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Autoimmune regulator: characterization of thymic gene regulation and promoter methylation

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LIST OF ORIGINAL PUBLICATIONS

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I **Kont V**, Laan M, Kisand K, Merits A, Scott HS, Peterson P. Modulation of Aire regulates the expression of tissue-restricted antigens. Mol Immunol. 2008 Jan;45(1):25–33.

II Laan M, Kisand K, **Kont V**, Möll K, Tserel L, Scott HS, Peterson P. Autoimmune regulator deficiency results in decreased expression of CCR4 and CCR7 ligands and in delayed migration of CD4+ thymocytes. J Immunol. 2009 Dec 15;183(12):7682–91.

III **Kont V**, Murumägi A, Tykocinski LO, Kinkel SA, Webster KE, Kisand K, Tserel L, Pihlap M, Ströbel P, Scott HS, Marx A, Kyewski B, Peterson P. DNA methylation signatures of the AIRE promoter in thymic epithelial cells, thymomas and normal tissues. Mol Immunol. 2011 Oct;

Contribution of Vivian Kont to original publications is following:

Study I: Participation in creating experimental designs, construction of plasmid and virus, performing laboratory experiments, analyzing the data;

Study II: Contribution with viral experiments and mouse samples;

Study III: Participation in creating experimental designs, performing the experiments, analyzing the data and writing the manuscript;

ABBREVIATIONS

AIRE/Aire	the human/mouse autoimmune regulator gene
AIRE/Aire	the human/mouse autoimmune regulator protein
ALPS	autoimmune lymphoproliferative syndrome
AP-1	activator protein 1
APECED	autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APC	antigen presenting cell
APS	autoimmune polyglandular syndrome
bp	basepair
CARD	caspase-recruitment domain
CBP	CREB-binding protein
CCL	CC-chemokine ligand
CHD	chromodomain, helicase, DNA binding
CpG	cytosine guanine dinucleotide
cTEC	cortical thymic epithelial cell
CycT1	cyclin Cemt1
DAXX	death-domain-associated
DC	dendritic cell
DN	double negative
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNMT	DNA methyltransferase
DSB	DNA-double stranded break
ES	embryonic stem
Ets	E-twenty-six
FACT	Facilitates Chromatin Transcription
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
HET	heterozygous
HDAC	histone deacetylase
HLA	human leukocyte antigen
HSR	homogenously staining region
IL-2Rα	interleukin-2 receptor alpha-chain
INO80	inositol requiring 80
Ins2	insulin 2
IPEX	immunodysregulation polyendocrinopathy enteropathy and X-linked inheritance syndrome
SWI/SNF	switching defective/sucrose nonfermenting
iPS	induced pluripotent stem cell
KPNB1	karyopherin B1
kb	kilobase
MCJ	methylation controlled DNAJ

MG	myasthenia gravis
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
mTEC	medullary thymic epithelial cell
Mup1	major urinary protein 1
NES	nuclear export signal
NFκB	nuclear factor κB
NF-Y	nuclear factor Y
NLS	nuclear localization signal
NUP93	nucleoporin 93
PARP1	poly(ADP-ribose) polymerase family, member 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHD	plant homeodomain
PIAS1	protein inhibitor of activated STAT 1
PML	promyelocytic leukemia
PRR	proline rich region
PTM	posttranslational modification
p-TEFb	positive transcription elongation factor b
RING	really interesting new gene
RTOC	reaggregated thymic organ culture
RUVBL2	RUVB-like protein 2
SLE	systemic lupus erythematosus
SMC1	structural maintenance protein 1
Sp-1	specificity protein 1
Spt1	Salivary protein 1
SWI/SNF	switching defective/sucrose nonfermenting
TCR	T cell receptor
TNF	tumor necrosis factor
TOP2a	topoisomerase (DNA) II alpha
TRIM28	tripartite motif-containing 28
TSA	tissue specific antigen
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
WT	wild type
XPO1	exportin 1

1. INTRODUCTION

Our immune system is composed of different specialized molecules, cells, tissues and organs, evolved to defend us against foreign substances and diseases. Simultaneously identifying foreign molecules the immune system must be sophisticated enough to differentiate between host's cells and remain tolerant to its components. Tolerance mechanisms are obligatory because our immune system randomly produces diversity of antigen-specific receptors, some of which might be self-reactive; and tolerance prevents harmful reactions against the body's own tissues. Failure of the immunological tolerance against self may lead to autoimmune diseases. The etiology of many autoimmune diseases is genetically complex and many genes act in concert to induce autoimmunity, and only rarely mutations in single gene cause autoimmune disease.

Recently it was found that mutations in a *AIRE* (Autoimmune regulator) gene are sufficient to elicit a rare multiorgan autoimmune disease known as APECED (Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy) syndrome, which is a good monogenic model to study development of tolerance towards self components and autoimmunity. APECED is characterized by a loss of immunological self-tolerance towards several endocrine organs including parathyroid and adrenal glands. AIRE is mainly expressed in medullary thymic epithelial cells (mTECs), where it directs the promiscuous (also known as open) gene expression of peripheral tissue-specific antigens by presenting them to developing thymocytes and thereby driving the negative selection of autoreactive T cells. Presentation of self-proteins via MHC on mTECs is necessary for central tolerance establishment. The additional involvement of thymic chemokines in the establishment of central tolerance has been proposed but the mechanisms are not clear. In the context of the tissue specific expression, it has been proposed that AIRE might be regulated on the level of epigenetic modifications including CpG methylation, chromatin modification, but the exact mechanisms remain still enigmatic.

Current thesis focuses on the role of mouse Aire gene in peripheral tissue-specific antigen and chemokine expression; and human AIRE promoter analysis in thymic epithelial cell populations, thymomas and normal peripheral tissue samples in order to gain insight, whether AIRE mTEC restricted expression is dependent on genomic methylation.

2. REVIEW OF LITERATURE

2.1. Immune system and tolerance

The immune system has developed to protect host from invading extracellular and intracellular pathogens. The immune tolerance is a property of the immune system not to attack specific antigens. Antigens are defined as molecules that are recognized by lymphocyte receptors on B and T cells. The tolerance can be devided into two types: central tolerance and peripheral tolerance. Central tolerance is related to lymphocyte development and operates mainly in the thymus and it eliminates majority of potentially dangerous T cells [1]. Peripheral tolerance develops outside the thymus and controls self-reactive T cells that have escaped central tolerance in the thymus [2]. For B cells, the central tolerance occurs in the bone marrow (central tolerance) and in periphery (peripheral tolerance), and B cells do not react against majority of antigens without T cells help [3].

2.1.1. Thymus, homing chemokines and their function in T cell maturation

Thymus is an essential primary lymphoid organ that develops from the third pharyngeal pouch of the anterior gut and supports the differentiation and selection of T cells and establishment of self-tolerance [4]. Thymus is bilobed organ in the thoracic cavity overlying the heart and major blood vessels. The two distinct lobes are joined by a connective tissue isthmus. Both lobes are organized into lobules subdivided from each other by connective tissue trabeculae. In addition to thoracic thymus, a functional cervical thymus in mice has been reported [5]. The thymus is proportionally the largest during infancy and subsequently involutes, which is defined as a decrease in the size and weight with progressing age. Involution starts at puberty and during this process the epithelial compartment atrophies and is replaced by abundant adipose tissue and lymphocytes [6].

Thymus is organized into outer cortex of immature T cells and inner medulla of more mature T cells, implying a differentiation gradient from cortex to medulla. The cortex consists mostly of immature thymocytes and in smaller numbers, of epithelial cells and macrophages. The medulla contains predominantly of epithelial cells and mature thymocytes, Hassall's corpuscles, macrophages and dendritic cells (DC) but also fibroblasts [4]. The epithelial cells of the Hassall's corpuscles produce thymic stromal lymphopoietin (TSLP), which activates maturation of thymic DCs and that in turn induces differentiation of regulatory T cells (Tregs) in the medulla of human thymus [7]. TSLP-activated DC secrete chemokines CCL22 and CCL17 [8], which are important in guiding the traffic of developing immature T cells to the medulla [9, 10]. Hassall's corpuscles may play significant role in DC mediated central tolerance.

Lympho-stromal crosstalk (interactions between T cells and nonhematopoietic cells) in the thymus has a critical role in the regulation of T cell development and selection. During the T cell development in the thymus the proper dynamic relocation of T cells at the right places and in the right order is needed, and also

close and remote crosstalk between the developing T cells and thymic stromal cells is obligatory. These processes include: first, the entrance of developing lymphocytes into the thymus; second, the generation of $CD4^+CD8^+$ double-positive (DP) thymocytes at the outer cortex of the thymus; third, the positive and negative selection of DP in the cortex; forth, interactions of mTECs with positively selected thymocytes for final development and central tolerance; fifth, export of mature thymocytes [11]. Thymic stromal cells produce chemokines as a homing cross-talk signal, which guides migration of developing thymocytes through individual microenvironments in the thymus, and developing thymocytes express different chemokine receptors for chemotactic guidance [11].

Chemokines are a family of small chemotactic cytokines, or proteins secreted by cells. The major role of chemokines is to induce directed chemotaxis in nearby responsive cells. They share structural characteristics such as small size (approx. ~8–14 kDa) and by the spacing of 4 conserved cysteins forming two essential disulphide bonds (Cys1-Cys3 and Cys2-Cys4). In CC-chemokine family, the first 2 cysteines are adjacent, whereas in CXC family they are separated by a non-conserved residue [12, 13]. Many chemokines are constitutively expressed in the thymus and in lymph nodes and spleen, including CCL17 [14], CCL19 [15–17], CCL21 [15, 18], CCL22 [19, 20] and CCL25 [21]. Their thymic expression implies that they play role in thymocyte migration and thymic development.

Entry of lymphoid progenitor cells into the thymus occurs as early as embryonic day 11.5 (E11.5) in mice and during the eighth week of gestation in humans [22, 23]. The entrance occurs in waves during embryogenesis and in adulthood [24]. The thymus is seeded at least by two different pathways: the vasculature-independent pathway, which probably occurs during the early stage of embryonic development before vascularization and is regulated by chemotactic attraction, and the vasculature-dependent pathway, which probably takes place in the late stage of emryogenesis and postnatally after vascularization. Chemokines, CCL21 and CCL25, are partially involved in the vasculature-independent colonization of early stage of fetal thymus. In postnatal thymus, the lymphoid progenitor cells enter the thymus through the area around the cortico-medullary junction [11]. The chemokine receptors CCR9, CCR7 and CXCR4 cooperatively regulate homing of hematopoietic progenitors to the embryonic mouse thymus [25], whereas in adult thymus CCR7 and CCR9 recruit hematopoietic progenitors [26].

After their entrance to thymus, the lymphoid progenitors start to develop into $CD4^-CD8^-$ double negative (DN) cells and relocate to the subcapsular zone in the cortex (Figure 1). Chemokine receptors CXCR4, CCR7 and CCR9 have been suggested to guide movement of immature thymocytes. Along with this movement immature DN thymocytes drive thymic stromal differentiation and cause the development of the cortical-epithelial environment. On the route to the subcapsular zone, the DN thymocytes rearrange T-cell receptor (TCR) gene segments, move inwards cortex and develop to $CD4^+CD8^+$ double positive (DP) cells. The cortical DP thymocytes have TCRs with a broad repertoire of antigen specificities. Since the generation of TCRs is highly random, a large number of different TCRs are made and thymocytes that make either nonfunctional receptor or recognise self-proteins die.

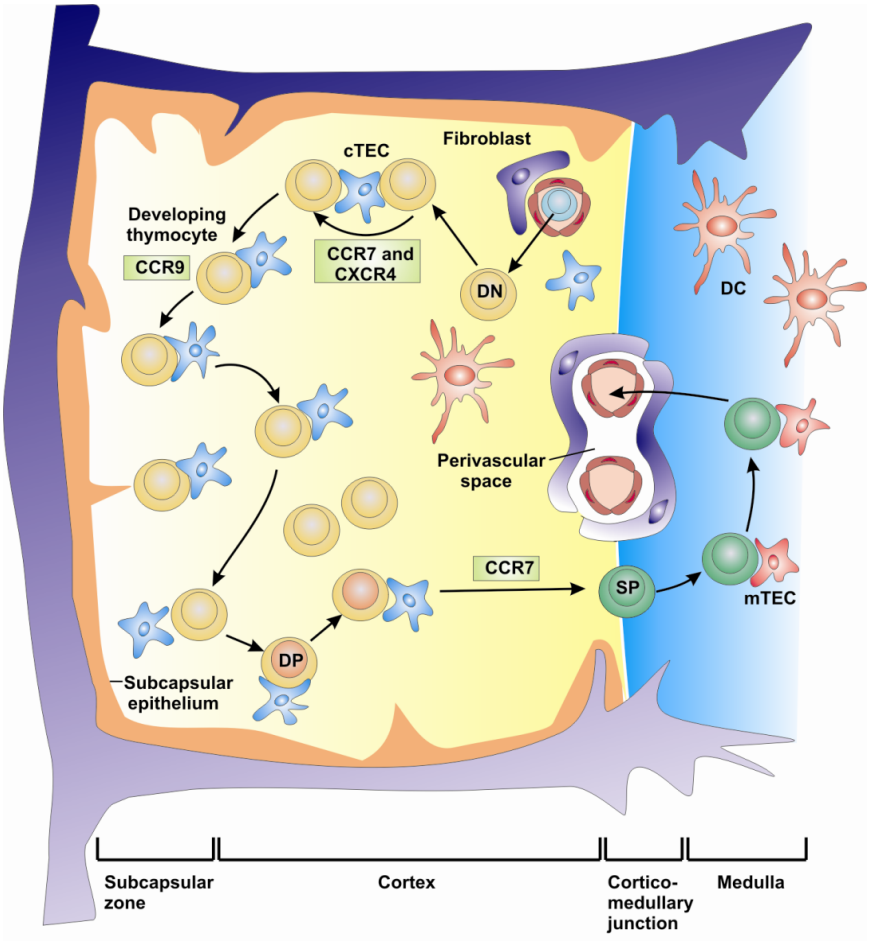

Figure 1. Traffic of thymocytes inside of the thymic compartments. In the postnatal thymus, circulating developing lymphocytes migrate into the thymus through the blood vessels in corticomedullary junction and migrate outwards to the capsule by chemokine signals through CXCR4 and CCR7 receptors. Further outward migration to the subcapsular region is mediated by CCR9 signals. Positively selected $CD4^+CD8^+$ DP differentiate into $CD4^+$ and $CD8^+$ T cells, and express CCR7 receptors, through which they are attracted to the medulla. In the medulla, SP thymocytes are further selected and tissue-specific-antigen-reactive T cells are deleted. Mature SP thymocytes are migrating back to the circulation. Modified from [11].

The functional and self-tolerant T-cell repertoire is achieved by presentation of different self-peptides bound to the major histocompatibility complex (MHC) molecules that are expressed by antigen-presenting cells (APCs). T cells interact via TCR with peptide-MHC complexes expressed by cTECs and DCs in the cortex. After T cells recognize peptide-MHC complex at intermediate avidity interaction, DP thymocytes receive signals for survival and differentiation into $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) thymocytes. This process, which is known as positive selection, ensures the elimination of T cells with nonfunctional receptor. T cells with very high or without receptor affinities towards self MHC undergo apoptosis and die in the cortex. Only 3–5% thymocytes survive positive selection. Positively selected thymocytes are induced to express CCR7 and are attracted to its ligands, CCL19 and CCL21, which are produced by mTECs and attracted to medulla via CCR7-mediated chemotaxis. Tolerance to self antigens is achieved in medulla, where self-reactive thymocytes that have passed positive selection in cortex are additionally screened. Thymocytes with TCRs that bind peptide-MHC complex with high avidity undergo apoptosis and are deleted. This process is known as negative selection and prevents emigration of self reactive thymocytes to periphery. Although the negative selection is highly efficient in eliminating the vast majority of self reactive thymocytes, some cells still escape from thymus to the periphery and their activation can lead to autoimmune diseases [11, 27].

In medulla, many different cell types present self-antigens to developing T cells (Figure 2). Thymic DCs are the most efficient APCs in thymus and bring peripheral antigens into the thymus [28, 29] and cross-present antigens to both $CD4^+$ and $CD8^+$ T cells [30]. mTECs are unique cell type that express wide range of tissue specific antigens (TSAs) that are usually expressed only in specific tissues [31], which leads to the elimination of self-reactive thymocytes [32, 33] and to presentation of these antigens to $CD4^+$ and $CD8^+$ medullary thymocytes [27]. During mTEC differentiation AIRE (Autoimmune Regulator) protein induces apoptosis and it is suggested that apoptosis is induced via direct induction of apoptotic program or indirectly via TSA upregulation that overload protein synthesis machinery to provoke death through endoplasmatic reticulum stress [34]. Thymic epithelial cells are capable to transfer membrane material, including MHC molecules, between epithelial cells and transfer MHC class I (MHCI) and MHC class II (MHCII) to DC but not reciprocally [35, 36]. mTECs, in addition to being an antigen reservoir, also function as APCs by directly presenting antigens to developing T cells [37]. The promiscuous (also known as open) gene expression of TSAs broadens the scope of self-antigens presented to developing thymocytes to facilitate central tolerance towards self [27].

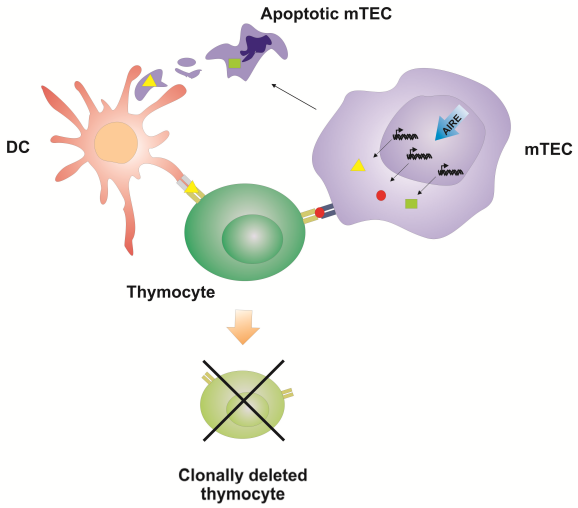

Figure 2. Self-antigens presetation to developing T cells. In mTECs, AIRE induces the expression of a wide repertoire of TSAs, which are processed to peptides and then presented on cell surface through MHC/HLA molecules. mTECs die by apoptosis after the induction of AIRE and TSAs, and apoptotic mTECs can be engulfed by DCs. Mature thymocytes translocate to medulla scanning for presented MHC:TSA complexes on mTECs or DCs and if their TCRs recognise presented complex with appropriate affinity, they will be deleted from the repertoire.

2.1.2. Autoimmunity

Autoimmunity is the failure of an organism to recognise its components as self, which results immune recognition and reaction against its own cells and tissues. Autoimmunity is associated with many diseases, which can be either organ-specific or with systemic distribution meaning that the response is primarily against particular organ specific antigens or against widespread antigens. Altogether 3–5% of population is affected by autoimmune diseases and women are affected to greater extent than men (sex ratio 3:1) [38]. The most common organ specific autoimmune diseases are Hashimoto's throiditis (targeted organ: thyroid gland), myasthenia gravis (muscle), pernicious anemia (stomach), Addison's disease (adrenal gland), insulin-dependent diabetes mellitus (pancreas). In systemic non-organ-specific diseases, such as systemic lupus erythematosus, scleroderma and SLE, rheumatoid arthritis, psoriasis, symptoms are often present in the skin, joints, kindney and muscle, which can be considerably affected. Autoimmune diseases can overlap and one individual may have more than one disease. Autoimmune diseases have a complex genetic basis, which means that many genes and triggering environmental factors act in concert to elicit auto-immunity [39, 40]. Autoimmune diseases caused by single-gene defects are very rare, but enable to study how defects in the immune system lead to the develop-ment of autoimmunity. Currently recognized monogenic autoimmune diseases are

IPEX (defect in *Foxp3* gene) (immunodysregulation polyendocrinopathy enteropathy and X-linked inheritance syndrome) (OMIM 304790), ALPS (defect in *FAS* gene) (autoimmune lymphoproliferative syndrome) (OMIM 601859), IL-2Rα deficiency (interleukin-2 receptor alpha-chain deficiency) (OMIM 606367) and APECED (defect in AIRE gene) (autoimmune polyendocrinopathy candidiasis and ectodermal dystrophy) (OMIM 240300) [38].

2.1.3. APECED

Autoimmune polyendocrinopathy candidiasis and ectodermal dystrophy (APECED), also known as autoimmune polyglandular syndrome type 1 (APS1), is a monogenic polyendocrine disease childhood onset and caused by mutations in the AIRE gene [41, 42]. Over 60 APECED-associated mutations have been reported in AIRE gene and APECED clinical picture varies in severity and in the number of disease components [78]. APECED is a rare disease, with high frequency among Iranian Jews (1:9000) [43], Sardinians (1:14 400) [44], Finns (1:25 000) [45] and Norwegians (1:90 000) [46]. The three major clinical manifestations associated with APECED classically involve hypoparathyroidism, chronic mucocutaneous candidiasis and primary adrenocortical insufficiency (Addison's disease), which vary in scope and timing. Two manifestations out of 3 are needed to fulfil the clinical diagnostic criteria. In addition, other autoimmune diseases might be present, such as hypothyroidism, primary hypogonadism, insulin-dependent diabetes mellitus, chronic active hepatitis, juvenile-onset pernicious anemia, vitiligo, alopecia and ectodermal dysplasia. The presence of autoantibodies emphasizes the role of autoimmunity in the pathogenesis of APECED. The autoantibodies are against several defined antigens, most often tissue-specific enzymes in the affected tissue. Nevertheless, tissue destruction is mediated by autoreactive T cells [47]. Also, the patients have autoantibodies against cytokines such as IL-17A and IL-22, which might explain the development of chronic mucocutaneous candidiasis [48, 49]. The early finding of high titer autoantibodies (interferon-ω and $-\alpha_2$) has created a useful diagnostic test for APECED diagnosis [50]. APECED patients have Treg defect, which is associated with loss of naive precurors [51], although in Aire deficient mouse model peripheral Treg cells seem normal [52]. Functional Tregs are the final checkpoint in the pathogenetic events and defect in Tregs can guide to impaired control of the pathological processes leading to APECED.

2.2. AIRE and central tolerance

2.2.1. AIRE

The human *AIRE* gene locates on chromosome 21q22.3 with 14 exons spanning 11.9 kb of genomic DNA, the encoded protein consists of 545 amino acid residues and the molecular weight is 57.7 kDa [41, 42]. The mouse *Aire* gene

has also 14 exons but spans over 13kb on chromosome 10 and encodes protein of 552 amino acids. Mouse and human AIRE share 71% similarity at the protein level [53–55]. AIRE contains several structural domains intrinsic for transcriptional regulators and chromatin-binding proteins (Figure 3).

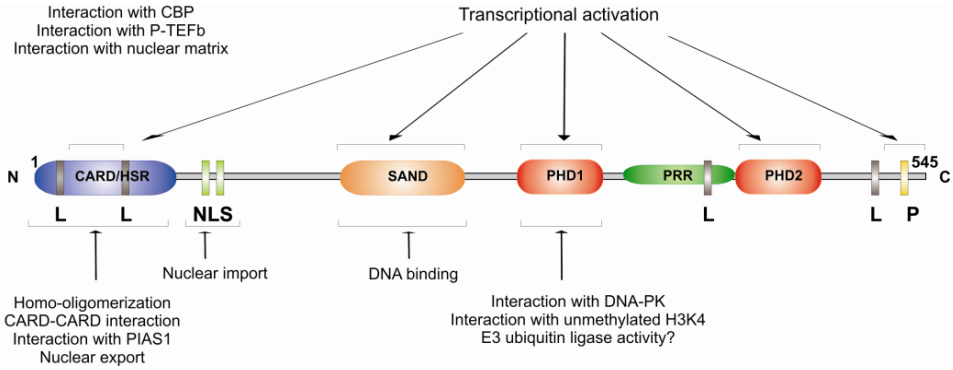

Figure 3. Domain structure of the AIRE protein. AIRE protein contains several domains and functional elements such as CARD, NLS, SAND, PHD, PRR, LXXLL (indicated in different colours). Functions of AIRE are shown with arrows pointing to the respective domain. Modified from [56].

The N-terminal region of AIRE gene contains caspase-recruitment domain (CARD) [57], overlaps with a homogenously staining region (HSR) domain, which is responsible for dimerization of the protein [58] or in interaction with other transcriptional control proteins, like CBP [57]. CARD is followed by SAND domain, termed by Sp100, AIRE, NucP41/75 and DEAF-1, responsible for the DNA-binding [59]. SAND domain is found in a number of nuclear proteins, many of which function in chromatin-dependent transcriptional control. The DNA-binding properties are centred around the conserved KDWK core motif [60]. Two plant homeodomain (PHD) zinc fingers are also found in AIRE and are separated by proline rich region (PRR). Mutations in PHD fingers reduce the transcriptional activation capacity of the AIRE protein [61–64].The PHD1 finger of the AIRE protein has been suggested to function as an E3 ubiquitin ligase [65], albeit subsequent study argues against [66]. C-terminal 30 amino acids, including the proline-rich PXXPXP motif, are important for transactivation [63]. Both the N- and C-terminal regions of the protein contain nuclear hormone binding LXXLL motifs (L is leucine and X any amino acid) [42]. AIRE has two functional nuclear localization signals (NLS), the consensus NLS of the N-terminus and another NLS in the C-terminus, whereas the nuclear export signal (NES) resides in the N-terminal HSR domain of AIRE [58].

2.2.2. The trancriptional control of AIRE gene

During the characterization of human AIRE gene, a putative promoter with a TATA box and a CpG island was discovered, and a GC box upstream of first exon was found [41]. Altogether, five GC boxes are located in human AIRE but none in the mouse promoter sequence. Based on computational analysis, within the 600 bp upstream of the ATG translation site several conserved transcription factor binding sites were discovered for thymus specific (Ets-1 and Gfi1) and hematopoietic transcription factors [54]. Also, it included sites for essential components of the basal transcriptional complex such as AP-4, an inverted CCAAT box and a typical TATA box [54].

Several years later, Murumägi et al. carried out studies on characterization of regulatory elements and methylation pattern of the AIRE promoter. They identified that human *AIRE* gene promoter contains indeed a CpG island, a typical TATA box and functional binding sites for positive *cis*-regulatory elements (for minimal promoter activity), such as the ubiquitously expressed transcription factors specificity protein 1 (Sp1), nuclear factor Y (NF-Y), activator protein 1 (AP-1) and E-twenty-six (Ets) family of transcription factors [67, 68]. The CpG island is highly hypermethylated in several AIRE-negative cell lines such as HeLa, TEC1A3, THP-1, U937 [68]. Furthermore, AIRE expression was detected in methyltransferase-defective cell line, suggesting the regulation of AIRE gene expression via CpG methylation [69].

2.2.3. AIRE expression pattern

AIRE is highly expressed in a subpopulation of mTECs within the thymus. In addition to thymus, AIRE is expressed at low levels in spleen, lymph node paracortex, fetal liver [41, 42, 70, 71], and in ovary and testis [52, 72, 73]. Recently, AIRE-expressing cells have been found in human peripheral lymphoid tissues, including tonsils and gut-associated lymphoid tissue (GALT) [74]. Extrathymic $AIRE^+$ cells show DC morphology [74], which agrees with the previous findings that AIRE is expressed in peripheral blood monocytes [70, 75] and in differentiated DCs [75]. In mice, Aire expression at the early embryo before emergence of the three germ cell layers (i.e. before gastrulation at embryonic day E6.5) was recently described [76]. Also, Aire was present at the two-cell stage, blastocycts and ES cells. In addition, ES cells and induced pluripotent stem (iPS) cells of human origin expressed AIRE [76].

At the subcellular level, AIRE protein is located in the cell nucleus and in nuclear dots resembling promyelocytic leukemia (PML) bodies. Whereas, AIRE nuclear bodies localize separate from PML bodies [58, 70]. The function of AIRE nuclear bodies is not known. AIRE subcellular location and its structural features support its role as a transcriptional activator and involvement in the regulation of gene expression [77].

2.2.4. AIRE function in thymus and self-tolerance

The development of Aire-deficient mouse models for APECED provides new hints about AIRE role in immune regulation and the pathogenesis of APECED. Similarly to APECED patients, Aire-deficient mice develop lymphocytic infiltrations (in retina of eye, salivary gland, stomach, ovary, pancreas, thyroid), circulating organ-specific autoantibodies and infertility [52, 79, 80]. Although, it has been reported that Aire-deficient mice do not develop the same profile of autoantibodies as in the patients with APECED, the finding suggests that AIRE might control a different set of genes in mice and in humans [81].

AIRE expression is defined to end-stage matured mTECs [34, 82], characterized by high expression levels of MHC class II molecules and costimulatory molecules CD80, CD86 and CD40 [71, 83]. mTECs specialize in promiscuous expression of hundreds of TSAs, which are intrinsic to different peripheral tissues [31]. Conducted gene-expression microarray of mTECs from Aire-deficient and wild-type (WT) mice revealed, that Aire directs the expression of peripheral TSAs. In mTECs, Aire activates the expression of approximately 200–1200 different genes [52]. The complexity of promiscuous gene expression in mTECs is differentiation dependent and increases from immature $CD80^{lo}$ to mature $CD80^{hi}$ mTECs [83]. AIRE regulated genes have stochastic expression and 1–3% of total mTECs express a particular TSA [84]. Loss of Aire and thereby inefficient antigen presentation by mTECs causes failure to negatively select organ-specific T cells in the thymus [32, 33]. To date, the significance of thymic expression of TSAs has been shown by different studies, which link specific thymic TSAs and autoimmune phenotype. And the lack of a single TSA in the thymus is suffient to trigger organ-specific autoimmunity [85–87]. Clearly, the promiscuous expression driven by Aire is obligatory for proper negative selection and protection against autoimmunity.

2.2.5. Thymomas

Thymomas are histologically diverse neoplasms of thymic epithelial cells and are classified according to their architectural features and content of lymphocytes into several subtypes [88]. The 2004 World Health Organization (WHO) classification divides thymomas into types A, AB, B1, B2 and B3 based on the morphology of the neoplastic cells and the relative amount of a non-neoplastic lymphocytic component [89]. Stage of their metastatic spread, in addition to complete resection, has demonstrated to be the best single prognostic marker for thymoma therapy. Approximately over 80% of thymomas are thymopoietic and generate SP $CD4^+$ and $CD8^+$ T cells and generation of T cells varies according to the thymoma subtype: it is minimal or absent in type A thymomas; it is prominent but scattered and relatively inefficient in type AB generating few mature SP T cells; it is abundant and almost normal in types B1 and B2; and it is sparse but still normal in type B3. The most common autoimmune manifestation in thymoma patients is myasthenia gravis (MG), which is found approximately in 10–50% of thymoma

patients. Immunohistochemical analysis have shown that the expression of MHCII was strongly or moderately reduced in the vast majority of thymomas, but regrouping according to presence or lack of MG, demonstrated that MG occurs predominantly in thymomas with high MHCII expression levels [90]. About 95% of thymomas lack the AIRE and AIRE-dependent antigen expression, which has maintained in their remnant thymic tissue and lymph nodes [91]. Only type B1 thymoma contains $AIRE^+$ mTECs and has a tissue architecture resembling normal thymus with the most clear-cut corticomedullary differentiation among thymomas, frequent Hassall's corpuscles, normal thymopoiesis, myoid cells and minimal genomic gains and losses. Lack of AIRE in thymomas very rarely leads to the typical manifestations caused by AIRE mutations in APECED, except for highly prevalent anti-IFNα and rare anti-Th17 cytokine autoantibodies [92]. AIRE deficiency in thymomas might help to create a dangerous environment, where thymocytes are activated against autoantigens, which are aberranty expressed by neoplastic epithelial cells in most thymomas [89].

2.2.6. Aire's molecular mechanisms

Several observations conclude that AIRE functions as a transcriptional regulator, but the precise molecular mechanisms are poorly understood. AIRE protein functional domains and its subcellular localization to nuclear bodies reveal its possible role in regulating transcription. So far, all Aire domains have reported to participate in transcriptional activation. The transcriptional activation region has been placed to both PHD fingers [61, 64], CARD and SAND domains [57, 62, 93] since mutations in these domains decrease the transactivation capacity of AIRE.

Initially, it was hypothesized that AIRE might act as a direct transcriptional regulator by binding to DNA. A study by Halonen *et al.* showed that SAND domain of AIRE contains NKAR motif and predicted it to be important for DNA-binding [62]. AIRE has been found to bind to two consensus sequences: (i) a tandem repeat of the ATTGGTTAA (G-box) sequence and (ii) a sequence similar to the TATA box- TTATTA (T-box) [94], whereas AIRE PHD domains can bind to the ATTGGTTAA sequence motif and SAND domain recognizes TTATTA motif, respectively [95]. AIRE binding to these motifs was proposed to regulate thymic expression of different genes such as cytokines, transcription factors and posttranslational modifiers [96]. However, the results by Koh *et al.* demonstrated Aire's affinity for nonspecific DNA sequences [97]. Taking into account the large number and chromosomal clustering of AIRE regulated TSAs [98, 99], AIRE seems to have a more general function rather than being a direct transcriptional regulator. Indeed this notion was confirmed when Aire was introduced into diverse cell types and very limited transcripts overlapped. This shows that the cellular environment dictates which genes are AIRE regulated [100].

Several interacting partners for AIRE have been identified in the context of transcriptional activation. First the common transcriptional coactivator CREB-binding protein (CBP) was shown to interact with AIRE through the CH1 and

CH3 conserved domains [64]. AIRE and CPB preferentially colocalize to nuclear bodies (NB) in cells that lack or have low levels of PML protein [93]. CBP and AIRE cooperation might be important for mTEC differentiation because signalling through RANK (induces $AIRE^-$ cells differentiation into $AIRE^+$ cells) translocates CBP from the cytoplasm to the nucleus and co-accumulation of CBP and AIRE in nuclear dots [57].

Following nuclear matrix-associated proteins have been described as partners of AIRE: protein inhibitor of activated STAT (PIAS1) [101] and the DNA-dependent protein kinase (DNA-PK) [102]. PIAS1 and DNA-PK proteins have shown to bind to nuclear-matrix-associated DNA sequences [101, 103]. The expression of AIRE enhances the formation of PIAS1 NBs. AIRE and PIAS1 NBs are localized adjacent to each other. The concurrent expression of PIAS1 and AIRE activates the human insulin promoter and SP-RING (RING finger-like domain) is obligatory to conduct this activation. Possibly AIRE and PIAS1 associate with a common nuclear complex through other components of the nuclear matrix to regulate AIRE target genes [101]. Another AIRE interacting partner DNA-PK, a Ser/Thr kinase, is formed by two Ku regulatory subunits, Ku70 and Ku80, and a large catalytic subunit DNA-PKc in the presence of DNA [102]. DNA-PK recognises and repairs DNA-double stranded breaks (DSB) and phosporylates several proteins involved in regulation of cell cycle and transcription [104]. Additionally it was shown that PIAS1 is able to promote the reparation of DSBs [105]. DNA-PK phophorylates AIRE protein and mutations in AIRE protein phoshorylation sites decrease the capacity of AIRE to activate transcription from reporter promoters [102].

AIRE has been reported to associate with a number of multi-protein complexes involved in transcriptional elongation and the conversion of unspliced pre-mRNAs to mature mRNAs. AIRE recruits positive transcription elongation factor b (P-TEFb) to promoters of target genes with poised RNA polymerase II (RNAPII) to enable efficient elongation of primary transcripts [106]. pTEF-b consists of the CycT1 and cyclin-dependent kinase 9 (Cdk9) [107]. Active p-TEFb heterodimer of CycT1 and Cdk9 interacts with AIRE, and CycT1 partially colocalizes with AIRE in the same nuclear dots [106]. Mammalian gene transcription is initiated by the binding of RNA polymerase II to gene promoters for correct formation of transcription pre-initiation complex. Particular complex initiates transcription but does not elongate unless P-TEFb forms a complex with RNApolII [108]. Genome-wide studies have established that majority of genes have engaged RNApolII at their promoters regardless of their activity. Poised RNApolII is mediated by the action of negative elongation factors and recruitment of p-TEFb stimulates the transition of RNApolII from pausing to productive elongation [107].

In a study, Abramson *et al*. identified AIRE interacting proteins by co-immunoprecipitation followed by mass spectrometry. The identified wide set of Aire associated proteins fall into four major functional classes: 1) nuclear transport (e.g., XPO1, NUP93, KPNB1), which are likely to mediate the shuttling of Aire into or out of the nucleus; 2) chromatin binding and structure

(e.g., histones, the cohesin complex), which could affect the structure or spatial organization of chromatin in response to Aire and serve as an access point for Aire binding; 3) transcription (DNA-PK, PARP1, TOP2a, FACT and RNApolII), which possibly modify the nucleosome environment in response to elongating RNApolII; 4) pre-mRNA processing (including several splicing factors and RNA helicases). In series of experiments the authors demonstrated that AIRE promotes TOP2a-initiated DSBs, which would recruit and activate multiple members such as DNA-PK, Ku80, PARP1, and H2AX, as well as several other Aire-associated molecules, including SMC1, TRIM28, MSH2/6 and RUVBL2. TOP2a-initiated DSBs ease superhelical tensions generated by advancing RNApolII during transcriptional elongation, which helps it to proceed more smoothly through the unwinding helix and providing local relaxation of the chromatin structure resulting in more efficient mRNA synthesis. Additionally, Aire enhances processing of pre-mRNAs to mature mRNA [109].

AIRE cooperates with p63 in HLA class II expression of mTECs, which is linking AIRE and its ability to control the representation of wide set of genes from peripheral tissues and organs in the thymus. AIRE and p63 interact through the association of a SAND domain of AIRE and transactivation (TA) domain of p63. A naturally occurring mutation in SAND domain of AIRE abolishes the binding of AIRE to p63 [110]. The expression of HLA class II is controlled by a class II master regulator (CIITA) [111]. It was shown that AIRE and p63 complex has regulatory role in CIITA expression in mTECs [110].

Recently, a new Aire interacting protein called the death-domain-associated (DAXX) protein, which exerts strong repressive role on AIRE transcriptional activity, was discovered [112]. DAXX is ubiquitously expressed throughout the body with particular high expression in the thymus and testes. In the nucleus DAXX is found to associate with PML NBs and together with PML the protein influences apoptosis and transcription, whereas in cytoplasma interacts with various proteins involved in cell death regulation [113]. DAXX is known to associate directly and repress several transcription factors, e.g. ETS1 [114], NFkB [115], p53 and its family members p63 and p73 [116]. DAXX has been found to take part of a large multiprotein complex that includes core histones (H2A, H2B, H3 and H4) and histone deacetylase II (HDAC II) and DAXX-mediated transcriptional repression is due to interaction with HDAC [117]. AIRE and DAXX colocalize in the nucleus, and DAXX represses the transcriptional activation property of AIRE to induce the expression of the insulin promoter. AIRE is able to interact with HDAC1 and HDAC2 and thereby it is suggested that histone deacetylation by HDACs is involved in DAXX-mediated repression of AIRE transcriptional activity [112].

AIRE has been reported to act as a histone code reader sensing epigenetic chromatin modifications. Two studies demonstrated that AIRE binds directly to unmethylated histone H3 lysine 4 (H3K4) by PHD1 domain and binding is necessary for the up-regulation of genes encoding TSAs [97, 118]. Next, AIRE was shown preferentially activate tissue-specific genes that are characterized by low levels of initial expression and lack active chromatin marks, such as histone

H3 trimethylation (H3K4me3) and acetylation (AcH3) on their promoters. During activation by AIRE, the target genes acquire histone H3 modifications associated with transcription and RNApolII, and H3K4me3 levels are increased on AIRE promoter during mTEC differentiation [119]. Transcriptional regulation by AIRE through its interactions with histone H3 is confined to the first N-terminal residues in H3 tail [120]. Overexpression of Aire and the H3K4-specific demethylase yielded unexpected results, showing that Aire's transcriptional footprint remained largly unchanged, with only a partial derepression of a subset of genes already accessed by Aire. These results argue that Aire's PHD1 and H3K4 interaction is not sufficient for Aire to productively target a given gene [121]. It would be important to biochemically confirm upstream and downstream interacting proteins which cooperate with AIRE to have more profound understanding of Aire targeting and function in context of expression of TSAs and mediating central tolerance.

2.3. Epigenetic modifications in regulation of gene expression

Epigenetics has emerged to explain how cells with a limited number of genes can differentiate into different cell types and how phenotype is passed to daughter cells. Epigenetics is defined as the study of stable and heritable changes in gene expression that do not involve alterations in DNA sequence. Epigenetic processes control gene expression patterns during cell cyle, differentiation and development in response to environmental and biological modifications [122]. Epigenetic state changes with age and maybe be critical determinant of cellular senscence and organism aging [123]. Epigenetic mechanisms have many layers of complexity, including chromatin remodelling and RNA transcripts and their encoded proteins. Epigenetic modifications fall into two main categories such as DNA methylation and posttranslational modification of histones.

2.3.1. Chromatin

Chromatin is the combination of genomic DNA and histone proteins, in which DNA is packed approximately 10 000-fold. The basic repeat unit for chromatin is the nucleosome that is typically composed of an octamer of the four core histones H2A, H2B, H3 and H4 and 146 bp of DNA wrapped twice around the histones. The protein octamer is divided into four dimers defined by H2A-H2B and H3-H4 histone pairs [124]. Noncondensed nucleosomes are connected by small segments of linker DNA forming "beads on a string" structure, which is stabilized by the histone H1. Histone H1 seals two rounds of DNA on the surface of the nucleosome core and repeating nucleosomal units are folded into higher-order chromatin fibers [125]. Each core histone is composed of a structured domain and an unstructured amino-terminal tail of 25–40 residues that surrounds the nucleosomes. Histone tails provide sites for different post-translational modifications (PTMs) such as methylation, acetylation and

phosphorylation, which are all involved in gene-specific regulation [126]. Nucleosomes are organized, mobilized and remodeled by regulatory proteins, chromatin remodelling factors and histone modifying enzymes, which determine the accessibility of DNA to other regulatory factors (Figure 4).

Chromatin remodelling complexes (remodellers) are specialized multi-protein machines that enable access to the nucleosomal DNA by altering the structure, composition and positioning of nucleosomes and influence the regulation of gene expression. The remodellers use the energy of ATP hydrolysis for breaking histone-DNA contacts, making DNA more accessible for various regulatory proteins. There are currently four different families of chromatin remodellers: SWI/SNF (switching defective/sucrose nonfermenting), ISWI (imitation switch), CHD (chromodomain, helicase, DNA binding), and INO80 (inositol requiring 80), which have different compositions and execute different tasks. In addition, remodellers are also utilized for many steps of DNA repair and DNA replication [127].

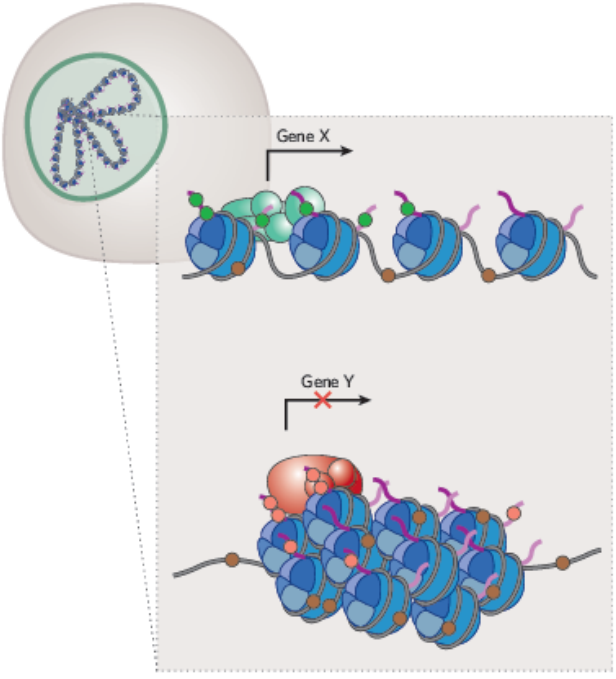

Figure 4. Chromatin structure and gene regulation. X is a transcriptionally active gene with an open chromatin structure and sparse DNA methylation (brown circles), Y is a transcriptionally silent gene with a closed chromatin structure and dense DNA methylation (brown circles). Histone tails provide sites for different posttranslational modifications (green and pink circles). Nucleosomes are organized, mobilized and remodeled by regulatory proteins, chromatin remodelling factors and histone modifying enzymes (green and red protein complex). Modified from [128].

2.3.2. Histone modifications

The surface of nucleosomes is covered with multiplicity of histone modifications. So far 8 different classes of modifications have been described such as acetylation, methylation (lysines and arginines), phosphorylation, ubiquitylation, SUMOylation, ADP ribosylation, deimination and proline isomerization. Different combinations of modifications gives wide variety of functional responses, which in turn depend on the signalling conditions within the cell. After stimulus, transcription factors are engaged to the promoter of specific genes and result in modification events. Modification events can be divided into those that correlate with activation such as acetylation, methylation phoshorylation and ubiquitination; and those that correlate with repression, for example, methylation, ubiquitination, SUMOylation, deimination and proline isomerization. Acetylation, methylation and phosphorylation are the best studied modifications [129].

The regulation of gene expression within euchromatin involves the delivery of chromatin-modifying enzymes by DNA-bound transcription factors. Chromatin modifying complexes are responsible for adding or removing covalent modifications on histone proteins. Most studied chromatin modifying complexes are histone acetyltransferases (HAT) and histone deacetylases (HDAC).

2.3.2.1. Histone acetylation and deacetylation

One of the features of actively transcribed genes is the acetylation of the amino-terminal tails of histones, which is found mostly at promoters and the 5'regions of genes, with the level of acetylation correlating with the rate of transcription. Different acetylations may target different regions of genes. For instance, H3K9ac is located in the region surrounding the transcription start site, whereas H3K4ac is elevated in the promoter and transcribed regions of active genes [130]. Acetylation of lysine residues results in neutralization of charge of histone tails and leads to loose contact between histone and DNA that makes DNA more accessible for transcription factors. Histone acetylation is catalyzed by class of enzymes known as HATs, which use acetyl-CoA as a substrate to acetylate lysine residues in histones. Several HAT families are indentified, including GNAT (Gcn5 N-acetyltransferases), p300/CBP and MYST [131]. All known HATs contain bromodomain, which functions as acetyl-lysine binding domain [132]. Histone acetylation performs a crucial role in DNA replication, recombination and repair. Various signalling events may change the association partners from HATs to HDACs [133].

Histone deacetylation has an opposite effect to acetylation and is generally associated with the transcriptional respression by the removal of acetyl groups from histone tails. HDACs are recruited to promoters via interaction with specific DNA-binding transcription factors. There are four families of HDACs (classes I to IV) [134]. Previously HDACs have been associated with inactive genes, but genome-wide mapping of HATs and HDACs revealed that both HATs and HDACs were found at active genes with acetylated histones, and targeted to transcribed regions by phosphorylated RNApolII. Inactive but so

called poised genes acquired H3K4 methylation before dynamic cycling of acetylation and deacetylation by transient HAT/HDAC binding, preventing RNApolII binding but poising for future activation [135].

2.3.2.2. Histone phosphorylation

Core histone phosphorylation has been described to have a role in transcription, mitosis, DNA repair and apoptosis. The most characterized phosphorylation events are phosphorylation of serine 10 and serine 28 in histone H3 and phosphorylation of the H2A.X, whereas several tyrosine phosphorylations within the H3 protein have been recently described. Each phospho-H3 modification has a specific kinase that mediates phosphorylation of a particular residue in H3. In mammalian cells, the phosphorylation is believed to have two opposite roles: one in the initiation of mitotic chromosome condensation and the other in localized chromatin decondensation leading to gene activation [136]. The contribution of phosphorylation to transcriptional activation is not well understood. It is thought that the addition of negatively charged phosphate groups to histone tails neutralizes the basic charge of histone tails and reduces their affinity towards DNA.

2.3.2.3. Histone methylation

There are two types of histone methylation, targeting either lysine or arginine residues. Modifications by lysine methyltransferases (MLL, SUV39, SET, etc) either activate or repress of transcription [137]. Lysine methylation can have different effects depending on which residue is modified. Three methylation sites on histones are associated with transcription activation such as H3K4, H3K36 and H3K79. The last two modifications are marks of transcriptional elongation. Histone modifications H3K9, H3K27 and H4K20 are connected to transcriptional repression by recruiting methylating enzymes to repressed genes [129]. Methylation is antagonized by histone demethylases (LSD, JHDM, JMJD, etc) selective for mono-, di-, or trimethylated lysines, which allows larger functional control of lysine methylation [138].

Histone arginine methylation is also involved in activation or repression of transcription and catalyzed by protein arginine methyltransferases (PRMTs). In mammals, PRMT1- and CARM1-catalyzed histone arginine dimethylation is involved in gene activation, while PRMT5-catalyzed histone arginine dimethylation is associated with gene repression. Arginine methylation is required for development, cellular proliferation and differentiation [139].

2.3.3. DNA methylation

Mammalian genome globally has less CpG dinucleotides than expected, except at short DNA stretches called CpG islands (CGI), which are associated with gene promoters. Originally CpG islands were defined as regions of at least 200 bp with a (GC)-content of at least 50% and a ratio of observed CpG frequency

to expected frequency of at least 0.6 [140]. In recent years a large class of CpG islands (orphan CGIs) were discovered that are remote from annotated transcription start sites, but show evidence for promoter function [141, 142]. These novel findings, the colocalisation of a CGI with bound RNA polymerase II, highlight the strong connection between CGIs and transcription initiation.

The accurate performance of gene expression during differentiation and development requires three types of regulatory DNA elements: core promoters, upstream promoter elements and enhancers [143]. Core promoters support the formation of relatively large multiprotein complexes that direct the RNA polymerase II to start transcription at accurate site [144]. Upstream promoter elements are situated within the first 100–200 nucleotides upstream of the core promoter and contain transcription factor binding sites to increase the rate of transcription by promoting assembly of pre-initiation complexes [145]. Enhancers locate either downstream or upstream of the promoter, on the same or different chromosomes, and promote the rate of transcription from promoter and define the expression pattern of the genes [146]. Mammalian transcription factor-binding sites are more CG-rich than the bulk genome and many contain CpG in their recognition site. The studies imply that the CpG-containing transcription factor-binding sites regulate the expression of genes with important roles in pathways leading to cell-type-specific gene expression and in pathways controlled by the complex networks of signalling systems [147].

Furthermore, it has become clear that chromatin is a dynamic and active participant in transcriptional regulation and its structure determines the accessibility of a gene`s promoter and depends on DNA methylation and histone modifications. During the development, DNA methylation and specific histone modifications influence each other: histone methylation may direct DNA methylation patterns, and DNA methylation directs the establishment of certain histone modifications. Early studies have demonstrated that isolated CGI chromatin showed high levels of histone H3 and H4 acetylation, which represents transcriptionally active chromatin. The genome-wide studies have revealed that trimethylation of H3K4 is a signature histone mark of CGI promoters even in the state of inactivity [148]. In mammals, four DNA methyltransferases (DNMTs) have been identified: DNMT1, DNMT3A, DNMT3B, and DNMT3L. DNMT1 acts mainly as a "maintenance“ methyltransferase during the DNA synthesis, and DNMT3A and DNMT3B have preference for unmethylated CpG dinucleotides and perform *de novo* methylation during the development. DNMT3L increases the ability of *de novo* methyltransferases to bind to the methyl-group donor and stimulate their activity, whereas DNMT3L has no catalytic activity itself [149]. Trimethylation of H3K9, H3K27, and H4K20 has been suggested to be needed as a prior condition for subsequent DNA methylation. In the context of H3K9 and H3K27, the relevant histone lysine methyltransferases, SUV39H1/2 and EZH2, interact directly with DNMT1, DNMT3A and DNMT3B. The additional link between DNA methylation and the histone code is methyl-CpG-binding proteins (MBDs), which interact specifically with methylated DNA and mediate transcriptional repression [149].

3. AIMS OF THE STUDY

The aims of the present study were:

1. To examine whether modulation of Aire regulates the expression of tissue specific antigens.

2. To study if Aire infuences the expression of ligands of chemokine receptor CCR4 and CCR7.

3. To determine DNA methylation signatures of the AIRE promoter in thymic epithelial cells, thymomas and normal tissues.

4. MATERIALS AND METHODS

4.1. Mice and cell culture

Aire deficient mice (C57BL/6J and Balb/c background) were generated at The Walter and Eliza Hall Institute (Melbourne, Australia) via homologous recombination of targeting vectors in mouse embryonic stem cells [150]. Thymuses and/or inguinal lymph nodes from 4- to 6-week old WT, Aire heterozygote (HET) and Aire-deficient (KO) mice were used. Embryonic (E13.5, E15.5 and E17.5), newborn, neonatal D11 and adult (6 weeks, 6 months and 12 months) mouse tissues were used in developmental dynamics analysis in study 1. In development analysis in study 2, the ages of mice were the same with exception of neonatal D10. The CCR7-deficient C57BL/6 mice were obtained from The Jackson Laboratory. All mice used in the studies 1 and 2 were bred and maintained at the mouse facility of the Institute of Molecular and Cell Biology, University of Tartu.

Mouse thymic epithelial cells TEC 1C6 (kindly provided by G. Holländer, University of Basel, Switzerland), human embryonic kidney HEK293 (obtained from ATCC) and thymic primary culture (made in-house as in 4.3. Mouse thymic stromal cell isolation and cell sorting) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Gibco BRL).

4.2. EGFP and Aire adenovirus construction and infection

The pAdTrack-CMV (Stratagene, La Jolla, CA, USA) vector expressing enhanced green fluorescence protein (EGFP) gene was used as pAd-GFP plasmid. The mouse *Aire* gene was amplified from pCAIRE vector [72] using the primers: mAire-5-SalI and mAire-3-NotI stop (Table 2) and cloned into SalI and NotI sites of pAdTrack-CMV resulting in AdAire-GFP. HEK293 cells (Invitrogen, Carlsbad, CA, USA), which constitutively express AdEasy deleted E1 genes *in-trans*, were used for expression analysis of adenoviral vectors and for virus growth. To make recombinant adenoviruses, pAd-GFP and pAdAire-GFP plasmids were recombined with pAdEasy-1. The amplification and harvest process was repeated to generate higher titer viral stocks. Subsequently, the lysates from several amplification steps were purified by CsCl gradient centrifugation [151]. Virus bands was collected and mixed with 2× preservation buffer (10mM Tris pH 8.0, 100mM NaCl, 0.1% BSA, 50% glycerol). We quantified the Ad-GFP and AdAire-GFP virus particles by absorbance measurement at 260 nm to be equivalent to 1012 particles with virus titers >2×108 pfu/ml. The adenoviruses were next verified for their expression by infection of HEK293 cells for 48 h and analyzed by immunoblotting with rabbit polyclonal anti-mouseAire and mouse monoclonal anti-G3PDH (Ambion, Austin, Texas, USA). The signal

was detected by Supersignal® West Pico Chemoluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

For adenoviral infection of thymic primary culture and TEC 1C6, the cells were infected with Ad-GFP and AdAire-GFP at approximately 70% confluence. Cells were incubated in 500 µl serum-free OptiMEM (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B) and infected with equal amounts of virus for 1 h. The infection rate was further assessed by quantitative real-time PCR analyzing intake of viral genomic DNA.

The cells were then collected after 48h using trypsin-EDTA and assessed for infection rate by quantifying the percentage of GFP-positive cells in Ad-Aire-GFP- or Ad-GFP infected TECs by a FACSCalibur (BD Biosciences, San Jose, CA, USA). RNA was isolated from the cells using TRIzol followed by reverse transcription and qPCR as described above [152].

4.3. Mouse thymic stromal cell isolation

Small cuts were made into the capsules of thymi dissected from eight to twelve 4-week-old mice and thymocytes were released by repetitive pipeting. The remaining thymic fragments were incubated in 0.5 mg/ml dispase/collagenase (Roche, Basel, Switzerland) and 1.5µg/ml DNase I (AppliChem, Darmstadt, Germany) in PBS at 37 °C for 20 min, with gentle agitation using a Pasteur pipette every 5 min. Released cells were collected to separate fractions and fresh enzyme solution was added four times. The cells were resuspended in 5mM EDTA in 10ml of RPMI-1460. Isolated cells were used either for stromal cell isolation or for growing thymic primary culture. For thymic primary culture, the isolated cells were cultured for 4 days in DMEM supplemented with 10% FCS and incubated 37°C, 5% CO2. For stromal cell isolation cells were counted in each fraction. To obtain 2×108 cells required for isolation, the final fraction was used first and then collected backwards through the collected fractions. The fractions were pooled and passed through a 100 µm mesh to remove clumps.

4.4. Human tissue samples and isolation of thymic cell populations

Normal peripheral tissue samples (cerebellum, uterus, lung, kidney, myocardium, gray matter, spleen and liver from 4 donors (marked as D1-D4) over 50 years of age) were obtained from the Tartu University Tissue Bank, Estonia. Thymomas (six WHO type A thymomas, five type AB thymomas and five type B3 thymomas, age unknown) were supplied by the cryo-archives of the Institute of Pathology, University of Würzburg, Germany. In addition to tumor tissue, residual normal non-neoplastic thymus tissue of adult (32–67 years old) thymoma patients was analyzed, supplied by the University of Heidelberg. TECs were purified from thymus samples of children undergoing corrective heart surgery (6 weeks to 5 years old) at the Department of Heart Surgery, Medical School

Heidelberg and Tartu University Clinic (Table 1). The study was approved by the local ethical committees of the Universities of Heidelberg, Würzburg and Tartu. Human medullary thymic epithelial cells (mTECs) and cortical thymic epithelial cells (cTECs) were purified as described previously [98].

For AIRE promoter methylation studies we purified epithelial cell subsets from human thymus tissue. We used the CD45 marker to deplete human thymic samples from all cells of hematopoietic origin. Subsequent cell sorting with EpCAM and CDR2 markers was used to divide thymic epithelial cell populations corresponding to immature MHC class II^{lo} mTEC ($EpCAM^{hi}$, $CDR2^{lo}$, MHC class II^{lo}), mature MHC class II^{hi} mTEC ($EpCAM^{hi}$, $CDR2^{lo}$, MHC class II^{hi}) and cTEC ($EpCAM^{lo}$, $CDR2^{hi}$).

For Q2chip experiment thymic pieces from 2-wk to 6-month old patients were dissected and collected into RPMI 1640. Connective tissue was removed and small cuts were made into the capsule of thymic piece and the thymocytes were released by repetitive pipetting. The remaining thymic fragments were incubated in 0.5 mg/ml dispase/collagenase (Roche, Basel, Switzerland) and 5 µg/ml DNase I (AppliChem, Darmstadt, Germany) in PBS at 37°C for 20 min with gentle agitation. The released cells were collected as separate fractions and fresh enzyme solution was added, this cycle was repeated four times. All cell fractions were pooled and subsequently enriched for $CD45^-$ cells by a depletion step using CD45 microbeads (Miltenyi Biotec, Hilden, Germany) and the AutoMACS system (Miltenyi Biotec, Hilden, Germany) according to the manufacturer's instructions. The positive fraction was collected and served as a thymocyte-enriched fraction for the Q2chip experiments. The CD45-negative fraction was subsequently stained with anti-EpCAM microbeads (Miltenyi Biotec, Hilden, Germany) and positively MACS-sorted. This fraction was highly enriched in mTECs.

Table 1. Human tissue samples and thymic cell populations by age and source.

Material	Age	Number of patients	Source
cerebellum	>50 years	2	Tartu University Tissue Bank
uterus	>50 years	2	Tartu University Tissue Bank
lung	>50 years	2	Tartu University Tissue Bank
kidney	>50 years	2	Tartu University Tissue Bank
myocardium	>50 years	3	Tartu University Tissue Bank
gray matter	>50 years	2	Tartu University Tissue Bank
spleen	>50 years	1	Tartu University Tissue Bank
liver	>50 years	1	Tartu University Tissue Bank
thymoma	unknown	6 A type, 5 AB type, 5 B3 type	University of Würzburg
normal (non-neoplastic) thymus	32–67 years	5	University of Heidelberg
TEC	6 weeks to 5 years	>10*	Medical School Heidelberg Tartu University Clinic

* means TECs used in different experiments such as cell sorting and Q2ChIP.

4.5. Immunofluorescence and microscopy

Cryostat sections (5 μm) of fresh-frozen 10-day-old mouse thymus were thaw-mounted onto SuperFrost Plus microscopeslides (Menzel–Gläzer) and fixed in cold acetone (−20 ◦C) for 5 min. Sections were permeabilized in PBS/0.5% Triton X- 100/1% normal goat serum (DAKO) for 15 min. Slides were blocked with 1% normal goat sera for 20 min at room temperature and incubated with rat monoclonal G8.8 (1:100) (provided by Andy Farr, University of Washington, USA) or rabbit polyclonal anti-mAIRE (1:2000) [72] and then incubated with Alexa Fluor 594-conjugated goat anti-rat IgG (H + L) or Alexa Fluor 488-conjugated goat anti-rabbit $(Fab)_2$ (both from Invitrogen- Molecular Probes Inc, Eugene, OR, USA), followed by washing six times in PBS. The slides were incubated with 15 μg/ml DAPI (Roche, Basel, Switzerland) and mounted with fluorescent mounting medium (DAKO). The images were acquired by fluorescence microscopy (Eclipse TE2000–4; Nikon, Melville, NY, USA).

4.6. Thymic reaggregate organ culture

Reaggregated thymic organ-cultures were done as described previously [153]. Briefly, thymic stromal cells from E17.5 C57BL/6 mice were prepared by disaggregating fetal thymic lobes, which had been previously cultured for 7 days in 1.45mM deoxyguanosine (Sigma, St. Louis, MO, USA) and dissagregation was done by using 1× trypsin 30 min (Life Technologies, Grand Island, NY, USA). Reaggregates were formed by mixing together stromal cells and thymocytes at a 3:1 cell ratio and cultured for 3 days at 37 °C.

4.7. Quantitative real time PCR

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA, Life Technologies) and reverse-transcribed to cDNA using the SuperScriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA, Life Technologies). Real-time PCR was performed with the ABI Prism 7900HT Fast Real-Time PCR System instrument (Applied Biosystems, Foster City, CA, USA) using qPCR SYBR Green Core Kit (Eurogentec, Liege, Belgium) according to the manufacturer's instructions except that 2mM $MgCl_2$ concentration was used. The amplification program included an initial denaturation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, and annealing and extension at 60 ◦C for 1 min, for 45 cycles. SYBR Green fluorescence was measured after each extension step, and the specificity of amplification was evaluated by melting curve analysis. The relative gene expression levels were calculated using the comparative *C*t (ΔCt) method (according to Applied Biosystems, Foster City, CA, USA), where the relative expression is calculated as $2^{-\Delta\Delta C\mathrm{t}}$, and where *C*t represents the threshold cycle. Every sample was run in three parallel reactions. Quantitative qRT-PCR primer sequences used in the study are listed in Table 2.

In chemokine expression assessment in whole thymi and lymph nodes, and over-expression of Aire induces chemokine expression the Student t-test, $p<0.05$, was used (GraphPad Prism 5 version).

4.8. DNA isolation and bisulfite genomic DNA sequencing

Genomic DNA was extracted from peripheral tissues, sorted thymic cell populations using JETQUICK Tissue DNA Purification Kit (Genomed, Löhne, Germany) or by phenol/chloroform extraction (briefly, add equal volume on phenol/chloroform to DNA solution, mix the top layer with equal volume of chloroform and precipitate DNA with 96% ethanol, wash with 70% ethanol and dissolve the material in destilled water). The DNA was bisulfite treated using EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. This reaction converts all non-methylated cytosine bases into uracil. Traditional sodium bisulfite treatment was performed on neoplastic thymus samples and normal thymuses from cardiac surgery patients. The conversion efficiency of EpiTect Bisulfite kit was compared with traditional conversion method using HeLa cell-line and the kit was used to treat sorted thymic cell population. Briefly, 2 µg of genomic DNA was denaturated with 2 M NaOH for 10 min, followed by incubation with 3 M sodium bisulfite and 10 mM hydroquinone for 16h at 50°C. After treatment, DNA was purified using a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany), precipitated with ethanol, and resuspended in 30 µl of distilled water. Two µl of the aliquot were used as a template for PCR. AIRE promoter region was amplified using BITSA primers as listed in [68]. Amplified DNA fragments were purified with NucleoSpin Extract II kit, cloned into pGEM-T (Promega, WI, USA) or TOPO TA (Invitrogen, Carlsbad, CA, USA, Life Technologies) vector and individual clones were sequenced in University of Melbourne and University of Tartu. Sequence alignment was performed using ClustalW (http://www.ebi.ac.uk/clustalw/).

4.9. Q2ChIP

Quick and quantitative ChIP (Q2ChIP) was carried out as described earlier [154] using protein G-sepharose beads (GE Healthcare, Munich, Germany) preblocked with 100 µg/ml BSA and 500 µg/ml salmon sperm DNA. Cells were fixed with formaldehyde and sonicated as in [119]. Sonicated supernatant containing chromatin of 17,6–22,4 x 10^3 cells, depending on the size of donated thymus and sorting efficiency, was immunoprecipitated using antibodies to H3 (ab1791), H3K4me3 (ab8580) (Abcam, MA, USA); H3K27me3 (Millipore, Billerica, MA, USA); purified rabbit IgG (PP64) (Millipore). Material from 18 000–20 000 cells was used for one immunoprecipitation. Precipitated DNA was extracted with the mix of phenol-chloroform isoamylalcohol (25:24:1). ChIP samples were analyzed in triplicates by quantitative PCR using Maxima

SYBR Green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania) on ABI Prism 7900HT (as in 4.7. Quantitative real time PCR) . The results were analyzed using comparative *Ct* method (Applied Biosystems, Foster City, CA, USA). Datasets of each primer pairs were normalized to ChIP input values and thereafter the histone modification results were normalized to H3. Sequences of the qRT-PCR primers used in this study are listed in Table 2.

4.10. Primer sequences

The sequences of all primers (TAG Copenhagen, Copenhagen, Denmark) used in the study are listed in Table 2.

Table 2. Primers used in the study together with corresponding sequence, species, gene and application of use.

Oligo name	Sequence	Species	Gene	Application
mAire-5-SalI	tttgtcgac agatggcaggtggggatggaatg	mouse	Aire	PCR
mAire-3-NotI stop	tttgcggccgctcaggaagagaagggtggtgtc	mouse	Aire	PCR
K2–8 sense	aggagctcattccgtagctg	mouse	K2–8	qPCR
K2–8 antisense	tctgggatgcagaacatgag	mouse	K2–8	qPCR
Aire 11/12	ccccgccggggaccaatctc	mouse	Aire	qPCR
Aire 12/13	ccccgccggggaccaatctc	mouse	Aire	qPCR
Tff3 sense	tacgttggcctgtctccaag	mouse	Tff3	qPCR
Tff3 antisense	cagggcacatttgggatact	mouse	Tff3	qPCR
Ins2 sense	gacccacaagtggcacaac	mouse	Ins2	qPCR
Ins2 antisense	tctacaatgccacgcttctg	mouse	Ins2	qPCR
Mup1 sense	tctgtgacgtatgatggattcaa	mouse	Mup1	qPCR
Mup1 antisense	tctggttctcggccatagag	mouse	Mup1	qPCR
Spt1 sense	aacttctggaactgctgattctg	mouse	Spt1	qPCR
Spt1 antisense	gaggcctcattagcagtgttg	mouse	Spt1	qPCR
CCL-5 sense	gtgcccacgtcaaggagtat	mouse	CCL5	qPCR
CCL-5 antisense	cccacttcttctctgggttg	mouse	CCL5	qPCR
CCL-17 sense	agtggagtgttccagggatg	mouse	CCL17	qPCR
CCL-17 antisense	ccaatctgatggccttcttc	mouse	CCL17	qPCR
CCL-22 sense	ctgatgcaggtccctatggt	mouse	CCL22	qPCR
CCL-22 antisense	ggagtagcttcttcacccag	mouse	CCL22	qPCR
CCL-19 sense	ctgcctcagattatctgccat	mouse	CCL19	qPCR
CCL-19 antisense	cttccgcatcattagcaccc	mouse	CCL19	qPCR
CCL-21 sense	ccctggacccaaggcagt	mouse	CCL21	qPCR
CCL-21 antisense	aggcttagagtgcttccggg	mouse	CCL21	qPCR
CCL-25 sense	gtgctgtgagattctacttcc	mouse	CCL25	qPCR
CCL-25 antisense	tatggtttgacttcttcctttcag	mouse	CCL25	qPCR
β2m sense	tgagactgatacatacgcctgca	mouse	β2m	qPCR
β2m antisense	gatgcttgatcacatgtctcgatc	mouse	β2m	qPCR
AIREprF	caagcgaggggctgccagtg	human	AIRE	Q2ChIP
AIREprR	gcgatctccgtgcggtgcag	human	AIRE	Q2ChIP
chAIRE 4int F	tccagagaccccctatctcc	human	AIRE	Q2ChIP
chAIRE 4int R	aggaaggcatgaccaatgac	human	AIRE	Q2ChIP
chAIRE 4int R	ttaagagcatggcgtttggt	human	AIRE	Q2ChIP

Oligo name	Sequence	Species	Gene	Application
chAIRE 8ex F	cattcccagtggatccttga	human	AIRE	Q2ChIP
chAIRE 8ex R	caaggtcagtgccgcagggg	human	AIRE	Q2ChIP
chAIRE 12int F	caaggtcagtgccgcagggg	human	AIRE	Q2ChIP
chAIRE 12int R	gcaagccctcgaagctggca	human	AIRE	Q2ChIP
chAIRE 14ex F	cttgtcagtgctcggctgta	human	AIRE	Q2ChIP
chAIRE 14ex R	agtaggtcaccaggcaagga	human	AIRE	Q2ChIP
hGAPDH_prom_F	tactagcggttttacgggcg	human	AIRE	Q2ChIP
hGAPDH_prom_R	acaggaggagcagagag cga	human	AIRE	Q2ChIP

5. RESULTS

5.1. Modulation of Aire regulates the expression of tissue-specific antigens (I)

5.1.1. Tissue-specific antigen expression is dependent on the Aire gene copy number

Intrathymic expression of TSAs has been viewed as the key element in the induction of central tolerance and a central role for the Aire has been suggested in this process. In 2002, Anderson *et al.* showed that the Aire gene promotes the promiscuous expression of peripheral TSAs. The conducted study demonstrated autoimmune manifestations in Aire KO mice that depended on lack of Aire in mTECs and specific reduction in mRNA levels of peripheral TSAs in mTECs [52]. It was followed by array results that showed decreased TSA mRNA levels in Aire KO mouse [83]. Thereof, we selected four genes; trefoil factor 3 (Tff3), insulin 2 (Ins2), salivary protein 1 (Spt1) and major urinary protein 1 (Mup1) representing TSAs downregulated in Anderson *et al*. and Derbinski *et al*. array data, to study whether in the level of the thymus the TSA expression is dependent on Aire. The qRT-PCR values were normalised to the expression level of keratin 8 (K8) mRNA, which in thymus is specifically expressed in epithelial cell fraction and according to microarray data is not influenced by Aire gene. To exclude possible genetic background effects on TSA expression levels, the 4 TSAs were also analyzed in thymi from Balb/c WT, Aire heterozygous (HET) and Aire-deficient (KO) mice (Figure 5).

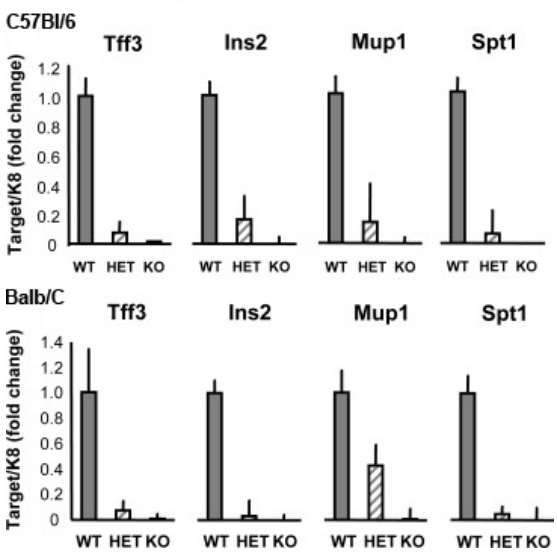

Figure 5. Dose-dependent effect of Aire on TSA expression in C57Bl/6 and Balb/c mice. The whole thymuses from WT, Aire heterozygous (HET) and Aire-deficient (KO) mice were analyzed for TSA gene expression by qRT-PCR.

The qRT-PCR analysis showed almost complete absence of TSA mRNA signal in C57BL/6 and Balb/c Aire-deficient thymus samples for all 4 TSAs studied (Figure 4). Moreover, *Aire* allele dose-dependency was observed, as heterozygous mouse thymus consistently had lower expression levels compared to the WT thymus levels. The expression level of all four TSAs in heterozygous mice thymus was around 10–20% of the expression level in WT mice in both strains, which does not correlate with Aire gene copy number for unknown reason. The results showed a clear Aire allele dose-dependency for Tff3, Ins2, Spt1 and Mup1 and confirmed that genetic background has no influence on Aire-dependent TSAs.

Next we quantified the TSA expression in lymph nodes from C57Bl/6 mouse to determine if Aire has similar effect on TSA expression (Figure 6) in the peripheral immune system.

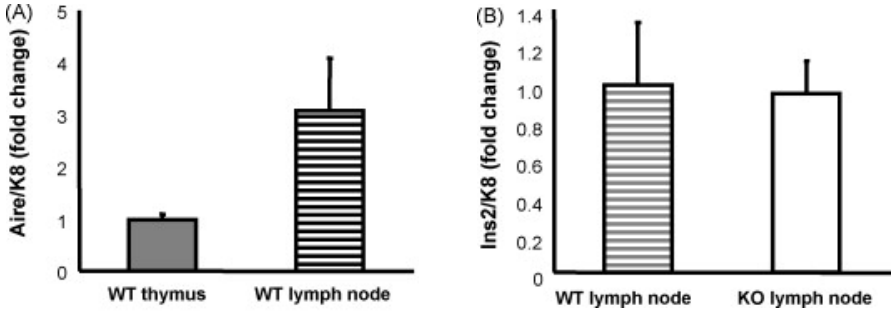

Figure 6. Expression of Aire and Ins2 in the lymph nodes. Thymuses or inguinal lymph nodes were collected from 4 to 6-week-old C57BL/6 mice and analyzed for Aire or Ins2 expression by qRT-PCR. (A) Aire expression was higher in the lymph nodes compared to the whole thymus of WT mice. (B) The expression of Ins2 was unaffected in the Aire KO mice compared to the WT mice. Data are mean with S.E.M. of triplicate measurements of one out of two representative experiments.

The detected Aire mRNA level in the lymph nodes was even higher than the one of the whole thymus (Figure 5A), whereas most of the analyzed TSAs were undetectable or close to the detection limit. The increase was in relative Aire mRNA level, since the expression of the epithelial cell marker K8 was very low in the lymph nodes. However, the mRNA signal for the Ins2 was present in lymph node but was unaltered in the Aire KO compared to the WT mice. Thus, Ins2 expression in lymph nodes did not depend on the presence of Aire (Figure 5B).

To assess Aire co-localization in the thymus with cell-surface marker EpCAM the thymus sections were stained with anti-EpCAM and anti-Aire antibodies, and nuclei were identified with DAPI (Figure 7A). The medullary compartment of thymus was distinctly characterized by high EpCAM expression and Aire-positive cells. Next, thymuses were enzyme digested and FACS-sorted to mTEC and cTEC population to analyse the expression level of Aire and TSAs by qRT-PCR (Figure 7B).

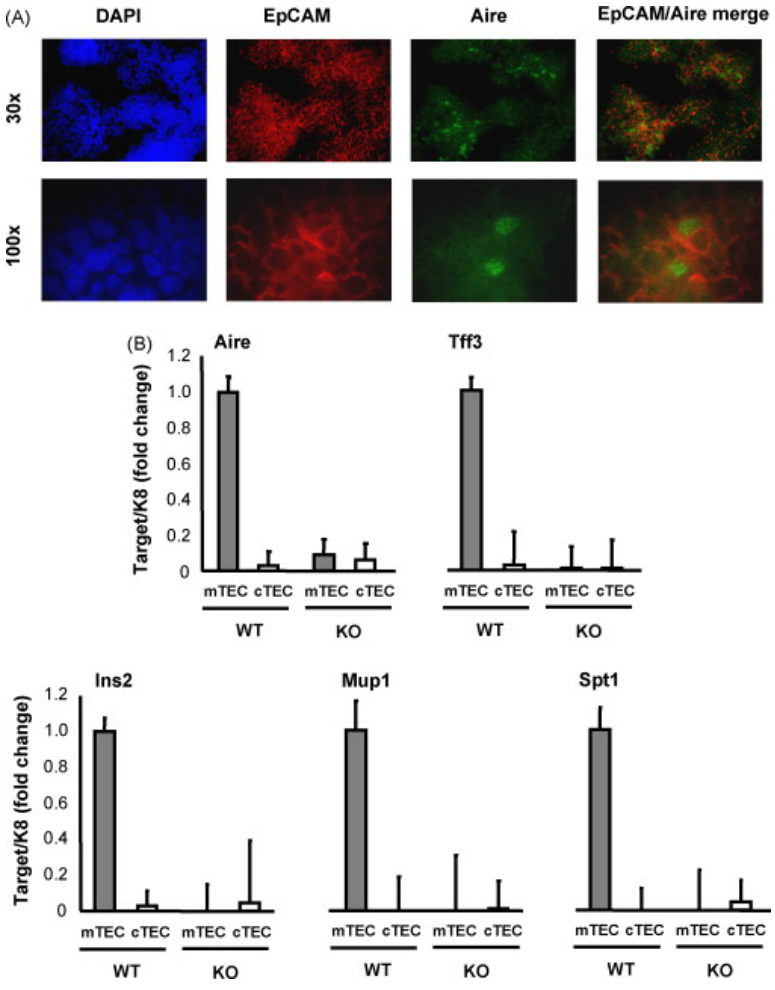

Figure 7. TRA expression in mTEC vs. cTEC populations of WT and Aire KO mice. (A) Thymuses stained with anti-EpCAM and anti-Aire antibodies were analyzed by immunofluorescent microscopy or (B) enzyme digested and FACS-sorted respectively to the EpCAM expression and analyzed for the expression level of TSAs in mTEC and cTEC populations of WT and Aire KO mice by qRT-PCR. Data in (B) are mean with S.E.M. of triplicate measurements of one out of two representative experiments.

The expression of the Tff3, Ins2, Mup1 and Spt1 antigens was limited to the mTEC population, i.e. the cell population of Aire expression. The cTEC population demonstrated very low expression for all four TRAs studied, both in the WT as well as Aire KO mouse.

Combined, these data show that Aire dose-dependently regulates TSA expression in thymus (but not in the lymph nodes), and confirms by qRT-PCR the previously published microarray data, suggesting that both Aire and TSAs are predominantly expressed by mTECs in thymus.

5.1.2. TSAs follow the expression of Aire during thymus development and involution

Hypothesizing that the expression of TSAs is directly dependent on *Aire* gene, this should be evident also throughout the development of thymic tissue. However, the thymic cell content and volume changes significantly during development leading to thymus decrease in size, weight and activity by the process of involution. Thymuses from different embryonic, neonatal, young or adult developmental stages were analyzed for the Aire and TSA expression by qRT-PCR (Figure 8).

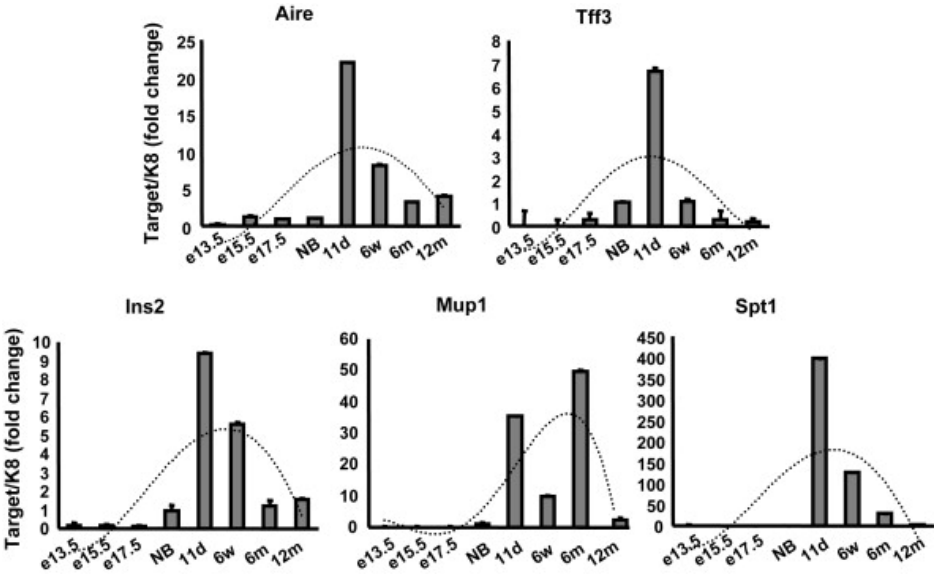

Figure 8. Aire and TSA expression during development. Thymuses were collected from normal WT mice at indicated time-points and the gene expression levels were analyzed by qRT-PCR. Aire expression, as well as the expression of Tff3, Ins2 and Spt1 genes reached their peak at day 11 (D11), Mup1 at 6 months (6m) after birth followed by a gradual decrease. Data are mean with S.E.M. of triplicate measurements of one out of two representative experiments. Dotted line corresponds to polynomial estimation.

Very low Aire expression was detectable already at day E13.5, which significantly increased at E15.5. Therefore, we propose that the start of Aire expression overlaps with the entry of the first wave of the hematopoietic cells into the thymus. In addition, we observed the highest expression level at postnatal D11 and a gradual decrease thereafter until the very last time-point studied. Aire expression was, however, clearly present even in 12-month-old mice. Thus, the expression of TSAs closely followed the pattern of Aire reaching their peak at D11 and followed by a significant decrease, suggesting a role for Aire in TSA regulation.

5.1.3. The impact of Aire on over-expression of TSA

Although Aire deficiency has been shown to reduce TSA mRNA levels, the ability of Aire to act as a single factor to up-regulate TSA expression has not been demonstrated. We asked whether the TSA expression is influenced by Aire in the conditions of Aire over-expression. Therefore, murine Aire gene was amplified from pcAire plasmid (made by Niko Sillanpää [72]) and cloned into pAdTrack-CMV plasmid, which was incorporated to adenoviral backbone plasmid pAdEasy-1. The recombinant plasmids were transfected into adenovirus packaging cell line HEK293 and recombinant Aire-expressing adenoviruses (AdAire-GFP; expresses green marker) (Figure 9A) were generated. The negative control (AdGFP) without insert was constructed similarly. Western blotting confirmed that transfected cells express Aire protein (Figure 9B) and it can be used as a tool to study Aire influence on the tissue-specific antigen expression. AdAire-GFP and negative control were transduced into primary stromal epithelial cells (which had lost Aire expression) and thymic medullary epithelial cell line TEC 1C6 and again TSA expression was studied by qRT-PCR (Figure 9C, D).

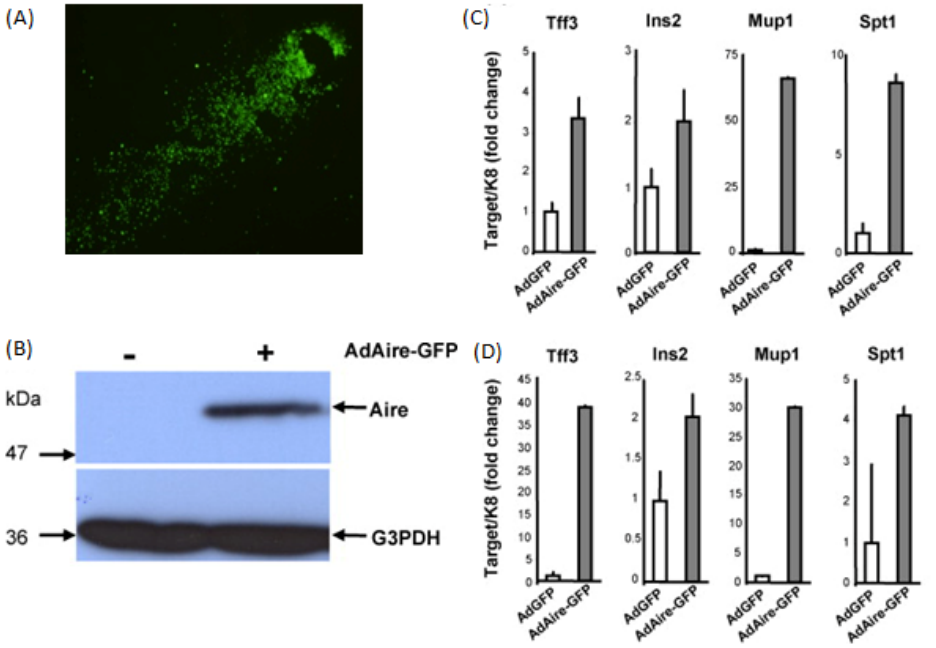

Figure 9. Over-expression of Aire induces TSA expression in primary thymic stromal cells and in thymic epithelial cell line. (A) Transduction of AdAire-GFP in HEK293 resulted in production of Aire-GFP protein. (B) Aire detection in AdAire-GFP transduced HEK293 cell line by *Western blot.* (C) Monolayers of primary thymic stromal cells or (D) thymic medullary epithelial cell-line TEC 1C6 were infected with equal amounts of Ad-GFP or AdAire-GFP. The Aire influence on the expression of TSAs was assessed by qRT-PCR. Data are mean with S.E.M. of triplicate measurements of one out of two representative experiments.

The addition of Aire increased the expression of Tff3, Ins2, Mup1 and Spt1 in monolayer culture of primary stromal cells and thymic medullary epithelial cell-line TEC 1C6. Altogether, Aire is sufficient for TSA induction in thymic epithelial cells in conditions where thymic cell signals are absent.

5.1.4. Ex vivo thymus culture and TSAs

During the cultivation of primary thymic monolayer culture we observed that the Aire expression fades after the disruption of 3-dimentional structure of the thymus. In addition, it has been shown that the 3D structure of thymus is needed for mTEC functions [155]. In order to gain insight into how the microenvironment affects Aire and TSA expression, we cultivated 2-dimensional (monolayer) disaggregated (thymocyte depleted) culture and reaggregated thymic organ culture (RTOC; thymocytes added). Thymuses from E17,5 embryos of C57BL/6 mice were harvested and used as intact thymus (3D lobes), disaggregated (3D>2D) or reaggregated by mixing freshly purified thymocytes with disaggregated thymic stromal cells (Figure 10).

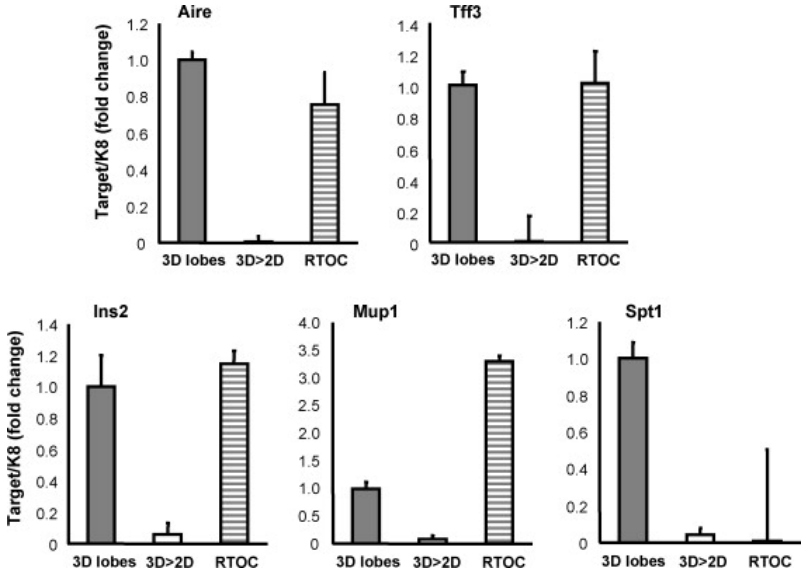

Figure 10. Thymic microenvironment is required for Aire and TSA expression. Thymuses were used as intact thymus (3D lobes), dissaggregated (3D>2D) or reaggregated (RTOC) cultures. Disaggregation resulted in loss of expression of Aire and TSA, which was recovered after reaggregation (except Spt1). Data are mean with S.E.M. of triplicate measurements of one out of two representative experiments.

The data show that the disruption of 3D structure of the thymus results in dramatical decrease of Aire and 4 TSAs, which was recovered by some extent by reaggregation suggesting a major role for the thymic microenvironment in Aire and TSA expression.

5.2. Aire infuences chemokine receptor CCR4 and CCR7 ligand expression (II)

5.2.1. Expression of Aire and thymic chemokines in thymus and lymph nodes

Since Aire has an ability to modulate TSA expression in thymic epithelial cells we were asked whether Aire can regulate the expression of thymic chemokines and influence the thymocyte migration. Since CCR4 and CCR7 have been shown to participate in cortico-medullary migration and negative selection, we choose to characterize the expression of 3 ligands for CCR4 (CCL5, CCL17 and CCL22), and 2 ligands for CCR7 (CCL19 and CCL21). We also selected CCR9 ligand CCL25, which is a chemokine known to mediate the outward movement of DN thymocytes in thymus [11]. We analyzed the mRNA transcript levels of these 6 chemokines in thymuses and inguinal lymph nodes from mice with different genotypes such as WT and Aire KO (Figure 11). To detect expression of chemokines in the epithelial cell population only, the data was normalized to the expression level of keratin 8 (K8) mRNA, which is expressed selectively by thymic epithelial cells.

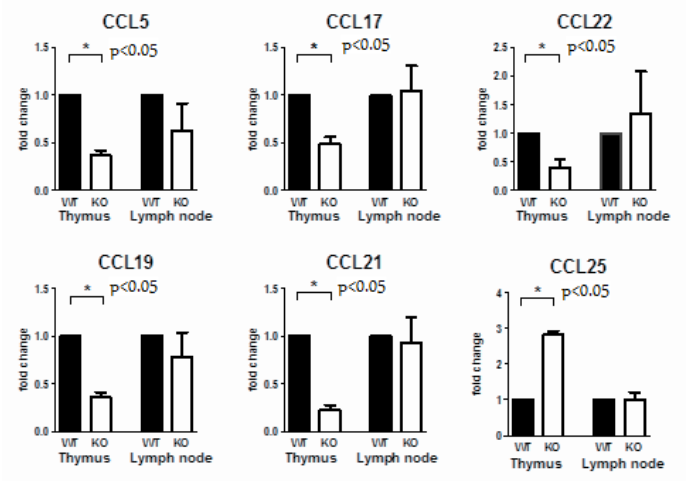

Figure 11. Chemokine expression in thymus and inguinal lymph nodes. Thymi and inguinal lymph nodes were dissected from 6–8 weeks old wild type (WT) and Aire KO (KO) mice and analyzed by qRT-PCR. Data are mean with S.E.M. *, Student t-test, $p<0.05$; all other comparisons between WT and Aire KO are not significant, n=3–5.

The expression of CCL5, CCL17, CCL22 (CCR4 ligands) and CCL19, CCL21 (CCR7 ligands) was down-regulated in thymi from Aire KO mice, whereas the expression of the CCR9 ligand, CCL25, showed an increased expression in the Aire KO thymi. The expression of the chemokines in lymph nodes remained unchanged.

5.2.2. Thymic chemokines follow the expression of Aire during thymus development and involution

As the TSAs followed Aire expression during thymus development (Figure 8) we asked whether thymic chemokines could also correlate the Aire expression. For this reason thymi from various mouse embryonic (E13,5-E17,5), neonatal (10 days), young (6 weeks) or adult developmental (6 months) stages were analyzed for Aire and thymic chemokine expression (Figure 12).

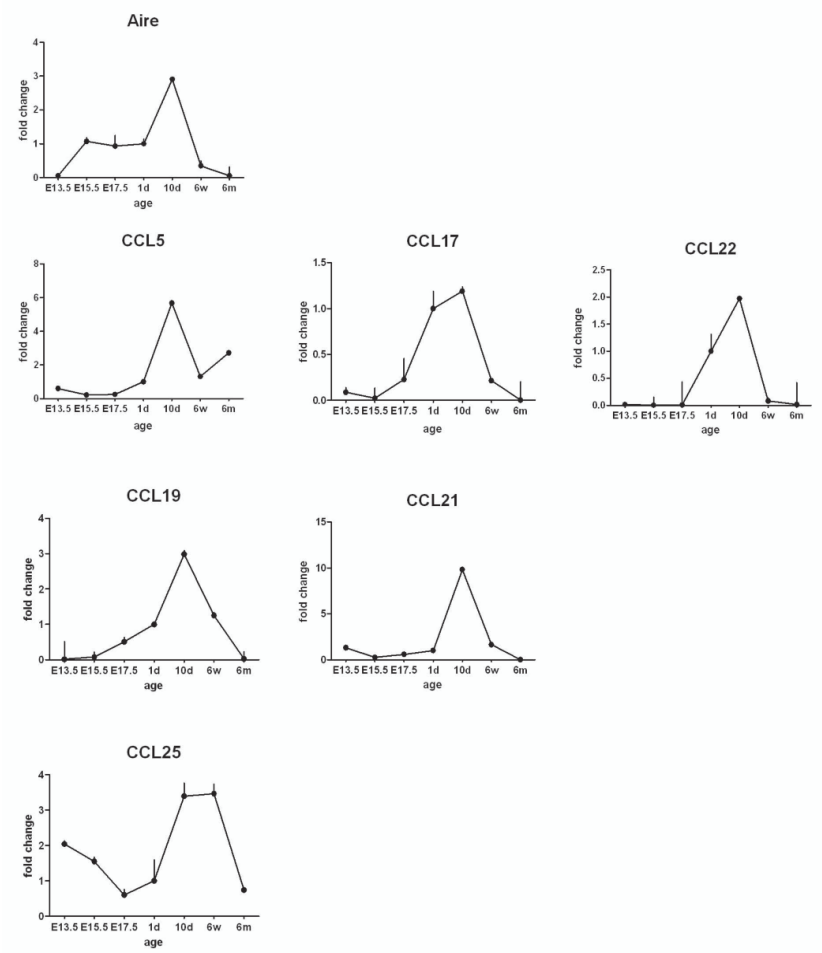

Figure 12. Aire and chemokine expression during development. Thymuses were collected from normal WT mice at indicated embryonic (E) and post-natal (d-day, w-week, m-month) time-points and the gene expression levels were assessed by qRT-PCR. Data are mean with S.E.M. of triplicate measurements of one out of two representative experiments. Line corresponds to polynomial estimation.

The results demonstrate that the chemokines CCL5, CCL17, CCL21 and CCL25 were already expressed at the E13.5, though Aire showed little expression at this time-point. At the postnatal stage the expression level of most of the

thymic chemokines followed the pattern of Aire, with a peak at day 10 followed by gradually decrease in expression. The only exceptional chemokine was the CCR9 ligand, CCL25, whose expression started very high at E13.5, then decreased gradually at E17.5 time-point, and reached a second peak at post-natal day 10, which decreased until 6 weeks of age.

5.2.3. The impact of Aire over-expression on thymic chemokine expression

Next, we asked whether Aire, as a single factor, can influence the thymic chemokine expression. And again, we used an adenoviral expression system (AdAire-GFP vs. Ad-GFP) for the overexpression of Aire. Infecting the TEC 1C6 cells with AdAire-GFP or Ad-GFP control viruses resulted in a virtually equal infection rate as determined using FACS by evaluating the percentage of GFP positive cells at 48 hours (AIRE-GFP: 38.1±4.3% vs. ad-GFP: 42.8±2.8%; mean with S.E.M., n=3, data not shown). Infected cells were harvested for RNA purification and the expression was analyzed by qRT-PCR (Figure 13).

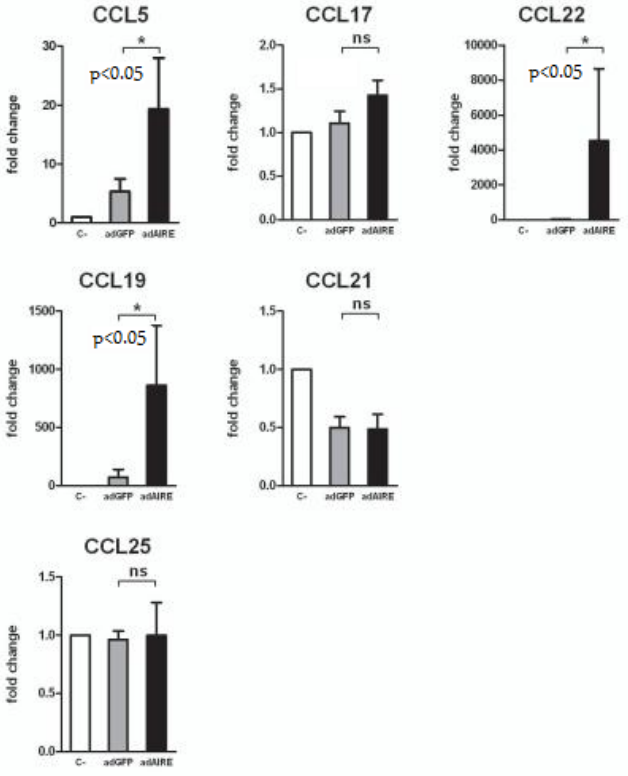

Figure 13. Over-expression of Aire induces chemokine expression in thymic epithelial cells. Infected TEC 1C6 cells with AdAire-GFP (adAIRE) or Ad-GFP control (adGFP) viruses, C – uninfected TEC 1C6 cells. Data are mean with S.E.M. *, Student t-test, $p<0.05$; ns= not significant, n=3–5.

Infection of TEC 1C6 cells with AdAire-GFP resulted in a significant increase in the expression of CCL5, CCL22 and CCL19, whereas there was no change in the expression level of CCL17, CCL21 and CCL25 chemokines. Obtained results demonstrate that in the absence of signals provided by other cell-types present in thymus, Aire is sufficient to boost the expression of several thymic chemokines in TECs.

5.3. DNA methylation signatures of the AIRE promoter in thymic epithelial cells, thymomas and normal tissues (III)

5.3.1. AIRE promoter methylation in thymus

The presence of CpG island in *AIRE* promoter was suggested in 1997, when AIRE gene was identified [41]. In accordance with this, Murumägi *et al.* mapped the CpG island of AIRE gene, which starts from approximately 300 bp upstream of translation start site and encompasses the first exon (Figure 14). Additionally, it was demonstrated that the CpG island in AIRE gene is highly hypermethylated in several AIRE-negative cell lines [68]. Furthermore, AIRE expression was induced in methyltransferase DNMT1 and DNMT3b double-deficient cell lines suggesting the regulation of AIRE gene expression via CpG methylation [69].

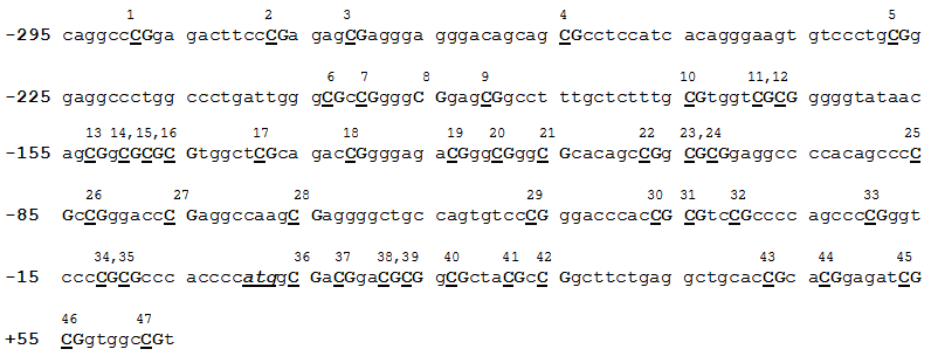

Figure 14. Distribution of CpG positions within the AIRE promoter region. The positions of CpG dinucleotides in AIRE promoter CpG island (-295 to +65, positions relative to the atg of the AIRE gene) are indicated in bold and by numbers. The translation start site is indicated by the atg codon in boldface. Modified from [68].

First we analyzed the methylation of 47 CpG dinucleotide positions within the CpG island of the human *AIRE* gene in 5 normal thymus tissues (Figure 15) adjacent to thymomas by bisulfite sequencing.

The analyzed 5 thymus samples showed highly variable methylation patterns from relatively moderate (20–60%) methylation across investigated positions to high (80–100%) methylation. In conclusion, these results confirm the presence of variable methylation pattern of the AIRE promoter on the level of normal thymus tissue.

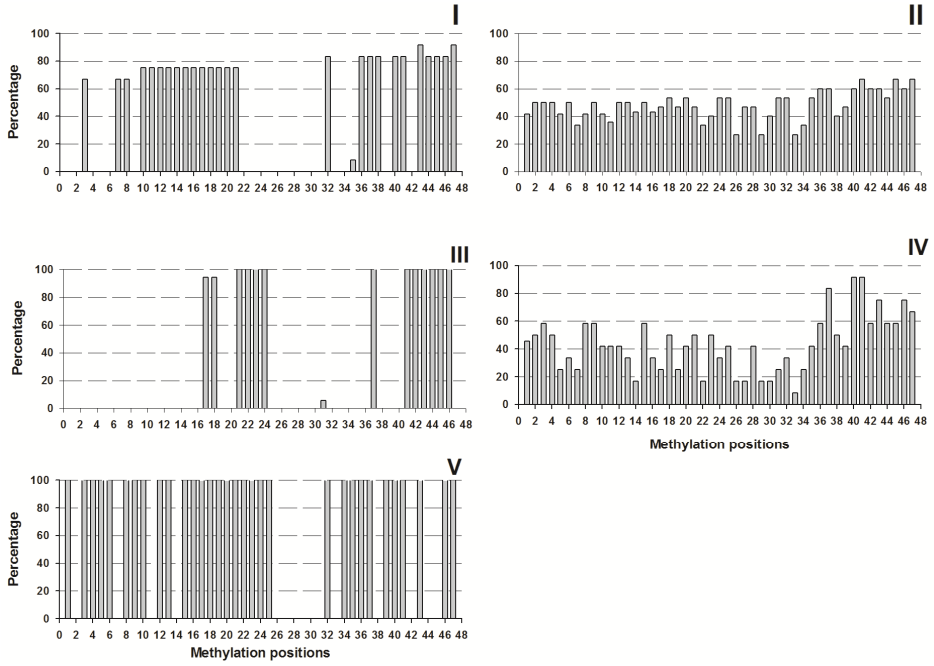

Figure 15. AIRE promoter methylation pattern in normal thymus tissue across 47 CpG positions analyzed. Each bar represents the methylation of a single CpG motif.

5.3.2. AIRE promoter methylation in human thymic epithelial cells

The highly variable methylation pattern of the AIRE promoter in normal thymus tissues might be the result of differences in cell composition. Taking into account that thymocytes form the vast majority of all cell types in thymus, the observed results most likely reflect the thymocyte specific methylation pattern. Therefore, we purified epithelial cell subsets from human thymus tissue and measured AIRE expression in $CD45^{+}$ cells, cTECs, immature MHC class II^{lo} and mature MHC class II^{hi} mTECs and normal thymus (Thy1) (Figure 16).

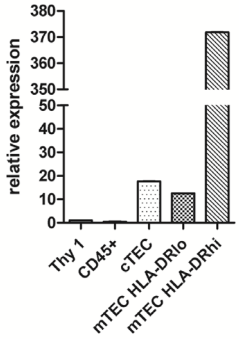

Figure 16. Analysis of AIRE expression in normal thymus, sorted thymic epithelial cell and thymocyte populations. AIRE expression was analyzed by qRT-PCR in CD45+, cTEC, mTEC HLA-DR^{lo}, mTEC HLA-DR^{hi} cells.

The obtained results confirm that the predominant cell population expressing AIRE in the thymus is terminally differentiated (mature) CD80^{hi}/MHC class II^{hi} mTEC. We therefore asked further whether the CpG island within the human AIRE promoter is less methylated in the MHC class II^{hi} mTEC population when compared to the other thymic epithelial subsets. To this end, we conducted the methylation analysis of purified genomic DNA on respective populations (Figure 17).

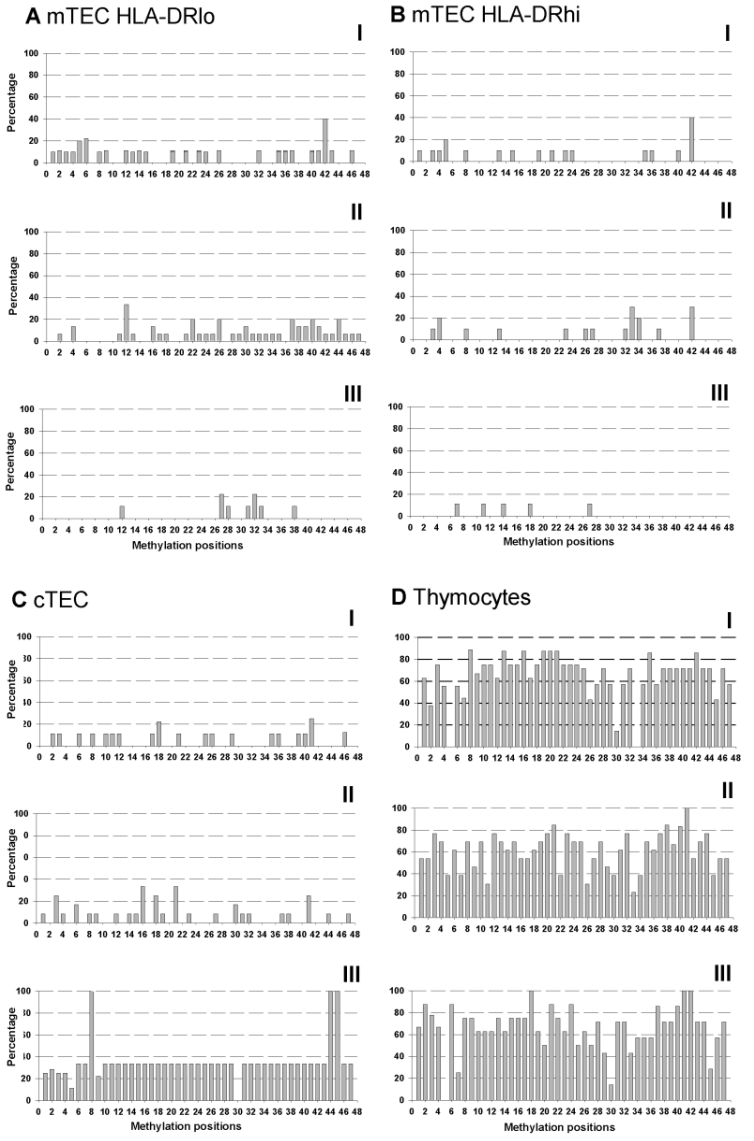

Figure 17. Analysis of the human AIRE promoter methylation pattern in sorted thymic cell populations. The methylation was analyzed in the following samples: (A) mTEC HLA-DR^{lo} (population representing low AIRE expression, I–III) and (B) mTEC HLA-DR^{hi} (population representing high AIRE expression, I–III); (C) cTEC (I–III); (D) thymocytes (I–III). Each bar represents methylation of a single CpG position.

The methylation analysis of purified genomic DNA in three individual donors showed relatively uniform intraindividual methylation pattern between MHC class II^{lo} and MHC class II^{hi} mTEC subsets. Thymocyte population had relatively high methylation (60–80%) in positions analyzed. These results indicate that inspite of the strong induction of Aire expression during terminal differentiation of MHC class II^{lo} to MHC class II^{hi} mTECs is not associated with significant changes in the methylation of the AIRE promoter.

5.3.3. AIRE promoter methylation in thymomas

Thymomas are neoplasms of thymic epithelial cells and are classified into several subtypes according to the WHO classification [88], based on their morphology and the content of lymphocytes. AIRE expression is lost in 95% of thymomas and only 5% of thymomas, mainly representing a subset of the rare organoid type B1, express AIRE and their architecture largely resembles normal thymus [91]. To investigate, whether the absence of expression in thymomas might be the result of high methylation levels, we analyzed AIRE promoter methylation by bisulfite sequencing in 6 samples of type A (medullary type), 5 samples of type B3 (cortical type) and 5 samples of type AB (mixed medullary and cortical type) thymomas (Figure 18).

Current analysis demonstrated that all thymomas had highly diverse methylation patterns with a clear prevalence of demethylation in the majority of sequenced positions. Based on the results, thymomas could be subdivided into 2 groups: one group with complete methylation at single CpG positions (A, I and IV; B, III and IV; C, I and V), and a second group in which the promoter was highly demethylated (A, II, III and VI; B, II and V; C, II-IV). The only exception to this classification was thymoma IV from type A, which had a high degree of methylation (70–80%) in almost all CpG positions analyzed. We could not identify any methylation pattern in the *AIRE* promoter specific to types of thymomas. On the contrary, obtained data indicate that the thymoma samples despite of their type showed methylation patterns in the *AIRE* promoter that may reflect their clonal origin.

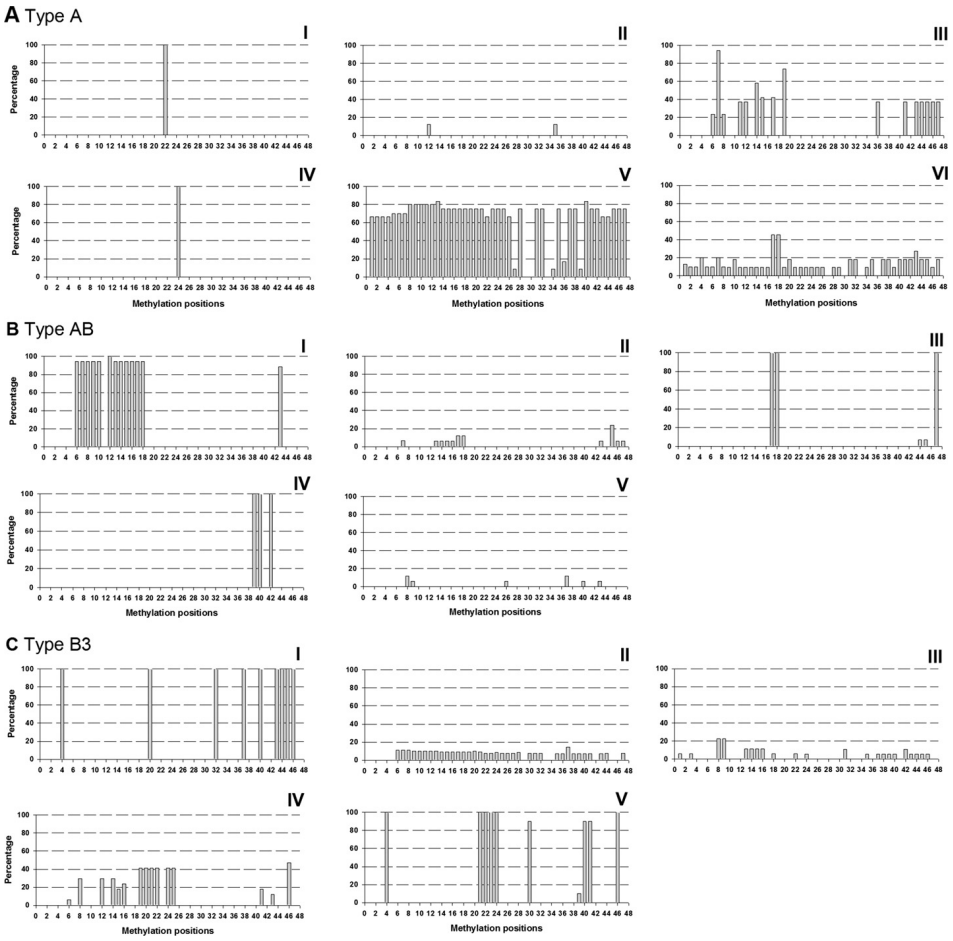

Figure 18. Human *AIRE* promoter methylation pattern in thymomas. (A) Type A (I–VI), (B) type AB (I–V) and (C) type B3 (I–V) thymomas were analyzed for *AIRE* promoter methylation. Each bar under thymoma sample represents methylation map of a single CpG position.

5.3.4. AIRE mRNA expression and promoter methylation in peripheral tissues

Previous results suggested a role for DNA methylation in the regulation of the *AIRE* gene in cell lines: treatment with DNA methyltransferase inhibiting reagent 5-aza-2'-deoxycytidine upregulated the AIRE expression in different cell lines [68], and cells deficient in both *DNMT1* and *DNMT3b* showed higher levels of AIRE expression than wild-type controls [69]. Hence, AIRE expression might be repressed in peripheral tissues due to hypermethylation in the *AIRE* promoter. We tested this hypothesis by correlating mRNA expression levels (Figure 19) with methylation in 14 normal tissues (Figure 20).

AIRE expression in thymic cell populations such as CD45+, cTEC, mTEC HLA-DR^{lo}, mTEC HLA-DR^{hi} cells (Figure 19) was compared to the following samples: cerebellum, uterus, lung, kidney, myocardium, gray matter, spleen and liver. Expression analysis of peripheral tissue samples shows a lack of AIRE expression in most tissues compared to mature mTEC HLA-DR^{hi} cells.

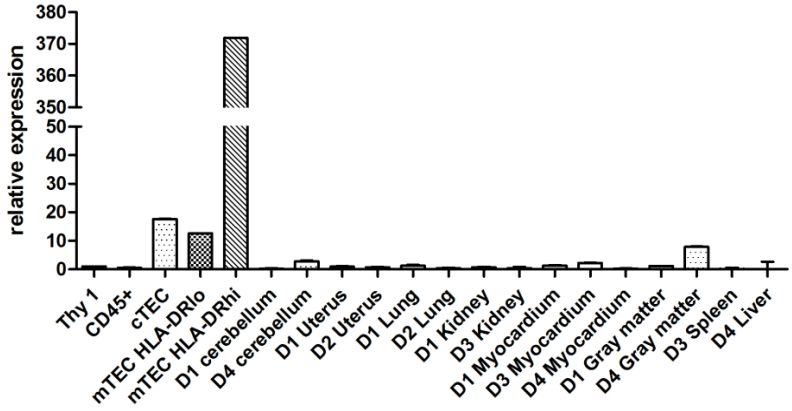

Figure 19. AIRE mRNA expression level in thymus, thymic epithelial cell populations (CD45+, cTEC, mTEC HLA-DR^{lo}, mTEC HLA-DR^{hi} cells), peripheral tissues such as cerebellum, uterus, lung, kidney, myocardium, gray matter, spleen and liver from 4 donors (D1-D4). The expression was normalized to β-actin.

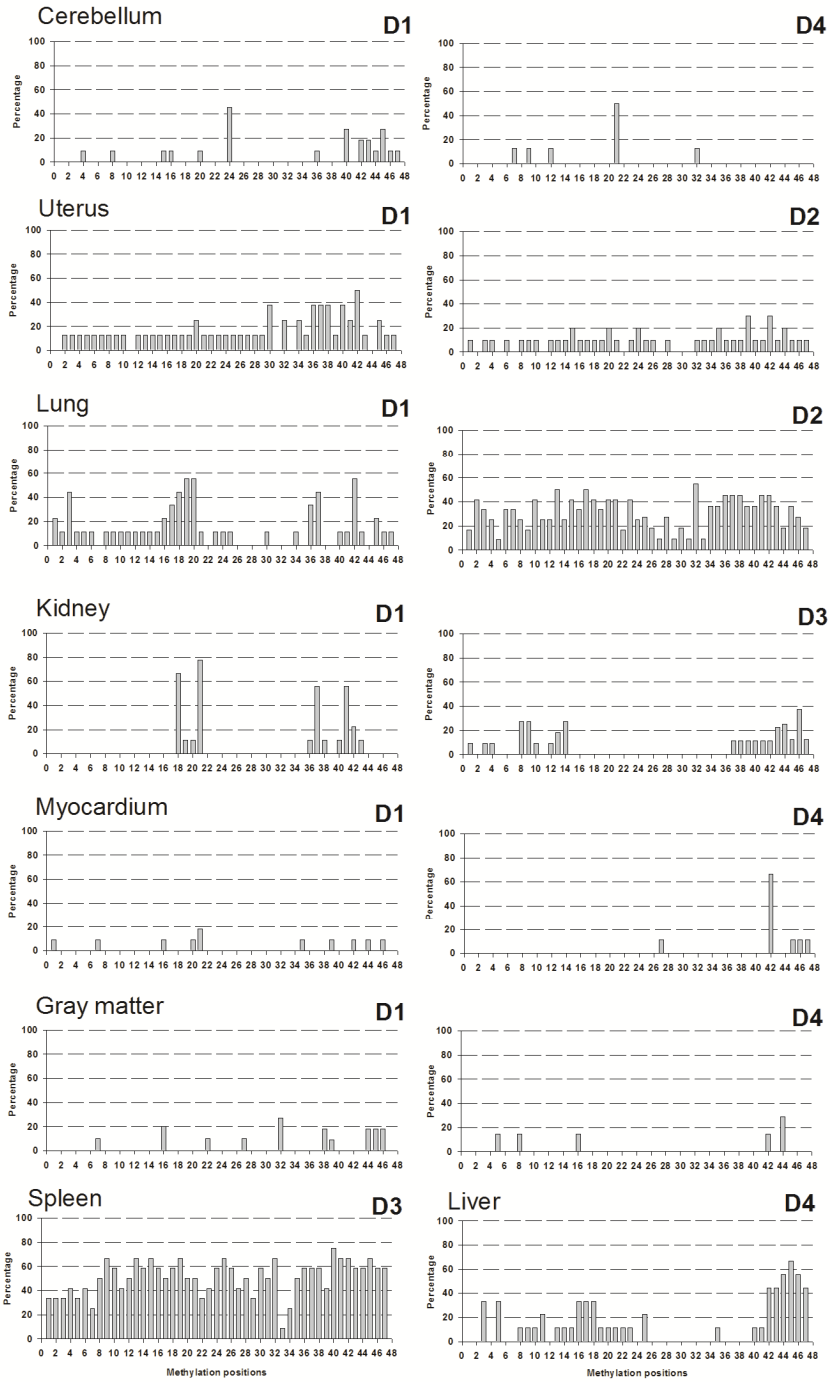

Figure 20. Analysis of the human *AIRE* promoter methylation in the peripheral tissues such as cerebellum, uterus, lung, kidney, myocardium, gray matter, spleen and liver from 4 donors (D1-D4). Each bar represents the methylation of a single CpG motif.

The *AIRE* promoter in tissues such as cerebellum, kidney, myocardium, gray matter and liver had a very low degree of methylation scattered across all positions studied. Uterus and lung samples showed a modest degree of methylation (10–40%) in the majority of analyzed positions. By contrast, the spleen had the greatest extent of methylation (about 60–70%) across the majority of CpG positions. In addition to tissue-specific patterns, the analysis demonstrated inter-individual differences in methylation pattern in several tissues. Alltogether, these data show that hypermethylation of the *AIRE* promoter does not account for the lack of AIRE expression in most peripheral tissues.

5.3.5. AIRE expression correlates with permissive promoter histone marks

Previous studies showed increased AIRE transcription upon treatment of human cell lines with trichostatin A [68] and increased H3K4me3 levels on the mouse *Aire* promoter during mTEC differentiation from $CD80^{lo}$ to $CD80^{hi}$ cells [119]. We investigated H3K4me3 and H3K27me3 chromatin modifications on human sorted cells. Due to limitations in the number of purified mTEC subsets from individual human thymic samples, we sorted for hemopoietic cells (i.e., $CD45^+$, $EpCAM^-$) and TECs (i.e., $CD45^-$, $EpCAM^+$). Firstly, AIRE expression levels were measured in $EpCAM^+$ cells and $CD45^+$ thymocytes (Figure 21, A). Secondly, we conducted Q2ChIP analysis of H3K4me3 and H3K27me3 modifications on the *AIRE* promoter and gene region (covering promoter; intervening sequences and exons based on random selection) in $EpCAM^+$ cells and $CD45^+$ thymocytes (Figure 21, B).

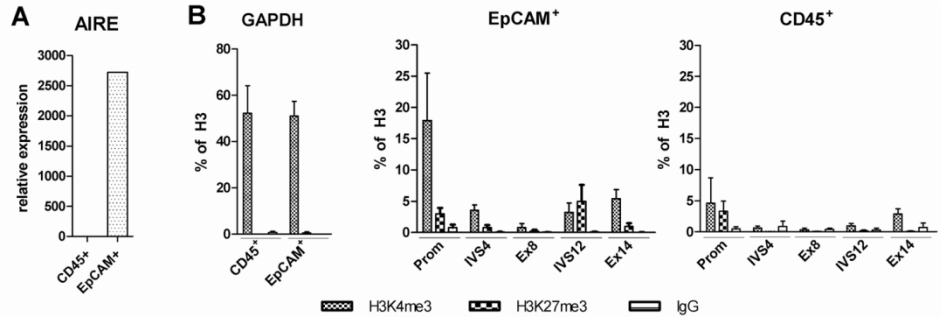

Figure 21. Chromatin modifications in the human *AIRE* promoter and gene. (A) AIRE expression levels were assessed in $EpCAM^+$ cells and $CD45^+$ thymocytes. (B) Q2ChIP analysis of the *AIRE* promoter and gene region (prom- promoter, IVS- intervening sequence, Ex- exon) in $EpCAM^+$ cells and $CD45^+$ thymocytes. The average values of 3 independent experiments including SEM are shown.

AIRE expression is confined to mTEC population, which also express high levels of EpCAM. In order to detect histone modifications in the human *AIRE* gene in $EpCAM^+$ TECs and $CD45^+$ thymocytes, we applied the Q2ChIP method and used specific antibodies against H3K4me3 and H3K27me3, which mark transcriptionally active and silenced chromatin, respectively [129]. An antibody against the histone H3 C-terminus was used as an internal control. The *AIRE* gene promoter had a higher level of H3K4me3 in $EpCAM^+$ cells than in $CD45^+$ thymocytes. In $EpCAM^+$ TECs, the H3K4me3 level was lower in intervening sequence 4 and exon 8 but slightly higher in intervening sequence 12 and exon 14 towards the 3'end of the *AIRE* gene. The H3K27me3 levels in $EpCAM^+$ TECs and $CD45^+$ thymocytes were similarly low in the promoter region, further decreasing in the middle of the gene, and negligible in the intervening sequence 12 at the 3' end of the gene. The promoter of the housekeeping gene *GAPDH* served as a positive control. Thus, there is link between AIRE expression and H3K4me3 permissive histone modification on the *AIRE* promoter.

6. DISCUSSION

The understanding of thymic expression of self-antigens started to evolve in the beginning of 1990s after the hypothesis presented by Boon and van Pel [156], suggesting that the thymus expresses many tissue-specific genes as genomic fragments. They proposed that promiscuous expression of peripheral proteins in the thymus allows immature thymocytes to become tolerant to self-proteins which they would encounter in peripheral sites within body. Consistent with this hypothesis Hanahan and colleagues demonstrated in 1997 that rare thymic medullary cells express pancreatic genes (i.e. insulin) [157] challenging general rules of cell-type specific gene expression. Although the significance of promiscuous gene expression had been proposed long time ago, its role was strongly established with the discovery of how the lack of AIRE promotes autoimmunity in human and mice. The establishment of purification protocol for thymic stromal cell populations [31] boosted investigation attempts towards characterization of peripheral tissue-specific antigens in thymus. The extraordinary finding was that mTECs are specialized to express in random manner a highly diverse set of genes representing all tissues of the body and their expression is fully maintained in the involuting thymus of aging mice [31]. Our obtained results confirmed that the expression of four TSAs; trefoil factor 3 (Tff3), insulin 2 (Ins2), salivary protein 1 (Spt1) and major urinary protein 1 (Mup1), was limited to the mTEC population, i.e. the cell population of Aire expression. Unexpectedly, the expression level of all four TSAs in heterozygous mice thymus was around 10–20% (instead of expected 50% with 1 Aire gene copy) of the expression level in WT mice in both strains studied and the finding does not correlate with Aire gene copy number for unknown reason. It is possible that only one Aire copy is not sufficient for the functional activity of the gene product or that Aire has allele-specific expression. At the same time we detected Aire allele dose-dependency of 2 copies for Tff3, Ins2, Spt1 and Mup1 and confirmed that difference in mouse strains has no influence on Aire-dependent TSAs. We observed that during development that the expression of TSAs closely followed the pattern of Aire reaching their peak at day 11 followed by a significant decrease, suggesting a role for Aire in TSA regulation. Lack of Aire has been shown to reduce TSA mRNA levels [52, 83], but the ability of Aire to act as a sufficient factor to up-regulate TSA expression has not been demonstrated before our study. It is difficult to speculate how many copies of Aire inside the transduced cells are needed to promote TSA expression in monolayer cultures, which had previously lost the ability to express Aire and Aire-dependent TSAs. As overexpression of Aire in primary thymic stromal cells and thymic epithelial cell-line TEC 1C6 results in somewhat different expression level of TSAs, we can speculate that each culture has their own available combination of transcription factors needed for expression.

The thymus provides specialized microenvironments that direct and support T cell differentiation and selection, which in turn influence the maturation of thymic epithelial compartment and the disruption of normal thymic architecture

changes the expression pattern and functionality of thymus [158]. The maturation of thymic epithelial stroma during the fetal period occurs independently of thymocyte-derived signals and is mainly regulated by thymic mesenchyme [159, 160], thymocytes deliver signaling molecules needed to maintain the normal thymic microenvironment. In this study, we show that the disruption of the three-dimensional thymic meshwork into two-dimensional culture results in rapid loss of Aire and TSA expression, whereas Aire as well as TSA expression was restored in reaggregated thymic organ culture. In the current case it could mean that interactions between mTECs and hematopoietic cells determine the scope of TSA expression.

It also appears that the array of genes induced by Aire varies by cell type. The comparison of Aire activity, when ectopically expressed in various cell types, shows that Aire has the tendency for promoting TSA expression in a variety of cellular contexts, but a different set of TSAs can be activated in each case [100, 109, 161]. Interesting is the notion that although Aire induces the expression of thousands of TSAs in mature $CD80^{hi}$ mTECs, numerous TSAs are also expressed in conditions of Aire-deficiency and these Aire-induced and –independent genes tend to cluster in the genome [83]. The results from the same study demonstrated that promiscuously expressed genes can be divided into 4 subgroups: (a) genes that are expressed at similar levels in cTECs and mTECs and at much lower levels, if at all, in hematopoietic cells (e.g., *PLP* or thyroglobulin); (b) genes that are only expressed in mTECs, irrespective of the maturation stage (e.g., *CRP*); (c) genes that are strongly induced in $CD80^{hi}$ mTECs dependent on Aire (e.g., insulin or *casein* α); and (d) genes that are induced in $CD80^{hi}$ in the absence of Aire (e.g., *GAD67* or *casein* β). In the context of promiscuous gene expression the definition of tissue-specific (or tissue-restricted) genes are defined as genes that are expressed in fewer than 5 of 45 tissues are defined as tissue-specific [83]. Aire-dependent TSAs seem to be transcribed differently than they are in the peripheral organ in which they are normally expressed [162]. One obvious difference between the thymic and peripheral tissue expression of TSAs is that the levels of TSA expression within mTECs are generally low when compared with the peripheral organ associated with a given TSA [31, 85]. This suggests that remarkably low level of antigen expression is sufficient for negative selection. The main mechanism by which Aire has been proposed to induce central tolerance is the promotion of the expression of peripheral tissue-specific genes in mTECs thus making them available for presentation. Current observations have suggested that there are two modes of presentations: firstly, known as antigen spreading, according to which TSAs are transferred to and cross-presented by thymic DC and secondly, self-antigen presentation by the mTEC themselves. Despite the thymic deletion of autoreactive T cells, a significant number of autoreactive T cells evade thymic deletion, which suggests that the process is incomplete. Therefore the mechanism of peripheral tolerance must exist to provide additional control to the process of thymic deletion. In our study, we detected the expression of both Aire and Ins2 antigen in inguinal lymph nodes, although the expression of Ins2

expression was not affected by the lack of Aire. After this it was published by other group that Aire in secondary lympoid organs is required for expression of several TSAs and these extrathymic Aire-expressing cells delete naive autoreactive T cells [161], which supports the immune tolerance by preventing the maturation of autoreactive T cells that escape thymic negative selection. Extrathymic Aire-expressing cells may play important role during aging and thymus involution shifting self-tolerance from thymus to periphery.

In addition to the modulation of TSA expression, Aire influences the production of the thymic chemokines involved in cortico-medullary migration and thus plays a role in intrathymic thymocyte migration and maturation. The cortico-medullary migration depends on the expression of two chemokine receptors, CCR4 and CCR7, and their corresponding ligands [11]. CCR4 and CCR7 are predominantly expressed on DP and SP CD4+ thymocytes [9, 10, 163], while their ligands are produced predominantly in the thymic medulla [9, 10, 164, 165]. Since the negative selection takes place in thymic medulla, the coordinated expression of CCR4 and CCR7 and their corresponding ligands is likely to direct the induction of central tolerance [11]. Moreover, array data from Aire KO mice suggests that in addition to down-regulation of TSAs, there is also an aberrant expression of a number of chemokines in the mTEC population [52, 83]. We currently showed that in Aire-deficient mice there is a down-regulation of the ligands for CCR4 and CCR7 (CCL5, CCL19 and CCL21), which are responsible for cortico-medullary migration. Whereas the expression of the chemokine, CCL25 [166], known to mediate the outward movement of DN thymocytes is elevated in the Aire KO mice, suggesting that the coordinated migration of thymocytes in Aire KO may be influenced at a different level of maturation. In this context we demonstrated that at the level of whole tissue, the majority of chemokines were not affected by Aire KO lymph nodes. Our results are in agreement with a report that stated that the number of Aire-target genes is different and more limited in the periphery [161] and also indicated a minor role for Aire in the regulation of peripheral chemokines.

In concordance with Aire and TSA expression during development, the CCR4 and the CCR7 ligands followed the expression pattern of Aire by having the highest expression peak at day 10 and gradually declined thereafter. However, a clear signal for many chemokines at E13.5 was detected, before the obvious increase in Aire signal at day E15.5, which suggests that in early development, mechanisms other than Aire trigger the chemokine expression needed for migration of early thymocytes. We also showed that the overexpression of Aire as a single factor induces the expression of the CCR4 ligands (CCL5 and CCL22), as well as the CCR7 ligand (CCL19). CCL17 and CCL21, although having clearly Aire-dependent expression, were not induced in other experimental settings, suggesting that optimal chemokine induction requires additional signals, maturation or cell-types. Likewise, the CCR9 ligand, CCL25, although negatively regulated in Aire-deficient situation, was not reduced by Aire overexpression, suggesting again an indirect mechanism behind this effect.

As Aire is predominantly expressed in the mature epithelial cells in thymus, we decided to investigate whether this tissue-specific expression is associated with CpG methylation signature in the promoter of the AIRE gene. Previous studies have demonstrated an induction of AIRE expression upon experimental global hypomethylation and increased AIRE expression levels in DNMT1 and DNMT3b double-deficient cell lines, which suggested that genomic methylation might be a critical mechanism for *AIRE* gene regulation [68, 69]. In mixed thymic cell populations we found partial methylation of the *AIRE* promoter with inter-individual differences in their methylation pattern and interestingly, this partial methylation was also present in purified thymic epithelial cell populations. One of the weaknesses of the experimental setup is the small sample size due to the limited availability of human thymic material. Despite this, our results support an earlier report about a high inter-individual variation in CpG methylation in eleven human somatic tissues [167]. We registered hypomethylation in AIRE-negative cTECs as well as in MHC class II^{lo} and MHC class II^{hi} mTECs in studied individuals. Regarding the strong induction of AIRE in MHC class II^{hi} mTECs upon terminal differentiation, our results argue against the hypothesis that DNA methylation plays an essential role in the regulation of AIRE expression. This conclusion is supported by our results showing that the *AIRE* promoter is hypomethylated in most of the AIRE-negative extrathymic tissues (cerebellum, uterus, lung, kidney, myocardium, gray matter, spleen and liver) and in AIRE-negative thymomas. As extrathymic tissues originate from donors over 50 year of age (thymoma donor age unknown), it could be that hypomethylation increases with age. In addition, we could not identify methylation patterns in the *AIRE* promoter region specific to thymoma type, yet we could identify tumor-specific methylation patterns that may reflect their clonal origin. During investigation of histone modifications in the human AIRE gene we detected H3K4me3 modification, which is mark of transcriptionally active chromatin, on AIRE promoter in EpCAM positive cells (enriched for Aire expressing mTECs). Taking into account that AIRE has high CpG motif content in investigated promoter region and it has tissue-specific expression pattern it is suggested that AIRE belongs to the subset of genes controlling developmental processes. Typically genes controlling developmental processes have low levels of CpG methylation and are enriched in H3K4me3 marks, which fits with AIRE recently described role in embryonic development and in mTEC maturation [76].

7. CONCLUSIONS

The following results are presented within the current thesis:

1. Current study characterized the expression of 4 TSAs in conditions where Aire was down- or upregulated: Aire had an allele dose-dependent effect on TSA expression in the thymuses of mice from two strains, C57BL/6J and Balb/c, but had no effect on TSA expression in the lymph nodes. Within the thymus, Aire and TSAs were both localized in the medulla and co-expressed during normal development and involution. Furthermore, over-expression of Aire gene resulted in an increase in TSA expression. The manipulation of in vitro organ-cultures demonstrated that thymic microenvironment plays a dominant role in Aire expression whereas TSAs follow the same expression pattern.
2. We showed that Aire has a dose-dependent effect on CCR4 and CCR7 ligand expression in the thymus. The CCR4 ligands are regulated by Aire in the $CD80^{hi}$ mTECs, whereas the CCR7 ligands are upregulated in adjacent cells. Overexpression of Aire induced the expression of CCL5, CCL22 and CCL19.
3. During the studies of Aire promoter methylation signatures in different cells and tissues we found that the AIRE promoter was hypomethylated in mTECs and cTECs but highly methylated in thymocytes. Furthermore, the AIRE promoter was hypomethylated in AIRE-negative thymic epithelial tumors (thymomas) and in peripheral tissues. Positive correlation was found in human TECs between AIRE expression and an active chromatin mark, histone H3K4me3 in the AIRE promoter.

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SUMMARY IN ESTONIAN

Autoimmuunregulaator: Tüümuse Geeniregulatsiooni ja Promootori Metülatsiooni Iseloomustamine

Meie immuunsüsteem koosneb erinevatest spetsialiseerunud molekulidest, rakkudes, kudedest ja organitest, mis on arenenud kaitsmaks meid võõraste osakeste ja haiguste eest. Samaaegselt võõraste osakeste äratundmisega peab organism olema suuteline vahet tegema peremehe enda rakkude ja antigeenide vahel ja säilitama tolerantsuse enda komponentide suhtes. Terved indiviidid on tolerantsed nende endi antigeenide suhtes. Lümfotsüütide arengu käigus toimub juhuslik retseptorite kokkupanek, mis on võimelised ära tundma nii võõraid kui ka organismi enda komponente ja tolerantsus enda komponentide suhtes säilitatakse hoides ära potentsiaalselt ohtlike organismi enda komponente äratundvate (ehk autoreaktiivsete) lümfotsüütide küpsemise ja aktivatsiooni, vastasel juhul areneb autoimmuunsus. Tolerantsi peamiseks kujunemiskohaks on tüümus, kus küpsevatele lümfotsüütidele esitletakse ainult organismi enda antigeene, kuna võõrad antigeenid viiakse perifeersetesse lümfoidorganitesse nagu lümfisõlmed, põrn jne. Tüümuses leiab aset negatiivne selektsioon ehk autoreaktiivsete T-rakkude selektiivne elimineerimine. Autoreaktiivsete T-rakkude elimineerimise eest vastutavad antigeene presenteerivad rakud, siia kuuluvad dentriitrakud ja tüümuse medullaarsed epiteelirakud (mTEC), kes esitlevad oma pinnal MHC molekuli-peptiidi komplekse T rakkude retseptoritele (TCR) T rakkude pinnal. Antud väitekirja kontekstis on eriti olulised antigeene presenteerivatest rakkudest just tüümuse medullaarsed epiteelirakud, millel on erakordne võime ekspresseerida suurel hulgal erinevaid geene, mis on omased teatud spetsiifilistele perifeersetele kudedele. Immunoloogilise tolerantsuse tekkimiseks ja säilitamiseks on eriti oluline mTECides toimuv koespetsiifiliste geenide ekspressioon, mida tuntakse ka avatud geeniekspressioonina, mis peaks tagama pideva autoreaktiivsete T-rakkude elimineerimise elu käigus.

Medullaarsed epiteelirakud ekspresseerivad AIRE (Autoimmuunregulaator) geeni, mis suunab perifeersete koespetsiifiliste geenide avatud geeniekspressiooni. Hiljuti avastati, et mutatsioonid AIRE geenis toovad endaga kaasa haruldase mitmeid kudesid mõjutava autoimmuunhaiguse APECED (Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy), millega kaasneb immunoloogilise tolerantsuse kadumine mitmete endokriinorganite suhtes. Mutatsioonide tulemusena kaotab AIRE valk funktsionaalsuse koespetsiifiliste geenide ekspresseerimisel ja autoreaktiivsete T rakkude elimineerimine pole enam efektiivne ja selle tulemusena pääsevad autoreaktiivsed T rakud tüümusest perifeeriasse autoimmuunseid reaktsioone põhjustama. Käesolev dissertatsioon keskendub Aire geenist sõltuva koespetsiifiliste antigeenide ja kemokiinide ekspressiooni uurimisele, ja AIRE geeni promootori regulatsiooni metülatsiooni sõltuvuse analüüsimisele. Koespetsiifiliste geenide töös uurisime, kas Aire lisamine või puudumine viib koespetsiifiliste geenide

ekspressiooni muutusteni ja kas see on limiteeritud tüümuse epiteeli rakkudega, ning kuidas muutub Aire ja koespetsiifiliste geenide ekspressioon tüümuse arengu käigus ja kas see on sõltuv tüümuse mikrokeskkonnast. Me leidsime, et koespetsiifiliste geenide ekspressioon on sõltuv Aire geeni koopiaarvust tüümuses kuid mitte perifeerses lümfisõlmes. Aire kui ka koespetsiifilised geenid ekspresseerusid tüümuse medullas ja neil oli sarnane avaldumismuster hiire tüümuse erinevatel arenguetappidel. Aire geeni üleekspresseerimise tingimustes tõusis ka koespetsiifiliste geenide avaldumine. Tüümuse struktuuri lõhkumise tulemusena langes kõigi uuritud geenide ekpressioon. Käesolevad tulemused näitavad, et Aire geenil on otsene roll koespetsiifiliste geenide ekspressioonil ja läbi Aire geeni on võimalik mõjutada tsentraalse tolerantsuse või autoimmuunsuse tekkimist.

Kuna tüümuse kemokiinid on olulised tüümusesse sisenevate tümotsüütide kortiko-medullaarsel migratsioonil, siis uurisime Aire geeni rolli tüümuse kemokiinide tootmises. Leidsime, et tüümuses Aire geeni puudumise tulemusena langevad CCR4 ja CCR7 ligandide tasemed ning CCR4 ligandid on Aire poolt reguleeritud $CD80^{hi}$ mTEC rakkudes. Sarnaselt koespetsiifilistele antigeenidele jälgivad ka tüümuse kemokiinid Aire avaldumismustrit sünnijärgsel perioodil. Aire geeni üleekspresseerimise tingimustes tõusis CCL5, CCL22 ja CCL19 kemokiinide tase. Antud leidude puhul on tegemist Aire uudse rolliga tsentraalse tolerantsuse kujunemise kontekstis.

AIRE promooteri metülatsiooni uurides leidsime, et AIRE promooter oli hüpometüleeritud mTECides ja cTECides ning CpG metülatsiooni tase tõusis AIRE negatiivsetes tümotsüütides. Samas oli AIRE promooter hüpometüleeritud Aire negatiivsetes tüümuse kasvajates ja perifeersetes kudedes. Ning leidsime positiivse korrelatsiooni AIRE ekspressiooni ja AIRE promooteril asuva aktiivse kromatiini märgise H3K4me3 vahel.

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PUBLICATIONS

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1. Kont V, Laan M, Kisand K, Merits A, Scott HS, Peterson P. "Modulation of Aire regulates the expression of tissue-restricted antigens". Mol Immunol. 2008 Jan; 45(1): 25–33. Epub 2007 Jun 28
2. Laan M, Kisand K, Kont V, Möll K, Tserel L, Scott HS, Peterson P. "Autoimmune regulator deficiency results in decreased expression of CCR4 and CCR7 ligands and in delayed migration of CD4+ thymocytes". J Immunol. 2009 Dec 15;183(12):7682–91
3. Kont V, Murumägi A, Tykocinski LO, Kinkel SA, Webster KE, Kisand K, Tserel L, Ströbel P, Scott HS, Marx A, Kyewski K, Peterson P. "DNA methylation signatures of the AIRE promoter in thymic epithelial cells, thymomas and normal tissues". Mol Immunol. 2011 Dec;49(3):518–26. Epub 2011 Oct 27

4. Morrot A, Terra-Granado E, Pérez AR, Silva-Barbosa SD, Milićević NM, Farias-de-Oliveira DA, Berbert LR, De Meis J, Takiya CM, Beloscar J, Wang X, Kont V, Peterson P, Bottasso O, Savino W. "Chagasic thymic atrophy does not affect negative selection but results in the export of activated CD4+CD8+ T cells in severe forms of human disease". PLoS Negl Trop Dis. 2011 Aug;5(8):e1268. Epub 2011 Aug 16

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Peamised uurimisvaldkonnad: Koespetsiifiliste antigeenide avatud ekspressioon tüümuses ja Aire geeni regulatsioonimehhanismid

Neli teaduspublikatsiooni:

1. Kont V, Laan M, Kisand K, Merits A, Scott HS, Peterson P. "Modulation of Aire regulates the expression of tissue-restricted antigens". Mol Immunol. 2008 Jan; 45(1): 25–33. Epub 2007 Jun 28
2. Laan M, Kisand K, Kont V, Möll K, Tserel L, Scott HS, Peterson P. "Autoimmune regulator deficiency results in decreased expression of CCR4 and CCR7 ligands and in delayed migration of CD4+ thymocytes". J Immunol. 2009 Dec 15;183(12):7682–91
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4. Morrot A, Terra-Granado E, Pérez AR, Silva-Barbosa SD, Milićević NM, Farias-de-Oliveira DA, Berbert LR, De Meis J, Takiya CM, Beloscar J, Wang X, Kont V, Peterson P, Bottasso O, Savino W. "Chagasic thymic atrophy does not affect negative selection but results in the export of activated CD4+CD8+ T cells in severe forms of human disease". PLoS Negl Trop Dis. 2011 Aug;5(8):e1268. Epub 2011 Aug 16

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Organisatsiooniline ja erialane tegevus: 5th EuroThymaide Annual Assembly and Training Day, Tallinn (2008).

Õppetöö

Juhendamine: Karin Kõndi bakalaureusetööd „NFkB mõju uurimine AIRE geeni regulatoores piirkonnas“.

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