subtypes is a more controversial issue. The expression of Helios and Neuropilin 1 (Nrp1) in steady state has been suggested to differentiate tTregs from pTregs (Sugimoto et al. 2006, Round & Mazmanian 2010, Yadav et al. 2012). Both markers have separately been shown to be upregulated in activated pTreg, therefore the use of either marker alone as a tTreg marker is controversial (Lin et al. 2013, Szurek et al. 2015). A review discussing these issues (Lin et al. 2013) suggests that until more robust markers are identified, tTregs should be defined as $Foxp3^+$ cells co-expressing Nrp1 and Helios.

Both tTreg and pTreg present imperative populations in the maintenance of peripheral tolerance and immune homeostasis. tTregs are mainly thought to induce peripheral tolerance to self-antigens and pTregs predominantly induce tolerance to foreign antigens in the mucosal tissues (Plitas & Rudensky 2016). Antigens for pTreg additionally include allergens, antigens from food and microbiota and paternal alloantigens during fetal development (Samstein et al.

2012, Plitas & Rudensky 2016, Kalekar & Mueller 2017). There are several mechanisms by which Tregs as a bulk population suppress the activation of Th cells and CTL. Ectoenzymes CD39 and CD73 expressed on Treg mediate the conversion of extracellular ATP to adenosine, which directly inhibits the proliferation of effector T cells and suppresses the activity of DCs and myeloid cells (Plitas & Rudensky 2016). Other molecules expressed by Treg to directly suppress T cell activation include Il10, TGFβ and programmed death 1 receptor (PD-1) and ligand (PD-1L) (La Rocca et al. 2014). Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expressed on Treg can have a dual effect on APC that is presenting a TSA. First, it can induce DC to express the enzyme indoleamine 2, 3-dioxygenase, which catabolizes conversion of tryptophan to kynurenine that is toxic to T cells (Grohmann et al. 2002). Secondly CTLA-4 has been shown to downregulate the surface expression of CD80 and CD86 on DC thus inhibiting the costimulatory signal required for T cell activation (Wing et al. 2008). IL2 has been shown to be critical for Treg differentiation and function (Benoist & Mathis 2012), however recent evidence suggests that responsiveness to IL2 is required only for Treg mediated suppression of autoreactive CTL, but is dispensable for regulating Th activation (Chinen et al. 2016).

2.4. Aire

2.4.1. AIRE gene and protein

The *AIRE* gene was cloned by two separate groups in 1997 (Consortium 1997, Nagamine et al. 1997) as a gene mutated in APS1. Aside from mTEC, where its expression was first described (Heino et al. 1999), mouse *Aire* has been found to be expressed in rare peripheral APC and testicular germ cells, and most recently in thymic B cells (Schaller et al. 2008, Yamano et al. 2015). Aire expression in mTEC is required for the induction and maintenance of thymic central tolerance and thymic Aire-positive B cells are thought to provide additional level of tolerance induction, however the role of Aire expression in peripheral cells is less clear (Anderson et al. 2002, Gardner et al. 2008, Yamano et al. 2015, Abramson & Husebye 2016).

The *AIRE* gene sequence and the functional domains of the encoded protein are highly conserved (Saltis et al. 2008). The human *AIRE* gene is located in chromosome 21q22.3 and it codes for a 545 aa protein (Consortium 1997, Nagamine et al. 1997). The functional domains of the AIRE protein include a N-terminal caspase-recruitment domain (CARD), nuclear localization sequence (NLS), SAND (SP100, AIRE1, NucP41/P75 and DEAF1) domain, two plant homeodomain zinc fingers (PHD1 and PHD2), a proline-rich region (PRR) and four leucine rich regions (Peterson et al. 2008). On cellular level AIRE has a specific nuclear location where it forms structures called nuclear dots (Björses et al. 1999, Heino et al. 1999). The formation of these dots depends on the N-

terminal CARD-domain of the AIRE protein (Ferguson et al. 2008). Mutations in the conserved amino acids of the CARD domain have been shown to disrupt the homo-oligomerization of Aire and dimerization of Aire with other factors such as CBP and P-TEFb resulting in the loss of nuclear dots and AIRE-mediated transcription (Peterson et al. 2008). The SAND domain in AIRE is required to interact with ATF7ip/MBD1 complex to locate to Aire-responsive genes (Waterfield et al. 2014). The two PHD-fingers of the AIRE protein have distinct roles in AIRE-mediated transcriptional activation. First of the two PHD-fingers binds the unmethylated lysine 4 of histone 3 (H3K4me0) near AIRE target genes (Koh et al. 2008, Org et al. 2008), whereas the second PHD-finger interacts with several AIRE-interacting proteins indicated in DNA-damage response (DDR) (Abramson et al. 2010, Yang et al. 2013). The LXXLL nuclear-receptor-binding motifs (X for any amino acid) present in the AIRE protein are also likely to be involved in transcriptional activation (Savkur & Burris 2004).

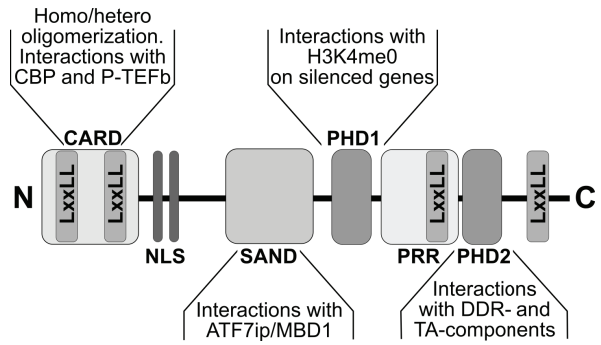

Figure 3. The structural domains of the AIRE protein and their general function. CARD (caspase recruitment domain); NLS (nuclear localization signal); SAND (SP100, AIRE1, NucP41/P75 and DEAF1 domain), PHD (plant homeodomain); PRR (proline-rich region). CBP – CREB-binding protein; PTEFb – positive transcription elongation factor b; H3K4me0 – unmethylated lysine 4 of histone 3; DDR – DNA-damage response; TA – transcription activation; ATF7ip/MBD1 – activating transcription factor 7–interacting protein/ methyl-CpG binding domain protein 1.

Over a hundred APS1-related mutations have been found in the *AIRE* locus. APS1 is a rare autoimmune disorder characterized by a clinical triad – chronic mucocutaneous candidiasis, hypoparathyroidism and Addison's disease (adrenal insufficiency) (Abramson & Husebye 2016). For diagnosis, two of the three components must develop but several other autoimmune complications may also occur in APS1 patients such as type 1 diabetes, ovarian failure, vitiligo etc. The disease-causing mutations have been identified throughout the *AIRE* gene, but even siblings having the same mutation can develop different clinical manifestations (Abramson & Husebye 2016). APS1-patients can also develop auto-

antibodies towards up to a hundred self-proteins including target tissue enzymes and immune modulators such as Th17-associated interleukins IL17 and IL22 and interferons (Kisand et al. 2008, Kisand et al. 2010, Meyer et al. 2016). Surprisingly, the occurrence of high-titer interferon autoantibodies correlates with lower incidence of type 1 diabetes in APS1-patients (Meyer et al. 2016). Recent data demonstrate that certain mono-allelic mutations in AIRE can give rise to "non-classical APS-1", characterized by a broad scale of organ-specific autoimmune disorders, which typically do not match the diagnostic criteria of classical APS-1 (Oftedal et al. 2015), making disease-inflicting *AIRE*-mutations much more common than previously though

2.4.2. Transcriptional regulation of *Aire* in mTEC

In the thymic epithelial compartment *Aire* expression is limited to the mTEC^{hi} population that is positive for the co-stimulatory markers *CD80*, *CD86* and *CD40*, and that has high levels of *MHCII* expression (Heino et al. 1999, Zuklys et al. 2000, Derbinski et al. 2001). *Aire* expression is induced only for a few days (Wang et al. 2012) in response to RANK-signaling (Akiyama et al. 2008, Hikosaka et al. 2008, Irla et al. 2008). Downstream of RANK ligation both the classical and alternative nuclear factor kappa B (NF-κB) pathways are activated. The transcriptional activation of genes depending on the classical NF-κB pathway involves the assembly of the IKK-α, IKK-β, and IKK-γ protein complexes and consequent nuclear translocation of the RelA/p50. The prerequisite for genes activated by the alternative NF-κB is the phosphorylation of IKK-α by NF-κB inducing kinase (NIK) and nuclear translocation of the RelB/p52 complex (Akiyama et al. 2012). Although the disruption of either NF-κB pathway results in aberrant TEC-profiles, decreased *Aire* expression or both (Akiyama et al. 2012), the studies concerning NF-κB in mTEC have been unable to distinguish between the effects these pathways have on mTEC maturation and *Aire* expression. The initiation of *AIRE* transcription requires hypomethylation of a CpG island in *AIRE* promoter that is heavily methylated in cells outside the TEC lineage (Murumägi et al. 2003, Kont et al. 2011). Transcription factors from the E26 transformation-specific family have been shown to bind to the regulatory elements of *AIRE* proximal promoter (Murumägi et al. 2006). Additionally, the *Aire* distal promoter contains a highly conserved region of 90 nucleotides suggested to have a role in regulating *Aire* expression (Blechschmidt et al. 1999). Recently *AIRE* expression was shown to be controlled by multiple transcription factors including Irf4, Irf8, Tbx21, Tcf7 and Ctcfl (Herzig et al. 2017). Adding another layer to the tight control of Aire expression is the discovery that Jmjd6, a lysyl-hydroxylase, is required for Aire protein expression (Yanagihara et al. 2015). *Aire* mRNA expression was not altered in Jmjd6-deficiency, but due to inefficient splicing of the intron 2 of the *Aire* mRNA, mature Aire protein levels were severely decreased and mice with transplanted Jmjd6-deficient thymus developed multiorgan autoimmunity.

2.4.3. Aire-dependent promiscuous gene expression

Each tissue in a body has its own specific gene expression profile. In addition to ubiquitously expressed genes, the genes encoding TSA are also expressed and are specific for a given tissue. mTEC^{hi} is a unique cell population as it expresses thousands of TSA and more than 3000 different genes are expressed in an Aire-dependent manner (Sansom et al. 2014). This process is called promiscuous (ectopic) gene expression (PGE). During negative selection, mTEC^{hi} present peptides in combination with MHC molecules on the cell surface to identify autoreactive thymocytes (Klein et al. 2014). In mTEC the genes that encode TSAs often bear epigenetic marks inherent to transcriptionally silenced chromatin such as H3K4me0 and H3K27me3 (Org et al. 2009, Sansom et al. 2014). The PHD1 of AIRE protein acts as a histone code reader specifically binding to H3K4me0 to activate the expression of silent genes (Koh et al. 2008, Org et al. 2008). Multiple proteins have been identified that interact with AIRE in TSA expression, several of which have a role in DDR [reviewed in (Abramson & Husebye 2016)]. Examples of Aire-induced TSA in mTEC^{hi} include Insulin 2 (Ins2) which in the periphery is restricted to pancreatic beta cells and Salivary protein1 (Spt1) restricted to salivary and lacrimal glands (Derbinski et al. 2005, Kont et al. 2008, Org et al. 2008). Failure to induce sufficient levels of Ins2 in mTEC^{hi} results in the escape of pro-insulin specific autoreactive T cells (Chentoufi & Polychronakos 2002). In double-transgenic mice with hen egg lysosome specific T cells that had hen egg lysosome expressed as a neo-self antigen under the control of the rat insulin promoter, Aire-deficiency results in almost a complete failure of hen egg lysosome-specific autoreactive thymocyte deletion often accompanied by fatal neonatal diabetes (Liston et al. 2003, Liston et al. 2004). Recent evidence suggests that rather than deleting thymocytes with self-antigen specific TCR, Aire expression in mTEC^{hi} is required to promote the differentiation of Tregs from these autoreactive thymocytes (Malchow et al. 2016).

Aire also influences the expression of genes involved in thymocyte and DC migration and TEC differentiation. Aire-KO mice exhibit delayed medullary migration of CD4^{+} SP cells due to decreases in the mTEC specific expression of ligands for Ccr4 (Ccl5, Ccl17, Ccl22) and Ccr7 (Ccl19 and Ccl21) (Laan et al. 2009). Aire regulates the expression of Xcl1, which is required for medullary accumulation of thymic DC (Lei et al. 2011). Xcl1-KO mice have a normal distribution of thymic DC subpopulations, but they fail to migrate to the medulla and promote tTreg differentiation, resulting in decreased tTreg numbers and thymocytes that elicit inflammation when transferred into nude mice (Lei et al. 2011). Aire-KO mTEC^{hi} fail to differentiate into keratinocyte-like terminally differentiated cells. Instead, in Aire-KO mice, mTEC accumulate in the mTEC^{hi} stage and display aberrant expression of keratinocyte markers such as *Dsg3*, *Krt6*, *Krt10* and *Ivl* (Yano et al. 2008, Wang et al. 2012).

Although Aire is responsible for the induction of up to half of the genes in PGE, there are still thousands of TSA expressed in an Aire-independent manner

(Sansom et al. 2014). Recently, LTβR-dependent transcription factor Fezf2 was shown to regulate the expression of a subset of Aire-independent TSA in TEC that are specific for peripheral tissues such as the lung and the liver. Fezf2-deficiency results in peripheral autoimmunity in respective tissues as well as autoantibodies specific for lung, retina and skin (Takaba et al. 2015).

2.5. Pregnancy and tolerance

From a purely immunological point of view the developing fetus represents a semiallogenic transplant that should be rejected by the host's immune system. It has become clear that substantial changes take place in the peripheral immune system of women during pregnancy. One of the main cell population indicated in inducing tolerance towards the fetus is Treg that show an extensive growth in numbers in the peripheral blood and decidua during pregnancy (Aluvihare et al. 2004, Arck & Hecher 2013). In mice pTregs specific for paternal antigens are induced in uterine draining lymph nodes upon encountering seminal plasma antigens and these cells accumulate to the uterus ready to suppress inflammatory reactions towards paternal antigens before the blastocyst implants (Aluvihare et al. 2004, Ruocco et al. 2014, Clark 2016). Pregnant mice, who specifically lack pTreg, but not tTreg, display increased embryonic resorption and immune cell infiltrations in the arteries supplying blood to the endometrium (Samstein et al. 2012).

Peripheral immune system and its role in tolerance induction are rather well described. However, relatively little is known about the effects pregnancy has on central tolerance and *vice versa* – what role does the central tolerance have in pregnancy-related tolerance induction. Amelioration of several autoimmune diseases, including rheumatoid arthritis and multiple sclerosis has been observed during human pregnancy, indicating that certain tTreg clones may become more prominent among the bulk Treg population (Arck & Hecher 2013). One well-described effect that pregnancy inflicts on central tolerance is the temporary decrease in thymic volume (Chambers & Clarke 1979, Shinomiya et al. 1991). Thymic involution during pregnancy occurs in all the mammals examined and at least in pregnant mice lack of pregnancy-related thymic involution (PRTI) results in decreased fertility (Clarke & Kendall 1994, Tibbetts et al. 1999). During murine pregnancy serum progesterone level is upregulated and reaches plateau at mid-pregnancy (Chung et al. 2012). Progesterone signaling is at least partially responsible for PRTI and for the developmental arrest of DN1 thymocytes (Tibbetts et al. 1999). Estrogen serum levels peak at the third trimester (Chung et al. 2012) resulting in decreased thymocyte proliferation and thymic output at late stage pregnancy (Zoller et al. 2007). Thymocyte differentiation and proliferation relies on signals received from thymic stromal cells (TEC, fibroblast, DC). Pregnancy is known to call forth certain phenotypic changes related to proliferation and phagocytic activity in these cells (Kendall & Clarke 2000), but pregnancy-related transcriptional

changes have not been described in the thymic stroma. In age-related thymic involution (ARTI), mTEC and cTEC have been shown to downregulate the expression of *E2f3*, a transcription factor critical for cell proliferation (Ki et al. 2014). Additionally the expression of a Notch1 ligand *Dll4* decreases in thymic stroma during aging. Thymic DC on the other hand increase the expression of proinflammatory molecules such as IL1, IL6 (Ki et al. 2014). Whether ARTI and PRTI share common traits remains to be determined.

2.6. Irf4

2.6.1. Irf4 gene and protein

In mammals the Interferon regulatory factor (IRF)-family consists of 9 members with a variety of functions in the immune system, metabolism, cancer and disease progression (Savitsky et al. 2010, Zhao et al. 2015). Irf4 was first identified in 1992 as a co-factor for PU.1 that binds to the immunoglobulin κ 3' enhancer (Pongubala et al. 1992). The *Irf4* gene was cloned by two separate groups in 1995 (Eisenbeis et al. 1995, Matsuyama et al. 1995) and the first *in vivo* study in 1997 revealed that Irf4-KO mice completely lack germinal center B cells and antibody secreting plasma cells (Mittrücker et al. 1997). These initial studies identified Irf4 as a lymphoid-specific transcription factor. Following publications have implicated Irf4 to be one of the key regulators of cell differentiation and function not only in other immune cells (Honma et al. 2005, Lehtonen et al. 2005, Mohapatra et al. 2015) but also in cells outside the immune system (Eguchi et al. 2011, Nakashima & Haneji 2013, Praetorius et al. 2013, Guo et al. 2014)

In human the *IRF4* gene codes for a 450 aa protein that bears a 92% similarity with the mouse *Irf4* (Grossman et al. 1996). As a transcriptional regulator, Irf4 is generally located in the nucleus (Brass et al. 1996) but can also be found in the cytoplasm (Negishi et al. 2005). Similarly to other IRF-proteins, Irf4 has a roughly 115 aa long N-terminal DNA-binding domain (DBD), that is characterized by five conserved tryptophan repeats that are separated by 10-18 aa (Matsuyama et al. 1995). The DBD of all IRFs binds to conserved IFN-stimulated response elements via its helix-loop-helix domain (Fujii et al. 1999). Within the DBD of Irf4 lies the NLS (Lau et al. 2000), which is required for Irf4 translocation from cytoplasm following extracellular stimuli such as activation of macrophages (Mϕ) by LPS (Negishi et al. 2005). The C-terminal IRF-association domain is required for forming homodimers (Ochiai et al. 2013) or heterodimers with other transcription factors such as Irf8 or PU.1 (Brass et al. 1999, Lu et al. 2003). When binding IFN-stimulated response elements as a homodimer, the interactions between Irf4 and DNA are weak probably due to the C-terminal auto-inhibitory domain of Irf4 hampering the binding of the DBD (Brass et al. 1996). However, as a homodimer, Irf4 can repress the transcription of IFN-stimulated genes by dislocating other IRF-family members Irf1 and Irf2 (Brass et al. 1996, Yamagata et al. 1996). When binding DNA with

transcriptional co-activators such as PU.1, the avidity of Irf4 binding to DNA is increased as strong interactions are formed between the auto-inhibitory domain of Irf4 and the phosphorylated PEST domain of PU.1 (Pongubala et al. 1992, Brass et al. 1999).

2.6.2. Transcriptional regulation of *Irf4* in the immune system

Although initially defined as a lymphoid lineage specific member of the IRF family (Eisenbeis et al. 1995, Matsuyama et al. 1995), Irf4 is expressed in several cell types of both innate and adaptive immune system (Nam & Lim 2016) (Figure 4). Contrary to several other IRF-transcription factors that are induced by type I IFN-signaling, Irf4 activation in fact, results in the repression of IFN-stimulated genes (Brass et al. 1996, Yamagata et al. 1996). In immune cells *Irf4* expression is activated cell-specifically by extracellular stimuli generally leading to the activation of the classical NF-κB pathway (Shaffer et al. 2009). The *Irf4* promoter binds several members of STAT and NF-κB in human monocytes differentiating into DC following antigen encounter, GM-CSF and IL4 co-stimulation or TNFα stimulation (Lehtonen et al. 2005). In T- and B lymphocytes *Irf4* is induced during both maturation and activation. The most prominent cell-extrinsic signal leading to *Irf4* induction in lymphocytes is the signaling downstream of their antigen binding receptors (TCR and BCR) and the consequent activation of c-Rel (Grumont & Gerondakis 2000). Additionally, IL4 and CD40 co-stimulation has been shown to upregulate *Irf4* expression in B cells (Gupta et al. 1999). Alone, IL4-signaling can induce *Irf4* in Mϕ (Honma et al. 2005) where its expression is further controlled by a histone demethylase Jmjd3 (Satoh et al. 2010).

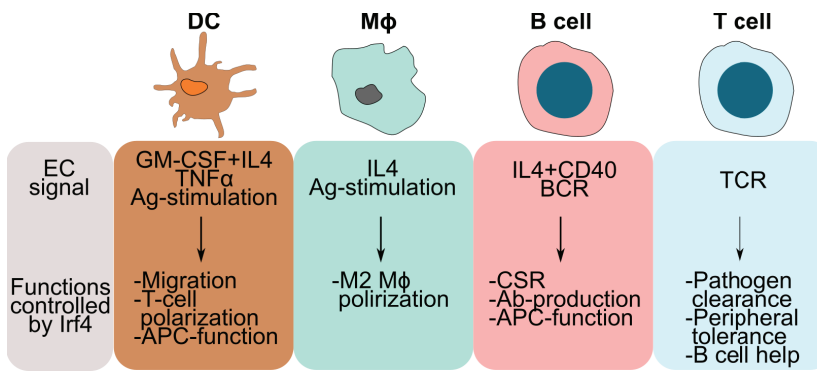

Figure 4. The induction and function of Irf4 in immune cells. Cell-specific extracellular (EC) signaling leads to the activation of the classical NF-κB, which in turn upregulates Irf4 expression. Cell-specific functions for Irf4 in peripheral dendritic cells (DC), macrophages (Mϕ) and in B and T cells. GM-CSF – granulocyte macrophage colony-stimulating factor IL4 – interleukin 4, TNFα – tumor necrosis factor α; TCR – T cell receptor; BCR – B cell receptor; CSR – class switch recombination; Ab – antibody.

2.6.3. Irf4 in the innate immune system

In mammals, the first immune cells to be activated upon encountering new pathogens originate from the innate immune system. The outcome of the infection depends on complex interactions between APCs and effector cells from both the innate and the more specific adaptive immune system. Several studies have shown that Irf4 functions as a key regulator of differentiation and function in several cell types with APC and/or effector capacity from both hands of the immune system [Figure 4 and reviewed in (Nam & Lim 2016)].

The majority of research concerning Irf4 in innate immunity has concentrated on its role in DC and Mϕ that phagocytose pathogens, infected or apoptotic cells, cell debris *etc*. Both these cell-types link innate and adaptive immune systems by presenting antigens to their lymphocytic counterparts (Savina & Amigorena 2007).

Irf4 is induced in M2 Mϕ polarization and activates the expression of several M2 Mϕ marker genes required for pathogen phagocytosis (Satoh et al. 2010). Following peritoneal administration of chitin, a M2 polarizing agent, only eosinophil but not Mϕ recruitment was impaired in Irf4-KO mice. However, the expression of M2 Mϕ marker genes such as *Arg1*, *MR* and *Ym1* was significantly downregulated in chitin induced Mϕ from Irf4-KO mice. Recently Irf4 was shown to control the development of a peritoneal monocyte-derived Mϕ population (Kim et al. 2016). Although the exact function of these Mϕ is yet unknown, they express high levels of MHCII and their differentiation was blocked by both Irf4-deficiency and antibiotic treatment, indicating that they might act as APC mediating signals between the microbiome and the immune system.

In DC Irf4 is crucial for the function of the $CD11b^+$ conventional DC and monocyte derived DC (Suzuki et al. 2004, Schlitzer et al. 2013). Both subtypes serve as potent APC for T lymphocytes and can promote both Th and CTL responses (Mildner & Jung 2014). As $CD11b^+$ conventional DC are a heterogeneous group of cells, Irf4-deficiency only partially affects their function in certain tissues (Mildner & Jung 2014). For instance, in cutaneous inflammation Irf4-deficient $CD11b^+$ conventional DC fail to migrate into lymph nodes (Bajaña et al. 2012). Irf4 promotes the survival of mucosal $CD11b^+$ conventional DC and is required for IL23 production in steady state and upon fungal challenge (Schlitzer et al. 2013). In inflammation and fungal challenge, DC are capable of phagocytosis, but fail to upregulate *MHCII* expression and migration is obstructed by the lack of Irf4-dependent *Ccr7* expression. Altogether the described Irf4-deficient $CD11b^+$ conventional DC no longer act as APC and fail to promote Th17 and Th2 responses in disease. Irf4 is dispensable for $CD11b^+$ conventional DC that cross-present antigens to CD8 T cells but in monocyte-derived DC, Irf4 is needed for these cells to differentiate into APC capable of cross-priming CD8 T cells (Briseño et al. 2016).

2.6.4. Irf4 in the adaptive immune system

Studies have shown that in most B cell differentiation stages Irf4 functions redundantly with Irf8, but Irf4 is a crucial factor for the development of antigen-presenting and antibody producing B cells (Mittrücker et al. 1997, Falini et al. 2000, Lu et al. 2003, Klein et al. 2006). In the absence of both Irf4 and Irf8, early B cells fail to exit the cell cycle and consequently cannot differentiate into the immature B cell stage (Lu et al. 2003). Irf4 is also required for receptor editing of the Igκ and Igλ loci in bone marrow B cells that react to self-antigen (Pathak et al. 2008) indicating a role for Irf4 in B cell tolerance induction.

In mature B cell stages the redundancy between Irf4 and Irf8 disappears as Irf4-deficiency, but not Irf8 deficiency, results in aberrant activation of mature B cells, germinal center formation and failure to commit to class switching (De Silva et al. 2012, Willis et al. 2014). Germinal center formation between mature B cells and follicular Th is diminished both in Irf4-KO mice and in mice where Irf4 is conditionally deleted in CD23-expressing mature B cells (Mittrücker et al. 1997, Willis et al. 2014). Irf4-dependent germinal center formation, however, is not intrinsic only to B cells as transferred Irf4-KO CD4 T cells in *L. major* infection model do not to upregulate *Bcl6* and *Icos* and hence fail to differentiate into follicular Th cells (Bollig et al. 2012).

Mature naïve B cells in peripheral lymphoid tissues express IgM and IgD as their BCR. The differentiation step during which antigen-stimulated B cells reorganize the constant region of the Ig heavy chain and acquire the ability to express other Ig molecules (e.g. IgG) is called class switch recombination (CSR) (Zan & Casali 2015). A key factor in this process is an enzyme AID (transcribed from *Aicda* – activation induced cytidine-deaminase), which introduces mutations into the Ig heavy chain locus. In addition to regulating *Aicda* in CSR following CD40 and IL4 stimulation, Irf4 together with Blimp1 also controls the expression of *Xbp1* which is required for plasma cell generation (Klein et al. 2006). In addition, the complex involving Irf4/8, PU.1 and E47 is shown to regulate CIITA in B cells, rendering Irf4-deficient B cells incapable of presenting antigens (van der Stoep et al. 2004).

Irf4 is required for the effector function of both Th and CTL, but in the thymus it has shown to be induced only in TCR-signaled $CD69^{hi}$ SP thymocyte (Cao et al. 2010, Nayar et al. 2012). However, both full Irf4-KO and CD4-specific Irf4-conditional KO (cKO) have normal-sized thymi with WT-like distribution of thymocytes (Mittrücker et al. 1997, Nayar et al. 2012). This indicates that redundancy exists between Irf4 and (most likely) Irf8 in thymocyte development as it does in early B lymphocyte development (Lu et al. 2003).

Although dispensable for the thymic development of T cells, Irf4 expression is an absolute necessity for most, if not all TCR-signaled peripheral Th and CTL populations (Huber & Lohoff 2014). The peripheral T cell pool consists of different Th-populations and Tregs from the $CD4^+$ lineage and CTLs and cytokine-secreting Tc cells (Tc9 and Tc17, so far) from the $CD8^+$ lineage

(Huber & Lohoff 2014). Irf4-deficiency in *L. monocytogenes* infection model results in impaired Th1 differentiation of the transferred Irf4-KO $CD4^+$ T cells and decreased Ifnγ expression (Mahnke et al. 2016). Other groups carried out similar experiments with transferred Irf4-KO $CD8^+$ T cells in viral and bacterial infections (Man et al. 2013, Yao et al. 2013) and found that Irf4-deficient CTLs fail to kill the infected cells and clear the infection despite acquiring an activated phenotype. In Th2 cells Irf4 upregulates GATA3 and Gfi1 required for Th2 differentiation and, along with Nfat factors stimulates IL4 expression (Nam & Lim 2016). Both the Th and CTL lineages have effector cells in human and mouse that express IL9 or IL17 (Th9, Th17 and Tc9, Tc17, respectively). As the expression of IL9 and IL17 is directly regulated by Irf4 in T cells (IL9 in mast and innate lymphoid cells as well), these subtypes fail to function as effector cells in Irf4-deficient conditions (Huber & Lohoff 2014, Nam & Lim 2016). The IL17-expressing subtypes Th17 and Tc17 have been indicated in multiple sclerosis (Huber et al. 2009, Burkett et al. 2015). The lack of either population in Irf4-KO mice, however, renders the mice resistant to experimental autoimmune encephalomyelitis in mouse model (Brüstle et al. 2007, Huber et al. 2013, Yang et al. 2015a).

Perhaps the most drastic systemic effect of Irf4-deficiency on effector $CD4^+$ T cells is seen in Treg, where Irf4 regulates the genes required to control Th2 and Th1 responses (Zheng et al. 2009, Cretney et al. 2011). Treg-specific Irf4-cKO mice develop a severe autoimmune phenotype soon after weaning and die as early as 3-4 months of age (Zheng et al. 2009). These mice have high levels of activated $CD4^+$ T cells, aberrant Th2 responses and show high levels of serum IgG1 and IgE in response to IL4. Additionally, they develop severe autoimmune infiltrations in several tissues with notable plasma cell contents.

2.6.5. Irf4 in cancer and other tissues

In addition to orchestrating the responses to pathogens in multiple immune system cell types, Irf4 has a role in the pathogenesis of several conditions, most notably malignant tumors and hematopoietic cancers. Interestingly, only a few tumor-related mutations have been described for *Irf4*, rather its oncogenic capacity derives from anomalous expression. Recently a single nucleotide polymorphism was identified in intron 4 of *Irf4* (Praetorius et al. 2013). This single nucleotide polymorphism lies in *Irf4* enhancer and in addition to affecting pigmentation in humans (Praetorius et al. 2013) it is shown to either increase or decrease *Irf4* expression in different hematopoietic cancers and reduce *Irf4* expression in melanoma (Gibbs et al. 2016). The oncogenic potential of IRF4 is best described in multiple myeloma, where BLIMP1 fails to downregulate *MYC* expression resulting in positive transcriptional feedback loop between the proto-oncogene MYC and IRF4 (Shaffer et al. 2008). Thus the plasma cells in multiple myeloma become highly proliferating, but die following the suppression of *IRF4 in vitro*.

Outside the hematopoietic system Irf4 has, for instance, been shown to inhibit lipogenesis in adipocytes (Eguchi et al. 2011). In these cells *Irf4* expression is induced only in the absence of insulin by FoxO1 to promote lipolysis and is quickly downregulated following feeding. Mice with adipocyte-specific Irf4-deficiency become cold-intolerant and gain significantly more weight on high-fat diet than WT mice. In bone tissue, Irf4 regulates the formation of osteoclasts in a RANKL-dependent manner (Nakashima & Haneji 2013). Mice that were injected with RANKL alone showed greater osteoclast formation and decreased bone mineral density compared to controls (mice injected with RANKL and RANK-inhibitor simvastatin). The group behind the study suggested that Irf4-dependent activation of *Nfatc1* downstream of RANKL is required for osteoclast formation from bone Mϕ. Irf4 is expressed on a basal level in neurons, where it can be upregulated by ischemic stroke (Guo et al. 2014). Irf4-deficient neurons fail to upregulate several genes such as *FosB* and *Egr1* which are required for the neuron to survive the stroke. Neuron-specific Irf4 transgenic mice on the other hand develop milder lesions and lower neuronal apoptosis following a stroke.

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The induction of central tolerance depends on elaborate interactions between maturing T cells and thymic stromal cells. These interactions in turn depend on already activated transcriptional mechanisms in these cells and determine the downstream differentiation events taking place in interacting cells. *Aire* expression depends on the interactions between $RANKL^{+}$ thymocytes and $RANK^{+}$ mTEC (Akiyama et al. 2008, Hikosaka et al. 2008, Irla et al. 2008). Aire protein in antigen-presenting $mTEC^{hi}$, on the other hand serves as a crucial factor regulating the expression of thousands of TSA required in thymocyte negative selection (Anderson et al. 2002, Sansom et al. 2014). Although recent publications have broadened our understanding on how Aire induces PGE in thymus [reviewed in (Anderson & Su 2016, Abramson & Anderson 2017), the transcriptional mechanisms underlying the specific activation of *Aire* itself in $mTEC^{hi}$ in response to RANK-signaling are currently unknown. The authors who identified the murine homologue of the human *AIRE* gene, also found a highly conserved region upstream of the *AIRE* gene and theorized that it may have a role in modulating *AIRE* expression (Blechschmidt et al. 1999). But until present day this hypothesis has not been validated.

During pregnancy the thymic volume and thymocyte output are drastically decreased in mammals (Chambers & Clarke 1979, Shinomiya et al. 1991, Clarke & Kendall 1994, Tibbetts et al. 1999). In pregnant mice this process appears to be required for the normal fetal development and has been speculated to benefit to the maternal-fetal tolerance (Tibbetts et al. 1999). The effects of pregnancy on thymocytes have been described (Tibbetts et al. 1999, Zoller et al. 2007), but whether pregnancy also affects the thymic stromal compartment, responsible for the induction of central tolerance is unknown. It is also unknown

whether the hormonal changes during pregnancy have a direct effect on thymocytes or is thymic involution, at least in part, mediated by transcriptional events called forth in thymic stromal cells.

Irf4 is a transcriptional regulator initially thought to be expressed exclusively in lymphocytes (Pongubala et al. 1992, Matsuyama et al. 1995, Grossman et al. 1996, Yamagata et al. 1996). However, it has been found to be expressed in multiple cell-types in- and outside of the immune system (Eguchi et al. 2011, Nakashima & Haneji 2013, Praetorius et al. 2013, Guo et al. 2014, Nam & Lim 2016) Regardless of the lineage of the specific Irf4-expressing cell-type, Irf4 appears to act as a key regulator of development and/or function in these cells. As several peripheral APC rely on Irf4 in their function (Nam & Lim 2016), we asked, whether this protein might also have a role in the differentiation or function of TEC which represent a rare population of APC of the epithelial lineage.

3. AIMS OF THE STUDY

1. To characterize the *in vivo* role of a highly conserved noncoding sequence upstream of the *Aire* coding region.
2. To describe the changes in the stromal compartment during PRTI and to determine the specific transcriptional changes in thymic stroma contributing to PRTI during normal murine pregnancy.
3. To determine the *in vivo* role of Irf4, a master regulator of peripheral immune cell differentiation and function, in thymic epithelial cells.

4. MATERIALS AND METHODS

4.1. Mice

All mice were maintained at the Vivarium of the Institute of Biomedicine and Translational Medicine, University of Tartu. CNS1-KO mice were generated at the Laboratory Animal Centre of Tartu University by targeted disruption of the CNS1 region. The vector was electroporated into 129S6/SvEvTac (W4) ES cells followed by G418 selection and in vitro Cre-recombination. Achieved clones were injected into C57BL/6J embryos and obtained chimeras were crossed with C57BL/6 mice. The mice were maintained on mixed 129sv/C57BL/6 background and crossed as heterozygous $CNS1^{+/-}$ mice. C57BL/6 mice deficient for the *Aire* gene were generated at the Walter and Eliza Hall Institute for Medical Research by a targeted disruption of Aire gene in exon 8. The mice were maintained and crossed as $Aire^{+/-}$ mice. TEC-specific conditional Irf4-deficient mouse strain was generated by crossing mice with the *Irf4* gene flanked by loxP sites ($Irf4^{fl/fl}$) (B6.129S1-Irf4tm1Rdf/J, The Jackson laboratory) (named WT here) and FoxN1:Cre mice (a kind gift from Thomas Boehm). F1 mice heterozygous for FoxN1:Cre and $Irf4^{fl/fl}$ were crossed with $Irf4^{fl/fl}$ resulting in mice lacking the first two exons of *Irf4* and expressing GFP in FoxN1:Cre expressing tissues. In experiments concerning knockout mice male and female mice were used in equal proportions. In Study I 4-6-week-old Aire-KO/CNS1-KO and WT littermate controls were used. In Study III 8-10-week-old Irf4-cKO mice and WT controls were used for all studies except determining of mononuclear infiltrations from aged mice. In Study II, pregnancy was determined by the presence of a vaginal plug and thymi were collected at three time points during the pregnancy: during the first (3–5 days), second (10–12 days), and third (17–19 days) trimesters. Age-matched virgin female mice were used as controls. All animal experiments were approved by the ethical committee of animal experiments at the Ministry of Agriculture, Estonia.

4.2. Flow cytometry and FACS

The antibodies used in the studies are listed in the Table 1. For purification, thymi were minced and gravity-sedimented several times in RPMI-1640 media containing 2% FBS and 20 mM HEPES. The enriched stromal compartment from each genotype was pooled and enzymatically digested in RPMI-1640 media containing collagenase 2 (125 U/mL; Gibco) and DNase1 (15 U/mL; AppliChem) for 20 min at room temperature followed by two 20-minute digestions with collagenase 2, DNase1 and Dispase (0.75 U/mL; Gibco). Following FcR blocking in 2.4G2 hybridoma medium, thymocytes and stromal cells were counted and stained for FACS-sorting or analysis. For thymocyte and lymphocyte analysis thymi and spleens were homogenized using glass-slides, erythro-

cytes in the spleens were lysed using osmotic shock: cells were resuspended in 900 µL deionized water and 100 µL 10 × PBS was added after 10 s. Cells were strained, counted, incubated in 2.4G2 FcR-blocking medium and stained with respective antibodies. All experiments were analyzed using LSRFortessa flow-cytometer with FACSDiva software (BD Biosciences) or FCS Express 5 Flow (De Novo Software)

Table 1. Antibodies used in flow cytometry and FACS

Fluorescence-activated cell sorting and flow cytometry antigens and antibody clones	**Conjugate**	**Company**
Aire (5H12)	eFluor660	eBioscience
B220 (MCA1258F)	FITC	Serotec
CD11c (N418)	BV421	Biolegend
CD25 (PC61.5)	APC	eBioscience
CD3 (145.2C11)	PerCP-Cy5.5	eBioscience
CD4 (GK1.5)	APC-eFluor 780	eBioscience
CD4 (RM4-5)	APC	BD Biosciences
CD44 (IM7)	PerCP-Cy5.5, PE	eBioscience
CD45 (30-F11)	PerCP-Cy5.5	eBioscience
CD62l (MEL-14)	Pe-Cy7	eBioscience
CD8 (53-6.7)	eFluor 650NC	eBioscience
EpCAM (G8.8)	Pe-Cy7	eBioscience
ERTR7 (ab51824)*		Abcam
FoxP3 (FJK-16s)	FITC	eBioscience
Helios (22F6)	APC	eBioscience
I-A/I-E (M5/114.15.2)	APC	eBioscience
I-A/I-E (M5/114.15.2)	BV510	Biolegend
Irf4 (3E4)	BV450	eBioscience
Ki67 (B56)	PE	BD Biosciences
Ly51 (BP1)	PE	eBioscience
Nrp1 (3E12)	BV421	Biolegend
UEA-1-lectin	FITC	Vector labs

* Conjugated in-house with Alexa Fluor 647 Monoclonal Andibody Labeling Kit (Invitrogen).

4.3. RNA Purification and RT-PCR

RNA purification was carried out by either TRIzol–chloroform extraction (whole tissues) or with RNeasy Micro Kits (Qiagen; sorted cells and FTOCs) according to manufacturers' protocols, followed by reverse transcription using SuperScript III Reverse Transcriptase (Life Technologies). Primers used in the study are listed in Table 2. All RT-PCRs were carried out on ViiA™ 7 real-time PCR system. Every sample was run in three parallel

reactions. Relative gene expression levels were calculated using the comparative Ct (ΔΔCt) method (according to Applied Biosystems), where the relative expression is calculated as $2^{-\Delta\Delta Ct}$, and where Ct represents the threshold cycle. β2-microglobulin and cytokeratin 8 were used as house-keeping genes for normalization.

Table 2. Primer sequences used for gene expression analysis in RT-PCR

Oligo name	**Sequence 5' → 3'**	**Gene**
CCL17 F	AGTGGAGTGTTCCAGGGATG	Ccl17
CCL17 R	CCAATCTGATGGCCTTCTTC	Ccl17
CCL19atg F	CTGCCTCAGATTATCTGCCAT	Ccl19
CCL19atg Rnew	CTTCCGCATCATTAGCACCC	Ccl19
CCL20 F	CAGAAGCAAGCAACTACGAC	Ccl20
CCL20 R	TCTTCGTGTGAAAGATGATAGC	Ccl20
CCL21 F	CCCTGGACCCAAGGCAGT	Ccl21
CCL21 R	AGGCTTAGAGTGCTTCCGGG	Ccl21
CCL22 F	CATTCTTGCTTCCCTGGAGA	Ccl22
CCL22 R	TCAGAGAGGAAAAGCGATCC	Ccl22
CCL25 F	GTGCTGTGAGATTCTACTTCC	Ccl25
CCL25 R	TATGGTTTGACTTCTTCCTTTCAG	Ccl25
CCL5 F	GTGCCCACGTCAAGGAGTAT	Ccl5
CCL5 R	CCCACTTCTTCTCTGGGTTG	Ccl5
CD40 F	GCTATGGGGCTGCTTGTTGA	Cd40
CD40 R	ATGGGTGGCATTGGGTCTTC	Cd40
CD86 F	TGAGTATGGTGATAACATGCAG	Cd86
CD86 R	ACTCTTGAGTGAAATTGAGAGG	Cd86
Crp F	CCTTCTGATCATGATCAGCT	Crp
Crp R	CATAAGAGAAGACACTGAAGCTG	Crp
CSNb F	GGCACAGGTTGTTCAGGCTT	Csn2
CSNb R	AAGGAAGGGTGCTACTTGCTG	Csn2
CXCL12 F	CATCAGTGACGGTAAACCAG	Cxcl12
CXCL12 R	CACAGTTTGGAGTGTTGAGG	Cxcl12
DLL4 F	AGGTGCCACTTCGGTTACAC	Dll4
DLL4 R	GGGAGAGCAAATGGCTGATA	Dll4
Dnmt3l	CGGTGGAACTGGAACAT	Dnmt3l
Dnmt3l F1	AACGCTGAAGTACGTGGA	Dnmt3l
Dsg1 f	CTTAGGATTGGTTCCATTCCTG	Dsg1
Dsg1 R	CCATTAGTTCAAATCCTGTGGT	Dsg1
DSG3 F	TTCGGAAGGTACAATTCATCAG	Dsg3
DSG3 R	TGCAAACTTCAGAGCTTTCC	Dsg3
GAD1 F	ATAGAAAGGGCCAATTCAGTC	Gad1
GAD1 R	TGCATCCTTGGAGTATACCC	Gad1
IL1B F1	CAACCAACAAGTGATATTCTCCATG	Il1b
IL1B R1	GATCCACACTCTCCAGCTGCA	Il1b
IL6 F	TTAAGGAGCTTATTGAGGAGCTG	Il6
IL6 R	CTACACAGAACCCGCCA	Il6
IL7 F	TGATGATACAAAGGAAGCTGC	Il7

Oligo name	Sequence 5' → 3'	Gene
IL7 R	GCCTTGTGATACTGTTAGTAAGTG	Il7
Irf4 ex2 F	TCTTCAAGGCTTGGGCATTG	Irf4
Irf4 ex2 R	CACATCGTAATCTTGTCTTCCAAGTAG	Irf4
Kit L F	TCAACATTAGGTCCCGAGAAA	Kit l
Kit L R	ACTGCTACTGCTGTCATTCCTAAG	Kit l
KRT10 R	GTAGTTCAATCTCCAGACCC	Krt10
KTR10 F	GTTCAATCAGAAGAGCAAGGA	Krt10
KRT2-8 F	AGGAGCTCATTCCGTAGCTG	Krt8
KRT2-8 R	TCTGGGATGCAGAACATGAG	Krt8
KRT6b F	CAAACTCACATCTCAGACAC	Krt6b
KRT6b R	GCAGCTCCTCATATTTAGTC	Krt6b
QmAIRE3/4 5f	CCTCAAAGAGCGTCTCCAG	Aire
QmAIRE3/4 5r	TGGTCTGAATTCCGTTTCCA	Aire
mAire_ex7/9 F	GTTAACCAGAAGAACGAGGATGAG	Aire
mAire_ex7/9 R	AGCAGGAGCATCTCCAGAG	Aire
mB2mF	TGAGACTGATACATACGCCTGCA	β2m
mB2mR	GATGCTTGATCACATGTCTCGATC	β2m
mCD80 F	GTCCATCAAAGCTGACTTCTC	Cd80
mCD80 R	ATGCCAGGTAATTCTCTTCCA	Cd80
mE2F3_f	CAAGGTGCAGAAGAGGAGGA	E2f3
mE2F3_r	GAGGTCCAGGGTACAGCTTT	E2f3
mH2-Aa F	CTCAGAAATAGCAAGTCAGTC	H2-Aa
mH2-Aa R	AATCTCAGGTTCCCAGTG	H2-Aa
mIns2 F1	GCTTCTTCTACACACCCATGTC	Ins2
mIns2 R1	AGCACTGATCTACAATGCCAC	Ins2
mINV F	GTGAGTTTGTTTGGTCTACAG	Ivl
mINV R	GAAAGCCCTTCTCTTGAATCTC	Ivl
mTff3_exp_F	TGTTGGCTGTGAGGTCTTTA	Tff3
mTff3_exp_sh2_R	CTGTGCAGTGGTCCTGAA	Tff3
Mup3 F	GCAGCAGCAGCTTCATTTT	Mup3
Mup3 R	AGGTAACATGCTGGAGAGAA	Mup3
mXcl1 F	TCTTGATCGCTGCTTTCACC	Xcl1
mXcl1 R	GAAGTCCTAGAAGAGAGTAGC	Xcl1
S100A8 F	TTTGTCAGCTCCGTCTTCAA	S100a8
S100A8 R	AGACAGTCACATAGCCCTAC	S100a8
Saa1 F	AGGAGACACCAGGATGAAGC	Saa1
Saa1 R	CCAGTTAGCTTCCTTCATGTCAG	Saa1
Spt1_F	AACTTCTGGAACTGCTGATTCTG	Spt1
Spt1_R	GAGGCCTCATTAGCAGTGTTG	Spt1

4.4. Immunofluorescence

Immunofluorescence was performed on frozen sections fixed with acetone or 4% formaldehyde. Formaldehyde-fixed tissues were permeabilized with 0.1% Triton X-100 in PBS. A 30 min blocking step at RT with 1% normal goat serum was used. Sections were incubated overnight with indicated primary Ab at 4°C (listed in Table 3), washed in PBS, and incubated with a respective secondary Ab (1:1000) for 60 min at room temperature. Slides were washed four times with PBS, nuclei stained with DAPI (1μg/mL) for 10 min, washed once more in PBS, and covered with fluorescent mounting medium (Dako) and coverslips. Images were obtained with LSM710 microscope (Zeiss).

Table 3. Antibodies used in immunofluorescence experiments

Antigens targeted and antibody clones	**Company**
Aire (M-300)	Santa Cruz
Ivl (ab28057)	Abcam
K14 (10143-1-AP)	Proteintech
K8 (Troma-1)	(provided by J. Dooley)
UEA-1-lectin conjugated with FITC	Vector Labs
Irf4 (3E4) conjugated with Brilliant Violet 450	eBioscience
Alexa Fluor 488 Goat anti rat IgG	Invitrogen
Alexa Fluor 594 Goat anti rabbit IgG	Invitrogen

4.5. Mononuclear infiltrations

Livers, pancreases and salivary glands from young (8-10) or aged WT and Irf4-cKO mice (40-48w) were isolated and fixed in 4% paraformaldehyde in PBS, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. Images were obtained with Eclipse C*i* microscope (Nikon).

4.6. Statistics

Statistical significance for flow cytometry and RT-PCR analysis was determined by a two-tailed unpaired t-test and for infiltrates by two-tailed Mann-Whitney U-test using Prism GraphPad Software (La Jolla, CA).

5. RESULTS

5.1. Study I

5.1.1. CNS1 is indispensable for the thymic expression of *Aire* and *Dnmt3l*

A highly conserved region of 90 nucleotides speculated to regulate *Aire* expression lies halfway between the *Aire* and the *Dnmt3l* genes on chromosome 10 in the mouse genome (Blechschmidt et al. 1999). This CNS (named CNS1 here) contains two well-conserved 10 bp motifs (Figure 5) separated by 7-9 nucleotides in most mammals analyzed (41/48). JASPAR database analysis of transcription factor binding sites identified three NF-κB factors (c-rel, Rela and p50) to be the most likely candidates to bind the CNS1 in the distal promoters of both *Aire* and *Dnmt3l* (Study I).

To determine whether this region has an *in vivo* function in regulating *Aire* expression, we generated mice with a deletion of 45 bp containing both conserved sites (Figure 5) and studied *Aire* expression in the thymus and peripheral tissues that have been shown to contain *Aire*-expressing cells (Gardner et al. 2008, Schaller et al. 2008, Yamano et al. 2015). The expression of *Aire* was abolished in the thymi, spleens and lymph nodes of CNS1-KO (Figure 6A), but unlike in Aire-KO (Figure 6B), was not affected in the testis. We saw a similar pattern in the expression of *Dnmt3l* (Figure 6C), which is a tissue-specific methyltransferase expressed mainly in gonocytes (Webster et al. 2005) but has also been detected in thymus (Aapola et al. 2000).

We next verified the decreased *Aire* expression in sorted TEC-subsets. *Aire* mRNA was highly expressed in the $mTEC^{hi}$ population from WT mice, but was barely detectable in the $mTEC^{hi}$ from CNS1-KO mice (Figure 6D). The low-level *Aire* mRNA expression identified in WT $mTEC^{lo}$ most likely derives from the carryover of $mTEC^{hi}$ fraction during cell purification or by the residual expression of Aire in the $mTEC^{lo}$ subset. We also performed flow cytometry analysis to verify whether the absence of *Aire* mRNA in CNS1-KO was also mirrored on protein level. Analysis of Aire expression in UEA^{+} mTEC revealed the $mTEC^{hi}$ from CNS1-KO also lacked the expression of Aire protein (Figure 6E). Collectively, these results demonstrate that *Aire* expression in lymphoid tissues critically depends on CNS1.

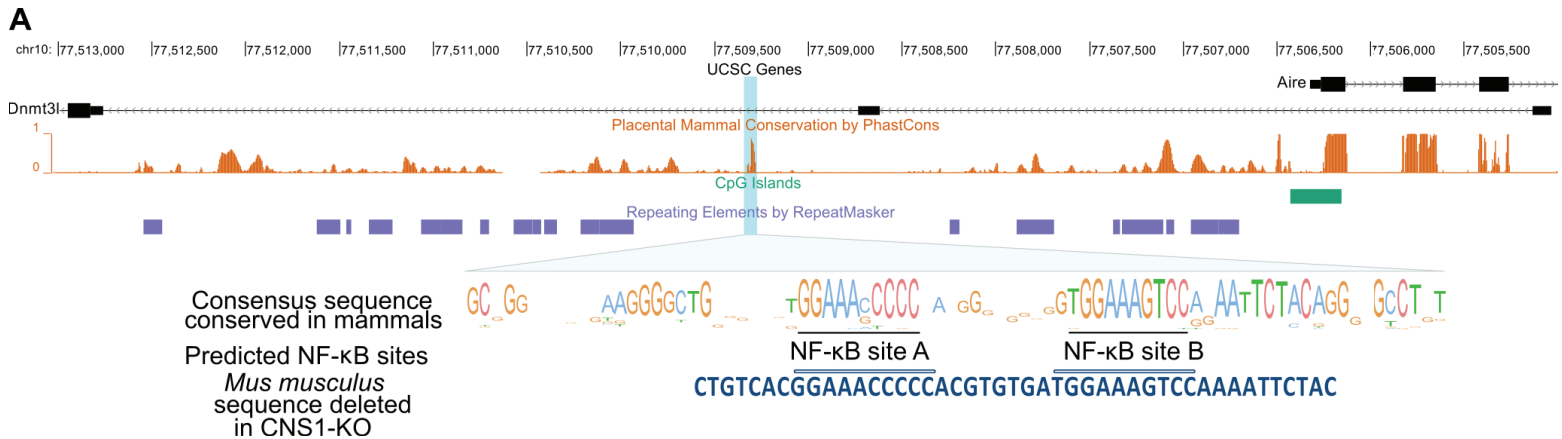

Figure 5. The region upstream of the *Aire* gene contains conserved region with two NF-κB binding sites (modified from Study I). UCSC Genome Browser view of the mouse genomic region between the *Aire* and *Dnmt3l* genes. The area highlighted light blue depicts the CNS1 region that contains two highly conserved binding sites for NF-κB factors (site A, site B).

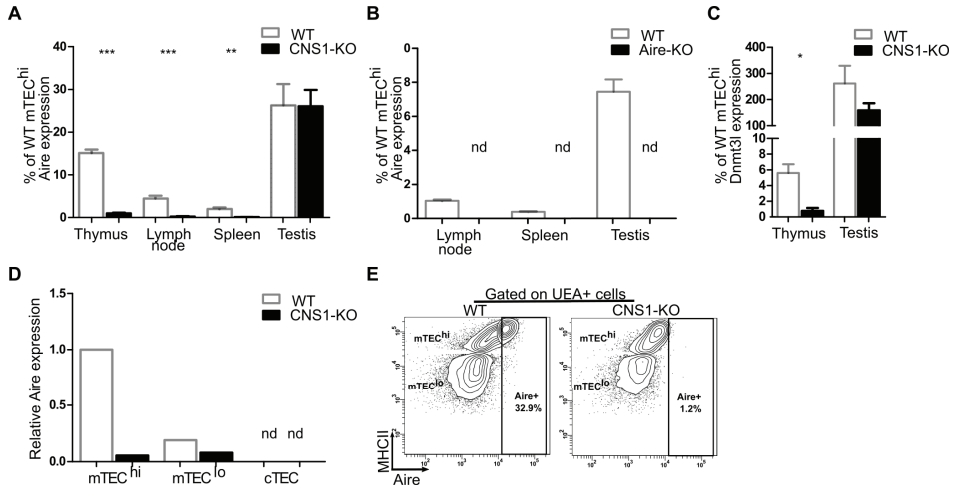

Figure 6. The deletion of CNS1 region blocks *Aire* expression in the mouse immune cells. The relative *Aire* mRNA expression from whole tissues of WT and CNS1-KO **(A)** and Aire-KO mice **(B)** normalized to WT mTEC^{hi} expression. **(C)** The relative *Dnmt3l* mRNA expression from whole tissues of WT and CNS1-KO mice. Data **(A-C)** are shown as the means + SEM from six (spleens, lymph nodes) or three mice (thymi, testes) per genotype from a single experiment. **(D)** Relative *Aire* mRNA expression in sorted thymic stromal cells from WT and CNS1-KO mice was determined by RT-PCR; nd, not detected. **(E)** Percentage of Aire-expressing cells in WT and CNS1-KO UEA^{+} populations from six pooled thymi was determined by flow cytometry. **p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, unpaired t-test, two-tailed.

5.1.2. Deletion of the Aire CNS1 results in an Aire-KO thymic phenotype

In mTEC^{hi} thousands of genes are induced by Aire (Sansom et al. 2014). Multiple genes involved in mTEC differentiation (Yano et al. 2008, Wang et al. 2012) and thymocyte migration (Laan et al. 2009, Lei et al. 2011) are expressed in an Aire-dependent manner, but most notably Aire is known to regulate the expression of TSA (Anderson et al. 2002, Sansom et al. 2014). To determine whether the lack of Aire expression in the thymi of CNS1-KO results in a similar phenotype as Aire-KO, we analyzed the mTEC subtypes by flow cytometry and studied the expression of Aire target genes in sorted mTEC^{hi} by RT-PCR. Our flow cytometry analysis of UEA^{+} cells revealed the mTEC^{hi} population to be increased in CNS1-KO (Figure 7A). This is similar to our results from Aire-KO (Figure 7B) and those reported earlier (Anderson et al. 2002, Gray et al. 2007a).

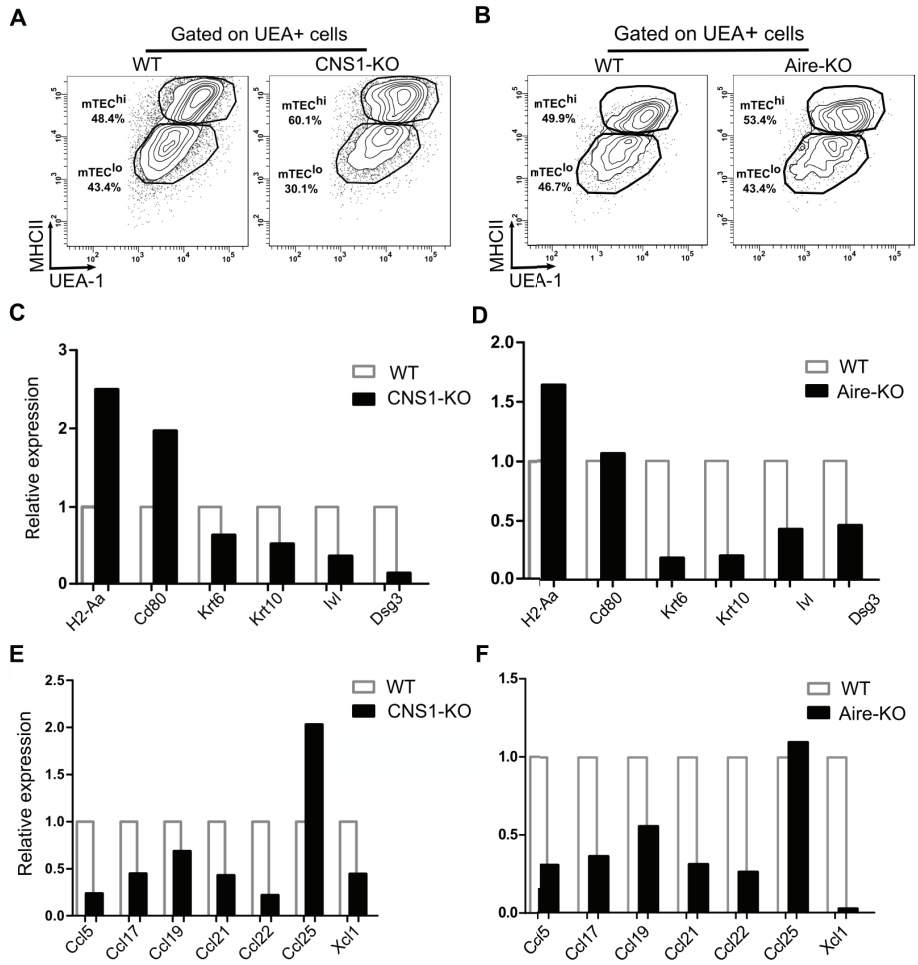

Figure 7. CNS1-KO and Aire-KO mice have similar thymic phenotypes. The percentages of mTEC subpopulations in WT and CNS1-KO **(A)** and Aire-KO mice **(B)** were evaluated by flow cytometry. Plots shown are representative of three independent experiments with pooled thymi (4-6 mice per genotype). Relative expression of Aire-dependent maturation markers in CNS1-KO **(C)** and Aire-KO mice **(D)** sorted mTEC^{hi} population was measured by RT-PCR. The relative expression of Aire-dependent chemokines in sorted mTEC^{hi} populations from CNS1-KO (**E**) and Aire-KO mice (**F**) was determined by RT-PCR.

mTEC^{hi} are thought to differentiate past the Aire-positive stage (Nishikawa et al. 2010) with Aire expression being a pre-requisite for the terminal differentiation of mTEC^{hi} (Yano et al. 2008, Laan et al. 2009). Similarly to Aire-KO the expression of mTEC^{hi} terminal differentiation markers *Krt6*, *Krt10*, *Ivl* and *Dsg3* were decreased in the sorted mTEC^{hi} of CNS1-KO mice whereas the expression of *H2-Aa* (a MHCII gene) and *CD80* were increased (Figure 7C and

Figure 7D). The decreased expression of *Ccl5*, *Ccl17*, *Ccl22* (Ccr4 ligands); *Ccl19*, *Ccl21* (Ccr7 ligands); and *Xcl1* in CNS1-KO also phenocopies the mTEC^{hi} from Aire-KO along with increased *Ccl25* expression (Figure 7E and Figure 7F). Transcript analysis of mTEC^{hi} from CNS1-KO and Aire-KO show the expression of Aire-dependent TSA such as *Tff3*, *Spt1*, *Ins2*, *Saa1* to be substantially downregulated with negligible changes in the expression of *Csn2*, an Aire-independent TSA (Study I, Figure 3A and SI Figure 3A).

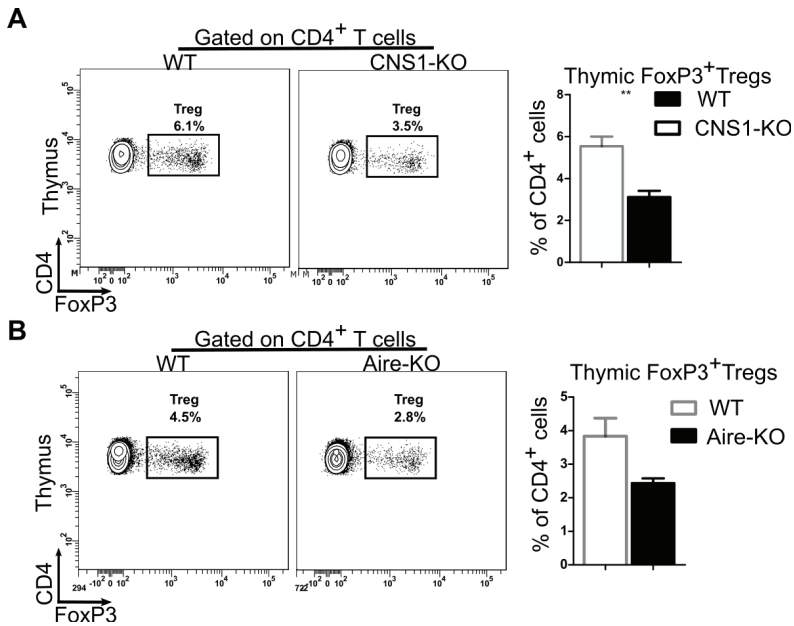

Figure 8. The percentages of Tregs in the thymi of WT and CNS1-KO **(A)** and Aire-KO mice **(B)** were determined by flow cytometry. Data are shown as the means + SEM of six mice per group from a single experiment. **$p \leq 0.01$, unpaired t-test, two-tailed.

Aire-dependent PGE is crucial in central tolerance homeostasis (Anderson et al. 2002). In addition to mediating thymocyte negative selection, presenting peptides from the induced TSA is required to drive the thymic differentiation of Tregs (Malchow et al. 2013, Yang et al. 2015b, Malchow et al. 2016). Consistent with previous findings (Malchow et al. 2013), the thymi of both CNS1-KO and Aire-KO harbored fewer Foxp3$^+$ cells among the CD4$^+$ T cell population (Figure 8A and Figure 8B). Surprisingly, when studying peripheral Treg populations, we found the Treg in the spleen to be more plentiful among CD4$^+$ T cells in CNS1-KO and Aire-KO (Study I, Figure 3I and SI Figure 3H). Thus, we conclude that the deletion of the newly described NF-κB-responsive enhancer element is sufficient to result in a thymic phenotype indistinguishable from Aire-KO.

5.2. Study II

5.2.1. Pregnancy results in a progressive loss of thymocytes and thymic nonlymphoid cells

During pregnancy the thymus decreases drastically mainly due to the reduction in thymocyte numbers (Clarke & Kendall 1994, Tibbetts et al. 1999, Zoller et al. 2007). We first verified the inverse correlation between body mass and thymic involution during pregnancy by weighing the mice and their thymi at each trimester. When compared to non-pregnant controls thymic involution becomes apparent during the second trimester (Study II, Figure 1A), peaking during the third trimester accompanied by drastic decreases in both the $CD45^+$ and $CD45^-$ populations (Figure 9A).

The decrease in thymic volume is ascribed to the universal drop in the total numbers of all major thymocyte populations accompanied by a development block at the DN1-DN2 thymocyte stage (Tibbetts et al. 1999, Zoller et al. 2007). We found no major changes in the distribution of the classic thymocyte populations (DN, DP, SP) during pregnancy, but the total numbers of these populations showed a progressive decrease when compared to non-pregnant controls. Our data showing the proportional increase of DN1 thymocytes within DN cells is in compliance with the previously reported developmental block of DN1-DN2 thymocytes during pregnancy (Tibbetts et al. 1999). However, the decreased total numbers of DN1 thymocytes indicate a reduction in TLP seeding (Study II, Figure 2A, and 2B).

One of the major mechanisms behind pregnancy induced immune tolerance is the accumulation of Tregs to the maternal-fetal interface (Aluvihare et al. 2004, Ruocco et al. 2014). The main Treg population responsible for inducing tolerance towards the semi-allogenic fetus is pTreg (Samstein et al. 2012). As a whole, the peripheral Treg population expands during pregnancy (Aluvihare et al. 2004), but whether this increase can only be attributed to the induction of pTreg is unknown. Therefore we evaluated the proportions and total numbers of Tregs in the thymus. Among thymic $CD4^+$ T cells, the proportion of $Foxp3^+$ T cells showed a slight but statistically significant increase by the third trimester in comparison with non-pregnant controls (Figure 9B and Study II, Figure 2C). By the third trimester, however, the total number of Treg had significantly declined (Figure 9C) in comparison with non-pregnant controls (Student's *t*-test: $p < 0.05$, $n = 6$).

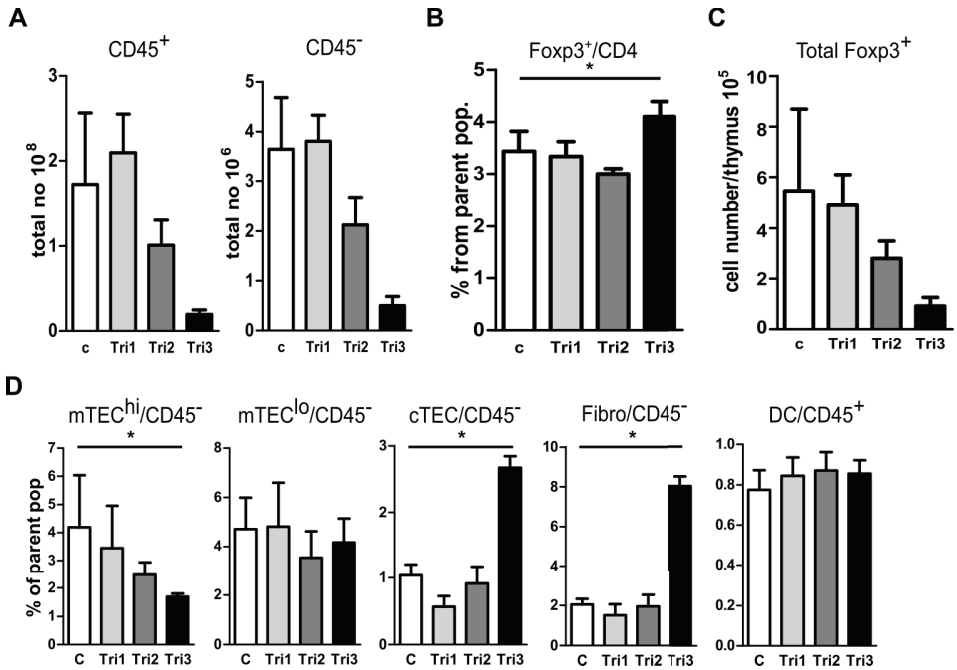

Figure 9. The effect of pregnancy on relative and total numbers of lymphoid and stromal cells. (A–D) C57BL/6 nonpregnant control mice (c) and mice during the first (Tri1), second (Tri2), and third (Tri3) trimesters of pregnancy were studied **(A)** Total numbers of thymic $CD45^+$ and $CD45^-$ cells were analyzed by flow cytometry (Spearman rank correlation between $CD45^+$ and the length of pregnancy: $p < 0.05$, $r = -0.6$, and between $CD45^-$ and the length of pregnancy: $p < 0.05$, $r = -0.8$). Relative numbers **(B)** and total numbers **(C)** of thymic $Foxp3^+$ Treg cells are shown (Spearman rank correlation between the length of pregnancy and the total number of Treg cells: $p < 0.05$, $r = -0.9$). Data (A, B, C) are shown as mean + SEM (n = 3 mice/group) and are from a single experiment. **(D)** Relative numbers of the indicated stromal cell populations were measured by flow cytometry (Spearman rank correlation between the length of pregnancy and the total numbers of all stromal cell populations measured: $p \leq 0.05$, $r \geq -0.6$. *Student's *t*-test, $p < 0.05$). Data are shown as mean + SEM ($n = 3$ mice/group) and are from a single experiment.

As with thymocytes, the effect of normal pregnancy on the thymic non-lymphoid cells becomes apparent during the second trimester of pregnancy. The total numbers of all the five major stromal cell populations analyzed ($mTEC^{hi}$, $mTEC^{lo}$, cTEC, fibroblasts and DC) showed a progressive decrease during pregnancy (Study II, Figure 3A). However, substantial differences arise in the cellular composition of thymic non-lymphoid cells between the non-pregnant controls and mice during the third trimester of pregnancy. There were no changes in the proportions of DC among $CD45^+$ cells. $mTEC^{hi}$ decreases drastically in numbers and also in proportion among $CD45^-$ cells. The pro-

portion of mTEC^{lo} remained unchanged whereas the proportions of cTEC and fibroblast were significantly increased by the third trimester (Figure 9D).

5.2.2. Pregnancy hinders TEC proliferation and TLP seeding

In ARTI the loss of thymic tissue is associated with a downregulation of the activity of a transcription factor *E2f3* in mTEC^{lo} and cTEC resulting in nonproliferating TEC-populations (Ki et al. 2014). Similarly we found *E2f3* mRNA to be critically downregulated in the same highly proliferative cell populations in PRTI (Figure 10A). Contrary to ARTI (Ki et al. 2014) we witnessed a slight decrease in the expression of inflammatory cytokines *IL1b* and *IL6* in DC and fibroblasts, respectively. In addition to the decreased expression of *Dll4* reported in ARTI (Ki et al. 2014), we found the expression level of *Kitl* and *IL7* also to be downregulated in late pregnancy, suggesting a potential role for the stromal cell-provided microenvironment in the pregnancy-induced block in thymocyte development and proliferation.

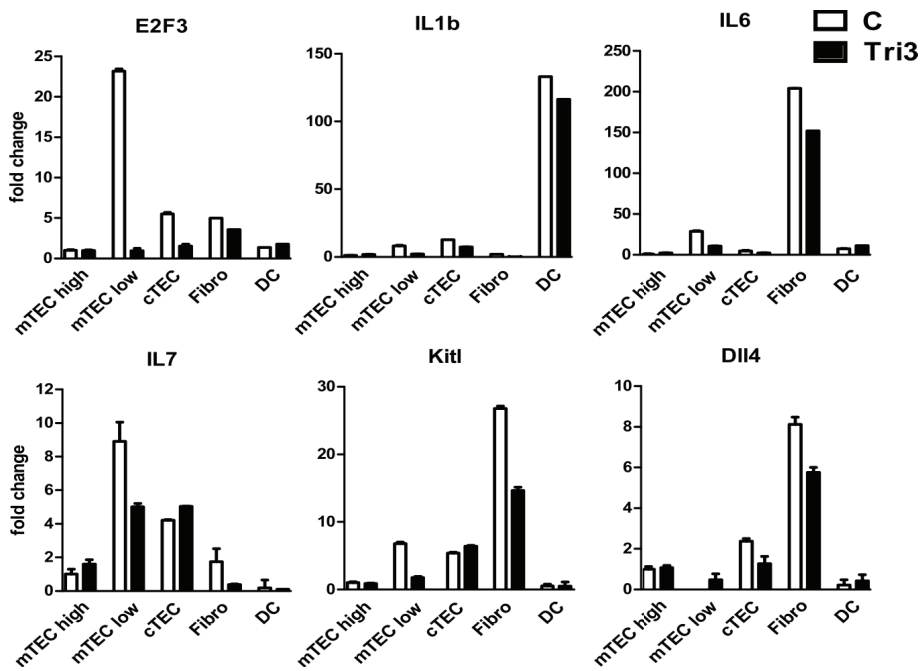

Figure 10. The effect of pregnancy on thymic nonlymphoid cells and dendritic cells (DCs). Gene expression profiles in different stromal cell populations in control mice (C) and in pregnant mice during the third trimester (Tri3). Data are shown as means + SEM of three technical replicates of one experiment performed in a pooled sample from four to six mice per group.

5.2.3. Pregnancy induces a decrease in the expression of ligands for Ccr9, Cxcr4, and Ccr7

In order to delineate the contribution each stromal subset has in producing chemokines required for TLP homing, we analyzed the expression of ligands for Ccr7 (*Ccl19* and *Ccl21*), Ccr9 (*Ccl25*) and Cxcr4 (*Cxcl12*) in the purified thymic stromal sets. Each chemokine measured had its distinct cellular source: *Ccl25* was predominantly produced by cTEC, *Cxcl12* by fibroblasts and cTEC, *Ccl21* by mTEC^{lo}, and *Ccl19* by mTEC^{hi} (Figure 11). In the same populations isolated from pregnant mice we observed a decrease in the production of all chemokines by their respective predominant cellular source by late pregnancy (Figure 11).

Previous reports have indicated either estrogen or progesterone in pregnancy-induced thymic involution (Tibbetts et al. 1999, Zoller et al. 2007). To determine whether either of the hormones mediates the changes in thymic stromal chemokine expression pattern, we analyzed purified thymic stromal cells from non-pregnant mice treated with either estrogen or progesterone. Contrary to estrogen treatment, short-term treatment with progesterone resulted in a significant decrease in *Ccl25* and *Cxcl12* expression in cTEC and *Ccl21* expression in mTEC^{lo} (Study II, Figure 5) suggesting that the pregnancy-induced suppression in chemokine expression is at least partly mediated by progesterone.

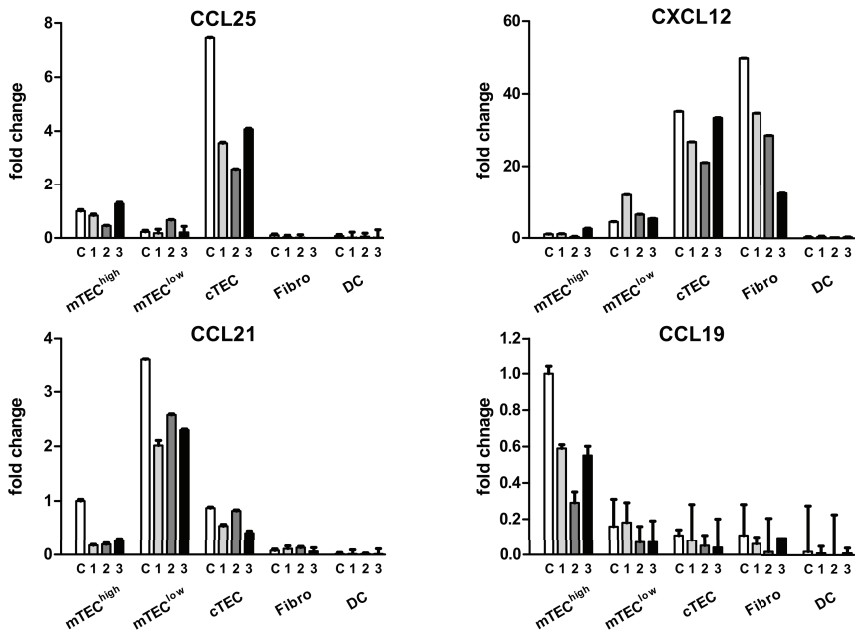

Figure 11. The effect of pregnancy on chemokine expression in thymic stromal cells. The expression levels of chemokines were measured by RT-PCR in different thymic stromal populations purified from the thymi of non-pregnant C57BL/6 control mice (C) and from pregnant mice during the first (1), second (2), and third (3) trimesters. Data are shown as mean + SEM of three technical replicates of one experiment performed in a pooled sample from four to six mice per group.

Collectively, these data indicate that in thymic stromal subsets the events during pregnancy result in a widespread downregulation in the expression of multiple proteins required for the homing and progenitor niche of the TLP.

5.3. Study III

5.3.1. RANK signaling induces Irf4 in the thymic epithelium

As the differentiation and antigen presenting capacity of peripheral APC such as DC, B cells and Mϕ is impaired in Irf4-deficiency (Nam & Lim 2016), we aimed to characterize the role of Irf4 in TEC that represent epithelial APC with pivotal role in central tolerance induction. Our RT-PCR analysis from sorted WT TEC revealed that all the major TEC-subsets express *Irf4* mRNA with highest levels in mTEC^{hi} (Figure 12A). The differential expression of *Irf4* mRNA was also mirrored on the protein level with roughly two-thirds of the mTEC^{hi} and up to a third of both mTEC^{lo} and cTEC being positive for Irf4 expression (Figure 12B).

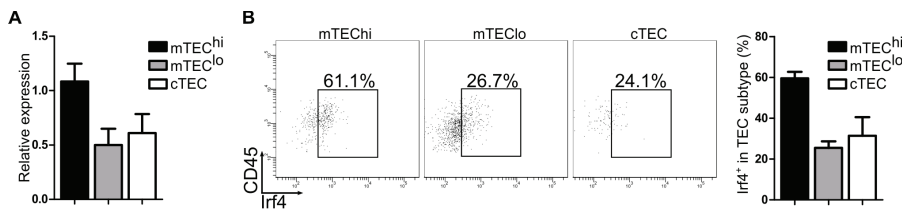

Figure 12. Thymic epithelium constitutively expresses Irf4. **(A)** Relative *Irf4* mRNA expression level in WT TEC subsets. **(B)** Irf4 protein expression in WT TEC subsets. Data are representative of two experiments from pooled WT thymi from four mice and are shown as means + SEM.

Developing thymocytes produce several proteins shown to regulate the maturation and gene expression profile of the thymic epithelial compartment (Akiyama et al. 2012). To identify the specific ligands for TNFSF receptors that drive *Irf4* expression in the thymic epithelium we used fetal thymic organ cultures (FTOCs), which we stimulated with several ligands known to have a functional role in TEC maturation. Among the TNFSF-members analyzed (RANKL, CD40L, Light, TNFα, LTβR agonist), we found RANKL to be the only TNFSF-member to induce *Irf4* mRNA in thymocyte-depleted FTOC (Study III, Figure 1C). The costimulation of FTOC with RANKL alongside with CD40 or LTβR has been shown to have a synergistic effect on the expression of *Aire* and *Fezf2* genes in TEC (Bichele et al. 2016); however *Irf4* expression was not increased in FTOC following either of the costimulations (Study III, Figure 1C).

As RANK-signaling can activate both the classical and non-classical NF-κB pathway, we wanted to determine the predominant pathway in thymic epithelium-specific *Irf4* expression. Therefore we treated RANK-induced FTOCs either with TPCA-1, a classical NF-κB pathway inhibitor with high selectivity for IKK-β over IKK-α or a selective NIK inhibitor (isoquinoline-1,3(2H, 4H)-dione), which blocks the alternative NF-κB pathway. Even low concentrations of the IKK-β inhibitor had a substantial effect on *Irf4* expression (Study III, Figure 1D), whereas using the NIK inhibitor resulted in a non-significant but strongly trending decrease in *Irf4* expression.

Based on these results we conclude that the so far undescribed Irf4 expression in TECs is constitutive and components from the classical NF-κB pathway are critical for the activation of Irf4 downstream of RANK-RANKL binding.

5.3.2. Irf4 regulates the maturation of thymic epithelium

To establish the role of Irf4 in thymic epithelium we next generated TEC-specific Irf4-deficient mice by crossing $Irf4^{fl/fl}$ mice (Klein et al. 2006) (referred from hereon as WT) with FoxN1:Cre mice (kind gift from T. Boehm). According to Mendelian distribution, a proportion of the F2 progeny of these mice are deficient in Irf4 expression in FoxN1:Cre expressing cells (referred to here as Irf4-cKO) and express GFP in tissues co-expressing FoxN1:Cre and Irf4. We verified the induction of GFP expression in TEC subtypes by flow-cytometry and found the levels of TEC subtypes in Irf4-cKO expressing GFP (Figure 13A) and Irf4-expressing TEC in the WT mice (Figure 12B) to be comparable.

According to our immunofluorescence analysis (Figure 13B), in WT mice Irf4 is predominantly expressed in the thymic medullary compartment by $Krt14^{+}$ cells. In Irf4-cKO, however, Irf4 is expressed on a substantially lower level and its expression is mainly restricted to $Krt14^{-}$ cells that could possibly represent $CD69^{hi}$ T cells or thymic DC (Cao et al. 2010, Yamamoto et al. 2011, Nayar et al. 2012). We carried out a flow cytometric analysis of the thymic stromal compartment of WT and Irf4-cKO to find the only differences in the stromal compartment to be the significantly decreased $mTEC^{lo}/mTEC^{hi}$ ratio in the Irf4-cKO mice (Figure 13C and Study III, SI Figure 1D). Our immuno-fluorescence analysis for mTEC marker Krt14, mature mTEC marker UEA-1, or terminally differentiated TEC positive for Ivl revealed no robust differences between the genotypes (Study III, Figure 2C). Therefore, based on our flow cytometry results we conclude that Irf4 regulates the differentiation from the $mTEC^{lo}$ subpopulation to $mTEC^{hi}$.

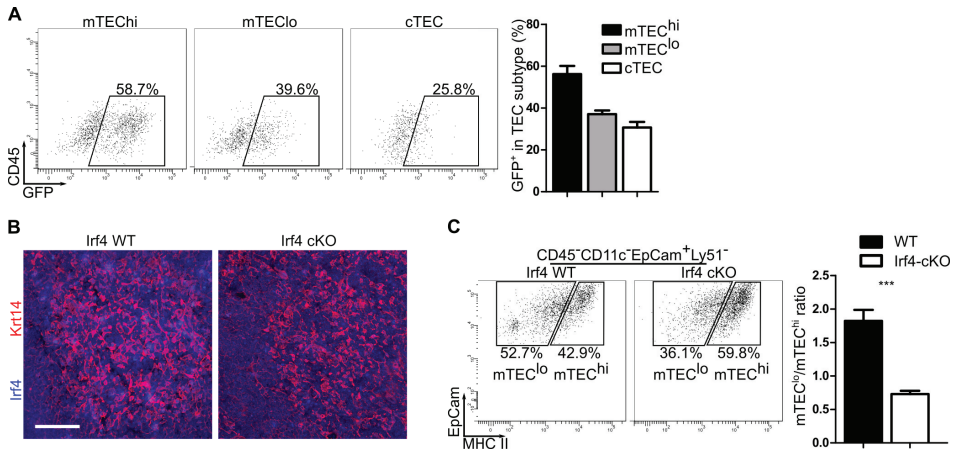

Figure 13. Irf4 regulates mTEC maturation. **(A)** GFP-protein expression in Irf4-cKO TEC subsets occurring after the excision of the first two exons of *Irf4*. **(B)** Thymic sections from WT and Irf4-cKO mice were stained for Irf4 (blue) and Keratin 14 (red). Images shown are representative of three mice per genotype from two independent experiments. Scale bar is 100 µm. **(C)** The percentages of mTEC subpopulations in WT and Irf4-cKO mice were evaluated by flow cytometry. Plots shown for **(A)** and **(C)** are representative of four independent experiments. Data for **(A)** and **(C)** are shown as the means + SEM from four independent experiments with pooled thymi (n = 3–6). ***$p \leq$ 0.001, unpaired t test, two-tailed.

5.3.3. Irf4 expression is essential for priming thymic epithelium into efficient Treg inducers

To establish whether the altered Irf4-deficient mTEC profile influences thymocyte differentiation, we analyzed thymocyte and peripheral T cell subpopulations by flow cytometry. Our results from 8-10-week-old mice imply that the intrathymic differentiation of the major thymocyte populations (DN subpopulations, DP; and $CD4^+$ and $CD8^+$ thymocytes) is unperturbed in Irf4-cKO. Likewise, we found no differences in the distribution of mature $CD4^+$ and $CD8^+$ T cell populations in the spleens and inguinal lymph nodes of the Irf4-cKO mice (Study III, Figure 3A; and data not shown). However, analysis of the thymic $Foxp3^+$ expressing Tregs among $CD4^+$ T cells revealed a 50% decrease in this population in Irf4-cKO (Figure 14A). This phenotype did not extend to the periphery, as we found the percentages of splenic Tregs among $CD4^+$ T cells in Irf4-cKO to be restored to WT levels (Study III, Figure 3C). Arguing for a homeostatic peripheral compensation is the fact that the total Treg numbers that were decreased in the thymi of Irf4-cKO were also restored to WT levels in the spleens (Figure 14B).

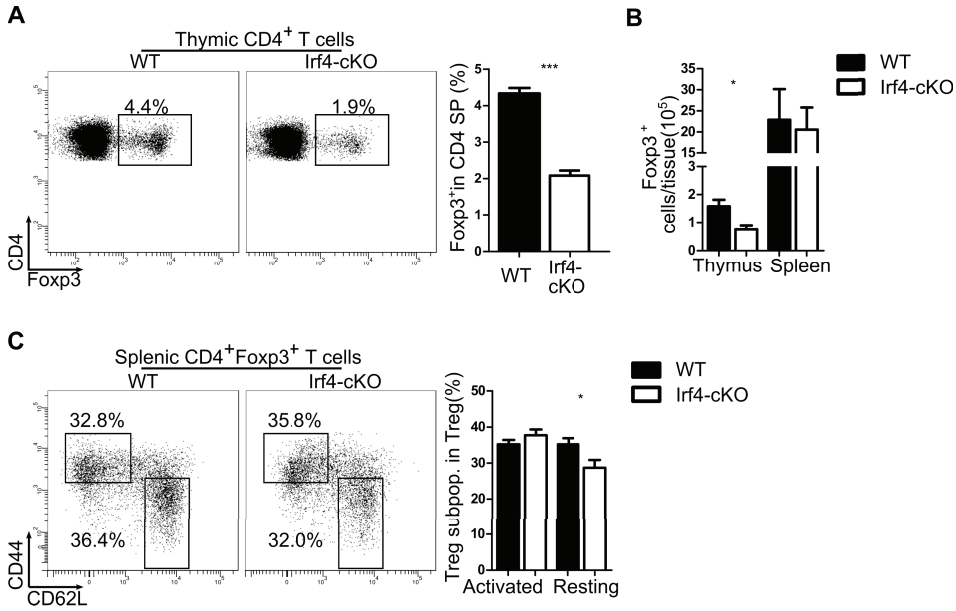

Figure 14. Irf4 primes TEC for Treg induction. Percentages of Foxp3$^+$ Tregs among thymic CD4$^+$ T cells **(A)** were evaluated by flow cytometry. Plots shown are representative of three independent experiments (n = 4–6). **(B)** Absolute numbers of Foxp3$^+$ Tregs from the thymi and spleens of WT and Irf4-cKO mice from two independent experiments (n = 4). **(C)** Distribution of activated and resting Tregs in the spleens of WT and Irf4-cKO mice. Plots shown are representative of two independent experiments (n = 4–6). Data (A–C) are shown as the means + SEM. *$p \leq 0.05$, ***$p \leq 0.001$, unpaired t test, two-tailed.

We hypothesized that the decrease in Tregs observed in the thymus could be the result of decreased proliferation; therefore we analyzed Ki-67 expression in Tregs. Contrary to our expectations, Tregs in both the thymus and spleens of Irf4-cKO expressed higher levels of the proliferation marker Ki-67 (Study III, Figure 3E). To differentiate between the resting and activated Treg phenotypes, we also analyzed the expression of CD44 and CD62L on the splenic Tregs from WT and Irf4-cKO. We found no differences in the activated CD44$^+$CD62L$^-$ Tregs. However we witnessed a subtle but significant decrease in the resting CD44$^-$CD62L$^+$ splenic Tregs in Irf4-cKO (Figure 14C).

5.3.4. Irf4-deficiency results in an increased induction of pTregs and peripheral mononuclear infiltrations

tTreg that are induced by central tolerance in the thymus express both Nrp1 and Helios in steady state, whereas pTregs, induced by peripheral tolerance mechanisms, do not. Using either protein alone as a tTreg marker has been

shown to be controversial (Lin et al. 2013, Szurek et al. 2015), therefore we defined tTregs as Tregs positive for both these markers as suggested in (Lin et al. 2013). To determine the mechanisms leading to the peripheral compensation of Tregs, we assessed the prevalence of Helios^{+}Nrp^{+} tTregs among all Tregs in WT and Irf-cKO mice. We found no differences in the proportions of tTregs or pTregs among the bulk Treg population (Study III, Figure 4A). However, whereas among thymic CD4^{+} T cells both pTregs and tTregs had decreased, only the proportion of pTregs (Figure 15A) showed a significant increase among the splenic CD4^{+} T cells. More specifically, we found this increase to be associated with the increase of activated pTregs among Tregs in Irf4-cKO (Figure 15B). Furthermore, we found the decrease seen in resting bulk splenic Treg population (Figure 14C) to derive from the reduced population of resting tTregs in Irf4-cKO (Figure 15B).

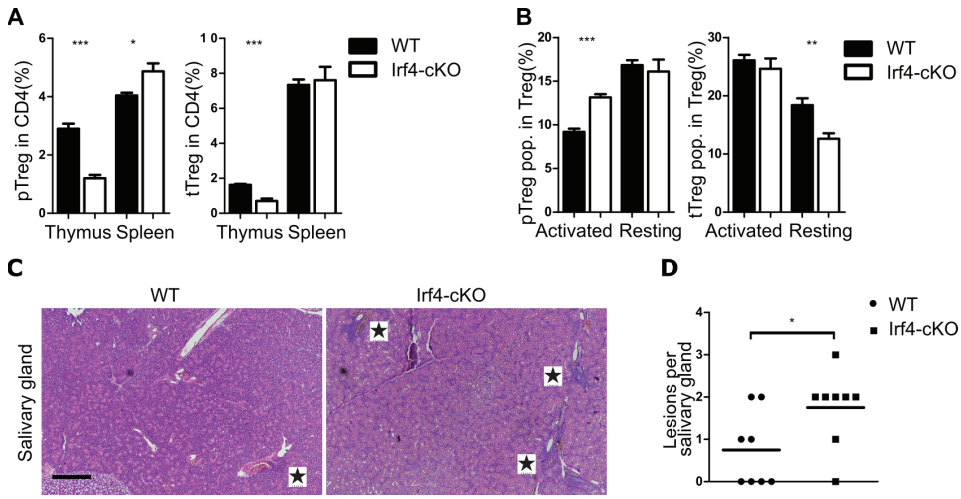

Figure 15. Irf4-cKO mice have increased induction of pTregs and mononuclear infiltrations in the salivary gland. **(A)** Distribution of pTregs and tTregs among thymic and splenic CD4^{+} SP cells was evaluated by flow cytometry. **(B)** Distribution of activated and resting tTregs and pTregs among Tregs in the spleens of WT and Irf4-cKO mice was evaluated by flow cytometry. Data (A–B) are shown as the means + SEM from a single experiment with six mice per genotype. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, unpaired *t* test, two-tailed. **(C)** H&E-stained tissue slides from aged (40–48 wk.) WT and Irf4-cKO salivary glands. Infiltrations in the salivary glands are marked by a Blackstar. Images shown are representative of four mice per genotype from two independent experiments. Scale bar 500 µm. **(D)** Quantitation of salivary gland lesions from aged (40–48 w) WT and Irf4-cKO mice. Data are shown for salivary glands of individual mice with median (horizontal line). *$p \leq 0.05$, Mann–Whitney *U* test, two-tailed.

Although the peripheral Treg pool is compensated for in Irf4-cKO, we found aged (40–48 wk.) but not young (8–10 wk.) Irf4-cKO mice to develop mild inflammatory lesions in their salivary glands (Figure 15C) but not livers or pancreases (Study III, Figure 4D). The incidence of salivary gland inflammatory lesions in Irf4-cKO was significantly higher than in age-matched WT animals, whose lesions in salivary glands were less frequent (Figure 15D).

5.3.5. Irf4 induces Treg differentiation autonomously of Aire by altering chemokine and costimulatory molecule expression levels

Multiple aspects of the phenotype witnessed in Irf4-cKO resemble those present in Aire-KO, specifically the altered $mTEC^{lo}/mTEC^{hi}$ ratio (Matsumoto 2011), a reduced Treg population in the thymus (Malchow et al. 2013, Yang et al. 2015b) and inflammatory lesions in the salivary gland (Anderson et al. 2002). Aire and Aire-dependent promiscuous gene expression in $mTEC^{hi}$ has been shown to be involved in Treg differentiation through rescuing autoreactive thymocytes from apoptosis and diverting them toward the Treg lineage (Malchow et al. 2016). As we found *Irf4* to be upregulated in sorted $mTEC^{hi}$ from Aire-KO mice (Figure 16A), we asked whether the two phenotypes could be interconnected with one transcriptional regulator controlling the expression of the other. However, our RT-PCR from sorted $mTEC^{hi}$ and flow cytometric analysis of the $mTEC^{hi}$ population from Irf4-cKO failed to reveal differential expression of Aire both on mRNA (Figure 16B) and protein level with ~40% $mTEC^{hi}$ from both WT and Irf4-cKO expressing comparable levels of Aire protein (Study III, Figure 5C). Furthermore, although there were differences in the expression of Aire-dependent and independent antigens in $mTEC^{hi}$ of Irf4-cKO, there was no clear up- or downregulation of the groups of genes studied in Irf4-cKO (Study III, Figure 5D). Multiple proteins indicated in end-stage mTEC differentiation are controlled by Aire (Yano et al. 2008, Wang et al. 2012), however our RT-PCR analysis revealed the maturation programs of Irf4-cKO and Aire-KO to differ from one another. We found the $mTEC^{hi}$ from Irf4-cKO to express slightly decreased levels of the MHCII molecule *H2-Aa* and increased expression of terminal differentiation markers *Krt6* and *Ivl* (Figure 16C). In Aire-KO on the other hand, mTEC are arrested in the $mTEC^{hi}$ stage expressing high levels of *H2-Aa* and express significantly lower levels of *Krt6* and *Ivl* when compared to WT (Yano et al. 2008, Wang et al. 2012).

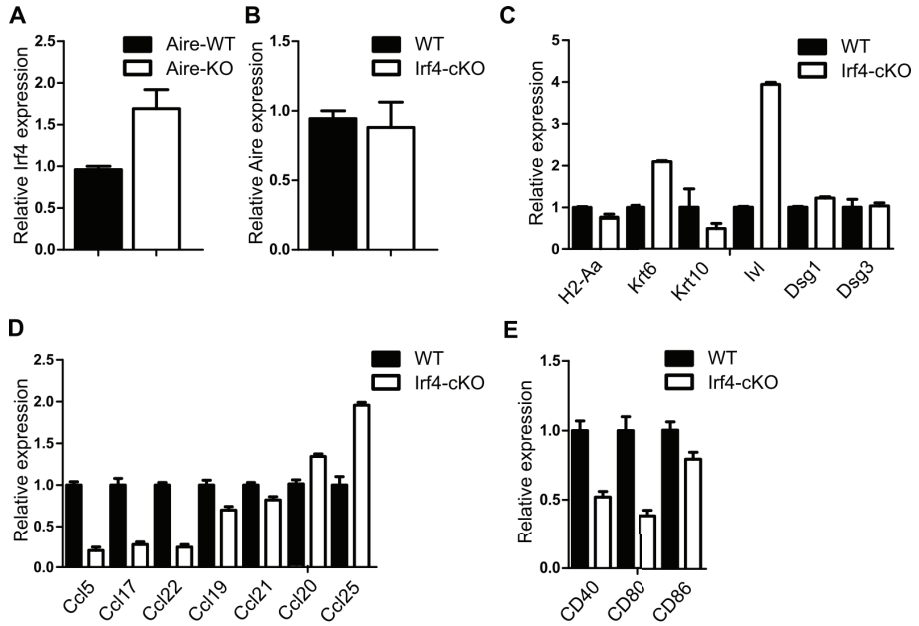

Figure 16. Irf4 regulates chemokine and costimulatory molecule expression in mTEC^{hi} independently of Aire. **(A)** Relative expression of *Irf4* mRNA in sorted mTEC^{hi} from Aire-KO. **(B)** Relative expression of *Aire* mRNA in sorted mTEC^{hi} from Irf4-cKO. Data (A and B) are shown as the means + SEM from two independent experiments from pooled thymi (n = 3–6). Relative expression of Aire-dependent and -independent maturation marker **(C)** and Aire-dependent chemokine **(D)** mRNA in the sorted mTEC^{hi} of WT and Irf4-cKO was measured by RT-PCR. Relative expression of costimulatory molecules **(E)** in the sorted mTEC^{hi} of WT and Irf4-cKO was measured by RT-PCR. Data (C-E) are shown as the means + SEM of three technical replicates from a single experiment from pooled sample (n = 8) and are representative of two experiments.

Our flow cytometric analysis dissecting the different thymocyte maturation stages showed the cTEC-dependent thymocyte differentiation steps to be unperturbed in Irf4-cKO (Study III, Figure 3A). Hence, we sought to determine the mTEC-specific factors regulating the migration and end-stage maturation steps of SP thymocytes. The entry of SP thymocytes into thymic medulla depends on the mTEC-specific secretion of several chemokines (Laan et al. 2009) that act as ligands for chemokine receptors expressed on thymocytes. The generation of Tregs in the medulla has been shown to depend on interactions between costimulatory molecules such as CD40, CD80 and CD86 expressed on mTEC^{hi} and their respective ligands on thymocyte cell surface (Williams et al. 2014). Therefore we used RT-PCR to analyze the gene expression levels of chemokines indicated in thymocyte entry to the medulla and costimulatory molecules required for Treg induction therein. The levels of *Ccl5*, *Ccl17*, and *Ccl22* that direct thymocyte migration into the medulla were drastically

decreased in the sorted mTEC^{hi} population from Irf4-cKO, with a subtle but consistent drop in the levels of *Ccl19 and Ccl21* (Figure 16D). The levels of *Ccl20* remained unaltered, whereas *Ccl25* was increased in the sorted mTEC^{hi} of Irf4-cKO. Our RT-PCR analysis of costimulatory molecule expression on mTEC^{hi} revealed a decrease in the expression of *CD40* and *CD80* with smaller changes in the expression of *CD86* (Figure 16E), which has common receptors on Tregs with CD80.

In conclusion, our analysis suggests that the thymic Treg phenotype witnessed in Irf4-cKO derives from the altered chemokine and costimulatory molecule expression pattern in the mTEC^{hi} population of Irf4-cKO mice.

6. DISCUSSION

6.1. CNS1 enhancer region regulates *Aire* expression in lymphoid tissues

The Aire protein represents an exceptional transcriptional regulator central in immune tolerance induction. It is well-established that Aire-mediated PGE is crucial in the generation of an immunocompetent T cell pool (Klein et al. 2014). This is best exemplified by the fact that mutations in the *Aire* gene are sufficient to cause grave immunodeficiency in human patients as well as in Aire-KO murine models (Consortium 1997, Nagamine et al. 1997, Anderson et al. 2002). However our understanding of the tightly controlled transcriptional mechanisms leading to *Aire* expression in mTEC^{hi} is only beginning to expand. For instance, it is known that in mTEC^{hi}, *Aire* expression is specifically upregulated following RANK-stimulation. RANK-signaling results in the non-specific activation of the classical and alternative NF-κB pathways, both of which have been shown to be required for mTEC maturation and Aire-expression in mTEC (Rossi et al. 2007, Akiyama et al. 2008, Hikosaka et al. 2008, Akiyama et al. 2012). Our study dissecting the role of a highly conserved genomic region in the *Aire* promoter is, however, the first to provide direct evidence of the differentiation-independent effect of the classical NF-κB pathway on *Aire* expression in mTEC^{hi}.

We show here that in mice lacking the CNS1 the expression of *Aire* is abolished in all lymphoid tissues where *Aire* expression has previously been detected. Interestingly, CNS1 appears to serve as an immune system specific enhancer for both *Aire* and *Dnmt3l* as we did not see a decrease in the expression of these genes in the testis of CNS1-KO. The function of Aire in the testis has not been described, but in general APS1-patients and Aire-KO mice are subfertile (Perheentupa 2006) and this phenomenon has been attributed to autoimmune rather than developmental effects (Kekäläinen et al. 2015). Nevertheless, contrary to Aire-KO, the preserved *Aire* expression in the testis from CNS1 mice makes this mouse model unique in studying the testis-specific activation and function of Aire.

In addition to mediating the transcription of various TSA genes in mTEC^{hi}, several genes indicated in mTEC maturation, thymocyte migration and thymic DC migration are expressed in an Aire-dependent manner (Yano et al. 2008, Laan et al. 2009, Nishikawa et al. 2010, Lei et al. 2011, Wang et al. 2012). It has also become clear that Aire is one of the mTEC-specific factors required for intrathymic Treg differentiation (Malchow et al. 2013, Yang et al. 2015b, Malchow et al. 2016). We show that similarly to Aire-KO, mTEC in CNS1-KO are skewed towards the mTEC^{hi} population with elevated levels of *H2-Aa* and *CD80* expression. The expression of genes inherent to terminally differentiated mTEC, on the other hand is decreased in CNS1-KO, indicating developmental block in mTEC differentiation similar to Aire-KO. Similarly, the aberrant

expression of Aire-dependent chemokines and TSA in mTEC^{hi} of CNS1-KO is close to indistinguishable from that of Aire-KO. Furthermore, both Aire-deficient mouse strains have a reduced proportion of thymic but not splenic Tregs suggesting a defect in Treg development dependent on central tolerance. Our study in CNS1-KO did not address the peripheral autoimmune phenotype present in Aire-KO mice. However, another group generated CNS1-KO mice similar to ours and demonstrated that these mice develop infiltrates in the retina as well as lacrimal and salivary glands already at 14-16 weeks of age (LaFlam et al. 2015).

In summary, our study identified a conserved non-coding element that is critical in mediating RANK-dependent upregulation of *Aire* in thymus and secondary lymphoid tissues.

6.2. Thymic involution during pregnancy is associated with changes in chemokine expression

The intrathymic differentiation of developing thymocytes is tightly governed by contacts established with thymic stromal cells. In addition, thymic stromal cells produce multiple molecules required for TLP seeding, as well as further migration and differentiation of thymocytes (Takahama 2006). It is known that during pregnancy thymic involution results in a substantial drop in all thymocyte populations (Tibbetts et al. 1999). What is unknown however is the role of thymic stromal cells in this process. Here we show that PRTI results in a substantial decrease in the numbers of all major thymic non-lymphoid cell populations. In addition, we identify the cellular sources of several key mediators of thymocyte maturation and migration in adult thymi and delineate the transcriptional changes taking place in these cell types during normal pregnancy.

TLP seeding to the embryonic thymus through the CMJ relies on the expression of Ccr9, Cxcr4, and Ccr7. After entering the thymus, early TLP are recruited towards the cortex, and migrate to the medulla following positive selection (Takahama 2006). As we identified cTEC and fibroblasts to be the predominant sources for Ccl25 and Cxcl12, we suggest the cortical entry of TLP to rely on the expression of Ccr9 and Cxcr4 in adult thymus. Our data also indicates that in adult thymus mTEC direct the migration of Ccr7-expressing positively selected thymocytes by producing Ccl19 and Ccl21. We demonstrate that during PRTI the mRNA levels of all these chemokines are progressively decreased in their respective predominant cellular source. In addition, we show short-term progesterone treatment to have an impact on the expression of *Ccl25* and *Cxcl12* in thymic stroma.

Thymic involution during aging is associated with thymic stroma-specific changes in the transcriptional profile mediating proliferation and TLP seeding (Ki et al. 2014). In both ARTI and PRTI the levels of *E2f3* are substantially decreased in TEC, resulting in a proliferative arrest of these cells. The only

molecule indicated in the reduced TLP seeding in ARTI is a Notch1-ligand Dll4 (Ki et al. 2014). In late pregnancy the levels of *Dll4* were reduced along with *Kitl* and *IL7* all of which are required for a functional thymocyte progenitor niche. As the numbers of all thymic stromal subsets are decreased by late pregnancy, the overall reduced production of the above-listed molecules by these cell types is likely to be a major contributor to the reduction in TLP seeding and differentiating thymocyte numbers during PRTI.

Newly generated pTregs specific for paternal antigens have a major role in inducing tolerance towards the developing fetus during pregnancy (Aluvihare et al. 2004, Samstein et al. 2012, Ruocco et al. 2014). In agreement with previous a report (Zoller et al. 2007) we found the proportion of bulk Foxp3$^+$ Tregs in the thymus to be slightly increased by late pregnancy. However, considering the five-fold reduction in total Treg numbers in the thymus, this proportional increase is unlikely to have a major contribution to the increased numbers of Tregs in the peripheral blood during pregnancy (Aluvihare et al. 2004, Ruocco et al. 2014). These results indicate that the elevated numbers of the peripheral Treg pool during pregnancy derive from the increased induction and/or proliferation of newly induced pTregs, increased proliferation of existing tTregs or the combination.

Collectively, we demonstrate that in addition to affecting TLP seeding to the thymus and all subsequent thymocyte populations, PRTI also results in a profound reduction in the cell numbers of all major thymic non-lymphoid cell populations. We propose that the progressive decrease of cTEC and fibroblasts in combination with their altered transcriptional phenotype provide a possible link between the effect progesterone has on TLP seeding and the developmental block at the DN1 stage during PRTI.

6.3. Irf4 shapes Treg homeostasis by regulating the expression of thymic chemokines and costimulatory molecules

The development of Tregs in the thymus and periphery requires a tolerogenic environment provided by APCs in the respective tissue or organ. Central tolerance induction in the thymus relies mainly on TEC, a rare and exceptional population of epithelial APC. However, our understanding of the transcriptional mechanisms rendering these cells capable of inducing tolerance in an autoreactive T cell is far from complete. Here we propose a model by which RANK-dependent activation of Irf4 and its target genes in TEC is required for the generation of Foxp3$^+$ Tregs in the thymus. Thus we provide another example of interdependent maturation and signaling of thymocytes and TEC in the thymus (Anderson et al. 2007, Rossi et al. 2007, Akiyama et al. 2008, Hikosaka et al. 2008, Klein et al. 2014).

We show that the expression of *Irf4* in TEC is specifically controlled by RANK-signaling which has been described in osteoclasts (Nakashima & Haneji 2013) but not in the immune system. Independent of the cell-specific external signaling, Irf4 induction in peripheral immune cells requires NF-κB activation. Previous studies have mainly implicated the importance of the classical NF-κB pathway in *Irf4* induction in peripheral APCs and lymphocytes (Grumont & Gerondakis 2000, Lehtonen et al. 2005). Although the extracellular stimuli leading to *Irf4* expression differ in peripheral immune cells (Gupta et al. 1999, Lehtonen et al. 2005, Saito et al. 2007, Carreras et al. 2010) and TECs, results from our NF-κB pathway inhibition experiment also indicate the dominant role of the classical NF-κB pathway in *Irf4* regulation in TECs. Additionally, inhibiting the alternative NF-κB pathway also lead to a statistically insignificant, although considerable reduction in *Irf4* expression in TEC. Therefore the alternative NF-κB pathway, shown to regulate *Irf4* in peripheral T cell lymphoma (Boddicker et al. 2015), must also be considered in TEC-specific *Irf4* induction.

TEC-specific Irf4-expression is required for the maintenance of the thymic Treg population, as we observed a 50% decrease in this population in our Irf4-cKO mice. We found no significant perturbations in thymocyte differentiation stages prior to the $CD4^+$ $Foxp3^+$ T cells. Thus, we theorized Irf4 to be specifically required for the function of mTEC that is the main population indicated in Treg differentiation. The Treg phenotype witnessed in the thymi of Irf4-cKO did not extend to the periphery, as we only found a slight decrease in the resting splenic Treg population. We found the peripheral compensation to rely on the increased proliferation of Tregs. Similarly to the thymic Treg population from mice deficient in the CNS3 of Foxp3 (Feng et al. 2015), thymic Tregs in the Irf4-cKO expressed higher levels of a proliferation marker Ki67 despite their decreased percentages. This finding indicates that although Tregs from both these KO-mice might be more prone to proliferate than their WT counterparts, mechanisms exist in the thymus that render them incapable of advancing into mitosis. Recently it was suggested that Tregs that recirculate back to the thymus inhibit *de novo* generation of Tregs (Thiault et al. 2015). However it is currently unknown whether these recirculating Tregs inhibit the induction or the proliferation of the already differentiated Treg cells. Either way, if intact in Irf4-cKO this repressive mechanism might prove significant in the Treg phenotype witnessed in Irf4-cKO.

Indicating that peripheral mechanisms have a dual role in the homeostatic compensation of splenic Treg population is the finding that Irf4-cKO mice have elevated levels of pTregs among $CD4^+$ T cells. The reduced resting tTreg population among splenic Tregs is likely to reflect the decreased thymic output, as the levels of activated tTregs were unchanged in Irf4-cKO.

Although Irf4-cKO mice have a significantly increased susceptibility to mononuclear infiltrations in the salivary gland, we did not find any other robust changes in the overall peripheral phenotype even in aged Irf4-cKO mice. This mild phenotype could possibly derive from Irf4 having overlapping functions with Irf8 or the genetic background of the C57BL/6 mice used in this study. The

increased susceptibility to salivary gland infiltrations along with the thymic phenotype of Irf4-cKO (skewed mTEC populations, decreased thymic Tregs) is reminiscent of the Aire-KO mice (Anderson et al. 2002, Malchow et al. 2013, Yang et al. 2015b). In addition, we found the chemokine expression profile of the mTEC^{hi} from both KO-mice to be similar. However, our analysis of the expression of mTEC maturation markers indicates, that unlike in Aire-KO, mTEC^{hi} in Irf4-cKO are not arrested in the mTEC^{hi} stage, as they also express high levels of terminally differentiated TEC markers such as *Ivl* and *Krt6*. Additionally, we did not find a consistent change in the expression of Aire-dependent and -independent antigens, suggesting that the Treg phenotype in Irf4-cKO does not occur due to aberrations in PGE. Providing a possible mechanism for the decreased Treg population in the thymus is our finding that mTEC^{hi} from Irf-cKO express substantially decreased levels of *CD40* and *CD80* mRNA when compared to WT. The changes in the expression levels of *CD86*, considered largely to be redundant with *CD80*, were considerably smaller.

In conclusion, our results show that thymic Treg induction requires RANK-dependent Irf4 expression in TEC, which in turn regulates the expression of several molecules indicated in Treg induction.

6.4. General discussion

The studies included in this thesis dissect the transcriptional mechanisms that shape central immune tolerance in the thymus. In the first study, we demonstrated *Aire* expression in mTEC to depend on an intergenic NF-κB-responsive enhancer element. Secondly, we delineated the effects pregnancy and pregnancy-related hormones impose on thymic stromal cells and how these effects might contribute to thymic involution during pregnancy. Lastly, we identified Irf4 expression in mTEC to be required for thymic Treg homeostasis. To emphasize how our research contributes to the studies of central tolerance induced in the thymus, the following section is dedicated to the connections between the studies and we also propose ideas for further research.

One of the most noticeable connections we found was the similarities between the thymic phenotypes of mice deficient in Aire (Aire-KO and CNS1-KO) and Irf4. For instance, the mTEC^{hi} populations in these mice have strikingly similar aberrations in their chemokine expression profile. In Aire-KO the decrease in mTEC-specific expression of Ccr4 and Ccr7 ligands results in delayed medullary migration of CD4^{+} thymocytes (Laan et al. 2009) and is likely to cause a similar effect in Irf4-cKO. Both these mice display decreased thymic Treg populations (Malchow et al. 2013, Yang et al. 2015b) (Study I, Study III). One of the factors determining the fate of an autoreactive thymocyte during negative selection is the presence of co-stimulation by surface molecules such as CD40 and CD80/CD86 on mTEC (Williams et al. 2014). Based on results presented here (Study I, Study III) and previously (Anderson et al. 2002), Aire is required for the gene and surface expression of the TSA, whereas

Irf4 appears to target the genes required to provide the co-stimulation to differentiate the antigen-bound autoreactive thymocyte into a Treg. Thus, these transcription factors control distinct steps in T cell-dependent central tolerance induction. In this respect it would be of interest to determine the effect on the thymic Treg population in mice deficient in both Aire and Irf4.

The expression of Ccr7 ligands was also perturbed in the mTEC from end-stage pregnant mice without a substantial effect on the proportions of Th or Treg populations (Study II). As these mice also presented decreased expression of TLP-homing chemokines in cTEC and fibroblasts, the acquired mTEC-specific chemokine expression pattern is possibly an adaptation to the overall decrease in the numbers of early thymocyte populations. Determining the transcription factors involved in the thymic stroma mediated PRTI however requires further research. Although it is known that all thymocyte subsets are decreased in PRTI, the transcriptional profile of the RANKL-producing T cells in pregnancy is unknown. Delineating the transcriptional networks in thymic crosstalk during pregnancy would expand our knowledge on what part does the central tolerance have in inducing and/or maintaining maternal-fetal tolerance. Furthermore, as PRTI and ARTI also have common phenotypic features in the thymic stroma, studies in this field might lead to treatments increasing thymic function in elderly patients suffering from various infections. In this light, it would be important to delineate the factors required for thymic tissue restoration in the mice that have recently given birth. *Ex vivo* administration of RANKL following bone marrow transfer was recently shown to boost the regeneration of TEC subsets and *de novo* thymopoiesis in mice (Lopes et al. 2017). In TEC, the expression of both *Aire* and *Irf4* are controlled by the classical NF-κB pathway, downstream of RANK-signaling (Study I, Study III), making them likely candidates contributing to the regeneration seen in these mice. However, as RANKL also induces osteoclast formation in an Irf4-dependent manner (Nakashima & Haneji 2013), using RANKL as a treatment in aged patients requires stringent optimization.

Collectively, the studies included in the current thesis broaden our general understanding of central tolerance mechanisms dependent on a miniscule epithelial compartment present in the thymus.

7. CONCLUSIONS

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

1. The highly conserved region found in the distal promoter of the *Aire* gene is a critical transcriptional regulator of *Aire* expression *in vivo*. The deletion of this region results in a thymic phenotype identical with that of Aire-KO mice.
2. PRTI results in a substantial decrease in the total numbers of all thymic non-lymphoid cell types with significant changes in their proportions within thymic stroma. This decrease is accompanied by changes in the transcriptional patterns of these cells. Most importantly, the cells responsible for TLP seeding downregulate the expression of molecules required for the homing and early differentiation of thymocyte progenitors.
3. Irf4 acts as a critical transcriptional regulator in murine TEC. In addition to controlling mTEC differentiation, it activates the transcription of several chemokines and costimulatory molecules critical for central tolerance dependent Treg induction.

8. SUMMARY IN ESTONIAN

Tsentraalse tolerantsuse eest vastutavad transkriptsioonilised protsessid tüümuses

Immuunsüsteemi toimimise üheks peamiseks alustalaks on võime eristada patogeenidest pärinevaid antigeene kehaomastest või ka organismile ohtu mitte kujutavatest võõrantigeenidest. Selle eristusvõime tagavad immuuntolerantsuse mehhanismid, mis jagatakse tolerantsust indutseeriva keskkonna põhjal kaheks: tsentraalne ja perifeerne immuuntolerantsus. Tsentraalse immuuntolerantsuse kujunemise kohaks on primaarsed lümfoidorganid; tüümus ja luuüdi. T-rakuline immuuntolerantsus kujuneb välja tüümuses, kus T-rakkude eellased (tümotsüüdid) läbivad nö „koolitusprogrammi“ ehk selektsiooni, mille käigus eliminеeritakse apoptoosi teel need T-rakud, mis reageerivad kehaomastele antigeenidele ja võiksid põhjustada autoimmuunvastust omaenda kudede vastu. Teise võimalusena suunatakse autoimmuunsed T-rakud supresseeriva funktsiooniga rakkudeks – regulatoorseteks T rakkudeks (Treg).

Oluliseks rakutüübiks tüümuses toimuva selektsiooniprotsessi tagamisel on tüümuse epiteelirakud, mis asetsevad organi koore- ja säsiosas ja tüümuse dendriitrakud. Selektsiooniprotsessi käigus moodustavad T-rakud rakk-rakk kontakte tüümuse epiteeli- ja dendriitrakkudega. Rakkudevahelised kontaktid tüümuse kooreosa epiteeliga määravad tümotsüütide arengu varasemad etapid, samas kui kontaktid tüümuse säsiosa epiteeliga on olulised autoimmuunsete tümotsüütide selekteerimiseks. Tüümuse epiteelirakud tagavad autoimmuunsete tümotsüütide selekteerimise selle kaudu, et nad ekspresseerivad suurt osa organismi koespetsiifilistest geenidest. Nende geenide poolt kodeeritud valkudest pärinevaid peptiide esitletakse epiteeli raku pinnal koesobivusantigeenide (MHC; *major histocompatibility complex*) poolt arenevatele tümotsüütidele. Autoimmuunse potentsiaaliga tümotsüütides käivituvad seejärel signaalirajad, mis suunavad need rakud apoptoosi või diferentseeritakse nad Treg rakkudeks. Kuna ka Treg rakkude funktsioon organismis on autoimmuunsete reaktsioonide ärahoidmine, siis toetavad mõlemad mehhanismid immuuntolerantsuse kujunemist.

Laialdase avatud geenieкspressiooni eest tüümuse säsiepiteelis vastutab valk nimega Autoimmuunsuse Regulaator (AIRE, hiires vastavalt Aire). AIRE-geenil on väga spetsiifiline avaldumise muster olles kõige kõrgemalt ekspresseerunud tüümuse epiteelis. Samas on seni olnud teadmata, kuidas reguleeritakse AIRE-geeni spetsiifilist ekspressiooni. See teema moodustab antud väitekirja esimese artikli. Väitekirja teine osa käsitleb tüümuses toimuvaid geeni avaldumise muutusi hiire tiinuse käigus. Areneval lootel on pooled geenid päritud isalt ja seega on loode ema immuunsüsteemi jaoks osaliselt võõrsiirik. Seega eeldab loote normaalne arenemine muutusi ema immuuntolerantsuse mehhanismides. Varasemast on teada, et nii tiinuse kui ka vananemise korral väheneb märgatavalt tüümuse maht ehk toimub tüümuse involutsioon. Samas ei ole seni uuritud tiinuse jooksul toimuvaid muutusi geenide avaldumises tüümuse epiteelis. Kolmandas osatöös on uuritud transkriptsioonifaktor Irf4 mõju tüümuse epiteelile ja Tregide diferentseerumisele.

Uurimistöö eesmärgid

1. Iseloomustada Aire-geeni oletatavas enhaanserjärjestuses asuvat evolutsiooniliselt konserveerunud regulaatorala funktsioon.
2. Uurida tiinusega seotud tüümuse involutsioonis toimuvaid muutusi erinevates tüümuse rakupopulatsioonides ning määrata tiinuse mõju nende rakkude geenikspressioonile.
3. Määrata Irf4 transkriptsioonifaktori roll tüümuse epiteeli arengus ja tsentraalse tolerantsuse indutseerimisel.

Uurimistöö peamised tulemused ja järeldused

Esimese töö eesmärk oli uurida *Aire* ekspressiooni reguleeriva enhaanseri funktsiooni. Eesmärgi täitmiseks aretati Tartu Ülikooli Katseloomakeskuses geenmodifitseeritud hiireliin, mille genoomist puudus oletatavast enhaanserjärjestusest 45-aluspaari pikkune DNA-lõik. Oma töös andsime antud piirkonnale nimeks konserveerunud mittekodeeriv järjestus 1 (CNS1; *conserved noncoding sequence*). Enhaanserala olulisuse määramiseks *Aire* geeni regulatsioonis analüüsisime reaalaja-PCRi abil CNS1-puudulikest (CNS1-KO) ja metsiktüüpi hiirte tüümusest, põrnast, lümfisõlmedest ja munanditest eraldatud mRNA baasil sünteesitud cDNAst *Aire* transkripti olemasolu. Kõigis CNS1-KO hiire kudedes, va munandites oli *Aire* ekspressioon oluliselt madalam kui metsiktüüpi hiire vastavates kudedes. Edasised voolutsütomeetrial ning immuunfluorestsentsil põhinevad meetodid kinnitasid, et CNS1-KO hiired ei ekspresseeri tüümuses ka Aire valku. Lisaks kinnitas FACSi meetodil (*fluorescence-activated cell-sorting*) sorditud tüümuse epiteeli rakkudele tehtud reaalaja-PCRi analüüs, et sarnaselt Aire-KO hiirele on CNS1-KO hiire tüümuse epiteelis häirunud avatud geenikspressioon. Täheldasime ka muutusi säsi epiteelirakkude diferentseerumises ja nende rakkude poolt vahendatavas kemokiinide ekspressioonis. Tümotsüütide voolutsütoomeetriline analüüs tuvastas ka, et sarnaselt Aire-KO hiirega on CNS1-KO hiire tüümuses oluliselt vähem Treg rakke. Kokkuvõtvalt võib öelda, et CNS1 piirkond on keskne *Aire* geeni avaldumiseks tüümuse epiteelis ning et Aire-puuduliku fenotüübi tekitamiseks piisas CNS1-piirkonna eemaldamisest hiire genoomist.

Teise eesmärgi saavutamine eeldas erinevate tüümuse rakupopulatsioonide isoleerimist kontrollhiirtest ja kolmes erineva tiinuse staadiumis olevatest hiirtest (1.–3. trimester). Tümotsüütide voolutsütomeetriline analüüs kinnitas varasemaid tulemusi, mille kohaselt väheneb tiinuse käigus kõigi tümotsüütide alapopulatsioonide arvukus. Uue leiuna kirjeldasime rasedusaegse involutsiooni mõju tüümuse epiteeli rakkudele ja nende omavahelistele proportsionaalsetele suhetele. Tuvastasime, et tüümuse epiteeli rakkude arvukus väheneb sarnaselt tümotsüütidele ja kolmandaks trimestriks on oluliselt muutunud ka epiteeli rakkude alapopulatsioonide omavaheline suhe. Leidsime, et rasedus/tiinus mõjutab suurel määral tüümuse epiteeli alapopulatsioonide geenikspressiooni profiili. Geenide hulgas, mille ekspressioon oli vähenenud, oli mitmeid kemokiine

ja faktoreid, mis meelitavad T-rakkude eellasi tüümusesse ja koordineerivad nende migratsiooni ja diferentseerumist tüümuse koore- ja säsiosas. Lisaks tiinusele toimub sarnane tüümuse involutsioon ka vananemise ajal. Vananemisest tulenevat tüümuse koe kadu ja sellega kaasnevat tüümuse funktsiooni langust peetakse üheks eakatel inimestel esinevate nakkushaiguste raske kulu põhjuseks. Leidsime, et kahel nii erineval füsioloogilisel põhjusel toimuvatel muutustel tüümuses on mitmeid ühiseid jooni tüümuse epiteeli geenide avaldumises. Seega võiksid antud töös kirjeldatud mehhanismid lisaks rasedusaegse immuuntolerantsuse muudatuste kirjeldamisele aidata mõista ja mõjutada ka tüümuse aktiivsuse langust eakatel patsientidel.

Kolmanda eesmärgi saavutamiseks ristasime omavahel geneetiliselt muundatud hiiri, mille tulemusel saime katseloomad, kellel puudus tüümuse epiteelirakkudes funktsionaalne *Irf4*-geen. Neis katseloomades, kelle tüümuse epiteelis *Irf4*-geenilt valku ei kodeeritud, tuvastasime mitmeid immunoloogilisi kõrvalekaldeid metsiktüüpi hiirtest. Ühe peamise leiuna avastasime, et nende hiirte tüümustes on Tregide populatsioon vähenenud kaks korda. Kuna Treg rakkude osakaal on oluline immuuntolerantsuse kujunemises, viitab selline muutus võimalikule autoimmuunsele protsessile ning näitab *Irf4* tähtsust autoimmuunvastuse vältimises. Võimaliku autoimmuunse reaktsiooniga kooskõlas leidsime neis hiirtes immuunrakkude infiltratsioone süljenäärmes. Töös otsisime ka põhjusi, miks on neis hiirtes vähem Treg rakke. Geenikspressiooni analüüsiga tuvastasime FACSi meetodil sorditud tüümuse epiteelist, et Irf4-puudulikes rakkudes on metsiktüüpi hiire rakkudega võrreldes oluliselt langenud mitmete Treg rakkude diferentseerumiseks vajalike kemokiinide ja kostimulatoorsete molekulide ekspressioon. Seega on Irf4 Aire kõrval üks väheseid teadaolevaid transkriptsioonifaktoreid, mille ekspressioon tüümuse epiteelis on vajalik Treg rakkudest sõltuva tsentraalse tolerantsuse tekitamiseks.

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Multiple aspects of supervising someone come with experience. I consider myself lucky enough to have been supervised by people who, in addition to having this experience, appear to have some sort of an inborn intuition on how to deal with inexperienced students. I believe the sole function of a plethora of neurons in my brain's cortex is to serve as a warehouse for information on how I have been supported and supervised by Prof. Pärt Peterson and Dr. Martti Laan during my PhD studies and earlier by Dr. Tõnis Org. These people have provided an excellent example on how to treat and oversee someone aspiring to find their way in science. This example will stay with me and I intend to act by it when supervising someone within or outside the academia.

During my years in the Molecular Pathology group I have received a massive amount of help from Dr. Kai Kisand. The way she relates to and does research and still finds time to mentor (almost?) all the students in our group is beyond admirable. I suspect that she has found some kind of a wormhole to a whole new dimension and operates outside our common understanding of time and space.

Our lives in the lab would be quite different without the support from our technical personnel. Thank you! I would especially like to thank our extremely versatile technician Maire Pihlap, without whom the often 12-hour experimental days would have extended much further into the night. Also, the incubation times during these days would have been immensely more boring.

Over the years I have understood that the general atmosphere we have in our lab deserves a lot of appreciation. Therefore I would thank all the former and present colleagues in our group for providing this environment of mutual respect and willingness to help each other entwined with an environment where we also remember to have a laugh at suited and ill-suited subjects.

I am greatly indebted to Prof. Adrian Liston and Dr. James Dooley from University of Leuven for their contribution to the Irf4-project. However, the effect of the experience I received while visiting the Translational Immunology laboratory extends much further than this single project. This experience has already, and will continue to positively influence my work in research.

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For many people, PhD studies involve periods of "ups" and "downs". For me, the studies began with a close to a 3-year period of the latter which, at times, would have been insufferable had it not been for Mariliis Haljasorg. The occurrence of the last 3 years of mainly "ups" of my PhD studies owes a great deal to her support, the time spent together, events we attended, hours of

thought-provoking arguments, cursing and laughing (at the fate of a PhD student, among other things). The fact that we still continue to do all these things has made the “ups”-period even better. Another person I hold highly responsible for recent upsides is our son Huko Haljasorg, who has a significant role in elevating the levels of endorphins and endocannabinoids (and sometimes adrenaline) in our bodies already for the better half of the “ups”-period.

PUBLICATIONS

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