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The role of NF-kappa B signaling in establishment of central tolerance

Úloha NF-kappa B signalizace v ustanovení centrální tolerance

BACHELOR THESIS

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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. 5. 2017 Jiří Březina

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Abstract:

The central tolerance, which is established in the thymus, significantly reduces the self-reactive repertoire of T cells and thereby prevents the development of auto-immune diseases. Essential for this process is the protein Auto-immune regulator (Aire), which is expressed by rare medullary thymic epithelial cells (mTEC). Aire drives the promiscuous gene expression of thousands of tissue restricted antigens which are critical for efficient negative selection of self-reactive T cells and the selection of T regulatory cells. The research of the last two decades have uncovered the role of NF-kappa B signaling in establishment of central tolerance. Here I summarize the most important evidence revealing the key role of NF-kappa B signaling in mTECs development and direct regulation of Aire gene expression, which argues for previously unappreciated fact that NF-kappa B signaling is the master regulator of processes that guide the development, maintenance and function of central tolerance.

Key words: *Central tolerance, NF-kappa B, Aire, mTEC, thymus*

Abstrakt:

Centrální tolerance, která je ustanovena v thymu, významně redukuje repertoár auto-reaktivních T lymfocytů a tím umožňuje předcházet rozvoji autoimunitních onemocnění. Pro tento proces je nezbytný protein Autoimunitní regulátor (Aire), který je exprimovaný unikátními medulárními epiteliálními buňkami thymu (mTEC). Aire řídí promiskuitní genovou expresi tisíců tkáňově specifických antigenů, která je zásadní pro účinnou negativní selekci auto-reaktivních T lymfocytů a selekci T regulačních lymfocytů. Výzkum posledních dvou dekád poukazuje na roli NF-kappa B signalizace v ustanovení centrální tolerance. V této práci předkládám nejdůležitější poznatky dokládající její klíčovou roli ve vývoji mTEC buněk a v přímé regulaci genové exprese Airu, které hovoří ve prospěch doposud nedoceněné skutečnosti, že NF-kappa B signalizace je hlavním regulátorem procesů podílejících se na vývoji, udržování a funkci centrální tolerance.

Klíčová slova: *Centrální tolerance, NF-kappa B, Aire, mTEC, thymus*

List of abbreviations:

Aire…… *Autoimmune regulator*

Aly/Aly…… *Alymphoplasia*

APC…… *Antigen presenting cell*

APECED…… *Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy*

APS-1…… *Autoimmune polyendocrine syndrome type 1*

ATF7ip- MBD1…….. *Activating transcription factor 7–interacting protein-Methyl CpG-binding protein 1*

Brd4…… *Bromodomain containing protein 4*

CARD…… *Caspase-recruitment domain*

CBP…… *Creb-binding protein*

CCL19…… *Chemokine C-C motif ligand 19*

CCL21…… *Chemokine C-C motif ligand 21*

CCR7…… *C-C chemokine receptor type 7*

CD40L…… *CD40 ligand*

Cld3,4…… *Claudine 3 and 4*

CLP…… *Common lymphoid progenitor*

CMJ…… *Cortico-medullary junctions*

CNS1…… *Conserved non-coding sequence 1*

cTEC…… *Cortical thymic epithelial cell*

DC…… *Dendritic cell*

DN…… *Double negative*

DNA-PK…… *DNA protein kinase*

DP…… *Double positive*

FTOC…… *Fetal thymic organ culture*

HEL…… *Hen egg lysozyme*

Hnrnpl…… *Heterogeneous nuclear ribonucleo-protein l*

Ii…… *Invariant chain*

IKKα…… *IκB kinase α*

IRBP…… *interphotoreceptor retinoid-binding protein*

jTEC…… *Junctional thymic epithelial cell*

KO…… *Knock out*

Lt…… *Lymphotoxin*

LtβR…… *Lymphotoxin β receptor*

mDC…… *Migratory DC*

MHC..... *Major histocompatibility complex*

mTEC..... *Medullary thymic epithelial cell*

mTECSC..... *mTEC-stem cell*

NFκB..... *Nuclear factor-kappa B*

NIK..... *NFκB-inducing kinase*

NLS..... *Nuclear localization signal*

OPG..... *Osteoprotegerin*

pDC..... *Plasmacytoid DC*

PGE..... *Promiscuous gene expression*

pMEC ..... *Precursor of Aire^{+}mTEC*

pMHC..... *Peptide- MHC complex*

pro-pMEC..... *Progenitor of pMEC*

RANK..... *Receptor activator of NFκB*

RANKL..... *RANK ligand*

RIP..... *Rat insulin promoter*

RNApII..... *RNA polymerase II*

Sirt1..... *Sirtuin-1*

SP..... *Single positive*

tB cell..... *Thymic B cell*

TCR..... *T cell receptor*

tDC..... *Thymic derived DC*

TEC..... *Thymic epithelial cell*

TF..... *Transcription factor*

tg..... *Transgenic*

TOP2..... *Topoisomerase 2*

TRA..... *Tissue restricted antigen*

TRAF3/TRAF6..... *TNF receptor associated factor 3/6*

T_{Reg}..... *T regulatory cell*

TSS..... *Transcription start site*

TSSP..... *Thymus-specific serine protease*

UEA1..... *Ulex Europaeus Agglutinin 1*

WT..... *Wild type*

3DS..... *Three-dimensional scaffold*

-/-..... *Knockout*

Table of contents:

1. Introduction

Vertebrates and especially mammals possess the adaptive immunity which endows them with highly specific immune responses and immunological memory against a vast array of pathogens. On the other hand, the adaptive immunity, embodied via the specificity of their lymphocyte and especially T cell receptors (TCR), also exhibits the capacity to recognize self-antigens and attack host own tissues which, in turn, can lead to the development of autoimmunity. To prevent this undesirable effect, several mechanisms of central tolerance exist that are capable to severely limit the auto-reactive repertoire of T cells and thus protect individuals against the onset of autoimmune attack. The past twenty years brought a new insight into the mechanisms underpinning central tolerance. Functional integration of these mechanisms and their sub-compartmentalization into distinct thymic microenvironments where they monitor the development of naïve T cells (thymocytes) and selects which of them are suitable to enter the periphery is the basic operational definition of central tolerance. The objective of my thesis is to summarize the most relevant results of contemporary research which have characterized these mechanisms with emphasis on the role of NFκB signaling in establishment, maintenance and function of central tolerance.

2. T cell central tolerance

2.1. Thymus and T cell development

The proper function of the thymus remained elusive for quite a long time. The breakthrough came in 1960´s when neonatal thymectomized mice were firstly used to assess thymus functions in newborns (Miller, 2002). These experiments revealed severe dysfunctions of lymphopoiesis and susceptibility to infections in neonatal mice suggesting that thymus play an indispensable role in immunity of postnatal life and does not merely function as a “graveyard” of dead thymocytes, as previously thought (Miller, 2002). Subsequent experiments pointed to the critical role of thymus in the development and maturation of T cells. T cells are derived from common lymphoid progenitors (CLP) differentiated from the hematopoietic stem cells residing in the bone marrow. CLPs then migrate to the thymus where they undergo further development. After entering the thymus, in cortico-medullary junctions (CMJ), developing T cells pass through the cortex and medulla, where they accomplish their maturation process and are subjected to positive and negative selection, respectively (Petrie, 2002). T cells, as representatives of adaptive immunity, are capable to recognize inconceivably wide range of antigens via their TCRs. This capacity is endowed by the VDJ gene recombination (Roth, 2014), during which recombinases, RAG1 and RAG2, rearrange gene segments constituting the α and β chains of TCRs (Brandt and Roth, 2008). The cortex is an initial site of proliferation and differentiation of mainly double negative (DN) $CD4^-CD8^-$ thymocytes which progressively rearrange their TCRs and transit to double

positive (DP) stage, characterized by the expression of both $CD4^+$ and $CD8^+$ co-receptors and completed rearrangement of αβ TCR (Germain, 2002). During their transition from DN to DP stage, thymocytes interact with several cell types residing in the thymic cortex, including cortical thymic epithelial cells (cTECs) which together with thymic fibroblasts condition the microenvironment important for this process by expression of DLL4 (delta-like notch ligand 4), cytokines IL-7 (interleukin 7), TGFβ (tumor growth factor β) or stem cell factor and chemokines CCL25 (chemokine C-C motif ligand 25), CXCL12 (C-X-C motif chemokine 12) or CCRL1 (C-C chemokine receptor type 1) etc. (Ohigashi, Kozai and Takahama, 2016). Once thymocytes reach their DP stage they are subjected to processes that test the functionality and self- specificity of their TCRs, positive and negative selection, respectively.

2.2. Affinity model of thymocytes selection

Positive and negative selections are crucial processes for establishment of T cell central tolerance. The common denominator of these two processes is a widely accepted "affinity model" of selection which is depicted in ***Figure 1***. Basically, this model proposes that the fate of thymocytes is determined by the affinity of its TCR to peptide fragments presented in the context of major histocompatibilty complex (MHC) on various thymic antigen presenting cells (APC).

About 90% of all TCRs reveal no or only a negligibly low affinity to presented peptides. Thus, these thymocytes are not capable to interact via TCR with surrounding cells and die by neglect (Palmer, 2003). It is postulated that low affinity interactions of TCRs with peptide-MHC complexes (pMHC) result in positive selection, whereas high affinity interactions result in negative selection of developing thymocytes (Klein *et al.*, 2014). Importantly, T cells with TCR affinities falling in between positive and negative selection range have been implicated in their conversion into T regulatory cells (T_{Reg}) (Jordan *et al.*, 2001). Notably, according to several studies (reviewed in (Bains *et al.*, 2013)), T_{Reg}s are selected through intermediate and/or higher affinity interactions compared with a high affinity of negatively selected thymocytes. However, it seems that affinities of T_{Reg}s and negatively selected TCRs overlap to a large extent (Lee *et al.*, 2012).

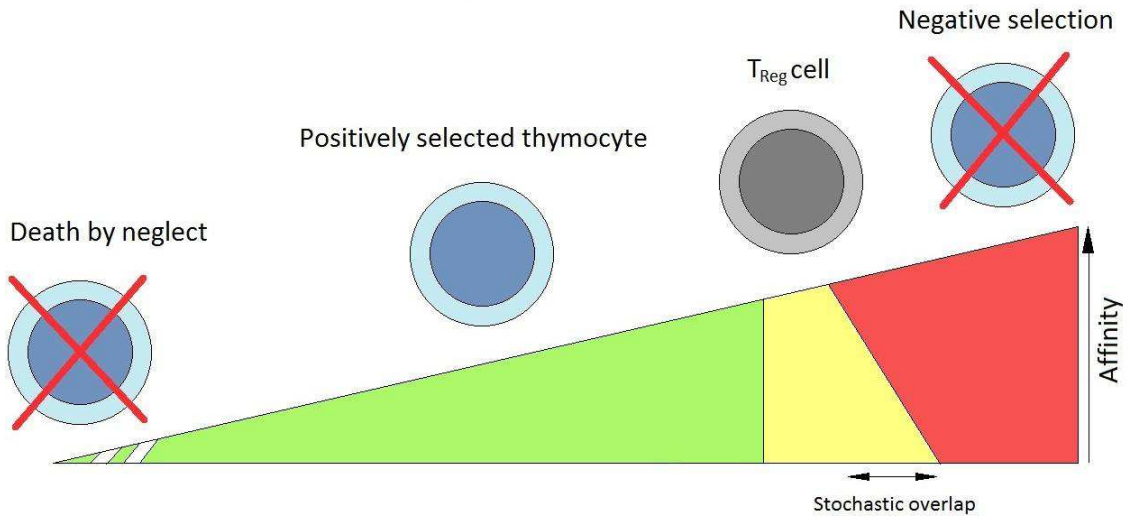

Figure 1) Affinity model of thymocytes selection: *Crosshatched peak on the left symbolizes almost none affinity of thymocytes to pMHCs resulting in death by neglect. Low affinity interactions in green allow positive selection, whereas high affinity interactions in red lead to apoptosis. T_{Reg} cell selection affinity range in yellow sits between positive and negative selection and shares stochastic affinity overlap with negative selection. Inspired by* (Klein *et al.*, 2014).

Importantly, while the affinity model is generically applicable for both positive and negative selection processes, caution must be exercise as this model largely ignores distinct cellular microenvironments and parameters of pMHC presentation in the cortex an medulla, respectively (Klein *et al.*, 2014).

2.3. Positive selection in the thymic cortex

DP thymocytes, with fully matured TCRs, are first tested for the ability to bind pMHCs presented by cTECs in a process referred to as positive selection. As described previously, only low affinity TCR/pMHC interactions lead to the survival of developing thymocytes (Klein *et al.*, 2014). During the interaction with cTECs, DP thymocytes become single positive (SP) which means that they lose one co-receptor expression from the pair CD8/CD4. If the TCR on DP cell interacts with pMHCI, thymocyte becomes CD8 restricted. Conversely, interaction with pMHC II results in the development of $CD4^+$ thymocytes (Palmer, 2003). It was also described that CD8/CD4 restriction is dependent on specific transcription factors, specifically Runx3 (for CD8 restriction) (Setoguchi *et al.*, 2008) and Th-POK (He *et al.*, 2005) which drives the differentiation into $CD4^+$ thymocytes and also repress the expression of Runx3 (Luckey *et al.*, 2014).

It is still not fully understood how peptides are generated and presented in the context of MHC molecules during positive selection. Several studies, which are discussed below, revealed that cTECs utilize unique proteolytic pathways for chopping available peptides, which suggest the presentation of unique peptide ligands (ligandome) on their MHC molecules which, by definition, differ from peptides generated and presented by mTECs. While, there is some evidence for unique cTEC ligandome, its more systematic characterization is still required (Lo *et al.*, 2009; Santori *et al.*, 2002; Sasaki *et al.*, 2015).

Unique ligandome of cTECs was shown to be produced by the unique enzymatic apparatus involved in MHC II processing machinery. Notably, cTECs express cathepsin L, encoded by Ctsl gene, instead of cathepsin S, which is produced by most of the APCs including mTECs (Nakagawa *et al.*, 1998). Cathepsins are lysosomal proteases specific for the cleavage of invariant chain (Ii) from MHC II molecules (Honey and Rudensky, 2003). However, Cathepsin L, exclusively expressed by cTECs, exhibits an additional capability to cleave peptides for MHC II presentation and thus enlarge the cTEC ligandome (Honey *et al.*, 2002). In the context of positive selection, analyses of $Ctsl^{-/-}$ mice revealed a severe reduction in the frequency and repertoire of positively selected $CD4^+$ thymocytes and also led to the impairment of Ii degradation (Nakagawa *et al.*, 1998). Moreover, the analysis of double knockout ($Ctsl^{-/-}Ii^{-/-}$) mice suggested that repertoire reduction in positively selected thymocytes was not due to absence of Ii

degradation, but rather due to changes in specific peptide generation by cTECs (Honey *et al.*, 2002). Another unique cTEC enzyme is thymus-specific serine protease (TSSP) encoded by Prss16 gene which also shapes its MHC II ligandome (Bowlus *et al.*, 1999). $TSSP^{-/-}$ mice revealed reduced repertoire of positively selected $CD4^+$ thymocyte TCRs, likely due to loss of presentation of some peptides which were TSSP dependent (Gommeaux *et al.*, 2009).

Specific feature of thymic epithelial cells (TECs) is high constitutive macroautophagy (Mizushima *et al.*, 2004). During this process, a portion of cytoplasm, which contain some organelles and nucleus, fuse with endosomes or lysosomes and content of these newly formed autophagosomes is cleaved to small peptides (Feng *et al.*, 2014). cTECs utilize macroautophagy for MHC II presentation during positive selection instead of classical exogenous loading of peptides on MHC II molecules which is inefficient in cTECs and more generally in TECs (Klein, Roettinger and Kyewski, 2001). Transplantation of the $Atg5^{-/-}$ thymus with abrogated macroautophagy, into variety of TCR transgenic (tg) mice, whose TCRs are for instance restricted to specific peptide, revealed the reduction of positive selection of $CD4^+$ thymocytes. Thus, this study suggests that the generation of certain peptides utilized in positive selection is strictly dependent on macroautophagy (Nedjic *et al.*, 2008).

cTECs also utilized a specialized enzymatic machinery for MHC I antigen processing and presentation, which participates in positive selection of $CD8^+$ thymocytes. Specifically, cTECs express a unique subunit of proteasome called β5t, which is encoded by Psmb11 gene and defines a specialized type of proteasome termed thymoproteasome (Murata *et al.*, 2007). $Psmb11^{-/-}$ mice showed only slightly reduced frequency of positively selected $CD8^+$ thymocytes (Murata *et al.*, 2007), but these cells possessed a limited TCR repertoire (Nitta *et al.*, 2010) and demonstrated impaired immunological properties including antigen responsiveness and reduced ability to maintain naïve T cell population in the periphery (Takada *et al.*, 2015). Concerning positively selecting peptides it has been suggested that β5t reduced the chymotrypsin-like activity of thymoproteasomes, which points to the ability of cTECs to generate mainly low affinity ligands for TCRs (Murata *et al.*, 2007). Such findings were confirmed by recent article demonstrating that peptides cleaved by thymoproteasome are frequently enriched by low affinity ligands to TCRs that enable cTECs to support the low affinity TCR signaling which, in turn, induces positive selection of $CD8^+$ thymocytes (Sasaki *et al.*, 2015).

Thus, positive selection examines the functionality of TCR clones through the presentation of unique peptides. Because the VDJ recombination is a stochastic process which may give rise to TCR clones recognizing either physiologically important foreign peptides or self-peptides, the recognition of which may lead to the development of autoimmunity, the mechanism which eliminates these self-reactive clones was established in the thymic medulla and is called the negative selection. To get to the medulla, SP thymocytes which underwent the positive selection start to express the chemokine receptor CCR7 (C-C chemokine receptor type 7) and migrate via the concentration gradient of mTEC-derived

chemokines CCL19 and CCL21 (Chemokine (C-C motif) ligand 19 and 21) (Kurobe *et al.*, 2006; Ueno *et al.*, 2004).

2.4. The thymic medulla and its selection processes

2.4.1. Promiscuous gene expression

The main requirement for the efficient negative selection in the thymic medulla is a presence of large repertoire of self-peptides which could be recognized by potential auto-reactive thymocytes. The mechanism which creates the medullary "showcase of the periphery" is called the ectopic or synonymously promiscuous gene expression (PGE) (Klein *et al.*, 2009). The promiscuously expressed self-peptides belong to the group of so called tissue restricted antigens (TRA) which are originally expressed in less than five tissues from sixty tested. Linsk *et al.* came with the hypothesis that presence of this peripheral TRA "patch quilt" in the thymus pointed to its possible role in negative selection of developing T cells (Linsk *et al.*, 1989). The first notion of PGE in the thymus resulted from the experiments with RIP-Tag transgenic mouse which express T antigen (Tag) under rat insulin promoter (RIP). The expression of Tag, the production of which in the periphery is restricted to pancreatic-β cells , was detected also in the thymus and led to the establishment of central tolerance against this antigen (Jolicoeur, Hanahan and Smith, 1994). The existence of PGE in the thymus was then confirmed by detection of expression of additional tissue specific transcripts such as brain specific antigens (Pribyl *et al.*, 1996), thyroid specific genes (Sospedra *et al.*, 1998) and C-reactive protein (Klein *et al.*, 1998). However, these studies ignored the identification of cellular source responsible for PGE in the thymus. Turning point surrounds the study which revealed that PGE is exclusively mediated by mTECs and most efficiently by their mature Ulex Europaeus agglutinin 1^{+} (UEA1) subset (Derbinski *et al.*, 2001).

The detailed mechanisms of mTEC-mediated PGE was demonstrated by the introduction of mice lacking the Autoimmune regulator (Aire) (Anderson *et al.*, 2002), which was shown to be the major regulator of PGE specifically in the mature MHC II^{Hi} $CD80^{Hi}$ mTECs ($mTEC^{Hi}$) (Derbinski *et al.*, 2005). Microarray analyses of distinct thymic cell types revealed that $mTECs^{Hi}$ express the largest cluster of TRAs. Nevertheless, smaller clusters of TRAs were found to be expressed also by MHC II^{lo} $CD80^{lo}$ mTECs ($mTEC^{lo}$) or by cTECs (Derbinski *et al.*, 2005). Moreover, the repertoire of TRAs expressed in the thymus contained not only Aire-dependent but also Aire-independent genes, suggesting that Aire is not the sole PGE regulator (Derbinski *et al.*, 2005). Indeed, recent study has suggested that Fezf2 is a long sought Aire-independent regulator of PGE (Takaba *et al.*, 2015). Although the repertoire of TRAs expressed in the thymus is broad and covers almost all peripheral epitopes, several organs, for example testis and brain, are represented in TRA pool significantly less than other tissues (Derbinski *et al.*, 2005). According to the recent study, TECs are capable to drive the expression of almost 20000 genes, among which 3980 belong to Aire-dependent TRAs. Single $mTEC^{Hi}$ expressed on average nearly 5300 genes from which

approximately 150 were TRAs regulated by Aire and about 600 TRAs were shown to be Aire independent (Sansom *et al.*, 2014), indicating that contribution of other unknown PGE regulators is substantial. Based on the fact that the single cell PCR analysis of mTECs revealed that individual TRA is expressed only by 1-3% of mTECs, it was suggested that PGE is a stochastic process (Derbinski *et al.*, 2008). However, recent studies revealed steady co-expression patterns between either Aire-dependent or -independent TRA genes localized in close proximity, suggesting that PGE takes place rather with "ordered stochasticity" (Brennecke *et al.*, 2015; Rattay *et al.*, 2016).

2.4.2. Promiscuous gene expression on molecular level- structure and properties of Aire

The human AIRE was identified as a gene which mutations cause a rare auto-immune syndrome called Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), also known as autoimmune polyendocrine syndrome type 1 (APS-1). The AIRE gene is formed by 14 exons, is localized on chromosome 21 and encodes a protein that consists of 545 amino acids (Aaltonen *et al.*, 1997; Nagamine *et al.*, 1997). The murine Aire gene is localized on chromosome 10 and demonstrates about 80% similarity with human AIRE (Blechschmidt *et al.*, 1999). APECED is a rare autosomal recessive disease with higher incidence in populations of Finns, Sardinians and Iranian jews and could be characterized by chronic mucocutaneous candidiasis, hypoparathyroidism and Addison´s disease. However, these manifestations are usually accompanied by various organ-specific auto-immunities, for example by the Type 1 diabetes (Kisand and Peterson, 2015). To provide a more comprehensive insight into the mechanisms of Aire function and pathogenesis of diseases accompanying Aire dysfunctions, the $Aire^{-/-}$ mouse strains were generated. In this context it is important to emphasize that similar to human APECED, the phenotype of autoimmunity in $Aire^{-/-}$ mice differs between individuals and its severity is highly dependent on the genetic background of a particular strain (Anderson *et al.*, 2002; Hubert *et al.*, 2009; Jiang *et al.*, 2005; Kuroda *et al.*, 2005; Ramsey *et al.*, 2002).

Aire protein is structurally composed of several functional domains **(*Figure 2*)**, often associated with nuclear proteins and transcriptional regulators. One of these domains is the caspase-recruitment domain (CARD) which is localized to the N-terminal end of the Aire and was shown to be crucial for the formation of Aire homo-dimer or homo-tetramer complexes which are necessary for its transactivation activities (Kumar *et al.*, 2001; Pitkänen *et al.*, 2000). Further, Aire contains two nuclear localization signals (NLS) (Pitkanen, 2001) and a SAND domain (named by Sp100, Aire, NucP41/75 and Deaf1) (Gibson *et al.*, 1998) through which Aire binds the ATF7ip-MBD1 (Activating transcription factor 7–interacting protein-Methyl CpG-binding protein 1) repressor complex that is crucial for the selectivity of Aire dependent genes (Waterfield *et al.*, 2014). The C-terminal end of Aire contains two PHD zinc-finger domains which typically act as readers of epigenetic marks (Musselman and Kutateladze, 2011). PHD1 domain was shown to interact with un-methylated histone H3 lysine 4 (H3K4me0) which is considered

to be a marker of silenced chromatin. It is suggested that PHD1 binds to inactive chromatin which contains genes that are considered to encode Aire-dependent TRAs. Thus, Aire recognizes its target genes via their epigenetic markers rather than through the specific DNA-binding consensus sequence as conventional TFs (Koh *et al.*, 2008; Org *et al.*, 2008). On the other hand, a precise function of PHD 2 domain remains still elusive. Nevertheless, its ablation implicated this domain in the interaction with several binding partners involved mainly in chromatin structure and/or transcription (Yang *et al.*, 2013). Aire protein also contains LXXLL motifs which were shown to mediate interactions with various transcriptional co-activators and co-repressors (Plevin, Mills and Ikura, 2005).

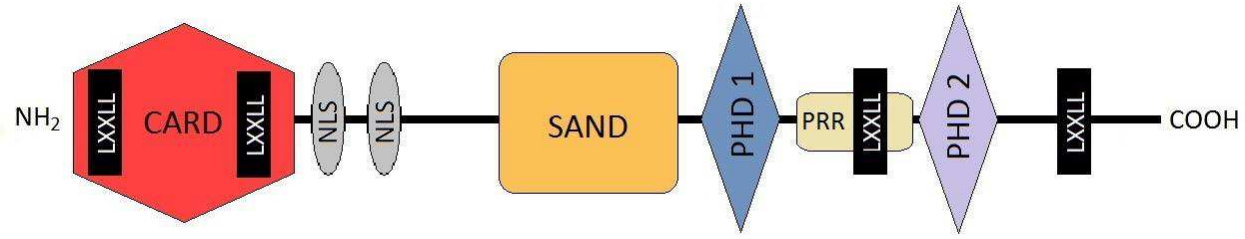

Figure 2) Structure of Aire: *The CARD domain and two LXXLL motifs constitute the N-terminal end of the Aire protein. In close proximity to CARD domain are localized two nuclear localization signals followed by a SAND domain. The C-terminal end comprises a proline rich region (PRR) and two distinct PHD1 and PHD 2 domains, intermingled with two LXXLL motifs. Inspired by* (Mathis and Benoist, 2009).

It is known that Aire drives the expression of approximately 4000 genes (Sansom *et al.*, 2014), nevertheless, it is not completely clear how Aire specifically targets them. Aire-dependent TRA genes are enriched with H3K4me0 marks which can be recognized and bound by PHD1 domain (Koh *et al.*, 2008; Org *et al.*, 2008). Although this mode of gene recognition is definitely crucial for efficient function of Aire, there are other mechanisms important for recognition of Aire specific genes (Koh *et al.*, 2010). Recent study revealed that Aire dependent TRAs could be also characterized by the absence of trimethylated histone H3 lysine 4 (H3K4me3) marks and enrichment of trimethylated histone H3 lysine 27 (H3K27me3) marks. It is clear, that Aire is not capable to directly recognize H3K27me3 marked genes which points to the role of Aire-interacting partners in recruitment to target genes (Sansom *et al.*, 2014). One important study in this field showed that Aire interacts with ATF7ip-MBD1 repressor complex. ATF7ip-MBD1 binds specifically to methylated CpG islands (associated with transcriptionally silenced genes) that are specifically enriched in Aire-dependent TRAs. In support of this notion, $MBD1^{-/-}$ mice suffer from the manifestations of auto-immune diseases (Waterfield *et al.*, 2014).

While the Aire resides in the nucleus, it interacts with the nuclear matrix (Akiyoshi *et al.*, 2004) and aggregates into characteristic formations called nuclear speckles which are probably localized in active transcription regions (Su *et al.*, 2008). To efficiently regulate the transcription of Aire-dependent genes,

Aire physically interacts with many TFs and regulators which form the complex of Aire interacting partners.

The first discovered partner of Aire was Creb-binding protein (CBP) which is a crucial co-activator of gene transcription (Pitkänen *et al.*, 2000). Cooperation between Aire and CBP strongly enhances the transactivation properties of Aire. CBP also reveals the acetyl-transferase activity and acetylates lysines of the SAND domain which leads to the increased stability of Aire. Acetylated Aire also selects different TRA genes in comparison with non-acetylated Aire (Saare *et al.*, 2012). Indeed, recent study confirmed that the activity of Aire is dependent on its acetylation status. It was found that the expression of Aire-dependent TRAs is highly dependent on the presence of deacetylase Sirtuin-1 (Sirt1) in Aire expressing mTECs and it was demonstrated that $Sirt1^{-/-}$ mice suffer from Aire-associated autoimmune manifestations (Chuprin *et al.*, 2015).

Using various experimental approaches including co-immunoprecipitation, mass spectometry analyses and RNAi- based methods, approximately 50 Aire binding partners were identified and divided into four functional clusters: I) nuclear transport II) chromatin binding/structure III) transcription IV) pre-mRNA processing (Abramson *et al.*, 2010; Giraud *et al.*, 2014). The transcription of Aire-dependent TRAs was found to be highly dependent on DNA damage response proteins such as DNA protein kinase (DNA-PK) and Topoisomerase 2 (TOP2), the members of the third cluster (Abramson *et al.*, 2010; Guha *et al.*, 2017; Žumer *et al.*, 2012). It was suggested that Aire after the binding to H3K4me0 recruits TOP2 which induces double strand DNA breaks. These breaks attract DNA-PK together with other detected DNA damage response proteins and they form a multiprotein complex which participates in the relaxation of surrounding chromatin to enhance the elongation of transcription (Abramson *et al.*, 2010). Aire mainly affects the elongation phase of transcription, because it binds the Cdk9 and CycT1 which together form the elongation complex p-TEFb (Oven *et al.*, 2007) which is recruited to transcription start sites (TSS) of Aire-dependent TRA genes. The recruitment of p-TEFb is necessary for the release of stalled RNA II polymerases (RNApII) and for initiation of the elongation phase of TRAs transcription (Giraud *et al.*,2012). Moreover, additional study revealed that Heterogeneous nuclear ribonucleo-protein I (Hnrnpl), which is a splicing factor, binds both, p-TEFb and Aire and its presence is important for the elongation phase of TRAs transcription (Giraud *et al.*, 2014). Aire was also found to interact with bromodomain containing protein 4 (Brd4) which was shown to build a “molecular bridge” connecting pTEFb and Aire and promotes their interaction. In addition, the binding of Brd4 to Aire is dependent on the acetylation of CARD domain by CBP (Yoshida *et al.*, 2015).

Finally, independent yeast-two-hybrid screens revealed additional Aire partners whose specific contribution to the Aire function still remains uncertain. Namely the ubiquitously expressed death associated protein 6 (DAXX) (Meloni *et al.*, 2010), the Homeodomain-interacting protein kinase 2 (HIPK2) (Rattay *et al.*, 2015) and the protein inhibitor of activated STAT (PIAS1) (Ilmarinen *et al.*, 2008).

Altogether, Aire recruits dozens of interaction partners for its efficient function. Those partners that have been already established are depicted in model of Aire-mediated gene transcription **(*Figure 3*)**.

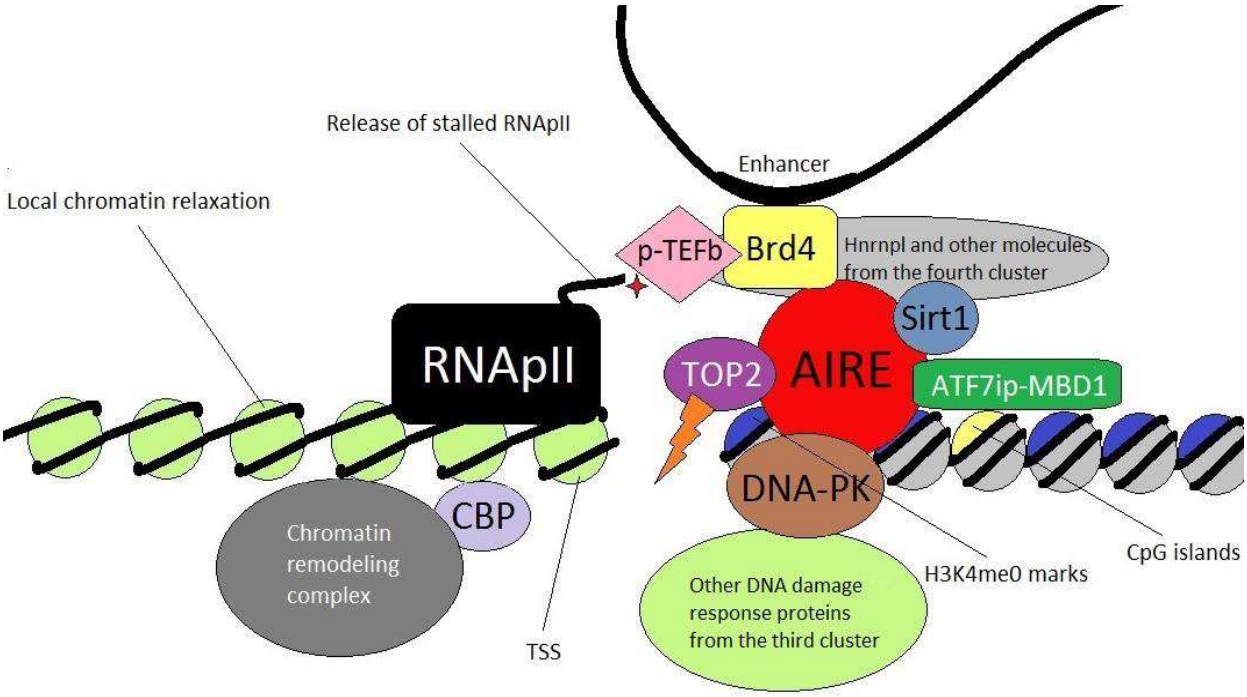

Figure 3) Model of Aire-mediated gene transcription: *Aire recognizes the signature of inactive chromatin, specifically H3K4me0 histone-marks and ATF7ip-MBD1 repressor molecules which bind methylated CpG di-nucleotides, and subsequently recruits TOP2 to induce double strand breaks at the TSSs of Aire´s regulated genes. These breaks recruit DNA-PK and other partners from the "third cluster" which participate in the relaxation of surrounding chromatin and regulate the recruitment of proteins responsible for Aire transcriptional transactivation capacity like CBP and Sirt1. These events are followed by binding of downstream regulators of gene expression, such as Brd4 and elongation factor p-TEFb which releases RNApII and starts the transcription of Aire regulated genes. In addition, Aire recruits Hnrnpl and other molecules from the "fourth cluster" which with ongoing elongation phase simultaneously splice the nascent mRNA. Inspired by* (Abramson and Husebye, 2016)

It´s obvious from the above, that the presence of Aire has a tremendous impact on the landscape of cellular gene expression. But importantly, the gene expression of Aire is inherent only to a rare subset of cells and therefore is tightly regulated in a cell specific manner. However, the exact regulation of Aire gene expression is still not entirely clear and is the subject of current research. Recent article demonstrated that the Aire gene locus in mTECs^{Hi} shows demethylation of specific region spanning the exon 2. The demethylation of this region leads to the increased accessibility for the multimolecular complex consisting key TFs: Irf4, Irf8, Tcf7 and Tbx21 that directly enhance the expression of Aire, specifically in mTECs^{Hi} (Herzig *et al.*, 2016). More relaxed chromatin of the Aire locus in mTECs^{Hi} is accessible also to TFs of the nuclear factor-kappa B (NFκB) signaling pathway which is a key player in the

regulation of Aire gene expression (Haljasorg *et al.*, 2015; LaFlam *et al.*, 2015) and is further discussed below. Nevertheless, the following chapter is focused especially on the contribution of Aire to the negative selection and T_{Reg} selection.

2.4.3. Negative selection and T_{Reg} selection are mediated by several distinct types of APCs

Because TCRs are generated by a random genetic recombination process, achieving the state of immunological tolerance requires that potentially dangerous auto-reactive T cells are either removed from the repertoire through negative selection (recessive tolerance) or kept in check by regulatory mechanisms (dominant tolerance). Both modes of tolerance are achieved by selection processes during T cell development in the thymus and result in the elimination of auto-reactive T cells or their deviation into the T_{Reg} lineage. Various types of APCs in thymic medulla, such as mTECs, dendritic cells (DC) and B cells, play a critical role in these processes (Perry and Hsieh, 2016). This sub-chapter provides a short overview of our knowledge in this rapidly advancing field.

The seminal hypothesis, that Aire drives the PGE was experimentally confirmed by the generation of $Aire^{-/-}$ mice which suffer from multiorgan autoimmunity. This study also mapped the expression of Aire to mTECs, whereby its deficiency resulted in alterations of gene expression profile, and translated mainly to the loss of some TRAs. This led to conclusion that TRA expression is at least partly dependent on Aire and its absence negatively impacts processes of central tolerance, which in turn leads to the development of autoimmunity (Anderson *et al.*, 2002). Direct role of Aire in negative selection of self-specific T cells was demonstrated by experiments with 3A9 TCR- insHEL double-tg mice (Liston *et al.*, 2003). These mice express the hen egg lysozyme (HEL), a neo-self antigen produced under the Aire-dependent RIP promoter specifically in mTECs (thus mimicking Aire-dependent TRA) as well as in pancreatic-β cells. Simultaneously, these mice generate T cells with transgenic 3A9 TCR specific to HEL. Results showed that if 3A9 TCR- insHEL mice express Aire, they present HEL by mTECs and display almost no mature T cells bearing TCR specific to HEL, suggesting their efficient negative selection. On the other hand, $Aire^{-/-}$ 3A9 TCR- insHEL mice showed abrogated expression of HEL in mTECs, which translated into impaired negative selection of HEL-specific T cells and their abundant persistence in the periphery (Liston *et al.*, 2003). Similar conclusions were made in another transgenic systems: I) OT I/II: RIP-mOVA mice which carried tg TCRs OT I and OT II on CD8 and CD4 T cells respectively, which were specific for membrane-bound ovalbumin (mOVA) expressed under RIP promoter (Anderson *et al.*, 2005), and II) in experiments with interphotoreceptor retinoid-binding protein (IRBP) (DeVoss *et al.*, 2006) which were further recapitulated in polyclonal experimental system (Taniguchi *et al.*, 2012). Thus, these data demonstrated the role of $Aire^{+}$mTECs in the recessive tolerance.

The deviation of thymocytes into T_{Reg}s (T_{Reg} selection) was also found to be largely dependent on the presentation of antigen by $Aire^{+}$ mTECs. In experiments with mice expressing in mTECs the influenza

hemagglutinin (HA) antigen produced under the Aire promoter which is presented to developing HA specific thymocytes, the author showed their partial deviation into T_{Reg} lineage. This was in sharp contrast to, TCR HA tg mice that lacks the presentation of HA in the thymus and therefore failed to generate any HA-specific T_{Reg}s (Aschenbrenner *et al.*, 2007). The substantial role of mTECs as APCs that contribute to both recessive and dominant tolerance was further confirmed in *in vivo* experiments, using MHC II silencing by RNAi in mTECs (Hinterberger *et al.*, 2010). Recently, the direct role of Aire in T_{Reg} selection was confirmed by demonstrating that generation of naturally expressed MJ23 (prostate cancer specific antigen) specific T_{Reg}s is fully dependent on Aire-driven expression and presentation of MJ23 antigen (Malchow *et al.*, 2013). Along the same lines, the necessity of Aire expression for T_{Reg} selection was confirmed in another recent study which showed that most of the auto-reactive T cells in the periphery of $Aire^{-/-}$ mice are developed from thymocytes which, in the presence of $Aire^{+}$ mTECs, would destined for T_{Reg} selection (Malchow *et al.*, 2016). Similarly, one additional study revealed the importance of the Aire expression for the generation of perinatal T_{Reg}s that functionally differ from those generated in the adulthood (Yang *et al.*, 2015).

Recently, two independent studies investigated the link between the repertoire of peptides presented in thymic medulla and the selection mechanism of central tolerance. They suggested that the decision whether the mechanism of central tolerance inclines to negative selection and/or T_{Reg} selection depends on the peripheral origin of the presented peptides (Legoux *et al.*, 2015) and on the abundance and scope of their expression in the thymic medulla (Malhotra *et al.*, 2016).

However, $Aire^{+}$ mTECs are not the only APCs involved in the establishment of central tolerance. The indispensable role of DCs in the mechanisms of central tolerance was documented by their ablation which led to the development of fatal autoimmunity (Ohnmacht *et al.*, 2009). DCs usually accumulate in the close proximity to mTECs which express Aire-dependent chemokine XCL-1 and attract XCL-1 receptor expressing DCs (Lei *et al.*, 2011). It has been recently suggested that due to this proximity, DCs are able to acquire mTECs-derived antigens (TRAs) and present them to developing thymocytes which is indispensable for their efficient negative selection and T_{Reg} selection (Leventhal *et al.*, 2016; Perry *et al.*, 2014). The latter study suggested that thymic derived $CD8\alpha^{+}$ DCs (tDC) is a predominant subset capable to acquire mTEC-derived antigen and therefore is responsible for selection processes (Perry *et al.*, 2014). Nevertheless, selection of the previously described tumor-specific T_{Reg}s (Malchow *et al.*, 2013) was found to be independent on tDCs (Leventhal *et al.*, 2016). Thus, it is not entirely clear which subset of DCs plays crucial role in selection processes. In this respect it is necessary to mention that DCs in the thymus can be subdivided into three distinct subsets: previously discussed $CD8\alpha^{+}$ tDCs, $Sirp\alpha^{+}$ migratory DCs (mDC) and plasmacytoid DCs (pDC) (Perry and Hsieh, 2016). In contrast to tDCs, mDCs and pDCs are capable to directly present antigens collected in the periphery. They migrate to the thymic medulla

through different pathways and participate in negative selection of auto-reactive thymocytes independently on PGE and Aire (Bonasio *et al.*, 2006; Hadeiba *et al.*, 2012).

Thymic B cells, displaying high expression of MHC II and CD80, are also competent APCs capable to impose negative selection (Perera *et al.*, 2013) and T_{Reg} selection (Walters *et al.*, 2014). This notion is further supported by a recent finding that demonstrates the expression of Aire in thymic B cells. Thus, thymic B cells, together with $mTECs^{Hi}$, represent unique Aire expressing APCs in the thymus. Moreover, their repertoire of Aire-dependent TRAs seems to be partially non-overlapping with that of $mTECs^{Hi}$, suggesting that their repertoires complement each other (Yamano *et al.*, 2015). The contribution of various APCs to the selection processes is summarized in ***Figure 4***.

Before the mTECs become efficient APCs that express a broad repertoire of TRAs, they undergo a complex developmental pathway which is described in the following chapter.

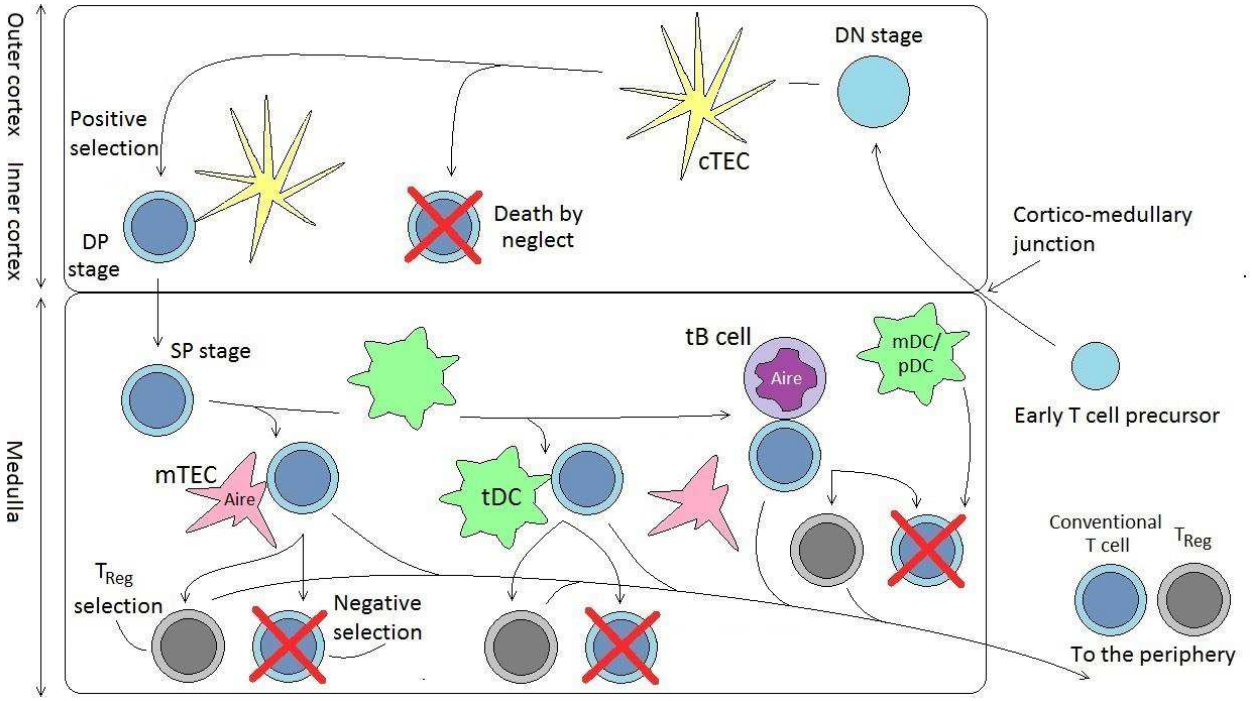

Figure 4) T cell development and selection processes: *Early T cell precursors enter the thymus in cortico-medullary junction. Thymocytes first migrate through the cortex, where they undergo the process of positive selection and differentiate from DN to DP stage. Most of the developing thymocytes do not pass the positive selection and die by neglect. Positively selected SP thymocytes migrate to the medulla, where they encounter mTECs, tDCs and thymic B cells (tB cell). Interaction with those APCs can lead to the negative selection or their deviation into T_{Reg}s. In addition, mDCs and pDCs are also capable to induce the negative selection. Thymocytes which successfully underwent the selection processes then subsequently enter the periphery as conventional T cells or T_{Reg}S.*

2.4.4. mTECs development

As discussed above, mTECs could be subdivided into $mTEC^{Hi}$ and $mTEC^{lo}$ subsets which could be defined according to the expression levels of MHC II and CD80 (Derbinski *et al.*, 2005). Several studies suggested that during the embryonic development $mTEC^{lo}$ subset differentiate to $mTECs^{Hi}$ (Gäbler, Arnold and Kyewski, 2007; Gray *et al.*, 2006; Rossi *et al.*, 2007). However, in the adult thymus only part of the $mTEC^{lo}$ population probably serves as an immature reservoir for $mTECs^{Hi}$ (Gäbler, Arnold and Kyewski, 2007). It is clear that individual cells forming $mTECs^{lo}$ population differ by the expression of CCL21 and involucrin and represent mature cell subsets which participate in attracting of positively selected thymocytes into medulla (Lkhagvasuren *et al.*, 2013) or perform terminally differentiated stage of mTECs, which is called "post Aire" mTECs (Metzger *et al.*, 2013; Nishikawa *et al.*, 2014).

$mTECs^{Hi}$ population also exhibits a certain level of heterogeneity mainly based on the expression of Aire (Gray *et al.*, 2007). $Aire^{+}$ $mTECs^{Hi}$ are characterized by a rapid turnover, likely caused by the expression of Aire which accelerates the apoptosis of these cells (Gray *et al.*, 2007). Hence, mTECs represent a dynamic population of proliferating, continuously differentiating as well as apoptosing cells (Gray *et al.*, 2006). In addition, there is also evidence that the presence of Aire expression is crucial for proper mTEC development, since $Aire^{-/-}$ mTECs display globular shape, altered localization and the inability to reach the terminally differentiated stage (Gillard *et al.*, 2007; Yano *et al.*, 2008). In summary, mTEC population contains functionally and phenotypically distinct cell subsets representing developmental continuum which links their maturation path from $mTECs^{lo}$ to $mTECs^{Hi}$ stages.

TECs in general originate from the endoderm, more specifically from the embryonic third pharyngeal pouch (Gordon *et al.*, 2004) which points to the fact that cTECs and mTECs share a common endodermal origin. Indeed, early studies suggested that embryonic progenitors referred to as $MTS20/24^{+}$ $PLET1^{+}$ cells (Bennett *et al.*, 2002; Depreter *et al.*, 2008) have the capacity to give rise to all TEC subsets (Bennett *et al.*, 2002). Then, the suggestion proposing the existence of a common bipotent TECs progenitor came along with cell-tracing models which demonstrated the ability of single progenitor cell to generate both cTECs and mTECs (Bleul *et al.*, 2006; Rossi *et al.*, 2006). However, these embryonic bipotent progenitors show very limited proliferative and self-renewal potential (Jenkinson *et al.*, 2008), which are important features of stem cells. Bipotent progenitors were shown to express Foxn1 (Corbeaux *et al.*, 2010), the major regulator of thymic organogenesis (Vaidya, Briones Leon and Blackburn, 2016) as well as typical cTEC markers, including CD205 and β5t (Baik *et al.*, 2013; Ohigashi *et al.*, 2013). A current article has even suggested that $mTECs^{Hi}$ can arise from common cTECs which, in the embryonic thymus, exhibit high cellularity and proliferation rate (Brunk *et al.*, 2017).

On the other hand, there is also evidence describing the existence of lineage-committed unipotent progenitors which make up mTEC islands derived from a single cell (Rodewald *et al.*, 2001). It was shown, that embryonic progenitors can be defined by the expression of claudin 3 and claudin 4 (Cld3,4)

(Hamazaki *et al.*, 2007). The stem cell like potential of these cells was confirmed by the expression of SSEA-1 and it was demonstrated that SSEA-1^+ Cld3,4^+ cells possess the ability to generate mTECs but not cTECs. Hence, these unipotent progenitor cells were thereafter named mTEC stem cells (mTECSCs) (Sekai, Hamazaki and Minato, 2014). However, mTECSCs were shown to contribute to the maintenance of mTECs mainly during embryonic and early postnatal development, and only to a very limited , if any extend in adult thymus (Sekai, Hamazaki and Minato, 2014). It was thus suggested that the maintenance of mTECs in the adult thymus is dependent on progenitors distinct from those detected in embryo. Moreover, recent article revealed that mTECs are maintained by unipotent progenitors which, even though are β5t^-, previously expressed β5t marker (Ohigashi *et al.*, 2015). Thus, this finding pointed to the fact that β5t^- unipotent progenitors are derived from embryonic β5t^+ bipotent progenitors. Moreover, β5t^- unipotent progenitors also expressed Cld3,4 (Ohigashi *et al.*, 2015) that are considered as markers of unipotent progenitors of mTECs in the embryo. Importantly, Cld3,4^+ progenitors were previously shown to be dispensable for mTEC maintenance in adults (Hamazaki *et al.*, 2007; Sekai, Hamazaki and Minato, 2014). Contrary to these studies, other authors suggested that the maintenance of mTECs in the adult thymus is mediated by bipotent progenitors. However, since these studies diverged in the observed progenitor phenotypes (Mayer *et al.*, 2016; Meireles *et al.*, 2017; Ucar *et al.*, 2014; Ulyanchenko *et al.*, 2016; Wong *et al.*, 2014), more accurate gene expression profiling and proliferative potential of bipotent progenitors in adult mice still awaits its resolution.

Recent studies, which describe the above mentioned but also phenotypically distinct mTEC unipotent progenitors called junctional TECs (jTEC) or precursors of Aire$^+$ mTECs (pMECs) and possible downstream progenitors of pMECs (pro-PMECs), suggested that NFκB signaling pathway is crucial for their development into mTECs (Akiyama *et al.*, 2016; Baik *et al.*, 2016; Onder *et al.*, 2015; Wu *et al.*, 2017). Moreover, additional evidence suggests a key role of NFκB signaling in development of mTEC subsets and especially of Aire$^+$ mTECs^{Hi} (Akiyama *et al.*, 2008; Boehm *et al.*, 2003; Kajiura *et al.*, 2004). It has been also shown that NFκB signaling cooperates with other crucial molecules for mTEC development including histone deacetylase 3 (HDAC3) and signal transducer and activator of transcription 3 (STAT3) (Goldfarb *et al.*, 2016; Lomada *et al.*, 2016). Altogether, these findings underline the essential role of NFκB signaling in mTEC development.

3. The role of NFκB signaling in establishment of central tolerance

NFκB signaling pathway, which is evolutionary highly conserved, regulates the expression of essential genes which contribute to basic physiological functions as differentiation, proliferation and apoptosis and therefore it is considered indispensable for life. However, NFκB signaling is relevant also for more complex mechanisms, including the initiation of immune responses and establishment of central tolerance, the latter being considered quite a recent evolutionary invention. The mammalian NFκB

signaling is constituted by canonical and non-canonical pathways which activate different set of TFs that are all members of NFκB family. Specifically, mammals encode five members of this family: RelA, RelB, p50, p52 and c-Rel, each endowed with the capacity to bind to κB enhancers from which they can drive gene expression. Canonical pathway preferentially activates TFs RelA, p50 and c-Rel and is triggered mostly by receptors typical for innate immunity, for example Toll like receptors. On the other hand, non-canonical NFκB signaling pathway typically induces action of TFs RelB and p52 and is activated, in the context of central tolerance, by several members of the tumor necrosis factor receptor superfamily, namely, by the Receptor activator of NFκB (RANK), CD40 and Lymphotoxin β receptor (LtβR). It is important to emphasize that the non-canonical NFκB signaling can be sub-divided according to its intracellular transducers to classical (NIK, IKKα, TRAF3, RelB, p52) and non-classical (TRAF6, RelA, RelB, c-Rel) pathways (Sun, 2011). Importantly, while it is now clear that the crosstalk between both classical and non-classical non-canonical pathways is necessary for establishment of central tolerance, the most current evidence describes the role of classical non-canonical NFκB signaling in this phenomenon (van Delft, Huitema and Tas, 2015). Therefore the following chapters are mostly dedicated to classical non-canonical NFκB signaling. The next chapter introduces its molecular components in the context of signal transduction.

3.1. Signal transduction pathway of non-canonical NFκB signaling

The receptors of non-canonical NFκB signaling mentioned above, RANK, CD40 and LtβR, are activated by its ligands RANKL, CD40L and $Lt\alpha_1\beta_2$, respectively (Locksley, Killeen and Lenardo, 2001). If the receptors are not engaged by their cognate ligands, TRAF3-TRAF2-cIAP1/2 ubiquitin ligase complex binds NFκB-inducing kinase (NIK) via TNF receptor-associated factor 3 (TRAF3) (Liao *et al.*, 2004), thus causing NIK ubiquitination and its subsequent degradation by proteasome. Upon receptor engagement, the TRAF3-TRAF2-cIAP1/2 complex, instead of NIK, binds the activated receptors and ubiquitinates self on TRAF3 and TRAF2, which causes the degradation of this complex (Vallabhapurapu *et al.*, 2008; Zarnegar *et al.*, 2008). Unengaged and free NIK then activates its downstream kinase IκB kinase-α (IKKα) and both kinases phosphorylate the inactive precursor of p52 called p100 (Senftleben *et al.*, 2001; Xiao, Harhaj and Sun, 2001). p100, which on it C-terminus contains Ankyrin inhibitory domain (AID), resides in the cytoplasm, forms a heterodimer with RelB TF and blocks its nuclear translocation (Solan *et al.*, 2002). Phosphorylation of p100 attracts the SCF ubiquitin ligase which ubiquitinates p100, leading to a proteasomal removal of AID and truncation of p100 into p52 (Liang, Zhang and Sun, 2006). Newly formed p52 constitutes active heterodimer with RelB and they together translocate into the nucleus to enhance the expression of target genes via binding to their κB enhancers. The inactivation and activation of non-canonical NFκB signaling is depicted in **(*Figure 5*)**.

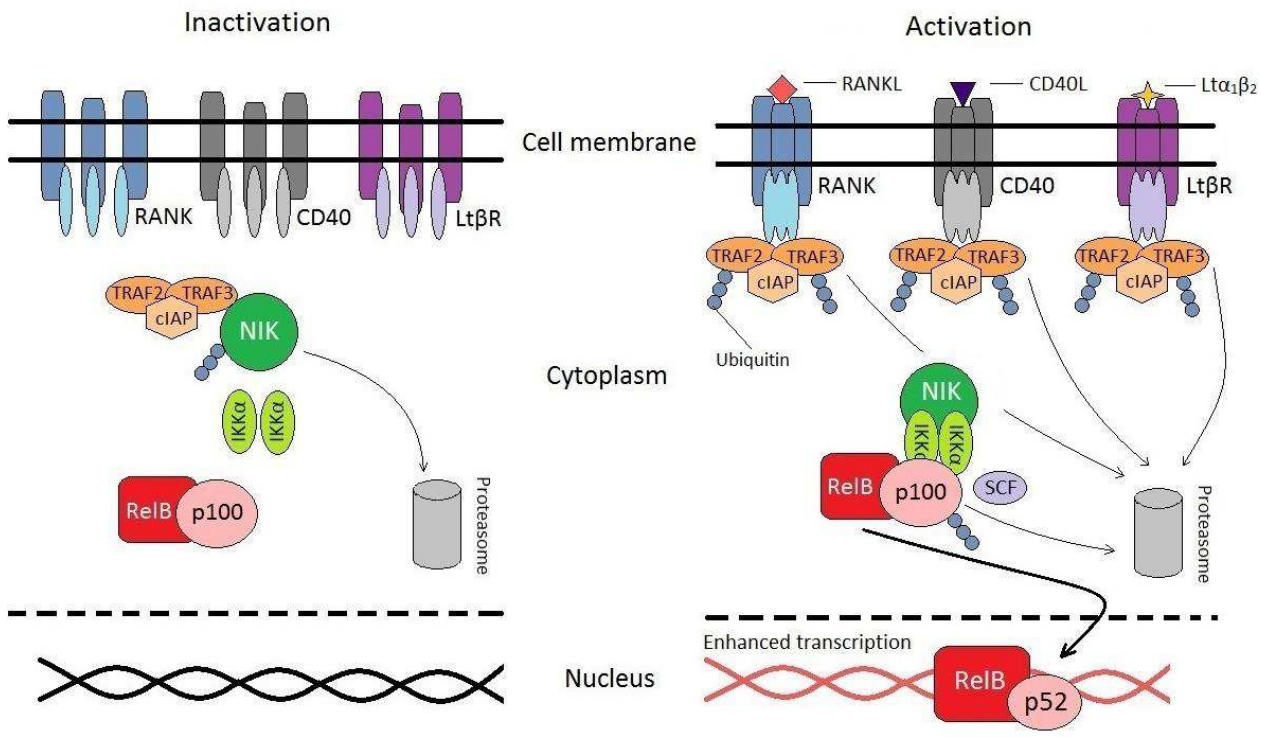

Figure 5) Inactivation and activation of non-canonical NFκB signaling: *Inactive receptors don´t bind the TRAF3-TRAF2-cIAP1/2 (cIAP) ubiquitin ligase complex which ubiquitinates NIK. Ubiquitinated NIK is degraded by proteasome. RelB/p100 heterodimer complex is not decayed and remains in the cytoplasm. The signal transduction is then suspended. When receptors are activated by their ligands, TRAF3-TRAF2-cIAP ubiquitin ligase complex binds to activated receptors and is self ubiquitinated what causes their degradation by proteasome. NIK phosphorylates IKKα and they cooperate in phosphorylation of p100 that constitutes binding site for SCF ubiquitin ligase. p100 is ubiquitinated by SCF which results to decay of p100 into p52. RelB/p52 heterodimers then translocate into nucleus to enhance the transcription of various genes. Inspired by* (Sun, 2011).

3.2. Contribution of individual components of NFκB signaling to central tolerance

The role of non-canonical NFκB signaling pathway in establishment of central tolerance was firstly documented by experiments with $RelB^{-/-}$ mice which revealed severe defects of thymic medulla and manifestations of multi-organ autoimmunity (Burkly *et al.*, 1995; Weih *et al.*, 1995). The following years brought comprehensive analyses of individual contributions of non-canonical NFκB signaling molecular components (mostly the classical, but also the non-classical) to central tolerance. Results of these experiments are summarized in the next chapter which is divided according to the function of these components in the signaling cascade to two sub-chapters: I) Receptors II) Intracellular transducers.

3.2.1. Receptors of the non-canonical NFκB signaling pathway

Each of the previously described receptors (RANK, CD40 and LtβR) transduces signals that cause different effects on the establishment of central tolerance. However, it is important to note that signaling through these receptors is also mutually interconnected and dependent on each other as it is discussed below.

mTECs and especially mTECs^{Hi} in the embryonic thymus were found to express RANK. The stimulation of RANK by RANK ligand (RANKL) resulted in increased mTEC^{Hi} cellularity and up-regulation of Aire-dependent TRAs expression which pointed to the fact that RANK signaling contributes to mTECs development. The role of RANK signaling in mTECs development was further confirmed by using RANK$^{-/-}$ mice which revealed almost complete absence of mTECs^{Hi}. In addition, the transplantation of RANK$^{-/-}$ thymus into athymic nude mice led to the development of severe autoimmunity (Rossi *et al.*, 2007). The following comprehensive study further demonstrated clear differences between RANK signaling in embryonic and adult thymus. While the embryonic thymus of RANKL$^{-/-}$ mice revealed the lack of mTECs, this insufficiency was partially restored few days after birth, suggesting additional requirement for signaling supporting mTEC development in postnatal/adult thymus (Akiyama et al., 2008). The receptor CD40, which was previously found to impact the development of mTECs and thymic medulla (Dunn et al., 1997; Gray et al., 2006) as well as T_{Reg} selection (Guiducci et al., 2005; Kumanogoh *et al.*, 2001; Spence and Green, 2008), showed its high expression in mTECs of adult mice and therefore was proposed to cooperate with RANK. This hypothesis was confirmed by the construction of double deficient RANKL$^{-/-}$ CD40$^{-/-}$ mice which demonstrated an altered thymic morphology including undefined CMJ and almost entire loss of UEA1^{+} mTECs, mTECs^{Hi}, Cld3,4^{+} mTEC progenitors and the expression of Aire, which is much more pronounced impact in comparison to RANKL$^{-/-}$ adult mice, suggesting abrogated development of whole mature mTEC compartment (Akiyama et al., 2008). The transplantation of double KO (RANKL$^{-/-}$ CD40$^{-/-}$) thymus into athymic nude mice revealed a more severe phenotype than the single RANKL$^{-/-}$ mice. On the other hand, single CD40$^{-/-}$ mice showed negligible defect in Aire^{+} mTECs^{Hi} and no autoimmune manifestations (Akiyama *et al.*, 2008). In addition, more recent study revealed that the CD40 signaling is directly triggered by RANK signaling, in the postnatal mice (Desanti et al., 2012).

Despite the fact that RANK and CD40 signaling are very important for Aire-driven mechanisms of central tolerance, the cellular sources of RANKL and CD40 ligand (CD40L) are still obscure. It was suggested that the source of RANKL in the embryo are lymphoid tissue inducer (Lti) cells and dendritic T cell epidermal progenitors (DETC) (Roberts *et al.*, 2012; Rossi *et al.*, 2007), whereas the source of RANKL and CD40L in the adult thymus should be positively selected T cells (Desanti et al., 2012; Hikosaka et al., 2008; Irla et al., 2008).

As disclosed above, RANK signaling crucially contributes to the development of mTECs and increases its cellularity. Nevertheless, targets of RANK signaling are affected also by Osteoprotegerin (OPG) which operates as its negative regulator (Hikosaka *et al.*, 2008). OPG is a soluble non-signaling RANKL decoy receptor expressed strictly by Aire^{+} mTECs^{Hi} in the thymus (McCarthy et al., 2015). The usage of OPG$^{-/-}$ mice clearly demonstrated altered cellularity of mTECs^{Hi} (Hikosaka et al., 2008*;* Khan *et al.*, 2014). OPG expression was shown to be highly dependent on TF Spi-b, the expression of which is triggered by RANK signaling. This suggests a negative feedback between RANK signaling and OPG which directly controls mTEC cellularity (Akiyama *et al.*, 2014).

Together with RANK and CD40, LtβR signaling also participates in establishment of central tolerance. First study focused on the LtβR signaling in this context revealed that LtβR$^{-/-}$ mice suffer from decreased mTEC cellularity and disrupted three-dimensional scaffold (3DS) of mTECs (Boehm *et al.*, 2003). However, the expression of Aire was found to be unaffected, in contrast to contemporary study (Chin *et al.*, 2003). Consistently with defects of medulla, LtβR$^{-/-}$ mice revealed signs of autoimmunity against several organs (Boehm *et al.*, 2003; Chin *et al.*, 2003). Microarray analysis of mTECs sorted from LtβR$^{-/-}$ mice confirmed that LtβR signaling doesn´t influence expression of the Aire and Aire-dependent TRAs (Venanzi *et al.*, 2007). Despite the fact that LtβR$^{-/-}$ and lymphotoxin α (Ltα$^{-/-}$) mice displayed decreased cellularity and disrupted 3DS of mTECs, the T_{Reg} selection was completely unaffected (Martins, Boehm and Bleul, 2008; Venanzi *et al.*, 2007; Zhu *et al.*, 2007). The additional study demonstrated that signaling through LtβR influenced the migration of thymocytes between cortex and medulla by regulation of chemokines CCL19 and CCL21 (Zhu *et al.*, 2007), which are also crucial for establishment of central tolerance (Kurobe *et al.*, 2006; Laan *et al.*, 2009). Indeed, qPCR analysis revealed that expression of CCL19 and CCL21 is reduced in LtβR$^{-/-}$ mice which furthermore revealed a comparable phenotype of cortex medulla migration with CCL19$^{-/-}$ and CCL21$^{-/-}$ mice (Zhu *et al.*, 2007). Altogether, these findings point to the fact that the breakdown of central tolerance in LtβR$^{-/-}$ mice could be caused by abrogated chemokine expression. Subsequent study specified that the deficiency in LtβR signaling causes the loss of mainly mature mTECs^{lo} rather than Aire^{+} mTECs^{Hi} and confirmed reduced expression of CCL19 and some Aire-independent TRAs, namely CRP and type-2 collagen (Seach *et al.*, 2008). Finally, previous findings were summarized in a recent study which suggested that LtβR signaling specifically influences newly established mature CCL21^{+} mTEC^{lo} subset and not the Aire^{+} mTECs^{Hi} (Lkhagvasuren *et al.*, 2013). Hypothetically, CCL21^{+} mTECs^{lo} could belong to the involucrin^{+} post-Aire expressing mTEC^{lo} subset, which also require LtβR signaling for their development (White *et al.*, 2010). Another recent study described the role of LtβR signaling in homeostasis of mTEC progenitors. Conditional KO of LtβR caused decreased mTEC cellularity, disruption of 3DS and impaired negative selection as it was shown in LtβR$^{-/-}$ mice. However, this effect was observed only in case of postnatal/adult mice and not during the embryonic development. This study concluded that LtβR signaling in the adult mice influences the

cellularity of previously discussed mTECSCs, probably by regulation of their differentiation from specific progenitors, and therefore the LtβR deficiency causes medullary defects (Wu *et al.*, 2017).

The contribution of RANK and LtβR signaling to the central tolerance could be summarized by a recent article which underlines their different roles. RANK signaling is considered to drive mainly the development of mTECs^{Hi} and the expression of Aire. On the other hand LtβR signaling which influences development of both mTECs^{Hi} and mTECs^{lo}, plays a crucial role in the regulation of expression of specific chemokines CCL19 and CCL21, which are involved in the cortex-medulla migration of thymocytes and establishment of central tolerance (Bichele *et al.*, 2016). This evidence doesn´t exclude the cooperation between these two signaling pathways mainly in the embryonic thymus, where the LtβR signaling enhances the expression of RANK (Bichele *et al.*, 2016; Mouri *et al.*, 2011) This observation is supported by the fact that double KO (LtβR$^{-/-}$ RANKL$^{-/-}$) mice showed even more pronounced medullary defect than that observed in single LtβR$^{-/-}$ or RANKL$^{-/-}$ mice alone (Mouri *et al.*, 2011).

3.2.2. Intracellular transducers of the non-canonical NFκB signaling pathway

The engagement of the receptors converges into the intracellular signaling cascade driven by kinases and TFs which are shared among all three (RANK, CD40 and LtβR) receptors of non-canonical NFκB signaling pathway. For this reason, KOs of intracellular transducers cause much more pronounced defects in establishment of central tolerance in comparison with KOs of individual receptors. As it was shown in chapter 3.1., the most upstream major intracellular component of non-canonical NFκB signaling pathway is NIK. The role of NIK in establishment of central tolerance was discovered using the Alymphoplasia (Aly/Aly) mice carrying non-functional mutation in NIK gene (NIK$^{Aly/Aly}$) (Shinkura *et al.*, 1999). These mice demonstrated a disturbed thymic architecture manifested by a smaller medulla, undefined CMJ and decreased number of mTECs^{Hi} (Kajiura *et al.*, 2004). Grafting of NIK$^{Aly/Aly}$ thymus into the nude mice led to the development of autoimmunity in the liver and pancreas. This was in agreement with the fact that NIK$^{Aly/Aly}$ thymus almost entirely lacks the expression of Aire and TRAs and consequently impacts both T_{Reg} and negative selections (Kajiura *et al.*, 2004; Mouri *et al.*, 2014; Murray, 2013). Moreover, NIK$^{Aly/Aly}$ mice also revealed reduced expression of TF RelB and increased frequency of p100 (Kajiura *et al.*, 2004). In addition, NIK-dependent signaling was found to be also crucial for development of DCs in the thymus and their efficient function in the central tolerance (Hofmann *et al.*, 2011; Mouri *et al.*, 2014). Another study also revealed that non-canonical NFκB signaling via NIK is crucial for the development of specific TEC subset, namely differentiation of previously mentioned jTECs, which reside in CMJ, into mTECs. Notably, medullary conditional KO of NIK caused accumulation of jTECs in whole medulla and completely blocked the generation of mature mTECs (Onder *et al.*, 2015). Similar phenotype with NIK$^{Aly/Aly}$ mice was observed in mice deficient for the non-classical component of non-canonical NFκB signaling pathway referred to as TNF receptor-associated factor 6 (TRAF6) (Akiyama

et al., 2005). In this context, it is important to emphasize that efficient signaling from RANK and CD40 receptors requires both TRAF6 and NIK components (Akiyama *et al.*, 2008; Mouri *et al.*, 2011), whereas LtβR signaling is dependent only on its classical component NIK (Mouri *et al.*, 2011).

Similar to TRAF6 KO, the mice deficient in NIK´s downstream kinase IKKα showed nearly identic phenotype as $NIK^{Aly/Aly}$ mice, resembling its medullary defects and autoimmune manifestations. IKKα deficiency moreover led to the reduced expression of chemokines CCL19 and CCL21 (Kinoshita *et al.*, 2006; Lomada *et al.*, 2007).

As it was discussed in chapter 3.1., the phosphorylation of p100 by NIK and IKKα leads to the translocation of RelB/p52 complex into the nucleus. The important role of RelB in the mechanisms of central tolerance was revealed by the detection of multi-organ autoimmunity in $RelB^{-/-}$ mice (Weih *et al.*, 1995). Additional studies revealed an atrophic thymic medulla, undefined CMJ, loss of $UEA1^{+}$ mTECs and thymic DCs and undetectable expression of Aire in $RelB^{-/-}$ mice (Burkly *et al.*, 1995; Heino *et al.*, 2000). Another insight into the role of RelB in the thymus was shown by using a conditional KO of RelB, in the thymus, constructed via LoxP sites flanking the RelB gene and Cre-recombinase expressed under Foxn1 promoter. The results showed the absence of both population of $mTECs^{Hi}$ ($Aire^{+}$ and $Aire^{-}$), decreased number of $mTECs^{lo}$ and also the loss of $Cld3,4^{+}$ mTEC progenitors, suggesting that the development of mTECs was stuck at very early developmental stage. Consistently with the loss of $mTECs^{Hi}$, the expression of Aire-dependent and independent TRAs was also reduced. Moreover, RelB deficiency in TECs caused reduction of T_{Reg}s and tDCs, suggesting developmental link between mTECs and tDC (Riemann *et al.*, 2017). Somewhat surprisingly, all the observed defects in RelB conditional KO were also, however to a lesser extent, detected in mice with TECs lacking RelA, the TF of non-classical non-canonical NFκB signaling pathway. RelA together with other components of this pathway namely, c-Rel and TRAF6 were found to enhance the expression of RelB (Riemann *et al.*, 2017). Hence, this study suggests that the role of non-classical non-canonical NFκB signaling pathway in establishment of central tolerance is to enhance the expression of RelB (Riemann *et al.*, 2017).

The deficiency in NFκB2, the gene encoding TF p52 and its precursor p100, was also found to influence mTECs development and central tolerance. However, it is important to note that the defects were not that pronounced as in $NIK^{Aly/Aly}$, $TRAF6^{-/-}$, $IKK\alpha^{-/-}$, or $RelB^{-/-}$ mice (Zhang *et al.*, 2006; Zhu *et al.*, 2006). $NFκB2^{-/-}$ mice revealed the reduction in $UEA1^{+}$ cells and almost complete lack of $mTECs^{Hi}$ (Zhang *et al.*, 2006; Zhu *et al.*, 2006). Furthermore, $NFκB2^{-/-}$ mice displayed breakdown of central tolerance, which was not caused by impaired T_{Reg} selection, followed by multi-organ autoimmunity (Zhang *et al.*, 2006; Zhu *et al.*, 2006).

The contribution of classical non-canonical NFκB components to the establishment of central tolerance is summarized in more detail in ***Table 1***.

KO	Medullary defects						Autoimmune manifestations						References
	Architecture	mTECs	mTECs Hi	Aire	TRA	T Reg	Li	Lu	P	K	SG	R	
RANK													Rossi et. al. 2007
RANKL													Akiyama et. al. 2008
RANKL/ CD40													Akiyama et. al. 2008
LtβR													Boehm et. al. 2003 Zhu et. al. 2007
$NIK^{Aly/Aly}$													Kajiura et. al. 2004
IKKα													Kinoshita et. al. 2006 Lomada et. al. 2007
RelB													Riemann et. al. 2017
NFκB2													Zhang et. al. 2006 Zhu et. al. 2006
CNS1			*										LaFlam et. al. 2015
Aire (B6)			*										Hubert et. al. 2009
Aire (BALBc)													Jiang et. al. 2005

Legend		
Not detected	**Detected**	**Undetermined**

Table 1) Contribution of individual non-canonical NFκB signaling components to establishment of central tolerance: *The table provides a summary of medullary defects and organ specific autoimmune manifestations (lymphocytic infiltrations) in mice with knocked out individual components of the classical non-canonical NFκB signaling pathway. CNS1 knockout mouse, which is the main topic of chapter 3.3., and Aire knockouts on different backgrounds are shown here for comparison. Abbreviations: * Increased $mTEC^{Hi}$ numbers; Li: Liver; Lu: Lung; P: Pancreas; K: Kidney; SG: Salivary gland; R: Retina*

The generation of KO animals with individual deficiencies in NFκB signaling components, enabled to gain insight into the processes underpinning the differentiation of mTEC progenitors into mTECs. Based on the evidence that RANK signaling directs the development of $Aire^+$ mTECs, the potential precursors of $Aire^+$ mTECs (pMEC), defined as $RANK^+UEA1^+MHC\ II^{Mid}CD80^-$, were identified. These precursors also expressed cTEC markers, such as keratin 8 and β5t (Akiyama *et al.*, 2016). Indeed, pMECs were found to give rise to $Aire^+$ mTECs after their addition into thymic culture from $NIK^{Aly/Aly}$ mice. The differentiation of pMECs into $Aire^+$ mTECs was found to be dependent on the expression of TRAF6, as the $TRAF6^{-/-}$ thymus revealed the absence of $Aire^+$ mTECs and accumulation of pMECs. On the other hand, the deficiency in RelB caused the developmental arrest on another precursor stage defined as $UEA1^+RANK^{lo}MHC\ II^{lo}$ $CD24^{Hi}$ (CD24 is a stem cell marker). These cells were named progenitors of pMECs (pro-pMECs), as they could hypothetically give rise to pMECs, and they were found to give rise to $Aire^+$ mTECs. Finally, the

progressive differentiation of pro-pMECs was shown to require also RANK and LtβR signaling. Altogether, this study suggested that the differentiation of pro-pMECs into the downstream developmental stages requires RelB, RANK and LtβR, suggesting the contribution of classical non-canonical NFκB signaling pathway to this process. On the other hand, the differentiation of pMECs into Aire^{+} mTECs was found to be RANK- and TRAF6-dependent which indicates the involvement of non-classical non-canonical NFκB signaling pathway (Akiyama *et al.*, 2016). Another recent study also mapped the expression of RANK in Cld3,4^{+} progenitors and revealed that their previously discussed SSEA-1^{+} subset, here referred to as mTECSCs (Sekai, Hamazaki and Minato, 2014), don´t express RANK and emerge independently on RelB. Contrary, their Cld3,4^{+} SSEA-1^{-} descendants (Hamazaki *et al.*, 2007) were found to be RANK^{+} and their presence and further differentiation into immature mTECs^{lo} and mTECs^{Hi} was shown to be entirely dependent on the expression of RelB. Thus, the differentiation of mTECSCs into mTECs requires non-canonical NFκB signaling pathway for its implementation (Baik *et al.*, 2016). Hence, although the knowledge about the nature, phenotype and gene expression landscape of mTEC progenitors still remains uncertain, as discussed above, it is clear that NFκB signaling is critically required for their differentiation into mTECs.

Altogether, the chapter 3.2. summarizes data which provide strong evidence that NFκB signaling is essential for establishment of central tolerance. In addition, the past two years brought two new reports that described a new phenomenon related to a direct regulation of Aire expression by NFκB via binding to its κB enhancers. To this discovery is dedicated the last chapter of my thesis.

3.3. Direct regulation of Aire expression by NFκB signaling

As it was described in a more detail in the chapter 2.4.2., the recent study analyzed the Aire gene methylation pattern in various cell types. mTECs and especially mTECs^{Hi} showed strong demethylation in the region of Aire gene, arguing for the presence of chromatin regions accessible to TFs. Indeed, the demethylation of Aire gene in mTECs^{Hi} enabled recruitment of multimolecular complex of TFs which induced its expression (Herzig *et al.*, 2016). Presumably, a relaxed structure of chromatin in mTECs^{Hi} could be more accessible also to TFs of NFκB family which might induce the expression of Aire. This assumption was strongly supported by the fact that the deficiency in majority of the above described individual NFκB signaling components resulted in the reduction or loss of Aire expression and Aire-dependent TRAs. These studies, however, assumed that impaired Aire expression is the consequence of mainly developmental defects of mTECs caused by defected NFκB signaling.

Recently, the hypothetical cis-regulatory element of Aire gene, already described in early study (Blechschmidt *et al.*, 1999), underwent further functional analysis. It revealed that this region, located approximately 3 kilo-base pairs upstream from the Aire promoter, contains two highly conserved κB enhancer sites, indicating possible direct role of NFκB signaling in the inducement of Aire gene

expression (Haljasorg *et al.*, 2015). This region was named as the conserved non-coding sequence 1 (CNS1) (also referred to as ACNS1). To further scrutinize its function, $CNS1^{-/-}$ mouse strain was constructed. $CNS1^{-/-}$ mice exhibited a complete loss of Aire expression on both, mRNA and protein level. Consistently with loss of Aire, the expression of Aire-dependent TRAs was severely reduced, whereas the expression of Aire-independent TRAs remained on its normal levels. Similarly to $Aire^{-/-}$ mice, the cellularity of CD4/CD8 SP thymocytes in the $CNS1^{-/-}$ thymus was comparable with WT mice (Anderson *et al.*, 2002), suggesting their normal development. Nevertheless, the deviation of thymocytes into T_{Reg} lineage in $CNS1^{-/-}$ mice was impaired (Haljasorg *et al.*, 2015). Furthermore, the $CNS1^{-/-}$ mice revealed unaffected thymic architecture, with slightly increased number of $mTECs^{Hi}$ (Haljasorg *et al.*, 2015), which is again typical phenotype of $Aire^{-/-}$ mice (Hubert *et al.*, 2009). On top of that, mTECs from the $CNS1^{-/-}$ thymus were found unable to reach the terminal differentiation stage (Haljasorg *et al.*, 2015).

Several studies detected the expression of Aire also in the periphery, specifically in spleen, lymph nodes and testes (Gardner *et al.*, 2008; Schaller *et al.*, 2008). Besides the absence of Aire expression in the thymus, $CNS1^{-/-}$ mice did not reveal expression of Aire in the spleen and lymph nodes, whereas its expression was detectable in testes. This suggests, that CNS1 specifically regulates the expression of Aire in lymphoid organs (Haljasorg *et al.*, 2015).

To verify, that CNS1 directly enhances the expression of Aire (CNS1 proximal gene) after the stimulation of various TFs of NFκB family, the reporter, which contained CNS1 upstream of the interferon-β minimal promoter followed by luciferase, was constructed and transfected into human embryonic kidney 293 (HEK 293) cells together with vectors expressing NFκB TFs (Haljasorg *et al.*, 2015). While the classical components of non-canonical NFκB signaling pathway, the RelB and p52, were found to enhance the expression of luciferase only negligibly, the components of the non-classical non-canonical pathways, the RelA, c-Rel and p50, considerably enhanced the activity of luciferase, from both κB enhancers. Moreover, the mutations of individual or both κB enhancers completely abrogated the enhancement of luciferase activity, even in the presence of RelA and p50, suggesting that κB enhancers act synergistically and the presence of both is needed for their proper function (Haljasorg *et al.*, 2015).

Previous studies, focused on RANK signaling, revealed that stimulation of mTECs with RANKL increased the number of Aire expressing mTECs (Hikosaka *et al.*, 2008; Rossi *et al.*, 2007). To find out, whether RANK signaling directly induces the expression of Aire in $CNS1^{-/-}$ mTECs , thymocyte-free fetal thymic organ culture (FTOC) was stimulated by RANKL. Data showed no up-regulation of Aire expression, suggesting that RANK signaling directly triggers the expression of Aire which acts via κB enhancers **(*Figure 6*)** (Haljasorg *et al.*, 2015).

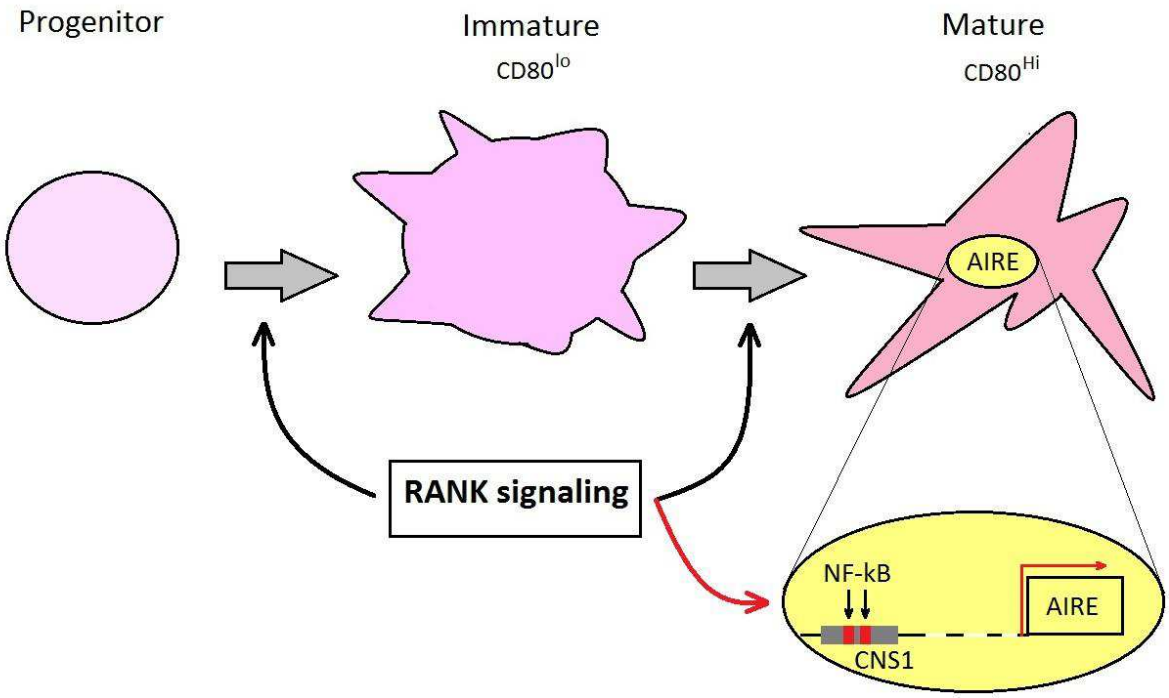

Figure 6) Dual role of RANK signaling in establishment of central tolerance: *RANK signaling is required for development of mTEC progenitors into its immature subset which gives rise to mature mTECs. The expression of Aire in mTECs^{Hi} is also induced by RANK signaling which translates into the binding of NFκB TFs to the CNS1. Hence, RANK signaling plays a dual role in mTECs^{Hi}: first it mediates their maturation, and second, it directly induces the expression of Aire. Inspired by* (Matsumoto, 2015)

While the above discussed study failed to detect signs of autoimmunity against any organ tested (Haljasorg *et al.*, 2015), the second study, also using CNS1$^{-/-}$ mice on "autoimmune-resistant" C57BL/6 genetic background, detected a mild lymphocytic infiltrates in lacrimal glands, salivary glands and retina (LaFlam *et al.*, 2015). Moreover, specific autoantibodies against retinal TRA IRBP (DeVoss *et al.*, 2006) were also detected (LaFlam *et al.*, 2015). In this context, it is important to emphasize that Aire$^{-/-}$ mice constructed on the same genetic background as CNS1$^{-/-}$ (C57BL/6), revealed very comparable signs of autoimmunity (Hubert *et al.*, 2009).

Apart from differences in autoimmune manifestations, the phenotype of thymic medulla was comparable in both studies using CNS1$^{-/-}$ mice. Nevertheless, in contrast with Haljasorg *et. al.*, using analogous in vitro reporter transfections into HEK 293 cells in the second mentioned study revealed a significant enhancement of the proximal gene via CNS1 by RelB and p52, the members of classical non-canonical NFκB signaling pathway (LaFlam et al., 2015).

Thus, even though it is not entirely clear which TFs of the previously discussed NFκB pathways play primary role in triggering the expression of Aire or whether they act simultaneously in this process, it is apparent that NFκB signaling, triggered by RANK, is a major inducer of Aire expression in mTECs.

4. Summary and discussion

The past two decades brought fundamental knowledge related to T cell central tolerance. The turning point came in 1997 with the discovery of the human Autoimmune-regulator (AIRE) gene, the mutations of which cause severe autoimmune syndrome, referred to as APECED. Subsequently, the murine Aire gene was found to be a homologue of human AIRE and mouse was suggested to be a suitable model for studying Aire structure and functions. In the meantime, during 1990´s, the ectopic promiscuous gene expression (PGE) of tissue restricted antigens (TRA) was detected in the thymic medulla and this phenomenon in the beginning of the millennium was attributed to medullary thymic epithelial cells (mTECs). Crucial point for further studies in the field of central tolerance was the introduction of Aire knouckout ($Aire^{-/-}$) mice in 2002 which revealed that Aire is the missing link, which is specifically expressed by mTECs, where it acts as a key regulator of PGE. $Aire^{-/-}$ mice also revealed signs of multi-organ autoimmunity, indicating that the absence of Aire has a negative impact on selection processes associated with mechanisms of central tolerance. This hypothesis was confirmed by various studies which were based mostly on transgenic mice models which generated T cells specific to a given neo-self antigen that functionally mimics the TRA in these studies. Aire was found to be crucial for both selection mechanisms the negative selection of self-reactive T cells as well as their deviation into T-regulatory cells (T_{Reg}). To fulfill these tasks, mTECs were characterized as very efficient antigen presenting cells (APC) expressing high levels of MHC molecules and auxiliary co-stimulatory receptors necessary for these selection processes.

Concurrently with the above analyzed discoveries, the research focused on the involvement of NFκB signaling pathway in central tolerance took place. It was found out that both the classical and non-classical non-canonical NFκB signaling pathways, which differ in their intracellular transducers, participate and shape the outcome of central tolerance. Specifically, both share the key role in establishment of central tolerance, the role which has been illustrated using various mice models with tissue specific deficiency in a particular component of NFκB signaling. These experiments provided a consensus that impaired NFκB signaling leads to altered organization of medulla, impaired development of mTEC subsets and loss of Aire and TRAs expression. These mice furthermore displayed multi-organ autoimmunity which resembles the phenotype observed in $Aire^{-/-}$ mice.

More recent studies focused on the mode of Aire on molecular level revealed that it requires nearly fifty interaction partners for its proper function. Furthermore, the role of thymic DCs in central tolerance was re-evaluated with emphasis on their contribution to T_{Reg} selection. In addition, thymic B cells were shown to play an important role in establishment of central tolerance as well, which is enhanced by the fact that thymic B cells can be licensed to express Aire. Importantly, extensive knowledge concerning

mTECs development has been also accumulated in the literature which provides multitude of important cues for further advancement in this rapidly developing field of research.

Nevertheless, the crucial knowledge and somewhat unexpected discoveries directly related to the topic of my thesis were brought by studies from the past two years which have described direct role of NFκB signaling in two essential processes for central tolerance. First, NFκB signaling was shown to be indispensable for maintenance and progressive differentiation of mTEC stem cells (mTECSC), mTEC progenitors and in general immature mTECs to mature mTECs, including the $Aire^{+}$ $mTEC^{Hi}$ subset. Second, NFκB signaling was found to drive the expression of Aire gene from the conserved non-coding sequence 1 (CNS1) which contains two κB enhancer sites. When these sites were knocked out, affected mice displayed phenotype comparable with that of $Aire^{-/-}$ mice, suggesting that NFκB signaling pathway is the major regulator of Aire expression. Given that NFκB signaling plays a dual role in central tolerance by being indispensable for the generation of mTECs as well as expression of Aire in these cells, these recent findings implicate NFκB signaling as the master regulator of critical processes associated with the development, establishment and function of central tolerance in thymic medulla, either directly or indirectly.

Because the current evidence, which suggests direct regulation of Aire expression by NFκB signaling, is mostly based on *in vitro* experiments, future studies should further disclose for example which transcription factors (TF) of NFκB family specifically trigger the expression of Aire via CNS1 and many other aspects accompanying the regulation of Aire gene expression in more physiological conditions. The future discovery of more specific phenotypes of mTEC progenitors and precise description of their developmental lineage up to mature mTECs will on the other hand support to broaden the knowledge about the role of NFκB signaling in mTECs development. In general, the research of central tolerance is largely limited by the experimentally available number of its main cell type mTECs, which, in addition, are very tricky to isolate and work with as they rapidly change their gene expression profile after being withdrawn from their natural thymic environment. For these and other reasons, a large junk of data are inherently burden with relevant experimental uncontrollable factors that might lower their informative value.

Finally, it is important to mention that due to only recent acquisition of the knowledge about processes regulating mechanisms of central tolerance and Aire expression, their clinical utility has been so far unexplored. However, given the rapid progress in this field, exploitation of available data for translational medicine might not be that remote and unreachable in a very near future.

5. References

Aaltonen, J., Bjorses, P., Perheentupa, J., Horelli-Kuitunen, N., Palotie, A., Paltonen, L. and Su Lee, Y. (1997) 'An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains.', *Nature genetics*, 17, pp. 399–403. doi: 10.1016/j.cell.2009.12.030.

Abramson, J., Giraud, M., Benoist, C. and Mathis, D. (2010) 'Aire's Partners in the Molecular Control of Immunological Tolerance', *Cell*. Elsevier Ltd, 140(1), pp. 123–135. doi: 10.1016/j.cell.2009.12.030.

Abramson, J. and Husebye, E. S. (2016) 'Autoimmune regulator and self-tolerance - molecular and clinical aspects', *Immunological Reviews*, 271(1), pp. 127–140. doi: 10.1111/imr.12419. **Review**

Akiyama, N., Shinzawa, M., Miyauchi, M., Yanai, H., Tateishi, R., Shimo, Y., Ohshima, D., Matsuo, K., Sasaki, I., Hoshino, K., Wu, G., Yagi, S., Inoue, J., Kaisho, T. and Akiyama, T. (2014) 'Limitation of immune tolerance-inducing thymic epithelial cell development by Spi-B-mediated negative feedback regulation.', *The Journal of experimental medicine*, 211(12), pp. 2425–38. doi: 10.1084/jem.20141207.

Akiyama, N., Takizawa, N., Miyauchi, M., Yanai, H., Tateishi, R., Shinzawa, M., Yoshinaga, R., Kurihara, M., Demizu, Y., Yasuda, H., Yagi, S., Wu, G., Matsumoto, M., Sakamoto, R., Yoshida, N., Penninger, J. M., Kobayashi, Y., Inoue, J. and Akiyama, T. (2016) 'Identification of embryonic precursor cells that differentiate into thymic epithelial cells expressing autoimmune regulator', *J Exp Med*, 213(8), pp. 1441–1458. doi: 10.1084/jem.20151780.

Akiyama, T., Maeda, S., Yamane, S., Ogino, K., Kasai, M., Kajiura, F., Matsumoto, M. and Inoue, J. (2005) 'Dependence of Self-Tolerance on TRAF6-Directed Development of Thymic Stroma', *Science*, 308(5719), pp. 248–251. doi: 10.1126/science.1105677.

Akiyama, T., Shimo, Y., Yanai, H., Qin, J., Ohshima, D., Maruyama, Y., Asaumi, Y., Kitazawa, J., Takayanagi, H., Penninger, J. M., Matsumoto, M., Nitta, T., Takahama, Y. and Inoue, J. ichiro (2008) 'The Tumor Necrosis Factor Family Receptors RANK and CD40 Cooperatively Establish the Thymic Medullary Microenvironment and Self-Tolerance', *Immunity*, 29(3), pp. 423–437. doi: 10.1016/j.immuni.2008.06.015.

Akiyoshi, H., Hatakeyama, S., Pitkanen, J., Mouri, Y., Doucas, V., Kudoh, J., Tsurugaya, K., Uchida, D., Matsushima, A., Oshikawa, K., Nakayama, K. I., Shimizu, N., Peterson, P. and Matsumoto, M. (2004) 'Subcellular expression of autoimmune regulator is organized in a spatiotemporal manner', *Journal of Biological Chemistry*, 279(32), pp. 33984–33991. doi: 10.1074/jbc.M400702200.

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(2), pp. 227–239. doi: 10.1016/j.immuni.2005.07.005.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A. A., Benoist, C., Mathis, D., Boehmer, H. von, Bronson, R., Dierich, A. A., Benoist, C. and Mathis, D. (2002) 'Projection of an immunological self shadow within the thymus by the Aire protein', *Science*, 298(5597), pp. 1395–1401. doi: 10.1126/science.1075958.

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.', *Nature immunology*, 8(4), pp. 351–358. doi: 10.1038/ni1444.

Baik, S., Jenkinson, E. J., Lane, P. J. L., Anderson, G. and Jenkinson, W. E. (2013) 'Generation of both cortical and Aire+ medullary thymic epithelial compartments from CD205+ progenitors', *European Journal of Immunology*, 43(3), pp. 589–594. doi: 10.1002/eji.201243209.

Baik, S., Sekai, M., Hamazaki, Y., Jenkinson, W. E. and Anderson, G. (2016) 'Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK+ medullary epithelial progenitors', *European Journal of Immunology*, 46(4), pp. 857–862. doi: 10.1002/eji.201546253.

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 Computational Biology*, 9(7). doi: 10.1371/journal.pcbi.1003102. **Review**

Bennett, A. R., Farley, A., Blair, N. F., Gordon, J., Sharp, L. and Blackburn, C. C. (2002) 'Identification and characterization of thymic epithelial progenitor cells', *Immunity*, 16(6), pp. 803–814. doi: 10.1016/S1074-7613(02)00321-7.

Bichele, R., Kisand, K., Peterson, P. and Laan, M. (2016) 'TNF superfamily members play distinct roles in shaping the thymic stromal microenvironment', *Molecular Immunology*. Elsevier Ltd, 72, pp. 92–102. doi: 10.1016/j.molimm.2016.02.015.

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 research*, 9(2), pp. 158–66. doi: 10.1101/gr.9.2.158.

Bleul, C. C., Corbeaux, T., Reuter, A., Fisch, P., Mönting, J. S. and Boehm, T. (2006) 'Formation of a functional thymus initiated by a postnatal epithelial progenitor cell.', *Nature*, 441(7096), pp. 992–996. doi: 10.1038/nature04850.

Boehm, T., Scheu, S., Pfeffer, K. and Bleul, C. C. (2003) 'Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR.', *The Journal of experimental medicine*, 198(5), pp. 757–769. doi: 10.1084/jem.20030794.

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.', *Nature immunology*, 7(10), pp. 1092–100. doi: 10.1038/ni1385.

Bowlus, C. L., Ahn, J., Chu, T. and Gruen, J. R. (1999) 'Cloning of a novel MHC-encoded serine peptidase highly expressed by cortical epithelial cells of the thymus.', *Cellular immunology*, 196, pp. 80–86. doi: 10.1006/cimm.1999.1543.

Brandt, V. L. and Roth, D. B. (2008) 'G.O.D.'s Holy Grail: Discovery of the RAG Proteins', *The Journal of Immunology*, 180(1), pp. 3–4. doi: 10.4049/jimmunol.180.1.3. **Review**

Brennecke, P., Reyes, A., Pinto, S., Rattay, K., Nguyen, M., Kuchler, R., Huber, W., Kyewski, B. and Steinmetz, L. M. (2015) 'Single-cell transcriptome analysis reveals coordinated ectopic gene-expression patterns in medullary thymic epithelial cells', *Nat Immunol*, 16(9), pp. 933–941. doi: 10.1038/ni.3246\rhttp://www.nature.com/ni/journal/v16/n9/abs/ni.3246.html#supplementary-information.

Brunk, F., Michel, C., Holland-Letz, T., Slynko, A., Kopp-Schneider, A., Kyewski, B. and Pinto, S. (2017) 'Dissecting and modelling the emergent murine TEC compartment during ontogeny', *European Journal of Immunology*, accepted a, pp. 1–21. doi: 10.1002/eji.201747006.

Burkly, L., Hession, C., Ogata, L., Reilly, C., Marconi, L. a, Olson, D., Tizard, R., Cate, R. and Lo, D. (1995) 'Expression of relB is required for the development of thymic medulla and dendritic cells', *Nature*, 373(6514), pp. 531–536. doi: 10.1038/373531a0.

Corbeaux, T., Hess, I., Swann, J. B., Kanzler, B., Haas-Assenbaum, A. and Boehm, T. (2010) 'Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage.', *Proceedings of the National Academy of Sciences of the United States of America*, 107(38), pp. 16613–16618. doi: 10.1073/pnas.1004623107.

van Delft, M. A. M., Huitema, L. F. A. and Tas, S. W. (2015) 'The contribution of NF-kB signalling to immune regulation and tolerance', *European Journal of Clinical Investigation*. doi: 10.1111/eci.12430. **Review**

Depreter, M. G. L., Blair, N. F., Gaskell, T. L., Nowell, C. S., Davern, K., Pagliocca, A., Stenhouse, F. H., Farley, A. M., Fraser, A., Vrana, J., Robertson, K., Morahan, G., Tomlinson, S. R. and Blackburn, C. C. (2008) 'Identification of Plet-1 as a specific marker of early thymic epithelial progenitor cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(3), pp. 961–966. doi: 10.1073/pnas.0711170105.

Derbinski, J., Gabler, J., Brors, B., Tierling, S., Jonnakuty, S., Hergenhahn, M., Peltonen, L., Walter, J., Kyewski, B., 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.', *The Journal of experimental medicine*, 202(1), pp. 33–45. doi: 10.1084/jem.20050471.

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.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(2), pp. 657–62. doi: 10.1073/pnas.0707486105.

Derbinski, J., Schulte, A., Kyewski, B. and Klein, L. (2001) 'Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self.', *Nature immunology*, 2(11), pp. 1032–9. doi: 10.1038/ni723.

Desanti, G. E., Cowan, J. E., Baik, S., Parnell, S. M., White, A. J., Penninger, J. M., Lane, P. J. L., Jenkinson, E. J., Jenkinson, W. E. and Anderson, G. (2012) 'Developmentally regulated availability of RANKL and CD40 ligand reveals distinct mechanisms of fetal and adult cross-talk in the thymus medulla.', *Journal of immunology (Baltimore, Md. : 1950)*, 189(12), pp. 5519–26. doi: 10.4049/jimmunol.1201815.

DeVoss, J., Hou, Y., Johannes, K., Lu, W., Liou, G. I., Rinn, J., Chang, H., Caspi, R. R., Caspi, R., Fong, L. and Anderson, M. S. (2006) 'Spontaneous autoimmunity prevented by thymic expression of a single self-antigen.', *The Journal of experimental medicine*, 203(12), pp. 2727–35. doi: 10.1084/jem.20061864.

Dunn, R. J., Luedecker, C. J., Haugen, H. S., Clegg, C. H. and Farr, a G. (1997) 'Thymic overexpression of CD40 ligand disrupts normal thymic epithelial organization.', *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 45(1), pp. 129–141. doi: 10.1177/002215549704500116.

Feng, Y., He, D., Yao, Z. and Klionsky, D. J. (2014) 'The machinery of macroautophagy.', *Cell research*. Nature Publishing Group, 24(1), pp. 24–41. doi: 10.1038/cr.2013.168. **Review**

Gäbler, J., Arnold, J. and Kyewski, B. (2007) 'Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells', *European Journal of Immunology*, 37(12), pp. 3363–3372. doi: 10.1002/eji.200737131.

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. and Anderson, M. S. (2008) 'Deletional tolerance mediated by extrathymic Aire-expressing cells.', *Science (New York, N.Y.)*, 321(5890), pp. 843–7. doi: 10.1126/science.1159407.

Germain, R. N. (2002) 'T-cell development and the CD4-CD8 lineage decision.', *Nature reviews. Immunology*, 2(5), pp. 309–322. doi: 10.1038/nri798. **Review**

Gibson, T. J., Ramu, C., Gemũnd, C. and Aasland, R. (1998) 'The APECED polyglandular autoimmune syndrome protein, AIRE-1, contains the SAND domain and is probably a transcription factor [1]', *Trends in Biochemical Sciences*, 23(7), pp. 242–244. doi: 10.1016/S0968-0004(98)01231-6.

Gillard, G. O., Dooley, J., Erickson, M., Peltonen, L. and Farr, A. G. (2007) 'Aire-Dependent Alterations in Medullary Thymic Epithelium Indicate a Role for Aire in Thymic Epithelial Differentiation', *The Journal of Immunology*, 178(5), pp. 3007–3015. doi: 10.4049/jimmunol.178.5.3007.

Giraud, M., Jmari, N., Du, L., Carallis, F., Nieland, T. J. F., Perez-Campo, F. M., Bensaude, O., Root, D. E., Hacohen, N., Mathis, D. and Benoist, C. (2014) 'An RNAi screen for Aire cofactors reveals a role for Hnrnpl in polymerase release and Aire-activated ectopic transcription.', *Proceedings of the National Academy of Sciences of the United States of America*, 111(4), pp. 1491–6. doi: 10.1073/pnas.1323535111.

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', *Proceedings of the National Academy of Sciences*, 109(2), pp. 535–540. doi: 10.1073/pnas.1119351109.

Goldfarb, Y., Kadouri, N., Levi, B., Sela, A., Herzig, Y., Cohen, R. N., Hollenberg, A. N. and Abramson, J. (2016) 'HDAC3 Is a Master Regulator of mTEC Development', *Cell Reports*. The Authors, 15(3), pp. 651–665. doi: 10.1016/j.celrep.2016.03.048.

Gommeaux, J., Grégoire, C., Nguessan, P., Richelme, M., Malissen, M., Guerder, S., Malissen, B. and Carrier, A. (2009) 'Thymus-specific serine protease regulates positive selection of a subset of CD4+ thymocytes', *European Journal of Immunology*, 39(4), pp. 956–964. doi: 10.1002/eji.200839175.

Gordon, J., Wilson, V. A., Blair, N. F., Sheridan, J., Farley, A., Wilson, L., Manley, N. R. and Blackburn, C. C. (2004) 'Functional evidence for a single endodermal origin for the thymic epithelium.', *Nature immunology*, 5(5), pp. 546–553. doi: 10.1038/ni1064.

Gray, D., Abramson, J., Benoist, C. and Mathis, D. (2007) 'Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire.', *The Journal of experimental medicine*, 204(11), pp. 2521–2528. doi: 10.1084/jem.20070795.

Gray, D. H. D., Seach, N., Ueno, T., Milton, M. K., Liston, A., Lew, A. M., Goodnow, C. C. and Boyd, R. L. (2006) 'Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells', *Blood*, 108(12), pp. 3777–3785. doi: 10.1182/blood-2006-02-004531.

Guha, M., Saare, M., Maslovskaja, J., Kisand, K., Liiv, I., Haljasorg, U., Tasa, T., Metspalu, A., Milani, L. and Peterson, P. (2017) 'DNA breaks and chromatin structural changes enhance the transcription of Autoimmune Regulator target genes', *Journal of Biological Chemistry*, (3), p. jbc.M116.764704. doi: 10.1074/jbc.M116.764704.

Guiducci, C., Valzasina, B., Dislich, H. and Colombo, M. P. (2005) 'CD40/CD40L interaction regulates CD4+CD25+ T reg homeostasis through dendritic cell-produced IL-2', *European Journal of Immunology*, 35(2), pp. 557–567. doi: 10.1002/eji.200425810.

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*. Elsevier Inc., 36(3), pp. 438–450. doi: 10.1016/j.immuni.2012.01.017.

Haljasorg, U., Bichele, R., Saare, M., Guha, M., Maslovskaja, J., Kõnd, K., Remm, A., Pihlap, M., Tomson, L., Kisand, K., Laan, M. and Peterson, P. (2015) 'A highly conserved NFkB responsive enhancer is critical for thymic expression of Aire in mice', *European Journal of Immunology*, 45(12), pp. 3246–3256. doi: 10.1002/eji.201545928.

Hamazaki, Y., Fujita, H., Kobayashi, T., Choi, Y., Scott, H. S., Matsumoto, M. and Minato, N. (2007) 'Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin.', *Nature immunology*, 8(3), pp. 304–311. doi: 10.1038/ni1438.

He, X., He, X., Dave, V. P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B. A. and Kappes, D. J. (2005) 'The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment.', *Nature*, 433(7028), pp. 826–33. doi: 10.1038/nature03338.

Heino, M., Peterson, P., Sillanpää, N., Guérin, S., Wu, L., Anderson, G., Scott, H. S., Antonarakis, S. E., Kudoh, J., Shimizu, N., Jenkinson, E. J., Naquet, P. and Krohn, K. J. E. (2000) 'RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD mouse', *European Journal of Immunology*, 30(7), pp. 1884–1893. doi: 10.1002/1521-4141(200007)30:7<1884::AID-IMMU1884>3.0.CO;2-P.

Herzig, Y., Nevo, S., Bornstein, C., Brezis, M. R., Ben-Hur, S., Shkedy, A., Eisenberg-Bord, M., Levi, B., Delacher, M., Goldfarb, Y., David, E., Weinberger, L., Viukov, S., Ben-Dor, S., Giraud, M., Hanna, J. H., Breiling, A., Lyko, F., Amit, I., Feuerer, M. and Abramson, J. (2016) 'Transcriptional programs that control expression of the autoimmune regulator gene Aire', *Nature Immunology*, 18(December), pp. 1–14. doi: 10.1038/ni.3638.

Hikosaka, Y., Nitta, T., Ohigashi, I., Yano, K., Ishimaru, N., Hayashi, Y., Matsumoto, M., Matsuo, K., Penninger, J. M., Takayanagi, H., Yokota, Y., Yamada, H., Yoshikai, Y., Inoue, J. ichiro, Akiyama, T. and Takahama, Y. (2008) 'The Cytokine RANKL Produced by Positively Selected Thymocytes Fosters Medullary Thymic Epithelial Cells that Express Autoimmune Regulator', *Immunity*, 29(3), pp. 438–450. doi: 10.1016/j.immuni.2008.06.018.

Hinterberger, M., Aichinger, M., da Costa, O. P., Voehringer, D., Hoffmann, R., Klein, L., Prazeres, O., Voehringer, D. and Hoffmann, R. (2010) 'Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance.', *Nature immunology*, 11(6), pp. 512–519. doi: 10.1038/ni.1874.

Hofmann, J., Mair, F., Greter, M., Schmidt-Supprian, M. and Becher, B. (2011) 'NIK signaling in dendritic cells but not in T cells is required for the development of effector T cells and cell-mediated immune responses', *The Journal of Experimental Medicine*, 208(9), pp. 1917–1929. doi: 10.1084/jem.20110128.

Honey, K., Nakagawa, T., Peters, C. and Rudensky, A. (2002) 'Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands.', *The Journal of experimental medicine*, 195(10), pp. 1349–58. doi: 10.1084/jem.20011904.

Honey, K. and Rudensky, A. Y. (2003) 'Lysosomal cysteine proteases regulate antigen presentation', *Nature Reviews Immunology*, 3(6), pp. 472–482. doi: 10.1038/nri1110. **Review**

Hubert, F.-X., Kinkel, S. A., Crewther, P. E., Cannon, P. Z. F., Webster, K. E., Link, M., Uibo, R., Bryan, M. K. O., Meager, A., Forehan, S. P., Smyth, G. K., Antonarakis, S. E., Heath, W. R. and Scott, H. S. (2009) 'Aire- Deficient C57BL/6 Mice Mimicking the Common Human 13-Base Pair Deletion Mutation Present with Only a Mild Autoimmune Phenotype', *The Journal of ImmunologyJ*. doi: 10.4049/jimmunol.0802124.

Chin, R. K., Lo, J. C., Kim, O., Blink, S. E., Christiansen, P. A., Peterson, P., Wang, Y., Ware, C. and Fu, Y. (2003) 'Lymphotoxin pathway directs thymic Aire expression.', *Nature immunology*, 4(11), pp. 1121–1127. doi: 10.1038/ni982.

Chuprin, A., Avin, A., Goldfarb, Y., Herzig, Y., Levi, B., Jacob, A., Sela, A., Katz, S., Grossman, M., Guyon, C., Rathaus, M., Cohen, H. Y., Sagi, I., Giraud, M., McBurney, M. W., Husebye, E. S. and Abramson, J. (2015) 'The deacetylase Sirt1 is an essential regulator of Aire-mediated induction of central immunological tolerance.', *Nature immunology*, 16(7), pp. 737–745. doi: 10.1038/ni.3194.

Ilmarinen, T., Kangas, H., Kytömaa, T., Eskelin, P., Saharinen, J., Seeler, J. S., Tanhuanpää, K., Chan, F. Y. L., Slattery, R. M., Alakurtti, K., Palvimo, J. J. and Ulmanen, I. (2008) 'Functional interaction of AIRE with PIAS1 in transcriptional regulation', *Molecular Immunology*, 45(7), pp. 1847–1862. doi: 10.1016/j.molimm.2007.10.045.

Irla, M., Hugues, S., Gill, J., Nitta, T., Hikosaka, Y., Williams, I. R., Hubert, F. X., Scott, H. S., Takahama, Y., Holländer, G. A. and Reith, W. (2008) 'Autoantigen-Specific Interactions with CD4+ Thymocytes Control Mature Medullary Thymic Epithelial Cell Cellularity', *Immunity*, 29(3), pp. 451–463. doi: 10.1016/j.immuni.2008.08.007.

Jenkinson, W. E., Bacon, A., White, A. J., Anderson, G. and Jenkinson, E. J. (2008) 'An Epithelial Progenitor Pool Regulates Thymus Growth', *The Journal of Immunology*, 181(9), pp. 6101–6108. doi: 10.4049/jimmunol.181.9.6101.

Jiang, W., Anderson, M. S., Bronson, R., Mathis, D. and Benoist, C. (2005) 'Modifier loci condition autoimmunity provoked by Aire deficiency.', *The Journal of experimental medicine*, 202(6), pp. 805–15. doi: 10.1084/jem.20050693.

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.', *Proceedings of the National Academy of Sciences of the United States of America*, 91(14), pp. 6707–11. doi: 10.1073/pnas.91.14.6707.

Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., Naji, A. and Caton, A. J. (2001) 'Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist selfpeptide.', *Nature immunology*, 2(4), pp. 301–306.

Kajiura, F., Sun, S., Nomura, T., Izumi, K., Ueno, T., Bando, Y., Kuroda, N., Han, H., Li, Y., Matsushima, A., Takahama, Y., Sakaguchi, S., Mitani, T. and Matsumoto, M. (2004) 'NF-kB-Inducing Kinase Establishes Self-Tolerance in a Thymic Stroma-Dependent Manner', *The Journal of Immunology*, 172(4), pp. 2067–2075. doi: 10.4049/jimmunol.172.4.2067.

Khan, I. S., Mouchess, M. L., Zhu, M.-L., Conley, B., Fasano, K. J., Hou, Y., Fong, L., Su, M. A. and Anderson, M. S. (2014) 'Enhancement of an anti-tumor immune response by transient blockade of central T cell tolerance.', *The Journal of experimental medicine*, 211(5), pp. 761–8. doi: 10.1084/jem.20131889.

Kinoshita, D., Hirota, F., Kaisho, T., Kasai, M., Izumi, K., Bando, Y., Mouri, Y., Matsushima, A., Niki, S., Han, H., Oshikawa, K., Kuroda, N., Maegawa, M., Irahara, M., Takeda, K., Akira, S. and Matsumoto, M. (2006) 'Essential Role of IκB Kinase α in Thymic Organogenesis Required for the Establishment of Central Tolerance', *The Journal of Immunology*, 176(10), pp. 3995–4002. doi: 10.4049/jimmunol.176.7.3995.

Kisand, K. and Peterson, P. (2015) 'Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy', *Journal of Clinical Immunology*, 35(5), pp. 463–478. doi: 10.1007/s10875-015-0176-y. **Review**

Klein, L., Hinterberger, M., Wirnsberger, G. and Kyewski, B. (2009) 'Antigen presentation in the thymus for positive selection and central tolerance induction.', *Nature reviews. Immunology*. Nature Publishing Group, 9(12), pp. 833–844. doi: 10.1038/nri2669. **Review**

Klein, L., Klein, T., Rüther, U. and Kyewski, B. (1998) 'CD4 T cell tolerance to human C-reactive protein, an inducible serum protein, is mediated by medullary thymic epithelium.', *The Journal of experimental medicine*, 188(1), pp. 5–16. doi: 10.1084/jem.188.1.5.

Klein, L., Kyewski, B., Allen, P. M. and Hogquist, K. (2014) 'Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see).', *Nature reviews. Immunology*. Nature Publishing Group, 14(6), pp. 377–91. doi: 10.1038/nri3667. **Review**

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', *European Journal of Immunology*, 31, pp. 2476–2486.

Koh, A. S., Kingston, R. E., Benoist, C. and Mathis, D. (2010) 'Global relevance of Aire binding to hypomethylated lysine-4 of histone-3.', *Proceedings of the National Academy of Sciences of the United States of America*, 107(29), pp. 13016–21. doi: 10.1073/pnas.1004436107.

Koh, A. S., Kuo, A. J., Park, S. Y., Cheung, P., Abramson, J., Bua, D., Carney, D., Shoelson, S. E., Gozani, O., Kingston, R. E., Benoist, C. and Mathis, D. (2008) 'Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(41), pp. 15878–83. doi: 10.1073/pnas.0808470105.

Kumanogoh, a, Wang, X., Lee, I., Watanabe, C., Kamanaka, M., Shi, W., Yoshida, K., Sato, T., Habu, S., Itoh, M., Sakaguchi, N., Sakaguchi, S. and Kikutani, H. (2001) 'Increased T cell autoreactivity in the absence of CD40-CD40 ligand interactions: a role of CD40 in regulatory T cell development.', *Journal of immunology (Baltimore, Md. : 1950)*, 166(1), pp. 353–360. doi: 10.4049/jimmunol.166.1.353.

Kumar, P. G., Laloraya, M., Wang, C. Y., Ruan, Q. G., Davoodi-Semiromi, A., Kao, K. J. and She, J. X. (2001) 'The Autoimmune Regulator (AIRE) Is a DNA-binding Protein', *Journal of Biological Chemistry*, 276(44), pp. 41357–41364. doi: 10.1074/jbc.M104898200.

Kurobe, H., Liu, C., Ueno, T., Saito, F., Ohigashi, I., Seach, N., Arakaki, R., Hayashi, Y., Kitagawa, T., Lipp, M., Boyd, R. L. and Takahama, Y. (2006) 'CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance', *Immunity*, 24(2), pp. 165–177. doi: 10.1016/j.immuni.2005.12.011.

Kuroda, N., Mitani, T., Takeda, N., Ishimaru, N., Arakaki, R., Hayashi, Y., Bando, Y., Izumi, K., Takahashi, T., Nomura, T., Sakaguchi, S., Ueno, T., Takahama, Y., Uchida, D., Sun, S., Kajiura, F., Mouri, Y., Han, H., Matsushima, A., Yamada, G. and Matsumoto, M. (2005) 'Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice', *J Immunol*, 174(4), pp. 1862–1870. doi: 174/4/1862 [pii].

Laan, M., Kisand, K., Kont, V., Möll, K., Tserel, L., Scott, H. S. and Peterson, P. (2009) 'Autoimmune regulator deficiency results in decreased expression of CCR4 and CCR7 ligands and in delayed migration of CD4+ thymocytes.', *Journal of immunology (Baltimore, Md. : 1950)*, 183(12), pp. 7682–7691. doi: 10.4049/jimmunol.0804133.

LaFlam, T. N., Seumois, G., Miller, C. N., Lwin, W., Fasano, K. J., Waterfield, M., Proekt, I., Vijayanand, P. and Anderson, M. S. (2015) 'Identification of a novel cis-regulatory element essential for immune tolerance', *Journal of Experimental Medicine*, 212, pp. 1993–2002. doi: 10.1084/jem.20151069.

Lee, H. M., Bautista, J. L., Scott-Browne, J., Mohan, J. F. and Hsieh, C. S. (2012) 'A Broad Range of Self-Reactivity Drives Thymic Regulatory T Cell Selection to Limit Responses to Self', *Immunity*. Elsevier Inc., 37(3), pp. 475–486. doi: 10.1016/j.immuni.2012.07.009.

Legoux, F. P., Lim, J. B., Cauley, A. W., Dikiy, S., Ertelt, J., Mariani, T. J., Sparwasser, T., Way, S. S. and Moon, J. J. (2015) 'CD4+ T Cell Tolerance to Tissue-Restricted Self Antigens Is Mediated by Antigen-Specific Regulatory T Cells Rather Than Deletion', *Immunity*. Elsevier Inc., 43(5), pp. 896–908. doi: 10.1016/j.immuni.2015.10.011.

Lei, Y., Ripen, A. M., Ishimaru, N., Ohigashi, I., Nagasawa, T., Jeker, L. T., Bösl, M. R., Holländer, G. A., Hayashi, Y., Malefyt, R. D. W., Nitta, T. and Takahama, Y. (2011) 'Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development.', *The Journal of experimental medicine*, 208(2), pp. 383–394. doi: 10.1084/jem.20102327.

Leventhal, D. S., Gilmore, D. C., Berger, J. M., Nishi, S., Lee, V., Malchow, S., Kline, D. E., Kline, J., Vander Griend, D. J., Huang, H., Socci, N. D. and Savage, P. A. (2016) 'Dendritic Cells Coordinate the Development and Homeostasis of Organ-Specific Regulatory T Cells', *Immunity*. Elsevier Inc., 44(4), pp. 847–859. doi: 10.1016/j.immuni.2016.01.025.

Liang, C., Zhang, M. and Sun, S. C. (2006) 'beta-TrCP binding and processing of NF-kB2/p100 involve its phosphorylation at serines 866 and 870', *Cellular Signalling*, 18(8), pp. 1309–1317. doi: 10.1016/j.cellsig.2005.10.011.

Liao, G., Zhang, M., Harhaj, E. W. and Sun, S. C. (2004) 'Regulation of the NF-kB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation', *Journal of Biological Chemistry*, 279(25), pp. 26243–26250. doi: 10.1074/jbc.M403286200.

Linsk, R., Gottesman, M., Pernis, B., Sommer, S. and Sarkar, G. (1989) 'Are Tissues a patch quilt of ectopic gene expression?', *Science*, 246, p. 261.

Liston, A., Lesage, S., Wilson, J., Peltonen, L. and Goodnow, C. C. (2003) 'Aire regulates negative selection of organ-specific T cells.', *Nature immunology*, 4(4), pp. 350–354. doi: 10.1038/ni906.

Lkhagvasuren, E., Sakata, M., Ohigashi, I. and Takahama, Y. (2013) 'Lymphotoxin beta receptor regulates the development of CCL21-expressing subset of postnatal medullary thymic epithelial cells', *Journal of Immunology*, 190(10), pp. 5110–5117. doi: 10.4049/jimmunol.1203203.

Lo, W.-L., Felix, N. J., Walters, J. J., Rohrs, H., Gross, M. L., Allen, P. M., Cd, T., Lo, W.-L., Felix, N. J., Walters, J. J., Rohrs, H., Gross, M. L. and Allen, P. M. (2009) 'An endogenous peptide positively selects and augments the activation and survival of peripheral CD4+ T cells.', *Nature immunology*. Nature Publishing Group, 10(11), pp. 1155–61. doi: 10.1038/ni.1796.

Locksley, R. M., Killeen, N. and Lenardo, M. J. (2001) 'The TNF and TNF receptor superfamilies: Integrating mammalian biology', *Cell*, 104(4), pp. 487–501. doi: 10.1016/S0092-8674(01)00237-9. **Review**

Lomada, D., Jain, M., Bolner, M., Reeh, K. A. G., Kang, R., Reddy, M. C., DiGiovanni, J. and Richie, E. R. (2016) 'Stat3 Signaling Promotes Survival And Maintenance Of Medullary Thymic Epithelial Cells', *PLoS Genetics*, 12(1). doi: 10.1371/journal.pgen.1005777.

Lomada, D., Liu, B., Coghlan, L., Hu, Y. and Richie, E. R. (2007) 'Thymus medulla formation and central tolerance are restored in IKKalpha-/- mice that express an IKKalpha transgene in keratin 5+ thymic epithelial cells.', *Journal of immunology (Baltimore, Md. : 1950)*, 178(2), pp. 829–837. doi: 178/2/829 [pii].

Luckey, M. a, Kimura, M. Y., Waickman, A. T., Feigenbaum, L., Singer, A. and Park, J.-H. (2014) 'The transcription factor ThPOK suppresses Runx3 and imposes CD4(+) lineage fate by inducing the SOCS suppressors of cytokine signaling.', *Nature immunology*, 15(7), pp. 638–45. doi: 10.1038/ni.2917.

Malhotra, D., Linehan, J. L., Dileepan, T., Lee, Y. J., Purtha, W. E., Lu, J. V, Nelson, R. W., Fife, B. T., Orr, H. T., Anderson, M. S., Hogquist, K. A. and Jenkins, M. K. (2016) 'Tolerance is established in polyclonal CD4+ T cells by distinct mechanisms, according to self-peptide expression patterns', *Nature Immunology*, 17(2), pp. 187–95. doi: 10.1038/ni.3327.

Malchow, S., Leventhal, D. S., Lee, V., Nishi, S., Socci, N. D. and Savage, P. A. (2016) 'Aire Enforces Immune Tolerance by Directing Autoreactive T Cells into the Regulatory T Cell Lineage', *Immunity*. Elsevier Inc., 44(5), pp. 1102–1113. doi: 10.1016/j.immuni.2016.02.009.

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., Socci, N. D. and Savage, P. a (2013) 'Aire-Dependent Thymic Development of Tumor Associated Regulatory T Cells', *Science*, 339, pp. 1219–1224.

Martins, V. C., Boehm, T. and Bleul, C. C. (2008) 'LtBR Signaling Does Not Regulate Aire-Dependent Transcripts in medullary thymic epithelial cells', *The Journal of Immunology*, 181, pp. 400–407.

Mathis, D. and Benoist, C. (2009) 'Aire', *Annual Review of Immunology*, 27(1), pp. 287–312. doi: 10.1146/annurev.immunol.25.022106.141532. **Review**

Matsumoto, M. (2015) 'Switching on the Aire conditioner', *European Journal of Immunology*, 45(12), pp. 3237–3240. doi: 10.1002/eji.201546098. **Commentary**

Mayer, C. E., Žuklys, S., Zhanybekova, S., Ohigashi, I., Teh, H. Y., Sansom, S. N., Shikama-Dorn, N., Hafen, K., Macaulay, I. C., Deadman, M. E., Ponting, C. P., Takahama, Y. and Holl??nder, G. A. (2016) 'Dynamic spatio-temporal contribution of single β5t+ cortical epithelial precursors to the thymus medulla', *European Journal of Immunology*, 46(4), pp. 846–856. doi: 10.1002/eji.201545995.

McCarthy, N. I., Cowan, J. E., Nakamura, K., Bacon, A., Baik, S., White, A. J., Parnell, S. M., Jenkinson, E. J., Jenkinson, W. E. and Anderson, G. (2015) 'Osteoprotegerin-Mediated Homeostasis of Rank+ Thymic Epithelial Cells Does Not Limit Foxp3+ Regulatory T Cell Development.', *The Journal of Immunology*, 195(6), pp. 2675–82. doi: 10.4049/jimmunol.1501226.

Meireles, C., Ribeiro, A. R., Pinto, R. D., Leitão, C., Rodrigues, P. M. and Alves, N. L. (2017) 'Thymic crosstalk restrains the pool of cortical thymic epithelial cells with progenitor properties', *European Journal of Immunology*, pp. 1–12. doi: 10.1002/eji.201746922.

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', *Journal of Biological Chemistry*, 285(17), pp. 13012–13021. doi: 10.1074/jbc.M109.037747.

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 Reports*. The Authors, 5(1), pp. 166–179. doi: 10.1016/j.celrep.2013.08.038.

Miller, J. F. A. P. (2002) 'The discovery of thymus function and of thymus-derived lymphocytes', *Immunological Reviews*, 185(1), pp. 7–14. doi: 10.1034/j.1600-065X.2002.18502.x. **Review**

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', *Molecular biology of the cell*, 15(March), pp. 1101–1111. doi: 10.1091/mbc.E03.

Mouri, Y., Nishijima, H., Kawano, H., Hirota, F., Sakaguchi, N., Morimoto, J. and Matsumoto, M. (2014) 'NF-κB-inducing kinase in thymic stroma establishes central tolerance by orchestrating cross-talk with not only thymocytes but also dendritic cells.', *Journal of immunology (Baltimore, Md. : 1950)*, 193(9), pp. 4356–67. doi: 10.4049/jimmunol.1400389.

Mouri, Y., Yano, M., Shinzawa, M., Shimo, Y., Hirota, F., Nishikawa, Y., Nii, T., Kiyonari, H., Abe, T., Uehara, H., Izumi, K., Tamada, K., Chen, L., Penninger, J. M., Inoue, J., Akiyama, T. and Matsumoto, M. (2011) 'Lymphotoxin signal promotes thymic organogenesis by eliciting RANK expression in the embryonic thymic stroma.', *Journal of immunology (Baltimore, Md. : 1950)*, 186(9), pp. 5047–5057. doi: 10.4049/jimmunol.1003533.

Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y. and Tanaka, K. (2007) 'Regulation of CD8+ T cell development by thymus-specific proteasomes', *Science*, 316, pp. 1349–1353.

Murray, S. E. (2013) 'Cell-Intrinsic Role for NF-kappa B-Inducing Kinase in Peripheral Maintenance but Not Thymic Development of Foxp3+ Regulatory T Cells in Mice', *PLoS ONE*, 8(9), pp. 1–11. doi: 10.1371/journal.pone.0076216.

Musselman, C. A. and Kutateladze, T. G. (2011) 'Handpicking epigenetic marks with PHD fingers', *Nucleic Acids Research*, 39(21), pp. 9061–9071. doi: 10.1093/nar/gkr613. **Review**

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., Kawasaki, K., Asakawa, S., Ito, F. and Shimizu, N. (1997) 'Positional cloning of the APECED gene.', *Nature genetics*, 17, pp. 393–398. doi: 10.1038/ng1297-393.

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(5362), pp. 450–453. doi: 10.1126/science.280.5362.450.

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(7211), pp. 396–400. doi: 10.1038/nature07208.

Nishikawa, Y., Nishijima, H., Matsumoto, M., Morimoto, J., Hirota, F., Takahashi, S., Luche, H., Fehling, H. J., Mouri, Y. and Matsumoto, M. (2014) 'Temporal lineage tracing of Aire-expressing cells reveals a requirement for Aire in their maturation program', *J Immunol*, 192(6), pp. 2585–2592. doi: 10.4049/jimmunol.1302786.

Nitta, T., Murata, S., Sasaki, K., Fujii, H., Ripen, A. M., Ishimaru, N., Koyasu, S., Tanaka, K. and Takahama, Y. (2010) 'Thymoproteasome Shapes Immunocompetent Repertoire of CD8+ T Cells', *Immunity*. Elsevier Ltd, 32(1), pp. 29–40. doi: 10.1016/j.immuni.2009.10.009.

Ohigashi, I., Kozai, M. and Takahama, Y. (2016) 'Development and developmental potential of cortical thymic epithelial cells', *Immunological Reviews*, 271(1), pp. 10–22. doi: 10.1111/imr.12404. **Review**

Ohigashi, I., Zuklys, S., Sakata, M., Mayer, C. E., Hamazaki, Y., Minato, N., Hollander, G. A. and Takahama, Y. (2015) 'Adult Thymic Medullary Epithelium Is Maintained and Regenerated by Lineage-Restricted Cells Rather Than Bipotent Progenitors', *Cell Reports*. The Authors, 13(7), pp. 1432–1443. doi: 10.1016/j.celrep.2015.10.012.

Ohigashi, I., Zuklys, S., Sakata, M., Mayer, C. E., Zhanybekova, S., Murata, S., Tanaka, K., Hollander, G. A. and Takahama, Y. (2013) 'Aire-expressing thymic medullary epithelial cells originate from beta5t-expressing progenitor cells', *Proc Natl Acad Sci U S A*, 110(24), pp. 9885–9890. doi: 10.1073/pnas.1301799110.

Ohnmacht, C., Pullner, A., King, S. B. S., Drexler, I., Meier, S., Brocker, T. and Voehringer, D. (2009) 'Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity.', *The Journal of experimental medicine*, 206(3), pp. 549–59. doi: 10.1084/jem.20082394.

Onder, L., Nindl, V., Scandella, E., Chai, Q., Cheng, H. W., Caviezel-Firner, S., Novkovic, M., Bomze, D., Maier, R., Mair, F., Ledermann, B., Becher, B., Waisman, A. and Ludewig, B. (2015) 'Alternative NF-kB signaling regulates mTEC differentiation from podoplanin-expressing presursors in the cortico-medullary junction', *European Journal of Immunology*, 45(8), pp. 2218–2231. doi: 10.1002/eji.201545677.

Org, T., Chignola, F., Hetényi, C., Gaetani, M., Rebane, A., Liiv, I., Maran, U., Mollica, L., Bottomley, M. J., Musco, G. and Peterson, P. (2008) 'The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression.', *EMBO reports*, 9(4), pp. 370–6. doi: 10.1038/sj.embor.2008.11.

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.', *Molecular and cellular biology*, 27(24), pp. 8815–23. doi: 10.1128/MCB.01085-07.

Palmer, E. (2003) 'Negative selection--clearing out the bad apples from the T-cell repertoire.', *Nature reviews. Immunology*, 3(5), pp. 383–391. doi: 10.1038/nri1085. **Review**

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.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(42), pp. 17011–6. doi: 10.1073/pnas.1313001110.

Perry, J. S. A. and Hsieh, C. S. (2016) 'Development of T-cell tolerance utilizes both cell-autonomous and cooperative presentation of self-antigen', *Immunological Reviews*, 271(1), pp. 141–155. doi: 10.1111/imr.12403. **Review**

Perry, J. S. A., Lio, C. W. J., Kau, A. L., Nutsch, K., Yang, Z., Gordon, J. I., Murphy, K. M. and Hsieh, C. S. (2014) 'Distinct contributions of Aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus', *Immunity*. Elsevier Inc., 41(3), pp. 414–426. doi: 10.1016/j.immuni.2014.08.007.

Petrie, H. T. (2002) 'Role of thymic organ structure and stromal composition in steady-state postnatal T-cell production.', *Immunological reviews*, 189(1), pp. 8–19. doi: 10.1034/j.1600-065X.2002.18902.x. **Review**

Pitkanen, J. (2001) 'Subcellular Localization of the Autoimmune Regulator Protein. CHARACTERIZATION OF NUCLEAR TARGETING AND TRANSCRIPTIONAL ACTIVATION DOMAIN', *Journal of Biological Chemistry*, 276(22), pp. 19597–19602. doi: 10.1074/jbc.M008322200.

Pitkänen, J., Doucas, V., Sternsdorf, T., Nakajima, T., Aratani, S., Jensen, K., Will, H., Vähämurto, P., Ollila, J., Vihinen, M., Scott, H. S., Antonarakis, S. E., Kudoh, J., Shimizu, N., Krohn, K. and Peterson, P. (2000) 'The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein', *Journal of Biological Chemistry*, 275(22), pp. 16802–16809. doi: 10.1074/jbc.M908944199.

Plevin, M., Mills, M. and Ikura, M. (2005) 'The LxxLL motif: a multifunctional binding sequence in transcriptional regulation', *Trends in Biochemical Sciences*, 30(2), pp. 66–69. **Review**

Pribyl, T. M., Campagnoni, C., Kampf, K., Handley, V. W. and Campagnoni, a T. (1996) 'The major myelin protein genes are expressed in the human thymus.', *Journal of neuroscience research*, 45(6), pp. 812–9. doi: 10.1002/(SICI)1097-4547(19960915)45:6<812::AID-JNR18>3.0.CO;2-X.

Ramsey, C., Winqvist, O., Puhakka, L., Halonen, M., Moro, A., Kämpe, O., Eskelin, P., Pelto-Huikko, M. and Peltonen, L. (2002) 'Aire deficient mice develop multiple features of APECED phenotype and show altered immune response', *Hum Mol Genet*, 11(4), pp. 397–409. doi: 10.1093/hmg/11.4.397.

Rattay, K., Claude, J., Rezavandy, E., Matt, S., Hofmann, T. G., Kyewski, B. and Derbinski, J. (2015) 'Homeodomain-Interacting Protein Kinase 2, a Novel Autoimmune Regulator Interaction Partner, Modulates Promiscuous Gene Expression in Medullary Thymic Epithelial Cells', *The Journal of Immunology*, 194(3), pp. 921–928. doi: 10.4049/jimmunol.1402694.

Rattay, K., Meyer, H. V., Herrmann, C., Brors, B. and Kyewski, B. (2016) 'Evolutionary conserved gene co-expression drives generation of self-antigen diversity in medullary thymic epithelial cells', *Journal of Autoimmunity*. Elsevier Ltd, 67, pp. 65–75. doi: 10.1016/j.jaut.2015.10.001.

Riemann, M., Andreas, N., Fedoseeva, M., Meier, E., Weih, D., Freytag, H., Schmidt-Ullrich, R., Klein, U., Wang, Z.-Q. and Weih, F. (2017) 'Central immune tolerance depends on crosstalk between the classical and alternative NF-κB pathways in medullary thymic epithelial cells', *Journal of Autoimmunity*. Elsevier Ltd, pp. 1–12. doi: 10.1016/j.jaut.2017.03.007.

Roberts, N. A., White, A. J., Jenkinson, W. E., Turchinovich, G., Nakamura, K., Withers, D. R., McConnell, F. M., Desanti, G. E., Benezech, C., Parnell, S. M., Cunningham, A. F., Paolino, M., Penninger, J. M., Simon, A. K., Nitta, T., Ohigashi, I., Takahama, Y., Caamano, J. H., Hayday, A. C., Lane, P. J. L., Jenkinson, E. J. and Anderson, G. (2012) 'Rank Signaling Links the Development of Invariant gamma delta T Cell Progenitors and Aire + Medullary Epithelium', *Immunity*. Elsevier Inc., 36(3), pp. 427–437. doi: 10.1016/j.immuni.2012.01.016.

Rodewald, H. R., Paul, S., Haller, C., Bluethmann, H. and Blum, C. (2001) 'Thymus medulla consisting of epithelial islets each derived from a single progenitor.', *Nature*, 414(6865), pp. 763–768. doi: 10.1038/414763a.

Rossi, S. W., Jenkinson, W. E., Anderson, G. and Jenkinson, E. J. (2006) 'Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium.', *Nature*, 441(7096), pp. 988–991. doi: 10.1038/nature04813.

Rossi, S. W., Kim, M. Y., Leibbrandt, A., Parnell, S. M., Jenkinson, W. E., Glanville, S. H., McConnell, F. M., Scott, H. S., Penninger, J. M., Jenkinson, E. J., Lane, P. J. and Anderson, G. (2007) 'RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla', *J Exp Med*, 204(6), pp. 1267–1272. doi: 10.1084/jem.20062497.

Roth, D. B. (2014) 'V(D)J Recombination : Mechanism , Errors , and Fidelity', *Microbiology Spectrum*, 2(6), pp. 1–11. doi: 10.1128/microbiolspec.MDNA3-0041-2014.f1. **Review**

Saare, M., Rebane, A., Rajashekar, B., Vilo, J. and Peterson, P. (2012) 'Autoimmune regulator is acetylated by transcription coactivator CBP/p300', *Experimental Cell Research*, 318(14), pp. 1767–1778. doi: 10.1016/j.yexcr.2012.04.013.

Sansom, S. N., Shikama-Dorn, N., Zhanybekova, S., Nusspaumer, G., Macaulay, I. C., Deadman, M. E., Heger, A., Ponting, C. P. and Holländer, G. A. (2014) 'Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia', *Genome Research*, 24(12), pp. 1918–1931. doi: 10.1101/gr.171645.113.

Santori, F. R., Kieper, W. C., Brown, S. M., Lu, Y., Neubert, T. A., Johnson, K. L., Naylor, S., Vukmanović, S., Hogquist, K. A. and Jameson, S. C. (2002) 'Rare, structurally homologous self-peptides promote thymocyte positive selection', *Immunity*, 17(2), pp. 131–142. doi: 10.1016/S1074-7613(02)00361-8.

Sasaki, K., Takada, K., Ohte, Y., Kondo, H., Sorimachi, H., Tanaka, K., Takahama, Y. and Murata, S. (2015) 'Thymoproteasomes produce unique peptide motifs for positive selection of CD8(+) T cells.', *Nature communications*. Nature Publishing Group, 6(May), p. 7484. doi: 10.1038/ncomms8484.

Seach, N., Ueno, T., Fletcher, A. L., Lowen, T., Mattesich, M., Engwerda, C. R., Scott, H. S., Ware, C. F., Chidgey, A. P., Gray, D. H. D. and Boyd, R. L. (2008) 'The Lymphotoxin Pathway Regulates Aire-Independent Expression of Ectopic Genes and Chemokines in Thymic Stromal Cells', *The Journal of Immunology*, 180(8), pp. 5384–5392. doi: 10.4049/jimmunol.180.8.5384.

Sekai, M., Hamazaki, Y. and Minato, N. (2014) 'Medullary thymic epithelial stem cells maintain a functional thymus to ensure lifelong central T cell tolerance', *Immunity*. Elsevier Inc., 41(5), pp. 753–761. doi: 10.1016/j.immuni.2014.10.011.

Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Krähn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, a, Sun, S. C. and Karin, M. (2001) 'Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway.', *Science (New York, N.Y.)*, 293(5534), pp. 1495–1499. doi: 10.1126/science.1062677.

Setoguchi, R., Tachibana, M., Naoe, Y., Muroi, S., Akiyama, K., Tezuka, C., Okuda, T. and Taniuchi, I. (2008) 'Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development.', *Science (New York, N.Y.)*, 319(5864), pp. 822–825. doi: 10.1126/science.1151844.

Shinkura, R., Kitada, K., Matsuda, F., Tashiro, K., Ikuta, K., Suzuki, M., Kogishi, K., Serikawa, T. and Honjo, T. (1999) 'Alymphoplasia is caused by a point mutation in the mouse gene encoding Nf-kappa b-inducing kinase.', *Nature genetics*, 22(may), pp. 74–77. doi: 10.1038/8780.

Schaller, C. E., Wang, C. L., Beck-engeser, G., Goss, L., Scott, H. S., Anderson, M. S. and Wabl, M. (2008) 'Expression of Aire and the Early Wave of Apoptosis in Spermatogenesis', *The Journal of Immunology*, 180(3), pp. 1338–43. doi: 10.4049/jimmunol.180.3.1338.

Solan, N. J., Miyoshi, H., Carmona, E. M., Bren, G. D. and Paya, C. V. (2002) 'RelB cellular regulation and transcriptional activity are regulated by p100', *Journal of Biological Chemistry*, 277(2), pp. 1405–1418. doi: 10.1074/jbc.M109619200.

Sospedra, M., Ferrer-francesch, X., Juan, M., Foz-sala, M. and Juan, M. (1998) 'Transcription of a Broad Range of Self-Antigens in Human Thymus Suggests a Role for Central Mechanisms in Tolerance Toward Peripheral Antigens', *The Journal of Immunology*, 161, pp. 5918–5929.

Spence, P. J. and Green, E. A. (2008) 'Foxp3+ regulatory T cells promiscuously accept thymic signals critical for their development.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(3), pp. 973–978. doi: 10.1073/pnas.0709071105.

Su, M. A., Giang, K., Žumer, K., Jiang, H., Oven, I., Rinn, J. L., DeVoss, J. J., Johannes, K. P. A., Lu, W., Gardner, J., Chang, A., Bubulya, P., Chang, H. Y., Peterlin, B. M. and Anderson, M. S. (2008) 'Mechanisms of an autoimmunity syndrome in mice caused by a dominant mutation in Aire', *Journal of Clinical Investigation*, 118(5), pp. 1712–1726. doi: 10.1172/JCI34523.

Sun, S.-C. (2011) 'Non-canonical NF-κB signaling pathway', *Cell Research*, 21177(21), pp. 71–8571. **Review**

Takaba, H., Morishita, Y., Tomofuji, Y., Danks, L., Nitta, T., Komatsu, N., Kodama, T. and Takayanagi, H. (2015) 'Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance', *Cell*. Elsevier Inc., 163(4), pp. 975–987. doi: 10.1016/j.cell.2015.10.013.

Takada, K., Laethem, F. Van, Xing, Y., Akane, K., Suzuki, H., Murata, S., Tanaka, K., Jameson, S. C., Singer, A. and Takahama, Y. (2015) 'TCR affinity for thymoproteasome-dependent positively selecting peptides conditions antigen responsiveness in CD8 + T cells', *Nature immunology*, 16(10), pp. 1069–1077. doi: 10.1038/ni.3237.

Taniguchi, R. T., DeVoss, J. J., Moon, J. J., Sidney, J., Sette, A., Jenkins, M. K. and Anderson, M. S. (2012) 'Detection of an autoreactive T-cell population within the polyclonal repertoire that undergoes distinct autoimmune regulator (Aire)-mediated selection.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(20), pp. 7847–52. doi: 10.1073/pnas.1120607109.

Ucar, A., Ucar, O., Klug, P., Matt, S., Brunk, F., Hofmann, T. G. and Kyewski, B. (2014) 'Adult thymus contains Foxn1- epithelial stem cells that are bipotent for medullary and cortical thymic epithelial lineages', *Immunity*. The Authors, 41(2), pp. 257–269. doi: 10.1016/j.immuni.2014.07.005.

Ueno, T., Saito, F., Gray, D. H. D., Kuse, S., Hieshima, K., Nakano, H., Kakiuchi, T., Lipp, M., Boyd, R. L. and Takahama, Y. (2004) 'CCR7 signals are essential for cortex-medulla migration of developing thymocytes', *The Journal of experimental medicine*, 200(4), pp. 493–505. doi: 10.1084/jem.20040643.

Ulyanchenko, S., O'Neill, K. E., Medley, T., Farley, A. M., Vaidya, H. J., Cook, A. M., Blair, N. F. and Blackburn, C. C. (2016) 'Identification of a Bipotent Epithelial Progenitor Population in the Adult Thymus', *Cell Reports*. The Authors, 14(12), pp. 2819–2832. doi: 10.1016/j.celrep.2016.02.080.

Vaidya, H. J., Briones Leon, A. and Blackburn, C. C. (2016) 'FOXN1 in thymus organogenesis and development', *European Journal of Immunology*, 46(8), pp. 1826–1837. doi: 10.1002/eji.201545814. **Review**

Vallabhapurapu, S., Matsuzawa, A., Zhang, W., Tseng, P.-H., Keats, J. J., Wang, H., Vignali, D. a a, Bergsagel, P. L. and Karin, M. (2008) 'Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling.', *Nature immunology*, 9(12), pp. 1364–70. doi: 10.1038/ni.1678.

Venanzi, E. S., Gray, D. H. D., Benoist, C. and Mathis, D. (2007) 'Lymphotoxin pathway and Aire influences on thymic medullary epithelial cells are unconnected', *J Immunol*, 179(9), pp. 5693–5700. doi: 10.4049/jimmunol.179.9.5693.

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.', *Journal of immunology (Baltimore, Md. : 1950)*, 193(1), pp. 170–6. doi: 10.4049/jimmunol.1302519.

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., Su, M. and Anderson, M. S. (2014) 'The transcriptional regulator Aire coopts the repressive ATF7ip-MBD1 complex for the induction of immunotolerance.', *Nature immunology*, 15(3), pp. 258–65. doi: 10.1038/ni.2820.

Weih, F., Carrasco, D., Durham, S. K., Barton, D. S., Rizzo, C. A., Ryseck, R. P., Lira, S. A. and Bravo, R. (1995) 'Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-??B/Rel family', *Cell*, 80(2), pp. 331–340. doi: 10.1016/0092-8674(95)90416-6.

White, A. J., Nakamura, K., Jenkinson, W. E., Saini, M., Sinclair, C., Seddon, B., Narendran, P., Pfeffer, K., Nitta, T., Takahama, Y., Caamano, J. H., Lane, P. J., Jenkinson, E. J. and Anderson, G. (2010) 'Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells', *J Immunol*, 185(8), pp. 4769–4776. doi: 10.4049/jimmunol.1002151.

Wong, K., Lister, N. L., Barsanti, M., Lim, J. M. C., Hammett, M. V., Khong, D. M., Siatskas, C., Gray, D. H. D., Boyd, R. L. and Chidgey, A. P. (2014) 'Multilineage potential and self-renewal define an epithelial progenitor cell population in the adult thymus', *Cell Reports*. The Authors, 8(4), pp. 1198–1209. doi: 10.1016/j.celrep.2014.07.029.

Wu, W., Shi, Y., Xia, H., Chai, Q., Jin, C., Ren, B. and Zhu, M. (2017) 'Epithelial LTβR signaling controls the population size of the progenitors of medullary thymic epithelial cells in neonatal mice', *Scientific Reports*. Nature Publishing Group, 7(February), p. 44481. doi: 10.1038/srep44481.

Xiao, G., Harhaj, E. W. and Sun, S. C. (2001) 'NF-kB-inducing kinase regulates the processing of NF-kB2 p100', *Molecular Cell*, 7(2), pp. 401–409. doi: 10.1016/S1097-2765(01)00187-3.

Yamano, T., Nedjic, J., Hinterberger, M., Steinert, M., Koser, S., Pinto, S., Gerdes, N., Lutgens, E., Ishimaru, N., Busslinger, M., Brors, B., Kyewski, B. and Klein, L. (2015) 'Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction', *Immunity*. Elsevier Inc., 42(6), pp. 1048–1061. doi: 10.1016/j.immuni.2015.05.013.

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.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(5), pp. 1833–8. doi: 10.1073/pnas.1222023110.

Yang, S., Fujikado, N., Kolodin, D., Benoist, C. and Mathis, D. (2015) 'Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance', *Science*, 348(6234), pp. 589–594. doi: 10.1126/science.aaa7017.

Yano, M., Kuroda, N., Han, H., Meguro-Horike, M., Nishikawa, Y., Kiyonari, H., Maemura, K., Yanagawa, Y., Obata, K., Takahashi, S., Ikawa, T., Satoh, R., Kawamoto, H., Mouri, Y. and Matsumoto, M. (2008) 'Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance.', *The Journal of experimental medicine*, 205(12), pp. 2827–38. doi: 10.1084/jem.20080046.

Yoshida, H., Bansal, K., Schaefer, U., Chapman, T., Rioja, I., Proekt, I., Anderson, M. S., Prinjha, R. K., Tarakhovsky, A., Benoist, C. and Mathis, D. (2015) 'Brd4 bridges the transcriptional regulators, Aire and P-TEFb, to promote elongation of peripheral-tissue antigen transcripts in thymic stromal cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 112(32), pp. E4448-57. doi: 10.1073/pnas.1512081112.

Zarnegar, B. J., Wang, Y., Mahoney, D. J., Dempsey, P. W., Cheung, H. H., He, J., Shiba, T., Yang, X., Yeh, W.-C., Mak, T. W., Korneluk, R. G. and Cheng, G. (2008) 'Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK.', *Nature immunology*, 9(12), pp. 1371–8. doi: 10.1038/ni.1676.

Zhang, B., Wang, Z., Ding, J., Peterson, P., Gunning, W. T. and Ding, H. F. (2006) 'NF-kB2 is required for the control of autoimmunity by regulating the development of medullary thymic epithelial cells', *Journal of Biological Chemistry*, 281(50), pp. 38617–38624. doi: 10.1074/jbc.M606705200.

Zhu, M., Chin, R. K., Christiansen, P. A., Lo, J. C., Liu, X., Ware, C., Siebenlist, U. and Fu, Y. X. (2006) 'NF-kB2 is required for the establishment of central tolerance through an Aire-dependent pathway', *Journal of Clinical Investigation*, 116(11), pp. 2964–2971. doi: 10.1172/JCI28326.

Zhu, M., Chin, R. K., Tumanov, A. V, Liu, X. and Fu, Y. X. (2007) 'Lymphotoxin beta receptor is required for the migration and selection of autoreactive T cells in thymic medulla', *The Journal of Immunology*, 179(12), pp. 8069–8075. doi: 179/12/8069 [pii].

Žumer, K., Low, A. K., Jiang, H., Saksela, K. and Peterlin, B. M. (2012) 'Unmodified histone H3K4 and DNA-dependent protein kinase recruit autoimmune regulator to target genes.', *Molecular and cellular biology*, 32(8), pp. 1354–62. doi: 10.1128/MCB.06359-11.