Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Biologie
Studijní obor: Imunologie

Bc. Matouš Vobořil

Aire-exprimující buňky v periferních tkáních imunitního systému
Aire-expressing cells in immune peripheral tissues

Diplomová práce

Školitel: RNDr. Dominik Filipp. CSc.

Praha, 2014

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 14. 08. 2014

Podpis:

Acknowledgments

I would like to express my thanks mainly to Dominik Filipp for the opportunity to work in his laboratory and for his valuable advice, assistance and patience. My great thanks also belong to all members of the Laboratory of Immunobiology in the Institute of Molecular Genetics, especially to Jan Dobeš for his unlimited patients, critical comments and enormous help in the creation of this thesis. My thanks also belong to Martina Dobešová, Jana Balounová and Odřej Ballek. for many advices and help in the laboratory. Many thanks also go to Zdeněk Cimburek for his technical assistance with FACS analysis and sorting. And last but not least, I would like to thanks everyone else who help me at work and supported me.

Abstract

Tolerance to “self” is the fundamental property of the immune system and its breakdown can lead to autoimmune diseases. In order to eliminate self-reactive T-cells during their development in thymus (central tolerance), Aire promotes the expression of peripheral self-antigens in medullary thymic epithelial cells (mTECs). Recently, Aire was suggested to fulfil a similar function in rare lymph node and spleen cells (peripheral tolerance). However, the detection, characterization and function of these extrathymic Aire-expressing cells is still obscure. The main objective of presented thesis was to investigate if Aire positive cells are also present in other lymphoid as well as non-lymphoid tissues. Using two independent mouse transgenic models we identified the Aire-reporter expressing cells in several lymphoid tissues such as Peyer´s patches, spleen and bone marrow as well as in one non-lymphoid organ, the lungs. We show here that based on the expression of B220, EpCAM and CD11c markers these heterogenic cells consist of at least five phenotypically distinct subpopulations, and with the exception of those from lungs, all of them are strictly of hematopoietic origin. This study also demonstrates that Aire on protein level is predominantly expressed by one of these subpopulations with $CD45^{+}MHCII^{+}B220^{-}CD11c^{-}EPCAM^{-}$ phenotype and thus the study provides detail phenotypic characterisation of Aire-protein expressing cells in the immune periphery. We have also demonstrated that peripheral Aire-expressing cells possess the capacity to produce the set of TRAs which largely overlaps with that of mTECs. This study thus brings a new perspective to Aire-regulated peripheral immune tolerance and contributes to better understanding of cellular and molecular events that prevent autoimmunity.

Key words:

Aire, Aire-expressing cells, autoimmunity, immune tolerance

Abstract (CZ)

Tolerance k vlastním tkáním je jednou ze základních vlastností imunitního systému a její narušení může vést ke vzniku autoimunitních onemocnění. Negativní selekce autoreaktivních klonů T-buněk během jejich vývoje v thymu (centrální tolerance) je závislá na expresi tkáňově specifických antigenů medulárními epiteliálními buňkami (mTECs), která je řízena pomocí proteinu Aire. V současné době se ukazuje, že Aire protein zastává podobnou funkci také ve vzácných buněčných populacích v lymfatických uzlinách a ve slezině (periferní tolerance). Nicméně detekce, charakteristika a funkce těchto buněk exprimujících Aire v periferních tkáních imunitního systému zůstává stále nejasná. Hlavním cílem této práce bylo zjistit, zda se tyto buňky exprimující Aire vyskytují v dalších lymfatických či nelymfatických tkáních. S použitím dvou na sobě nezávislých transgenních myších modelů jsme identifikovali tyto buňky ve většině lymfatických tkání - např. v Peyerových placích, ve slezině a v kostní dřeni. Ukázalo se také, že se tyto buňky vyskytují i ve tkáních nelymfatického původu a to především ve tkáni plicní. Na základě exprese povrchových markerů B220, EpCAM a CD11c jsme ukázali, že Aire exprimující buňky tvoří pět fenotypově odlišných populací. Ukázali jsme také, že kromě buněk exprimujících Aire v plicní tkáni jsou všechny tyto buňky čistě hematopoetického původu. Práce dále ukazuje, že subpopulace těchto buněk určená $CD45^{+}MHCII^{+}B220^{-}CD11c^{-}EPCAM^{-}$ fenotypem produkuje Aire také na proteinové úrovni.. V neposlední řadě jsme ukázali, že buňky exprimující Aire mají schopnost produkovat vybrané tkáňově specifické antigeny, jichž exprese se shoduje s buňkami mTECs. Tato práce přináší nový pohled na Aire regulovanou periferní toleranci a přispívá k lepšímu porozumění buněčných a molekulárních mechanizmů zabraňujícím vzniku autoimunitního onemocnění.

Klíčová slova:

Aire, buňky exprimující Aire, autoimunitní onemocnění, imunologická tolerance

Table of contents

List of abbreviations

(cat)-L	cathepsin L
8.3 T-cells	TCR specific for IGRP
Aire	Autoimmune regulator
APCs	antigen presenting cells
APECED	Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy
APS1	Autoimmune Polyglandular Syndrome type 1
ATF7ip-MBD1	activating transcription factor 7-interacting protein; methyl CpG-binding protein 1
BAC	bacterial artificial chromosom
BECs	blood endothelial cells
cAMP	cyclic adenosine monophosphate
CARD	caspase-recruitment domain
Casc3	cancer susceptibility candidate 3
CBP	CREB-binding protein
CCR	C-C chemokine receptor type
CD62L	T-cell L-selectin
CMC	chronic mucocutaneous candidiasis
Crp	C-reactive protein
cTECs	cortical thymic epithelial cells
CTLA4	cytotoxic T lymphocyte-associated antigen-4
DAXX	death-associated protein 6

DCs	migratory dendritic cells
Deaf1	deformed epidermal autoregulatory factor 1
Defcr24	enteric α-defensin 24
DN	double negative stage
DNA-PK	DNA-dependent protein kinase
DP	double positive stage
DSIF	DRB sensitivity-inducing factor
EpCAM	epithelial cell adhesion molecule
eTACs	extra-thymic Aire-expressing cells
FACS	fluorescent-activated cell sorting
FasL	Fas ligand
FDCs	follicular dendritic cells
FH	TCR specific for melanocytes tyrosinase
FRC	fibroblastic reticular cells
Gad65	glutamic acid decarboxylase 2
Gad67	glutamate decarboxylase 1
GAPDH	glyceraldehyd-3-phospate dehydrogenase
GFP	green fluorescent protein
gp38	podoplanin
H3K4	histon H3 lysin 4
HA	influenza hemagglutinin
hCD2	human CD2
HEL	hen egg lysozyme

Hnrnpl	heterogeneous nuclear ribonucleoprotein L
IAPs	integrin $\alpha7^+$ pericytes
IECs	intestinal epithelial cells
iFABP	intestinal fatty acid binding protein
IFNs	interferons
IGRP	islet-specific glucose-6-phosphatase-related protein
IHC	immunohistochemistry
Ii	invariant chain
IL	interleukin
Ins2	insulin II
IPTs	immune privilege tissues
LAG3	lymphocyte-activation gen 3
LECs	lymphatic endothelial cells
LNSCs	lymph node stromal cells
MHC	major histocompatibility complex
mTECs	medullary thymic epithelial cells
Muc6	mucin 6
NALP5	NACHT leucin-riche-repeat protein 5
NLS	nuclear localization signal
NOS2	nitric oxide synthase
OT-I	TCR specific for OVA
OVA	ovalbumin
pDCs	plasmacytoid dendritic cells

PD-L1	programmed cell death – 1 ligand
PHDs	plant-homeodomains
PIAS1	protein inhibitor of activated STAT1
pMHC	peptide-MHC
PML	promyelocytic-leukemia
PRR	prolin riche region
PRRs	pattern recognition receptors
P-TEFb	positive transcription elongation factor b
pTreg	peripheral T- regulatory cell
qRT-PCR	quantitative real-time polymerase chain reaction
RIP	rat insulin promoter
SAND	Sp100, Aire1, NucP41/P75 and Deaf1
SP	single positive stage
STAT1	signal transducer and activator of transcription
Tag	T antigen
TCR	T-cell receptor
tDCs	thymic-resident dendritic cells
TECs	thymic epithelial cells
TGF-β	transforming growth factor β
TIGIT	T-cell immunoreceptor with Ig and ITIM motifs
TLR3	Toll-like receptor 3
TNF	tumor necrosis factor
tOVA	truncated ovalbumin

TRAIL	TNF-related apoptosis-inducing ligand
TRAs	tissue restricted antigens
TSSP	thymus specific serine protease
tTreg	thymus-derived T-regulatory cells
WT	wild-type

1 Introduction

T-cells, as an integral part of adaptive immune system, participate as effector cells in the process of recognition and elimination of infectious agents and creation of lasting immunological memory of previously encountered pathogens. Mature T-cell receptors (TCRs) are generated by a random recombination process which endows T-cells to recognize practically any immunologically relevant antigen independently of its origin, i.e. non-self (foreign) or self-derived (self-antigen). While the recognition of foreign antigens derived from a pathogen is beneficial for the survival of infected organism, the recognition of self-antigens often leads to the tissue damage and autoimmune diseases. For this reason, the removal of self-reactive T cells is a critical step towards the establishment of immunological homeostasis.

An essential role in the mechanisms that remove these self-reactive TCR bearing T-cells plays the autoimmune regulator (Aire) protein. Mutations in *AIRE* coding gene leads in humans to the development of Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy disease (APECED). Similarly, mice that lack gene encoding *Aire,* in experimental conditions develop multiorgan autoimmune disease. The processes that prevent the development of autoimmune diseases are collectively called the immunological tolerance. The mechanisms that underpin these processes operate in two distinct anatomical places. Central tolerance is established by medullary thymic epithelial cells (mTECs) in the thymus and is responsible for negative selection of self-reactive thymocytes. Nevertheless, the partial incompleteness of this process necessitates the employment of another level of protection, peripheral tolerance. The role of Aire protein in mechanisms of central tolerance has been intensively investigated during the last fifteen years and its basic mechanisms and components have been described. In contrast, the extend of Aire expression, identity of cellular sources of its production and its role in the mechanism of peripheral tolerance is still poorly understood. The presence of Aire-expressing cells in immune peripheral tissues, their phenotypic characterization and involvement in the production of self-antigens are the main objectives of this thesis.

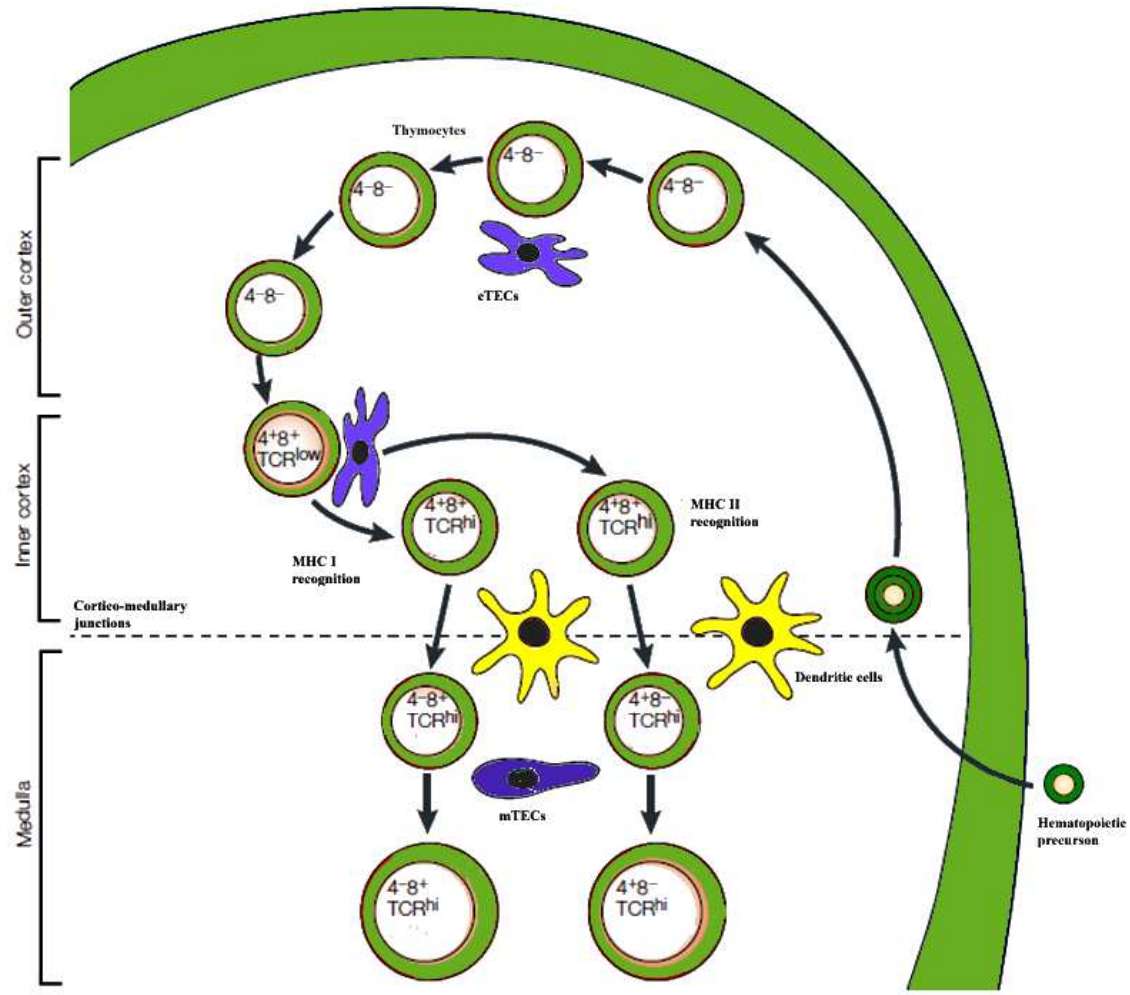
2 Overview of literature

This literature overview highlights the most relevant processes and mechanisms for the establishment of immunological tolerance. It is divided into four main chapters. While the first chapter deals with T-cell development, next chapters describe processes that guide thymic positive and negative selection, respectively, collectively called central tolerance. The last chapter provides an overview of current knowledge about peripheral tolerance with special emphasis on Aire expressing cells in peripheral tissues.

2.1 Thymus and T-cell development

T-cells are derived from lymphoid progenitors which developed from hematopoietic stem cells localized in the bone marrow. These immature T-cells progenitors migrate to the thymus in order to accomplish their maturation process, mainly into αβ T-cell receptor (TCR) bearing T-cells. Thymic development of T-cells can be divided into several stages which can be phenotypically defined by the cell-surface expression of CD4 and CD8 markers. Briefly, during the first stage of their development, T-cell progenitors do not produce either CD4 or CD8 molecules and therefore it is called a double negative (DN) stage. During the DN stage, TCR locus undergoes a process of VDJ recombination which ultimately leads to the surface expression of TCR. This event is likely followed by the clonal cell expansion (Hogquist et al., 2005). Developing T-cell with matured TCR starts to express both co-receptor molecules CD4 and CD8 and become double positive (DP). These DP T-cells interact with the antigen which is presented in context of the major histocompatibility complex (MHC) class I or II found on specialized antigen presenting cells (APCs) residing in the thymic cortex – cortical thymic epithelial cells (cTECs) and become $CD8^+$ or $CD4^+$ single positive (SP) (Anderson and Jenkinson, 2001; Zúñiga-Pflücker, 2004). The specific regions of the thymus through which T-cells during their development move has been anatomically demarcated. Specifically, lymphoid progenitors enter the thymus in cortico-medullary junction areas. Then, as immature T-cells, they progress into the cortex, where they become SP and migrate into the medulla where they accomplish their developmental process (Fig. 1) (Anderson and Jenkinson,

2001). This movement is chiefly regulated by the expression of chemokines and their gradient pattern. This argues that T-cells during their development require many specific interactions with thymic-resident cells (Norment and Bevan, 2000).



.

Figure 1: T-cell development in the thymus. *Thymus can be divided, based on its morphology, into three main parts: outer cortex, inner cortex and medulla. Each of these areas is characterized by a distinct stromal cell population and by thymocytes precursors present at different maturation stage. Interactions between stromal cells and thymocytes are important for T-cell maturation process which leads to generation of $CD4^+$ and $CD8^+$ T-cells. Immature T-cells pass through the cortex into the medullary part where their development terminates and from where they exit into the immune periphery. During the maturation, thymocytes differ phenotypically by expression of several markers, including CD4 and CD8 coreceptors. Adopted from (Anderson and Jenkinson, 2001) and modified.*

2.1.1 Affinity model of thymic selection

The affinity model of thymic selection is focused on the strength of interaction between TCR and self-peptide-MHC, which seems to be the critical determinant of T-cell fate during thymic development. This model suggests that weak or intermediate interactions with APCs are required to protect T-cells from cell death by neglect and to promote their positive selection. On the other hand, strong interactions cause T-cells death by apoptosis. Using TCR-transgenic systems it was also shown that high-affinity binding can cause clonal deviation and redirection of T-cells into thymus-derived T-regulatory cells (tTreg). So the demarcation between the clonal deletion (death by apoptosis) and clonal deviation appears to be plastic and seems to be affected by stochastic influences (Fig. 2) (Bains et al., 2013; Klein et al., 2014). The basic information about thymic selection processes are described in next chapters.

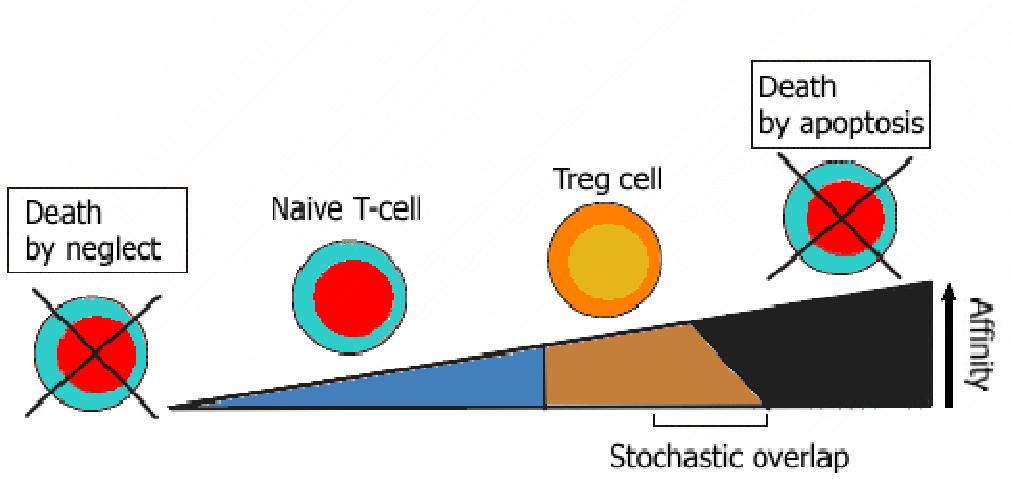


Figure 2: Affinity model of thymocytes selection. *The classical affinity model suggests that the fate of developing T-cells is determined by the strength of their TCR-self-peptide-MHC interactions. Adopted from (Klein et al., 2014) and modified.*

2.1.2 Thymic cortex and positive selection

In DP stage, developing thymocytes with fully rearranged and surface expressed TCRs interact with cTECs and test their ability to bind peptide-MHC complexes (pMHC) presented on these cells. If the DP cell is able to interact with pMHC class I complex it further differentiates into $CD8^+$ T-cell; if it recognizes pMHC class II

complex it becomes the $CD4^+$ T-cell. The classical affinity model suggests that only intermediate affinity binding of immature T cells to pMHC complexes leads to their survival and further development (Klein et al., 2014). This phenomenon is called positive selection. As indicated above, cTECs play a major role in this process by providing their pMHC complexes for the interactions with TCR of developing thymocytes. It is still not fully understood how the collection of self-peptides of cTECs is generated and presented on their MHC molecules during positive selection. It has been shown that cTECs are poorly phagocytic (Klein et al., 2001), so they mainly present antigens derived from endogenous proteins. There is accumulating evidence that cTECs use specific mechanisms for the generation of endogenous peptides and their loading on MHC molecules. Indeed, cTECs possess unique antigen processing enzymes, such as proteases like cathepsin-L (Cat)-L and Thymus Specific Serine Protease (TSSP) (Guerder et al., 2012). It has been demonstrated that Cat-L is exclusively expressed by cTECs, but not by mTECs, nor by thymic-resident dendritic cells (tDC) or migratory dendritic cells (DCs), which express primarily Cat-S. Cathepsins are lysosomal proteases responsible for the degradation of invariant chain (Ii), which protects MHC class II molecules against premature peptides loading in endoplasmic reticulum. The analysis of Cat-L and Ii double knock-out indicated crucial changes in the MHC ligand repertoire of cTECs (Nakagawa et al., 1998). Also deficiency in TSSP, which is responsible for the proteolytic generation of MHC class II peptides, leads to the alternations in T-cells TCR repertoire (Boehm, 2009). Furthermore, cTECs display high levels of constitutive macroautophagy, which can be also responsible for cytosolic peptides delivery into MHC class II molecules (Mizushima et al., 2004). Notably, Nedjic *et al.* proved that the disruption of gene essential for macroautophagy pathway, *Atg5*, impaired positive selection for some TCR specificities (Nedjic et al., 2008).

It was also shown that, in the context of MHC class I antigen presentation, cTECs express a unique catalytic subunit of the proteasome referred as β5t. This proteasome is called “thymoproteasome” because of the different substrate preferences in comparison to “housekeeping proteasomes” and “immunoproteasomes” (Florea et al., 2010). Together, these findings led to the hypothesis that cTECs utilize different proteolytic pathways only to test the ability of TCRs to interact with the set of MHC molecules expressed by the host. For this purpose, low affinity peptide fragments

have to be generated by proteolytic pathways distinct from those commonly used by other hosts cell types.

Because the VDJ recombination occurring during the T-cell development is a random process, TCRs with ability to recognize self-antigens are also generated. The self-antigen recognition can lead to the disruption of immune tolerance and development of autoimmune diseases. In order to overcome these undesirable events, regulatory processes that can effectively eliminate self-reactive T-cells or attenuate their effectors function must be in place. This critical immunological homeostatic process of central tolerance is called negative selection and is operating in the medullary part of the thymus (Anderson and Jenkinson, 2001).

2.1.3 Thymic medulla and Negative selection

Approximately only 5% to 10% of T-cells successfully accomplish the process of positive selection and they then proceed to the medulla region where they undergo the process of negative selection. For quite a long time, an accurate anatomical localization of negative selection to and within the thymus was problematic (Kyewski and Klein, 2006). Several ideas, including one that negative selection might occur in both cortex and medulla, were proposed (Baldwin et al., 1999; Spain and Berg, 1994; Volkmann et al., 1997). Later on, it became evident that only medulla is the *bona-fide* site of negative selection (Hogquist et al., 2005). The thymic medulla accommodates several types of cells with the capacity of antigen presentation: mTECs, DCs and tDCs, as well as B-cell, macrophages and granulocytes (Klein et al., 2009).

SP T-cells migrate to the medulla where they interact with various APCs, the most frequently with mTECs and DCs. Here, the ability, or rather inability of their TCR to recognize the self-antigen is strictly tested. mTECs are the only cell type in the thymus with the capacity to directly produce wide spectrum of self-peptides the expression of which is usually restricted to the immune periphery (Derbinski et al., 2001). The self-peptide-MHC complex recognition by thymocyte's TCR leads to the deletion or more accurately "negative selection" of self-reactive clones (Liston et al., 2003). It has been also shown that a relatively small percentage of self-reactive thymocytes is during negative selection converted to $CD4^+CD25^+Foxp3^+$ tTreg

(Aschenbrenner et al., 2007). Koble and Kyewski found that both tDCs and mTECs are able to present self-peptides. However, the presentation by tDCs depends largely on phagocytosis of dying mTECs and processing of their protein load (Koble and Kyewski, 2009). Negative selection can be also mediated by DCs which migrate to the thymus and present sequestered peripheral antigens to developing thymocytes (Bonasio et al., 2006; Guerder et al., 2013). The peripheral plasmacytoid dendritic cells (pDCs) can also home to the thymus and promote negative selection. It has been documented that the expression of C-C chemokine receptor 9 (CCR9) on pDCs provides them with thymic homing capacity and allows them to carry antigens from peripheral tissues in to the thymus (Hadeiba et al., 2012). Thymus also contains a population of B-cells which co-localize with tDCs and mTECs (Akashi et al., 2000; Isaacson et al., 1987; Perera et al., 2013). It has been shown that thymic B-cells express unique phenotypic markers compared with B-cells that are found in the periphery and show increased expression of MHC class II and co-stimulatory molecules. These abilities indicate that thymic B-cells have the potential to present antigens to developing T-cells. Indeed, studies have shown that thymic B-cells are able to induce negative selection of self-reactive T-cells (Frommer and Waisman, 2010; Perera et al., 2013). It was also reported that thymic B-cells can modulate thymic production of tTregs (Walters et al., 2014). The role of mTECs and transcriptional regulator Aire in the mechanism of central tolerance is described below.

2.1.4 Expression of Tissue restricted antigens in the thymus

The negative selection process is based on the premise of deletion of T-cell clones recognizing self-antigens in the context of MHC. In order to test the reactivity of TCRs towards self-antigens, there must be a cellular system in the thymus which produces and presents a wide range of self-antigens expressed ubiquitously or in a tissue specific manner in the body. Based on previous sequencing and data mining experiments which showed the presence of tissue-specific mRNAs in the thymus (Sarkar and Sommer, 1989), Linsk *et al.* hypothesized, that thymus represents a place of diverse so called “tissue restricted antigens” (TRAs) expression (Linsk et al., 1989). TRAs are defined as proteins, which are normally express in less than 5 of the 60 conventionally tested tissues (Klein et al., 2014). The first notion of TRA

transcription in the thymus resulted from the creation of transgenic mice expressing neo-self-antigen, which had originally been designed to study peripheral tolerance. Hanahan *et al.* constructed transgenic mouse model expressing T antigen (Tag) in pancreatic β cells under the control of the rat insulin promoter (RIP-Tag mice). It was shown that low levels of Tag mRNA were also detectable in the thymus (Hanahan, 1998; Jolicoeur et al., 1994). These results pointed to the existence of phenomenon of TRAs expression in the thymus. However, the cellular source of TRA expression has remained unknown for some time. The breakthrough came when Derbinski *et al.* established a new purification protocol that allowed the analysis of pure thymic stromal cells. This led to the identification of mTECs as almost exclusive source of 30 prototypic TRAs. The diversity of TRAs found in mTECs covered essentially all organs and included developmentally and temporally regulated genes (Derbinski et al., 2001). While the expression of the majority of TRAs is confined to mTECs, certain antigens are at very low levels expressed also in cTECs and tDCs (Derbinski et al., 2001; Kyewski et al., 2002). Based on the DNA microarrays analysis, gene expression in TECs can be divided into three distinct gene pools. Pool one includes genes expressed by cTECs and mTECs at the same levels. Pool two includes genes whose expression is restricted to immature mTECs lineages (defined as MHC class II^{lo} and $CD80^{lo}$) and pool three strictly belongs to mature mTECs (defined as MHC class II^{hi} and $CD80^{hi}$). The latter pool can be further subdivided according to their expression being depend or not on transcription regulator Aire (Derbinski et al., 2005; Kyewski and Klein, 2006) (Fig. 3). Taken together, these studies suggested that mTECs are the key source of TRA production which ultimately guides the negative selection of thymocytes during their thymic development. The structure and function of TRA-expression promoting factor Aire is described in the next chapter.

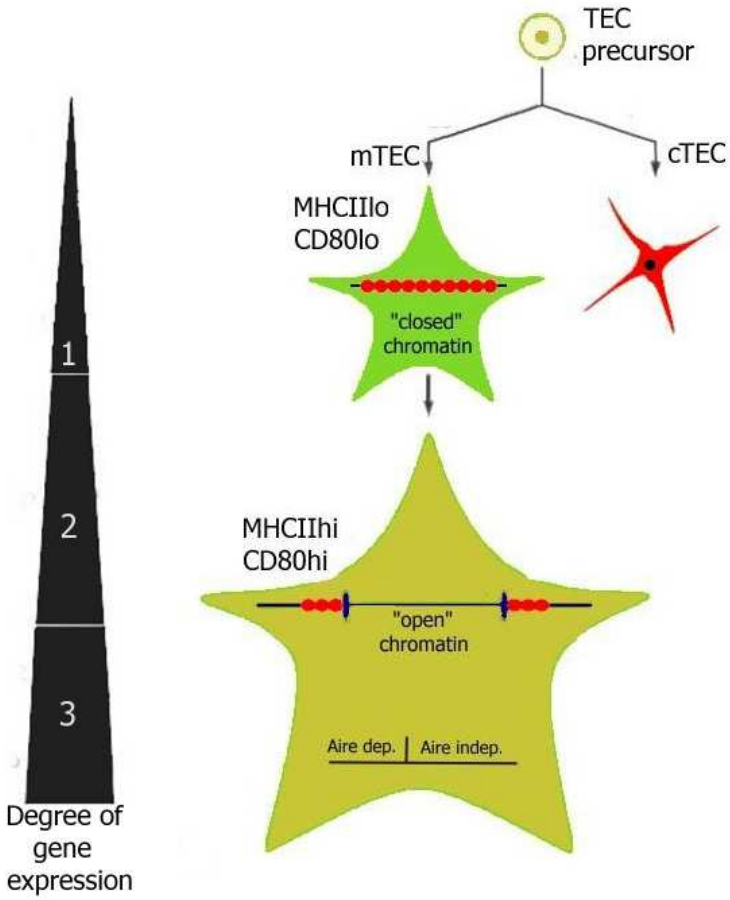


Figure 3: Differentiation model of promiscuous gene expression. *Progressive differentiation of mTECs correlates with the increase expression of TRAs and this process is reflected in the postulation of three distinct pools of genes expressed by various TECs (1, 2, and 3). Epithelial cells of the thymic cortex and medulla originate from a common endoderm-derived progenitor (TEC precursor) which differentiates into cTECs and mTECs. The model of TRAs expression is based on the assumption that the upregulation of MHC class II and CD80 leads to the progressive differentiation of mTECs. Promiscuous expression of TRAs in fully mature mTECs is either Aire-dependent or Aire-independent. Adopted from (Kyewski and Klein, 2006) and modified.*

2.1.5 Structure and properties of Aire

The human transcription regulator AIRE is encoded by the homonymous gene, *AIRE*, situated on q22.3 region of chromosome 21. Loss-of-function mutation in the *AIRE* gene causes a rare, inherited autoimmune syndrome called Autoimmune

Polyglandular Syndrome type 1 (APS1), which is also known as APECED (Consortium, 1997; Nagamine et al., 1997). The homologous murine *Aire* gene is located on 41.6 region of chromosome 10 and shows about 80% similarity with the human *AIRE* gene (Tab. 1) (Blechschmidt et al., 1999).

	mouse (*Mus musculus*)	human (*Homo sapiens*)
genomic context	chromosom: 10	chromosom: 21
	chromosom location: 10 41.6	chromosom location: 21q22.3
mRNA length	1936 bp	2257 bp
protein length	552AA	545 AA
protein weight	59,0 kDa	57,7 kDa

Table 1: Characteristic of Aire gene and protein. *The table shows the characteristics of Aire gene and Aire protein. Used abbreviations: bp - base pairs, AA - amino acids, kDa – kilodalton. The presented data were taken from PubMed database (http://www.ncbi.nlm.nih.gov/pubmed).*

Despite the fact, that APECED is a rare syndrome, it´s frequency is significantly increased among specific populations such as Iranian Jews (prevalence 1:9 000), Sardinians (1:14 000) and Finns (1:25 000) (Arstila and Jarva, 2013). APECED is clinically manifested by the presence of symptoms triad consisting of chronic mucocutaneous candidiasis (CMC), hypoparathyroidism and Addison´s disease. This triad is accompanied by several other endocrine and non-endocrine disturbances which affect a smaller fraction of the patients (Ahonen et al., 1990). CMC is usually the first clinical sign of APECED which occurs at the age of five years and it is the most common component of this disease, manifested nearly in all patients. Hypoparathyroidism and Addison´s disease are the most common endocrinopathies and are usually diagnosed between 4 and 12 years of patient's life. Any two diseases of this triad are required for APECED diagnosis (Husebye et al., 2009). Another very important feature in diagnosis of APECED is the presence of specific autoantibodies. Many of these autoantibodies are target against intracellular enzymes the expression of which, as TRAs, is confined to mTECs (Husebye and Anderson, 2010). Diagnostically very important are neutralizing antibodies against type I interferons (IFNs) which presence is detected in all APECED patients. Anti-IFNs antibodies are present even before development of any other symptom (Meloni et al., 2008). The presence of CMC in APECED patients correlates with the occurrence of

autoantibodies against interleukin-17 (IL-17) and IL-22. These autoantibodies are present in both APECED patients and also in CMC patients without APECED (Kisand et al., 2010). Forty nine percent of APECED patients with hypoparathyroidism are positive for NACHT leucin-riche-repeat protein 5 (NALP5) autoantibodies. This protein is normally expressed in the cytoplasm of parathyroid chief cells (Alimohammadi et al., 2008). The production of autoantibodies and occurrence of self-reactive T-cells propagate the destruction of the affected tissues as these are the key pathogenic factors of this disease (Arstila and Jarva, 2013). In addition, the detection of TRAs specific antibodies is a very useful diagnostic tool. However, and in this context, the results of clinical studies are always closely dependent on the particular cohort of patients and they do not provide a complete insight into a disease pathogenesis (Arstila and Jarva, 2013). To solve these problems, the mouse equivalent of the *Aire* gene was cloned (Wang et al., 1999) and several Aire deficient ($Aire^{-/-}$) mouse strains were generated. All of these strains recapitulate the APECED syndrome pathogenesis and the self-reactive T-cells and autoantibody-dependent autoimmunity is readily detectable. It is important to emphasize however, that the severity of autoimmune syndrome differ with the genetic background of a particular mouse strain (Anderson et al., 2002; Ramsey et al., 2002b).

Aire protein has a multidomain structure typical for the proteins possessing the ability to bind to chromatin and to regulate gene transcription. Aire contains caspase-recruitment domain (CARD), a nuclear localization signal (NLS), a Sp100, Aire1, NucP41/P75 and Deaf1 domain (SAND) (Gibson et al., 1998), two plant-homeodomains (PHDs), prolin riche region (PRR) and four LXXLL motifs (where L means leucin and X is any amino acid) (Fig. 3) (Perniola and Musco, 2014).

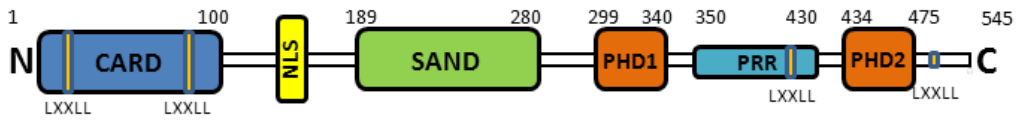


Figure 4: Domain structure of the Aire protein*. Numbers on the top represent length (in amino acids) of individual domains. Inspired by (Mathis and Benoist, 2009).*

Aire was shown to localize into small speckles uniformly distributed in the nucleus which are similar to promyelocytic-leukemia nuclear bodies (Su et al., 2008). These nuclear speckles are associated with the nuclear matrix, which provides a structural framework for chromatin organization and gene regulation (Tao et al., 2006). The N-terminal end of Aire was described as homogeneously-staining region domain (Pitkänen et al., 2000), but its structural re-analysis revealed the presence of CARD domain, which is predicted to fold into a six α-helical structure (Ferguson et al., 2008). The CARD domain has been shown to be involved in Aire oligomerization in nuclear speckles (Pitkänen et al., 2001; Ramsey et al., 2002a). CARD domains are also commonly present in several pro-apoptotic proteins like the initiator caspases (Hofmann et al., 1997). It has been shown that Aire protein-positive mTECs have quite a short life span (Gray et al., 2007; Metzger et al., 2013). Liiv *et al*. showed that the truncated form of Aire comprising only CARD and NLS domains is sufficient to induce apoptosis of mTECs and that HEK293 cells transfected with *Aire* exhibited nuclear accumulation of glyceraldehyd-3-phospate dehydrogenase (GAPDH) which reflects cellular stress and apoptosis (Liiv et al., 2012).

CARD domain is followed by NLS signal which provides the molecular instruction for the protein transport into the nucleus through nuclear pores. NLS is recognized by importin-α followed by the binding of importin-β to this complex and mediates the process of nuclear translocation (Pemberton and Paschal, 2005; Perniola and Musco, 2014).

SAND domain has a crucial role in the regulation of transcriptional machinery. For example, the dominant-negative mutation in glycin 228 in this domain is commonly found in APECED patients. This mutation disrupts Aire transactivation capacity, alters Aire subcellular localization and predisposes both human and mice to autoimmune thyroiditis (Ilmarinen et al., 2005; Su et al., 2008).

The C-terminal region of Aire contains two PHD domains separated by PRR. PHD zinc fingers are cystein-rich domains with two zinc atoms, which can “read” the epigenetic marks of chromatin, mainly the histones modifications (Musselman and Kutateladze, 2011). Specifically, the PHD1 domain recognizes the unmethylated histone H3 lysin 4 (H3K4). This interaction allows Aire to activate silenced chromatin and regulate the gene transcription (Org et al., 2008). The comparative

analysis of the wild-type (WT) and PHD2-deficient Aire showed that PHD2 domain was necessary for Aire interaction with a set of protein partners involved in chromatin binding and transcription regulation. It was also shown that PHD2 domain influences the ability of Aire to regulate the mTEC transcriptome, implying that it is important for the induction of central tolerance (Yang et al., 2013). Aire also contains four LXXLL motifs which were found to function as co-activators or co-repressors with the ability to bind nuclear receptors and influence the process of gene transcription (Plevin et al., 2005).

Biochemical studies of Aire properties have been paralleled by the discovery of its functional partners in the TRAs production. The first described example was CREB-binding protein (CBP) known as a common co-activator of gene transcription (Kwok et al., 1994). CBP was shown to co-localize with Aire within the nuclear speckles (Pitkänen et al., 2000). In subsequent studies, Aire was identified to interact with many transcription machinery proteins, such as the positive transcription elongation factor β (P-TEFβ) (Oven et al., 2007), the protein inhibitor of activated STAT1 (PIAS1) (Ilmarinen et al., 2008), p63 nuclear protein involved in control of cell proliferation and apoptosis (Tonooka et al., 2009), the death-associated protein 6 (DAXX) (Meloni et al., 2010) and the complex of DNA-dependent protein kinase (DNA-PK) (Liiv et al., 2008). In the year 2010, using the co-immunoprecipitation and mass spectrometry analyses, Abramson *et al.* identified an additional set of Aire-associated proteins. The identified proteins were divided into four major classes based on their function: nuclear transport, chromatin binding/structure, transcription and pre-mRNA processing (Fig. 5) (Abramson et al., 2010).

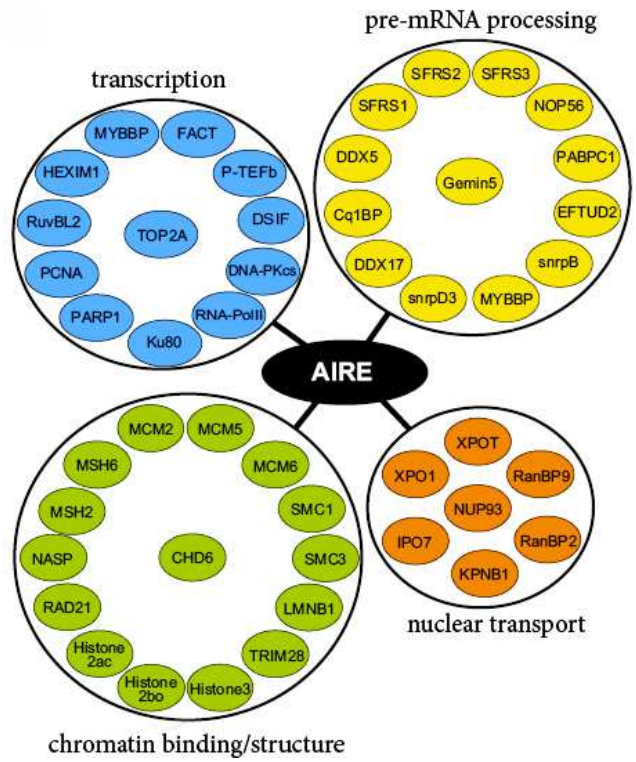


Figure 5: Aire-associated proteins network*. Identification of Aire partners by co-immunoprecipitation experiments. Taken from (Yang et al., 2013)*

2.1.6 Aire-mediated TRAs transcription activation and regulation

The Aire was shown to be an unusual transcriptional regulator: (i) it causes the thymic production of many TRAs whose expression pattern in their original parenchymal tissues is very different and usually tightly controlled; (ii) it lacks a clear DNA-binding motif; and (iii) it recognizes nonspecific marks of inactive chromatin such as the unmethylated H3K4 (Peterson et al., 2008). In the context of transcriptional activation, Aire binds PIAS1 protein and DNA-PK. These two proteins are known to bind nuclear matrix and in the complex with Aire are important for the formation of nuclear structures and for the activation of TRAs' promoters. DNA-PK can also phosphorylate Aire and collaborate with it in the formation of chromatin loops (Ilmarinen et al., 2008; Oven et al., 2007). Aire then interacts with CBP, which acetylates histones thus making the chromatin and DNA accessible for transcriptional machinery (Pitkänen et al., 2000). For the purpose of initiation of transcriptional elongation, Aire interacts with ATF7ip-MBD1 (activating transcription factor 7-interacting protein; methyl CpG-binding protein 1) complex

which recognizes methylated CpG dinucleotides and targets Aire to genetic loci encoding TRAs (Waterfield et al., 2014). The PHD1 domain of Aire binds to the unmethylated H3K4 (Org et al., 2008) and recruits the splicing factor Hnrnpl (Giraud et al., 2014) and elongation factor P-TEFβ. P-TEFβ removes the negative elongation factor and phosphorylates pause factors such as DSIF (DRB sensitivity-inducing factor) (Oven et al., 2007). This protein complex unleashes stalled RNA polymerase II to induce transcription of specific TRAs (Giraud et al., 2012).

These results suggest that Aire does not fulfill the criteria of a general transcription factor. Rather it functions as a transcription regulator with adaptor-like properties. In this sense, Aire regulates the transcription by recruiting large molecular complexes which are responsible for targeting transcriptional machinery to specific sites on DNA. Thus, Aire plays a major role in the initiation of TRAs transcription, transcript elongation and possibly also in transcript splicing.

2.1.7 The role of thymic Aire in central tolerance

To elucidate the function of Aire protein in the mechanisms of central tolerance, several Aire$^{-/-}$ mouse strains have been generated. It was shown that Aire$^{-/-}$ mice developed multi-organ autoimmunity with lymphoid infiltration of target tissues and serum autoantibodies (Anderson et al., 2002; Ramsey et al., 2002b). Constructing the bone marrow chimeras, Anderson *et al.* demonstrated that Aire deficiency in the radioresistant thymic stromal cells, but not in the cells of hematopoietic origin, leads to autoimmune diseases. Moreover, they demonstrated that Aire promotes the expression of a fraction of TRAs in mTECs (Anderson et al., 2002). The comparison of WT and Aire$^{-/-}$ mice confirmed that expression of some TRAs such as insulin was strictly Aire-dependent, while the expression of other genes, such as glutamate decarboxylase 1 (GAD67), was Aire-independent (Anderson et al., 2002; Derbinski et al., 2005). In addition, it has been shown that the expression of particular TRA is confined to only a small fraction (1-3%) of mTECs (Derbinski et al., 2001; Klein et al., 2001). This led to the hypothesis that Aire targeted genes are present in clusters which are activated in random fashion (Johnnidis et al., 2005). The analysis of transcriptom of individual mTECs by a single-cell qRT-PCR supported the view that genes from a particular locus are expressed in random combinations and thus further

argued for the support of the stochastic model of TRAs transcription regulation. As a result, there is now a general consensus that each mTEC can produce a different spectrum of TRAs (Derbinski et al., 2008). The hypothesis that expression of an individual self-antigen by mTECs can prevent the autoimmunity against specific tissue was confirmed by the identification of interphotoreceptor retinoid-binding protein as a target of eye-specific autoimmune disease in the $Aire^{-/-}$ mice (DeVoss et al., 2006). This and other findings (DeVoss et al., 2010; Shum et al., 2009) provide additional support for the importance of TRAs expression in preventing autoimmunity.

The discovery of the importance of Aire and TRAs expression within the thymus left open the question concerning how Aire-dependent TRAs expression prevents the onset of APECED disease. There are two potential mechanisms involved: the first one is through the process of negative selection of thymocytes specific for TRAs (recessive tolerance); the second one proposes the functional deviation of self-reactive T-cells into tTregs (dominant tolerance) (Metzger and Anderson, 2011). To address the question of how $Aire^{+}$ mTECs promotes central tolerance, several transgenic models of neo-self-antigens production were generated. Liston *et al.* constructed insHEL mice where the hen egg lysozyme is expressed in the thymus under the rat insulin promoter and crossed this transgenic animal with $Aire^{-/-}$ mouse. This transgenic system showed the increase of HEL-specific T cells in the periphery (Liston et al., 2003). Similar results were also shown in the ovalbumin (OVA) model, in which OVA expression in the thymus is driven by rat insulin promoter (RIP-mOVA) (Anderson et al., 2005).

The role of $Aire^{+}$ mTECs in generation of TRAs specific tTregs was shown by Aschenbrenner *et al*. They used two transgenic systems of Aire-driven neo-self antigen expression: Aire-HA (influenza hemagglutinin) and Aire-OVA, to reveal an antigen-specific differentiation of tTregs. The Aire-HA mouse was crossed with TCR-HA mouse expressing a transgenic TCR specific for HA. Results demonstrated that about 24% of TCR-HA specific $CD4^{+}$ T cell were converted to $Foxp3^{+}CD25^{+}$ tTregs. These results were also confirmed on Aire-OVA mouse model (Aschenbrenner et al., 2007). The phenomenon of naturally produced self-antigen specific T-cell deviation into tTreg lineage was finally confirmed by Malchow *et al*. by the identification of tTregs (MJ23tg) specific for prostate-associated antigen that

was present in both male and female tumor-free mice but were completely missing in Aire$^{-/-}$ mice (Malchow et al., 2013).

2.2 Mechanisms of peripheral tolerance

During the thymic development, about 95% of developing T-cells are eliminated and only 5% of them are released into the immune periphery (von Boehmer et al., 2003) Nevertheless, the mechanisms of negative selection do not work absolutely precisely and some self-reactive T-cell clones escape to the immune periphery (Kim et al., 2007).

There are at least two possible scenarios explaining how self-reactive T-cells escape from thymic selection. The first possibility is that the negative selection in the thymus most effectively eliminates thymocytes with high affinity/avidity TCRs for self pMHC complexes presented on mTECs and tDCs. This would then suggest that the mechanisms of peripheral tolerance are mostly acting on T-cell bearing low affinity/avidity TCRs for self pMHC which can escape to the periphery due to their less effective and/or ineffective elimination in the thymus (Liu et al., 1995; Mueller, 2010). Data supporting this situation came from Zehn *et al*. who, using RIP-mOVA mouse model, have demonstrated that relatively high number of low avidity OVA-MHC class I-specific CD8^{+} T-cells are present in the blood and secondary lymphoid organs (Enouz et al., 2012; Zehn and Bevan, 2006). Another mechanism explaining the presence of self-reactive T-cells in the periphery is based on the fact that some T-cells can express TCRs with high affinity/avidity for TRAs that are not expressed by mTECs nor there were brought to the thymus by DCs and they can´t be deleted in the thymus (Nichols et al., 2007).

There are several mechanisms employed in the immune periphery in order to prevent the autoimmune reaction against body-own tissues promoted by self-reactive T-cells which escaped from the thymic selection process, collectively called “peripheral tolerance”. One of these mechanisms is based on the existence of certain anatomical sites called “immune privilege tissues” (IPTs). These sites display the capacity to sequester antigens and protect tissue against damage caused by self-reactive T-cells and a collateral inflammation. Originally, the notion of IPTs was largely limited to a few tissues such as the brain and eyes, but it was later shown that it could also

include testis, livers and mucosa. It was reported that IPTs express critical molecules that regulate tolerogenic T-cell functions such as pro-apoptotic receptors TRAIL (TNF-related apoptosis-inducing ligand) and FasL (Fas ligand) or secreted molecules including TGF-β (transforming growth factor β) and IL-10 (Forrester et al., 2008).

Another mechanism maintaining immune peripheral tolerance was described as the model of ignorance of self-pMHC complexes. Briefly, the circulation of naïve T-cells from blood to lymphoid organs and then back to the blood is normally guided by CCR7 ligand gradient and through interactions with L-selectin (CD62L) expressed on T-cells (Mueller, 2010). Self-pMHC specific T cells are retained at sites with the high TRA expression only if their TCR engagement is accompanied by proper co-stimulatory conditions. Their absence or low levels lead to the diminishment of T-cells in non-lymphoid peripheral tissues and their limited capacity to interact with TRA-expressing cells. Also the expression of CCR7 and CD62L is dependent on co-stimulatory conditions. Thus, the down-regulation of CCR7 and CD62L on self-reactive T-cells limits their reentry into draining lymph nodes where they can interact with tissue-resident DCs, which may present high levels of self-antigens (Masopust et al., 2004; Mueller, 2010; Reinhardt et al., 2003).

The DCs are also very important players in the mechanism of peripheral tolerance. Under physiological circumstances all peripherally expressed antigens can be presented to naïve T-cells circulating through the secondary lymphoid tissue by DCs. This may lead to the generation of fully activated self-reactive T-cells specific for particular TRA. But in the absence of stimuli via pattern recognition receptors (PRRs), DCs expressed only a very low level of co-stimulatory molecules like CD80 (B7.1) and CD86 (B7.2) and exhibit a tolerogenic potential (Steinman et al., 2003). The recognition of a particular TRA by self-reactive T-cell without co-stimulation leads to the functional unresponsiveness also known as clonal anergy (Hawiger et al., 2001; Liu et al., 2002). In addition, the lack of co-stimulation in the presence of an appropriate cytokine milieu can, alternatively, lead to the generation of peripheral Tregs (pTregs) (Chen et al., 2003).

The co-stimulatory molecules from B7 family play a very important role in the maintenance of peripheral tolerance. Programmed cell death – 1 ligand (PD-L1) has been shown to regulate T-cell responses in the small intestine. The loss of the PD-1:

PD-L1 signaling leads to the expansion of self-reactive $CD8^+$ T-cells and breakage of $CD8^+$ tolerance to intestinal self-antigen (Reynoso et al., 2009). Similarly, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a structural homolog of CD28 which binds CD80 and CD86, after its interactions with B7 class co-stimulatory molecules, terminates T-cell proliferation, induces clonal anergy and this signaling can also lead to $Foxp3^+CD4^+$ pTreg generation (Walunas et al., 1994; Wing et al., 2008). It has been also shown, that the generation of pTregs depends on the presence of transforming grow factor β (TGF-β) and IL-2 (Kretschmer et al., 2005). Tolerogenic DCs may also have a role in TCR-specific control mechanisms. Using RIP-mOVA mice, it has been demonstrated that the recognition of OVA peptide presented by tolerogenic DCs to OT-I $CD8^+$ T-cells (TCR specific for OVA) leads to a functional inactivation and peripheral deletion of T cells in secondary lymphoid organs and thus prevents their autoreactivity towards self-pMHC complexes within the peripheral tissues (Kurts et al., 1997).

tTregs as well as pTregs absence leads to the lethal autoimmunity (Kim et al., 2007; Wing and Sakaguchi, 2010). Both types of Tregs share the expression of many cell-surface molecules and secreted proteins which have abilities to promote immune regulation. For example CD25, α-subunit of IL-2 receptor (IL-2R), is upregulated on Treg cells and could deprive effector T-cells of IL-2 and inhibit their proliferation (Fontenot et al., 2005; Pandiyan et al., 2007). Another Treg cell-specific surface molecule is aforementioned CTLA4 which is implicated in Treg cell-mediated suppression (Takahashi et al., 2000). Other cell surface molecules that are implicated in Treg cell suppression are two ectoenzymes CD39 and CD73. They facilitate the production of cyclic adenosine monophosphate (cAMP). cAMP signaling, initiated by Tregs, inhibits the proliferation of effector T-cells and also negatively impact the function of DCs (Deaglio et al., 2007). There are several others mechanisms of Tregs that are used to control DCs function. For instance, the presence of lymphocyte-activation gen 3 (LAG-3) (the CD4 homolog), with high affinity binding to MHC class II (Huang et al., 2004). The DCs dependent Treg cell-mediated immune regulation can be also supported by Ig family member TIGIT. Upon Treg interaction with DCs, TIGIT induces the production of tolerogenic cytokines IL-10 and TGF-β by DCs (Yu et al., 2009). While Tregs can also produce these tolerogenic cytokines,

they can also secrete other molecules like granzymes which cause the cytolysis and induce apoptosis of target cells (Josefowicz et al., 2012).

All these above described mechanisms involved in the maintenance of peripheral tolerance, with the exception of Treg-promoted tolerogenic function, are clearly dependent on non-inflammatory and normal physiological conditions in the immune periphery. Under the inflammatory conditions, tolerogenic DCs become fully activated upregulate co-stimulatory molecules and fully support the activation of T-cells. Also, the mechanism of immune privilege can be breached under perturbed physiological settings (Greter et al., 2005). Recently, a crucial role of additional cell types called lymph node stromal cells (LNSCs) in inducing peripheral tolerance of $CD8^+$ T-cell was described. This fact brought a new perspective to the importance of mechanisms of peripheral tolerance as LNSCs possess the ability to directly delete self-reactive T-cells (Lee et al., 2007).

2.2.1 Lymph node stromal cells induce peripheral tolerance

Lymph nodes are highly organized and complex structures which can be divided into cortical, paracortical and medullary areas as well as on T a B cell resident zones and each of these areas is characterized by the presence of specific non-hematopoietic stromal cells. LNSCs represent a heterogeneous population of cells which, based on the surface expression of the glycoproteins CD31 and podoplanin (gp38), can be subdivided into (i) fibroblastic reticular cells (FRCs; $gp38^+CD31^-$), (ii) lymphatic endothelial cells (LECs; $gp38^+CD31^-$), (iii) blood endothelial cells (BECs, $gp38^-$ $CD31^+$) and (iv) the fraction of double negative (DN, $gp38^-$ $CD31^-$) cells. The DN population contains follicular dendritic cells (FDCs), integrin $\alpha7^+$ pericytes (IAPs) and possibly other cell types (Malhotra et al., 2013). LECs and DN cells can be also identified by the expression of alpha-smooth muscle actin (Togoo et al., 2014).

The role of LNSCs in immune peripheral tolerance was described initially in 2007 with the use of iFABP-tOVA transgenic mouse model. In this model, the truncated cytosolic form of ovalbumin (tOVA) is expressed under the control of intestinal fatty acid binding protein (iFABP) locus. The expression of tOVA is in this mouse model targeted to mature intestinal epithelial cells (IECs) in jejunum and ileum, but it was also found in $CD45^-$ radio-resistant LNSCs. It was then shown that transfer of OT-I T-cells to iFABP-tOVA mice resulted in their high proliferation in response to

signals from LNSCs without their proper co-stimulation and later on to diminishment of these cells (Lee et al., 2007). Later, it was described that stromal cells predominantly responsible for tOVA expression in the iFABP-tOVA model are FRCs. *In vitro* stimulation assay confirmed the ability of FRCs to present this neo self-antigen to OT-I T-cells. It was also shown that FRCs have reduced capacity to produced TRAs and stimulate T-cells after Toll-like receptor 3 (TLR3) ligation (Fletcher et al., 2010). It was then reported that IFNγ and tumor necrosis factor (TNF) release from activated T-cells triggered the expression of inducible nitric oxid synthase (NOS2) by LNSCs. Notably, the production of nitric oxide by FRCs and LECs inhibited the proliferation and effector functions of activated T-cells (Lukacs-Kornek et al., 2011).

The role of LNSCs in $CD8^+$ T-cell peripheral tolerance was also confirmed using transgenic mouse model expressing a TCR specific for melanocytes tyrosinase (FH T-cells). These $CD8^+$ T-cells recognize their cognate antigen in the context of the MHC class I molecule AAD. It was demonstrated that FH^+AAD^+ tyrosinase-expressing mice (FH^+AAD^+ $tyrosinase^+$) has significantly less FH T-cells than FH^+AAD^+ $tyrosinase^-$ mice. It suggests that the transfer of FH T-cells into AAD^+ $tyrosinase^+$ mice resulted in activation, proliferation and deletion of these T-cells in all lymph nodes (Nichols et al., 2007). In 2010, Cohen *et al.* identified cells that express and directly present tyrosinase in lymph node stroma as LECs. They also established that, similar to FRCs and other LN stromal cells, LECs also express a set of TRAs (Cohen et al., 2010). It was then reported that LECs are able to induce peripheral tolerance by a direct contact with $CD8^+$ T-cells. LECs can mediate $CD8^+$ T-cells deletion via PD-L1 expression (Tewalt et al., 2012). Together with another study which used a transgenic mouse expressing HA peptide under the regulatory sequences of glial fibrillary acid protein (Magnusson et al., 2008), these data provided compelling evidence for the involvement of LNSCs in the maintenance of peripheral $CD8^+$ T-cell tolerance through expression of particular TRAs and active deletion of self-reactive cells.

Although mTECs and LNSCs shared the capacity to express TRAs, the mechanisms by which these cells regulate TRAs gene expression appear to be completely different. It was shown that transcriptional regulator Aire is not responsible for TRAs expression in FRCs, LECs and BECs. Interestingly, the majority of LNSCs were

found to express an Aire-like transcriptional regulator called Deaf1 (deformed epidermal autoregulatory factor 1). Deaf1 was shown to regulate the expression of genes encoding TRAs in LNSCs. Deaf1 is present in two isoforms. The canonical Deaf1 isoform is able to translocate to the nucleus and maintain the expression of TRAs, while a splice variant of Deaf1 is retained in the cytoplasm and inhibits activity of canonical Deaf1. The production of non-canonical isoform is significantly higher in the pancreatic lymph nodes of type 1 diabetes patients (Yip et al., 2009).

Numerous studies have demonstrated the involvement of LNSCs in $CD8^+$ T-cell tolerance, but the role of these stromal cells in $CD4^+$ T-cell tolerance was unclear. Malhotra *et al.* demonstrated that LNSCs also express a low level of MHC class II molecules and showed that FRCs, LECs and BECs can upregulate surface expression of MHC class II under inflammatory conditions which might give them the ability to interact with $CD4^+$ T-cell and promote their tolerance (Malhotra et al., 2012). This study was confirmed by Dubrot *et al.* who showed that endogenous expression of MHC class II by LNSCs is controlled by the IFNγ-inducible promoter IV of class II transactivator. So the LNSCs upregulate these molecules after being exposed to IFNγ (Dubrot et al., 2014).

A new era in the field of peripheral tolerance came in 2008, when it was reported that the DN fraction of LNSCs express high level of MHC class II under steady-state conditions. Importantly, it was also shown that these DN LNSCs express low levels of Aire (Fletcher et al., 2010; Gardner et al., 2008). The hunt for lymph node-residing Aire-expressing cells and the elucidation of their role in peripheral tolerance began.

2.2.2 The role of peripheral Aire expression in tolerogenic mechanisms

While the function of Aire in the mechanism of central tolerance is quite well established, its expression profile, cellular source and delivery of function in peripheral organs, including secondary lymphoid tissues, have been ill-defined and surrounded by a controversy. The initial experiment revealed *Aire* expression on transcript level in lymph nodes and spleen (Heino et al., 1999). Later on, qRT-PCR analysis detected expression of *Aire* mRNA in many others tissues including livers, lungs, small intestine and urinary system (Adamson et al., 2004). This finding was

also confirmed by immunohistochemistry (IHC) staining using polyclonal anti-Aire antibody which identified Aire$^+$ cells in many of these tissues including lymph nodes, spleen and bone marrow (Adamson et al., 2004; Halonen et al., 2001). Several additional studies also described Aire expression in DCs isolated from peripheral lymphoid tissues. Poliani *et al.* found Aire expressing cells in human peripheral tissues like lymph nodes, tonsils and gut-associated lymphoid tissues. These cells were described as CD11c$^+$ MHC class II$^+$ (HLA-DR) DCs (Poliani et al., 2010). Another two studies observed the presence of Aire$^+$ DCs in the spleen. These DCs were described as CD11c^{int} B220$^+$ (Grupillo et al., 2012) or as 33D1$^+$ DCs from marginal zone (Lindmark et al., 2013). Both of these studies also showed the expression of TRA insulin by these cells (Grupillo et al., 2012; Lindmark et al., 2013). Hubert *et al.* created a new rat monoclonal antibody specific for murine Aire. Using flow cytometry analysis and IHC staining, they were able to identify Aire$^+$ cell in the thymus but failed to detect any Aire protein in peripheral stromal tissues of C57Bl/6 mice (Hubert et al., 2008).

In 2008 Gardner *et al.* created bacterial artificial chromosome (BAC) transgenic mouse in which the expression of green fluorescent protein (GFP) is driven by Aire regulatory sequences (Adig mouse). This model facilitated the identification of GFP positive cells with an active Aire locus in both lymph nodes and spleen and led to the description of new population of extra-thymic Aire-expressing cells (eTACs). Flow cytometry analysis and the generation of bone-marrow chimeras revealed that eTACs are of stromal origin (CD45$^-$) and expressed the epithelial cell adhesion molecule (EpCAM) and inhibitory B7 family member PD-L1. It was also shown that eTACs lacked the expression of both CD80 and CD86 co-stimulatory molecules and were negative for the B-cell marker B220, the FRCs marker gp38 and the DCs marker CD11c. Importantly, eTACs, as well as mTECs, were found to express high levels of MHC class II molecules (Tab. 2).

The transgenic construct in Adig mouse was prepared in such a way that the GFP under Aire-regulatory site was expressed as a chimeric protein with islet-specific glucose-6-phosphatase-related protein (IGRP) which was identified previously as the type I diabetes autoantigen. It was shown that adoptive transfer of CD8$^+$ T-cell with TCRs specific for IGRP (8.3 T-cells) resulted in 8.3 T-cells proliferation in all secondary lymphoid organs and that such proliferation ultimately led to 8.3 T-cells

deletion. The role of eTAC-mediated neo-antigen MHC class I presentation in this mechanism was confirmed by the bone marrow reconstitution experiments. Specifically, MHC class I deficient mice which is not able to present or cross-present the IGRP antigen in the MHC class I context, was used as the donor of bone marrow cells and Adig mice as their acceptor. In this schematics, the presence or absence of bone marrow–derived MHC class I sufficient or deficient hematopoietic cells, and especially APCs, had no effect on the outcome of T cell-eTAC interactions which resulted in 8.3 T-cell proliferation and deletion.

After this seminal discovery of eTACs, it still remained to determine the role of Aire protein itself in eTACs. The microarray analyses of eTACs revealed the upregulation of 160 genes in the presence of Aire. When compared with the set of genes regulated by Aire in mTECs and it was clear that Aire-regulated TRAs in eTACs were distinct from those in the thymus. The authors thus suggested that peripheral Aire likely complements its role in the thymus, rather than intensifies deletion of the same TRAs that are expressed by mTECs. Together, these results showed the presence of eTACs in lymph nodes and spleen and described the capacity of eTACs to induce deletional tolerance among $CD8^+$ T-cell in secondary lymphoid organs. Although not experimentally tested in this paper, the expression of MHC class II by eTACs suggested that they may have a functional capacity to interact with $CD4^+$ T-cells (Gardner et al., 2008).

The final evidence of $CD4^+$ T-cell-eTACs interactions was provided by Gardner *et al.* five years later, in 2013. Contrary to their previous study, they described eTACs as a bone marrow-derived ($CD45^+$) cell population. Using the very same Adig transgenic mice described above, the FACS (fluorescent-activated cell sorting) analysis of $CD45^+$ eTACs revealed expression of MHC class II, EpCAM and CD11c. In comparison with mTECs and DCs, eTACs express very low levels of co-stimulatory molecules CD80 and CD86 (Tab. 2). To clarify the function of Aire in eTACs they also demonstrated the expression of some TRAs. To investigate the $CD4^+$ T-cells-eTACs interactions, the *Aire*-driven BDC-GFP (AbBDC) construct was used to generate a new transgenic mouse. The authors showed that the adoptive transfer of BDC2.5 TCR-transgenic $CD4^+$ T-cells into AbBDC mouse resulted in a robust proliferation and functional inactivation (anergy) of $CD4^+$ T-cells in secondary lymphoid organs. Next they showed that proliferation was also observed

in AdBDC mice reconstituted with MHC class II deficient bone marrow which suggested that antigen presentation by partially radioresistant and thus residual population of eTACs is sufficient to drive BDC2.5 T-cell proliferation. They also demonstrated that $CD4^+$ T cell-eTACs interactions led to the enrichment of $Foxp3^+$ Treg cells. However, it is not clear, if eTACs only enhance Tregs numbers by supporting their proliferation or they are able to convert BDC2.5 T-cell directly into Tregs. It is of note, that the mechanism of tolerance induced by eTACs (functional CD4 T-cell inactivation) was fully independent on the presence of Treg cells. Together these data suggest that bone marrow-derived population of eTACs is able to induce the peripheral tolerance of $CD4^+$ T-cells through their functional inactivation and Treg cells enrichment (Gardner et al., 2013).

Both of these studies revealed an interesting phenomenon of Aire expressing cells in immune periphery. They described so-called eTACs as cells having a very important role in maintenance of immune peripheral tolerance either through TCR specific functional inactivation of self-reactive $CD8^+$ or $CD4^+$ T-cell, or through enrichment of pTregs. However, despite these interesting studies, the existence of eTACs remains still quite controversial, mainly due to the fact that the very same group presented within 5 years quite opposing results which have not been independently confirmed or disputed by other labs. Even more importantly, neither of these two studies reliably demonstrated the occurrence of peripheral Aire at the protein level and failed to show a clear phenotypic characterization of these cells. Moreover, both studies are using rather artificial models of neo-self antigen presentation for the detection of recessive and dominant type of peripheral tolerance imparted by eTACs to cognate self-reactive T-cells.

In the following part of my thesis, I will describe the experimental journey to identify, isolate and characterize *bona fide* eTACs from various peripheral lymphoid and non-lymphoid tissues. These experiments were conducted for the purpose to contribute to better understanding of cellular and molecular processes that maintain our immune homeostasis.

	eTACs (Gardener et al., 2008)	eTACs (Gardener et al., 2013)	FRC (Fletcher et al., 2010)	LEC (Cohen et al., 2010)
gp38	–	–	+	+
CD31	–	–	–	+
EpCAM	+	+	–	–
CD11c	–	+	–	–
MHCII	+	+	–	–
CD45	–	+ /low	–	–
B220	–	–	–	–
PD-L1	+	+	+	+
CD80	–	low	low	–
CD86	–	low	–	–
AIRE mRNA	+	+	–	–
AIRE reporter	+	+	–	–
AIRE protein	?	?	–	–

Table 2: Comparison of eTACs and LNSCs populations. *eTACs were first described as DN (gp38$^-$, CD31$^-$) LNSCs population which is negative for DC marker CD11c (Gardner et al., 2008). In 2013 the same group described eTACs as non-conventional hematopoietic APCs which express CD45 and CD11c molecules (Gardner et al., 2013). Compared with eTACs, FRCs and LECs express stromal marker gp38 and are strictly CD45$^-$ (Cohen et al., 2010; Fletcher et al., 2010).*

3 Material and methods

3.1 Material

3.1.1 Animals

Animals were bred in the animal facility of The Institute of Molecular Genetics AS CR, v.v.i. in individually ventilated cages under specific-pathogen free conditions. Animals were tested regularly according to the FELASA guidelines. Aire-HCO Balb/c (Aschenbrenner et al., 2007) and Adig (Aire-GFP) Balb/c (Gardner et al., 2008) transgenic mice were used for the most of the experimental work. Both mice were generated with the use of bacterial artificial chromosome transgenic construct. The *Aire* start codon in Aire-HCO mouse was replaced by open reading frame encoding influenza hemaglutinin (HA), C-reactive protein (Crp), OVA and human CD2 (hCD2). The *Aire* locus in Adig mouse was modified to drive expression of green fluorescent protein (GFP) which is fused to islet-specific glucose-6-phosphatase-related protein (IGRP). Aire-KO ($Aire^{-/-}$) mice on Bablb/c background (Ramsey et al., 2002b) were used as negative control in qRT-PCR experiments. All these models were generously provided to us by Prof. Ludger Klein, AG Thymus function, Institute for Immunology, Medicine Faculty, LMU Munich, Germany. The MHC II-eGFP mice (Boes et al., 2002) were used for immunohistochemical experiments. This is a knock-in mouse, where eGFP construct is inserted into gene for MHC II. This mouse was provided to us by doc. Jan Černý, Laboratory of Cell Immunology, Department of Cell Biology, Faculty of Science, Charles University in Prague. Balb/c WT and C57BL/6 WT mice came from the animal facility of The Institute of Molecular Genetics and they were used as the WT control. In the case of transgenic and Aire deficient animals, wild-type littermates served as controls.

3.1.2 Antibodies

List of primary and secondary antibodies that were used in experimental part of the thesis.

Name	Clone	host	Reactivity	Conjugate	Manufacturer
Primary antibodies					
Aire	5H12	rat	mouse	Purified	eBioscience
Aire	5H12	rat	mouse	AF660	eBioscience
Aire	D-17	goat	mouse	Purified	Santa Cruz Biotechnology
B220	RA3-6B2	rat	mouse	AF647	in house
CD11c	N418	hamster	mouse	APC-Cy7	BioLegend
CD19	6D5	rat	mouse	PE-Cy5	BioLegend
CD45	30-F11	rat	mouse	biotin	BioLegend
EpCAM	G8.8	rat	mouse	PE	BioLegend
EpCAM	G8.8	rat	mouse	APC	eBioscience
hCD2	RPA-2.10	mouse	human	APC	BioLegend
I-A/I-E	2G9	rat	mouse	FITC	BDPharmigen
I-A/I-E	M5/114.15.2	rat	mouse	PE-Cy5	eBioscience
IgG2, κ Isotype Ctrl	RTK4174	rat	mouse	APC	BioLegend
secondary antibodies					
AF488	-	donkey	rat	AF488	Life technologies
AF647	-	chicken	rat	AF647	Life technologies
AF555	-	donkey	goat	AF555	Life technologies
SA-PE-Cy7	-	streptavidin		PE-Cy7	BioLegend
SA-PE-Cy7	-	streptavidin		PE-Cy7	eBioscince

Table 3: The list of used antibodies

3.1.3 Primers

The set of primers that were used in qRT PCR analysis. Primers were designed by Roche Universal ProbeLibrary Assay Design Center (version 2.45). Gad65 primers sequences were taken from (Derbinski et al., 2001).

Species	Target gene	Primer sequence	
		Forward (5´-3´)	**Reverse (5´-3´)**
mouse	Aire	AGGCTCCCACCTGAAGACTAA	CACGGTCACAGCTCTCTGG
mouse	Casc3	TTCGAGGTGTGCCTAACCA	GCTTAGCTCGACCACTCTGG
mouse	Defcr24	CTGAGCTGCTACTCACCAATCCTCC	AGCGCCTTCTGGGTCTCCAA

mouse	Gad65	TGGAATCCTCACAAGATGATGG	TCTTCATTGTCTTCCAGAGTGC
mouse	Gad67	ATACAACCTTTGGCTGCATGCT	TTCCGGGACATGAGCAGT
mouse	Ins2	CGAAGTGGAGGACCCACA	TGCTGGTGCAGCACTGAT
mouse	Muc6	CCGGACACCAACACAGAAG	CCCTTCACTGTGGTCGAAGT

Table 4: The set of used primers. *Used abbreviations:* ***Aire*** *(autoimmune regulator, Pubmed Gene ID: 11634),* ***Casc3*** *(cancer susceptibility candidate 3, Pubmed Gene ID: 192160),* ***Defcr24*** *(enteric α-defensin 24 (cryptdin 24), Pubmed Gene ID: 503491),* ***Gad65*** *(glutamic acid decarboxylase 2, PubmedGene ID: 14417),* ***Gad67*** *(glutamat decarboxylase 1, Pubmed Gene ID: 14415),* ***Ins2*** *(insulin II, Pubmed Gene ID: 16334), and* ***Muc6*** *(mucin 6, Pubmed Gene ID: 353328).*

3.2 Methods

3.2.1 Isolation of mouse tissues

The freshly obtained tissues (thymus, lymph nodes, spleen, Peyer's patches and lungs) were minced by scissors into small pieces or, in case of lymph nodes, punched by needle and prepared for enzymatic digestion. Mouse bone marrow cells were harvested from long bones (*femur, tibia*) by PBS flushing and the obtained suspension was filtrated through a 100µm cell-strainer. Minced solid tissues were put into 37°C RPMI medium and treated by 0,1 $mg.ml^{-1}$ Dispase I. (Gibco-Invitrogen, Carlsbad, California) on shaking thermoblock (37°C, 800rpm). After the first 30 minutes of incubation, the supernatant was collected and replenished with a new medium (RPMI + Dispase I.). The reaction was then pipetted up and down regularly. The medium replenishment was then repeated every 10 minutes. The enzymatic digestion was stopped by adding 3% FSC supplemented with 2mM EDTA (Gibco-Invitrogen, Carlsbad, California) to each collected supernatant which was then filtered through a 100µm cell-strainer. The reaction was terminated after complete digestion of the tissue. Finally, the collected supernatants were centrifuged (4°C, 8 minutes, 300g) and re-suspended in 3% FSC and 2mM EDTA in PBS. In case of mTECs isolation, CD45 magnetic beads were used to separate epithelial cells according to the expression of CD45 by autoMACS Pro Separator (all Myltenyi biotec, Bergisch Gladbach, Germany). Staining and separation was performed

according to manufacturer's instructions and $CD45^-$ fraction was used for further analysis.

3.2.2 Fluorescence-activated cell sorting

For fluorescence-activated cell sorting (FACS) analysis, single-cell suspension was stained with specific antibodies directly conjugated with fluorochromes or with biotinylated antibodies further visualized by fluorochrome conjugated streptavidin. The list of antibodies used in the study is presented in Table 3. To exclude death cells, cells were also stained by Hoechst 33258 dye or "Viability Dye" (eBioscience, San Diego, California). Cells were analyzed by LSR II FACS machine (BD Biosciences, San Jose, California) and data analyses were performed using FlowJo 9.7.5 software (Tree Star, Ashland, Oregon). For the purpose of RNA isolation or immunoflourescence, cells were sorted by BD Influx Cell Sorter (BD Biosciences, San Jose, California) and used for further analysis.

3.2.3 Intracellular FACS staining

Intracellular staining was used for the detection of Aire protein expressing cells in the cellular fraction. Target cells were isolated and stained according to the protocol described above. Cells were washed by 3% FSC in PBS and fixed with Foxp3 fix/perm solution for 20 minutes in room temperature, washed two times with Foxp3 washing solution (both solutions from BioLegend, San Diego, California) and stained with APC-conjugated anti-Aire antibody for 30 minutes at the room temperature. Cells were analyzed by flow cytometry. Isotype control staining was included for each sample.

3.2.4 The preparation of cryostat section and immunofluorescence

Small pieces of selected tissues were fixed in 4% paraformaldehyde for one hour and placed into the 30% sucrose overnight. The samples were then frozen in Tissue freezing medium (Leica Microsystems, Wetzlar, Germany) using a dry ice bath and placed into the minus 80°C. The 10µm thick tissues sections were made using CM1850 (Leica Microsystems, Wetzlar, Germany). The immunofluorescent analysis

was performed on cells isolated and sorted according to the protocol described above. Cells were sorted into a cultivation media and placed on microscope slide and let to adhere. Microscopic slides with tissues sections or sorted cells were fixed in PHEM fixative solution for 15 minutes and washed three times in PBS for 5 minutes each. PHEM fixative solution is based on PHEM buffer (Sobue et al., 1988) supplemented with paraformaldehyde (4%, Polysciencies, Warrington, Pennsylvania) and Triton X-100 (1%, Thermo Scientific, Rockford, Illinois). The samples were then incubated in blocking solution (1% BSA in antibody diluent composed of 4% sodium azid (Sigma-Aldrich, St. Louis, Missouri), 0,1% gelatine (Bio-Rad, Oslo, Norway) and PBS) for one hour and primary antibodies, diluted in primary antibody dilution solution (0.5% BSA (Serva, Heildelberg, Germnany) dissolved in PBS, antibody diluent), were applied overnight. Samples were washed three times in PBS and incubated with secondary antibodies conjugated with flourochromes diluted in antibody dilution solution for two hours. All images were taken using Leica DMI6000 TCS SP5 AOBS confocal microscope (Leica Microsystems, Wetzlar, Germany). Secondary antibody staining was used as a negative control.

3.2.5 RNA isolation from sorted cells

For RNA isolation, cells were sorted by BD Influx Cell Sorter directly into the RLT buffer and immediately processed by RNasy plus micro kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

3.2.6 Preparation of cDNA library

The cDNA library was prepared from isolated RNA by the reverse-transcription. RNA was incubated with random hexamers, 5X reaction buffer, RiboLock RNAse Inhibitor, 10mM dNTP Mix and RevertAid Reverse Transcriptase (all Thermo Scientific, Waltham, Massachusetts). The exact protocol is depicted bellow. XP cycler (Bioer Technology, Binjiang, China) was used for cDNA library construction and PCR reactions.

Mix 1

RNA	11,5 µl
random hexamers	1,0 µl
5 minutes	65°C
1 minute	chilled on ice

Mix 2:

reaction buffer	4,0 µl
RiboLock	0,5 µl
dNTPs (10mM)	2,0 µl
revers transcriptase	1,0 µl
10 minutes	25°C
60 minutes	45°C
10 minutes	75°C

3.2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression level of target genes was determined by qRT-PCR analysis. cDNA was obtained by reverse-transcription of RNA from FACS sorted cells as described above. The measurements were performed in duplicates or triplicates on LC480 or LC480 II cyclers (Roche Applied Science, Mannheim, Germany). PCR reactions were performed for up to 50 cycles using SYBR Green I master mix (Roche Applied Science, Mannheim, Germany) and validated primers (Table 4). Relative levels of target mRNA were calculated using housekeeping gene normalization (Cacs3; cancer susceptibility candidate 3, Pubmed Gene ID: 192160) using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.2.8 Statistical analysis

All statistical analysis and graphs that were used in this thesis were created by GraphPad Prism (GraphPad Software, version 5.04, San Diego, California).

4 Results

4.1 General consideration

The presence of Aire and its function outside the thymus has been for some time surrounded by highly contradictory results. That especially concerns two articles from the same research group describing the identification and phenotypic as well as functional characterization of eTACs using transgenic animal models (Gardner et al., 2008; Gardner et al., 2013). Results obtained recently in our and collaborating laboratory of Prof. Klein in Munich suggest that the population of Aire-reporter positive cells (GFP^+ from Adig mice or $hCD2^+$ from Aire-HCO mice) residing in lymph nodes is strictly of hematopoietic origin and much more heterogenic. At least four populations of Aire-reporter positive cells can be identified ($B220^+$, $EpCAM^+CD11c^+$, $EpCAM^-CD11c^+$ and $EpCAM^-CD11c^-$ also called DN cells). Interestingly, as only the population of DN cells produces a detectable amount of Aire on protein level, this data is in complete discordance with the two previous Gardner´s reports concerning eTACs which are mentioned above (Dobes, J., Yamano, T., Klein, L., Filipp, D., unpublished data). The main objective of presented thesis was to investigate if Aire-reporter positive cells are also present in other lymphoid as well as non-lymphoid tissues. This included the following goals:

1. Identify Aire-reporter expressing cells in lymphoid and non-lymphoid tissues using two transgenic mouse models.

2. Characterize these cells phenotypicaly by FACS.

3. Verify Aire expression in these cells on protein level using various methods.

4. Determine the ability of Aire protein expressing cells to produce TRAs and determine if these TRAs are Aire regulated in the same fashion as in thymic mTECs.

4.2 Aire-expressing cells are present in lymphoid and non-lymphoid tissues

Aire-HCO and Adig (Aire-GFP) transgenic mouse models (see Materials and Methods section for details) were used for identification of Aire-expressing cells in selected tissues. The expression of hCD2 on the cell surface (Aire-HCO) or GFP (Adig) reflects the activation of the Aire locus. FACS analysis of hCD2 or GFP expression was used to detect the presence of Aire-expressing cells in various tissues. WT littermate mice were used as a negative control. As it was reported, mTECs express high levels of Aire protein so, in these experiments, the thymus was used as a positive control (Derbinski et al., 2001). As illustrated in Fig. 6A, hCD2 positive cells were identified in the thymus, lymph nodes, Peyer´s patches, spleen, bone marrow and lungs. Other non-lymphoid tissues like the liver, the skin, the brain and the kidney were also tested for hCD2 expression, but the expression of transgene was not detectable or was not detected reproducibly (data not shown). The highest percentage of $hCD2^+$ cells showed the spleen, Peyer´s patches and lungs (Fig. 6A). Comparable results were obtained from Adig mice (Fig. 6B) but the highest percentage of GFP^+ cells showed lymph nodes, with lower, but comparable abundance in Peyer´s patches, in the thymus and in the spleen.

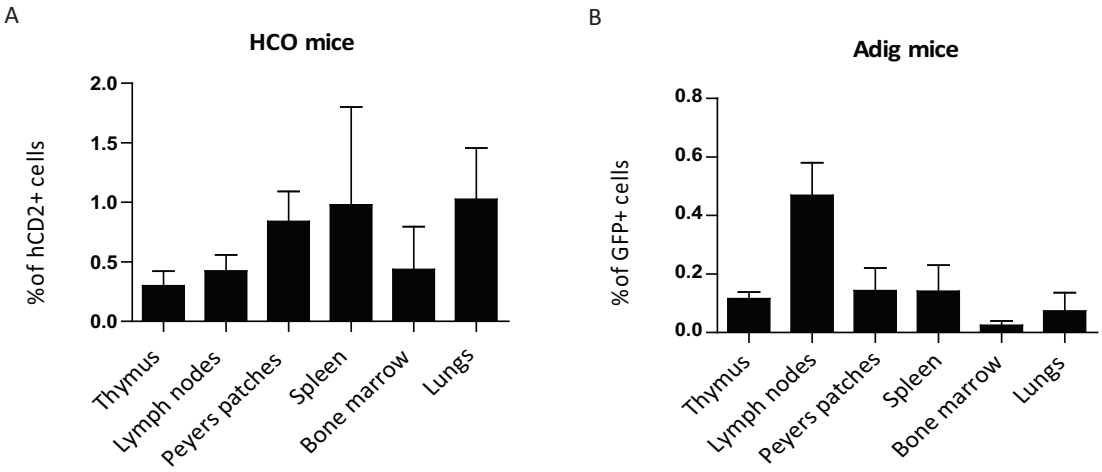


Figure 6: The FACS analysis of Aire expressing cells presence in various murine tissues. *FACS analysis of hCD2 expression in tissues of Aire-HCO mice reveals the presence of $hCD2^+$ cells in thymus, lymph nodes, Peyer´s patches, spleen, bone marrow and lungs* ***(A)****. Results derived from the same panel of tissues as in* ***(A)*** *show the analysis of GFP expressing cells in Adig mice* ***(B)****. Analysis is based on a minimum of three independent experiments (n=3), visualized as mean with standard deviation (SD).*

These results confirmed that these two transgenic models are largely comparable and useful for the identification of Aire-expressing cells in murine tissues. Importantly, they indicated that Aire-expressing cells are present not only in lymphoid (thymus, lymph nodes, spleen etc.) but also in non-lymphoid tissues such as lungs.

4.3 Phenotypic characterization of Aire-expressing cells in lymphoid tissues

Due to an easier and straightforward detection of Aire-expressing cells in lymphoid tissues, Adig mice were used for the next set of experiments. Cell fractions from particular tissues were obtained as described in the Materials and Methods section. FACS analysis was used for detection of GFP expressing cells in lymphoid tissues and for a basic phenotypic characterization of these cells. Based on previous studies, MHC class II, EpCAM, CD11c (Gardner et al., 2008; Gardner et al., 2013) as well as CD45RB (B220) (as some thymic B-cells produce Aire; Yamano, T., Klein, L., personal communication) were selectively used as phenotypic markers of GFP^+ cells. It is of technical note that, and as presented in Figures 7-11, B220 expressing cells show the variation in staining intensity. This was caused by the use of different batches of anti-B220 antibodies. However, in all cases, B220 negativity was strictly set by the intensity of unstained cell controls.

4.3.1 Aire-expressing cells in the thymus

Cells were isolated from a single whole thymus. As illustrated in figure 7, the thymus contains approximately 0.15% of GFP^+ MHC class II^+ cells, where about twenty percent of these cells express B-cell marker B220 and show that thymic B-cells are able to activate Aire locus and express GFP in Adig mouse model. $B220^-$ GFP^+MHCII^+ cells can be divided into four populations based on EpCAM and CD11c expression. $EpCAM^+CD11c^-$ cells represent the largest population which most likely resembles thymic mTEC population. These results also documented the presence of another two populations of GFP expressing cell types with $EpCAM^-$ $CD11c^-$ and $EpCAM^-CD11c^+$ phenotypes in the thymus. The presence of the latter population argues for the existence of GFP expressing DCs in the thymus (Fig. 7A, B).

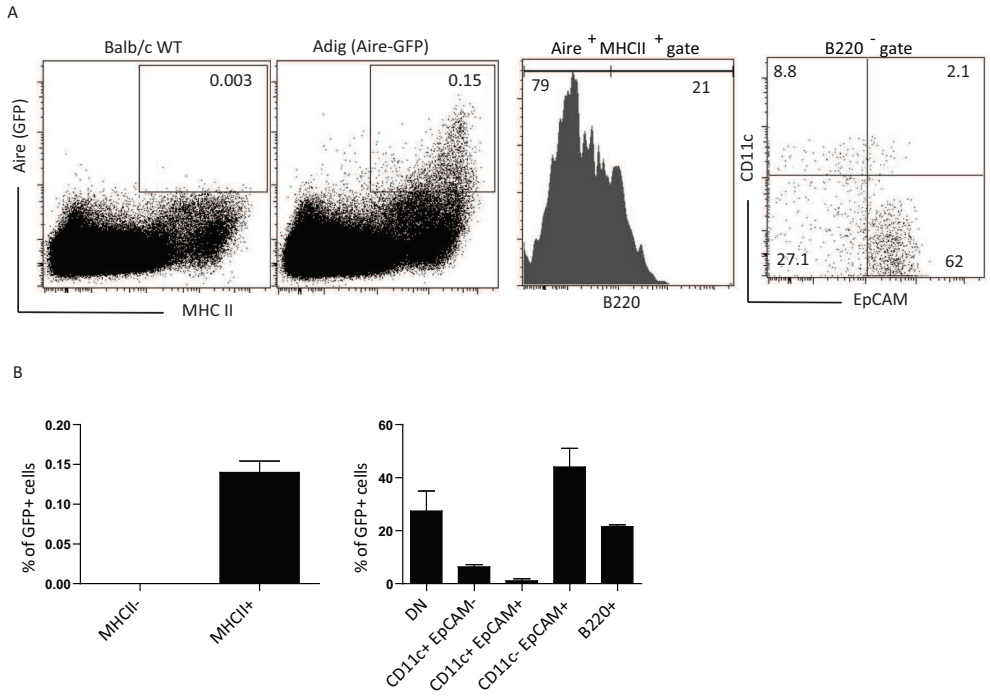


Figure 7: FACS analysis of GFP$^+$ (Adig) cells in the thymus*. Thymic cells were gated according to their GFP and MHC class II positivity (left two dot plots) and tested for their B220 expression. B220 negative cells were further subdivided according to the EpCAM and CD11c expression. Representative dot-plots depicting the gating strategy are shown* ***(A)****. Bar graphs represent the proportion of individual populations in percentage in the whole suspension, n=3* ***(B)****.*

4.3.2 Aire-expressing cells in lymph nodes

Cells were isolated from skin draining and mesenteric lymph nodes and pooled together from two Adig mice into one sample. Figure 8 shows that lymph nodes contain about 0.5 % of GFP$^+$ cell. Similar to thymic GFP$^+$ cells, the most of GFP$^+$ cells in lymph nodes also express MHC class II molecules. However, unlike the thymus, a minor population of GFP$^+$MHCII$^{neg/low}$ cells was detected in lymph nodes (Fig. 8A, B). These cells represented mainly the B220$^-$ and DN (EpCAM$^-$CD11c$^-$) population (data not shown). Compared to the thymus where B-cells (B220$^+$ cells) compromise approx. 20% of GFP$^+$MHCII$^+$ cells, in lymph nodes they represent only five percent. B220$^-$GFP$^+$MHCII$^+$ cells comprise of two equally sized populations of DN and EpCAM$^-$CD11c$^+$phenotype. The EpCAM$^+$CD11c$^+$ population possesses the very same phenotypic characteristic as eTACs, the main GFP expressing population in lymph nodes described by (Gardner et al., 2013). Our results indicate that

EpCAM^{+}CD11c^{+} cells are the least abundant GFP^{+} MHCII^{+} population in lymph nodes (Fig, 7A, B).

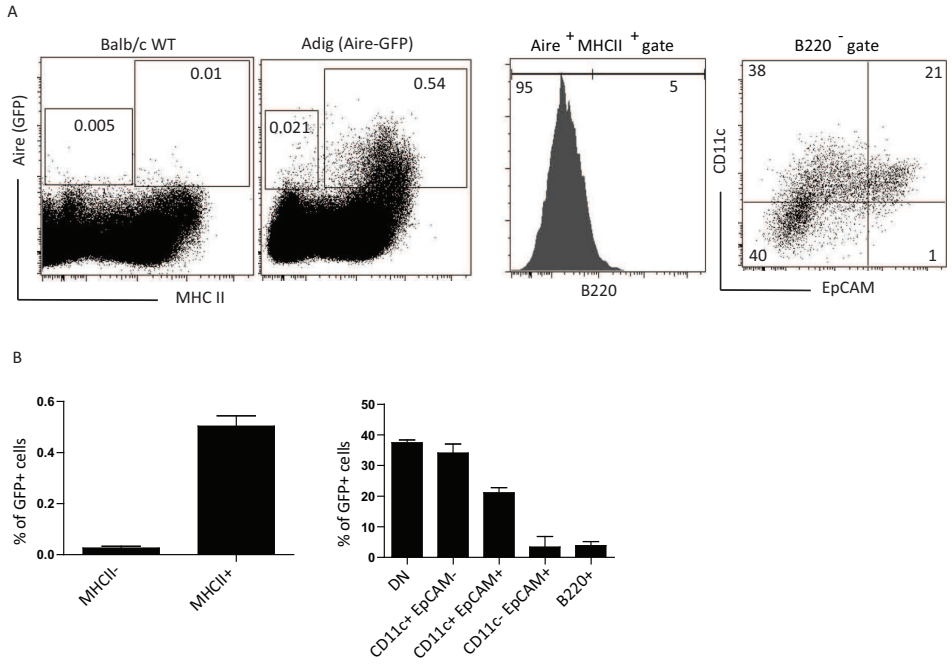


Figure 8: FACS analysis of GFP^{+} (Adig) cells in skin draining and mesenteric lymph nodes. *Cells isolated from lymph nodes were gated according to their GFP and MHC class II positivity and tested for B220, EpCAM and CD11c expression. Representative dot-plots are shown* ***(A)****. Bar graphs (mean with SD) represent the proportion of individual populations in percentage in the whole suspension, n=3* ***(B)****.*

These results indicate that the variety and enumeration of GFP^{+} subpopulations with relevant phenotypes in lymph nodes differ from that reported earlier (Gardner et al., 2008; Gardner et al., 2013). Our data for the very first time showed that there are several other phenotypically distinct GFP^{+} populations present in lymph nodes of Adig mice and that EpCAM^{+}CD11c^{+} population is far from being the dominant one.

4.3.3 Aire-expressing cells in Peyer´s patches

Peyer´s patches were isolated from small intestines obtained from two Adig mice and cleaned of surrounding intestinal tissue. Single-cell suspension was obtained according to protocol described in Materials and Methods section. Figure 9 illustrates that Peyer´s patches contain approximately 0.18 % of GFP^{+}MHCII^{+} cells. Cells expressing B220 marker (B-cells) are much more dominant in number than in the previously described tissues – thymus and lymph nodes. B220^{-} population of

MHCII^{+} GFP^{+} cells is accommodated mainly by EpCAM^{-} CD11c^{-} (DN) cells (Fig. 9A, B). While in Peyer´s patches the population of MHCII^{-} cells could be occasionally recognize (Fig. 9), its detection was not reproducible and thus was not further characterized.

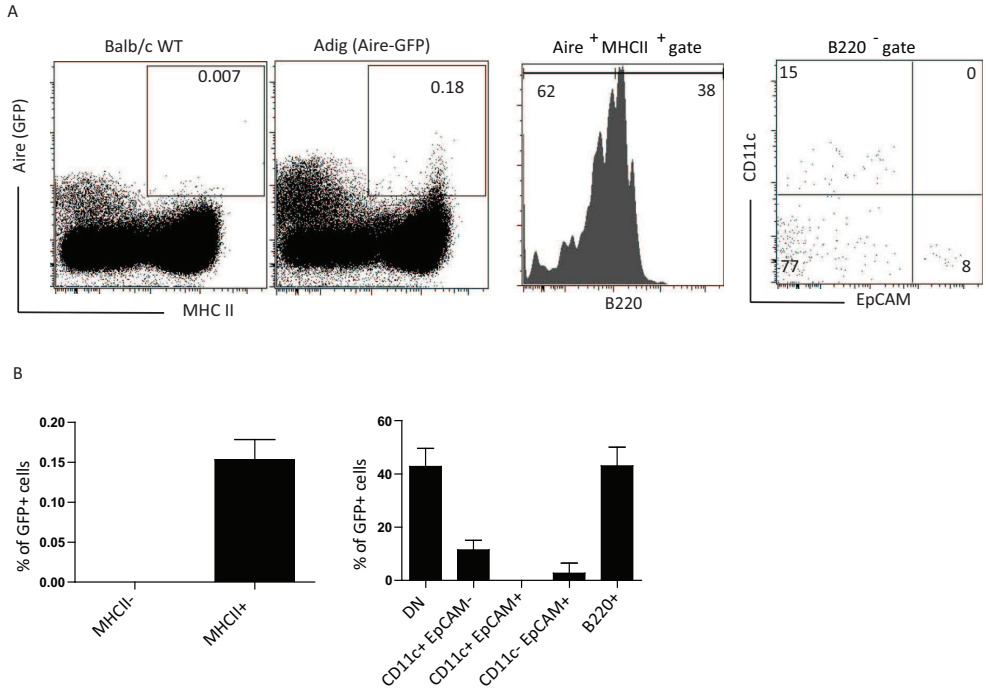


Figure 9: FACS analysis of GFP^{+} (Adig) cells in Peyer´s patches*. Cells obtained from Peyer´s patches were gated according to their GFP and MHC class II positivity and tested for the B220, EpCAM and CD11c expression. Representative dot-plots are shown* ***(A)****. The two Bar graphs (mean with SD) represent the proportion of individual populations in percentage in the whole suspension, n=3.* ***(B)****.*

4.3.4 Aire-expressing cells in the spleen

Cells were isolated from a single whole spleen, which was cut into small pieces. As illustrated in Fig. 10, the spleen contains two nearly equal populations of GFP^{+} cells which differ by the expression level of MHC class II. Almost fifty percent of GFP^{+}MHCII^{+} cells are B220^{+}, indicating a very high incidence of GFP^{+} B-cells in the spleen. B220^{-} GFP^{+}MHCII^{+} cells show similar pattern of distribution as was observed in Peyer´s patches. EpCAM^{+} CD11c^{+} cells have not been detected in the spleen (Fig. 10A, upper panels). Splenic GFP^{+}MHCII$^{neg/low}$ cells were also characterized. Notably, approximately more than half of these cells are B220^{+} cells

(Fig.10B). Almost all of the B220$^-$GFP$^+$MHCII$^{neg/low}$ cells display the DN (CD11c$^-$ EpCAM$^-$) phenotype (Fig. 10A, two lower panels).

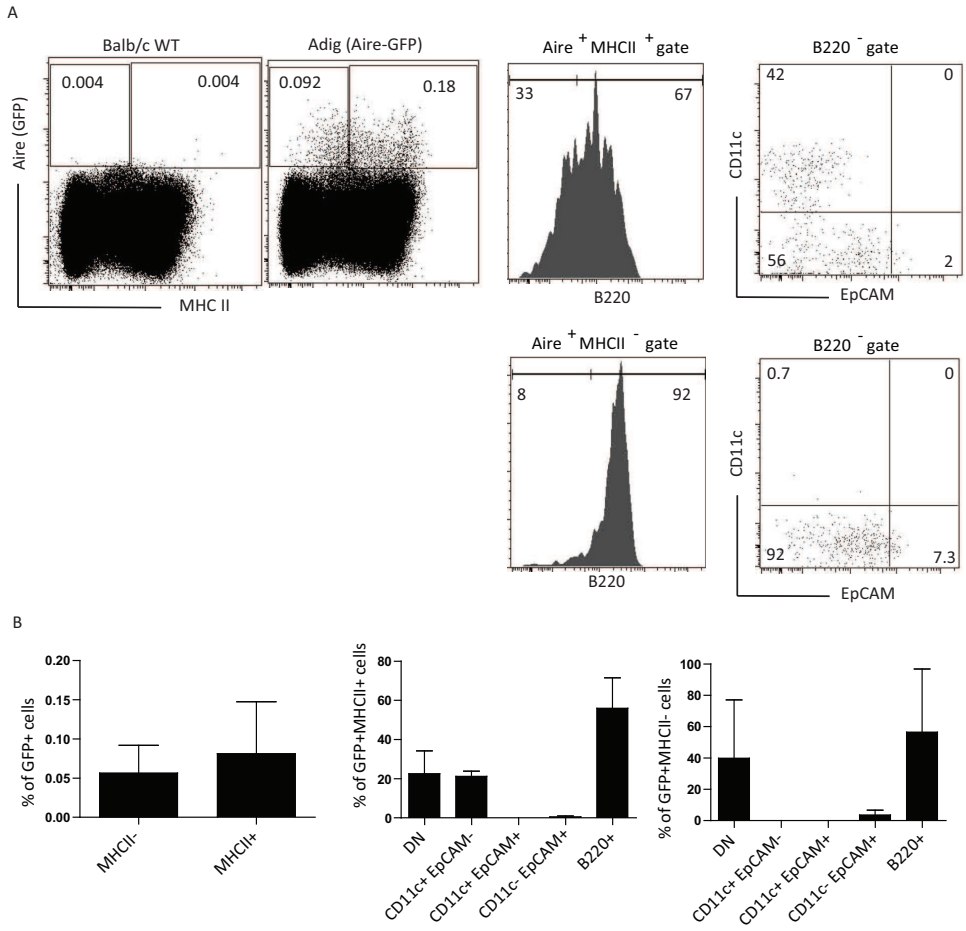


Figure 10: Phenotypic characterization of GFP$^+$ cells in the spleen. *FACS analysis of GFP$^+$ (Adig) cells in the spleen. Cells isolated from the spleen were gated according to their GFP and MHC class II positivity and tested for B220, EpCAM and CD11c expression. GFP$^+$MHCII$^+$cell-gated histograms of B220 expression and dot plots of its B220$^-$ gated CD11c and EpCAM expression are shown in the upper part of the figure. The same staining strategy is shown for GFP$^+$MHCII$^-$ cell-gated histogram in the lower part of the panel. Representative dot-plots are shown **(A)**. Bar graphs (mean with SD) represent the proportion of individual populations in percentage in the whole suspension, n=2, **(B)**.*

4.3.5 Aire-expressing cells in bone marrow

As in the case of GFP$^+$ cells in the spleen, bone marrow contains two populations of GFP$^+$ cells classified according to the MHC class II expression. The frequency of GFP$^+$ cells in the bone marrow is lower than in tissues described above. In both cases

of GFP$^+$ cells (MHCII$^+$ and MHCII$^-$), B220$^+$ cells represent a minor population of cells. As in the spleen, the DN cells are the most dominant population of GFP$^+$ cells in bone marrow (Fig. 11 A, B).

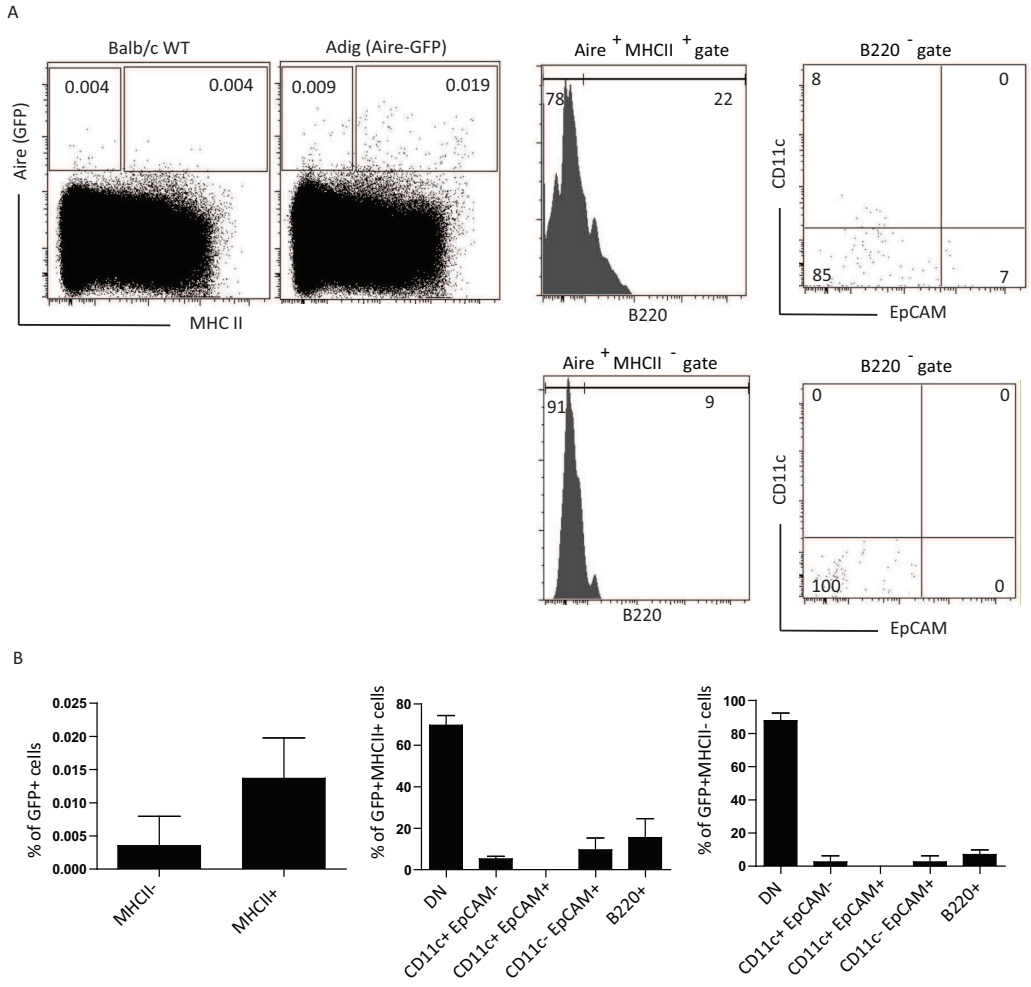


Figure 11: FACS analysis of GFP$^+$ (Adig) cells in bone marrow. *Cells isolated from bone marrow were gated according to their GFP and MHC class II positivity and tested for B220, EpCAM and CD11c expression. GFP$^+$MHCII$^+$ cells plots are shown in the upper part of the figure and plots for GFP$^+$MHCII$^-$ cells in the lower part. Representative dot-plots are shown* ***(A).*** *Bar graphs (mean with SD) represent the proportion of individual populations in percentage in the whole suspension, n=3* ***(B).***

Taken together these results describe the presence of various Aire-reporter positive populations and their abundance in lymphoid tissues. These results also show that based on their surface marker expression profiles, GFP$^+$ cells in lymphoid tissues are phenotypically heterogeneous. In all cases, except the spleen and thymus, the most

dominant population of GFP^+ cells is $MHCII^+B220^-CD11c^-EpCAM^-$ (DN). In the case of the spleen, $B220^+$ cells account for more than half of GFP^+ cells and, as expected in the thymus, the cells with mTECs characteristic are the most prominent.

4.4 Phenotypic characterization of Aire-expressing cells in lungs

Both mice models (Aire-HCO and Adig) were used to characterize Aire-expressing cells in lungs. WT littermate mice were used as negative controls. Cells were isolated from a single right lung lobe according to the protocol described in Materials and Methods section. FACS analysis was used to detect hCD2 and GFP expressing cells in this tissue and for phenotypic characterization of these cells we used anti-MHC II, anti-CD19 (Aire-HCO), anti-B220 (Adig), anti-EpCAM and anti-CD11c antibodies. The same gating strategy was used as described above for lymphoid tissues.

4.4.1 Detection of Aire-expressing cells in lungs using Aire-HCO mouse model

In the case of Aire-HCO model, lungs contain two populations of $hCD2^+$ cells classified according to the MHC class II expression. Unlikely to lymphoid tissues, the $hCD2^+MHCII^-$ population in lungs is clearly dominant as it is approximately five times more abundant than the $hCD2^+MHCII^+$ population. Almost fifty percent of $hCD2^+MHCII^+$ cells are $CD19^+$ cells, which indicate the existence of hCD2 expressing B-cells in lungs. $CD19^-$ cells show mainly the $EpCAM^-CD11c^-$ characteristics. The $hCD2^+MHCII^-$ population is negative for all tested markers (Fig. 12 A, B).

4.4.2 Aire-expressing cells in lung using Adig mouse model

Results obtained from the lungs of Aire-HCO model were compared with results derived from Adig mouse model. Both models show comparable results, differing only in the number of reporter positive cells in individual populations. Figure 13 illustrates the presence and phenotypic characterization of GFP^+ cells in the lung tissue. As in the case of Aire-HCO mice, Adig mice contain two populations of GFP^+ cells classified according to the MHC class II expression. The GFP^+MHCII^+ population shows the same pattern as observed in Aire-HCO mice, where DN and $B220^+$ cells were the most dominant populations of Aire-expressing $MHCII^+$ cells (Fig. 13 A, upper panel). The GFP^+MHCII^- cells also represent a larger population of GFP^+ cells than $MHCII^+$ cells (Fig. 13B), but compared with Aire-HCO mice, the percentage of these cells in Adig mice is diminished (Fig. 13 A, lower panel).

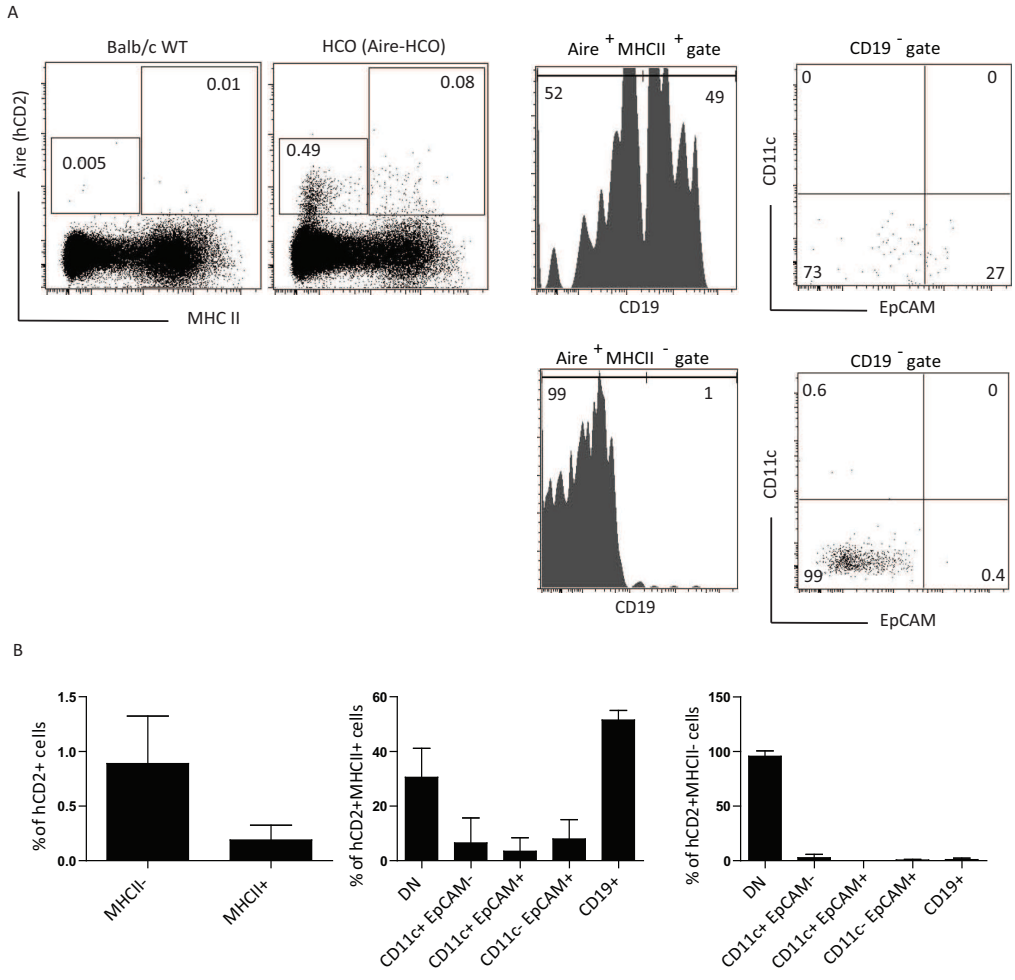


Figure 12: FACS analysis of hCD2$^+$ (Aire-HCO) cells in lungs. *Cells isolated from lungs were gated according to their hCD2 and MHC class II positivity and tested for B220, EpCAM and CD11c expression. hCD2$^+$MHCII$^+$gated plots are shown in the upper part of the figure and hCD2$^+$MHCII$^-$ gated plots in the lower part. Representative plots are shown* ***(A)****. Bar graphs (mean with SD) represent the proportion of individual populations in percentage in the whole suspension, n=3.* ***(B).***

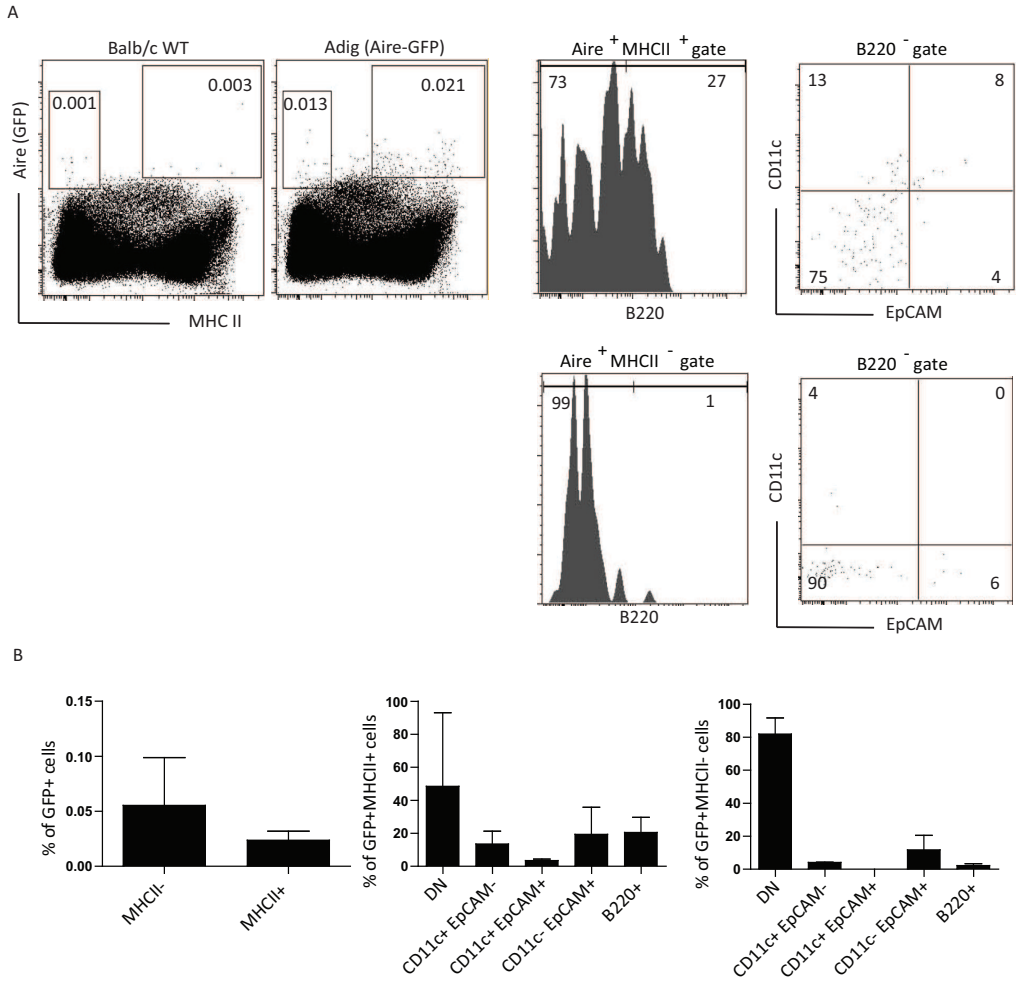


Figure 13: FACS analysis of GFP$^+$ (Adig) cells in lungs. *Cells isolated from lungs were gated according to their GFP and MHC class II positivity and tested for B220, EpCAM and CD11c expression. GFP$^+$MHCII$^+$ cells plots are shown in the upper part of the figure and GFP$^+$MHCII$^-$ cells plots in the lower part. Representative plots are shown* ***(A)****. Bar graphs (mean with SD) present the abundance of individual populations in percentage in the whole suspension, n=3* ***(B).***

Taken together these results show the existence of Aire-expressing cells in non-lymphoid tissue, specifically in lungs. Although both mice models differ in the relative numbers of cells in each population, in general, these results show that MHCII$^-$B220$^-$ (or CD19$^-$) EpCAM$^-$CD11c$^-$ population is the most dominant population in lungs.

4.5 The hematopoietic or stromal origin of Aire-expressing cells in the thymus and peripheral tissues

Two previously published studies by Gardner and colleagues showed opposite results concerning the origin of Aire-expressing cells in immune peripheral tissues. Notably, Gardner *et al.* proposed that eTACs are of hematopoietic origin (Gardner et al., 2013) which was contrary to their previous study from the year 2008 where they identified eTACs as non-hematopoietic stromal cells (Gardner et al., 2008; Gardner et al., 2013). To map the origin of these cells, FACS analysis of hematopoietic marker CD45 was used. Cells were isolated from Adig mice tissues according to the protocol described in Material and Methods section and stained by anti-CD45 antibody. In the case of the thymus, the population of $MHCII^+$ $EpCAM^+$ $CD11c^-$ cells is $CD45^-$ confirming them to be stromal mTECs. The results obtained also suggested the presence of GFP^+ hematopoietic cells in the thymus (Fig. 14).

In peripheral lymphoid tissues (Fig. 14) GFP^+ cells ($MHCII^+$ cells - green dots, $MHCII^-$ cells - red dots) showed the strict CD45 positivity, which strongly argues for the hematopoietic origin of Aire-expressing cells in lymph nodes, Peyer´s patches, spleen and bone marrow. Similar to thymus, lungs also contain two populations of GFP^+ cells in the context of CD45 expression. As illustrated in the last panel (lungs), the vast majority of the GFP^+ cells are $CD45^-$ what indicates their stromal origin (Fig. 14). These results were also confirmed by using the alternative Aire-HCO mouse model (data not shown).

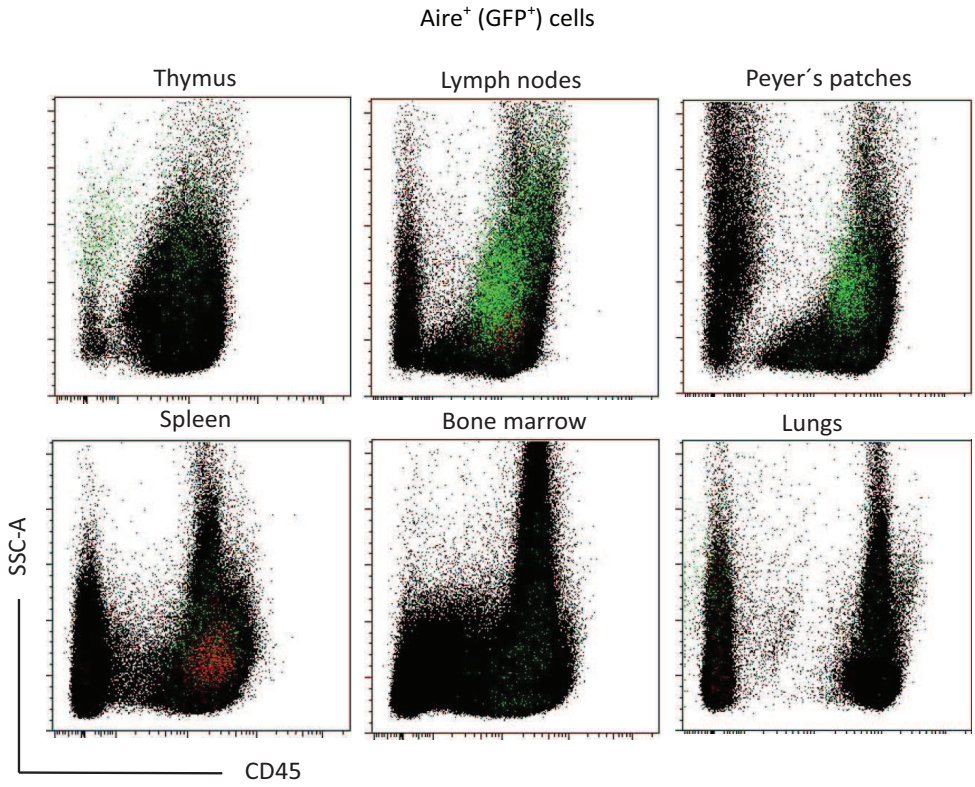


Figure 14: FACS analysis of CD45 expression on GFP$^+$ cells in the thymus and peripheral tissues. *Black dots represent all live cells in the indicated tissue. GFP$^+$MHCII$^+$ cells are represented by green dots and GFP$^+$MHCII$^-$ cells by red dots in all panels. GFP$^+$ cells in the thymus and lungs are most likely of two origins: stromal origin (CD45$^-$) and hematopoietic origin (CD45$^+$). All GFP$^+$ cells in the peripheral lymphoid tissues express CD45 marker indicating the hematopoietic origin of these cells. Representative dot plots are shown, n=3.*

These results strongly suggest that Aire-reporter expressing cell in immune periphery, described as eTACs, are of hematopoietic origin, which confirms results from the currently published study (Gardner et al., 2013). In case of thymus and lungs, Aire-reporter expressing cells are mainly of stromal origin what attests to the presence of mTECs in the thymus and describes a potentially new population of Aire-reporter expressing cells in the lung tissue.

4.6 Identification of Aire protein expression in selected tissues

Our results described above, suggests that Aire-expressing cells are present in many mouse tissues including lymphoid tissues and lungs. But the identification of these

cells was performed by FACS analysis of the transgenic construct production (hCD2 and GFP), which reflects the activation of Aire locus, but may not correlate with the expression of Aire on the protein level. Thus, we opted for alternative and more convincing methods of Aire expression detection. This included the intracellular FACS staining and immunofluorescence which detect not only the Aire expression pattern but also its localization within the cell.

4.6.1 Identification of Aire protein expressing cells using intracellular FACS staining

To identify the expression of Aire on protein level, an intracellular FACS staining was employed. These experiments were performed on Adig mouse model. Cells were isolated from two mice and according to Materials and Methods section, prepared for intracellular FACS staining. The cellular fractions were stained with anti-Aire (APC fluorochrome) antibody and correlated with the expression of GFP, which allow us to identify GFP^+Aire^+ cells. As illustrated in the figure 15, the expression of Aire protein was detected in GFP^+ cell in the thymus, lymph nodes and Peyer´s patches but not in the spleen, bone marrow and lungs (Fig. 15).

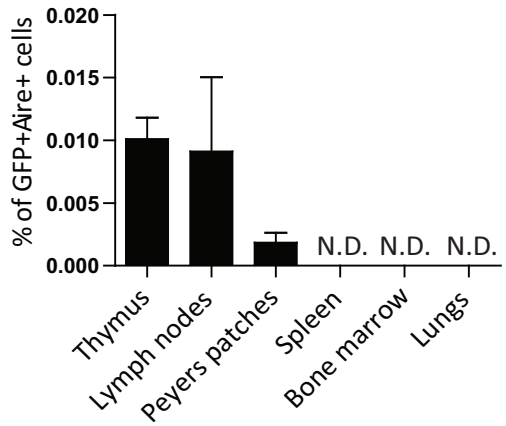


Figure 15: Intracellular FACS staining was used to identify expression of the Aire protein in selected tissues. *The isotype control staining was used as a negative control. Littermates WT mice were used for an accurate gating of GFP positivity. Cells positive for the Aire protein were identified in the thymus, lymph nodes and Peyer´s patches. In other tissues, $Aire^+$ cells were not detected (N.D.). Data are presented as bar graphs (mean with SD), n=2*

To characterize the GFP$^+$Aire-protein$^+$ cells in the thymus, lymph nodes and Peyer´s patches, the FACS analysis of EpCAM, CD11c, MHCII and B220 expression was used in combination with Aire intracellular staining. These markers were chosen in accordance with our above described results. In the thymus, only a small percentage of cells (0,011%) are double positive for GFP and Aire protein. Figure 16 shows that Aire$^+$ cells in the thymus are strictly positive for MHC class II and most of these cells are also CD11c$^-$EpCAM$^+$, which is in a full agreement with the expression of Aire protein in cells with mTECs phenotype. Importantly, these results strongly suggest that DN population is also positive for Aire protein. In case of B220$^+$ cells only the minority of these cells, compared with GFP expression, were identified as Aire$^+$ (Fig. 16).

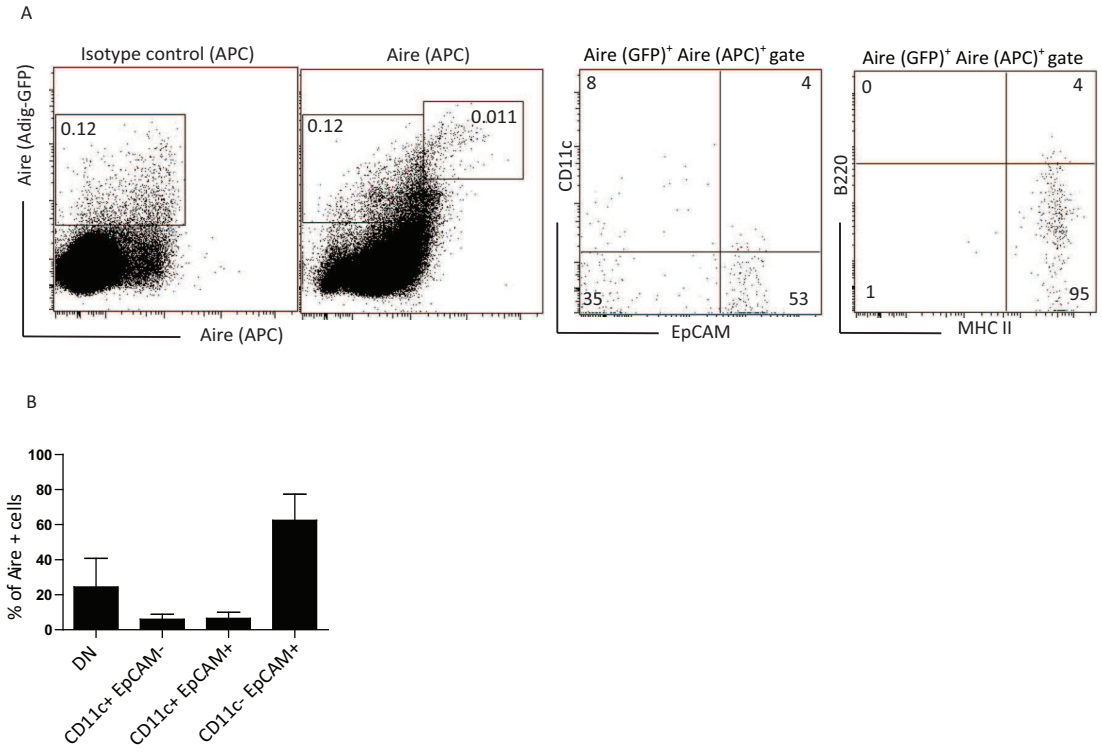


Figure 16: Aire protein expression in the thymus. *Intracellular FACS staining was used to identify the expression of the Aire protein in the thymus. The isotype control staining was used as a negative control. Littermate WT mice were used for accurate gating of GFP positivity (data not shown). Cells isolated from thymus were gated according to their GFP and Aire (APC) positivity and tested in parallel for the CD11c, EpCAM, B220 and MHCII expression. Representative plots are shown* ***(A)****. Bar graphs (mean with SD) show the abundance of each population in percentages, n=2* ***(B)****.*

The phenotypic characterization of GFP^+Aire^+ cells was performed also in lymph nodes and Peyer´s patches. As illustrated in Figure 17, lymph nodes contain a comparable percentage (0.013%) of Aire protein expressing cells as in the thymus. GFP^+Aire^+ cells in lymph nodes are almost strictly positive for MHC class II and the vast majority of them belong to the DN ($CD11c^-EpCAM^-$) population. The percentage of Aire protein expressing cells in others populations, including $B220^+$, is very low (Fig. 17).

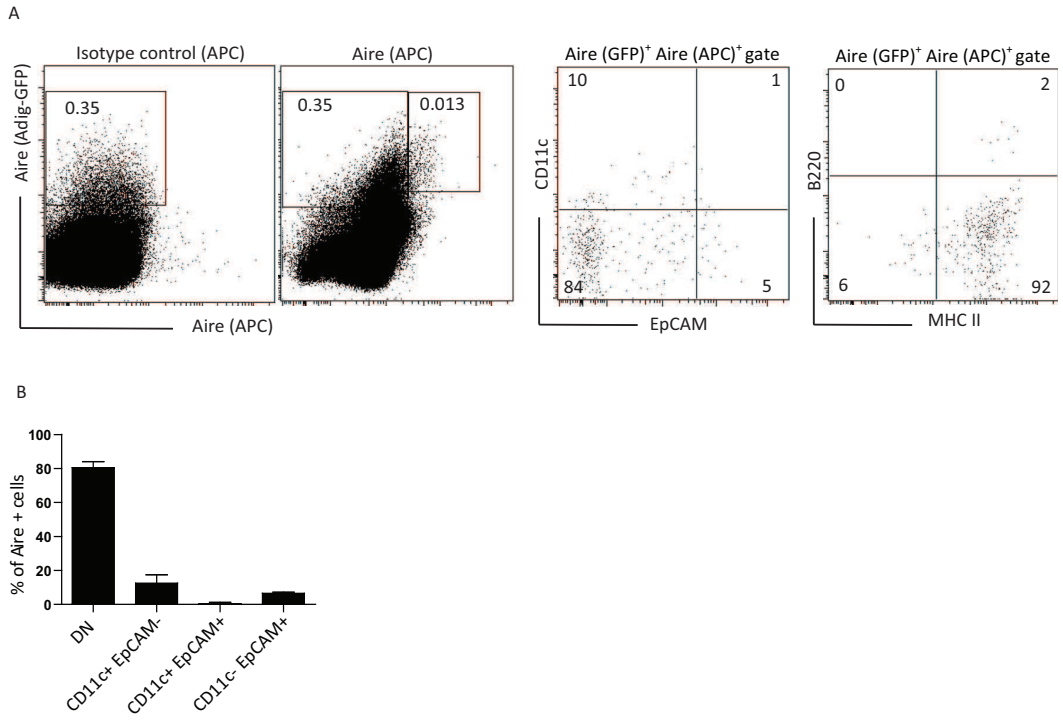


Figure 17: Aire protein expression in lymph nodes. *The expression of the Aire protein in lymph nodes was determined using the intracellular FACS staining. The isotype control staining was used as a negative control. Littermate WT mice were used for accurate gating of GFP positivity (data not shown). Cells obtained from lymph nodes were gated according to their GFP and Aire (APC) positivity and tested in parallel for the CD11c, EpCAM, B220 and MHCII expression. Representative plots are shown* ***(A)****. Bar graphs (mean with SD) show the percentage of each subpopulation, n=2* ***(B)****.*

The figure 18 illustrates the characterization of GFP^+Aire^+ cells in Peyer´s patches. Our results indicate that, compared to the thymus and lymph nodes, only a very small fraction (0,003%) of cells is of GFP^+Aire^+ phenotype. Compared with the phenotype

of GFP$^+$ cells in Peyer´s patches (Fig. 9), the Aire$^+$ cells show very similar results, which is also reflected in the percentage of B220$^+$ cells. (Fig. 18).

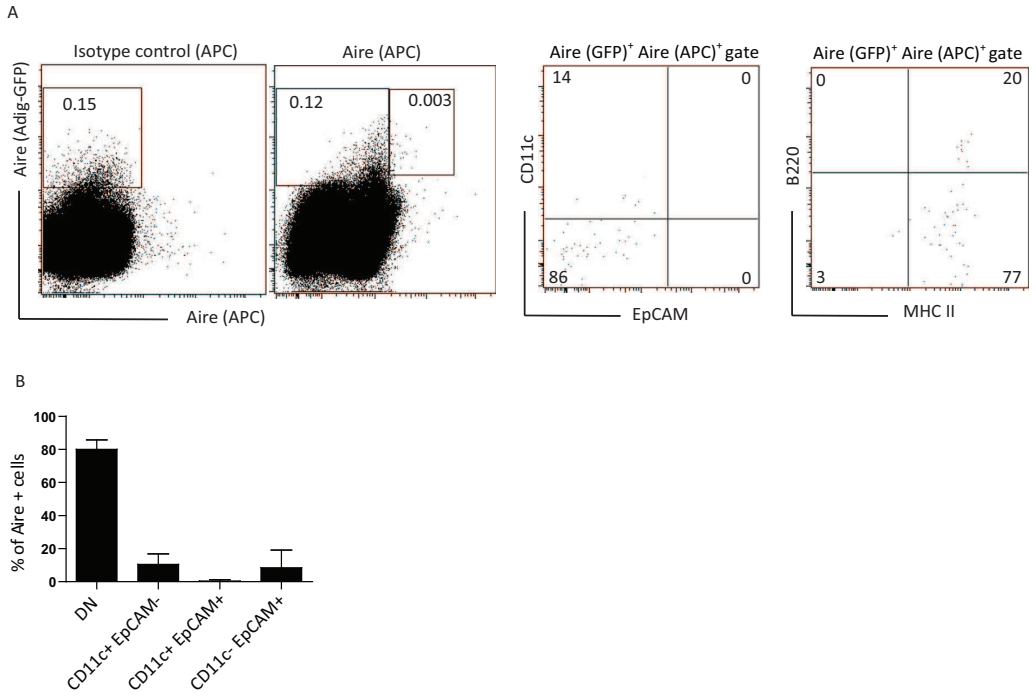


Figure 18: Aire protein expression in Peyer´s patches. *Intracellular FACS staining was used to identify the expression of the Aire protein in the Peyer´s patches. Isotype control staining was used as negative control. Littermate WT mice were used for precise adjustment of GFP positivity (data not shown). Cells isolated from Peyer´s patches were gated according to their GFP and Aire (APC) positivity and tested for the CD11c, EpCAM, B220 and MHCII expression. Representative plots are shown* ***(A)****. Bar graphs (mean with SD) present the abundance analysis depicted in percentages, n=2* ***(B)****.*

Taken together, these results demonstrate that Aire protein expressing cells can be identified in the thymus, lymph nodes and Peyer´s patches. But intracellular FACS staining failed to identify any major population of Aire$^+$ cells in the spleen, bone marrow and lungs. In case of lymph nodes and Peyer´s patches, these and results described above (Fig. 8, 9) confirmed the existence of Aire-expressing cells in immune periphery and characterized these cells as predominantly of CD45$^+$MHCII$^+$CD11c$^-$EpCAM$^-$B220$^-$ phenotype.

4.6.2 Identification of Aire protein using tissue immunohistochemistry staining

An immunohistochemistry staining was used to identify Aire-expressing cells in selected tissues. In this set of experiments, MHCII-eGFP and Balb/c WT mice models were used. The tissues were prepared as described in Materials and Methods section and stained with anti-Aire antibody (D-17 clone, Tab. 3) and DAPI. The secondary AF555-conjugated antibody (Tab. 3) was used as a negative control staining. In the case of the thymus, Peyer´s patches and the spleen, the MHCII-eGFP mouse model was used to identify the MHC class II expression. In case of lungs, the identification of Aire-expressing cells by immunohistochemistry staining depended on the genetic background of mouse model used. Notably, we failed to identify any $Aire^+$ cells in C57BL/6 mice, which prohibited us to employ the MHC-eGFP mouse model for this purpose (data not shown). Thus, the Balb/c WT mice were used to identify Aire-expressing cells in lung tissue. As illustrated in Figure 20, the expression of the Aire protein was detected in the thymus, Peyer´s patches, the spleen and also in lungs. In cases of lymphoid tissues, the Aire expressing cells also exhibited the surface expression of MHC class II, which perfectly correlates with the results from intracellular FACS staining. These results also confirmed the fact that Aire transcription regulator is predominantly detectable in the nucleus (Su et al., 2008). In this context, the figure 20C clearly demonstrates the co-staining of DAPI and Aire, which indicate the nuclear localization of Aire protein in these cells.

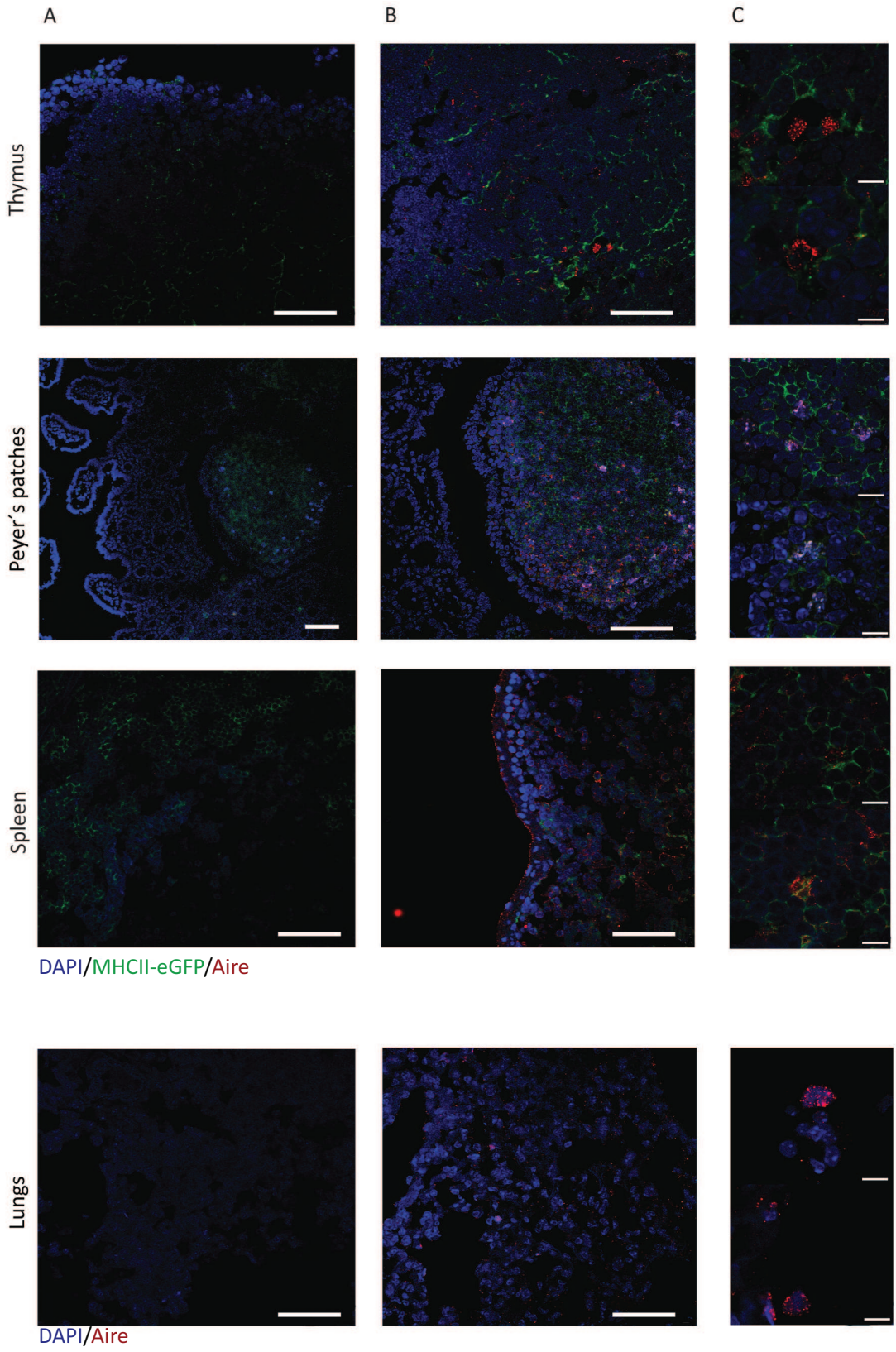


Figure 20: Identification of the Aire protein expressing cells in selected tissues. *Immunohistochemistry staining was used to identify the Aire protein presence in the thymus, Peyer´s patches, the spleen and lungs. Tissues were stained with DAPI*

*(blue) and Aire (red). The sections from the thymus, Peyer´s patches and spleen were isolated from MHCII-eGFP mice (green). AF555 secondary antibody staining was used as a negative control. Representative pictures are shown **(A)**. Aire-expressing cells are present in all these tissues **(B)**. Scale bars represent 50 µm. In all tissues, representative images of Aire-expressing cells showing nuclear localization of Aire protein were selected. Representative images of thymus, Peyer´s patches and spleen also demonstrate the co-staining of Aire and MHCII **(C)**. Scale bars represent 10 µm.*

Contrary to our results obtained by intracellular FACS staining, the immunohistochemistry staining identified Aire protein expressing cells also in the spleen and lungs. This data correlates with our results obtained on Aire-reporter expressing mouse models (Fig. 10, 12, 13).

4.6.3 Identification of Aire protein by immunofluorescent approach on sorted cells

The immunofluorescent approach was used to confirm the presence of Aire protein in Aire-reporter expressing cells from lymph nodes and Peyer´s patches. Adig mice were used for this experiment. Cells were FACS sorted according to GFP expression (described in Materials and Methods section) and stained with anti-Aire antibody (5H12 clone, Tab. 3) and DAPI. Secondary antibody staining (AF647, Tab. 3) was used as a negative control. Figure 21 shows the identification of Aire protein in GFP^+ cells from thymus, lymph nodes and Peyer´s patches. These results also demonstrated the nuclear localization of Aire protein (Fig. 21).

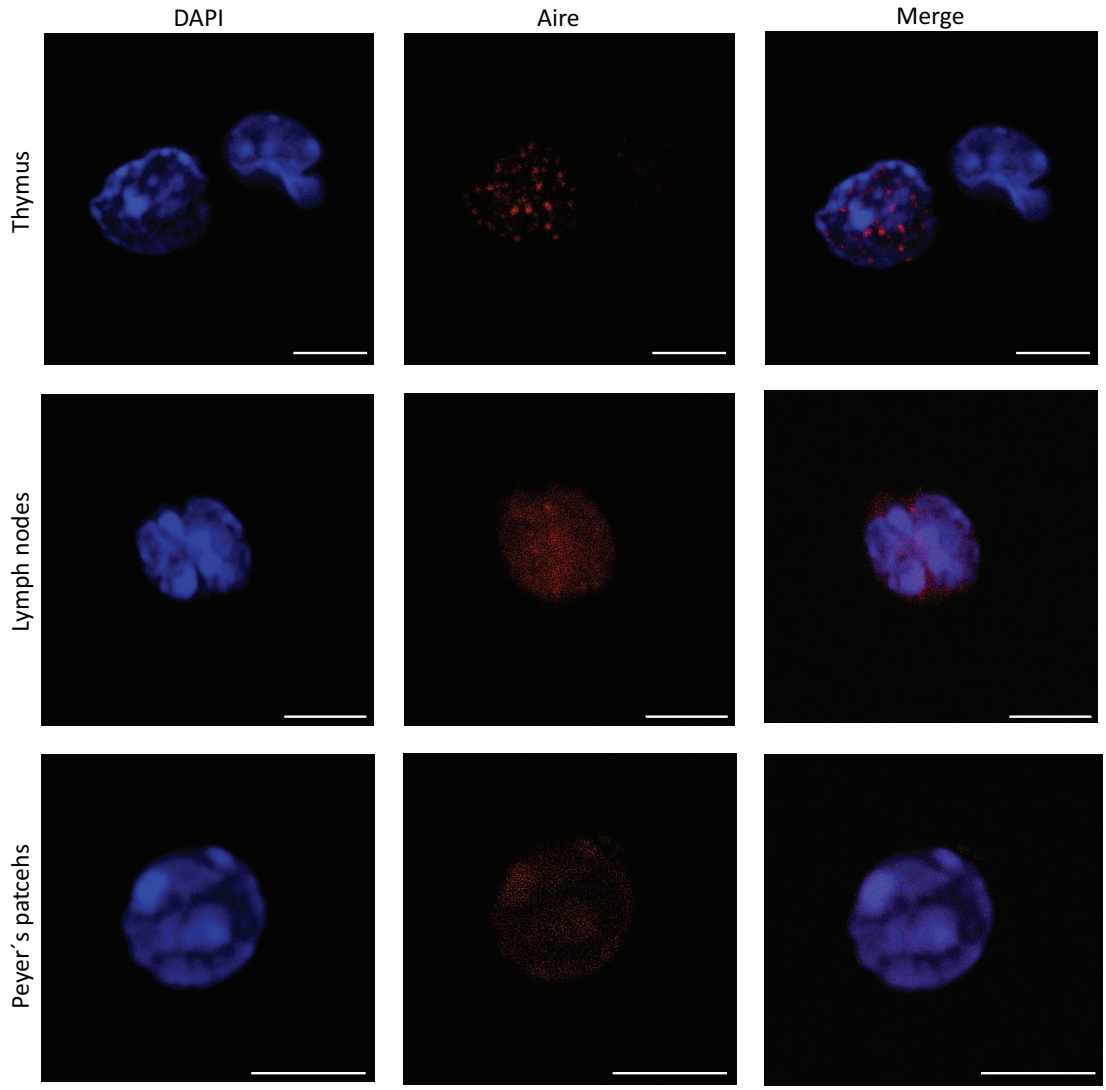


Figure 21: Identification of Aire protein expressing cells by immunofluorescent approach on FACS-sorted cells. *Cells were sorted out according to their GFP expression and stained with DAPI (blue) and Aire (red). Aire protein expression and its nuclear localization was clearly detected in the thymus, lymph nodes and Peyer´s patches-derived GFP$^+$ cells. Scale bars represent 5µm. Representative figures are shown.*

4.7 qRT-PCR analysis of TRAs expression in peripheral tissues

The previous results demonstrated the nuclear localization of Aire protein in Aire-expressing cells in the thymus and peripheral tissues. This led us to propose that Aire protein is involved in the expression of TRAs in peripheral tissues. To test this prediction, GFP$^+$ or hCD2$^+$ (in case of lungs) cells from selected tissues from Adig or

Aire-HCO mice, respectively, were sorted out and qRT-PCR analysis was used to identify the expression of selected TRAs. As mTECs express a set of Aire-dependent and Aire-independent TRAs (Derbinski et al., 2005; Derbinski et al., 2001), thymus-derived mTEC population served as a positive control. Cell sorted from Adig-Aire$^{-/-}$ mice (see Materials and Methods section for details) were used as a negative control. The Figure 22 demonstrate that GFP^{+} cells from the thymus, lymph nodes and Peyer´s patches express comparable levels of Aire mRNA. The expression of Aire mRNA was also demonstrated on hCD2^{+} cells from lungs, but compared with others tissues this expression is low. All tested tissues, including lungs, show the expression of selected TRAs (Gad65, Gad67 and Defcr24). In case of Gad67, the results confirm the Aire-independent character of this TRA (Derbinski et al., 2005). The expression of Ins2 was detected only in the thymus, lymph nodes and Peyer´s patches and expression of Muc6 was not tested in hCD2^{+} cells from lungs (Fig. 22).

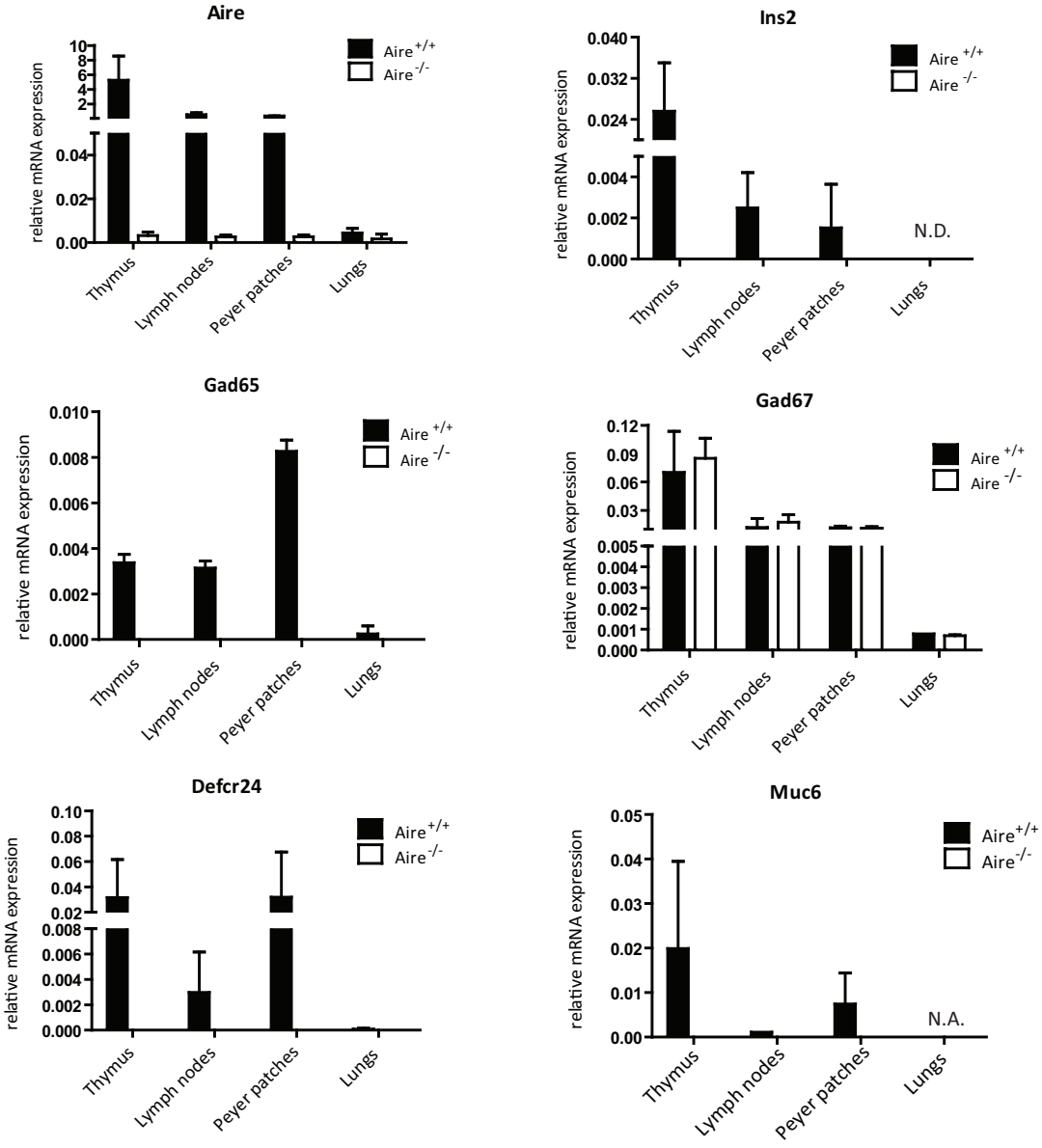


Figure 22: qRT-PCR analysis of TRAs expression in thy thymus, lymph nodes, Peyer´s patches and lung. *FACS sorting was used to isolate GFP$^+$ (Adig) or hCD2$^+$ (Aire-HCO, lungs) cells. All data were normalized to the expression of housekeeping gene Casc3 and compared with results from Aire-KO mice. qRT-PCR analysis revealed the expression of selected TRAs in thymus, lymph nodes, Peyer´s patches and lungs. N.A. – not analyzed.*

Taken together these results indicate that Aire-expressing cells in peripheral tissues are able to express at least some TRAs which are also produced by mTECs. This suggests that these cells possess the capacity to present self-antigens to CD8$^+$ or CD4$^+$ T cells in the immune periphery and promote the peripheral tolerance.

5 Discussion

Aire expression is a fundamental attribute of mTECs which lie at the basis of the mechanism of central tolerance. Its extrathymic expression, however, has been the subject of intense debates and controversies. The novelty of this work is that it clearly demonstrates for the first time the presence of Aire-expressing cells and their capacity to produce TRAs in tissues outside the thymus and lymph nodes.

Aire expressing cells were traced with the use of transgenic mouse models which allowed their identification based on the expression of a specific reporter construct. Aire-reporter expressing cells were found in all lymphoid tissues tested, such as the spleen, Peyer´s patches and bone marrow. In addition, and quite surprisingly, Aire-reporter positive cells were also found in a non-lymphoid tissue, specifically in lungs. Using different experimental settings, we also detected Aire expression on protein level in the majority of tested lymphoid tissues, which confirm the data obtained on the Aire-reporter expression in our mouse models.

The potential functional role of Aire protein-expressing cells in tolerogenic mechanisms was assessed by their ability to produce TRAs. We have shown that these cells are able to produce at least some of the well-established TRAs, which are prototypically express by mTECs. These and corollary results will be in this chapter further discussed and put into the context of current knowledge accumulated in the field of Aire-mediated tolerogenic processes in the thymus and immune periphery.

Our findings that Aire-reporter expressing cells are present in Peyer´s patches, spleen, bone marrow and in the lungs have not been so far described in the literature. Although several reports indicated the presence of Aire on mRNA or protein level in several tissues, these results are largely inconsistent when compared and cross-examined among themselves (Adamson et al., 2004; Halonen et al., 2001; Heino et al., 1999). Moreover, even though previous studies employing the very same mouse transgenic models identified Aire-reporter expressing cells in lymph nodes and partially also in the spleen, the presence of these cells in others lymphoid or non-lymphoid tissues have not been addressed (Gardner et al., 2008; Gardner et al., 2013). In this thesis, using two different transgenic mouse models, the Aire-HCO and Adig (Aire-GFP) mice, we identified Aire-expressing cells in selected tissues.

Notably, in both models we identified Aire-expressing cells in the thymus, lymph nodes, Peyer´s patches, spleen, bone marrow and lungs.

For the sake of clarity, it is necessary to emphasize that while these two transgenic mouse models provided convergent results, they differ in the percentage of reporter positive cells in some tissues. This was particularly apparent from the higher percentage of reporter positive cells in all tissues of Aire-HCO model, mainly in the spleen and lungs (Fig. 6). Several considerations can be put forward to explain this. First, the identification of Aire-reporter expressing cells in Aire-HCO transgenic model is dependent on hCD2 antibody surface staining, which might be more sensitive than the detection of intracellularly expressed GFP. Second, the genome integration site of a given construct most likely differ in each transgenic model, thus the vicinity of nearby-located repressor, activators and/or transcription factor binding sites might significantly affect the level of expression of the reporter gene.. Third, different BACs were used to prepare the recombinant constructs used for the generation of transgenic mouse. Data presented in the thesis are mainly derived from Adig model because of a simple and straightforward identification of GFP expression. Importantly, the usage of Adig model enables a direct comparison of obtained results with publically available data (Gardner et al., 2008; Gardner et al., 2013).

The phenotypic characterization of Aire-reporter expressing cells revealed the presence of five distinct subpopulations of these cells distinguished according to their B220, EpCAM and CD11c expression. Our results showed that the most dominant population of reporter positive cells in all tested lymphoid tissues are $MHCII^{+}EpCAM^{-}CD11c^{-}$ cells (DN). In the case of Aire-reporter positive cells in lymph nodes and spleen, two previous studies already attempted to describe their phenotype. In 2008, Gardner and colleagues made the phenotypic analysis of Aire-reporter positive cells using FACS approach and described them as $CD45^{-}MHCII^{+}B220^{-}CD11c^{-}EpCAM^{-}$ (Gardner et al., 2008). Later on, in 2013, the same group re-examined these experiments and came up with a significantly altered conclusion whereby Aire reporter positive cells represent a uniform population expressing CD45, MHCII, EpCAM and CD11c ($CD45^{+}MHCII^{+}EpCAM^{+}CD11c^{+}$) (Gardner et al., 2013). However, even these adjusted conclusions are inconsistent with inhere reported results obtained on the population of Aire-reporter positive cells

which are derived from the same Adig transgenic model. The substantial phenotypic differences might be explained by a different method of isolation of these cells from various tissues and by different gating strategy used for the FACS analysis. In our view, the critical role in these discrepancies might also play different genetic background of Adig mice models: C57BL/6 or NOD (Gardner et al., 2008; Gardner et al., 2013) vs. Balb/c (this thesis).

The FACS gating strategy used in this thesis revealed the existence of novel MHCII$^-$ Aire-reporter positive cells, which has not been reported so far. Based on previous studies which showed the existence of MHCII$^-$ mTECs as precursors of their mature MHCII$^+$ counterparts in the thymus (Gray et al., 2007; Metzger et al., 2013), the presence of this cell population suggests that a similar maturation pathway can take place in the immune periphery. These Aire-reporter positive MHCII$^-$ cells were identified mainly in the spleen and bone marrow. In the case of the former, the MHCII$^-$ cells showed a relatively high percentage of B220$^+$ cells, which may reflect relatively lower levels of MHCII expression by B-cells. Consistent with this notion, in all tested tissues, MHC class II low-expressing B220$^+$ cells were detected in the fraction of Aire-reporter positive cells.

The phenotypic characterization of lung tissue from Aire-HCO transgenic mice revealed that MHCII$^{-/low}$ Aire-reporter expressing cells are the dominant population among all Aire-reporter positive cells. However, this phenomenon is clearly observable only when using the Aire-HCO model. In Adig mouse model, Aire-reporter positive cells represent two nearly equal populations that differ by the expression level of MHC class II. Despite these discrepancies, the phenotype of MHCII$^{-/low}$ reporter population in lungs showed a very consistent pattern of being predominantly represented by the DN (EpCAM$^-$CD11c$^-$) cells.

The stromal/hematopoietic origin of eTACs, which was historically surrounded by a controversy (Gardner et al., 2008; Gardner et al., 2013) was also a subject of our investigation. It is of note that Gardner *et al.* studied the origin of eTACs by CD45 staining and bone marrow chimeras experiments. In the case of FACS CD45 staining, two populations of Aire-reporter expressing cells in lymph nodes (both positive for MHC class II): CD45$^-$ EpCAM$^+$ and CD45$^+$ CD11c$^+$ cells were shown. The presence and functional role of the latter, the population of hematopoietic origin,

was largely ignored and left out of the discussion. The bone marrow chimeras experiments provided evidence for stromal origin of the former $CD45^{-}$ $EpCAM^{+}$ population (Gardner et al., 2008). Contrary to this first study, the same group published the new study where they described eTACs as cells belonging to an unusual class of APCs of hematopoietic origin. The description was based on FACS analysis of CD45 expression which revealed that eTACs from lymph nodes are mainly $CD45^{int/+}$. The new bone marrow chimeras with the use of the same experimental setting as in their first study then confirmed the hematopoietic character of these cells (Gardner et al., 2013). It remains to believe that the discrepancies related to the cellular origin of eTACs between these two studies are due to the different FACS gating, interpretation of CD45 positivity or negativity and/or following the preconceived idea related to the parallel between mTECs and eTACs.

We tested the stormal or hematopoietic origin of Aire-reporter positive cells in all selected tissues. Our results demonstrate the expression of CD45 by Aire-reporter positive cells (Fig. 14) and thus confirmed the hematopoietic origin of Aire-reporter expressing cells in peripheral lymphoid tissues. It is of note that converging results pointing to the hematopoietic origin of eTACs were also obtained in our bone marrow chimeras experiment (Dobeš et.al, unpublished data).

An exception to this rule was the lung tissue, where CD45 staining revealed the stromal as well as hematopoietic character of Aire-reporter positive cells. According to our preliminary data, the population of $MHCII^{-}$ Aire-reporter positive cells is negative for CD45 marker. This fact also argues for the stromal character of this population. If confirmed, this population would represent so far not formally described population of peripheral Aire-reporter expressing cells. To verify their origin, the bone marrow chimeras experiment will be performed in the future.

Absolutely critical for the advancement of research in the field of peripheral tolerance is the identification of cells that express *bona fide* Aire on protein level and display other essential characteristics of tolerance-inducing cells. Traditionally, the identification of Aire expression on protein level in the peripheral tissues has been performed by the immunohistochemistry staining (Adamson et al., 2004; Halonen et al., 2001). These studies used the polyclonal anti-Aire antibody but did not document the nuclear localization of Aire protein inside the Aire-expressing cells. Moreover,

these studies failed to correlate the presence of Aire protein in peripheral tissues with phenotypic characteristics of Aire-expressing cells. Gardner and colleagues were first who tested the Aire protein expression in reporter positive cells. Using the immunofluorescent approach, they identified the expression of Aire protein in Aire-GFP^{+} cells, but they also did not show the nuclear localization or phenotypic characteristic of Aire-protein expressing cells. Moreover, it seemed that not all Aire-GFP^{+} cells were expressing Aire-protein (Gardner et al., 2008).

Then, Hubert and colleagues used intracellular FACS staining with newly generated monoclonal anti-Aire antibody and found out that Aire-protein expression is strictly restricted to mTECs. Importantly, they failed to identify any Aire protein expression in the stromal cells presented in the spleen and lymph nodes (Hubert et al., 2008). For these reasons the expression of Aire protein in immune peripheral tissues became highly controversial issue. Our results using intracellular FACS staining described the Aire protein expression within the Aire-reporter positive population in lymph nodes and Peyer´s patches. These data showed however that Aire protein is only detectable in the MHCII^{+}EpCAM^{-}CD11c^{-} (DN) population (Fig. 17, 18). Thus, eTAC population, as defined by Gardner *et al.* (Gardner et al., 2013), at least in our hands, does not express sufficient levels of Aire protein which could be readily detectable by FACS.

The immunohistochemistry staining confirmed the Aire protein expression in selected peripheral tissues and, in addition, using the co-staining with DAPI, revealed the proper nuclear localization of Aire. To confirm this result on a single cell level, we decided to sort these cells out and perform immunofluorescent staining with anti-Aire monoclonal antibody. A clear nuclear localization of Aire was confirmed only in the thymus, lymph nodes and Peyer´s patches. One of the main objectives of this experiment was also to show the co-staining of GFP (Adig reporter) and Aire protein. But because of the weak GFP signal in permeabilized and fixed cells, we were not able to demonstrate this. This experiment will be repeated with anti-GFP antibody staining. In the case of the spleen, bone marrow and lungs, we failed to sort out a sufficient number of cells from Adig mice to perform the immunofluorescent staining. Additional methods using combination of FACS and microscopy should be used in order to verify the nuclear localization of Aire in cells

derived from these tissues. Also, the staining of Aire$^{-/-}$ and WT mice should be used to unambiguously identify Aire expressing cells in selected tissues.

The previously described publication from Gardner and colleagues also compared the expression of TRAs in mTECs and eTACs using microarray analysis and qRT-PCR approach. They revealed that eTACs express a different set of Aire regulated TRAs in comparison to that of mTECs (Gardner et al., 2008). Our result obtained by qRT-PCR analysis revealed that Aire-reporter positive cells from thymus, lymph nodes and Peyer´s patches express the same or at least largely overlapping set of chosen TRAs (Fig. 20). To confirm this conclusion, more than five TRAs have to be tested. In case of Aire-reporter positive cells from lung, the expression level of chosen TRAs was much lower than that in lymphoid tissues. It seems that the proportion of Aire-reporter expressing cells in lungs which also produce Aire protein is very small and thus, the relative production of TRAs in sorted GFP^{+} lung cells is miniscule compared to other peripheral Aire^{+} lymphoid tissues. It is clear that there is a need to characterized Aire protein expressing cells in lung tissue more accurately. Nevertheless, our experiments identified for the very first time the population of Aire and TRAs expressing cells in the lungs. Interestingly, these cells, contrary to other Aire-reporter positive cell in immune periphery, are of stromal origin.

6 Conclusions

The thesis describes the presence of Aire-reporter expressing cells in the thymus, secondary lymphoid organs (lymph nodes, Peyer´s patches and spleen), bone marrow and even in lungs. FACS analysis of surface expressed markers revealed five populations of Aire-reporter expressing cells divided according to B220, EpCAM and CD11c expression. The DN ($EpCAM^{-}CD11c^{-}$) population was shown to be the dominant population of reporter positive cells in immune peripheral tissues. Anti-CD45 staining confirmed that majority of Aire-reporter expressing cells is of hematopoietic origin with the exception of unique Aire-reporter expressing population in the lung tissue, which showed the stromal origin. These findings thus, described a new population in non-lymphoid tissue of Aire-reporter expressing cells. The intracellular FACS staining and/or immunofluorescent approach confirmed the Aire expression on protein level in lymph nodes, Peyer's patches, spleen and lungs where its nuclear localization correlated with their capacity to express Aire-dependent TRAs.

Together, these results brought a new perspective to the research revolving around Aire-expressing cells in immune periphery a led to a more detail phenotypic and functional description of Aire-expressing cells. All these findings suggest that mouse lymphoid as well as non-lymphoid peripheral tissues harbour the population of Aire-expressing cells with the ability to present TRAs. If these TRAs are presented in the context of MHC molecules they can potentially promote peripheral tolerance and protect against autoimmunity. This data expands the potential importance of Aire protein expression in immune periphery and contributes to a deeper understanding of cellular and molecular processes maintaining the peripheral immune tolerance.

7 References

Abramson, J., Giraud, M., Benoist, C., and Mathis, D. (2010). Aire's partners in the molecular control of immunological tolerance. Cell *140*, 123-135.

Adamson, K.A., Pearce, S.H., Lamb, J.R., Seckl, J.R., and Howie, S.E. (2004). A comparative study of mRNA and protein expression of the autoimmune regulator gene (Aire) in embryonic and adult murine tissues. J Pathol *202*, 180-187.

Ahonen, P., Myllarniemi, S., Sipila, I., and Perheentupa, J. (1990). Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. N Engl J Med *322*, 1829-1836.

Akashi, K., Richie, L.I., Miyamoto, T., Carr, W.H., and Weissman, I.L. (2000). B lymphopoiesis in the thymus. J Immunol *164*, 5221-5226.

Alimohammadi, M., Björklund, P., Hallgren, A., Pöntynen, N., Szinnai, G., Shikama, N., Keller, M.P., Ekwall, O., Kinkel, S.A., Husebye, E.S., *et al.* (2008). Autoimmune polyendocrine syndrome type 1 and NALP5, a parathyroid autoantigen. N Engl J Med *358*, 1018-1028.

Anderson, G., and Jenkinson, E.J. (2001). Lymphostromal interactions in thymic development and function. Nat Rev Immunol *1*, 31-40.

Anderson, M.S., Venanzi, E.S., Chen, Z., Berzins, S.P., Benoist, C., and Mathis, D. (2005). The cellular mechanism of Aire control of T cell tolerance. Immunity *23*, 227-239.

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., *et al.* (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395-1401.

Arstila, T.P., and Jarva, H. (2013). Human APECED; a Sick Thymus Syndrome? Front Immunol *4*, 313.

Aschenbrenner, K., D'Cruz, L.M., Vollmann, E.H., Hinterberger, M., Emmerich, J., Swee, L.K., Rolink, A., and Klein, L. (2007). Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. Nat Immunol *8*, 351-358.

Bains, I., van Santen, H.M., Seddon, B., and Yates, A.J. (2013). Models of self-peptide sampling by developing T cells identify candidate mechanisms of thymic selection. PLoS Comput Biol *9*, e1003102.

Baldwin, K.K., Trenchak, B.P., Altman, J.D., and Davis, M.M. (1999). Negative selection of T cells occurs throughout thymic development. J Immunol *163*, 689-698.

Blechschmidt, K., Schweiger, M., Wertz, K., Poulson, R., Christensen, H.M., Rosenthal, A., Lehrach, H., and Yaspo, M.L. (1999). The mouse Aire gene: comparative genomic sequencing, gene organization, and expression. Genome Res *9*, 158-166.

Boehm, T. (2009). The adaptive phenotype of cortical thymic epithelial cells. Eur J Immunol *39*, 944-947.

Boes, M., Cerny, J., Massol, R., Op den Brouw, M., Kirchhausen, T., Chen, J., and Ploegh, H.L. (2002). T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. Nature *418*, 983-988.

Bonasio, R., Scimone, M.L., Schaerli, P., Grabie, N., Lichtman, A.H., and von Andrian, U.H. (2006). Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. Nat Immunol *7*, 1092-1100.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med *198*, 1875-1886.

Cohen, J.N., Guidi, C.J., Tewalt, E.F., Qiao, H., Rouhani, S.J., Ruddell, A., Farr, A.G., Tung, K.S., and Engelhard, V.H. (2010). Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. J Exp Med *207*, 681-688.

Consortium, F.-G.A. (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet *17*, 399-403.

Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., *et al.* (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med *204*, 1257-1265.

Derbinski, J., Gäbler, J., Brors, B., Tierling, S., Jonnakuty, S., Hergenhahn, M., Peltonen, L., Walter, J., and Kyewski, B. (2005). Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. J Exp Med *202*, 33-45.

Derbinski, J., Pinto, S., Rösch, S., Hexel, K., and Kyewski, B. (2008). Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism. Proc Natl Acad Sci U S A *105*, 657-662.

Derbinski, J., Schulte, A., Kyewski, B., and Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat Immunol *2*, 1032-1039.

DeVoss, J., Hou, Y., Johannes, K., Lu, W., Liou, G.I., Rinn, J., Chang, H., Caspi, R.R., Caspi, R., Fong, L., *et al.* (2006). Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. J Exp Med *203*, 2727-2735.

DeVoss, J.J., LeClair, N.P., Hou, Y., Grewal, N.K., Johannes, K.P., Lu, W., Yang, T., Meagher, C., Fong, L., Strauss, E.C., *et al.* (2010). An autoimmune response to odorant binding protein 1a is associated with dry eye in the Aire-deficient mouse. J Immunol *184*, 4236-4246.

Dubrot, J., Duraes, F.V., Potin, L., Capotosti, F., Brighouse, D., Suter, T., LeibundGut-Landmann, S., Garbi, N., Reith, W., Swartz, M.A., *et al.* (2014). Lymph

node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4+ T cell tolerance. J Exp Med *211*, 1153-1166.

Enouz, S., Carrié, L., Merkler, D., Bevan, M.J., and Zehn, D. (2012). Autoreactive T cells bypass negative selection and respond to self-antigen stimulation during infection. J Exp Med *209*, 1769-1779.

Ferguson, B.J., Alexander, C., Rossi, S.W., Liiv, I., Rebane, A., Worth, C.L., Wong, J., Laan, M., Peterson, P., Jenkinson, E.J., *et al.* (2008). AIRE's CARD revealed, a new structure for central tolerance provokes transcriptional plasticity. J Biol Chem *283*, 1723-1731.

Fletcher, A.L., Lukacs-Kornek, V., Reynoso, E.D., Pinner, S.E., Bellemare-Pelletier, A., Curry, M.S., Collier, A.R., Boyd, R.L., and Turley, S.J. (2010). Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. J Exp Med *207*, 689-697.

Florea, B.I., Verdoes, M., Li, N., van der Linden, W.A., Geurink, P.P., van den Elst, H., Hofmann, T., de Ru, A., van Veelen, P.A., Tanaka, K., *et al.* (2010). Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit beta5t. Chem Biol *17*, 795-801.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005). A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol *6*, 1142-1151.

Forrester, J.V., Xu, H., Lambe, T., and Cornall, R. (2008). Immune privilege or privileged immunity? Mucosal Immunol *1*, 372-381.

Frommer, F., and Waisman, A. (2010). B cells participate in thymic negative selection of murine auto-reactive CD4+ T cells. PLoS One *5*, e15372.

Gardner, J.M., Devoss, J.J., Friedman, R.S., Wong, D.J., Tan, Y.X., Zhou, X., Johannes, K.P., Su, M.A., Chang, H.Y., Krummel, M.F., *et al.* (2008). Deletional tolerance mediated by extrathymic Aire-expressing cells. Science *321*, 843-847.

Gardner, J.M., Metzger, T.C., McMahon, E.J., Au-Yeung, B.B., Krawisz, A.K., Lu, W., Price, J.D., Johannes, K.P., Satpathy, A.T., Murphy, K.M., *et al.* (2013). Extrathymic Aire-expressing cells are a distinct bone marrow-derived population that induce functional inactivation of $CD4^+$ T cells. Immunity *39*, 560-572.

Gibson, T.J., Ramu, C., Gemünd, C., and Aasland, R. (1998). The APECED polyglandular autoimmune syndrome protein, AIRE-1, contains the SAND domain and is probably a transcription factor. Trends Biochem Sci *23*, 242-244.

Giraud, M., Jmari, N., Du, L., Carallis, F., Nieland, T.J., Perez-Campo, F.M., Bensaude, O., Root, D.E., Hacohen, N., Mathis, D., *et al.* (2014). An RNAi screen for Aire cofactors reveals a role for Hnrnpl in polymerase release and Aire-activated ectopic transcription. Proc Natl Acad Sci U S A *111*, 1491-1496.

Giraud, M., Yoshida, H., Abramson, J., Rahl, P.B., Young, R.A., Mathis, D., and Benoist, C. (2012). Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells. Proc Natl Acad Sci U S A *109*, 535-540.

Gray, D., Abramson, J., Benoist, C., and Mathis, D. (2007). Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. J Exp Med *204*, 2521-2528.

Greter, M., Heppner, F.L., Lemos, M.P., Odermatt, B.M., Goebels, N., Laufer, T., Noelle, R.J., and Becher, B. (2005). Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. Nat Med *11*, 328-334.

Grupillo, M., Gualtierotti, G., He, J., Sisino, G., Bottino, R., Rudert, W.A., Trucco, M., and Fan, Y. (2012). Essential roles of insulin expression in Aire+ tolerogenic dendritic cells in maintaining peripheral self-tolerance of islet β-cells. Cell Immunol *273*, 115-123.

Guerder, S., Joncker, N., Mahiddine, K., and Serre, L. (2013). Dendritic cells in tolerance and autoimmune diabetes. Curr Opin Immunol *25*, 670-675.

Guerder, S., Viret, C., Luche, H., Ardouin, L., and Malissen, B. (2012). Differential processing of self-antigens by subsets of thymic stromal cells. Curr Opin Immunol *24*, 99-104.

Hadeiba, H., Lahl, K., Edalati, A., Oderup, C., Habtezion, A., Pachynski, R., Nguyen, L., Ghodsi, A., Adler, S., and Butcher, E.C. (2012). Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. Immunity *36*, 438-450.

Halonen, M., Pelto-Huikko, M., Eskelin, P., Peltonen, L., Ulmanen, I., and Kolmer, M. (2001). Subcellular location and expression pattern of autoimmune regulator (Aire), the mouse orthologue for human gene defective in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). J Histochem Cytochem *49*, 197-208.

Hanahan, D. (1998). Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity. Curr Opin Immunol *10*, 656-662.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M., and Nussenzweig, M.C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. J Exp Med *194*, 769-779.

Heino, M., Peterson, P., Kudoh, J., Nagamine, K., Lagerstedt, A., Ovod, V., Ranki, A., Rantala, I., Nieminen, M., Tuukkanen, J., *et al.* (1999). Autoimmune regulator is expressed in the cells regulating immune tolerance in thymus medulla. Biochem Biophys Res Commun *257*, 821-825.

Hofmann, K., Bucher, P., and Tschopp, J. (1997). The CARD domain: a new apoptotic signalling motif. Trends Biochem Sci *22*, 155-156.

Hogquist, K.A., Baldwin, T.A., and Jameson, S.C. (2005). Central tolerance: learning self-control in the thymus. Nat Rev Immunol *5*, 772-782.

Huang, C.T., Workman, C.J., Flies, D., Pan, X., Marson, A.L., Zhou, G., Hipkiss, E.L., Ravi, S., Kowalski, J., Levitsky, H.I., *et al.* (2004). Role of LAG-3 in regulatory T cells. Immunity *21*, 503-513.

Hubert, F.X., Kinkel, S.A., Webster, K.E., Cannon, P., Crewther, P.E., Proeitto, A.I., Wu, L., Heath, W.R., and Scott, H.S. (2008). A specific anti-Aire antibody reveals

aire expression is restricted to medullary thymic epithelial cells and not expressed in periphery. J Immunol *180*, 3824-3832.

Husebye, E.S., and Anderson, M.S. (2010). Autoimmune polyendocrine syndromes: clues to type 1 diabetes pathogenesis. Immunity *32*, 479-487.

Husebye, E.S., Perheentupa, J., Rautemaa, R., and Kampe, O. (2009). Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. J Intern Med *265*, 514-529.

Ilmarinen, T., Eskelin, P., Halonen, M., Rüppell, T., Kilpikari, R., Torres, G.D., Kangas, H., and Ulmanen, I. (2005). Functional analysis of SAND mutations in AIRE supports dominant inheritance of the G228W mutation. Hum Mutat *26*, 322-331.

Ilmarinen, T., Kangas, H., Kytömaa, T., Eskelin, P., Saharinen, J., Seeler, J.S., Tanhuanpää, K., Chan, F.Y., Slattery, R.M., Alakurtti, K., *et al.* (2008). Functional interaction of AIRE with PIAS1 in transcriptional regulation. Mol Immunol *45*, 1847-1862.

Isaacson, P.G., Norton, A.J., and Addis, B.J. (1987). The human thymus contains a novel population of B lymphocytes. Lancet *2*, 1488-1491.

Johnnidis, J.B., Venanzi, E.S., Taxman, D.J., Ting, J.P., Benoist, C.O., and Mathis, D.J. (2005). Chromosomal clustering of genes controlled by the aire transcription factor. Proc Natl Acad Sci U S A *102*, 7233-7238.

Jolicoeur, C., Hanahan, D., and Smith, K.M. (1994). T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. Proc Natl Acad Sci U S A *91*, 6707-6711.

Josefowicz, S.Z., Lu, L.F., and Rudensky, A.Y. (2012). Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol *30*, 531-564.

Kim, J.M., Rasmussen, J.P., and Rudensky, A.Y. (2007). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. Nat Immunol *8*, 191-197.

Kisand, K., Bøe Wolff, A.S., Podkrajsek, K.T., Tserel, L., Link, M., Kisand, K.V., Ersvaer, E., Perheentupa, J., Erichsen, M.M., Bratanic, N., *et al.* (2010). Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. J Exp Med *207*, 299-308.

Klein, L., Hinterberger, M., Wirnsberger, G., and Kyewski, B. (2009). Antigen presentation in the thymus for positive selection and central tolerance induction. Nat Rev Immunol *9*, 833-844.

Klein, L., Kyewski, B., Allen, P.M., and Hogquist, K.A. (2014). Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat Rev Immunol *14*, 377-391.

Klein, L., Roettinger, B., and Kyewski, B. (2001). Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. Eur J Immunol *31*, 2476-2486.

Koble, C., and Kyewski, B. (2009). The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. J Exp Med *206*, 1505-1513.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M.C., and von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol *6*, 1219-1227.

Kurts, C., Kosaka, H., Carbone, F.R., Miller, J.F., and Heath, W.R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. J Exp Med *186*, 239-245.

Kwok, R.P., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bächinger, H.P., Brennan, R.G., Roberts, S.G., Green, M.R., and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature *370*, 223-226.

Kyewski, B., Derbinski, J., Gotter, J., and Klein, L. (2002). Promiscuous gene expression and central T-cell tolerance: more than meets the eye. Trends Immunol *23*, 364-371.

Kyewski, B., and Klein, L. (2006). A central role for central tolerance. Annu Rev Immunol *24*, 571-606.

Lee, J.W., Epardaud, M., Sun, J., Becker, J.E., Cheng, A.C., Yonekura, A.R., Heath, J.K., and Turley, S.J. (2007). Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. Nat Immunol *8*, 181-190.

Liiv, I., Haljasorg, U., Kisand, K., Maslovskaja, J., Laan, M., and Peterson, P. (2012). AIRE-induced apoptosis is associated with nuclear translocation of stress sensor protein GAPDH. Biochem Biophys Res Commun *423*, 32-37.

Liiv, I., Rebane, A., Org, T., Saare, M., Maslovskaja, J., Kisand, K., Juronen, E., Valmu, L., Bottomley, M.J., Kalkkinen, N., *et al.* (2008). DNA-PK contributes to the phosphorylation of AIRE: importance in transcriptional activity. Biochim Biophys Acta *1783*, 74-83.

Lindmark, E., Chen, Y., Georgoudaki, A.M., Dudziak, D., Lindh, E., Adams, W.C., Loré, K., Winqvist, O., Chambers, B.J., and Karlsson, M.C. (2013). AIRE expressing marginal zone dendritic cells balances adaptive immunity and T-follicular helper cell recruitment. J Autoimmun *42*, 62-70.

Linsk, R., Gottesman, M., and Pernis, B. (1989). Are tissues a patch quilt of ectopic gene expression? Science *246*, 261.

Liston, A., Lesage, S., Wilson, J., Peltonen, L., and Goodnow, C.C. (2003). Aire regulates negative selection of organ-specific T cells. Nat Immunol *4*, 350-354.

Liu, G.Y., Fairchild, P.J., Smith, R.M., Prowle, J.R., Kioussis, D., and Wraith, D.C. (1995). Low avidity recognition of self-antigen by T cells permits escape from central tolerance. Immunity *3*, 407-415.

Liu, K., Iyoda, T., Saternus, M., Kimura, Y., Inaba, K., and Steinman, R.M. (2002). Immune tolerance after delivery of dying cells to dendritic cells in situ. J Exp Med *196*, 1091-1097.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Lukacs-Kornek, V., Malhotra, D., Fletcher, A.L., Acton, S.E., Elpek, K.G., Tayalia, P., Collier, A.R., and Turley, S.J. (2011). Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. Nat Immunol *12*, 1096-1104.

Magnusson, F.C., Liblau, R.S., von Boehmer, H., Pittet, M.J., Lee, J.W., Turley, S.J., and Khazaie, K. (2008). Direct presentation of antigen by lymph node stromal cells protects against CD8 T-cell-mediated intestinal autoimmunity. Gastroenterology *134*, 1028-1037.

Malchow, S., Leventhal, D.S., Nishi, S., Fischer, B.I., Shen, L., Paner, G.P., Amit, A.S., Kang, C., Geddes, J.E., Allison, J.P., *et al.* (2013). Aire-dependent thymic development of tumor-associated regulatory T cells. Science *339*, 1219-1224.

Malhotra, D., Fletcher, A.L., Astarita, J., Lukacs-Kornek, V., Tayalia, P., Gonzalez, S.F., Elpek, K.G., Chang, S.K., Knoblich, K., Hemler, M.E., *et al.* (2012). Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. Nat Immunol *13*, 499-510.

Malhotra, D., Fletcher, A.L., and Turley, S.J. (2013). Stromal and hematopoietic cells in secondary lymphoid organs: partners in immunity. Immunol Rev *251*, 160-176.

Masopust, D., Vezys, V., Usherwood, E.J., Cauley, L.S., Olson, S., Marzo, A.L., Ward, R.L., Woodland, D.L., and Lefrançois, L. (2004). Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. J Immunol *172*, 4875-4882.

Mathis, D., and Benoist, C. (2009). Aire. Annu Rev Immunol *27*, 287-312.

Meloni, A., Fiorillo, E., Corda, D., Incani, F., Serra, M.L., Contini, A., Cao, A., and Rosatelli, M.C. (2010). DAXX is a new AIRE-interacting protein. J Biol Chem *285*, 13012-13021.

Meloni, A., Furcas, M., Cetani, F., Marcocci, C., Falorni, A., Perniola, R., Pura, M., Bøe Wolff, A.S., Husebye, E.S., Lilic, D., *et al.* (2008). Autoantibodies against type I interferons as an additional diagnostic criterion for autoimmune polyendocrine syndrome type I. J Clin Endocrinol Metab *93*, 4389-4397.

Metzger, T.C., and Anderson, M.S. (2011). Control of central and peripheral tolerance by Aire. Immunol Rev *241*, 89-103.

Metzger, T.C., Khan, I.S., Gardner, J.M., Mouchess, M.L., Johannes, K.P., Krawisz, A.K., Skrzypczynska, K.M., and Anderson, M.S. (2013). Lineage tracing and cell ablation identify a post-Aire-expressing thymic epithelial cell population. Cell Rep *5*, 166-179.

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., and Ohsumi, Y. (2004). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell *15*, 1101-1111.

Mueller, D.L. (2010). Mechanisms maintaining peripheral tolerance. Nat Immunol *11*, 21-27.

Musselman, C.A., and Kutateladze, T.G. (2011). Handpicking epigenetic marks with PHD fingers. Nucleic Acids Res *39*, 9061-9071.

Nagamine, K., Peterson, P., Scott, H.S., Kudoh, J., Minoshima, S., Heino, M., Krohn, K.J., Lalioti, M.D., Mullis, P.E., Antonarakis, S.E., *et al.* (1997). Positional cloning of the APECED gene. Nat Genet *17*, 393-398.

Nakagawa, T., Roth, W., Wong, P., Nelson, A., Farr, A., Deussing, J., Villadangos, J.A., Ploegh, H., Peters, C., and Rudensky, A.Y. (1998). Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. Science *280*, 450-453.

Nedjic, J., Aichinger, M., Emmerich, J., Mizushima, N., and Klein, L. (2008). Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. Nature *455*, 396-400.

Nichols, L.A., Chen, Y., Colella, T.A., Bennett, C.L., Clausen, B.E., and Engelhard, V.H. (2007). Deletional self-tolerance to a melanocyte/melanoma antigen derived from tyrosinase is mediated by a radio-resistant cell in peripheral and mesenteric lymph nodes. J Immunol *179*, 993-1003.

Norment, A.M., and Bevan, M.J. (2000). Role of chemokines in thymocyte development. Semin Immunol *12*, 445-455.

Org, T., Chignola, F., Hetenyi, C., Gaetani, M., Rebane, A., Liiv, I., Maran, U., Mollica, L., Bottomley, M.J., Musco, G., *et al.* (2008). The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression. EMBO Rep *9*, 370-376.

Oven, I., Brdicková, N., Kohoutek, J., Vaupotic, T., Narat, M., and Peterlin, B.M. (2007). AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells. Mol Cell Biol *27*, 8815-8823.

Pandiyan, P., Zheng, L., Ishihara, S., Reed, J., and Lenardo, M.J. (2007). CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol *8*, 1353-1362.

Pemberton, L.F., and Paschal, B.M. (2005). Mechanisms of receptor-mediated nuclear import and nuclear export. Traffic *6*, 187-198.

Perera, J., Meng, L., Meng, F., and Huang, H. (2013). Autoreactive thymic B cells are efficient antigen-presenting cells of cognate self-antigens for T cell negative selection. Proc Natl Acad Sci U S A *110*, 17011-17016.

Perniola, R., and Musco, G. (2014). The biophysical and biochemical properties of the autoimmune regulator (AIRE) protein. Biochim Biophys Acta *1842*, 326-337.

Peterson, P., Org, T., and Rebane, A. (2008). Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. Nat Rev Immunol *8*, 948-957.

Pitkänen, J., Doucas, V., Sternsdorf, T., Nakajima, T., Aratani, S., Jensen, K., Will, H., Vähämurto, P., Ollila, J., Vihinen, M., *et al.* (2000). The autoimmune regulator

protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein. J Biol Chem *275*, 16802-16809.

Pitkänen, J., Vähämurto, P., Krohn, K., and Peterson, P. (2001). Subcellular localization of the autoimmune regulator protein. characterization of nuclear targeting and transcriptional activation domain. J Biol Chem *276*, 19597-19602.

Plevin, M.J., Mills, M.M., and Ikura, M. (2005). The LxxLL motif: a multifunctional binding sequence in transcriptional regulation. Trends Biochem Sci *30*, 66-69.

Poliani, P.L., Kisand, K., Marrella, V., Ravanini, M., Notarangelo, L.D., Villa, A., Peterson, P., and Facchetti, F. (2010). Human peripheral lymphoid tissues contain autoimmune regulator-expressing dendritic cells. Am J Pathol *176*, 1104-1112.

Ramsey, C., Bukrinsky, A., and Peltonen, L. (2002a). Systematic mutagenesis of the functional domains of AIRE reveals their role in intracellular targeting. Hum Mol Genet *11*, 3299-3308.

Ramsey, C., Winqvist, O., Puhakka, L., Halonen, M., Moro, A., Kampe, O., Eskelin, P., Pelto-Huikko, M., and Peltonen, L. (2002b). Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. Hum Mol Genet *11*, 397-409.

Reinhardt, R.L., Bullard, D.C., Weaver, C.T., and Jenkins, M.K. (2003). Preferential accumulation of antigen-specific effector CD4 T cells at an antigen injection site involves CD62E-dependent migration but not local proliferation. J Exp Med *197*, 751-762.

Reynoso, E.D., Elpek, K.G., Francisco, L., Bronson, R., Bellemare-Pelletier, A., Sharpe, A.H., Freeman, G.J., and Turley, S.J. (2009). Intestinal tolerance is converted to autoimmune enteritis upon PD-1 ligand blockade. J Immunol *182*, 2102-2112.

Sarkar, G., and Sommer, S.S. (1989). Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. Science *244*, 331-334.

Shum, A.K., DeVoss, J., Tan, C.L., Hou, Y., Johannes, K., O'Gorman, C.S., Jones, K.D., Sochett, E.B., Fong, L., and Anderson, M.S. (2009). Identification of an autoantigen demonstrates a link between interstitial lung disease and a defect in central tolerance. Sci Transl Med *1*, 9ra20.

Sobue, K., Fujio, Y., and Kanda, K. (1988). Tumor promoter induces reorganization of actin filaments and calspectin (fodrin or nonerythroid spectrin) in 3T3 cells. Proc Natl Acad Sci U S A *85*, 482-486.

Spain, L.M., and Berg, L.J. (1994). Quantitative analysis of the efficiency of clonal deletion in the thymus. Dev Immunol *4*, 43-53.

Steinman, R.M., Hawiger, D., and Nussenzweig, M.C. (2003). Tolerogenic dendritic cells. Annu Rev Immunol *21*, 685-711.

Su, M.A., Giang, K., Zumer, K., Jiang, H., Oven, I., Rinn, J.L., Devoss, J.J., Johannes, K.P., Lu, W., Gardner, J., *et al.* (2008). Mechanisms of an autoimmunity

syndrome in mice caused by a dominant mutation in Aire. J Clin Invest *118*, 1712-1726.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T.W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med *192*, 303-310.

Tao, Y., Kupfer, R., Stewart, B.J., Williams-Skipp, C., Crowell, C.K., Patel, D.D., Sain, S., and Scheinman, R.I. (2006). AIRE recruits multiple transcriptional components to specific genomic regions through tethering to nuclear matrix. Mol Immunol *43*, 335-345.

Tewalt, E.F., Cohen, J.N., Rouhani, S.J., Guidi, C.J., Qiao, H., Fahl, S.P., Conaway, M.R., Bender, T.P., Tung, K.S., Vella, A.T., *et al.* (2012). Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. Blood *120*, 4772-4782.

Togoo, K., Takahama, Y., and Takada, K. (2014). Alpha-smooth muscle actin expression identifies subpopulations of mouse lymph node non-hematopoietic cells. Biochem Biophys Res Commun *449*, 241-247.

Tonooka, A., Kubo, T., Ichimiya, S., Tamura, Y., Ilmarinen, T., Ulmanen, I., Kimura, S., Yokoyama, S., Takano, Y., Kikuchi, T., *et al.* (2009). Wild-type AIRE cooperates with p63 in HLA class II expression of medullary thymic stromal cells. Biochem Biophys Res Commun *379*, 765-770.

Volkmann, A., Zal, T., and Stockinger, B. (1997). Antigen-presenting cells in the thymus that can negatively select MHC class II-restricted T cells recognizing a circulating self antigen. J Immunol *158*, 693-706.

von Boehmer, H., Aifantis, I., Gounari, F., Azogui, O., Haughn, L., Apostolou, I., Jaeckel, E., Grassi, F., and Klein, L. (2003). Thymic selection revisited: how essential is it? Immunol Rev *191*, 62-78.

Walters, S.N., Webster, K.E., Daley, S., and Grey, S.T. (2014). A Role for Intrathymic B Cells in the Generation of Natural Regulatory T Cells. J Immunol.

Walunas, T.L., Lenschow, D.J., Bakker, C.Y., Linsley, P.S., Freeman, G.J., Green, J.M., Thompson, C.B., and Bluestone, J.A. (1994). CTLA-4 can function as a negative regulator of T cell activation. Immunity *1*, 405-413.

Wang, C.Y., Shi, J.D., Davoodi-Semiromi, A., and She, J.X. (1999). Cloning of Aire, the mouse homologue of the autoimmune regulator (AIRE) gene responsible for autoimmune polyglandular syndrome type 1 (ASP1). Genomics *55*, 322-326.

Waterfield, M., Khan, I.S., Cortez, J.T., Fan, U., Metzger, T., Greer, A., Fasano, K., Martinez-Llordella, M., Pollack, J.L., Erle, D.J., *et al.* (2014). The transcriptional regulator Aire coopts the repressive ATF7ip-MBD1 complex for the induction of immunotolerance. Nat Immunol *15*, 258-265.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. Science *322*, 271-275.

Wing, K., and Sakaguchi, S. (2010). Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol *11*, 7-13.

Yang, S., Bansal, K., Lopes, J., Benoist, C., and Mathis, D. (2013). Aire's plant homeodomain(PHD)-2 is critical for induction of immunological tolerance. Proc Natl Acad Sci U S A *110*, 1833-1838.

Yip, L., Su, L., Sheng, D., Chang, P., Atkinson, M., Czesak, M., Albert, P.R., Collier, A.R., Turley, S.J., Fathman, C.G., *et al.* (2009). Deaf1 isoforms control the expression of genes encoding peripheral tissue antigens in the pancreatic lymph nodes during type 1 diabetes. Nat Immunol *10*, 1026-1033.

Yu, X., Harden, K., Gonzalez, L.C., Francesco, M., Chiang, E., Irving, B., Tom, I., Ivelja, S., Refino, C.J., Clark, H., *et al.* (2009). The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol *10*, 48-57.

Zehn, D., and Bevan, M.J. (2006). T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. Immunity *25*, 261-270.

Zúñiga-Pflücker, J.C. (2004). T-cell development made simple. Nat Rev Immunol *4*, 67-72.