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# AIRE-expressing cells in immune tolerance in health and disease

AIRE-exprimující buňky v imunitní toleranci ve zdraví a nemoci

Ph.D. Thesis

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**Declaration:**

I hereby declare that my dissertation thesis is a presentation of my original research work and that I have listed all the information sources and literature used. Neither this work nor a substantial part of it has been submitted for the award of any academic degree or diploma.

Prague, 26.06.2020

**Prohlášení:**

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V Praze, 26.06.2020

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## ABSTRACT

The process of self-nonself discrimination by the immune system is a fundamental attribute of healthy organisms. Since T-cell receptors (TCRs) are generated by the random process of somatic recombination without regard to its targets, the newly developed T-cell clones could recognize either self or nonself antigens. The mechanisms of central tolerance robustly limit the self-reactive repertoire within the T-cell population via deletion of clones that express self-reactive TCRs or their deviation into the regulatory T-cells (Tregs). These processes occur mainly in the thymic medulla where the TCR reactivity to self-antigens is tested by various types of antigen-presenting cells, mainly medullary thymic epithelial cells (mTECs), dendritic cells (DCs), and B-cells. The cooperation between these cell-types has been shown to be essential for the establishment of thymic tolerance. A key molecule regulating the production of self-antigens is the autoimmune regulator (AIRE), which is thought to be expressed primarily by mTECs and its mutations are associated with the development of severe autoimmune disorders. In this context, the presented thesis describes the novel regulatory pathways important for the development of a functional and "harmless" repertoire of T-cells and for enforcement of tolerance. First, we have shown that signaling through Toll-like receptors (TLRs) on mTECs leads to the influx of monocyte-derived DCs to the thymic medulla and subsequent regulation of Tregs development. Consistently, the abrogation of TLR signaling in TECs resulted in decreased frequency and functionality of Tregs, leading to aggravated mouse experimental colitis. Second, we demonstrated that gastrointestinal symptoms associated with AIRE loss-of-function are associated with the defective central tolerance to enteric α-defensins. Third, and consistent with the notion that the processes of central tolerance are complemented by the various mechanisms of peripheral tolerance, we have identified a novel population of peripheral lymph node resident AIRE-expressing cells, which share several characteristics with innate lymphoid cells type 3 (ILC3) and can efficiently present endogenously expressed antigen to peripheral $CD4^+$ T-cells. Lastly, we have developed a new mouse model that enables cell-specific depletion of AIRE and thus allows to study the function of AIRE in a much broader physiological context.

# ABSTRACT (CZ)

Rozpoznávání tělu vlastních a cizích struktur imunitním systémem je jedním ze základních dějů udržujích zdraví organismus. Jelikož T-buněčné receptory (TCRs) jsou vytvářeny náhodným necíleným procesem somatické rekombinace, nově vznikající klony T-buněk mohou rozpoznávat jak tělu-vlastní tak cizí antigeny. Mechanismy centrální tolerance mají schopnost omezovat autoreaktivní T-buněčný repertoár pomocí odstraňování klonů nesoucí autoreaktivní TCR nebo pomocí konverze těchto klonů na regulační T-buňky. Tyto procesy probíhají převážně ve dřeni brzlíku, kde dochází k testování autoreaktivity T-buněčných klonů pomocí různých typů buněk prezentujích antigen, jako jsou medulární epiteliální buňky brzlíku (mTECs), dendritické buňky a B-buňky. Ukazuje se také, že vzájemná spolupráce těchto buněčných typů je nepostradatelná pro správné fungování imunitní tolerance. Klíčovou úlohu v řízení produkce tělu vlastních antigenů zastává protein autoimunitní regulátor (AIRE), který je produkovaný převážně mTEC buňkami a jehož mutace vedou k rozvoji těžkých autoimunitních reakcí. V tomto kontextu předkládaná disertační práce popisuje nové regulační dráhy asociované s AIRE proteinem, které jsou důležité pro vývoj funkčního a bezpečného repertoáru T-buněk a ustanovení tolerance. Zaprvé jsme ukázali, že signalizace přes receptory rodiny Toll (TLR) na mTEC buňkách vede ke zvýšené migraci dendritických buněk derivovaných z monocytu do dřeně brzlíku, kde následně dochází k nárůstu počtu regulačních T-buněk. Shodně, také vypnutí TLR signalizace na mTEC buňkách vede ke snížení počtu a funkce regulačních T-buněk a to následně způsobuje výrazné zhoršení experimetálního zánětu tlustého střeva. Zadruhé jsme popsali, že symptomy asociované s trávícím traktem u pacientů s nefukčním proteinem AIRE, mohou být vysvětleny ztrátou centrální tolerance k enterickým α-defensinům. Zatřetí, a v návaznosti na funkci periferní tolerance, práce popisuje nový typ AIRE protein produkujících buněk v lymfatických uzlinách, které byly charakterizované jako přirozené lymfoidní buňky třetího typu (ILC3), a které jsou schopné efektivně prezentovat tělu vlastní antigeny $CD4^+$ T-buňkám. V neposlední řadě, se nám podařilo vyvinou nový myší model umožňující buněčně specifické vypnutí genu AIRE, což dovoluje studování jeho funkce v mnohem širším fyziologickém kontextu.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AHR | Aryl hydrocarbon receptor |
| AIRE | Autoimmune regulator |
| APCs | Antigen-presenting cells |
| APECED | Autoimmune polyendocrinophaty-candidiasis-ectodermal dystrophy |
| APS-1 | Polyendocrine syndrome type-1 |
| ASCL1 | Achaete-Scute Family BHLH Transcription Factor 1 |
| BATF3 | Basic leucine zipper transcriptional factor ATF-like 3 |
| BCR | B-cell receptor |
| BM | Bone marrow |
| BST2 | Bone marrow stromal cell antigen 2 |
| cAMP | Cyclic adenosine monophosphate |
| CARD | Caspase-recruitment domain |
| CAT | Cooperative antigen transfer |
| cDC1 | Classical dendritic cells type 1 |
| cDC2 | Classical dendritic cells type 2 |
| cDCs | Classical dendritic cells |
| CDP | Common dendritic cell progenitors |
| CLDN | Claudin |
| CLP | Common lymphoid progenitors |
| CMC | Chronic mucocutaneous candidiasis |
| CMJ | Cortico-medullary junction |
| CNS1 | Conserved non-coding sequence 1 |
| CpG | Cytidine-phosphate-guanosine |
| cTECs | Cortical thymic epithelial cells |
| CTLA-4 | Cytotoxic T-lymphocyte antigen 4 |
| DCLK1 | Doublecortin-like kinase I |

| | |
|---|---|
| DCs | Dendritic cells |
| DLL4 | Delta-like ligand 4 |
| DN | Double-negative |
| DP | Double-positive |
| ECM | Extracellular matrix |
| EECs | Enteroendocrine cells |
| ESAM | Endothelial cell adhesion molecule |
| eTACs | Extrathymic AIRE-expressing cells |
| FEZF2 | FEZ Family Zinc Finger 2 |
| FLT3L | FMS-like tyrosine kinase 3 ligand |
| FOXN1 | Forkhead box protein N1 |
| FOXP3 | Forkhead box P3 |
| GF | Germ-free |
| GFP | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| H3K4me0 | Unmethylated Histon H3 lysine 4 |
| HA | Influenza hemagglutinin |
| hCRP | Human C-reactive protein |
| HEL | Hen egg lysozyme |
| HMGB1 | Heat-shock proteins, and high-mobility group box 1 |
| IBDs | Inflammatory bowel diseases |
| iFABP | Intestinal fatty-acid binding protein |
| IL | Interleukin |
| ILC2s | Innate lymphoid cells type 2 |
| ILC3s | Innate lymphoid cells type 3 |
| iNKT | Invariant natural killer T-cells |
| IPEX | Immunodysregulation polyendocrinopathy enteropathy X-linked |
| IRAK | IL-1 receptor-associated kinase |

| | |
|---|---|
| IRF | Interferon regulatory factor |
| ITGA6 | Integrin subunit aplha 6 |
| ITGB4 | Integrin subunit β4 |
| IVL | Involucrin |
| KRT | Cytokeratin |
| LAG | Lymphocyte activation gene |
| LNSCs | Lymph node stromal cells |
| LPS | Lipopolysaccharide |
| LTβR | Lymphotoxin β receptor |
| M-CSF | Macrophage colony-stimulating factor |
| MERTK | Tyrosine-protein kinase MER |
| MHC | Major histocompatibility complex |
| mLN | Mesenteric lymph-nodes |
| moDCs | Monocytes-derived dendritic cells |
| mOVA | Membrane-bound chicken ovalbumin |
| mTECs | Medullary thymic epithelial cells |
| mTEPCs | Medullary thymic epithelial cell progenitors |
| NKT | Natural killer T-cells |
| NLS | Nuclear localization signal |
| NOD | Non-obese diabetic |
| OVA | Chicken ovalbumin |
| PAMPs | Pathogen-associated molecular patterns |
| PCs | Paneth cells |
| pDCs | Plasmacytoid dendritic cells classical DCs |
| PDPN | Podoplanin |
| PGE | Promiscuous gene expression |
| PHD | Plant-homeodomain |
| PLP | Proteolipid protein |

| | |
|---|---|
| pMHC | Peptide/major histocompatibility complex |
| POU2F3 | POU Class 2 Homeobox 3 |
| PRRs | Pattern-recognition receptors |
| pTregs | Peripheral regulatory T-cells |
| RA | Retinoic acid |
| RAG2 | Recombination activating gene 2 |
| RANK | Receptor activator of nuclear factor-kappa B |
| RBPJ | Recombining binding protein suppressor of hairless |
| RIP | Rat insulin promoter |
| RORγ | RAR-related orphan receptor gamma¨ |
| RUNX3 | Runt-related transcription factor 3 |
| SAND | Sp100, AIRE, NucP41/75, Deaf1 domain |
| SCFA | Short-chain fatty acids |
| SFB | Segmented filamentous bacteria |
| SOX4 | SRY-Box Transcription Factor 4 |
| Tconv | Conventional T-cells |
| TCR | T-cell receptor |
| TECs | Thymic epithelial cells |
| TGFβ | Transforming growth factor-β |
| TIGIT | T-cell immunoreceptor with Ig and ITIM domains |
| TIR | Toll-like receptor-interleukin 1 receptor |
| TLRs | Toll-like receptors |
| TNF | Tumor necrosis factor |
| TRAs | Tissue-restricted antigens |
| Tregs | Regulatory T-cells |
| TSPs | Thymic seeding progenitors |
| TSSP | Thymic-specific serine protease |
| tTregs | Thymic regulatory T-cells |

# LITERATURE OVERVIEW

## 1. INTRODUCTION

The coevolution of complex multicellular organisms, such as vertebrates, with microbes puts selective pressure on the development of functional barrier responsible for protection against potential microbial threats (Eberl, 2010). The evolutionary old defense strategy based on the mechanism of the innate immune system is mediated by the production of generic receptors that recognize conserved microbial structures and triggers the inflammatory response that limits pathogens invasion (Janeway and Medzhitov, 2002). Since these general microbial structures are common for both potential pathogens and commensals, the additional protective layer that enables the specific determinations of microbes have been developed. Thus the jawed vertebrates have developed an adaptive immune system that can initiate protective responses against any pathogens that can be immunologically recognized (Beutler, 2004).

This capacity of adaptive immunity is based on the random somatic recombination of genes which enables to generate immense diversity of surface receptors to recognize antigens. This was specifically attributed to a population of T and B lymphocytes bearing the T-cells (TCR) and B-cell receptors (BCR), respectively. The somatic gene rearrangement is mediated by the recombination of VDJ segments during the generation of antigen-specific receptors. Since this process is completely stochastic, the TCRs or BCRs that can recognize antigens derived from body own tissues, commensal microbiota, or food are generated (Hodgkin, 2018). Since the recognition of these antigens resulted in the development of devastating autoimmune disorders (now affecting 5-10% of Western population) (Cooper et al., 2009), the existence of a mechanism that recognizes and eliminates lymphocytes with “self” specific receptors is critical for the homeostatic function of the immune system (von Boehmer et al., 1989). Although the majority of autoimmune diseases were shown to be polygenic, recent studies have identified several monogenic causes that seem to be more common than previously thought (Marson et al., 2015). Surprisingly, several of these monogenic autoimmune diseases are linked to the

defects in central and peripheral tolerance and particularly to mutations in the autoimmune regulator (AIRE) gene (Abramson and Husebye, 2016).

The following section of the literature overview describes the complex mechanisms of immune tolerance and focuses on the role of AIRE-expressing cells in these processes. First, the intrathymic mechanisms controlling the development of functional and safe TCR repertoires are reviewed. Specifically, the exact function of diverse populations of thymic antigen-presenting cells (APCs) in positive and negative selection of developing T-cells are highlighted. This chapter is followed by the description of peripheral tolerance mechanisms with the emphasis on self-antigen presentation and the function of specific subtypes of APCs. The last part characterizes the Toll-like receptors (TLRs) and describes their function in immune processes.

## 2. IMMUNE TOLERANCE

Immune tolerance was postulated as a state of unresponsiveness of the immune system to the antigen that has the capacity to trigger an immune response in a given organism. The process of tolerance is operational at two levels: (i) central tolerance, which operates in the thymus for T-cell tolerance and in the bone marrow (BM) for B-cell tolerance; and (ii) peripheral tolerance that targets autoreactive T-cells and B-cells in secondary lymphoid and non-lymphoid tissues in the immune periphery.

### 2.1 Central tolerance

#### *2.1.1 Early T-cell development*

T-cell development in the adult (postnatal) thymus occurs in several distinct phases consisting of BM-derived lymphopoiesis, TCR mediated selection, differentiation, and functional maturation. In the adult organism, the blood-borne precursors of T-cells, the thymic seeding progenitors (TSPs), migrate to the thymic tissue and enter the organ through blood vessels at the cortico-medullary junction (CMJ) (Bhandoola et al., 2007; Lind et al., 2001). The process of TSPs differentiation and migration is driven by several mediators, specifically by chemotactic factors and ligands of Notch (Petrie and Zúñiga-Pflücker, 2007). During the BM-derived differentiation, the TSPs start to express the CCR9 and CCR7, the receptors for CCL25 and CCL19/CCL21, respectively, that are

essential for the recruitment of TSPs into the thymus (Zlotoff et al., 2010). Once entering the thymic tissue, TSPs overexpress CXCR4, the ligand of which drives their migration into the thymic cortex (Plotkin et al., 2003; Trampont et al., 2010). Here, under the influence of Notch ligands (Chen et al., 2019), the process of T-cell commitment is initiated. TSPs differentiate into double-negative thymocytes (DN1-DN4, $CD8^{-}CD4^{-}$) that undergo approximately 20 divisions. This massive expansion results in the generation of a huge amount of precursor cells (~5 x $10^7$) that are during the whole thymic selection process reduced to about 5%, which represents a daily thymic output (~2-3 x $10^6$) (Kyewski and Klein, 2006). The first selection checkpoint, called β-selection, is dependent on pre-TCR signaling and ensures that only those DN (DN2-DN4) thymocytes that undergo successful TCRβ locus rearrangement can progress to the $CD8^{+}CD4^{+}$ double-positive (DP) stage. After differentiation into the DP lineage, thymocytes are subjected also to the rearrangement of TCRα locus which accomplishes the process of VDJ recombination, and leads to the generation of TCR repertoire with enormous diversity (Hogquist et al., 2005; Roth, 2014). This event is subsequently followed by a massive proliferation and clonal cell expansion leading to the generation of a large pool of TCRs with diverse antigen specificities. The subsequent developmental decisions of thymocytes are determined by the TCR interaction with peptide/major histocompatibility complex (pMHC) complexes produced by thymic (APCs) (Klein et al., 2009). The cell interactions and journey of developing thymocytes through the thymic tissue is visualized in Figure 1.

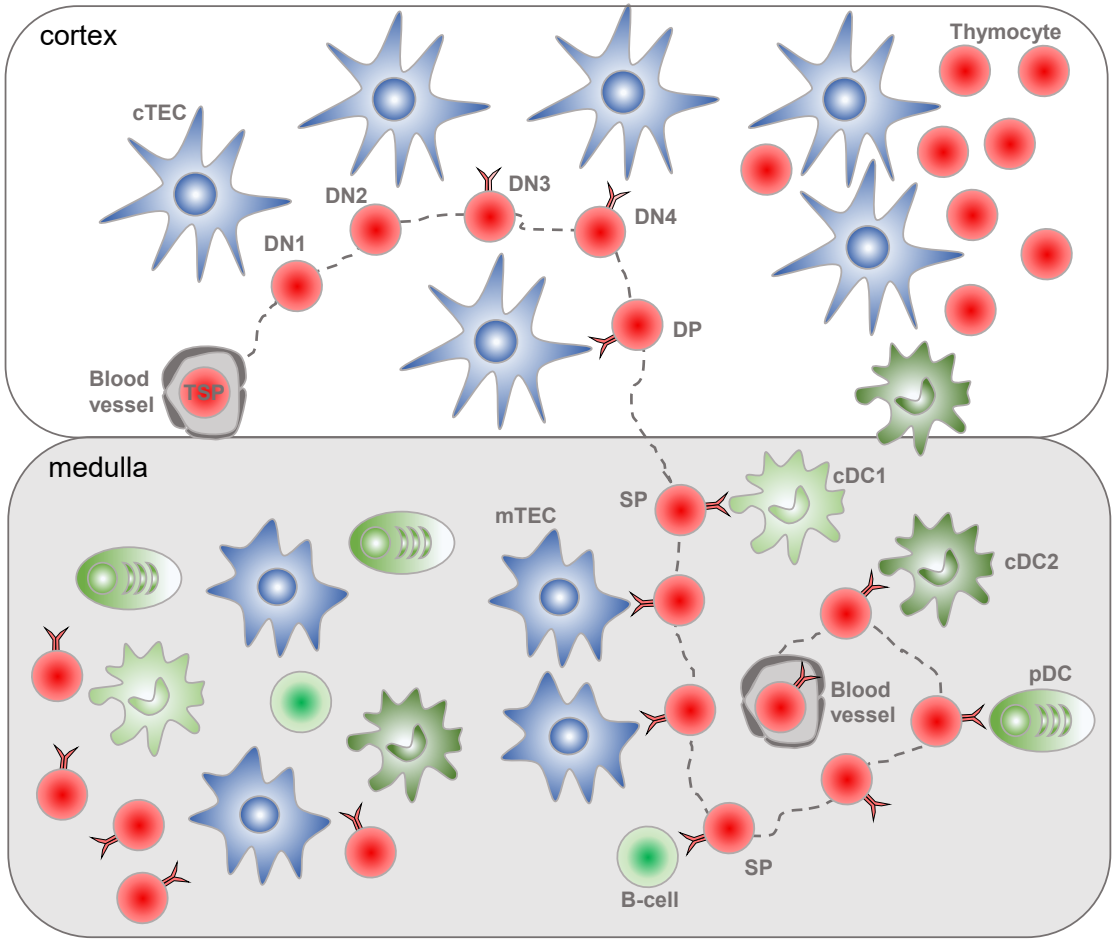


**Figure 1. Cell interactions in the thymus during T-cell development.** Thymic seeding progenitors (TSPs) enter the thymus at the cortico-medullary junction, become double-negative (DN, $CD4^{-}CD8^{-}$), and migrate towards the subcapsular zone. During this migration through the cortical region, thymocytes undergoing TCR rearrangement (DN2-DN4) and become double-positive (DP, $CD4^{+}CD8^{+}$). DP thymocytes scan the surface of cortical thymic epithelial cells (cTECs) for positive selection and after accomplishing this process, thymocytes leave the cortex and migrate to the medullary region as single positive (SP, $CD4^{+}$ or $CD8^{+}$). After entering the medulla SP thymocytes assume random walk during which they scan the surface of medullary thymic epithelial cells (mTECs), classical dendritic cells type 1, and 2 (cDC1 and cDC2), plasmacytoid dendritic cells (pDC), and B-cells and test their TCR for self-reactivity. It was measured that SP thymocytes perform several hundred contacts with antigen-presenting cells until they leave the medulla and migrate to the periphery (4-5 days). Inspired by *Klein et al.* (Klein et al., 2014).

### *2.1.2 Positive selection*

The process of positive selection is mostly determined by the certain window of affinity/avidity of the interaction between TCR and their pMHC ligands in the thymic

cortex (Werlen et al., 2003). Specifically, more than 90% of DP thymocytes due to the useless TCR specificities fail to interact with pMHC complexes within any affinity. The lack of MHC restriction results in the so-called "death by neglect" of the DP thymocytes (von Boehmer et al., 1989). This suggests that most apoptotic cell death in the thymus is associated with the failure of positive selection. On the other hand, strong or avid interaction also resulted in thymocyte cell death by apoptosis. This process, called clonal deletion, is mostly attributed to the mechanism of negative selection occurring in thymic medulla (Klein et al., 2009; von Boehmer et al., 1989). Nevertheless, the clonal deletion was also described in the thymic cortex (McCaughtry et al., 2008). Even though the notion of cortical clonal deletion still remains controversial, the CD28-mediated co-stimulation seems to be essential for this process in both anatomical locations of the thymus, cortex, and medulla (Breed et al., 2019).

Together with the co-stimulatory molecules expressed mainly by cortical thymic epithelial cells (cTECs) and F4/80$^+$ macrophages (Breed et al., 2019), the peptides presented to DP thymocytes in the context of MHC molecules are also crucial for the proper mechanism of positive selection in the cortex (Hogquist et al., 1994; Jameson et al., 1994). However, the nature and identity of those peptides are still elusive. Two possible scenarios were suggested: (i) cTECs and potentially also cells of hematopoietic origin (F4/80$^+$ macrophages) present ubiquitously expressed peptides, (ii) specifically cTECs present the unique epitopes also derived from ubiquitously expressed peptides. The latter hypothesis is supported by the fact, that cTECs were shown to use unique antigen-processing machinery to generate pMHC class I and also pMHC class II complexes (Klein et al., 2014).

In terms of the affinity/avidity model, those DP thymocytes that encounter pMHC ligands with low or intermediate affinity are positively selected and become single-positive (SP) CD8$^+$ or CD4$^+$ thymocytes (Klein et al., 2014). The CD8/CD4 determination is based on the MHC class I (MHCI) or MHC class II (MHCII) restriction and on the gradient of transcription factors. Specifically, the recognition of pMHC I and the presence of runt-related transcription factor 3 (RUNX3) drive the DP thymocytes to differentiate into CD8 SP lineage (Setoguchi et al., 2008). Reciprocally, the pMHC class II restriction and

overexpression of Th-POK determines the development of CD4 SP thymocytes (Luckey et al., 2014). After completing the process of positive selection, SP thymocytes upregulate CD69 and migrate into the medulla (Ross et al., 2014). Chemokine receptors CCR7 and CCR4 were shown to play an essential role in this relocation process (Hu et al., 2015; Kwan and Killeen, 2004; Ueno et al., 2004). CCR4 is produced shortly after positive selection and its ligands CCL17 and CCL22 are predominantly expressed by thymic dendritic cells (DCs) located in the medullary region. The CCR7 expression is among thymocytes restricted to SP lineage and ligands are produced mostly by medullary thymic epithelial cells (mTECs) (Misslitz et al., 2004). Thus the gradient of both chemokines emanates from the medullary region of the thymus (Lancaster et al., 2018).

### *2.1.3 Negative selection*

The main function of the negative selection process lies in the removal of potentially dangerous T-cell clones bearing the self-reactive TCRs (Kappler et al., 1987). This process of clonal deletion is operating mostly in the thymic medullary region and it is called “recessive tolerance” (Klein and Jovanovic, 2011). On the other hand, since the transplantation of thymic epithelium (Modigliani et al., 1995) or injection of a specific type of lymphocytes to naïve animals (Sakaguchi et al., 1995) was shown to be able to confer tolerance to donor tissue, there are other mechanisms than recessive tolerance operating in the thymus. This mechanism has been referred to as “dominant tolerance” and is characterized by the deviation of self-reactive T-cells to regulatory T-cells (Tregs) (Klein and Jovanovic, 2011; Klein et al., 2019). This cell lineage can suppress immune responses by interfering with the induction and proliferation of effector T-cells (Josefowicz et al., 2012). In general, the processes of negative selection including those involved in decision-making between recessive and dominant tolerance, are based on the several thymocyte-intrinsic and thymocyte-extrinsic determinants among which the affinity and/or avidity of the self pMHC-TCR interaction is the most crucial (Lee et al., 2012).

#### *2.1.3.1 Topology of self-antigen expression*

Both mechanisms of central tolerance are based on the premise that SP thymocytes interact by its TCRs with the pMHC complex presenting self-peptides. These self-antigens could be divided into two main groups: (i) antigens with the ubiquitous expression pattern and (ii) antigens whose expression is limited to only one or very few (maximum to 5) anatomical places (Anderson et al., 2002; Derbinski et al., 2001). These self-antigens are known as tissue-restricted antigens (TRAs) and their expression was specifically attributed to a rare population of mTECs. The process of ectopic gene expression by mTECs was called "promiscuous gene expression" (PGE) (Derbinski et al., 2001). The specifics and regulations of PGE are very different from those of standard gene regulation in the peripheral tissues and employ several characteristics: (i) TRAs, whose production is tightly regulated are expressed by a single mTEC in a stochastic manner (only 1-3% of all mTECs express given TRA at given time point) (Derbinski et al., 2008); (ii) TRA genes are often expressed from a single-allele using alternative transcriptional start sites (Villaseñor et al., 2008); (iii) sex-related genes, that are also attributed to as TRAs, are expressed by mTECs irrespectively of the gender (prostate antigens or β-casein expressed by mTECs in both males and females) (Derbinski et al., 2008; Malchow et al., 2013); (iv) TRAs contain several development-related genes that are expressed by mTECs with no connections to the developmental status of the organism (i.e., α-Fetoprotein expressed by the yolk sac and fetal liver) (Derbinski et al., 2001).

Using RNA sequencing analyses it was postulated that mTECs express more than 18,000 genes, which represent approximately 85% of the protein-coding genome (Danan-Gotthold et al., 2016; Sansom et al., 2014). Compared to this number in mTECs, all other cell types from different tissues typically express from 12,000 to 14,000 genes (i.e., 60-65% of coding genome) (Abramson and Anderson, 2017). Remarkably around 3,000-4,000 genes in mTECs are regulated by AIRE protein (Sansom et al., 2014). Thus, a set of mTEC-dependent TRAs can be expressed in an AIRE-dependent or AIRE-independent manner, where the AIRE-independent genes represent a larger fraction of TRA transcripts. Even the regulation of AIRE-independent PGE is still not completely understood, the transcription factor FEZ Family Zinc Finger 2 (FEZF2) was suggested to

play an important role in mediating immune tolerance to AIRE-independent TRAs (Takaba et al., 2015). In addition, and despite the fact, that the protein-coding genes and TRA transcripts corresponding to most peripheral tissues are highly represented in mTECs, the tissues of immunologically privileged sites, such as brain and testis, are dramatically underrepresented (Danan-Gotthold et al., 2016). This suggests that the strictness of central tolerance might be lower to organs that are not under constant immunological pressure. Also as touched above, a given TRA in a given time is expressed only by 1-3% of mTECs and one mTEC is able to co-express approximately 100-300 TRAs (Brennecke et al., 2015; Meredith et al., 2015). This is set by the fact that the neighboring TRA genes seem to cluster into co-expression groups. Correspondingly, it was postulated that 200-500 mTECs are sufficient to cover the entire TRA repertoire (Abramson and Anderson, 2017). Together this suggests that PGE is controlled by the rules of "ordered stochasticity", where the initial co-expression pattern of TRAs is stochastic but is then highly regulated by a coordinated set of events (Meredith et al., 2015).

#### *2.1.3.2 Characteristics of AIRE and APS-1*

As described above, AIRE has been determined as the major transcriptional regulator, which in the population of mTECs, promotes the expression of a substantial amount of TRAs (Anderson et al., 2002). AIRE is structurally composed of several domains often associated with transcriptional factors and nuclear proteins. It comprises a Caspase-recruitment domain (CARD) (Ferguson et al., 2008), a nuclear localization signal (NLS), a conserved SAND domain (Sp100, AIRE, NucP41/75, Deaf1) (Gibson et al., 1998), two plant-homeodomain (PHD 1 and PHD 2) zinc-fingers and LXXLL motifs (Perniola and Musco, 2014; Plevin et al., 2005). Importantly the CARD domain was shown to be essential for forming of AIRE homo-dimers and homo-tetramers, which is crucial for its physiological function (Huoh et al., 2020; Pitkänen et al., 2000; Waterfield et al., 2014). As described previously, AIRE promotes the expression of approximately 4000 genes, whose production is usually restricted to only a very few tissues and whose expression in mTECs is silenced (Sansom et al., 2014). In this scenario, AIRE binds to the transcriptional start site (TSS) of genes and its transcription-transactivation capacity is

regulated by deacetylase Sirtuin 1 (Chuprin et al., 2015). Using PHD1 finger domain, AIRE directly binds to unmethylated Histon H3 lysine 4 (H3K4me0), the epigenetic marker of repressive chromatin (Koh et al., 2008; Org et al., 2008). This binding allows the formation of the AIRE-mediated complex of proteins (TOP2, DNA-PK, CBP…) (Abramson et al., 2010; Org et al., 2008; Pitkänen et al., 2000) that promotes the breaks at the TSS of responsive genes and leads to the relaxation of chromatin (Abramson and Husebye, 2016; Guha et al., 2017). This is accompanied by the recruitment of several mediators of gene expression (pTEFb/BRD4 complex) (Oven et al., 2007; Yoshida et al., 2015) and by the release of stalled RNA polymerase that enables the transcription of AIRE-dependent genes (Giraud et al., 2012). In general, AIRE can potentially interact with more than fifty partners associated with nuclear transport, chromatin binding/structure, pre-mRNA processing, and transcription (Abramson et al., 2010).

The study of AIRE function in central tolerance was facilitated by the generation of *Aire*$^{-/-}$ mouse strains (Anderson et al., 2002; Hubert et al., 2009; Ramsey et al., 2002). It was shown that mutations in the *Aire* gene lead to the diminished expression of AIRE-dependent TRA in mTECs, escape of self-reactive (TRA-specific) T-cells to the immune periphery, and subsequent development of the severe autoimmune disease (Anderson et al., 2002). Human *AIRE* loss-of-function mutations lead to the development of an autoimmune disease called autoimmune polyendocrine syndrome type-1 (APS-1) or alternatively autoimmune polyendocrinophaty-candidiasis-ectodermal dystrophy (APECED, OMIM: 240300) (Consortium, 1997; Nagamine et al., 1997). APS-1 is a monogenic disease that could be inherited either by autosomal recessive or by the dominant way. The dominant inheritance is associated with the fact, that AIRE forms homo-oligomers where in the case of one defective AIRE allele, the entire oligomer loses its function (Oftedal et al., 2015). The prevalence of this disease differs between both types of mutations. The prevalence of an autosomal recessive form of APS-1 in general population is very low, with exception of some geographical pockets, where the prevalence is much higher, such as in Iranian Jews 1:9000, Sardinians 1:14400, Finns 1:25000 (Perheentupa, 2006) and Norwegians 1:80000). On the other hand, the frequency of dominant form across the population is much higher (1:1000) (Husebye et al., 2009). Also, the symptoms in the dominant form are usually much milder than in the case of

classical autosomal recessive APS-1 (Oftedal et al., 2015). The most common symptoms are chronic mucocutaneous candidiasis (CMC), hypoparathyroidism, and primary adrenal insufficiency (Addison's disease). The majority of the patients develop at least two of the three major components, which are frequently accompanied by additional symptoms such as type 1 diabetes, ovarian failure, vitiligo, enamel dysplasia, or gastrointestinal symptoms (Husebye et al., 2018). The APS-1 components affecting gastrointestinal tissues are present in approximately 25-30% of patients and are associated with the loss of tolerance to intestinal TRAs (Perheentupa, 2006). Specifically, the histidine decarboxylase (Sköldberg et al., 2003), tryptophan hydroxylase (Ekwall et al., 1998; Söderbergh et al., 2004) and tyrosine hydroxylase (Hedstrand et al., 2000), the molecules expressed by enteroendocrine cells (EECs), were shown to be targeted in APS-1 patients leading to the decrease or complete absence of EECs from patient's intestinal biopsies (Posovszky et al., 2012). Also, it was described that sera from APS-1 patients can cross-react with the secretory granules of Paneth cells (PCs) suggesting that antimicrobial peptides could be also targets of APS-1 associated autoimmune attack (Ekwall et al., 1998). The sera of APS-1 was also shown to contain high titers of neutralizing autoantibodies against many cytokines such as interferons type 1 and IL-17/IL-22 (Meyer et al., 2016). The presence of this blocking autoantibodies is often associated with the development of CMC in APS-1 patients (Kisand et al., 2010).

As described above, several *Aire*$^{-/-}$ mouse strains were generated. Although all of these strains to a certain extent recapitulate the phenotype of APS-1, the severity of the disease profoundly differs with the genetic background of mice. While the phenotype of BALB/c or non-obese diabetic (NOD) mice develop a very severe phenotype accompanied by immune cells infiltrates within the majority of the organs (Jiang et al., 2005b), the C57BL/6 mice show only very mild phenotype with the auto-inflammation in the retina, salivary glands, and pancreas (Anderson et al., 2002; Jiang et al., 2005b; Ramsey et al., 2002).

This data suggest that AIRE's major function is to regulate the expression of specifically silent genes in mTECs (Org et al., 2008). As described above, this is accomplished by the interference with the common transcriptional control that allows AIRE to regulate not

only the expression of TRA-associated genes but also several other molecules (Meredith et al., 2015; Sansom et al., 2014). It was described that AIRE regulates genes associated with differentiation of mTECs (Nishikawa et al., 2010; Yano et al., 2008), production of cytokines and chemokines (Fujikado et al., 2016; Hubert et al., 2011; Laan et al., 2009; Lei et al., 2011) and genes important for antigen handling and presentation (Anderson et al., 2005). By controlling the expression of these sets of genes, AIRE also regulates the intrathymic migration of cells, differentiation, and activation status of thymic APCs and thus comprehensively affects both, the negative selection of self-reactive T-cells (recessive tolerance) and positive selection of Tregs (dominant tolerance).

#### *2.1.3.3 Mechanisms of central tolerance: T-cell deletion versus Tregs selection*

The previously mentioned premise, that the recognition of epitopes derived from AIRE-dependent or AIRE-independent TRAs by self-reactive T-cells leads to their deletion, has been described using the neo-self-antigen technology. First, using the mTEC-restricted expression of human C-reactive protein (hCRP), it was confirmed that high-affinity hCRP specific transgenic T-cells are clonally deleted (Klein et al., 1998). The fact that mTECs also induce clonal deletion of AIRE-dependent TRA-specific TCR clones was described by the use of mice models in which the expression of hen egg lysozyme (HEL) (Liston et al., 2003) or membrane-bound chicken ovalbumin (mOVA) (Anderson et al., 2005) was driven by the rat insulin promoter (RIP). Since the activation of RIP is in the thymus completely dependent on AIRE, the expression of HEL or mOVA mimics the production of AIRE-dependent TRAs. By crossing these models with TCR-HEL or OT-II mice, where all T-cells were specific to HEL or mOVA, respectively, most of these neo-self specific T-cells were subjected to clonal deletion. Also, the crossing of the above transgenic mice to *Aire*$^{-/-}$ animals lead to the decreased expression of AIRE-dependent antigens in mTECs and the diminished clonal deletion of HEL of OVA-specific T-cells (Anderson et al., 2005; Liston et al., 2003).

Using TCR transgenic systems it was shown that similar to clonal deletion, Tregs differentiation can be induced by TCR agonist (Apostolou et al., 2002; Jordan et al., 2001). This was further corroborated by the study of *Aschenbrenner et al.* using AIRE-HA (Aire-driven influenza hemagglutinin) mice crossed with TCR-HA transgenic animals. In this

experimental system, almost one-fourth of HA-specific T-cells has deviated into Treg lineage. This suggests that AIRE-expressing mTECs play a crucial role in Tregs generation (Aschenbrenner et al., 2007). This was confirmed by the fact, that also organ-specific Tregs require AIRE-dependent expression of TRAs (Lin et al., 2016; Malchow et al., 2013). The importance of AIRE itself in shaping the Tregs repertoire was described by deep sequencing of the complete TCRα genes in Tregs and conventional T-cells (Tconv) isolated from AIRE-sufficient or –deficient mice. This experiment showed that in the absence of AIRE, the T-cell receptor sequences which were usually found among Treg lineage could be detected in the repertoire of Tconv cells (Malchow et al., 2016). However, several studies suggested that AIRE is essential for the mechanism of central tolerance mostly during the neonatal period (Guerau-de-Arellano et al., 2009). Specifically, it was described that the AIRE-dependent Treg repertoire is distinct during ontogeny (Stadinski et al., 2019) and that Tregs generated in the neonatal period are much more protective from the onset of autoimmunity than Tregs generated in adults (Yang et al., 2015).

As stated above, the decision between clonal deletion and Tregs generation is affected by several thymocyte-intrinsic and thymocyte-extrinsic determinants. The simplest models are based on the affinity and/or avidity of the pMHC-TCR interactions. Specifically, the high-affinity interaction leads to clonal deletion, whereas weaker interactions resulted in Tregs generation. This is in agreement with the fact that most of the T-cell transgenic systems specific to neo-self-antigens (Anderson et al., 2005; Liston et al., 2003) which exhibit a very high affinity are predestined to massive clonal deletion rather than Tregs deviation. On the other hand, using MHC-tetramer technology operating with natural TCR affinities provides evidence that the clonal deletion of TRA-specific thymocytes is far from being complete and is rather biased towards Treg selection (Hassler et al., 2019; Legoux et al., 2015; Malhotra et al., 2016; Taniguchi et al., 2012). Specifically, this phenomenon was described using the MHCII tetramers specific to neo-self-antigens, whose expression is restricted to either all (ubiquitous antigens) or various tissues (TRA-like expression pattern). It was shown that ubiquitous antigen recognition led to the massive deletion of antigen-specific T-cells, whereas the recognition of TRA-like antigens mostly promotes diversion to Treg lineage (Legoux et al., 2015; Malhotra et al., 2016).

This idea was recently corroborated by studying the tolerance to physiologically relevant TRA (proteolipid protein, PLP) at polyclonal level, which showed that PLP-specific T-cells are rather converted to Tregs than subjected to clonal deletion (Hassler et al., 2019). Since the expression of ubiquitous antigens in the thymus is much more abundant compared to mTEC-restricted TRAs expression, these studies also suggest that high-avidity interactions cause clonal deletion whereas low-avidity interaction results in Tregs generation. Additionally, it was hypothesized that T-cell clonal deletion can result from a "single hit" of antigen that promotes the longer dwelling time of thymocytes on the surface of APC, leading to amplified intensity of TCR signaling. By contrast, Tregs development seems to be associated with multiple antigen encounters (Klein et al., 2019). This could be explained by the fact, that mostly the APCs expressing low levels of pMHCII complexes are responsible for Tregs generation (Hu et al., 2017). The thymic models of T-cells fate are visualized in Figure 2.

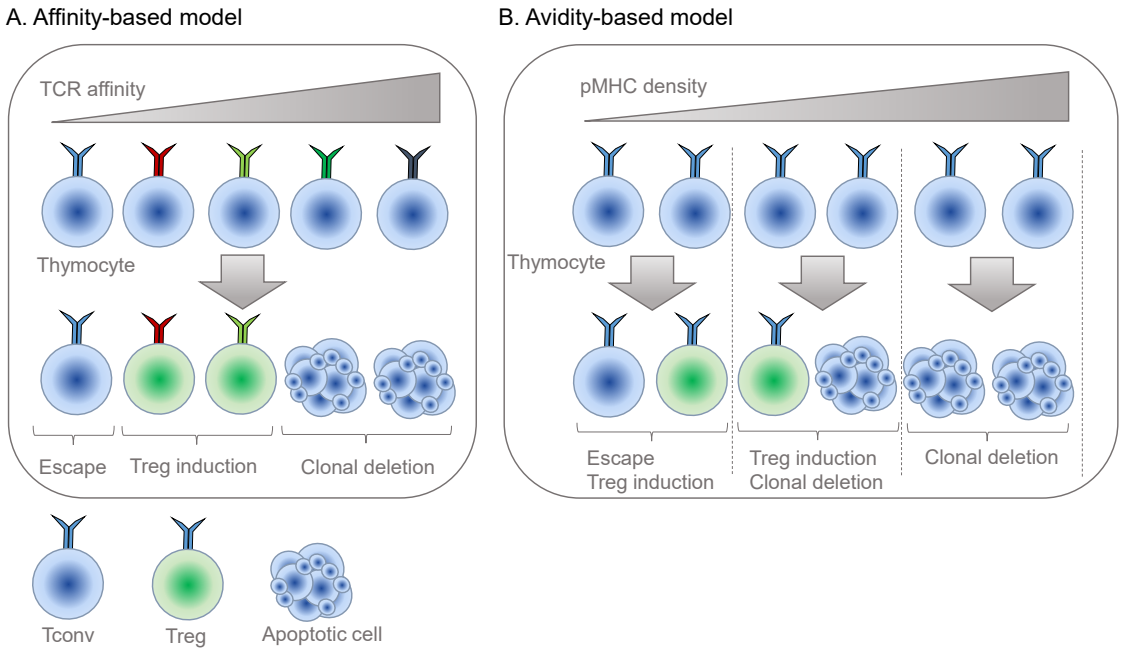


**Figure 2. Models of T-cell deletion versus Tregs selection. A.** An affinity-based model of T-cell development relies on the fact that T-cells bearing high-affinity T-cell receptors (TCRs) are removed from the repertoire by the process of clonal deletion, whereas T-cells bearing intermediate affinity TCRs are converted to regulatory T-cell (Treg) lineage. T-cell with low-affinity TCRs escape into the periphery and become conventional T-cells (Tconv). **B.** According to the avidity-based model, the density of the presented antigens determines the fate of developing T-cells. Decreasing the density of antigen presented on antigen-presenting cells is associated with

thymocytes escape to the periphery or with the selection of Tregs, whereas the increasing density of antigen leads to the clonal deletion. Inspired by *Klein et al.* (Klein et al., 2019).

Along with the properties of pMHC-TCR interactions and TCR signaling, the thymocytes-extrinsic factors such as CD28 co-stimulation (Tai et al., 2005) or cytokine signaling also represent determinants of deletion versus Tregs diversion. Together with the TCR stimulus, IL-2 plays an indispensable role in promoting Tregs differentiation. Specifically, the strong TCR signal promotes the upregulation of CD25 (IL-2Rα) which, in turn, enables the high affinity-binding of IL-2 leading to Forkhead box P3 (FOXP3) upregulation (Lio and Hsieh, 2008). Thus, the intrathymic levels of IL-2 seem to be a limiting factor for Tregs differentiation. Several studies demonstrated that IL-2, which is provided by both developing T-cells and thymic DCs, is important for the proper development of Tregs (Owen et al., 2018; Weist et al., 2015). Recently, it was shown that the major producers of IL-2 in the thymus are $CD25^+$ self-reactive T-cells (Hemmers et al., 2019). Alternatively, the IL-2 produced by these cells could be also captured and *trans*-presented by thymic DCs (Wuest et al., 2011). Interestingly the amount of IL-2 in the thymus could be also controlled by the recirculation of mature Tregs back to the thymus. It was hypothesized that this mechanism works as a negative feedback loop in which the mature Tregs capture intrathymic IL-2 and restrain the *de novo* generation of Tregs (Thiault et al., 2015). Recently, it was shown that the expression of CD73, a marker of mature T-cells, could distinguish between newly generated and recirculating Tregs (Owen et al., 2019). Interestingly, migration of these cells to the thymus was described as AIRE-dependent, since it is driven by CCL20 chemokine, whose expression in the thymus is restricted to the $AIRE^+$ mTECs (Cowan et al., 2018).

Transforming growth factor-β (TGFβ) was also demonstrated as an important factor for proper development of T-cells, since mice lacking TGFβ receptor on thymocytes from DP stage, develop severe multi-organ autoimmunity (Li et al., 2006; Marie et al., 2006). In terms of thymic Tregs development, it was suggested that TGFβ works as a pro-survival factor preventing the apoptosis of self-reactive T-cells and thus driving them to the Treg-lineage (Ouyang et al., 2010).

### *2.1.4 Antigen-presenting cells in the thymus*

As described in detail in the previous section, the mechanisms of thymic central tolerance, i.e positive selection occurring in the thymic cortex and clonal deletion and/or Tregs generation in the medullary region, is orchestrated by pMHC complexes presented by various thymic APCs. Thus, thymic APCs play a crucial role in determining the fate of developing T-cells. The thymus accommodates various types of non-hematopoietic and hematopoietic APCs, specifically thymic epithelial cells (TECs; cTECs and mTECs), thymic DCs, macrophages, and B-cells (Klein et al., 2009). In terms of APCs of hematopoietic origin, the population of thymic DCs is by far the most studied. The majority of DC are localized in the thymic medulla region (Sanos et al., 2011) and are comprised of two major categories: plasmacytoid DCs (pDCs) and classical DCs (cDCs), from which the latter can be subdivided into cDCs type 1 (cDC1) and type 2 (cDC2) (Li et al., 2009). Importantly, it became clear that developing T-cells encounter antigens presented by all these cell subtypes (Hinterberger et al., 2010; Ohnmacht et al., 2009; Yamano et al., 2015).

#### *2.1.4.1 Thymic epithelial cells*

Originally, TECs were subdivided according to the intrathymic position to cTECs, localized in the cortical region, and mTECs which are positioned in the medullary region of the thymus. TECs originate from the third pharyngeal pouch region which is formed by both ectoderm and endoderm. This suggests that cTECs and mTECs shared the dual germ layer origin and are differentiated from a common TEC bipotent progenitor (Rodewald, 2008; Rossi et al., 2006). The earliest steps of TEC differentiation are regulated by a group of transcription factors that drives thymic epithelial anlage from the third pharyngeal pouch and maintain the TEC program (Manley and Condie, 2010). Among these, the most crucial is the Forkhead box protein N1 (FOXN1), whose loss-of-function mutation is characterized by the presence of only small cystic thymus, almost complete absence of T-cells and a hair-loss (“nude mice” phenotype) (Blackburn et al., 1996; Nehls et al., 1996). FOXN1 expression is not only crucial for the development and maintenance of TEC compartment but also regulates the expression of functional molecules important for the proper selection of T-cell, such as CCL25 or delta-like ligand 4 (DLL4, the ligand of

Notch) (Calderón and Boehm, 2012; Žuklys et al., 2016). Although cTECs and mTECs arise from the same progenitors, they are functionally distinct.

#### *2.1.4.2 Cortical thymic epithelial cells (cTECs)*

cTECs are specific epithelial cell type (defined by the expression of EpCAM and LY51) arranged in the three-dimensional scaffold in the thymic cortex that enables very close interactions with DN and DP thymocytes and facilitates their differentiation and development (Takahama et al., 2017). Specifically, cTECs produce high levels of previously mentioned cytokines, CCL25 (ligand of CCR9) and CXCL12 (ligand of CXCR4) that are crucial for homing of blood-born TSPs (Gossens et al., 2009; Jenkinson et al., 2007; Plotkin et al., 2003) into the thymus and also DLL4 (Hozumi et al., 2008; Koch et al., 2008), IL-7 (Ribeiro et al., 2013) and stem cell factor (Buono et al., 2016), which promote proliferation and differentiation of the TSPs into T-cell lineage (Kadouri et al., 2020). Besides, cTECs can form multicellular clusters, referred to as thymic nurse cells that encompass several thymocytes and facilitate the β-selection and positive selection of thymocytes (Nakagawa et al., 2012).

As described above, cTECs used unconventional antigen-processing pathways that enable them to generate a unique ligandome library of pMHC class I and also pMHC class II complexes, which are essential for positive selection of thymocytes (Klein et al., 2009). In the case of MHCI peptide loading, cTECs express a unique subunit of the proteasome referred to as β5t. This type of proteasome is called “thymoproteasome” and it was shown that its substrate preference is distinct from the conventional type of proteasome (using β5 subunit) or “immunoproteasome” (using β5i subunit) (Florea et al., 2010). Moreover, the mice deficient for β5t show marked reduction of positively selected $CD8^+$ T-cells (Murata et al., 2007). Additionally, cTECs display high levels of macroautophagy that enables the unconventional loading of endogenous peptides to MHCII molecules (Nedjic et al., 2008).

Concerning the MHCII antigen presentation, cTECs express unique lysosomal proteases, the thymic-specific serine protease (TSSP) and cathepsin L. Deficiency in these proteases result in a defective selection of $CD4^+$ T-cells (Gommeaux et al., 2009; Nakagawa et al., 1998). Specifically, the deletion of Cathepsin L led to the dramatic diminishment of polyclonal $CD4^+$ T-cell (Nakagawa et al., 1998), whereas the abrogation of TSSP

expression affected only certain TCR clones in $CD4^+$ T-cell repertoire (Gommeaux et al., 2009). In aggregate, cTECs represent the TEC population which due to their unique properties enables the differentiation and selection of functional repertoire of T-cells.

#### *2.1.4.3 Medullary thymic epithelial cells (mTECs)*

As described above, mTECs play a critical role in mediating recessive and dominant tolerance to self-antigens through a unique capacity to express thousands of TRAs and present them to developing T-cell in the context of MHC molecules. However, recent studies described that mTECs are highly heterogeneous and comprise several subpopulations with very distinct functional properties (Bornstein et al., 2018; Dhalla et al., 2020; Metzger et al., 2013; Miller et al., 2018; Nishikawa et al., 2010). Specifically, using single-cell RNA sequencing it was shown that mTEC compartment consist of minimal four different subsets, referred to as mTEC I-IV (Bornstein et al., 2018).

Traditionally, the mTEC population was divided according to the expression levels of MHCII and CD80 to immature $mTECs^{low}$ ($MHCII^{low}CD80^{low}$) and mature $mTECs^{high}$ ($MHCII^{high}CD80^{high}$) (Derbinski et al., 2005). Recently the $mTECs^{low}$ population (referred to as mTEC I) was shown to be composed of at least two functionally distinct subsets (Bornstein et al., 2018; Kadouri et al., 2020). A small fraction of this population consists of mTECs progenitors (mTEPCs) that possess the differentiation capacity and give rise to mature $mTECs^{high}$ (Gray et al., 2007a; Rossi et al., 2007a). Functionally, these postnatal mTEPCs are very similar to those observed in the embryonic thymus and defined by the expression of tight junction components claudin 3 and 4 (CLDN3, CLDN4) (Hamazaki et al., 2007; Sekai et al., 2014). Interestingly, the postnatal thymus also consists of $CLDN3^+CLDN4^+$ mTECs, which contrary to embryonic ones do not have the progenitor capacity and are mostly restricted to mature $mTECs^{high}$ population (Hamazaki et al., 2007). Recently, these mTECs progenitors in the postnatal thymus were characterized by the high expression of podoplanin (PDPN) and attributed to previously described jTECs (TECs localized at the cortico-medullary junction) (Miragaia et al., 2018). It was suggested that these cells are also potential precursors of a mature fraction of $mTECs^{low}$ (Onder et al., 2015). These mature $mTECs^{low}$ were described as a large fraction of mTEC I subset and were characterized by the high expression of genes encoding CCR7 ligands

(*Ccl21a* and *Ccl21c*), integrin β4 (*Itgb4*) and mTEC-specific cytokeratins (*Krt5* and *Krt14*) (Bornstein et al., 2018). Due to the high levels of CCL21, the major function of this mTECs is to recruit positively selected CCR7 expressing thymocytes to the medullary region of the thymus (Kwan and Killeen, 2004; Lkhagvasuren et al., 2013).

Mature mTECs$^{high}$ population (mTEC II) is phenotypically defined by the upregulation of several molecules that are associated with their specific function. During the maturation these cells overexpress MHCII, co-stimulatory molecules CD80 and CD86, and AIRE (Bornstein et al., 2018; Derbinski et al., 2005). This enables mTEC$^{high}$ to produce and present TRA antigens to developing thymocytes and drive their clonal deletion or Tregs conversion. The development of these AIRE$^{+}$ mTECs$^{high}$ was shown to be largely dependent on a cross-talk with thymocytes. Specifically, the signaling through the tumor necrosis factor (TNF) receptor family, such as CD40, receptor activator of nuclear factor-kappa B (RANK) or lymphotoxin β receptor (LTβR), the ligands of which are produced mostly by thymocytes, is particularly important for mTECs differentiation (Akiyama et al., 2008; Boehm et al., 2003; Rossi et al., 2007b). Since these receptors activate mostly the non-canonical NF-κB signaling pathway the disruption of its downstream components, such as IKKα, Relb, TRAF6 or NIK resulted in an altered mTECs$^{high}$ development and onset of autoimmunity (Akiyama et al., 2005; Kajiura et al., 2004; Kinoshita et al., 2006; Riemann et al., 2017). Moreover, it was reported that a conserved non-coding sequence 1 (CNS1) positioned upstream of the *Aire* coding region contains two NF-κB binding sites. The specific depletion of CNS1 element prevents the expression of AIRE and leads to the development of autoimmunity (Haljasorg et al., 2015; LaFlam et al., 2015). This together suggests that the expression of AIRE and subsequent maturation of mTEC to AIRE$^{+}$ mTECs$^{high}$ requires NF-κB.

As described above, the AIRE-driven expression of TRAs is mediated by the formation of molecular complexes that generate multiple double-strand breaks in TSS of responsive genes (Guha et al., 2017). Due to this mechanism, the AIRE$^{+}$ mTECs$^{high}$ were regarded as a terminally differentiated cell type with a relatively short lifespan (approximately 3 days) (Gray et al., 2007a). Nevertheless, over the past several years it was described that AIRE$^{+}$ mTECs$^{high}$ can further differentiate into their terminal stage (Bornstein et al., 2018; Miller

et al., 2018; White et al., 2010; Yano et al., 2008). Specifically, it was shown that mature mTECs$^{high}$ differentiate into "post-AIRE" mTECs (mTEC III) that are characterized by the downregulation of AIRE and MHCII expression and upregulation of several molecules that are associated with cornified epithelial pathways such as cytokeratins (KRT1 and KRT10), involucrin (IVL) or desmogleins (Metzger et al., 2013; Wang et al., 2012). Interestingly, same as the terminally differentiated keratinocytes (corneocytes), the KRT10$^{+}$post-AIRE mTECs lose their nuclei and form specific thymic structures called Hassall's corpuscles (Farr et al., 2002; Wang et al., 2012). This suggests that the process of cornification represents an alternative programmed cell death pathway of AIRE$^{+}$ mTECs$^{high}$ (Kadouri et al., 2020). Even the exact function of the post-AIRE mTECs is poorly understood, the previously described data suggests that corneocyte-like mTECs$^{high}$ may serve as a reservoir of TRAs that can be potentially presented by other thymic APCs (Watanabe et al., 2005). This hypothesis is supported by the fact that Hassall's corpuscles show the enhanced expression of chemokines that are associated with the chemoattraction of DCs and other myeloid cells (Wang et al., 2019b).

The other subset of terminally differentiated mTECs was described as thymic tuft-cells (Bornstein et al., 2018; Miller et al., 2018). This unique population of mTECs was characterized by its dependence on the transcriptional factor POUF3, expression of doublecortin-like kinase I (DCLK1) and genes involved in the taste reception signaling pathway (*Plcb2*, *Trpm5*, *Gnb3*). This expression profile together with a flask-shaped morphology highly resembles the mucosal tuft-cells (Bornstein et al., 2018; Gerbe et al., 2012; Miller et al., 2018). Although the function of these thymic tuft-cells is unclear, it was suggested that they may interact with the innate type of lymphocytes such as innate lymphoid cells type 2 (ILC2s) or natural killer T-cells (NKT) through the expression of IL-25 (Bornstein et al., 2018; Miller et al., 2018; Schneider et al., 2019). It was also described, that due to the considerable expression of MHCII and the regulation of IL-4 expression, these cells could regulate the development of FOXP3$^{low}$ precursors of Tregs (Owen et al., 2019). The heterogeneity and differentiation pathway of mTECs is described in Figure 3.

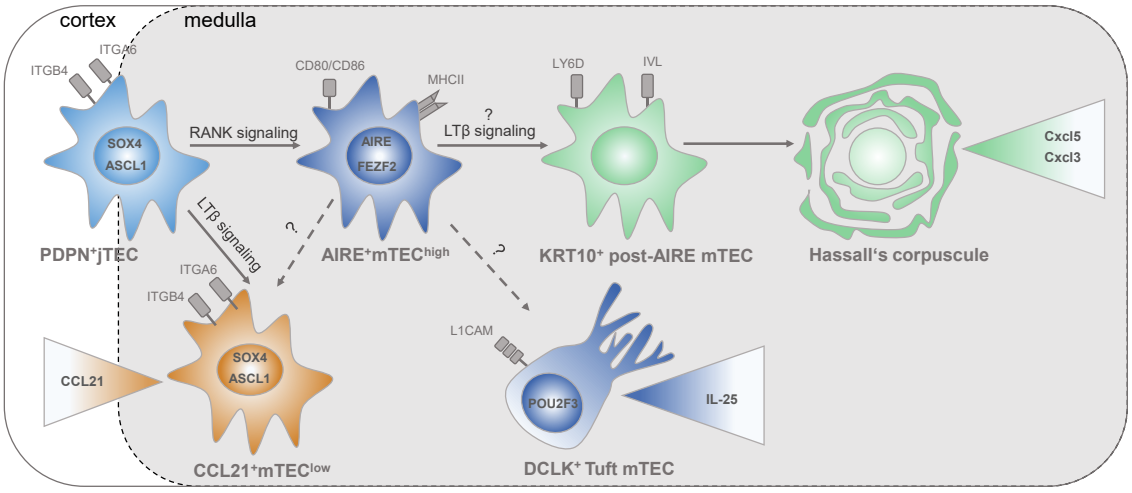


**Figure 3. Differentiation of medullary thymic epithelial cells.** The podoplanin$^{+}$ (PDPN) junctional thymic epithelial cells (jTECs) are the earliest subset of medullary TECs (mTECs). They have the capacity to give rice the mature CCL21$^{+}$mTEC$^{low}$ whose development is dependent on lymphotoxin β signaling (LTβ). Both CCL21$^{+}$mTEC$^{low}$ and PDPN$^{+}$ jTECs share expression of several molecules, including integrin subunit beta 4 (ITGB4), including integrin subunit alpha 6 (ITGA6) and SRY-Box Transcription Factor 4 (SOX4) and Achaete-Scute Family BHLH Transcription Factor 1 (ASCL1). PDPN$^{+}$ jTECs can also give rise to autoimmune regulator$^{+}$ (AIRE) mTECs$^{high}$. The differentiation is mainly driven by receptor activator of nuclear factor-kappa B (RANK) signaling. AIRE$^{+}$ mTECs$^{high}$ was shown to further differentiate into keratin 10$^{+}$ (KRT10) post-AIRE mTECs that share several markers with cornified epithelium, such as keratins or involucrin (IVL). During the differentiation, these cells lose their nuclei and form Hassall's corpuscles that overexpress chemokines from CXCL family. It was also suggested that AIRE$^{+}$ mTECs$^{high}$ can give rise to Doublecortin-like kinase I$^{+}$ (DCLK1) thymic tuft cells (Tuft mTECs) that are characterized by the dependence on POU Class 2 Homeobox 3 (POU2F3) transcription factor and production of IL-25. Inspired by *Kadouri et al.* (Kadouri et al., 2020).

#### *2.1.4.4 Plasmacytoid dendritic cells (pDCs)*

pDCs were described as a unique DC-lineage that is in the immune periphery dedicated to the production of type I interferons in response to viral stimulation (Cella et al., 1999). These cells are considered as part of the DC population because of the dependence on FMS-like tyrosine kinase 3 ligand (FLT3L) (D'Amico and Wu, 2003). Although they share several features with conventional cDCs, the exact origin of pDCs is still controversial. It was suggested that pDCs could originate either from common DC-progenitors (CDP) or common lymphoid progenitors (CLP) (Dress et al., 2019; Rodrigues et al., 2018). Nevertheless, the development of pDCs was shown to be completely dependent on transcription factor E2-2 that regulates the expression of other

molecules important for pDC lineage specification such as IFN-regulatory factor 7 (IRF7) and IRF8 as well as SPIB (Cisse et al., 2008; Ghosh et al., 2010). In general, pDCs can be also characterized by the surface expression of SiglecH, BM Stromal cell antigen 2 (BST2), or B220 (Swiecki and Colonna, 2015).

The exact function of pDCs in the thymus is still not completely clear. It was suggested that since the pDCs develop in the immune periphery, they can migrate to the thymus and present the peripheral antigens to the developing thymocytes (Bonasio et al., 2006; Li et al., 2009). This was further corroborated by the fact that OVA-pulsed pDCs can migrate to the thymic medulla and negatively select the OVA-specific T-cells (Bonasio et al., 2006; Hadeiba et al., 2012). Moreover, it was described that this migration is driven by the CCR9/CCL25 axis since the CCR9 abrogation results in an almost complete absence of pDCs in the thymus (Hadeiba et al., 2012). In this context it is interesting that CCR9 is indispensable for the migration of cells into the intestinal tissue (Kunkel et al., 2000). This may suggest that pDCs are able to transfer the food antigens or antigens derived from commensal bacteria to the thymus and subsequently induce central tolerance to harmless non-self-antigens (Hadeiba et al., 2012). In addition, it has been described that pDCs can directly interact with Hassall's corpuscles and participate in the generation of Tregs (Wang et al., 2019b; Watanabe et al., 2005).

#### *2.1.4.5 Classical DCs type 1 (cDC1)*

As suggested above, the development of all DCs subsets (pDCs, cDC1, and cDC2) from CDP is mostly dependent on the FLT3L (Waskow et al., 2008). The subsequent commitment to cDC1 lineage was shown to be dependent on basic leucine zipper transcriptional factor ATF-like 3 (BATF3) (Hildner et al., 2008), IRF8 (Schiavoni et al., 2002), and DNA-binding protein inhibitor ID2 (Hacker et al., 2003). This was formally proofed by the fact that mice lacking any of these factors exhibited a severe defect in cDC1 development (Hacker et al., 2003; Hildner et al., 2008; Schiavoni et al., 2002). Functionally, the cDC1 was shown to be very efficacious in the cross-presentation of exogenous antigens to MHCI molecules and subsequent activation of $CD8^+$ T-cells (den Haan et al., 2000). Also, due to the high expression of MHCII and cytokine IL-12, these cells also participate in the differentiation of $CD4^+$ T-cells to TH1 cells (Durai and

Murphy, 2016; Mashayekhi et al., 2011). Phenotypically the cDC1 can be easily defined by the expression of chemokine receptor XCR1 and CD8α (Guilliams et al., 2016).

Unlike the pDCs, the cDC1 develop within the thymic tissue from migrant precursor cells. Recently by using single-cell RNA sequencing, it was described that genes associated with cDC1 commitment were observed already in pre-DC population in BM. This suggests that precursors of cDC1 ($SiglecH^{-}Ly6C^{-}$) migrate to the thymus where they undergo intrathymic licensing leading to the upregulation of MHCII and CD80/86 expression (Liu et al., 2009; Schlitzer et al., 2015). It was described that CCR7/CCL21 chemokine axis is essential for the recruitment of cDC1 from BM (Cosway et al., 2018). Therefore the *Ccr7*$^{-/-}$ mice show a marked reduction of cDC1 in the thymus (Hu et al., 2017). Although the mechanisms controlling the recruitment of cDC1 into the thymus are known, the chemokines that control the intrathymic positioning are poorly defined. However, it was described that XCR1 expressing cDC1 are attracted towards the XCL1 ligand which is in the thymus exclusively expressed by $AIRE^{+}$ mTECs. Interestingly, the expression of XCL1 was shown to be dependent on AIRE (Lei et al., 2011). This suggests that cDC1 localize in the medullary region of the thymus in close contact with mTECs which enables them to cross-present mTEC-derived self-antigens to developing thymocytes (Lei et al., 2011; Perry et al., 2014). Since the peripheral cDC1 effectively cross-present exogenous antigens on MHCI molecules, it was suggested that thymic cDC1 are indispensable for clonal deletion of self-reactive $CD8^{+}$ T-cells. However, the deep TCR sequencing of $CD8^{+}$ T-cells isolated from *Batf3*$^{-/-}$ mice, which completely lack the cDC1 cells, show no changes in the TCR repertoire (MacNabb et al., 2019). This, together with other studies, proposes that cDC1 would rather be important for the deletion of $CD4^{+}$ T-cell or their conversion to Treg lineage (Lei et al., 2011; Perry et al., 2014). Specifically, it has been shown that the TCR repertoire of Tregs was altered in *Batf3*$^{-/-}$ mice suggesting a role of cDC1 in Tregs generation (Perry et al., 2014). On the other hand, several studies observed no Tregs phenotype in *Batf3*$^{-/-}$ animals (Herbin et al., 2016; Leventhal et al., 2016). Also, the specific depletion of MHCII in XCR1-producing cDC1 cells demonstrates no apparent effect on Tregs generation (Wohn et al., 2020). This discrepancy suggests that the exact role of cDC1 in Tregs generation awaits its resolution.

#### *2.1.4.6 Classical DCs type 2 (cDC2)*

cDC2 are defined by the surface expression of CD11b and CD172α (SIRPα) and comprise a heterogeneous population of cells defined by the production of MGL2 (CD301b), endothelial cell adhesion molecule (ESAM), and CLEC12A (Durai and Murphy, 2016; Kumamoto et al., 2013; Lewis et al., 2011) or by the transcription factors T-bet and RAR-related orphan receptor gamma (RORγ) (Brown et al., 2019). Compared to cDC1, the specific transcription factors driving the cDC2 commitment are poorly defined. Nevertheless, the IRF4, RELB, and recombining binding protein suppressor of hairless (RBPJ) were described to be important for cDC2 development (Caton et al., 2007; Lewis et al., 2011; Suzuki et al., 2004; Wu et al., 1998). Functionally, the cDC2 were shown to be effective in MHCII antigen loading and presentation and thus specifically attributed to the activation of $CD4^+$ T-cells (Dudziak et al., 2007). Due to the phenotypic similarity (CD11c, MHCII, CD11b and SIRPα expression) and analogous function, the monocytes-derived DCs (moDCs) are often incorporated among the cDC2 subsets. However, compared to cDCs and pDCs, the differentiation of moDCs fully depends on macrophage colony-stimulating factor (M-CSF) and not on FLT3 ligand (Hettinger et al., 2013). Also since the internal heterogeneity of those cells does not allow the specific gating, moDCs can be distinguished from the cDC2 by the expression of molecules often associated with macrophages or monocytes, such as tyrosine-protein kinase MER (MERTK), CD64 or chemokine receptor CX3CR1 (Guilliams et al., 2014).

Compared to cDC1, thymic cDC2s (defined as $XCR1^-CD8\alpha^-Sirp\alpha^+$) are of extrathymic origin and entering the tissue via vascular plexus connected to the thymic medulla (Li et al., 2009). Factors that drive the cDC2 migration into the thymus and also affecting the intrathymic positioning are largely unknown. The only described factors driving the migration of cDC2 to the thymic medulla are ligands of CCR2 (CCL2, CCL8, and CCL12) that are expressed by mTECs. It has been described that the number of cDC2 are reduced in *Ccr2*$^{-/-}$ mice (Baba et al., 2009). Also, it was suggested that cDC1 and cDC2 show different intrathymic localization. Whereas the cDC1 are distributed thorough the thymus parenchyma, the cDC2 remain close to the vascular region of the thymic medulla (Hu et

al., 2015; Perry and Hsieh, 2016). Several studies also reported that cDC2 can be localized in the thymic cortex (Baba et al., 2009; Stritesky et al., 2013).

Functionally, cDC2s were shown to efficiently present blood-borne antigens for the selection of developing T-cell (Atibalentja et al., 2009; Atibalentja et al., 2011; Baba et al., 2009; Proietto et al., 2008). This capacity is in concordance with the intrathymic position of cDC2 with preference to thymic vascular regions (Hu et al., 2015). Since these cells are of extrathymic origin they are capable of acquiring antigens in the periphery, transport them to the thymic medulla and subsequently present those antigens to developing T-cells (Bonasio et al., 2006; Li et al., 2009). Specifically, it was shown, that OT-II T-cells underwent clonal deletion mediated by immigrating cDC2 in a model where mOVA is exclusively produced by cardiomyocytes (Bonasio et al., 2006). As suggested above, cDC2s are the major source of CCR4 ligands (CCL17 and CCL22) that drive the migration of positively selected thymocytes from the cortex to the medulla. Interestingly the *Ccr4*$^{-/-}$ mice demonstrated that disruption of thymocyte-cDC2 interactions leads to the aberrant clonal deletion and development of autoimmunity (Hu et al., 2015). In addition to clonal deletion, cDC2s were shown to be efficient in the generation of Tregs (Proietto et al., 2008). Specifically, it has been demonstrated that cDC2s are important not only for the generation of polyclonal populations of Tregs but also for the differentiation of AIRE-dependent MJ23 TCR-specific T-cells to Treg lineage (Leventhal et al., 2016). This was further corroborated by the fact that an increased ratio of cDC2 to cDC1 cells in the thymus of *Ccr7*$^{-/-}$ mice led to the expansion of the thymic Tregs pool (Hu et al., 2017). This together suggests that cDC2, in contrast to cDC1 and pDCs, are important for Tregs generation. On the other hand, since there are conflicting reports about the function of cDC1 and cDC2 in Tregs selection, the contribution of these two subtypes to the mechanisms of dominant tolerance needs to be further investigated (Perry et al., 2014).

As suggested above, the moDCs or "classical" macrophages are phenotypically and functionally very similar to cDC2 (Guilliams et al., 2014). Thus, the exact function of this subset of cells is very poorly described and very often incorrectly assigned to conventional cDC2. Nevertheless, it was suggested that F4/80$^{+}$ macrophages preferentially localized in the thymic cortex and are essential for deletion (Breed et al., 2019) of cortical thymocytes

and scavenging of apoptotic T-cell (Esashi et al., 2003). Also, the monocyte/macrophage population was described to be important for the regulation of invariant natural killer T-cells (iNKT) in the thymus (Wang et al., 2019a).

Together it is clear that different populations of myeloid cells (pDCs, cDC1, cDC2, or moDCs) contribute to the mechanisms of central tolerance. Unfortunately, the clear functional determination of each DC-subset is still poorly defined and needs to be further explored. Especially, to describe the exact function of cDC2s, the mouse model enabling the specific targeting of these cells is needed to assess their contribution to both clonal deletion and Tregs selection. On the other hand, since the medullary localization of those cells is regulated by chemokines expressed predominantly by mTECs, the cooperation of those cell subsets seems to be essential for the proper functioning of central tolerance.

#### *2.1.4.7 Thymic B-cells*

Together with TECs, thymic populations of DCs, and macrophages, thymus also accommodates a specific population of thymic B-cells, which are localized mainly in the medulla and CMJ of the thymus and comprise a similar proportion of total thymic cells as DCs and mTECs (Lu et al., 2015; Perera et al., 2013). Although the proportion of these cells in the thymus is relatively high, not much is known about their function. Compared to peripheral B-cells, thymic B-cells are highly primed for antigen presentation and this is accompanied by an enhanced production of MHCII and co-stimulatory molecules CD80 and CD86 (Perera et al., 2013). This suggests that B-cells undergo a certain level of intrathymic licensing leading to the upregulation of previously mentioned molecules but also the expression of AIRE (Yamano et al., 2015). It was suggested that this licensing is mediated by interaction with thymocytes mostly through the CD40 signaling axis. Comparing the transcriptome of thymic B-cell isolated from *Aire*$^{-/-}$ and *Aire*$^{+/+}$ mice, it was revealed that B-cells are able to produce several AIRE-dependent TRAs (Yamano et al., 2015). This enables the thymic B-cells to regulate both, the clonal deletion and Tregs differentiation. In addition, thymic B-cells play an important role in driving tolerance to B-cell specific antigens that are presented in the context of MHC molecules to developing T-cells (Detanico et al., 2011).

### *2.1.5 Indirect presentation of self-antigens*

As described above the total population of mTECs produces thousands of TRAs. However, each individual mTEC produces and presents a distinct set of TRAs, which constitutes only 1-3% of the total TRA pool (Brennecke et al., 2015; Derbinski et al., 2008; Meredith et al., 2015). This, together with the limited number of mTECs in the thymic medulla, represents a certain limitation for the antigen encounter by developing T-cells. To overcome this limitation, there are two possible mechanisms in the thymus: (i) the motile thymocytes during the medullary development scan the surface of several hundreds of mTECs to encounter most of the displayed TRAs (Klein, 2009); and (ii) the TRAs are presented to developing T-cells not only directly by mTECs itself, but also indirectly by thymic DCs (Gallegos and Bevan, 2004). The indirect presentation of TRAs not only increases the number of presented TRA-peptides in the thymic medulla but also enables the presentation of the very same peptide by different cellular microenvironments. This, in general, more closely mimics the antigen presentation in the periphery and extend the scope and stringency of negative selection (Klein et al., 2014; Perry and Hsieh, 2016).

The essential role of DCs in the negative selection of self-reactive T-cells was firstly postulated by their genetic ablation using diphtheria toxin-mediated depletion of CD11c expressing cells (*CD11c*$^{Cre}$*ROSA26*$^{DTA}$ mice). In this experimental system, the general depletion of DCs led to impaired negative selection followed by the development of fatal autoimmunity (Ohnmacht et al., 2009). Also, several years ago it was postulated that negative selection of CD4$^+$ T-cells specific to mTEC-derived antigen requires its indirect presentation by DCs. Specifically, the deletion of OT-II$^+$ T-cells in the *RIP-mOVA* mouse model was shown to be dependent on indirect OVA presentation by BM-derived APCs (Gallegos and Bevan, 2004). Recently, using two-photon microscopy of thick thymic slices, it was proposed that the negative selection of CD8$^+$ (OT-I$^+$) T-cells in the *RIP-mOVA* system was mediated by antigen presentation by DCs (Lancaster et al., 2019). On the other hand, mTEC-specific MHCII knock-down mice model suggested that mTECs itself are sufficient for negative selection of OT-II$^+$ T-cells, regardless of DC depletion (Hinterberger et al., 2010). To distinguish the exact role of indirect TRA presentation to the T-cell selection, *Perry et al.* sequenced TCRα repertoire of CD4$^+$ T-cells from mice

either with down-regulated MHCII expression on mTECs or depleted MHCII presentation on BM APCs. This experimental system proofed that some of the TCR clones specific to mTEC-derived TRAs are selected only by BM APCs. Moreover, the TCR clones sensitive to indirect antigen presentation were shown to be mostly deviated into the Treg lineage (Perry et al., 2014). It was also described that some of the AIRE-dependent TRAs, produced by mTECs, require the indirect presentation by thymic DCs (Leventhal et al., 2016; Taniguchi et al., 2012). This, together with the fact that the genetic ablation of MHCII specifically on DCs (*CD11c$^{Cre}$MHCII$^{fl/fl}$* mice) impaired the Tregs selection (Leventhal et al., 2016), suggests that indirect presentation of self-antigens by thymic DCs plays an important role in the mechanisms of central tolerance.

#### *2.1.5.1 Cooperative antigen transfer*

The indirect presentation of mTEC-derived antigens by BM APCs is enabled by the antigen handover between these cell types. This process is called cooperative antigen transfer (CAT) and operates only in one direction: from mTECs to thymic DCs (Koble and Kyewski, 2009). Moreover, CAT from mTECs was specifically attributed only to the thymic population of DCs, since the DCs isolated from splenic tissues possess very limited capacity ty acquire mTEC-derived antigens (Kroger et al., 2017). This suggests that CAT is a tightly regulated process that requires specific signals and molecules produced by both mTECs and thymic DCs.

In general, the process of CAT was shown to be mediated by several mechanisms: (i) endocytosis or phagocytosis of mTEC's apoptotic vesicles (Koble and Kyewski, 2009; Perry et al., 2018); (ii) acquisition of MHCII molecules or parts of the plasma membranes by the process of trogocytosis (Kroger et al., 2017; Millet et al., 2008); and (iii) endocytosis of mTEC-derives exosomes (Skogberg et al., 2015). In the case of mice studies, it was suggested that CAT is mostly operational through cell-cell contact-dependent mechanisms such as trogocytosis and/or endocytosis of apoptotic bodies (Millet et al., 2008; Perry et al., 2018). Recently, the phagocytosis of apoptotic cells was described to be important for thymocyte negative selection (Kurd et al., 2019). This suggests that scavenger receptors expressed by thymic BM APCs should be the potent regulators of CAT. To this point, the CD36, a scavenger receptor predominantly expressed

by thymic cDC1, was shown to regulate the transfer of surface antigens from mTECs. Furthermore, the CD36-dependent antigen transfer was described to be important for both clonal deletion and Treg selection (Perry et al., 2018). Another level of CAT regulation is through the attraction of a particular DC population to the vicinity of mTECs (Hubert et al., 2011). As mentioned previously, the AIRE, together with TRAs, regulates also the production of several chemokines, which have the capacity to recruit DCs to the thymic medulla (Cosway et al., 2018; Hubert et al., 2011). Specifically, the XCL1, which attracts $XCR1^{+}$cDC1 to $AIRE^{+}$ mTECs, was suggested to be an important regulator of CAT (Lei et al., 2011).

It has been documented by several publications that the complexity of CAT is partially due to the heterogeneity of BM APCs in the thymus (Koble and Kyewski, 2009; Kroger et al., 2017; Perry et al., 2018). As suggested above, the thymic DCs seem to be a major population of cells with the ability to acquire the mTEC-derived antigens through the process of CAT (Lancaster et al., 2019; Leventhal et al., 2016; Perry et al., 2014). On the other hand, several studies reported that specific subtypes of thymic DCs vary in their capacity to acquire those antigens (Kroger et al., 2017; Perry et al., 2018). Using *in vitro* co-cultivation experiments it was suggested that transfer of MHC molecules from TECs to cDC1 and cDC2 occurred at the same efficiency, while the antigen transfer to pDCs is limited (Kroger et al., 2017). The marginal role of pDCs in the presentation of mTEC-derived antigens was confirmed by using pDC-depleted mouse models, where the selection of AIRE-dependent TCR clones of Tconv or Tregs was found unchanged (Leventhal et al., 2016; Perry et al., 2014). On the other hand and as suggested above, the cDC1 and cDC2 were shown to be indispensable for the selection of AIRE-dependent TCR clones of Tregs. Specifically, by using the *Batf3*$^{-/-}$ mice (cDC1 depletion) it was demonstrated that around 12% of Treg TCR specificities was controlled by cDC1 (Perry et al., 2014), whereas the others by cDC2 (Leventhal et al., 2016) or mTECs itself (Hinterberger et al., 2010). Importantly, even such a minor depletion of Treg clones in *Batf3*$^{-/-}$ mice was sufficient to provoke the onset of autoimmune disease (Perry et al., 2018). It was also described that amount of CAT to cDC1 and cDC2 cells differs in relation to nature of the antigen. While the transfer of surface antigens from mTECs to cDC1 or cDC2 occurred at the same efficiency, the transfer of intracellular green

fluorescent protein (GFP) antigen (*Aire-GFP* mouse model) was restricted mostly to cDC1 (Kroger et al., 2017; Perry et al., 2018). On the other hand, using the *RIP-OVA* mice model, the transfer of intracellular OVA was increased to cDC2 (Lancaster et al., 2019). Since the expression of GFP in *Aire-GFP* mouse model is in the thymus restricted only to $AIRE^{+}$ $mTECs^{high}$ population (Gardner et al., 2008), whereas the production of OVA (*RIP-OVA* mice) is attributed mostly to $mTECs^{low}$ or post-AIRE mTECs (Lancaster et al., 2019; Mouri et al., 2017), the cDC1 and cDC2 probably interact with different developmental stages of mTECs. This suggests that cDC1 presumably acquire the antigens from $mTECs^{high}$ while cDC2 from post-AIRE mTECs (Lancaster et al., 2019; Lei et al., 2011; Perry et al., 2014).

Taken together, it is clear that cooperation and antigen handover from mTECs to various APC populations determine the cell-fate of developing T-cell. The failure of a single component of this very complex system can lead to autoimmune reactions and potential development of autoimmune diseases. Although the process of thymic tolerance is designed to delete all self-reactive T-cells, some of them escape to the periphery, where they autoreactive potential is under surveillance of another layer of immune tolerance, peripheral tolerance (Kim et al., 2007b).

### 2.2 Peripheral tolerance

As alluded above, central tolerance is an extremely efficient process that allows only 5% of T-cells to exit to the immune periphery (von Boehmer et al., 2003). Despite this, some of the self-reactive T-cell clones which exhibit the low affinity/avidity TCRs or clones whose antigen is not expressed in the thymus, escape the mechanisms of central tolerance and are released to the periphery (Liu et al., 1995; Nichols et al., 2007; Zehn and Bevan, 2006). For this reason, the central tolerance is complemented by a set of mechanisms that have the potential to induce tolerance not only to self-antigens but also to harmless non-self-antigens in the immune periphery, such as food or commensal microbiota (Mueller, 2010).

### *2.2.1 Peripheral tolerance to self-antigens*

The first and the simplest barrier to self pMHC recognition is the process of antigen sequestration or self-antigen ignorance. This represents a state where self-antigens cannot be seen by the immune system due to the several reasons: (i) naïve T-cells are specifically excluded from non-lymphoid tissue in which the probability of contact with TRA is much higher than in lymphoid organs, (ii) self-antigen is primarily localized in the immune-privileged site where the high expression of pro-apoptotic molecules (TRAIL, FAS-Ligand) and secretion of anti-inflammatory cytokines (IL-10, TGFβ) block the immune cell activation (Forrester et al., 2008), and (iii) the amount of particular antigen is too low to trigger the immune response (Mueller, 2010). The function of antigen sequestration was nicely described by using transgenic mice expressing mOVA under the keratin-14 promotor (*K14-mOVA*), where the expression of mOVA is restricted to the skin, esophagus, tongue, and thymus. Interestingly, the adoptive transfer of OT-I and OT-II T-cells did not trigger autoimmunity. However, disruption of the skin in the very same model caused infiltration of the skin by OVA-specific T-cells and development of the autoimmune reaction (Bianchi et al., 2009). This suggests, that without tissue inflammation the presentation of OVA does not cause autoimmunity. Furthermore, the immune-privileged sites are also not resistant to the development of autoimmune reactions once inflammation within the organs occurs (Greter et al., 2005). Another mechanism of peripheral tolerance is based on the anergy, which was described as a state of T-cell unresponsiveness after the encounter of pMHC without proper co-stimulatory signal (Hawiger et al., 2001; Liu et al., 2002). The T-cell anergy state is often caused by the interaction with tolerogenic DCs that in steady-state conditions express only very low levels of CD80/CD86 molecules. Similar to the mechanisms described above, the tolerogenic potential of DCs could be disrupted by the local inflammatory reaction leading to DCs activation followed by upregulation of co-stimulatory molecules through the pattern recognition receptors (PRRs) stimulation (Steinman et al., 2003). Also, the lack of CD80/CD86 and the increased production of cytokines TGFβ, IL-10, or IL-2 give tolerogenic DCs the capacity to induce the differentiation of $CD4^+$ T-cells to peripheral Tregs (pTregs) (Kalekar et al., 2016; Kretschmer et al., 2005).

One of the most crucial mechanisms of peripheral immune suppression is mediated by Tregs. The population of Tregs consists of thymic Tregs (tTregs) and pTregs and the depletion of both leads to the development of fatal autoimmunity (Kim et al., 2007b; Wing and Sakaguchi, 2010). Also, the direct mutations in the *Foxp3* gene, which results in the absence of mature Treg-lineage, causes the development of multi-organ autoimmune disease in both mice and humans (Bennett et al., 2001; Fontenot et al., 2003). Specifically, the IPEX syndrome (immunodysregulation polyendocrinopathy enteropathy X-linked) that is manifested by severe enteropathy, dermatitis, endocrinopathy, nail dystrophy, and several other symptoms, is linked to the dysfunction of FOXP3, which was described as s master regulator of Treg function (Bennett et al., 2001; Fontenot et al., 2003). Functionally both types of Tregs express the specific cell-surface molecules and cytokines that were proposed to play an essential role in Treg-mediated cell suppression (Josefowicz et al., 2012). Tregs overexpress CD25, a subunit of the IL-2 receptor, which is essential for their development and homeostasis and also fulfills the major role in T-cell suppression since it enables the Tregs to deprive effector T-cells of IL-2 and thus block their proliferation (Fontenot et al., 2005; Pandiyan et al., 2007). Similarly, Tregs also produce high levels of cytotoxic T-lymphocyte antigen 4 (CTLA-4), T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and lymphocyte activation gene 3 (LAG-3), that block the activation of DCs and induce the production of tolerogenic cytokines IL-10 or TGFβ (Huang et al., 2004; Takahashi et al., 2000; Yu et al., 2009). The expression of two ectoenzymes CD39 and CD73 were also shown to be specifically enriched in Tregs. It was described that both these enzymes facilitate the secretion of cyclic adenosine monophosphate (cAMP) that inhibits the activation of DCs and blocks the proliferation of T-cells (Deaglio et al., 2007). In addition to surface molecules, several secreted proteins, such as cytokines IL-10, IL-35, and TGFβ or granzyme B, were shown to be important for Treg-mediated suppression (Josefowicz et al., 2012). Specifically, the genetic depletion of IL-10 in $FOXP3^{+}$ Tregs leads to the break of intestinal tolerance and the development of spontaneous colitis (Chaudhry et al., 2011; Rubtsov et al., 2008). Also, the depletion of IL-12p35 or EBI3, two major components of IL-35 complex, caused the development of inflammatory bowel disease (Collison et al., 2007).

Along with the previously mentioned mechanisms, the clonal deletion of self-reactive T-cells was suggested to be operational also in the immune periphery. This function was specifically attributed to a population of lymph node stromal cells (LNSCs) that apart of its skeletal function, i.e. they form the 3-D structure and architecture of lymphoid tissues, they also express a small number of TRAs (Lee et al., 2007; Malhotra et al., 2013). It was shown that LNSC can effectively deplete adoptively transferred OT-I T-cells in the mouse model were OVA is produced under the promotor of an intestinal fatty-acid binding protein (iFABP) (Lee et al., 2007). The ability to produce TRAs was associated with two types of LNSCs: fibroblastic reticular cells (FRCs) that were responsible for iFABP presentation (Fletcher et al., 2010) and lymphatic endothelial cells (LECs) that are able to produce and present melanocyte tyrosinase and effectively deplete tyrosinase-specific $CD8^+$ T-cells (Cohen et al., 2010; Nichols et al., 2007). Moreover, it was described that LNSCs can acquire pMHCII molecules from DCs and thus can potentially affect the peripheral selection of $CD4^+$ T-cells (Dubrot et al., 2014). This prediction was confirmed by the fact that the specific depletion of MHCII from LNSCs exhibited enhanced T-cell infiltration and increased production of autoantibodies to several tissues (Dubrot et al., 2018). Recently, the self-antigen presentation by LNSCs was shown to be able to induce the generation of antigen-specific pTregs (Nadafi et al., 2020).

Apart from LNSCs, the expression of TRAs was also found in the rare population of DC-like cells ($CD11c^+MHCII^+EpCAM^+$) residing in the border of T-cell and B-cell zone in the lymph nodes (Gardner et al., 2013). Interestingly it was described that this population expresses a reasonable amount of AIRE and is able to produce around two hundred TRAs that are almost non-overlapping with those expressed by mTECs (Gardner et al., 2008). Using neo-self antigen technology it was suggested that these extrathymic AIRE-expressing cells (eTACs) can clonally delete both the $CD8^+$ and $CD4^+$ T-cells (Gardner et al., 2008; Gardner et al., 2013). Furthermore, single-cell RNA sequencing and multiparametric flow cytometry experiments identified the presence of AIRE-expressing DC-like cells also in human tonsils. Nevertheless, compared to mouse eTACs, the expression of AIRE in DC-like human cells was not associated with the production of TRAs (Fergusson et al., 2018). Thus the exact function of AIRE in these cells is still unclear and remains to be determined.

The above described data from published reports collectively suggest that immune periphery encompasses several mechanisms that can inactivate or delete self-reactive T-cells and hence prevent the organism from the development of autoimmune diseases. Apart from tolerance to self-antigens, the immune periphery, such as the gastrointestinal tract, also possesses several specific mechanisms to tolerize immune system to harmless non-self-antigens.

### *2.2.2 Peripheral tolerance to non-self-antigens*

As suggested above, the presentation of antigens that are not encoded in the host genome, such as antigens derived from the intestinal microbiota or food, is very limited in the thymus. However, because these antigens are by nature associated with microbial presence and interact with the immune system on mucosal and epithelial surfaces, they can under certain circumstances erroneously activate it. For this reason, central tolerance is complemented by several other mechanisms with the capacity to tolerate these antigens (Nutsch and Hsieh, 2012). One such mechanism is described as oral tolerance. It is defined as a state of unresponsiveness to antigens acquired orally and is effective mostly to food antigens (Rezende and Weiner, 2017). In general, the presentation of food antigens largely depends on classical DCs in gut-draining mesenteric lymph-nodes (mLN) (Worbs et al., 2006). Specifically, these $CD103^+$ DCs are able to take up intestinal antigens in lamina propria and migrate along the gradient of CCR7 ligands to mLN (Jang et al., 2006; Schulz et al., 2009). Once entering the mLN, these $CD103^+$ DCs start the expression of metabolites of retinoic acid (RA) (vitamin A) that induces the production of gut-homing receptor CCR9 and integrin-α4β7 on antigen-experienced T-cells (Iwata et al., 2004). Moreover, the induction of gut-homing receptors and the presence of RA drives the expression of FOXP3 in antigen-specific T-cells and mediate their differentiation into pTregs (Benson et al., 2007; Sun et al., 2007). Interestingly, the abrogation of integrin-β7 or CX3CR1 signaling resulted in decreased selection of pTregs in the gut-associated tissues suggesting that migration of $CX3CR1^+CD103^+$ DCs and the gut-homing program is essential for the generation of pTregs specific to food antigens (Hadis et al., 2011).

Another step of peripheral immune tolerance to harmless non-self-antigens is the suppression of T-cells specific to commensal bacteria. It was suggested that Tregs can

inhibit responses to commensal microbiota caused by the $CD4^+$ T-cells (Atarashi et al., 2011; Lathrop et al., 2011). However, the origin of these Tregs is still controversial. Several studies have reported that these microbiota specific Tregs are generated directly in the thymus (Cebula et al., 2013; Hadeiba et al., 2012). Specifically, deep sequencing of TCRs from tTregs revealed the changes in their repertoire in germ-free animals (Cebula et al., 2013). It was also hypothesized that $CCR9^+$ pDCs and/or cDC2 cells that migrate to the thymus from periphery may present the microbiota-derived antigens to developing thymocytes and subsequently generate Tregs (Bonasio et al., 2006; Hadeiba et al., 2012). On the other hand, the presence of *Clostridia* species in the gut was shown to promote the generation of colonic pTregs (Atarashi et al., 2011). This effect has been specifically attributed to the production of microbial metabolites, the short-chain fatty acids (SCFA) that have the capacity to induce FOXP3 expression in intestinal $CD4^+$ T-cells (Arpaia et al., 2013; Mariño et al., 2017). In addition, the specific subtype of $FOXP3^+$ pTregs that co-express master regulator of Th17 response RORγ were shown to be highly dependent on the microbiota or microbial metabolites (Ohnmacht et al., 2015; Sefik et al., 2015; Song et al., 2020). Thus the induction of intestinal pTregs is mediated by specific subtypes of gut-associated APCs that are able to acquire and present microbial antigens to T-cells. Interestingly, the innate lymphoid cells type 3 (ILC3s) were described as the major population of intestinal APCs that have the capacity to tolerize microbiota-specific T-cells (Hepworth et al., 2015; Hepworth et al., 2013). These gut-related ILC3s express high levels of MHCII and efficiently present microbiota-derived antigens to intestinal T-cells (Hepworth et al., 2015). In this context, the depletion of MHCII specifically in ILC3s led to the development of T-cell dependent intestinal inflammation (Hepworth et al., 2013). This suggests that gut-related ILC3s can delete or inactivate microbiota-specific T-cells. Consistent with this, it was described that these $MHCII^+$ ILC3s secrete high levels of IL-2 and Aryl hydrocarbon receptor (AHR) which were shown important for driving $CD4^+$ T-cells to Treg-lineage (Hepworth et al., 2015; Qiu et al., 2013). Complementary to this, it has been shown that IL-1β-stimulated ILC3 upregulate the expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), which drives intestinal macrophages and DCs to produce IL-10, TGFβ, and RA and thus support the selection of microbiota-specific pTregs (Coombes et al., 2007; Mortha et al., 2014).

Together, it is clear that the mechanism of T-cell tolerance is a multilayered process that operates in very distinct anatomical places. The tolerance to body own self-antigens is mostly achieved by dominant or recessive tolerance in the thymus, supplemented by several mechanisms of peripheral tolerance in lymph nodes or spleen. On the other hand, the tolerance to microbiota or food-derived antigens is mostly enforced in gut-related tissues. Breakdown of any of these layers of tolerance can lead to the development of severe autoimmune symptoms such as APS-1, IPEX, or inflammatory bowel diseases (IBDs) (Filipp et al., 2018). The schematics of multilayered process of T-cell tolerance is depicted in Figure 4.

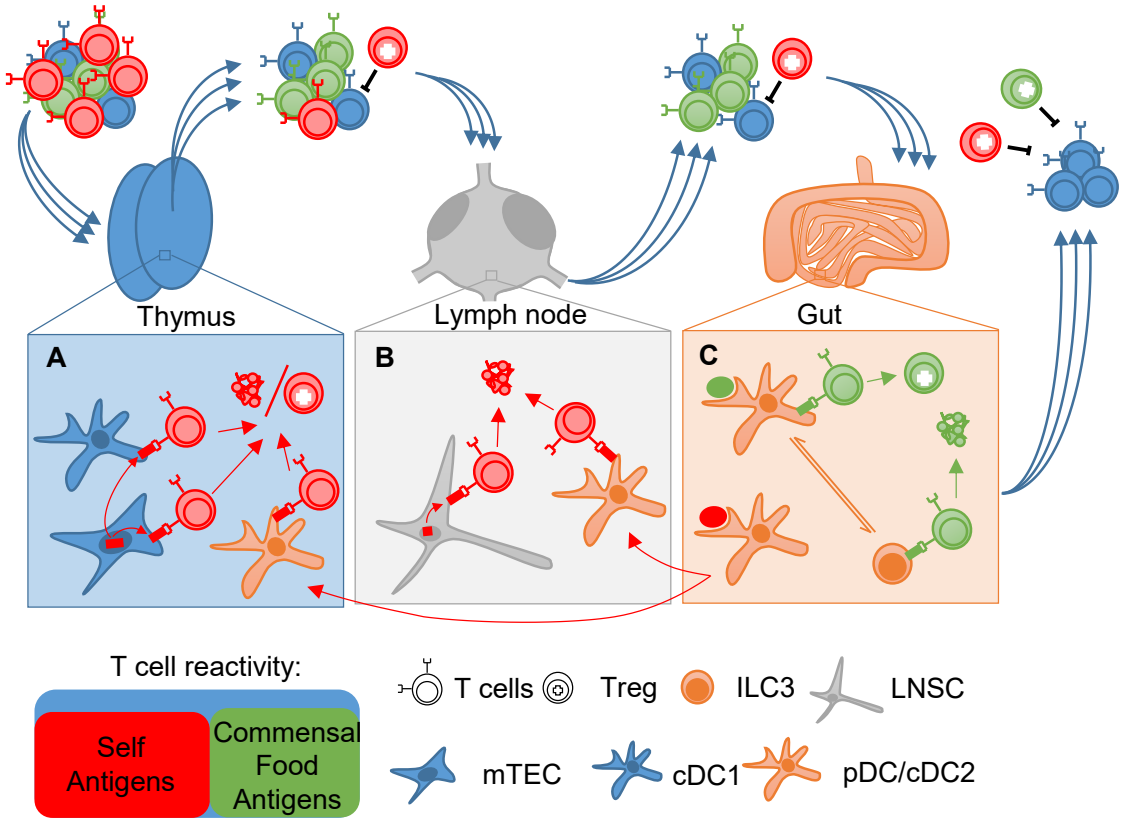


**Figure 4. Multilayered mechanism of T-cell tolerance.** The schema displays the consecutive steps of T-cell selection in the thymic tissue, lymph nodes, and the gut. Mixed T-cell populations are shown as clones that are left after each particular step of selection. Specificities of T-cell clones are color-coded: red - reactivity to self-antigens, green - reactivity to microbial or food antigens, blue – reactivity to remaining antigens. **A**. The thymus serves as a first T-cell selection checkpoint where cognate T-cells are either clonally deleted or converted to regulatory T-cells (Tregs). The selection occurred mostly to self-antigens that are presented directly by medullary thymic

epithelial cells (mTECs) or indirectly by classical dendritic cells type 1 (cDC1). Alternatively, the plasmacytoid dendritic cells (pDC) or classical dendritic cells type 2 (cDC2) can acquire self-antigens in the periphery and transferred them to the thymus. **B**. The next step of T-cell selection occurs in peripheral lymph nodes, where mostly the self-antigens are presented by cDC2 or lymph node stromal cells (LNSC). Cognate T-cells could be either deleted or anergized. **C**. T-cell reactive to microbial and food antigens are selected mostly in the gut tissue. Specifically, the innate lymphoid cell type 3 (ILC3) could directly delete the T-cells specific to microbial antigens, whereas the cDC2 mostly contribute to the generation of peripheral Tregs. The result of this complex process of tolerance is the presence of a functional and safe peripheral repertoire of T-cell clones. Adopted from *Filipp et al.* (Filipp et al., 2018).

## 3. TOLL-LIKE RECEPTORS

The development of the immune system is dependent on the co-evolution of microbes and the host. Thus the recognition of microbial structures is an essential mechanism for the initiation of immune responses and is mediated by germ-line encoded PRRs. These receptors recognize conserved molecular structures, referred to as pathogen-associated molecular patterns (PAMPs) that are shared among the microbes (Janeway and Medzhitov, 2002). The first identified and far the most studied PRRs are TLRs (Kawai and Akira, 2010). Structurally, TLRs are transmembrane proteins with surface domains containing leucine-rich repeats (LRRs) that recognize PAMPs, transmembrane domains, and intracellular TIR (TLR-interleukin 1 receptor) domains required for signal transduction (Botos et al., 2011; Medzhitov et al., 1997). Currently, 10 humans and 12 murine functional TLRs have been identified. TLR1-TLR10 was shown to be conserved in both species whereas the TLR11, 12, and 13 are present only in the mouse genome (Kawasaki and Kawai, 2014; Rock et al., 1998). Functionally, each TLR was shown to have a different function in terms of PAMP recognition and subsequent immune responses. On the other hand, the downstream signaling through all TLRs often leads to the increased secretion of pro-inflammatory cytokines, chemokines, type I interferons (IFNs), or antimicrobial peptides (Kawasaki and Kawai, 2014).

### 3.1 Ligands of Toll-like receptors

Based on the intracellular localization, the TLRs could be divided into two major groups: (i) TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 that are present on cell surfaces and

binds mainly components of microbial membranes such as carbohydrates, lipids or lipoproteins; the (ii) TLR3, TLR7, TLR8 and TLR9 which are expressed on membranes of intracellular compartments as endosomes or lysosomes and recognize microbial-derived nucleic acids (Kawai and Akira, 2010). TLR2 was shown to form heterodimers with TLR1 or TLR6 and is involved in the recognition of a broad range of PAMPs including lipopeptides from bacteria, zymosan from fungi or hemagglutinin protein from a virus (Barbalat et al., 2009; Jin et al., 2007; Kang et al., 2009). A founding member of the TLR family is TLR4, which forms a protein complex with MD2 and recognizes the bacterial lipopolysaccharide (LPS) (Park et al., 2009). The efficient binding of LPS to the TLR4-MD2 complex also requires the presence of LPS-binding protein (LBP) and CD14 (Akashi-Takamura and Miyake, 2008; Kim et al., 2007a). The TLR5 and TLR11 were shown to interact mainly with bacterial flagellin or molecules derived from *Toxoplasma gondii*, respectively (Uematsu et al., 2008; Yarovinsky et al., 2005). Intracellular TLRs were identified as molecules recognizing a viral or synthetic double-strand RNA (TLR3) (Choe et al., 2005; Tabeta et al., 2004), single-stranded RNA derived from RNA viruses or Poly(U) RNA (TLR7 and TLR8) (Hornung et al., 2005; Mancuso et al., 2009) and unmethylated 2′-deoxyribo-cytidine-phosphate-guanosine (CpG) DNA motifs derived from bacterial or viral DNA (TLR9) (Haas et al., 2008).

Several reports suggested, that TLRs can also sense endogenous ligands: host-derived molecules with the capacity to trigger immune responses (Beg, 2002). Most of these ligands are associated with the products of cell death, injury, or tumor growth including the components of extracellular matrix (ECM), heat-shock proteins, and high-mobility group box 1 (HMGB1) (Kawai and Akira, 2010; Tsan and Gao, 2009; Yang and Tracey, 2010). Moreover, the ribonucleoprotein complexes and host DNA released by necrotic or apoptotic cells can stimulate intracellular TLR7 and TLR9 and trigger the response (Vollmer et al., 2005). Specifically, the secreted components of ECM such as biglycan (Schaefer et al., 2005), hyaluronic acid (Jiang et al., 2005a), or versican (Kim et al., 2009) can activate TLR2 or TLR4 and induce the expression of pro-inflammatory cytokines and chemokines (Beg, 2002). The TLR recognition of self-derived RNA or DNA molecules should be blocked by the proper degradation of these molecules by serum nucleases in the endolysosomes (Barton et al., 2006). For example, TLR9 undergoes proteolytic cleavage

that prevents the binding of self-DNA before its degradation (Kim et al., 2008). It is also important to emphasize that viral and bacterial nucleic acids carry diverse modifications and employ different chemical marks then host nucleic acids (Chen et al., 2016). This suggests that in steady-state conditions, the self-derived nuclei acids should not activate the innate immune response. On the other hand, the inflammatory and autoimmune processes can destroy these safeguards and lead to the activation of innate immunity (Means et al., 2005; Means and Luster, 2005; Viglianti et al., 2003). As suggested above the HMGB1 release from dying cells could stimulate TLRs (Yang and Tracey, 2010). Specifically, it can bind to both pathogen and self-DNA and form the HMGB1-DNA complexes that are recognized by the RAGE receptor and delivered to early endosomes for TLR9 recognition (Tian et al., 2007). The very same mechanism was also demonstrated by the engagement of complexes of cathelicidin LL37 and self-DNA, which have the capacity to activate TLR7 and TLR9 signaling (Lande et al., 2007). This together suggests that activation of innate immune reactions through TLRs could be triggered by not only exogenous (pathogen-derived) but also endogenous (self-derived) ligands.

### 3.2 Toll-like receptor signaling

In general, TLR signaling can be classified as MyD88-dependent, which mostly drives the induction of pro-inflammatory cytokines or TRIF-dependent that is associated with the overexpression of type I interferons. MyD88-dependent signaling pathway is universally used by all TLRs with the exception of TLR3 and thus, MyD88 deficient mice are broadly used in experiments in which the effect of TLR signaling on a given physiological process is tested. For the purposes of cell-specific ablation of MyD88 protein, a mouse strain with a conditional MyD88 allele is also available (Hou et al., 2008).

TLR4 was shown to uses both the MyD88 and TRIF-dependent signaling pathways (Kawasaki and Kawai, 2014). After TLR engagement, by their cognate ligands, MyD88 recruits IL-1 receptor-associated kinase (IRAK) family members that are autophosphorylated (Kollewe et al., 2004; Lin et al., 2010), released from MyD88 and activate ubiquitin-ligase TRAF6 (Jiang et al., 2002). TRAF6 induces polyubiquitination of TAK1 that activates two different pathways: (i) IKK complex of NF-κB and (ii) MAPK pathway (Chen, 2012). Activated IKK phosphorylates IκBα, an inhibitor of NF-κB, which

undergo degradation allowing NF-κB translocation into the nucleus (Bhoj and Chen, 2009). TAK1 activation of MAPK family members resulted in the stabilization of the AP-1 transcription factor and induces pro-inflammatory responses (Kawagoe et al., 2008). The TRIF-dependent signaling pathway can activate both the NF-κB and IRF3 pathways. After ligand TLR3 ligand encounter, TRIF activates TRAF3 that induces IKK-mediates phosphorylation of IRF3 that induces the expression of type I IFNs (Oganesyan et al., 2006). In terms of TLR7 and TLR9 signaling the TRAF6 can also activate IRF7, which after the nuclear translocation also regulates the production of type I IFNs (Kawai and Akira, 2010).

Expression of TLRs is largely restricted to cells on the interphase between host and external environments, such as mucosal epithelial cells, keratinocytes, endothelial cells, and migratory cells of hematopoietic origin such as DCs, macrophages and monocytes (Andonegui et al., 2009; Baker et al., 2003; Janeway and Medzhitov, 2002; Price et al., 2018). Expression of TLRs on APCs of hematopoietic origin was shown to be particularly important for the host immunity. In general, the TLR engagement by microbial ligands initiates the maturation of APCs which results in the upregulation of MHC I and/or II, costimulatory molecules, and increased production of pro-inflammatory cytokines (IL-6, TNFα, etc.) (Banchereau and Steinman, 1998). In terms of epithelial or endothelial cells, the stimulation of TLRs is often associated with upregulation of chemokines attracting DCs, macrophages and/or neutrophils to the site of infection or lead to the overexpression of antimicrobial peptides that have the ability to opsonize or destabilize the membrane of potential pathogens (Abreu, 2010; Ayabe et al., 2000; Vaishnava et al., 2008). For this reason, the mutations in TLRs are being associated with several diseases such as IBDs or increased incidence of colorectal cancer (Cario and Podolsky, 2000; Fukata et al., 2007). TLRs were shown to be expressed also by hematopoietic stem cell (Burberry et al., 2014), embryonic cell types such as macrophages (Balounová et al., 2014) and erythro-myeloid progenitors (Balounová et al., 2019), or TECs (Bernasconi et al., 2005; Cavalcante et al., 2016; Huang et al., 2014). But the exact function of TLR-signaling in these cell-types remains mostly undetermined.

## THESIS AIMS

The general aim of the thesis is to characterize the role of AIRE-expressing cells in the mechanisms underpinning the central and peripheral immune tolerance to self-antigens. The main goal of the work is to describe the novel regulatory pathways and cell subsets that are essential for self-antigen presentation to T-cells and enforcement of tolerance. This includes several specific questions:

1. What is the specific role of TLR-signalling in the mTEC-driven mechanisms of central tolerance?

2. What is the role of enteric α-defensins expression by mTECs for the mechanism of central tolerance to intestinal antigens in mice and humans?

3. What is the identity and potential function(s) of AIRE-expressing cells in lymph nodes?

4. May the generation of a novel *Aire*$^{fl/fl}$ mouse strain allow to study a cell-specific function of AIRE in different tissues?

# RESULTS

The list of this author's publications, which collectively forms the essence of the presented thesis:

**Vobořil, M.**, T. Brabec, J. Dobeš, I. Šplíchalová, J. Březina, A. Čepková, M. Dobešová, A. Aidarova, J. Kubovčiak, O. Tsyklauri, O. Štěpánek, V. Beneš, R. Sedláček, L. Klein, M. Kolář, and D. Filipp. Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation. *Nature Communications, 2020; 11:2361*. **($IF_{2018}$ = 11.878)**

Dobeš, J., A. Neuwirth, M. Dobešová, **M. Vobořil**, J. Balounová, O. Ballek, J. Lebl, A. Meloni, K. Krohn, N. Kluger, A. Ranki, and D. Filipp. Gastrointestinal Autoimmunity Associated With Loss of Central Tolerance to Enteric α-Defensins. *Gastroenterology, 2015; 149:139-150*. **($IF_{2018}$ = 19.809)**

Yamano, T., J. Dobeš, **M. Vobořil**, M. Steinert, T. Brabec, N. Ziętara, M. Dobešová, C. Ohnmacht, M. Laan, P. Peterson, V. Benes, R. Sedláček, R. Hanayama, M. Kolář, L. Klein, and D. Filipp. 2019. Aire-expressing ILC3-like cells in the lymph node display potent APC features. *Journal of Experimental Medicine, 2019; 216:1027-1037.* **($IF_{2018}$ = 10.892)**

Dobeš, J., F. Edenhofer, **M. Vobořil**, T. Brabec, M. Dobešová, A. Čepková, L. Klein, K. Rajewsky, and D. Filipp. A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire. *European Journal of Immunology, 2018; 48:546-548.* **($IF_{2018}$ = 4.695)**

Filipp, D., T. Brabec, **M. Vobořil**, and J. Dobeš. 2019. Enteric α-defensins on the verge of intestinal immune tolerance and inflammation. *Seminars in Cell & Developmental Biology, 2019; 88:138-146.* **($IF_{2018}$ = 5.460)** (Review article)

Here, the author contributed to the review article describing the mechanisms of tolerance to intestinal antigens occurring on multiple layers of T-cell development and priming.

## 1. TOLL-LIKE RECEPTOR SIGNALING IN THYMIC EPITHELIUM CONTROLS MONOCYTE-DERIVED DENDRITIC CELL RECRUITMENT AND TREG GENERATION

Several studies suggested the link between AIRE and the noncanonical NF-κB signaling, as mice lacking either AIRE or several of the components of the NF-κB pathway show impaired induction of central tolerance and abnormal thymic structure (Haljasorg et al., 2015; LaFlam et al., 2015; van Delft et al., 2015). Specifically, signaling through TNF receptor family members was shown to be particularly important (Akiyama et al., 2008; Boehm et al., 2003). The RANK-RANKL interaction, provided mostly by lymphoid tissue inducer cells or positively selected T-cell, was described to be important for the development of $AIRE^+$ mTECs (Akiyama et al., 2008; Hikosaka et al., 2008; Rossi et al., 2007b). Also, the pharmacological blockade of RANKL results in a loss of AIRE expression and decrease numbers of Tregs (Khan et al., 2014). Since the CD40-CD40L interaction also activates the noncanonical NF-κB signaling, the depletion of CD40 affected the numbers of $AIRE^+$ mTECs. However, the thymic phenotype was shown to be milder than in the case of *Rankl*$^{-/-}$ mice (Akiyama et al., 2008). Furthermore, the signaling through LTβR was shown to be essential for the thymic medulla formation and differentiation of mTECs (Boehm et al., 2003). Besides, the LTβR seems to be also important in mTECs and DCs crosstalk and the generation of Tregs (Cosway et al., 2017). The exact function of NF-κB pathway in the expression of AIRE was further corroborated by the finding that the depletion of *Aire* CNS1 element that contains two NF-κB binding sites leads to the abrogated AIRE expression and development of autoimmunity (Haljasorg et al., 2015; LaFlam et al., 2015). Interestingly, the canonical NF-κB signaling was also proposed to play an important role in mTECs biology since the TEC-specific depletion of TRAF6 adaptor led to the development of autoimmune response against the liver (Bonito et al., 2013). This suggests that also receptors triggering the canonical NF-κB pathway may play a role in the process of central tolerance. Among them, the TLRs seem to be the most prominent since their expression was previously found on human TECs and specifically enriched in patients with myasthenia gravis (Bernasconi et al., 2005; Cavalcante et al., 2018).

In the presented study we have demonstrated that mTECs express a battery of TLRs, including TLR9, whose expression was shown to be comparable to conventional DCs. Interestingly the genetic ablation of MyD88 specifically in TECs (*MyD88$^{fl/fl}$Foxn1$^{Cre}$*) had no apparent impact on the frequency of general TECs subpopulations, however, it affects the development of post-AIRE mTECs. In association with this, the mTECs isolated from *MyD88$^{fl/fl}$Foxn1$^{Cre}$* mice showed reduced expression of chemokines attracting the cDC2 to the thymic medulla. In addition, the *in vivo* stimulation of mTECs through intrathymic injection of TLR9 ligand led to the enhanced differentiation of post-AIRE mTECs, increased production of chemokines, and subsequent enhancement of cDC2 in the thymic medulla. Using single-cell RNA sequencing, we have identified the population of thymic moDCs that were enriched in the thymus and effectively acquire mTEC-derived antigens in response to TLR9 signaling. Consistently, the cellularity of moDCs was shown to be decreased in *MyD88$^{fl/fl}$Foxn1$^{Cre}$* mice. Since the cellularity of DCs was shown to be important for the generation of Tregs in the thymus we tested the frequency and functionality of Treg-population in *MyD88$^{fl/fl}$Foxn1$^{Cre}$* mice. We found that these mice suffer from a decrease amount and reduced functionality of thymic Tregs, which renders the peripheral T-cell repertoire prone to induction of experimental colitis. Altogether, our model proposes that TLR/MyD88 signaling works as an important regulatory pathway of mTECs biology affecting the basic processes of central tolerance (Vobořil et al., 2020).

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ARTICLE

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# Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation

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The development of thymic regulatory T cells (Treg) is mediated by Aire-regulated self-antigen presentation on medullary thymic epithelial cells (mTECs) and dendritic cells (DCs), but the cooperation between these cells is still poorly understood. Here we show that signaling through Toll-like receptors (TLR) expressed on mTECs regulates the production of specific chemokines and other genes associated with post-Aire mTEC development. Using single-cell RNA-sequencing, we identify a new thymic CD14$^+$Sirpα$^+$ population of monocyte-derived dendritic cells (CD14$^+$moDC) that are enriched in the thymic medulla and effectively acquire mTEC-derived antigens in response to the above chemokines. Consistently, the cellularity of CD14$^+$moDC is diminished in mice with MyD88-deficient TECs, in which the frequency and functionality of thymic CD25$^+$Foxp3$^+$ Tregs are decreased, leading to aggravated mouse experimental colitis. Thus, our findings describe a TLR-dependent function of mTECs for the recruitment of CD14$^+$moDC, the generation of Tregs, and thereby the establishment of central tolerance.

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The establishment of tolerance is a fundamental attribute of a healthy immune system. Since T cell antigen receptors (TCRs) are generated by random somatic recombination, i.e. could be self or nonself-specific, T cells that express a self-reactive TCR must be removed from the conventional T cell repertoire. The critical part of this process occurs in the thymic medulla where the strength of TCR recognition of self-antigens is probed by various types of antigen presenting cells (APCs), mainly dendritic cells (DCs), B-cells, and highly specialized medullary thymic epithelial cells (mTECs)[1]. mTECs mediate the promiscuous expression of thousands of otherwise strict tissue-restricted self-antigens (TRAs), a large number of which are under the control of the transcriptional regulator Aire[2]. The presentation of TRAs by mTECs can result in either the deletion of self-reactive T cells[3] or their conversion into Tregs[4,5]

It has been recently demonstrated that the process of cooperative antigen transfer (CAT) from mTECs to DCs is essential for the establishment of thymic tolerance[6–11]. The complexity of CAT is foremost due to the heterogeneity of DCs in the thymus. These CD11c$^+$ cells are comprised of two major categories: B220$^+$ plasmacytoid DCs (pDC) and classical DCs (cDCs), the latter which can be subdivided into Xcr1$^+$CD8α$^+$Sirpα$^-$ classical type 1 DCs (cDC1) and Xcr1$^-$CD8α$^-$Sirpα$^+$ classical type 2 DCs (cDC2)[12,13]. While cDC1 arise primarily in the thymus, cDC2 and pDCs originate extrathymically and then migrate to the thymic medullary region[14,15]. mTEC-derived antigens are transferred both to thymic resident cDC1[6,10] and cDC2[16,17]. Although it has been shown that the migration of cDC1 and cDC2 to the vicinity of mTECs is affected by a gradient of Xcl1[18] and Ccr2/Ccr7 ligands, respectively[19,20], the potential involvement of other chemokines in the regulation of CAT still awaits resolution.

Toll-like receptors (TLRs) sense various immunologically relevant microbial ligands such as lipoproteins, carbohydrates, and nucleic acids. All TLRs, with the exception of TLR3, signal through the adaptor protein, MyD88, which via the activation of the NF-κB pathway induces the expression of pro-inflammatory cytokines, chemokines, and other inflammation-related molecules[21]. While the exact role of non-canonical NF-κB signaling in the development and function of mTECs has been previously demonstrated[22–24], the impact of TLR signaling via the canonical NF-κB pathway in the physiology of mTECs remains undetermined.

Here, we show that, among TLRs, mTECs abundantly express TLR9, and the stimulation of which leads to the influx of Xcr1$^-$Sirpα$^+$ cDC2 into the thymic medulla. RNA sequencing of stimulated mTECs reveals that the mechanism underpinning this phenomenon is related to the upregulation of a set of chemokines, whose receptors are predominantly expressed by a CD14$^+$ subset of thymic DCs, which have been identified as monocyte-derived DCs (CD14$^+$moDC). Furthermore, mice with MyD88-deficient TECs, which exhibit a deficiency in the recruitment of CD14$^+$ moDC, also suffer from a decreased thymic Treg output and functionality, which renders the peripheral T cell repertoire prone to colitis induction.

## Results

**mTECs express a set of TLRs and signaling adaptors**. The function of TLR signaling in the physiology of mTECs has not yet been studied in detail[25–27]. We first determined that both mTECs$^{low}$ and mTECs$^{high}$ subsets (Fig. 1a and Supplementary Fig. 1a) expressed TLR2, 3, 4, and 9 (Fig. 1b). Remarkably, TLR9, which recognizes bacterial, viral or altered DNA[21] and ligands associated with cellular stress[28], is highly expressed by mTECs$^{high}$ at levels comparable to thymic cDCs (Fig. 1a, b and Supplementary Fig. 1b). Transcripts of TLR adaptors *MyD88* and *Trif*[21] were also readily detectable (Fig. 1c). Although the levels of TLR4 and TLR9 were higher in mTECs$^{high}$, the major producers of Aire, our analysis of Aire$^{+/+}$ and Aire$^{-/-}$ mice revealed that TLRs are expressed in an Aire-independent manner (Fig. 1d).

To assess the significance of TLR/MyD88 signaling in TECs development, we crossed a thymic epithelial cell-specific Foxn1$^{Cre}$ driver[29] with a MyD88$^{fl/fl}$ transgenic mice[30] (hereafter called MyD88$^{\Delta TEC}$). In comparison to the control, MyD88$^{\Delta TECs}$ mice showed no significant differences in the frequency of all tested TEC subpopulations (Fig. 1e, f), suggesting that canonical NF-κB signaling through TLRs/MyD88 does not affect mTEC$^{high}$ maturation. Similarly, in all mTEC$^{high}$ subsets, the expression of CD80, CD86, PD-L1, CD40, and ICOSL on was not altered (Supplementary Fig. 1c).

Together, this data demonstrates that TLRs are broadly expressed by mTECs and MyD88-dependent signaling has no apparent impact on TEC subpopulation frequency.

**MyD88-dependent chemokine expression in mTECs$^{high}$**. Given the high expression of selected TLRs in mTECs$^{high}$ cells, we assessed the impact of the absence of TLR signaling in unperturbed conditions. RNA-sequencing of mTECs$^{high}$ (sorted as shown in Supplementary Fig. 1a) from wild type (MyD88$^{fl/fl}$) and MyD88$^{\Delta TECs}$ mice revealed MyD88-dependent transcriptional variance (Fig. 2a) defined by 303 differentially expressed transcripts (Fig. 2b and Supplementary Data 1 and 2). While 206 of these transcripts were induced and 97 repressed by MyD88, they were not enriched for Aire-dependent or Aire-independent TRA genes[31] (Supplementary Fig. 2a, left panel). Consistent with the role of TLR/MyD88 signaling in epithelial cells[21], we found several differentially expressed genes (DEGs) which fell into one of two categories: (i) *Il1f6* and *Csf2* cytokines, (ii) *Cd25*, *Cd4*, and *Cd24* chemokines. These mediators act through receptors that are primarily expressed by myeloid cells and DCs[32]. Specifically, IL36R, the receptor for IL1F6, is expressed by DCs and T cells[33] while Csf2r, the receptor for Csf2, is expressed mostly by monocytes, macrophages, and granulocytes[34]. The Ccr9, the receptor for Ccl25, is expressed by both thymocytes and pDCs driving their migration into the thymus[14,35]. Both Ccr5 (receptor for Ccl4) and Ccr3 (receptor for Ccl24) are expressed predominantly on granulocytes and DCs modulating their migration into inflamed tissues[32,36]. qRT-PCR analysis confirmed MyD88-regulated expression of selected genes in mTECs$^{high}$ (Fig. 2c). Since the TLRs were postulated to sense both microbial and endogenous molecules[21], we examined which of them could potentially act as a trigger. The analysis of mRNA expression of MyD88-dependent cytokines and chemokines (Fig. 2b, c) in the mTEC$^{high}$ population isolated from either Germ-free (GF) or specific-pathogen-free (SPF) mice was comparable (Supplementary Fig. 2b), indicating that these signals are likely of endogenous origin.

Next, we assessed the response of mTECs to TLR/MyD88 stimulation. Given the high expression of TLR9 (Fig. 1b), we stimulated mTECs$^{high}$ from MyD88-deficient (MyD88$^{-/-}$) and WT (MyD88$^{+/+}$) mice in vitro with CpG oligodeoxynucleotides (CpG ODN) or PBS. RNA-sequencing revealed significant changes in the transcriptional profile only in MyD88$^{+/+}$ cells. Notably, 347 DEGs were associated with TLR9 stimulation (Fig. 2d, e and Supplementary Data 3 and 4), and of these, 198 were upregulated while 149 were downregulated. However, the pattern of expression of TRA genes remained largely unchanged after in vitro CpG ODN stimulation (Supplementary Fig. 2a, right panel). Importantly, among the most upregulated DEGs were two sets of chemokines: (i) *Cxcl1*, *2*, *3*, and *5*, which signal via the Cxcr2 receptor, expressed predominantly on neutrophils[37] and (ii) *Ccl3*, *5* and *20* which signal via various chemokine receptors, including Ccr1, 3, 5, 6 which are expressed mostly on myeloid

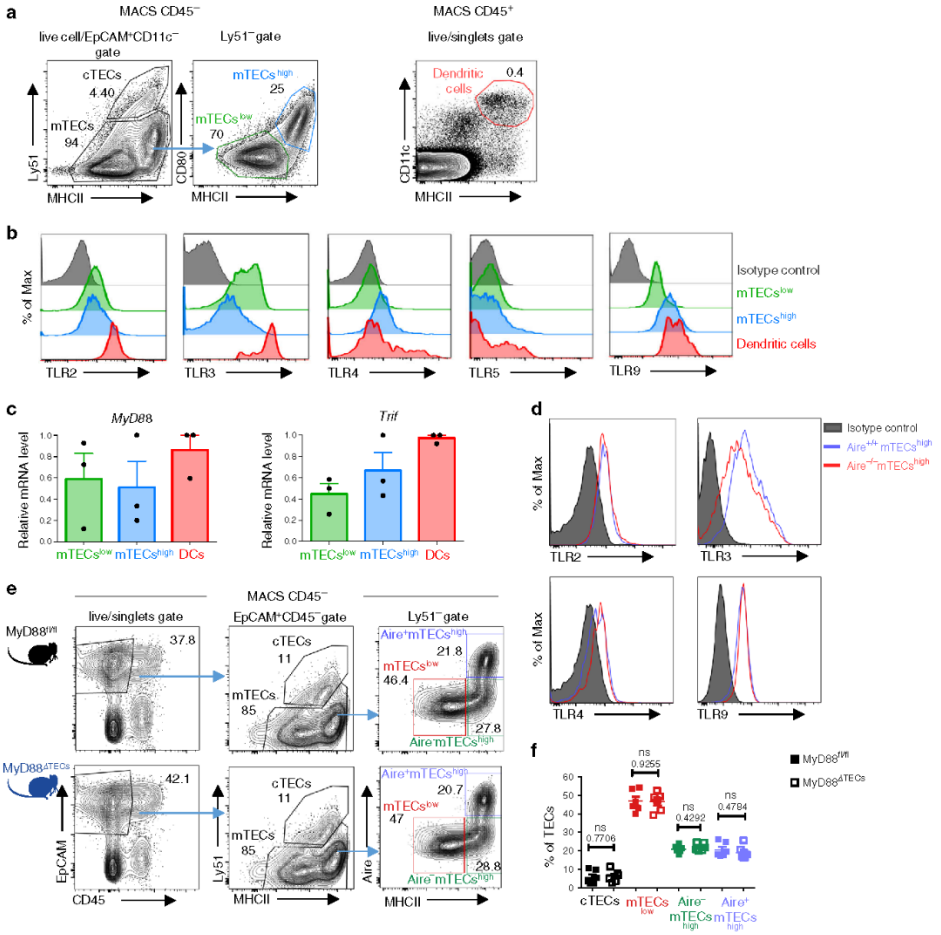



**Fig. 1 mTECs express a set of TLRs and their signaling adaptors independently of Aire. a** Gating strategy used for the analysis of TEC populations and general thymic conventional DCs. MACS enriched CD45⁻ and EpCAM⁺CD11c⁻ pre-gated cells were further divided into cTECs (Ly51⁺), mTECs$^{low}$ (MHCII$^{low}$CD80$^{low}$), and mTECs$^{high}$ (MHCII$^{high}$CD80$^{high}$). Thymic conventional DCs were gated as CD11c⁺MHCII⁺ from the CD45⁺ fraction. A more detailed gating strategy is found in Supplementary Fig. 1a, b. **b** Representative flow cytometry histograms of TLR expression on mTECs and DCs isolated from the thymus ($n = 3$ independent experiments). **c** *MyD88* and *Trif* mRNA expression is determined by qRT-PCR from FACS sorted mTECs and DCs. The expression is calculated relative to Casc3 and normalized to the highest value within each experiment=1 (mean ± SEM, $n = 3$ samples). **d** Representative flow cytometry histograms of TLR expression on mTECs from Aire$^{+/+}$ and Aire$^{-/-}$ mice, ($n = 3$ independent experiments). **e** Representative comparative flow cytometry plots of different TEC subpopulations in MyD88$^{fl/fl}$ and MyD88$^{\Delta TECs}$ mice. **f** Quantification of TEC frequencies from plots in e (mean ± SEM, $n = 6$ mice). Statistical analysis was performed by unpaired, two-tailed Student's t-test, p-values are shown. ns = not significant.

cells[32]. Cytokines (*Tnfα*, *Il-6*, *Il12a*, *Il1f6* and *Csf2*) and other genes (*Cd40*) were also found to be upregulated (Fig. 2e). The upregulation of *Cxcl1* and *Ccl5* chemokines after in vitro (Fig. 2f) as well as in vivo intrathymic TLR9 stimulation (Fig. 2g) was confirmed by qRT-PCR analysis. As shown in Supplementary Fig. 2c, repeated intraperitoneal (i.p.) injection of CpG ODN was insufficient for the upregulation of chemokines in mTECs$^{high}$. It is of note that in vitro stimulation of TLR4 on mTECs$^{high}$ by LPS also resulted in the upregulation of the previously noted chemokines, albeit at a lower level (Supplementary Fig. 2d).

In addition to TLRs, MyD88 also conveys signals generated by IL-1 family cytokines, such as IL-1β, IL-18 or IL-33[38]. Even though the receptors for these cytokines are expressed by mTECs$^{high}$ (Supplementary Fig. 3a), only in vitro stimulation with IL-1β lead to the upregulation of cytokines and chemokines induced by TLR9 stimulation (Supplementary Fig. 3b).

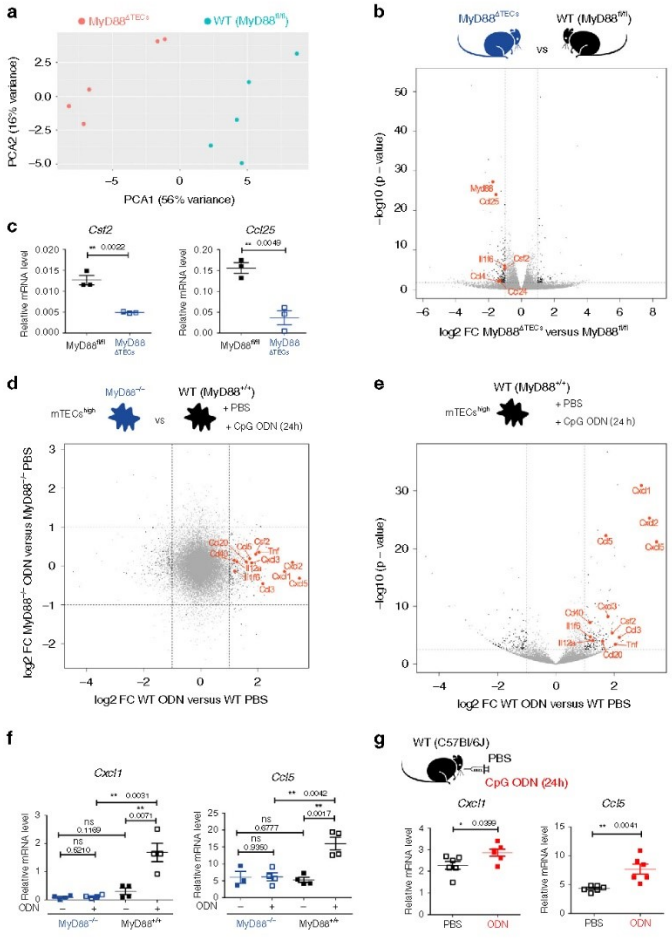



**Fig. 2 TLR/MyD88 signaling in mTECs$^{high}$ drives the expression of cytokines and chemokines. a** Principal component analysis of bulk RNA-sequencing data from mTECs$^{high}$ (sorted as in Supplementary Fig. 1a) derived from MyD88$^{fl/fl}$ and MyD88$^{\Delta TECs}$ mice. Data represents the analysis of $n = 5$ samples for each condition. **b** Volcano plot analysis of RNA-sequencing data described in **a**. Fold-change cutoff of log2 = ±1,0 and *p*-value: 0.05 are marked by dashed lines (also in **d**, **e**). Differentially expressed genes are depicted in black, genes of interest are in red, and other detected genes in grey. **c** qRT-PCR analysis of relative mRNA expression normalized to Casc3 of genes selected from b (mean ± SEM, $n = 3$ samples). **d** Fold-change fold-change plot of RNA-sequencing data from CpG ODN or PBS in vitro stimulated mTECs$^{high}$ (sorted as in Supplementary Fig. 1a) from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice ($n =$ 4 samples for each condition). Color code as in **b**. **e** Volcano plot analysis of RNA-sequencing data from d, comparing CpG ODN versus PBS in vitro stimulated mTECs$^{high}$ from MyD88$^{+/+}$ mice. Statistical analysis for b, d and e was performed by Wald test, *p*-value cutoff: 0.05. **f**, **g** qRT-PCR analysis of Cxcl1 and Ccl5 mRNA expression (normalized to Cacs3) from in vitro (mean ± SEM, $n = 4$ samples) and intrathymically (mean ± SEM, $n = 6$ mice), respectively, CpG ODN or PBS stimulated mTECs$^{high}$ from indicated animals. Statistical analysis for **c**, **f**, and **g** was performed by unpaired, two-tailed Student's *t*-test, $p \leq 0.05 = *$, $p \leq 0.01 = **$, ns not significant.

Besides chemokines and cytokines, TLR/MyD88 signaling in mTECs$^{high}$ (Fig. 2b) also regulated the expression of molecules associated with cornified epithelial pathway[39] (Supplementary Data 1–4). This specifically relates to genes that are associated with post-Aire mTECs[40,41], such as *Krt10*, *Krt77* and *Flg2* (Supplementary Fig. 3c). Moreover, previously published data has shown the enhanced expression of *Il1f6*, *Cxcl3* and *Cxcl5* in post-Aire mTECs[42]. Thus, we enumerated the total numbers of Involucrin$^{+}$EpCAM$^{+}$ cells in the medullary region of the CpG ODN intrathymically stimulated thymus. We did not observe any changes in the frequency

of general mTECs subsets (Supplementary Fig. 3d) although the total numbers of Involucrin$^+$ post-Aire mTECs were significantly increased (Supplementary Fig. 3e, f).

Together, these results show that TLR/MyD88 signaling in mTECs under physiological or stimulatory conditions regulates the differentiation of mTEC$^{high}$ cells into Involucrin$^+$ post-Aire stage. This stage is associated with the expression of a set of chemokines that signal via an overlapping set of chemokine receptors that are primarily expressed by DCs[32].

**TLR9/MyD88 signaling in mTECs targets Sirpα$^+$ cDC2.** Migration of different DC subsets into the thymus is orchestrated by distinct chemokines[14,18,19]. Thus, we next assessed which of these subsets would be the target for TLR9/MyD88-induced chemokines in TECs. We sorted three main subsets of CD11c$^+$MHCII$^+$ thymic DCs: B220$^+$ pDC, Sirpα$^-$Xcr1$^+$ cDC1, and Sirpα$^+$Xcr1$^-$ cDC2 (Supplementary Fig. 4a), along with Gr-1$^+$ granulocytes, CD4 and CD8 single positive thymocytes and performed qRT-PCR analysis of the chemokine receptors indicated above. Remarkably, apart from granulocytes, the chemokine receptors *Cxcr2*, *Ccr1*, *3*, *5*, and *6* were mostly expressed by DCs, specifically by cDC2 and pDC (Fig. 3a). This prompted us to quantify the relative frequencies of all thymic DC subsets in MyD88$^{\Delta TECs}$ in comparison to WT (MyD88$^{fl/fl}$) mice. In unstimulated conditions, TEC-intrinsic MyD88 signaling did not change the total frequency of CD11c$^+$MHCII$^+$ DCs (Fig. 3b, left plot). However, we observed alterations in the frequencies of DC subsets. While cDC1 were increased, the frequencies of pDC and cDC2 were diminished in the MyD88$^{\Delta TECs}$ thymus (Fig. 3b). In contrast, FACS analysis of TLR9-stimulated thymi revealed a significant increase in cDC2 accompanied by decreased cDC1 in the thymus of WT (MyD88$^{fl/fl}$) (Fig. 3c and Supplementary Fig. 4b, c) but not MyD88$^{\Delta TECs}$ animals (Fig. 3c). The frequencies of pDC remained comparable under these two conditions. This demonstrates that the recruitment of cDC2 to the thymus is attributable specifically to TLR9 signaling in TECs (Fig. 3c and Supplementary Fig. 4b). In agreement with medullary localization of cDC2 (Supplementary Fig. 4d), microscopically examined thymi from WT mice stimulated with CpG ODN showed an enrichment of CD11c$^+$Sirpα$^+$ cDC2 exclusively in the keratin14-rich medullary region (Figs. 3d, e).

Together, this data suggests that MyD88-driven chemokines expressed by mTECs$^{high}$, target receptors on thymic Sirpα$^+$ cDC2 and mediate their recruitment to the thymic medulla in steady state and TLR9 stimulatory conditions.

**TLR9/MyD88 signaling in mTECs recruits CD14$^+$moDCs.** Chemokine-dependent migration of DCs to the proximity of mTECs, which underpins the mechanisms of CAT[18], has been shown to be essential for the presentation of mTEC-derived antigens by DCs[6,10]. One prediction from the TEC-dependent TLR/MyD88-induced influx of Sirpα$^+$cDC2 to the thymic medulla is that the frequency of CAT to this subset would be enhanced.

To verify this prediction, we crossed Foxn1$^{Cre}$ mice with ROSA26$^{TdTOMATO}$ leading to TEC-specific, cytoplasmic expression of TdTOMATO (TdTOM) protein in the thymus. In agreement with a previous study[9] and as shown in Supplementary Fig. 5a, we found two major populations of TdTOM$^+$ cells: (i) a TdTOM$^{high}$ EpCAM$^+$ population which was CD45$^-$ and represented TECs expressing TdTOM endogenously (Supplementary Fig. 5b); and (ii) a CD45$^+$ TdTOM$^+$ population comprised of mainly CD11c$^+$ DCs (Supplementary Fig. 5a) which acquired TdTOM via CAT (Fig. 4a). Interestingly, these DCs were enriched for the EpCAM$^+$ marker (Fig. 4b) which was likely co-transferred with TdTOM[9]. Bone marrow (BM) chimeras of lethally irradiated Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice reconstituted with WT BM cells showed that around 6% of donor-derived DCs acquired TdTOM (Supplementary Fig. 5c–e). This formally demonstrates that TdTOM is transferred from TECs to DCs.

It has been previously documented that distinct subtypes of thymic DCs vary in their capacity to acquire antigens from TECs[6,10,11,16]. Whereas CAT of TdTOM from TECs to cDC1 and cDC2 is very potent in the Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ system, it is limited in the case of pDC (Fig. 4e, f). This result was also corroborated with the use of BM chimeras which were described above (Supplementary Fig. 5f). Flow cytometry imaging showed that transferred TdTOM in MHCII$^+$CD11c$^+$ DCs is localized intracellularly (Fig. 4g).

To determine the heterogeneity of all thymic DC subsets that participate in CAT, we performed single-cell RNA-sequencing (ddSEQ)[43] of Gr-1$^-$CD11c$^+$TdTOM$^+$ cells isolated from thymi of Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice. Two-dimensional tSNE projection clustering analysis revealed five different clusters of TdTOM$^+$ DCs (Fig. 5a). Based on their expression profiles and previously described signature genes of cells from mononuclear phagocyte system (MPS)[44], we designated the clusters in accordance with MPS nomenclature[13]: two cDC1 clusters (*Batf3*): a cDC1a (*Ccl5* and *Ccr7*) and cDC1b (*Cd8a*, *Itgae*, *Xcr1* and *Ppt1*)[45]; cDC2 cluster (*Sirpα*, *Mgl2* and *Cd209a*)[12], moDC cluster (*Sirpα*, *Cd14*, *Itgam*, *Cx3cr1* and *Ccr2*)[46]; and one pDC cluster (*Bst2*, *Ccr9*, *Siglech*, and *Ly6d*)[14] (Fig. 5b and Supplementary Data 5). This data allowed the clustering of DCs which participate in CAT according to their specific surface markers (Supplementary Fig. 6a). As shown in Fig. 5b, the previously defined thymic moDC subpopulation shared several markers with both cDCs (*Itgax*, *Itgam*, *Sirpα*, and *Irf4*) and classical tissue resident macrophages (*Lyz2*, *Mertk*, and *Mafb*). Due to the high mRNA expression of molecules associated with antigen processing and presentation by moDC subpopulation (Supplementary Fig. 6b), we tested their capacity to present mTEC-derived antigens and activate antigen specific T cells. Specifically, thymic CD14$^+$moDCs isolated from the Aire-HCO mouse model expressing influenza hemagglutinin (HA) under the control of Aire regulatory sequences[47], were co-cultivated with HA-specific CD4$^+$ T cell hybridoma cells (A5) carrying a GFP-NFAT reporter[4]. While the result demonstrated that thymic CD14$^+$moDCs can efficiently present mTEC-derived antigens to T cells (Supplementary Fig. 6c), it seems that their previous detection was obstructed by using the previously established gating strategy (Supplementary Fig. 4a), by which they are indistinguishable from a conventional Sirpα$^+$ cDC2 subset.

Next, we determined which of the five defined thymic DC clusters expressed the receptors for TLR9/MyD88-induced chemokines/cytokines from mTECs (Figs. 2b, d, e). The heat map analysis of chemokine receptors identified by ddSEQ analysis revealed that most of these receptors were expressed by the Sirpα$^+$CD14$^+$moDC cluster (Fig. 5c, left panel). Interestingly, each of the TdTOM$^+$ DC clusters expressed a specific set of chemokine receptors (Fig. 5c).

Having characterized the CAT system with participating subsets of DCs in Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice, we used it as a readout to determine the targeting specificity of TEC-dependent TLR9/MyD88 stimulation on these DC subsets. First, in general, TLR9 intrathymic stimulation of Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice boosted the frequency of total TdTOM$^+$ CD11c$^+$ DCs (Fig. 5d left graph and Supplementary Fig. 6d) as well as the mean fluorescent intensity (MFI) of TdTOM in these cells, demonstrating their enhanced rate of CAT under stimulatory conditions (Supplementary Fig. 6e). Second, as predicted, the observed increase in CAT was fully attributable to TdTOM$^+$Sirpα$^+$ DCs and not to other DCs populations (Fig. 5d right graph and Supplementary Fig. 6f). Third, and most importantly, the unsupervised flow cytometry tSNE

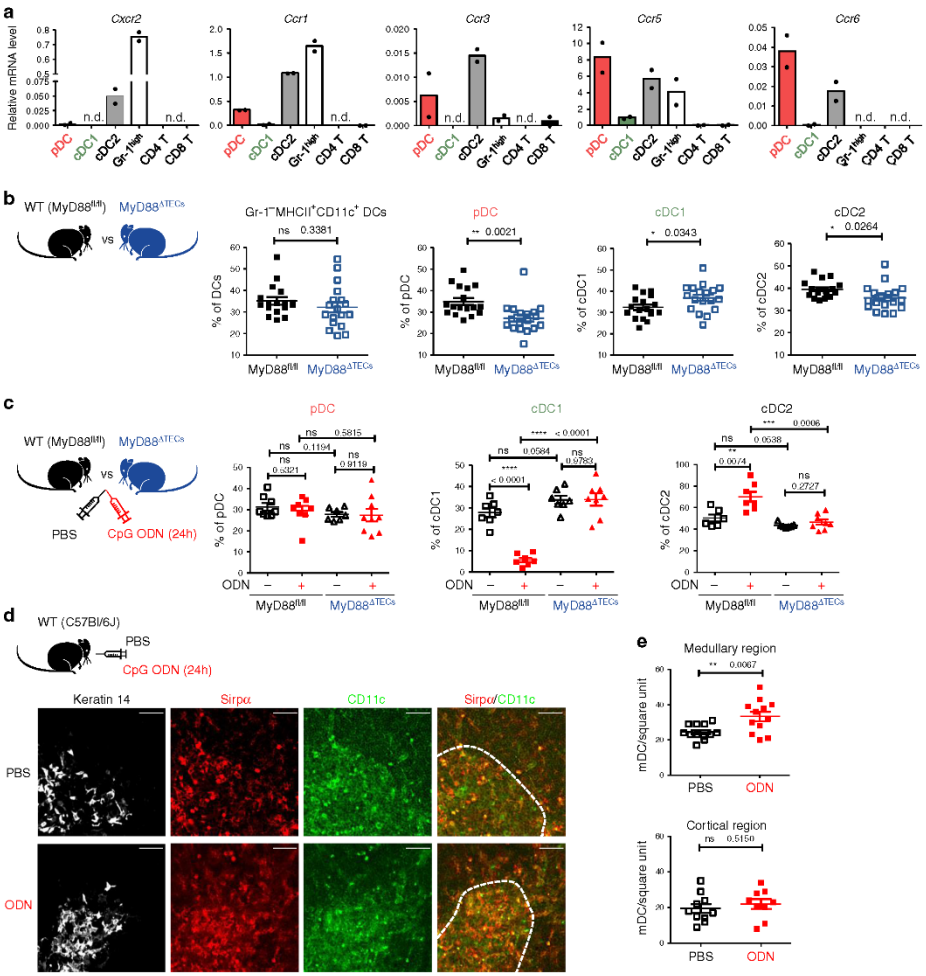



**Fig. 3 TLR/MyD88 signaling in mTECs$^{high}$ affects the migration of DCs into the thymic medulla. a** qRT-PCR analysis of the relative mRNA expression (normalized to Casc3) of indicated chemokine receptors on FACS sorted populations of thymic DCs; pDC plasmacytoid DCs, cDC1 classical type 1 DC, cDC2 classical type 2 DC, Gr-1$^{high}$ = neutrophils, CD4 T = CD4$^+$, and CD8 T = CD8$^+$ thymic T cells. Sorting protocol of thymic DC subsets is provided in Supplementary Fig. 4a. T cells were sorted as TCRβ$^+$ and either CD4 or CD8 single positive ($n = 2$ independent experiments). **b** Comparative flow cytometry analysis of total DCs (Gr-1$^-$CD11c$^+$MHCII$^+$) and different thymic DC subpopulations between MyD88$^{fl/fl}$ and MyD88$^{\Delta TECs}$ mice enumerated according to gating strategy shown in Supplementary Fig. 4a (mean ± SEM, $n = 17$ for MyD88$^{fl/fl}$ and $n = 18$ for MyD88$^{\Delta TECs}$ mice). **c** Flow cytometry analysis of different thymic DC populations (gated as in Supplementary Fig. 4a) isolated from CpG ODN or PBS intrathymically stimulated MyD88$^{fl/fl}$ or MyD88$^{\Delta TECs}$ mice (mean ± SEM, pDC graph: $n = 7$ for ODN$^-$MyD88$^{\Delta TECs}$ and $n = 8$ for other three displayed items; cDC1 graph: $n = 7$ for MyD88$^{fl/fl}$ and ODN$^-$MyD88$^{\Delta TECs}$ and $n = 8$ for ODN$^+$ MyD88$^{\Delta TECs}$ mice; cDC2 graph: $n = 6$ for ODN$^-$MyD88$^{fl/fl}$, $n = 7$ for ODN$^+$MyD88$^{fl/fl}$ and ODN$^-$MyD88$^{\Delta TECs}$ and $n = 8$ for ODN$^+$MyD88$^{\Delta TECs}$ mice). Statistical analysis in **b**, **c** was performed by unpaired, two-tailed Student's *t*-test, $p \le 0.05 = *$, $p \le 0.01 = **$, $p \le 0.001 = ***$, $p < 0.0001 = ****$, ns not significant. **d** Microscopic examinations of thymic sections isolated from CpG ODN or PBS intrathymically stimulated WT mice. Cryosections were stained with keratin 14 (white), Sirpα (red), and CD11c (green). Scale bar represents 50 μm. The white dashed line demarks keratin 14-rich medulla. **e** Quantification of CD11c$^-$Sirpα$^+$ cells in the medullar or cortical region of the cryosections shown in d (mean ± SEM, $n = 12$ counted square unites per medullary region; $n = 10$ and $n = 9$ counted square unites per PBS- and ODN-treated cortical region, respectively. Data are derived from three independent experiments). Statistical analysis was performed by unpaired, two-tailed Student's *t*-test, $p \le 0.01 = **$, ns not significant.

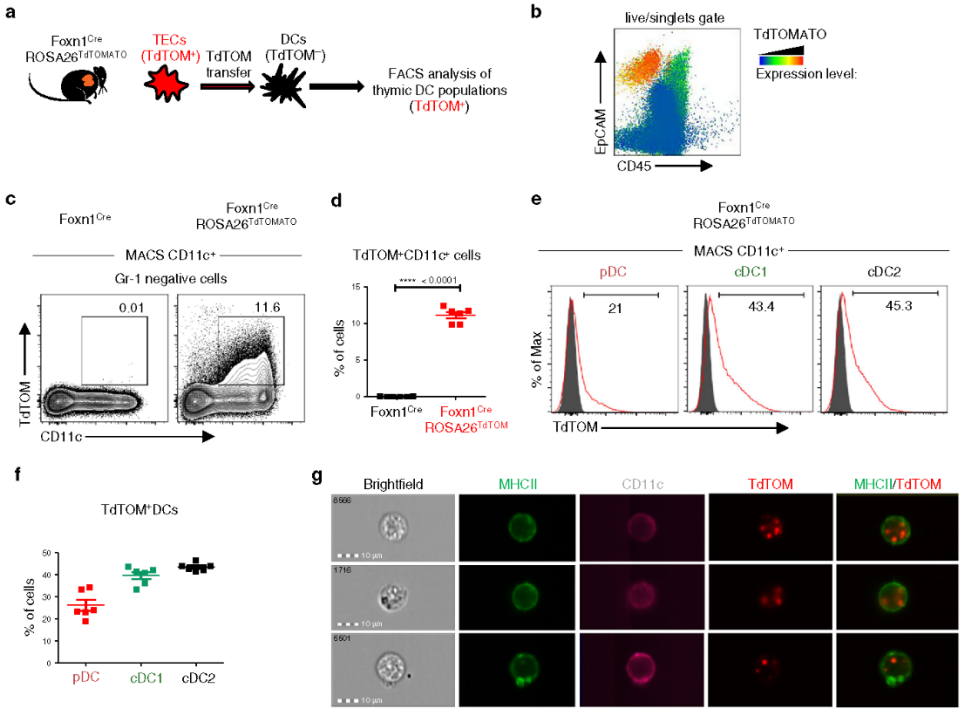


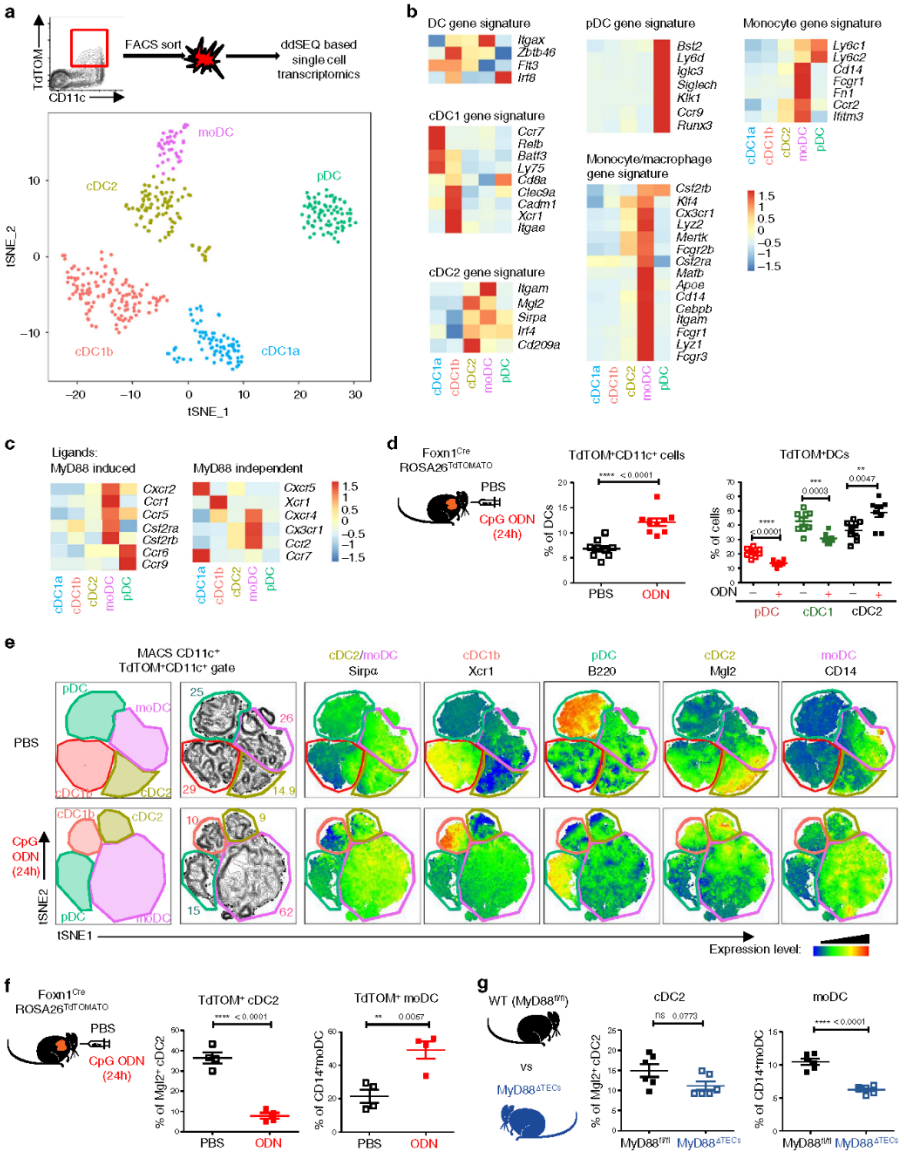

**Fig. 4 Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ as a model of thymic cooperative antigen transfer. a** Experimental design. **b** Flow cytometry heat-map analysis showing the intensity of TdTOM fluorescence among MACS TCRβ-depleted cells from the thymus of the Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mouse. **c** Representative flow cytometry plots comparing the frequency of TdTOM$^+$CD11c$^+$ cells in the thymic MACS-enriched CD11c$^+$ cells between the WT (Foxn1$^{Cre}$) and Foxn1$^{Cre}$ROSA26$^{TdTOM}$ mouse. Cells were pre-gated as live, singlets, and Gr-1$^-$. **d** Quantification of TdTOM$^+$CD11c$^+$ cells from c (mean ± SEM, $n = 6$ mice). Statistical analysis was performed by unpaired, two-tailed Student's *t*-test, $p < 0.0001 =$ ****. **e** Representative flow cytometry histograms showing the frequency of TdTOM$^+$ cells among pDC, cDC1, and cDC2 (gated as in Supplementary Fig. 4a). Gray histograms = Foxn1$^{Cre}$ (control) mice, red histograms = Foxn1$^{Cre}$ROSA26$^{TdTOM}$ mice. **f** Quantification of frequencies of TdTOM$^+$ DCs among the indicated DC subsets (mean ± SEM, $n = 6$ mice). **g** Representative images from the Imagestream analysis showing intracellular localization of transferred TdTOM in MHCII$^+$CD11c$^+$ DCs from the thymus of Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ ($n = 400$ measured cells).

analysis of the main DC subsets defined by markers revealed by ddSEQ analysis showed that the increase of TdTOM$^+$ DCs was mostly due to the specific enrichment of CD14$^+$moDCs (Figs. 5e, f), which also co-express chemokine receptors for ligands induced by TLR9/MyD88 signaling in mTECs (Figs. 2b, e and 5c). Concomitantly, we observed a decrease in Mgl2$^+$ cDC2, Xcr1$^+$ cDC1b, and B220$^+$ pDC (Fig. 5e, f and Supplementary Fig. 6g). Importantly, and further confirming the need of MyD88 signaling for its recruitment, the decreased frequency of total Sirpα$^+$ DCs in the thymus of non-manipulated MyD88$^{\Delta TECs}$ mice (Fig. 3b) was shown to be accounted specifically by the diminishment of the CD14$^+$moDC subset (Fig. 5g).

To find which of the chemokines described (in Fig. 2b, e) were responsible for CD14$^+$moDC migration to the thymus, we crossed Cxcr2$^{fl/fl}$ mice with the pan-hematopoietic driver Vav1$^{Cre}$ to abrogate the signaling of its cognate ligands Cxcl1, 2, 3, and 5 that were among the most upregulated genes in mTECs after TLR9 stimulation. We observed no changes in the recruitment of CD14$^+$moDC after TLR9 stimulation between Cxcr2$^{fl/fl}$Vav1$^{Cre}$ and WT mice (Supplementary Fig. 6h). This suggests, that together with ligands of Ccr2, (i.e. Ccl2, 7, 8, and 12)[19], the ligands of Ccr1, Ccr3 or Ccr5, or their combinations[36], regulate the entry of CD14$^+$moDC into the thymic medulla.

Together, TLR9/MyD88-dependent chemokine signaling in mTECs specifically targets the recruitment and subsequent CAT from the mTECs to Sirpα$^+$CD14$^+$moDC subpopulation which exhibits a tangible capacity for antigen presentation.

**TLR9/MyD88 signaling in mTECs affects Treg development.** Previous studies have suggested that the development of thymic Tregs is dependent on antigen presentation by both mTECs and DCs[6,17,47]. Specifically, antigen presentation by Sirpα$^+$DCs[17] and/or alternatively by CD8α$^+$cDC1[6,10] was implied in the development of organ-specific Tregs. It has been also suggested that the increased ratio of Sirpα$^+$DCs to CD8α$^+$cDC1 leads to an enhanced production of thymic CD25$^+$Foxp3$^+$ Tregs[17,20]. Since a decreased frequency of Sirpα$^+$DCs (Fig. 3b), specifically CD14$^+$moDCs (Fig. 5g) was observed in the thymus of




MyD88$^{\Delta TECs}$ mice, we tested whether these effects would impact the development of the major thymocyte populations and Tregs. While the DN (CD8$^-$CD4$^-$), DP (CD8$^+$CD4$^+$), and CD8$^+$ T cells frequencies were comparable between MyD88$^{\Delta TEC}$ and WT mice, CD4$^+$ T cells, and more specifically CD25$^+$Foxp3$^+$ Tregs were significantly reduced in 4-week-old MyD88$^{\Delta TECs}$ mice (Fig. 6a–c and Supplementary Fig. 7a). Since it has been reported that in 4 week-old-mice nearly one half of CD25$^+$Foxp3$^+$ thymic cells consist of mature recirculating Tregs[48,49], we used CD73 protein staining to determine if Tregs reduced in MyD88$^{\Delta TECs}$ mice were newly generated (CD73$^-$) or recirculating (CD73$^+$)[50]. As shown in Fig. 6d, e, the abrogation of MyD88 signaling in mTECs affected mainly the generation of CD25$^+$Foxp3$^+$ thymic Tregs and not their recirculation. On the other hand, the CD25$^+$Foxp3$^+$ thymic Tregs

**Fig. 5 TLR/MyD88 signaling increases cooperative antigen transfer between TECs and the CD14$^+$moDC subpopulation. a** Two-dimensional tSNE plot from ddSEQ single-cell RNA-sequencing from FACS sorted Gr-1$^-$CD11c$^+$TdTOM$^+$ DCs from the thymus of Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice. The color code represents different cell clusters based on the mRNA expression profile of each cell. **b** Heat-map analysis of the expression of signature genes determining each subset defined in **a**. **c** Heat-map analysis of the expression of chemokine receptors by DC subsets defined in **a**. **d** Quantification of TdTOM$^+$CD11c$^+$ DC subsets (defined as in Supplementary Fig. 4a) in CpG ODN or PBS intrathymically stimulated Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice (representative flow cytometry plots are shown in Supplementary Fig. 6d, f) (mean ± SEM, $n = 9$ mice). Statistical analysis was performed using unpaired, two-tailed Student's *t*-test, $p \leq 0.01 = **$, $p \leq 0.001***$, $p < 0.0001****$. **e** Representative flow cytometry tSNE analysis of TdTOM$^+$CD11c$^+$cell population in PBS or CpG ODN intrathymically stimulated Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice. tSNE analysis was performed using FlowJO software, based on the FSC-A, SSC-A, CD11c, MHCII, Sirpα, Xcr1, B220, Mgl2 and CD14 markers ($n = 2$ independent experiments). **f** Quantification of frequencies of TdTOM$^+$CD14$^+$ moDC or TdTOM$^+$Mgl2$^+$cDC2 from CpG ODN or PBS intrathymically stimulated Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice (representative flow cytometry plots are shown in Supplementary Fig 6g) (mean ± SEM, $n = 4$ mice). **g** Flow cytometry analysis comparing the frequency of cDC2 (Sirpα$^+$Mgl2$^+$) and moDC (Sirpα$^+$CD14$^+$) between MyD88$^{fl/fl}$ and MyD88$^{\Delta TECs}$ mice (mean ± SEM, $n = 6$ mice). Total Sirpα$^+$ DC population was gated as shown in Supplementary Fig 4a. Statistical analysis in f and g was performed by unpaired, two-tailed Student's *t*-test, $p \leq 0.01 = **$, $p < 0.0001****$, ns not significant.

were not reduced in newborn MyD88$^{\Delta TECs}$ (Supplementary Fig. 7b) or GF mice (Supplementary Fig. 7c) when compared to their WT SPF littermates. This, in association with unchanged chemokine expression in mTECs from GF mice, (Supplementary Fig. 2b) further strengthens the notion that the ligands that regulate the mTEC-mediated MyD88-dependent cellularity of Tregs is not likely of exogenous origin.

To further explore the MyD88-dependent regulation of Tregs generation, we tested our prediction that TLR9/MyD88 stimulation of mTECs would lead to the opposite effect, i.e. boosted number of Tregs. Indeed, seven days after intrathymic injection of CpG ODN, we observed a significant increase in the frequency and total number of CD25$^+$Foxp3$^+$ thymic Tregs (Fig. 6f and Supplementary Fig. 7d–f). Importantly, this increase was completely dependent on TEC-intrinsic MyD88 signaling (Fig. 6f). Compared to the decreased numbers in CD73$^-$ Tregs in MyD88$^{\Delta TEC}$, intrathymic injection of CpG ODN led to increased numbers of not only CD73$^-$ newly generated Tregs but also recirculating CD73$^+$ Tregs (Fig. 6g and Supplementary Fig. 7g). This suggests that there are other mTEC-dependent mechanisms which after CpG ODN stimulation can affect the recirculation of Tregs into the thymus. One outstanding question related to the results from the above experiments (Figs. 3c and 5d–f and Supplementary Fig. 6c) is whether the increased generation of thymic CD25$^+$Foxp3$^+$CD73$^-$ thymic Tregs is dependent on the antigen presenting capacity of DCs. To resolve this query, we intrathymically injected CpG ODN into H2-Ab1$^{fl/fl}$Itgax$^{Cre}$ (H2-Ab1$^{\Delta DCs}$) mice, where antigen presentation by DCs has been abrogated. As demonstrated in Fig. 6h, i and Supplementary Fig. 7h, the presentation of antigen by DCs is essential for the increase in numbers of newly generated CD73$^-$ CD25$^+$Foxp3$^+$ thymic Tregs after TLR9 stimulation.

Next, we tested the physiological consequences of the decrease in production of Tregs in MyD88$^{\Delta TECs}$ mice. We took advantage of a T cell induced colitis model, where the adoptive transfer of naïve, Treg depleted CD4$^+$ T cells into Rag1-deficient mice induces severe colitis[51]. In this experimental setup, and as illustrated in Fig. 7a, the i.p. injection of the CD4$^+$ T cell population isolated from peripheral lymph nodes of either MyD88$^{\Delta TEC}$ or MyD88$^{fl/fl}$ mice was compared to colitis-inducing transfer of CD4$^+$CD45RB$^{high}$CD25$^-$ cells isolated from WT mice.

Strikingly, mice that received CD4$^+$ T cells from MyD88$^{\Delta TECs}$ began to lose weight ~4 weeks after adoptive transfer, behaving identically to the positive control. In contrast, mice that received CD4$^+$ T cells from WT mice continuously gained weight over time (Fig. 7b). The clinical signs of colitis in mice receiving CD4$^+$ T cells from MyD88$^{\Delta TEC}$ and in the positive controls were manifested by the presence of inflammatory infiltrates in the colon lamina propria, increased bowel wall thickness, presence of abscesses in colon tissue (Fig. 7c), increased spleen weight (Supplementary Fig. 8a, b), and a higher colon weight/length ratio (Fig. 7d and Supplementary Fig. 8a). To confirm the persistence of the transferred T cell population, we also analyzed Tregs frequencies in all conditions. We found that both positive controls and mice that received CD4$^+$ T cells from MyD88$^{\Delta TECs}$ had severely diminished Tregs compared to WT controls (Fig. 7e). The very similar phenotype of mice that received CD4$^+$ T cells from MyD88$^{\Delta TECs}$ and those which received CD4$^+$CD45RB$^{high}$CD25$^-$ suggested, that Tregs in MyD88$^{\Delta TECs}$ were not only reduced in numbers but also functionally altered. Along with the decreased expression of CD25 (Fig. 7f), Tregs from MyD88$^{\Delta TECs}$ mice showed a significantly reduced capacity to suppress the proliferation of OVA-specific OT-II T cells in vitro (Fig. 7g, h) and prevent the early onset of diabetes caused by activated KLGR1$^+$ OT-I T cells in a RIP-OVA dependent autoimmune mouse model[52] (Supplementary Fig. 8c–e).

Taken together, these results demonstrate that TLR/MyD88 signaling in TECs affects the development of thymic CD25$^+$Foxp3$^+$ Tregs. Specifically, in mice with MyD88-deficient TECs, the frequency and functionality of thymic CD25$^+$Foxp3$^+$ Tregs was decreased and unable to prevent T cell induced colitis.

## Discussion

Present study lends a support for the role of TLR signaling in the mechanism of central tolerance. First, we found that mTECs$^{high}$ express TLRs, including TLR9, whose signaling is functionally wired to the expression of chemokines and genes associated with their post-Aire development. Second, the receptors for these chemokines are predominantly expressed by the Sirpα$^+$ thymic population of CD14$^+$moDCs whose enrichment in the thymus and subsequent CAT is positively regulated by mTEC-intrinsic TLR/MyD88 signaling. Third, TLR/MyD88 signaling in mTECs is important for the proper development of thymic CD73$^-$CD25$^+$Foxp3$^+$ Tregs since its abrogation resulted in a decreased number and the functionality of Tregs, associated with pathological effects in the mouse model of colitis.

The importance of TLR/MyD88 signaling in Aire-dependent autoimmunity was suggested in experiments conducted with MyD88$^{-/-}$Aire$^{-/-}$ double-knockout mice. These mice develop more severe symptoms of autoimmunity than Aire$^{-/-}$ single KO animals indicating the positive regulatory role of MyD88 signals in tolerance induction. Strikingly, neither the enhancement of MyD88 signals by an i.p. injection of TLR ligands, nor their diminishment in mice from GF conditions altered the severity of Aire-dependent autoimmunity[53]. Our data advocates for a scenario in which the worsening of autoimmunity in MyD88$^{-/-}$ Aire$^{-/-}$ mice could be caused by the lack of MyD88 signaling in mTECs$^{high}$, downregulation of their chemokines needed to

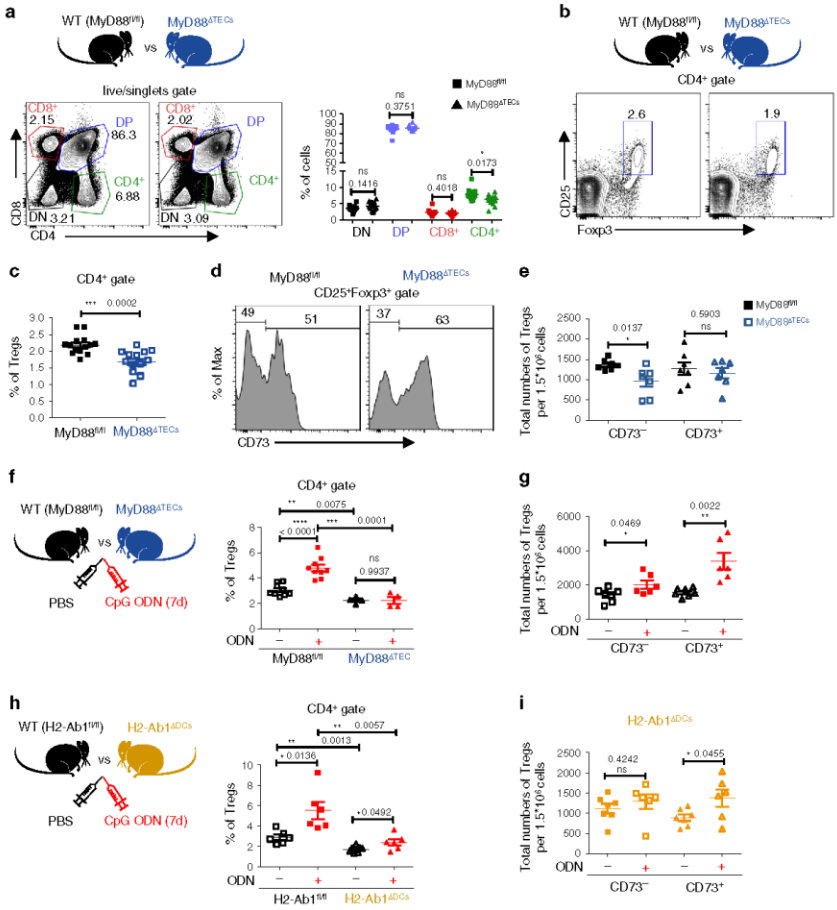



**Fig. 6 Development of thymic Tregs is impaired in MyD88$^{\Delta TECs}$ mice. a** Representative flow cytometry plots (left plot) and their quantification (right plot) comparing the frequencies of main thymic T cell populations between MyD88$^{fl/fl}$ and MyD88$^{\Delta TECs}$ mice (mean ± SEM, $n = 14$ mice). **b** Representative flow cytometry plots comparing the frequencies of CD4$^+$CD25$^+$Foxp3$^+$ thymic Tregs between MyD88$^{fl/fl}$ and MyD88$^{\Delta TECs}$ mice. **c** Quantification of frequencies from b (mean ± SEM, $n = 14$ mice). **d** Representative flow cytometry histograms showing the expression of CD73 by CD4$^+$ CD25$^+$Foxp3$^+$ thymic Tregs (gated as in b). **e** Quantification of the total numbers of CD73$^-$ and CD73$^+$ thymic Tregs from d (mean ± SEM, $n = 7$ mice). **f** Quantification of the frequencies of thymic Tregs from CpG ODN or PBS intrathymically stimulated (7 days) MyD88$^{fl/fl}$ or MyD88$^{\Delta TECs}$ mice (mean ± SEM, $n = 4$ for MyD88$^{\Delta TECs}$ and $n = 9$ for MyD88$^{fl/fl}$ mice). **g** Quantification of the total numbers of CD73$^-$ and CD73$^+$ thymic Tregs from CpG ODN or PBS intrathymically stimulated (7 days) WT (C57Bl/6J) mice (mean ± SEM, $n = 6$ for ODN$^+$ and $n = 7$ for ODN$^-$ mice). **h** Quantification of frequencies of thymic Tregs from CpG ODN or PBS intrathymically stimulated (7 days) H2-Ab1$^{fl/fl}$ or H2-Ab1$^{fl/fl}$Itgax$^{Cre}$ (H2-Ab1$^{\Delta DCs}$) mice (mean ± SEM, $n = 6$ for H2-Ab1$^{fl/fl}$ and ODN$^+$ H2-Ab1$^{\Delta DCs}$ and $n = 7$ for ODN$^-$ H2-Ab1$^{\Delta DCs}$ mice). **i** Quantification of the total numbers of CD73$^-$ and CD73$^+$ thymic Tregs from CpG ODN or PBS intrathymically stimulated (7 days) H2-Ab1$^{\Delta DCs}$ mice (mean ± SEM, $n = 6$ for ODN$^+$ and $n = 7$ for ODN$^-$ mice). Statistical analysis in **a**, c, **e**–**i** was performed by unpaired, two-tailed Student's *t*-test, $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 ***$ $p < 0.0001 ****$, ns not significant.

recruit CD14$^+$moDCs and, consequently, suboptimal production of thymic Tregs. Consistent with the previous report[53], we confirmed that the extrathymically enhanced (i.p. CpG ODN) or the lack of bacterially-derived MyD88 signals (GF mice) had no effect on the expression level of these chemokines and cytokines in WT mice. This was further corroborated by the fact that GF mice displayed normal numbers of Tregs[50] (Supplementary Fig. 7c). This data demonstrates that the ligand triggering TLR9/MyD88 signaling in mTECs$^{high}$ is likely of endogenous thymic-derived origin.

Since MyD88 also conveys signals from the receptors of IL-1 family cytokines (IL-1β, IL-18, IL-33)[38], we tested in vitro whether their signaling in mTECs$^{high}$ could trigger chemokine responses similar to those observed upon TLR9 stimulation. Of

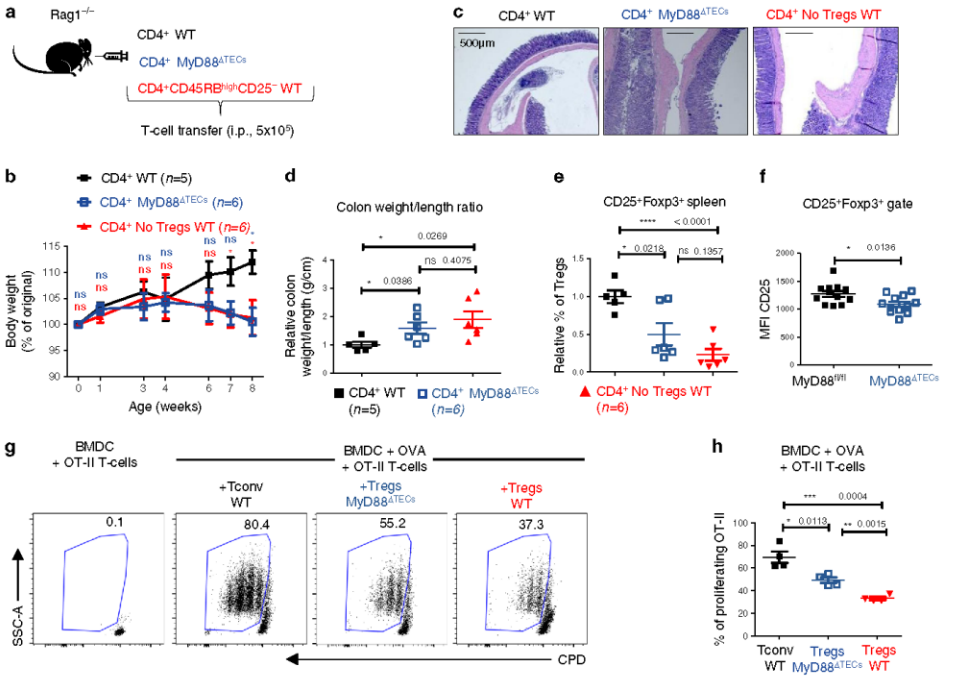



**Fig. 7 Tregs from MyD88$^{\Delta TECs}$ mice have reduced suppressive capacity and failed to prevent the T cell induced colitis. a** Experimental design of induced colitis. **b** Relative quantification of mice weight normalized to its value on day 0 (100% of original weight) after T cell transfer over the time-course of the colitis experiment (mean ± SEM, $n = 5$–6 mice) Statistical analysis was performed by unpaired, two-tailed Student's *t*-test comparing the relative weight of WT CD4$^+$ with MyD88$^{\Delta TECs}$ CD4$^+$ transferred mice (blue) or with WT CD4$^+$CD45RB$^{high}$CD25$^-$ transferred mice (red), $p \leq 0.05 = *$, ns not significant. **c** Representative H&E-stained slides of colon sections performed 8 weeks after T cell transfer. Scale bar represents 500 µm ($n = 5$ for CD4$^+$ WT and $n = 6$ for CD4$^+$MyD88 $^{\Delta TECs}$ and CD4$^+$ No Tregs WT mice). **d** Relative quantification (normalized to average of control mice from each experiment) of colon weight/length ratio of T cell induced colitis experimental mice (mean ± SEM, $n = 5$–6 mice). **e** Relative quantification of the frequencies (normalized to average of control mice from each experiment) of CD4$^+$CD25$^+$Foxp3$^+$ Tregs isolated from the spleens of experimental mice 8 weeks after T cell transfer (mean ± SEM, $n = 5$–6 mice). **f** Quantification of the Means fluorescent intensity (MFI) of CD25 protein expression in CD25$^+$Foxp3$^+$ Tregs (gated as in Fig. 6b) in MyD88$^{fl/fl}$ and MyD88$^{\Delta TECs}$ mice (mean ± SEM, $n = 12$ mice) Statistical analysis in **b**, **d**–**f** was performed by unpaired, two-tailed Student's *t*-test, $p \leq 0.05 = *$, $p < 0.0001$****, ns not significant. **g** Representative flow cytometry plots showing the frequency of proliferating OT-II T cells, co-cultivated with OVA pulsed BMDC and CD4$^+$CD25$^+$ Tregs cells (alternatively with CD4$^+$CD25$^-$ Tconv cells, black) isolated from LNs of MyD88$^{fl/fl}$ (WT control, red) or MyD88$^{\Delta TECs}$ (blue) for 72 h. **h** Quantification of frequencies of proliferating OT-II Tcells form h (mean ± SEM, $n = 4$ wells from two independent experiments).

this trio of cytokines, only IL-1β exhibited this capacity. This indicates that IL-1β could act as a co-regulator of chemokines and cytokine expression in mTECs$^{high}$. However, two observations suggest that TLR9/MyD88 signaling axis can act independently of IL-1β: (i) a direct, in vitro, stimulatory capacity of CpG ODN induces chemokine expression in sorted mTECs$^{high}$; and (ii) both in vivo intrathymic stimulation of TLR9/MyD88 signaling axis as well as its downregulation in MyD88$^{\Delta TECs}$ cells impacts the recruitment of the very same subsets of CD14$^+$moDCs.

It has been postulated that Aire$^+$ mTECs further differentiate into post-Aire cells, which downregulate the expression of MHCII and Aire, upregulate a set of genes, such as keratins (Krt1, 10, 77) or involucrin and form Hassall's corpuscules[40,41,54]. However, the regulatory mechanism(s) guiding this differentiation process remains poorly understood[55]. Our transcriptomic results are consistent with the idea that TLR/MyD88 signaling establishes an expression profile that is associated with the differentiation of mTECs$^{high}$ into post-Aire mTECs. Notably, TLR9 stimulation not only increased the number of Involucrin$^+$ post-Aire mTECs (Supplementary Fig. 3e, f), but also lead to the upregulation of cytokines and chemokines (*Il1f6*, *Lcn2*, *Cxcl3*, and *Cxcl5*) associated with Hassall´s corpuscles[42] which attract CD14$^+$moDCs. Together with the fact that they serve as a reservoir of a large amount of Aire-dependent TRAs, post-Aire mTECs could hold central position in the mechanism of transfer of mTEC-derived antigens to thymic DCs.

As described above, TLR/MyD88 signaling in mTECs$^{high}$ drive the expression of chemokines which act on an overlapping set of receptors[32] predominantly expressed by CD14$^+$moDCs (Cxcr2, Ccr1, Ccr3, Ccr5) and pDCs (Ccr5, Ccr6, and Ccr9). A correlative nature between the frequency of CD14$^+$moDC in the thymus of MyD88$^{\Delta TECs}$ and of WT stimulated with CpG, underpins the

importance of these chemokines in controlling the migration of these cells into the thymic medulla. However, the deletion of Cxcr2 on hematopoietic cells, the common receptor for Cxcl1, Cxcl2, Cxcl3 and Cxcl5, did not yield any changes in the enrichment of CD14$^{+}$moDC in the thymus (Supplementary Fig. 6h). This observation, in conjunction with previous reports[18,56], allows one to predict that while the ligands of Ccr3 and/or Ccr5 (Ccl3, Ccl4, Ccl5 or Ccl24) likely regulate the entry of CD14$^{+}$moDC into the thymus[19], Cxcl-chemokines may regulate the positioning of these cells in close proximity of post-Aire mTECs. Interestingly, with the decreased frequency of CD14$^{+}$ moDCs in the thymus of MyD88$^{\Delta TEC}$, pDCs were similarly diminished. However, in contrast to CD14$^{+}$moDCs, the number of pDCs did not increase after TLR9 intrathymic stimulation. This is consistent with the fact that the migration of pDCs to the thymus is driven by Ccl25 (ligand of Ccr9 receptor)[14], the expression of which was diminished in MyD88$^{\Delta TEC}$ but was not upregulated in WT mTECs after TLR9 stimulation.

It has been previously documented that specific subtypes of thymic DCs vary in their capacity to acquire antigens from TECs. Notably, while the transfer of MHC molecules from TECs to CD8α$^{+}$ cDC1 and Sirpα$^{+}$DCs occurred at the same efficiency[16], the transfer of intracellular GFP was restricted mainly to CD8α$^{+}$cDC1[10]. In comparison, our data shows that cytoplasmic TdTOM from Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ could to certain extent, be transferred to all major subtypes of thymic DCs. This may be explained by the robustness of the Foxn1$^{Cre}$-dependent system where, compared to Aire-GFP model, the production of TdTOM is not restricted only to Aire-expressing mTECs but to the entire thymic TEC population. Importantly, since the CAT of TdTOM after CpG ODN intrathymic injection is increasingly targeted to CD14$^{+}$moDC subpopulation, the efficiency of CAT correlates not only with the broadness of antigen expression but also with the frequency of a given DC subtype in the medulla. On the other hand, since TECs constitute a relatively rare cell population of thymic cells[57], the amount of antigen, which can be potentially transferred to DCs, is fairly limited. This could explain the fact that even when the entire population of thymic pDCs is not affected by intrathymic TLR9 stimulation, the frequency of TdTOM$^{+}$ pDCs is significantly decreased, due to the increased competition for TdTOM uptake by CD14$^{+}$moDCs.

It has become clear that developing thymocytes encounter self-antigens presented by various types of thymic APC, including mTECs[47], B-cells[58], pDCs[14], and cDCs[11,59]. Although the generation of thymic Tregs was shown to be dependent on antigen presentation by both mTECs and DCs[4,47], thymic cDCs seem to be particularly important for this process[6,17,60]. Along with self-antigen presentation, thymic cDCs express high levels of co-stimulatory molecules CD80/86 as well as CD70 which play a crucial role in promoting thymic Treg development[61,62]. Among cDCs, Sirpα$^{+}$DCs are the most efficient in supporting Treg generation[17,20,63]. In this context, our data demonstrates that the development of thymic CD25$^{+}$Foxp3$^{+}$ Tregs is boosted by TLR/MyD88 signaling in TECs, which produce a chemokine gradient driving the migration of CD14$^{+}$moDCs into the thymus. We also found that mTEC-intrinsic TLR9/MyD88 signaling increased the cell ratio of Sirpα$^{+}$DCs to Xcr1$^{+}$cDC1, which correlated with an increased production of thymic Tregs. These findings accurately recapitulate the thymic phenotype of Ccr7$^{-/-}$ mice where the increased ratio of Sirpα$^{+}$DCs to cDC1 correlated with the increased generation of thymic Tregs[20]. This data, together with the fact that abrogation of MHCII-antigen presentation specifically in DCs, resulted in a reduced number of thymic Tregs in unstimulated[17] as well as in CpG stimulated thymus (Fig. 6h), suggest that TLR/MyD88-dependent generation of thymic Tregs is mediated by antigen-presentation by DCs.

Our results also show that TLR/MyD88 signalling in mTECs drives the recirculation of mature CD73$^{+}$CD25$^{+}$Foxp3$^{+}$ Tregs into the thymus. Compared to newly generated CD73$^{-}$ Tregs, their increased number in the TLR9 stimulated thymus was not dependent on MHCII presentation by DCs. Together, with the fact that recirculation of CD73$^{+}$ Tregs was not abrogated in MyD88$^{\Delta TECs}$ mice, suggests that Ccl20, the ligand for Ccr6, which is highly expressed by recirculating Tregs[64] regulates the increased recirculation of CD73$^{+}$CD25$^{+}$Foxp3$^{+}$ Tregs into the thymus after TLR9 intrathymic stimulation (Figs. 2d and e).

Altogether, our model proposes that TLR/MyD88 signaling in mTECs regulates the generation of Tregs. The mechanism involves TLR-induced chemokine production and subsequent chemotactic recruitment of CD14$^{+}$moDC to the thymic medulla, which predicates the developmental output of Tregs. Although this study explores only TLR9 signaling in mTECs, questions surrounding the nature of potential thymic-derived endogenous ligands for TLR/MyD88 signals in mTECs remains enigmatic and warrant further study.

## Methods

**Mice**. A majority of the mice used in this study were of C57BL/6J genetic background and housed in the animal facility at the Institute of Molecular Genetics of the ASCR v.v.i. under SPF conditions. Mice were fed with irradiated standard rodent high energy breeding diet (Altromin 1314 IRR) and given reverse osmosis filtered and chlorinated water ad libitum. Light were adjusted to a 12 h/12 h light/dark cycle; temperature and relative humidity were maintained at 22 ± 1°C and 55 ± 5%, respectively. Experimental protocols were approved by the ethical committee of the Institute of Molecular Genetics and by the ethical committee of the Czech Academy of Science. Aire$^{-/-}$ (B6.129S2-Aire$^{tm1.1Doi}$/J, stock# 004743)[2], Foxn1$^{Cre}$ (B6(Cg)-Foxn1$^{tm3(cre)Nrm}$/J, stock# 018448)[29], MyD88$^{fl/fl}$ (B6.129P2(SJL)-Myd88$^{tm1Defr}$/J, stock# 008888), MyD88$^{-/-}$ (B6.129P2(SJL)-Myd88$^{tm1.1Defr}$/J, stock# 009088)[30], Rag1$^{-/-}$ (B6.129S7-Rag1$^{tm1Mom}$/J, stock# 002216)[65], Ly5.1 (B6.SJL-Ptprc$^{a}$Pepc$^{b}$/BoyJ, stock# 002014)[66], Cxcr2$^{fl/fl}$ (C57BL/6-Cxcr2$^{tm1Rmra}$/J, stock# 024638)[67], H2-Ab1$^{fl/fl}$ (B6.129×1-H2-Ab1$^{tm1Koni}$/J, stock# 013181)[68], and Itgax$^{Cre}$ (B6.Cg-Tg(Itgax-cre)1-1Reiz/J, stock# 008068)[69] mice were purchased from Jackson Laboratories. Rosa26$^{TdTOMATO}$ (B6;129S6-Gt(ROSA)26$^{Sortm14(CAG-tdTomato)Hze}$/J, stock# 007908)[70] and Vav1$^{Cre}$ (B6.Cg-Commd10$^{Tg(Vav1-icre)A2Kio}$/J, stock# 008610)[71] were kindly provided by V. Kořínek (Institute of Molecular Genetics of the ASCR, Prague, Czech Republic). Aire-HCO (Balb/c)[4] were provided by L. Klein. Cd3e$^{-/-}$[72], RIP-OVA[73], OT-I$^{+}$Rag2$^{-/-}$[74] (all C57BL/6J) were provided by O. Štěpánek. OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J, stock# 004194)[75] mice were kindly provided by T. Brdička (Institute of Molecular Genetics of the ASCR, Prague, Czech Republic). C57BL/6J GF and control C57BL/6J SPF mice were kindly provided by M. Schwarzer (Institute of Microbiology of the ASCR, Nový Hrádek, Czech Republic. Both GF and control SPF mice were subject to the SSNIFF V1124-300 diet. Thymic cell populations were isolated from 3–6-week-old mice with the exception of newborn mice (4 days old) used in Supplementary Fig. 7b. For the purpose of BM chimera experiments, 5–6-week-old mice were irradiated and analysed between 11 and 13 weeks of age. Comparative analysis used age-matched cohorts regardless of sex and caging. Where possible, littermates were used as the controls. For the purpose of tissue isolation, mice were euthanized by cervical dislocation.

**Tissue preparation and cell isolation**. Thymic antigen presenting cells, TECs and DCs, were isolated as follows. Thymus was minced into small pieces and treated with Dispase II (Gibco), dissolved in RPMI at concentration 0.1 mg ml $^{-1}$. Tissue was homogenized by pipetting and after 10 min of incubation (37°C), the supernatant was collected and the reaction was stopped by adding 3% FSC and 2 nM EDTA. The process was repeated until all thymic fragments were digested. For detail description see[76]. For thymic epithelial cells isolation, the whole thymic cell suspension was depleted of CD45$^{+}$ cells by CD45 microbeads staining (Miltenyi biotec). Thymic dendritic cells were isolated using MACS enrichment for CD11c$^{+}$ cells through staining with biotinylated CD11c antibody, followed by Ultrapure Anti-Biotin microbeads staining (Miltenyi biotec). For isolation of T cell, thymus, peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN) or spleen were mechanically mashed through 40 µm Cell strainer (Biologix) and cell suspensions were passed through 50 µm filters (Sysmex). The resulting cell suspension was spun down (4 °C, 400 g, 10 min) and erythrocytes were removed using ACK lysis buffer.

**Flow cytometry analysis and cell sorting**. Flow cytometry (FACS) analysis and cell sorting were performed using BD LSR II and BD Influx (BD Bioscience) cytometers, respectively. For surface staining, cells were incubated for 20–30 min at 4 °C with the indicated fluorochrome- or biotin-conjugated antibodies. Where necessary, cells were further incubated with streptavidin conjugates for 15 min.

Dead cells were excluded using Hoechst 33258 (Sigma) or viability dye eFlour 450 or 506 (eBioscience). For the intracellular staining of Aire and Foxp3, the cells were first stained for the targeted surface molecules, fixed, and permeabilized for 30 min at room temperature (RT) using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), then stained for 30 min at RT with fluorochrome-conjugated antibodies. FlowJO V10 software (Treestar) and BD FACSDiva™ Software v6.0 for BD™ LSR II (with HTS Option) was used for FACS data analysis including tSNE analysis shown in Fig. 5e. A complete inventory of staining reagents is listed in Supplementary Data 6.

**Imaging flow cytometry**. Imaging flow cytometry was performed at the Center for Advanced Preclinical Imaging (CAPI) with the use of AMNIS ImageStream X MkII (AMNIS). DCs isolated from Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ mice were stained for the surface markers MHCII and CD11c. Dead cells were excluded by Hoechst 33258 staining and bright field analysis. Cells were recorded using 40x magnification. Data was analyzed with Ideas 6.1 software (AMNIS). A complete list of staining reagents can be found in Supplementary Data 6.

**In vitro TLRs and cytokines stimulation assays**. mTECs$^{high}$ were gated as EpCAM$^+$CD11c$^-$Ly51$^-$MHCII$^{high}$CD80$^{high}$ and sorted into RPMI media (Sigma) containing 10% FSC and 1% Penicillin/Streptomycin (Gibco). Cells were then cultured in a 96-flat-well plate in 200 µL of 10% FSC RPMI with Penicillin/Streptomycin in the presence of Endotoxin-free TLR ligands (InvivoGen) or recombinant mouse cytokines: TLR9 ligand-CpG ODN (ODN 1826) (5 µM), TLR4 ligand-LPS (1 µg/ml), Il-1β (10 ng/ml), Il-33 (10 ng/ml) (both ImmunoTools) and Il-18 (10 ng/ml) (Biolegend). After 24 h, the supernatant was removed and the cells were resuspended in RNA-lysis buffer. Subsequently, RNA isolation was performed.

**In vivo TLR stimulation**. For intrathymic injections, mice were anesthetized by i.p. injection of Zoletil (Tiletamine (50 mg/ml) and Zolazepam (50 mg/ml), Virbac) dissolved in PBS at a dose of 50 mg/kg and 10–20 µl of 500 µM CpG ODN (ODN 1826, InvivoGen) or PBS was injected using an insulin syringe (29G) directly into the first intercostal space from the manubrium ~2 mm left of the sternum and 4 mm in depth. The angle of injection was from 25 to 30° relative to the sternum[77]. For systemic TLR9 stimulation, mice were injected by CpG ODN (ODN 1826, InvivoGen) (500 µM) or PBS at day 0 and day 1 into the peritoneum. Mice were then maintained under SPF conditions and euthanized at the indicated time point of an experiment.

**Immunofluorescent analysis of thymic cryosections**. The thymus was fixed overnight in 4% paraformaldehyde (Sigma) at 4 °C, washed three times in PBS, incubated overnight in 30% sucrose at 4 °C, and finally embedded in OCT compound (VWR). Cryoblocks were cut at 8 µm and blocked with PBS containing 5% BSA (w/v) and 0.1% Triton X-100 for 1 hour at room temperature. Samples were incubated overnight at 4 °C with the following primary antibodies: anti-keratin 14, Sirpα, and CD11c-biotin (Fig. 3d) or anti-Involucrin and anti-EpCAM-APC (Supplementary Fig. 3b). The samples were stained with secondary reagents, Goat anti-rat AF-568, goat anti-rabbit AF-647 and streptavidin FITC or goat anti-rabbit AF-488 for one hour at RT. Sections stained only with secondary reagents were used as negative controls. 4′,6-diamino-2-phenylindole (DAPI) was used to visualize cell nuclei. Stained sections were mounted in Vectashield medium (Vector Laboratories) and imaged using a Dragonfly 503 (Andor)—spinning disk confocal microscope with the immersion objective HC PL APO 20×/0.75. A complete list of staining reagents can be found in Supplementary Data 6. Z-stacks were composed using ImageJ and deconvolution was done by Huygens Professional. CD11c$^+$Sirpα$^+$ double positive cells were counted in multiple 300µmx300µm areas in keratin-14 rich (medulla) and keratin-14 negative (cortex) region. Counting was done as a blind experiment by three different investigators. Involucrin$^+$EpCAM$^+$ double positive cells were counted as number of cells per thymic medullary region (determined by DAPI staining).

**Gene expression analysis by qRT-PCR**. Total RNA from FACS-sorted cells was extracted using an RNeasy Plus Micro Kit (Qiagen) and reverse transcribed using RevertAid (ThermoFisher) transcriptase and random hexamers (ThermoFisher). Quantitative RT PCR (qRT PCR) was performed using the LightCycler 480 SYBR Green I Master mix (Roche) on a LightCycler 480 II (Roche). Each sample was tested in duplicate. Threshold cycles were calculated using LightCycler 480 1.5 software. Gene expression was calculated by the relative quantification model[78] using the mRNA levels of the housekeeping gene, *Casc3*, as a control. Primers were designed using Primer-BLAST (NCBI, NIH). Primers sequences are listed in Supplementary Data 6.

**Bone marrow chimera generation**. Bone marrow cells were isolated from the *femur* and *tibia* of Ly5.1 mice (CD45.1$^+$) and subsequently depleted of erythrocytes using ACK lysis buffer. Recipient mice (Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$, CD45.2$^+$) were irradiated with 6 Gy and reconstituted with $2 \times 10^6$ donor BM cells. These mice were maintained on water supplemented with gentamycin (1 mg/ml) for 10 days. Three weeks after irradiation, the frequency of blood cell reconstitution was measured by FACS using anti-CD45.1 and CD45.2 antibodies. If the reconstitution was higher than 80%, mice were euthanized 6 weeks after transfer and subjected to further analysis.

**RNA-sequencing and analysis**. mTECs were sorted according to the protocol described above and RNA was extracted using a RNeasy Plus Micro Kit (Qiagen). cDNA synthesis, ligation of sequencing adaptors and indexes, ribosomal cDNA depletion, final PCR amplification and product purification were prepared with a SMARTer® Stranded Total RNA-Seq – Pico Input Mammalian library preparation kit v2 (Takara). Library size distribution was evaluated on a Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent). Libraries were sequenced on a Illumina NextSeq® 500 instrument using a 76 bp single-end high-output configuration resulting in ~30 million reads per sample. Read quality was assessed by FastQC (0.11.9). Subsequent read processing including removing sequencing adaptors (Trim Galore!, version 0.4.5), mapping to the reference genome (GRCm38 (Ensembl assembly version 91)) with HISAT2 (2.1.0), and quantifying expression at the genetic level (featureCounts) was done via the SciLifeLab/NGI-RNAseq pipeline [https://github.com/SciLifeLab/NGI-RNAseq]. Final per gene read counts served as an input for differential expression analysis using a DESeq2 R Bioconductor (3.10). Prior to this analysis, genes that were not expressed in at least two samples were discarded. Genes exhibiting a minimal absolute log2-fold change value of 1 and a statistical significance (adjusted $p$-value < 0.05) between conditions were considered as differentially expressed for subsequent interpretation and visualization. All figures (volcano plots, etc.) were generated using basic R graphical functions. The raw sequencing data were deposited at the ArrayExpress database under accession numbers E-MTAB-8024 (for Fig. 2a, b) and E-MTAB-8025 (for Fig. 2d, e).

**Single-cell RNA sequencing**. DCs were sorted from Foxn1$^{Cre}$ROSA26$^{TdTOMATO}$ as Gr-1$^-$CD11c$^+$TdTOM$^+$ (described in detail in Supplementary Fig. 3a and Fig. 4c). Two independent samples (Sample 1 and 2) were used for further analysis. A single-cell library was prepared by Illumina/Bio-Rad single-cell RNA-seq system with a SureCell WTA 3′ Library Prep Kit according to the manufacturer's instructions. Total cell concentration and viability was ascertained using a TC20 Automated Cell Counter (Bio-Rad). A ddSEQ Single-Cell Isolator (Bio-Rad) was used to co-encapsulate single cells with barcodes and enzyme solutions for cDNA synthesis. Nextera SureCell transposome solution was used for cDNA fragmentation and ligation of sequencing indexes, followed by PCR amplification and short fragment removal. Finally, library fragment length distribution and concentration were analyzed on a Agilent Bioanalyzer 2100 using a High Sensitivity DNA Kit (Agilent). The resulting libraries were sequenced using a 68/75 paired-end configuration on a Illumina NextSeq® 500 instrument resulting in ~73 million reads per sample.

**Single-cell RNA sequencing analysis**. The quality of reads was assessed by FastQC. Cell identification was accomplished with cell barcodes and low-expression cells filtering using UMI-tools[79]. The analysis identified 202 cells in Sample 1 and 218 cells in Sample 2. Reads assigned to the selected cells were mapped to the GRCm38 genome assembly (Ensembl version 91) with HISAT2 (2.1.0). Gene expression was quantified using, featureCounts (2.0.0) after deduplication of per-gene assigned read counts by UMIs with UMI-tools. De-duplicated per-gene read counts were imported into R for exploration and statistical analysis using a Seurat[80] package (version 3.0). Counts were normalized according to total expression, multiplied by a scale factor (10,000), and log-transformed. For cell cluster identification and visualization, gene expression values were also scaled according to highly variable genes after controlling for unwanted variation generated by sample identity. Cell clusters were identified based on t-SNE of the first six principal components of PCA using Seurat's method, FindClusters, with a original Louvain algorithm and resolution parameter value of 0.3. To find cluster marker genes, Seurat's method, FindAllMarkers, along with a likelihood ratio test assuming an underlying negative binomial distribution suitable for UMI datasets was used. Only genes exhibiting a significant (adjusted $p$-value < 0.05) minimal average absolute log2-fold change of 1 between each of the clusters and the rest of the dataset were considered as differentially expressed. For t-SNE expression plots, normalized count data were used. Heatmaps of gene expression per cluster were generated based on gene z-score scaled raw counts. The raw sequencing data was deposited at the ArrayExpress database under accession number E-MTAB-8028.

**In vitro antigen presenting assay**. For the purpose of antigen presentation assay CD14$^+$moDCs were gated as CD11c$^+$MHCII$^+$B220$^-$Xcr1$^-$Cx3cr1$^+$CD14$^+$ and FACS sorted from Aire-HCO mice into DMEM high-glucose medium (Sigma) supplemented with 10% FCS and 1% Penicillin-Streptomycin (Gibco) and cultivated in a 96 well plate together with the A5 hybridoma cell line (HA-specific CD4 T cell hybridoma cells carrying a GFP-NFAT reporter) at a 1:5 ratio (10 000 of CD14$^+$moDC: 50 000 of A5 cells). As a positive control, CD14$^+$moDCs were pulsed with HA peptide (107-119; customized by Thermofisher) at a concentration of 1 µg/ml. After 20 h, the level of GFP expression by A5 hybridomas was analyzed by flow cytometry.

**Induction of T cell transfer colitis and histological analysis**. FACS-sorted $5\times10^5$ $TCR\beta^+CD4^+CD45RB^{high}CD25^-$ or complete $TCR\beta^+CD4^+$ were transferred by i. p. injection into $Rag1^{-/-}$ recipient mice (5-7 weeks old). The weight of mice was recorded weekly to monitor the progress of colitis. Mice were euthanized 8 weeks after transfer[51]. Spleens and colons of the animals were weighed and the length of the colon was measured. For histological analysis PBS washed colons were fixed in 4% paraformaldehyde (Sigma) and embedded into paraffin. Tissue sections were cut into 5µm thin slices, deparaffinized, and stained with hematoxylin and eosin (H&E).

**In vitro Tregs suppression assay**. BM-derived DCs (BMDCs) were prepared as follows. BM cells were flushed from femur and tibia of WT C57BL/6J mice and cultured in RPMI media (Sigma) containing 10% FSC and 1% Penicillin/Streptomycin (Gibco) supplemented with GM-CSF (5 ng/ml). Fresh media containing GM-CSF was added at day 3 and 5 of cultivation. After 7 days, BMDCs was pulsed with OVA cognate peptide 323-339 (irrelevant OVA 257–264 peptide was used as control) (InvivoGen) at a concentration of 1 µg/ml and co-cultivated with OVA-specific OT-II T cells and Tregs (10 000 BMDCs: 50 000 OT-II T cells: 50 000 Tregs). OT-II T cells were isolated from OT-II$^+$Rag1 $^/$ mice as MACS-enriched $CD4^+$ T cells ($CD4^+$ T Cell Isolation Kit, Miltenyi biotec). $CD4^+$ conventional T cells (Tconv) were used as a negative control. Tregs were isolated from LNs (pLN and mLN) of WT ($MyD88^{fl/fl}$) and $MyD88^{\Delta TECs}$ mice using subsequent Auto-MACS (Miltenyi biotec) procedure. CD4-enriched T cells ($CD4^+$ T Cell Isolation Kit, Miltenyi biotec) were stained by anti-CD25 biotin conjugated antibody and $CD4^+CD25^+$ Tregs were isolated using Anti-Biotin MicroBeads (Miltenyi biotec). Tconv cells were prepared using Auto-MACS as $CD4^+CD25^-$ cells. After 3 days of co-cultivation, cells were stained with anti-Vβ5 and anti-Vα2 antibodies to distinguish OT-II$^+$ T cells. Proliferation was measured by FACS using CPD670 staining.

**In vivo model of autoimmune diabetes**. $Cd3\varepsilon^{-/-}$-RIP-OVA mice (6–8 weeks old) were intravenously injected by MACS enriched $CD8^+$ T cells ($5\times10^5$ cells per mouse) isolated from lymph nodes and spleen of Rip-OVA Ly5.1 ($CD45.1^+$) mice at day 8. After 7 days (day 1) $Cd3\varepsilon^{-/-}$-RIP-OVA mice were intravenously injected, FACS sorted $CD4^+CD25^+$ Tregs were isolated from LNs (mLN and pLN) of WT ($MyD88^{fl/fl}$), $MyD88^{\Delta TECs}$ mice ($3\times10^5$ cells per mouse), OT-I {OT-I$^+$$Rag2^{-/-}$, 100 cells per mouse), and OT-II cells {OT-II$^+$$Rag1^{-/-}$, $1\times10^4$ cells per mouse). BMDCs (generated as described previously, 10 days of culture, media refreshment at day 4 and 7) were pulsed with OVA peptides (OVA 257–264, 2 mM and OVA 323-339, 100 µM, InvivoGen) in the presence of LPS (100 µg/ml, InvivoGen) for 3 h. In all, $1\times10^6$ of antigen-stimulated DCs were used for injection (at day 0). Glucose levels were monitored on a daily basis (between day 5 and 14) using test strips (Diabur-Test 5000, Roche or GLUKOPHAN, Erba Lachema, Czech Republic). The animal was considered to have developed autoimmunity when the concentration of glucose in the urine reached ≥10 mmol/l. At day 14, mice were euthanized and the frequency of splenic $KLGR1^+$ OT-1 T cells was measured by flow cytometry.

**Statistical analysis**. The statistical tests used to analyze the data are indicated in figure legends. Graph construction and statistical analysis were performed using Prism 5.04 software (GraphPad). Statistical analysis of RNAseq and scRNAseq data is indicated in the corresponding method section.

**Reporting summary**. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. The source data underlying Fig. 1c, f, 2c, f, g, 3a–c, e, 4d, f, 5d, f, g, 6a, c, e–i, 7b, d–h and Supplementary Figs. 2b–d, 3a, b, d, e, 4c, 5e, f, 6c, e, h, 7a–d, f, h and 8b, c, e are provided as a Source Data file. The raw RNA sequencing data are deposited at the ArrayExpress database [https://www.ebi.ac.uk/arrayexpress/] under accession numbers E-MTAB-8024 (Fig. 2a, b), E-MTAB-8025 (Fig. 2d, e) and E-MTAB-8028 (Fig. 5a–c).

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## References

1. Klein, L., Kyewski, B., Allen, P. M. & Hogquist, K. A. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* **14**, 377–391 (2014).
2. Anderson, M. S. et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395–1401 (2002).
3. Liston, A., Lesage, S., Wilson, J., Peltonen, L. & Goodnow, C. C. Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* **4**, 350–354 (2003).
4. Aschenbrenner, K. et al. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat. Immunol.* **8**, 351–358 (2007).
5. Malchow, S. et al. Aire-dependent thymic development of tumor-associated regulatory T cells. *Science* **339**, 1219–1224 (2013).
6. Perry, J. S. et al. Distinct contributions of Aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus. *Immunity* **41**, 414–426 (2014).
7. Leventhal, D. S. et al. Dendritic cells coordinate the development and homeostasis of organ-specific regulatory T cells. *Immunity* **44**, 847–859 (2016).
8. Gallegos, A. M. & Bevan, M. J. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* **200**, 1039–1049 (2004).
9. Koble, C. & Kyewski, B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J. Exp. Med.* **206**, 1505–1513 (2009).
10. Perry, J. S. A. et al. Transfer of cell-surface antigens by scavenger receptor CD36 promotes thymic regulatory t cell receptor repertoire development and allo-tolerance. *Immunity* **48**, 1271 (2018).
11. Lancaster, J. N. et al. Live-cell imaging reveals the relative contributions of antigen-presenting cell subsets to thymic central tolerance. *Nat. Commun.* **10**, 2220 (2019).
12. Li, J., Park, J., Foss, D. & Goldschneider, I. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. *J. Exp. Med.* **206**, 607–622 (2009).
13. Guilliams, M. et al. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat. Rev. Immunol.* **14**, 571–578 (2014).
14. Hadeiba, H. et al. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. *Immunity* **36**, 438–450 (2012).
15. Bonasio, R. et al. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. *Nat. Immunol.* **7**, 1092–1100 (2006).
16. Kroger, C. J., Spidale, N. A., Wang, B. & Tisch, R. Thymic dendritic cell subsets display distinct efficiencies and mechanisms of intercellular MHC transfer. *J. Immunol.* **198**, 249–256 (2017).
17. Leventhal, D. S. et al. Dendritic cells coordinate the development and homeostasis of organ-specific regulatory T cells. *Immunity* **44**, 847–859 (2016).
18. Lei, Y. et al. Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. *J. Exp. Med.* **208**, 383–394 (2011).
19. Baba, T., Nakamoto, Y. & Mukaida, N. Crucial contribution of thymic Sirp alpha+ conventional dendritic cells to central tolerance against blood-borne antigens in a CCR2-dependent manner. *J. Immunol.* **183**, 3053–3063 (2009).
20. Hu, Z. et al. CCR7 modulates the generation of thymic regulatory T cells by altering the composition of the thymic dendritic cell compartment. *Cell Rep.* **21**, 168–180 (2017).
21. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11**, 373–384 (2010).
22. Abramson, J. & Anderson, G. Thymic Epithelial Cells. *Annu Rev. Immunol.* **35**, 85–118 (2017).
23. Haljasorg, U. et al. A highly conserved NF-kappaB-responsive enhancer is critical for thymic expression of Aire in mice. *Eur. J. Immunol.* **45**, 3246–3256 (2015).
24. LaFlam, T. N. et al. Identification of a novel cis-regulatory element essential for immune tolerance. *J. Exp. Med.* **212**, 1993–2002 (2015).
25. Bernasconi, P. et al. Increased toll-like receptor 4 expression in thymus of myasthenic patients with thymitis and thymic involution. *Am. J. Pathol.* **167**, 129–139 (2005).
26. Cavalcante, P. et al. Toll-like receptors 7 and 9 in myasthenia gravis thymus: amplifiers of autoimmunity? *Ann. N. Y Acad. Sci.* **1413**, 11–24 (2018).
27. Huang, H. B. et al. TLR4 is constitutively expressed in chick thymic epithelial cells. *Vet. Immunol. Immunopathol.* **158**, 182–188 (2014).
28. Tian, J. et al. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat. Immunol.* **8**, 487–496 (2007).
29. Gordon, J. et al. Specific expression of lacZ and cre recombinase in fetal thymic epithelial cells by multiplex gene targeting at the Foxn1 locus. *BMC Dev. Biol.* **7**, 69 (2007).
30. Hou, B., Reizis, B. & DePranco, A. L. Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. *Immunity* **29**, 272–282 (2008).
31. Sansom, S. N. et al. Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia. *Genome Res.* **24**, 1918–1931 (2014).

32. Griffith, J. W., Sokol, C. L. & Luster, A. D. Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu Rev. Immunol.* **32**, 659–702 (2014).
33. Vigne, S. et al. IL-36R ligands are potent regulators of dendritic and T cells. *Blood* **118**, 5813–5823 (2011).
34. Becher, B., Tugues, S. & Greter, M. GM-CSF: from growth factor to central mediator of tissue inflammation. *Immunity* **45**, 963–973 (2016).
35. Zlotoff, D. A. et al. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* **115**, 1897–1905 (2010).
36. Dyer, D. P. et al. Chemokine receptor redundancy and specificity are context dependent. *Immunity* **50**, 378–389.e375 (2019).
37. Belperio, J. A. et al. Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J. Clin. Invest.* **110**, 1703–1716 (2002).
38. Fields, J. K., Günther, S. & Sundberg, E. J. Structural Basis of IL-1 Family Cytokine Signaling. *Front Immunol.* **10**, 1412 (2019).
39. Eckhart, L., Lippens, S., Tschachler, E. & Declercq, W. Cell death by cornification. *Biochim Biophys. Acta* **1833**, 3471–3480 (2013).
40. Miller, C. N. et al. Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development. *Nature* **559**, 627–631 (2018).
41. Bornstein, C. et al. Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells. *Nature* **559**, 622–626 (2018).
42. Wang, J. et al. Hassall's corpuscles with cellular-senescence features maintain IFNα production through neutrophils and pDC activation in the thymus. *Int Immunol.* **31**, 127–139 (2019).
43. Kolodziejczyk, A. A., Kim, J. K., Svensson, V., Marioni, J. C. & Teichmann, S. A. The technology and biology of single-cell RNA sequencing. *Mol. Cell* **58**, 610–620 (2015).
44. Biton, M. et al. T helper cell cytokines modulate intestinal stem cell renewal and differentiation. *Cell* **175**, 1307–1320.e1322 (2018).
45. Ardouin, L. et al. Broad and largely concordant molecular changes characterize tolerogenic and immunogenic dendritic cell maturation in thymus and periphery. *Immunity* **45**, 305–318 (2016).
46. Hettinger, J. et al. Origin of monocytes and macrophages in a committed progenitor. *Nat. Immunol.* **14**, 821–830 (2013).
47. Hinterberger, M. et al. Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. *Nat. Immunol.* **11**, 512–519 (2010).
48. McCaughtry, T. M., Wilken, M. S. & Hogquist, K. A. Thymic emigration revisited. *J. Exp. Med.* **204**, 2513–2520 (2007).
49. Thiault, N. et al. Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. *Nat. Immunol.* **16**, 628–634 (2015).
50. Owen, D. L. et al. Thymic regulatory T cells arise via two distinct developmental programs. *Nat. Immunol.* **20**, 195–205 (2019).
51. Mottet, C., Uhlig, H. H. & Powrie, F. Cutting edge: cure of colitis by CD4 +CD25+ regulatory T cells. *J. Immunol.* **170**, 3939–3943 (2003).
52. Drobek, A. et al. Strong homeostatic TCR signals induce formation of self-tolerant virtual memory CD8 T cells. *EMBO J* **37**, e98518 (2018).
53. Gray, D. H., Gavanescu, I., Benoist, C. & Mathis, D. Danger-free autoimmune disease in Aire-deficient mice. *Proc. Natl Acad. Sci. USA* **104**, 18193–18198 (2007).
54. Yano, M. et al. Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. *J. Exp. Med.* **205**, 2827–2838 (2008).
55. White, A. J. et al. Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells. *J. Immunol.* **185**, 4769–4776 (2010).
56. Lancaster, J. N., Li, Y. & Ehrlich, L. I. R. Chemokine-mediated choreography of thymocyte development and selection. *Trends Immunol.* **39**, 86–98 (2018).
57. Klein, L. Dead man walking: how thymocytes scan the medulla. *Nat. Immunol.* **10**, 809–811 (2009).
58. Yamano, T. et al. Thymic B cells are licensed to present self antigens for Central T cell tolerance induction. *Immunity* **42**, 1048–1061 (2015).
59. Ohnmacht, C. et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J. Exp. Med.* **206**, 549–559 (2009).
60. Román, E., Shino, H., Qin, F. X. & Liu, Y. J. Cutting edge: Hematopoietic-derived APCs select regulatory T cells in thymus. *J. Immunol.* **185**, 3819–3823 (2010).
61. Salomon, B. et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* **12**, 431–440 (2000).
62. Coquet, J. M. et al. Epithelial and dendritic cells in the thymic medulla promote CD4+Foxp3+ regulatory T cell development via the CD27-CD70 pathway. *J. Exp. Med* **210**, 715–728 (2013).
63. Proietto, A. I. et al. Dendritic cells in the thymus contribute to T-regulatory cell induction. *Proc. Natl Acad. Sci. USA* **105**, 19869–19874 (2008).
64. Cowan, J. E. et al. Aire controls the recirculation of murine Foxp3. *Eur. J. Immunol.* **48**, 844–854 (2018).
65. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869–877 (1992).
66. Janowska-Wieczorek, A. et al. Platelet-derived microparticles bind to hematopoietic stem/progenitor cells and enhance their engraftment. *Blood* **98**, 3143–3149 (2001).
67. Liu, L. et al. Functional defect of peripheral neutrophils in mice with induced deletion of CXCR2. *Genesis* **51**, 587–595 (2013).
68. Hashimoto, K., Joshi, S. K. & Koni, P. A. A conditional null allele of the major histocompatibility IA-beta chain gene. *Genesis* **32**, 152–153 (2002).
69. Caton, M. L., Smith-Raska, M. R. & Reizis, B. Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J. Exp. Med* **204**, 1653–1664 (2007).
70. Madisen, L. et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* **13**, 133–140 (2010).
71. de Boer, J. et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Eur. J. Immunol.* **33**, 314–325 (2003).
72. Sommers, C. L. et al. Function of CD3 epsilon-mediated signals in T cell development. *J. Exp. Med.* **192**, 913–919 (2000).
73. Kurts, C., Miller, J. F., Subramaniam, R. M., Carbone, F. R. & Heath, W. R. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J. Exp. Med.* **188**, 409–414 (1998).
74. Palmer, E., Drobek, A. & Stepanek, O. Opposing effects of actin signaling and LFA-1 on establishing the affinity threshold for inducing effector T cell responses in mice. *Eur. J. Immunol.* **46**, 1887–1901 (2016).
75. Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* **76**, 34–40 (1998).
76. Dobeš, J. et al. A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire. *Eur. J. Immunol.* **48**, 546–548 (2018).
77. Liu, L. L. et al. A simplified intrathymic injection technique for mice. *Biotech. Histochem.* **87**, 140–147 (2012).
78. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45 (2001).
79. Smith, T., Heger, A. & Sudbery, I. UMI-tools: modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy. *Genome Res.* **27**, 491–499 (2017).
80. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* **36**, 411–420 (2018).


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## Author contributions

M.V. co-designed and conducted the majority of the experiments and wrote the manuscript. T.B., J.D., and J.B. performed some experiments and provided technical help, I.Š. performed microscopic experiments. A.Č., M.D., and A.A. provided technical support for the work. O.T. and O.Š. performed the experiments using mouse diabetic model. M.K. and V.B. performed RNA sequencing. J.K. analyzed RNAseq and scRNAseq data. R.S. and L.K provided technical and experimental help, mice and material. D.F. designed experiments, supervised research, and edited the paper.

## Competing interests
The authors declare no competing interests.

## Additional information
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## 2. GASTROINTESTINAL AUTOIMMUNITY ASSOCIATED WITH LOSS OF CENTRAL TOLERANCE TO ENTERIC α-DEFENSINS

Human loss-of-function mutations in the *AIRE* gene lead to the development of rare APS-1 syndrome, which is relatively frequent in Finland, Sardinia, and Iran (Consortium, 1997; Nagamine et al., 1997; Perheentupa, 2006). Due to the defects in central tolerance and subsequent activation of self-reactive T-cells and autoantibodies in the immune periphery (Anderson et al., 2002), the APS-1 patient suffers by a dozen of autoimmune symptoms. The main components of APS-1 are CMC, hypoparathyroidism, and Addison's disease that are present in the majority of patients. These components are often accompanied by gastrointestinal symptoms that are manifested as constipation, diarrhea, and malabsorption (Husebye et al., 2018; Perheentupa, 2006). The development of these gut-related components of APS-1 was associated with the loss of central tolerance to intestinal self-antigens (histidine decarboxylase, tyrosine hydroxylase, and tryptophan hydroxylase) that are produced by EECs (Ekwall et al., 1998; Sköldberg et al., 2003; Söderbergh et al., 2004). It was also described that PCs could be also targeted by an autoimmune reaction in APS-1 patients since the sera from those patients cross-react with PCs secretory granules, suggesting enteric α-defensins as a potential autoantigen in APS-1 (Ekwall et al., 1998).

The presented study demonstrates that enteric α-defensins are expressed as AIRE-dependent TRAs by mTECs in mice and humans. Studying the APS-1 patients from Finland, Italy (Sardinia), and the Czech Republic we found that around 30% of patients were seropositive for DEFA5 (human α-defensins 5) antibody. Also, the presence of those autoantibodies correlates with the destruction of PCs and the presence of chronic diarrhea. To describe the mechanism of PC's destruction, the *Aire*$^{-/-}$ mice were used. Specifically, we showed that the transfer of α-defensin-specific T-cell isolated from *Aire*$^{-/-}$ mice to athymic nude mice resulted in the destruction of PCs, microbiota dysbiosis, and the upregulation of proinflammatory Th17 response in the small intestine. Together, this study provides a mechanism by which the loss of AIRE-dependent tolerance to intestinal self-antigen leads to gut inflammation in models of APS-1 (Dobeš et al., 2015).

Author's contribution: Western blot analysis of APS-1 seropositivity to intestinal antigens.

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# Gastrointestinal Autoimmunity Associated With Loss of Central Tolerance to Enteric α-Defensins

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**BACKGROUND & AIMS:** Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is an autoimmune disorder characterized by chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency, but patients also develop intestinal disorders. APECED is an autosomal recessive disorder caused by mutations in the autoimmune regulator (AIRE, which regulates immune tolerance) that allow self-reactive T cells to enter the periphery. Enteric α-defensins are antimicrobial peptides secreted by Paneth cells. Patients with APECED frequently have gastrointestinal symptoms and seroreactivity against secretory granules of Paneth cells. We investigated whether enteric α-defensins are autoantigens in humans and mice with AIRE deficiency. **METHODS:** We analyzed clinical data, along with serum and stool samples and available duodenal biopsies from 50 patients with APECED collected from multiple centers in Europe. Samples were assessed for expression of defensins and other molecules by quantitative reverse transcription polymerase chain reaction and flow cytometry; levels of antibodies and other proteins were measured by immunohistochemical and immunoblot analyses. Histologic analyses were performed on biopsy samples. We used *Aire*$^{-/-}$ mice as a model of APECED, and studied the effects of transferring immune cells from these mice to athymic mice. **RESULTS:** Enteric defensins were detected in extra-intestinal tissues of patients with APECED, especially in medullary thymic epithelial cells. Some patients with APECED lacked Paneth cells and were seropositive for defensin-specific autoantibodies; the presence of autoantibodies correlated with frequent diarrhea. *Aire*$^{-/-}$ mice developed defensin-specific T cells. Adoptive transfer of these T cells to athymic mice resulted in T-cell infiltration of the gut, loss of Paneth cells, microbial dysbiosis, and the induction of T-helper 17 cell-mediated autoimmune responses resembling those observed in patients with APECED. **CONCLUSIONS:** In patients with APECED, loss of AIRE appears to cause an autoimmune response against enteric defensins and loss of Paneth cells. *Aire*$^{-/-}$ mice developed defensin-specific T cells that cause intestinal defects similar to those observed in patients with APECED. These findings provide a mechanism by which loss of AIRE-mediated immune tolerance leads to intestinal disorders in patients with APECED.

*Keywords:* Mouse Model; Intestinal Crypt; DEFA; Microbiota.

The thymus is the site of T-cell lineage commitment and the place where T cells with a high affinity for self-antigens are removed from the developing T-cell pool through the process of negative selection. Critical cellular components of this process are medullary thymic epithelial cells (mTECs), which synthesize and present in the context of their major histocompatibility complex tissue restricted antigens (TRAs), the expression of which is otherwise restricted to peripheral organs.[1,2] The autoimmune regulator (AIRE) mediates the expression of a fraction of TRAs on the transcriptional level.[3] Presentation of TRAs by mTECs leads to the deletion of $CD4^+$ or $CD8^+$ T cells bearing a T-cell receptor that is reactive to the TRA-major histocompatibility complex[4] or conversion of such $CD4^+$ T cells into T-regulatory cells.[5]

Loss-of-function mutations in the *AIRE* gene cause a rare autosomal recessive syndrome called autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED; OMIM: 240300),[6,7] which is relatively frequent in Finland, Sardinia, and Iran.[1] Due to the loss of central tolerance and subsequent occurrence of self-reactive T cells and autoantibodies in the immune periphery,[8] these patients display up to a dozen clinical autoimmune components occurring in various combinations.[9] The main components of APECED are chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency,[10] but gastrointestinal symptoms, which occur intermittently and are still difficult to explain, are also fairly common.[9,11]

*Abbreviations used in this paper:* **AIRE, autoimmune regulator; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; cTECs, cortical thymic epithelial cells; EECs, enteroendocrine cells; FACS, fluorescence-activated cell sorting; IL, interleukin; mLN, mesenteric lymph node; mTECs, medullary thymic epithelial cells; pLN/mLN, peripheral/mesenteric lymph node; PCs, Paneth cells; SFB, segmented filamentous bacteria; TRAs, tissue restricted antigens; WT, wild-type.**


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Paneth cells (PCs), which reside at the bottom of small intestinal crypts, are long-lived cells that constitutively produce and secrete antimicrobial proteins and inflammatory mediators.[12] Among their bactericidal products, enteric α-defensins are the most abundant,[13] and exhibit a broad range of activities against bacteria, viruses, and fungi.[14] So far, 27 enteric α-defensins (also called cryptdins) have been identified in the mouse genome,[15] some of which are strain specific.[16] In contrast, only 2 enteric α-defensin genes, *DEFA5*[17] and *DEFA6*,[18] were recognized in humans. Enteric α-defensins protect the crypt's residential stem cells from microbial attack[19,20] and shape the composition of commensal microbiota.[21]

The gut-related autoimmune symptoms of APECED have been associated with the lack of tolerance to 2 self-antigens: tryptophan hydroxylase[22,23] and histidine decarboxylase,[24] both produced by enteroendocrine cells (EECs) of the gut epithelium. Interestingly, sera from APECED patients were shown to also cross-react with secretory granules of PCs.[23] In addition, the comparative microarray analysis between wild-type (WT) and Aire-deficient mTECs revealed that at least some members of the enteric α-defensin family are down-regulated in the latter.[3,25] Nevertheless, the potential role of PC's enteric α-defensins as self-antigens in gut-related autoimmunity of APECED patients has not been addressed so far. Here, we report that deficiencies in Aire-mediated expression of enteric α-defensins in mTECs are linked to cellular and molecular alterations in PCs-supported intestinal homeostasis and, in turn, to intestine-related autoimmunity.



## Materials and Methods

This information is provided in the Supplementary Materials.

## Results

### *Enteric α-Defensins are Present in the Human Thymus*

In the first series of experiments, we quantified the expression of the 2 enteric α-defensins *DEFA5* and *DEFA6* by quantitative reverse transcription polymerase chain reaction in the human small intestine, thymus, and spleen. As illustrated in Figure 1*A*, *DEFA5* and *DEFA6* expression in the thymus is readily detectable, although at much lower levels compared with that of the small intestine. Their expression in the spleen, used as a negative control, was barely detectable. When human thymic stromal $CD45^-$ cells were fluorescence-activated cell sorted (FACS) according to the scheme presented in Figure 1*B*, the expression of *DEFA5* and *DEFA6* was confined to the TEC population, and $CD45^+$ cells were negative (Figure 1*C*). Expression of DEFA5 protein exclusively in mTECs coexpressing AIRE was confirmed by immunofluorescence on FACS-sorted TECs (Figure 1*D*). Notably, of 150 AIRE-expressing cells, 12% (±3%, n = 3) stained positive for DEFA5. No positivity for DEFA5 was detected in AIRE-negative cells. These results demonstrated that mTECs are the exclusive cellular source of enteric α-defensins in the human thymus.

### *Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy Patients Produce Enteric α-Defensin-Specific Antibodies*

We hypothesized that expression of enteric α-defensins in human mTECs is AIRE-dependent, so the presence of enteric α-defensin–specific T cells in APECED patients might impact the integrity of defensin-expressing PCs. To address this question, paraffin-embedded duodenal biopsies from APECED patients and healthy controls were stained with anti-DEFA5 antibody (Figure 1*E*). Although the crypt structure and the composition of the entire epithelium remained seemingly unaffected, DEFA5-expressing PCs were decreased in 4 samples (Table 1) and undetected in 2 of 10 APECED samples (Figure 1*E* and Table 1). This lack of DEFA5 correlated with the absence of signal revealed by histologic Masson's trichrome staining of PCs[26] (Supplementary Figure 1). This suggested a link between AIRE-deficiency and pathologic changes in the PC compartment.

We next assessed the occurrence of autoantibodies with DEFA5 specificity in the sera of APECED patients by Western blot. In the first round, 7 of 30 APECED samples (27 from Finland, 3 from Czech Republic) (approximately 23%) were positive (Figure 2*A* and Supplementary Figure 2*A*); this group of patients is separated by *dashed line* in Table 1. It is of note that all 4 duodenal biopsies from DEFA5-seropositive patients (APS1-01, -20, -24, and -31) exhibited a decreased number of, or a lack of, PCs. The analysis of an additional 20 patient samples from Sardinia revealed 14 of a total of 50 APECED patients as seropositive (approximately 28%). None of the APECED patients was seropositive for DEFA6 autoantibodies (data not shown).

Next, using healthy duodenal sections, we compared the immunoreactivity of sera collected from healthy controls with those derived from APECED patients. Although the former showed no apparent staining of both PCs and intestinal sections, sera from APECED patients displayed a complex pattern of reactivity. Specifically, the highest serum reactivity toward PCs was observed in sera from patients with undetectable PCs, followed by those seropositive for DEFA5; the lowest was observed in DEFA5-seronegative patients (Figure 2*B*). It is of note that enteric α-defensin autoantibodies were of IgG, but not IgA, isotype (Supplementary Figure 2*B*). Remarkably, this pattern of staining intensity correlated with a number as well as the seroreactivity of additional yet unidentified intestinal autoantigens (Supplementary Figure 3*A*). These autoantigens are not ubiquitously expressed (Supplementary Figure 3*B*) and do not originate from contaminating traces of bacteria (Supplementary Figure 3*C*). In support of the notion of multi-targeted autoimmunity, when 10 intestine biopsies were probed for a specific marker of EECs, chromogranin A (Supplementary Figure 4), the reduced number of these cells was observed predominantly in DEFA5-seropositive samples (Table 1). The 2 patients who lacked PCs APS1-16 and -24, also lacked and showed a decreased number of EECs,

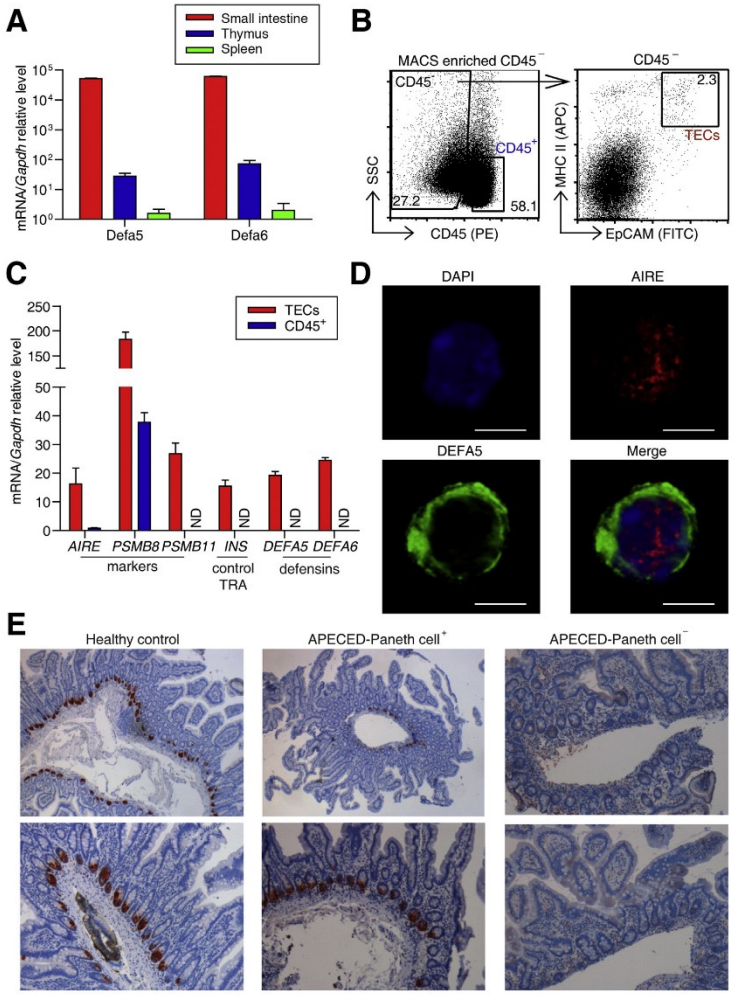


**Figure 1.** Human mTECs express enteric $\alpha$-defensins. (*A*) The relative messenger RNA levels of *DEFA5* and *DEFA6* expression in human small intestine, thymus, and spleen. (*B*) TECs were FACS-sorted as $CD45^-$ $MHCII^+$ $EpCAM^+$ cells. (*C*) The expression levels of lineage-specific markers (*AIRE*, *PSMB8*, and *PSMB11*), control TRA—insulin (*INS*) and *DEFA5* and *DEFA6* in sorted TECs. Data represent mean gene expression level ($\pm$ SD) measured in triplicate from 3 independent samples. ND, not detected. Thymic $CD45^+$ cells (*B*) were used as a control. (*D*) Microscopic detection of DEFA5 production by TECs. One representative image is shown. Scale bar = 5 $\mu$m. (*E*) The comparative antibody staining revealed the absence of DEFA5 in 2 APECED patients ($PC^-$). *Upper* and *bottom panels* represent images from 2 individuals.

respectively. These results suggest that intestine autoimmunity in patients with APECED progresses through several stages that correlate with DEFA5 seronegativity, followed by seropositivity, and culminate in the loss of PCs, the stage associated with the highest titer of multi-targeting autoantibodies.

APECED patients exhibit gastrointestinal symptoms and dysfunction.[27,28] We observed that idiopathic diarrhea was present in 9 of 14 (64.3%) DEFA5-seropositive patients (APS1-2p, -4p, -18p, -1, -3, -5, -24, -26, and -31), as well as in both patients who lacked PCs (APS1-16 and 24). Only 12 of 36 (33.3%; $P = .06$; Fisher's exact test) PC-positive DEFA5-seronegative patients suffered from this intestinal symptom. Overall, DEFA5-seropositive patients are approximately 2 times more likely to experience idiopathic diarrhea than their DEFA5-seronegative counterparts. Interestingly, when grouping Finnish and Czech patients together, the difference in the incidence of diarrhea among DEFA5-seropositive (6 of 7) compared with DEFA5-seronegative patients (6 of 23) is significant ($P = .0086$). No such association was found among Sardinian patients.

**Table 1.** Characteristics and Clinical Parameters of APECED Patients

| Patient | Nationality | Sex | Age, *y* | Diarrhea[a] | Age at the time of intestinal biopsy | CrA detection on intestinal biopsy | HD5 detection on intestinal biopsy | Anti-HD5 autantibodies in the blood |
|---|---|---|---|---|---|---|---|---|
| APS1-1p | I | M | 9 | No | — | — | — | Negative |
| APS1-5p | I | F | 20 | No | — | — | — | Negative |
| APS1-6p | I | F | 41 | No | — | — | — | Negative |
| APS1-7p | I | F | 35 | Yes | — | — | — | Negative |
| APS1-9p | I | F | 41 | Yes | — | — | — | Negative |
| APS1-11p | I | F | 37 | No | — | — | — | Negative |
| APS1-12p | I | M | 48 | Yes | — | — | — | Negative |
| APS1-13p | I | M | 17 | Yes | — | — | — | Negative |
| APS1-14p | I | F | 39 | No | — | — | — | Negative |
| APS1-15p | I | F | 7 | No | — | — | — | Negative |
| APS1-17p | I | M | 38 | Yes | — | — | — | Negative |
| APS1-19p | I | F | 24 | Yes | — | — | — | Negative |
| APS1-20p | I | M | 37 | Yes | — | — | — | Negative |
| APS1-02 | Finn | F | 42 | Yes | — | — | — | Negative |
| APS1-04 | Finn | M | 65 | Yes | — | — | — | Negative |
| APS1-06 | Finn | M | 32 | No | 24 | Normal | Normal | Negative |
| APS1-07 | Finn | F | 32 | No | — | — | — | Negative |
| APS1-08 | Finn | F | 56 | Yes | 51 | Decreased | Decreased | Negative |
| APS1-09 | Finn | F | 32 | No | — | — | — | Negative |
| APS1-10 | Finn | F | 25 | No | — | — | — | Negative |
| APS1-11 | Finn | F | 46 | No | — | — | — | Negative |
| APS1-12 | Finn | F | 50 | No | — | — | — | Negative |
| APS1-13 | Finn | M | 45 | No | 42 | Normal | NA | Negative |
| APS1-14 | Finn | M | 43 | No | — | — | — | Negative |
| APS1-15 | Finn | F | 17 | No | — | — | — | Negative |
| APS1-17 | Finn | F | 52 | No | 39 | NA | Normal | Negative |
| APS1-18 | Finn | M | 18 | No | — | — | — | Negative |
| APS1-19 | Finn | F | 53 | Yes | — | — | — | Negative |
| APS1-21 | Finn | F | 51 | No | — | — | — | Negative |
| APS1-22 | Finn | F | 8 | Yes | — | — | — | Negative |
| APS1-23 | Finn | F | 35 | No | 35 | Normal | Normal | Negative |
| APS1-25 | CZ | F | 19 | No | — | — | — | Negative |
| APS1-27 | CZ | M | 16 | No | — | — | — | Negative |
| APS1-28 | Finn | M | 44 | No | 36 | Normal | Normal | Negative |
| APS1-29 | Finn | F | 20 | No | — | — | — | Negative |
| APS1-2p | I | F | 24 | Yes | — | — | — | Positive |
| APS1-3p | I | M | 31 | No | — | — | — | Positive |
| APS1-4p | I | F | 18 | Yes | — | — | — | Positive |
| APS1-8p | I | M | 37 | No | — | — | — | Positive |
| APS1-10p | I | F | 8 | No | — | — | — | Positive |
| APS1-16p | I | M | 4 | No | — | — | — | Positive |
| APS1-18p | I | F | 39 | Yes | — | — | — | Positive |
| APS1-01 | Finn | F | 45 | Yes | 37 | Normal | Decreased | Positive |
| APS1-03 | Finn | F | 28 | Yes | — | — | — | Positive |
| APS1-05 | Finn | F | 46 | Yes | — | — | — | Positive |
| APS1-20 | Finn | F | 58 | No | 56 | Decreased | Decreased | Positive |
| APS1-26 | CZ | M | 9 | Yes | — | — | — | Positive |
| APS1-31 | Finn | F | 5 | Yes | 5 | Absent | Decreased | Positive |
| APS1-24 | Finn | M | 31 | Yes | 31 | Decreased | Absent | Positive |
| APS1-16 | Finn | M | 17 | Yes | 11 | Absent | Absent | Negative |

CZ, from Czech Republic; F, female; Finn, from Finland; I, from Italy (Sardinia); M, male; NA, not analyzed for technical reason.
[a]Diarrhea is defined as defecation >3 times a day or by unusual consistency of stools.

In general, the occurrence of autoantibodies is predicated by the presence of self-reactive T cells. We could detect defensin-specific T cells in the peripheral blood of a limited number of available DEFA5-seropositive and PC-lacking APECED patients (Supplementary Figure 5).

### *Autoimmune Regulator–Dependent Expression of Enteric α-Defensins (Cryptdins) in Mouse Medullary Thymic Epithelial Cells*

To obtain mechanistic insight into enteric α-defensin–driven gut autoimmunity, we explored Aire-deficient mice

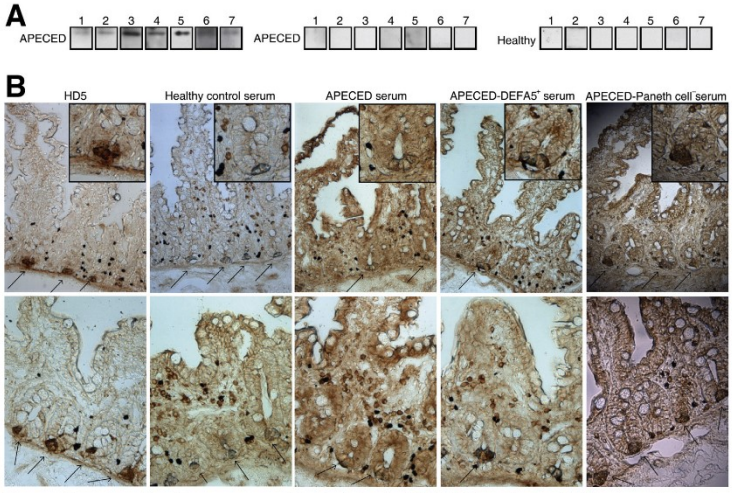


**Figure 2.** DEFA5-specific autoantibodies are present in the blood of APECED patients. (*A*) Western blot analysis revealed the reactivity of human sera to DEFA5 antigen. The seropositivity in 7 APECED samples is shown (*left panels*). For comparison, DEFA5-seronegative samples of APECED and healthy subjects are presented in the *middle* and *right panels*, respectively. (*B*). Immunohistochemistry of HD5 antibody on duodenal sections from a healthy donor, used as a positive control, revealed PCs staining on the bottom of intestinal crypts (*left panels*). Sera isolated from healthy controls show no staining (healthy control serum). Immunostaining of PCs and surrounding structures was most prominent in the serum sample derived from APECED patients lacking PCs (PCs serum), which was slightly decreased in DEFA5-seropositive APECED patients (DEFA5Ab$^{-}$ serum). Sera from the remaining APECED patients also showed elevated reactivity toward PCs and surrounding structure. Representative images from 2 patients from each group are shown.

(Aire$^{-/-}$) as an experimental model of APECED. We first determined whether expression of cryptdins in the WT mouse thymus recapitulates that of enteric $\alpha$-defensins in human mTECs. We found that members of the cryptdin family that are predominantly expressed in the intestine of C57BL/6 mice (*Defcr3, Defcr5, Defcr20, Defcr21*, and *Defcr24*)[16] are also expressed in the mouse thymus (Figure 3*A*).

To identify the cellular source of intrathymic cryptdin production, mTECs and cortical thymic epithelial cells (cTECs) were FACS sorted according to the scheme presented in Figure 3*B*. Quantitative reverse transcription polymerase chain reaction analysis of messenger RNAs derived from mTECs, cTECs, and MACS-enriched CD45$^{-}$ cells confirmed the expression of cell restricted markers for each of these populations: CD45$^{+}$—Cathepsin S (*Ctss*), $\beta$5i proteasome subunit (*Psmb8*); cTECs—$\beta$5t proteasome subunit (*Psmb11*); mTECs—Cathepsin S, $\beta$5i proteasome subunit, *Aire*, and TRAs, such as insulin (*Ins2*), mucin 6 (*Muc6*), and glutamic acid decarboxylase 67 kDa (*Gad67*). Importantly, the exclusive expression of cryptdin messenger RNAs in mTECs (Figure 3*C*) was on the protein level restricted to Aire-expressing mTEC cells only (Figure 3*D*). Notably, of 100 Aire-positive mTECs, 36% ($\pm$9%, n = 3) co-stained for cryptdins.

The presence of cryptdins in mTECs posed questions concerning the dependency of cryptdin expression on Aire. As presented in Figure 3*E*, the expression of *Defcr3, 5*, and *20* was completely abrogated and negligible levels of *Defcr21* and *24* (<1% of the WT) were detected in Aire$^{-/-}$ animals. *Ins2* and *Gad67*, well-characterized genes with Aire-dependent and independent expression, respectively, were used as controls. These data demonstrated that Aire controls the expression of cryptdins in mTECs.

### *Cryptdin-Specific T Cells in Autoimmune Regulator–Deficient Mice*

The lack of cryptdin expression in Aire$^{-/-}$ mTECs might result in impaired removal of cryptdin-specific T cells during negative selection in the thymus. To test this hypothesis, Aire$^{-/-}$ and WT littermate control mice were immunized with a mixture of cryptdin peptides and immune responses were analyzed 8 days later. As illustrated in Figure 4*A*, a sizeable fraction of CD4$^{+}$ and CD8$^{+}$ T cells derived from peripheral lymph nodes (pLNs) and mesenteric lymph nodes (mLNs) of cryptdin-immunized Aire$^{-/-}$, but not immunized WT mice or nonimmunized Aire$^{-/-}$, proliferated in response to antigen re-stimulation in vitro. In addition, the co-incubation of duodenum-derived CD8$^{+}$, but not CD4$^{+}$, T cells from cryptdin-immunized Aire$^{-/-}$ mice with FACS-sorted PCs increased the rate of cell death of the latter (Supplementary Figure 7). These results point to the presence of self-reactive cryptdin-specific T cells in the LNs of Aire$^{-/-}$ mice and their capacity to target and destroy PCs in vitro.

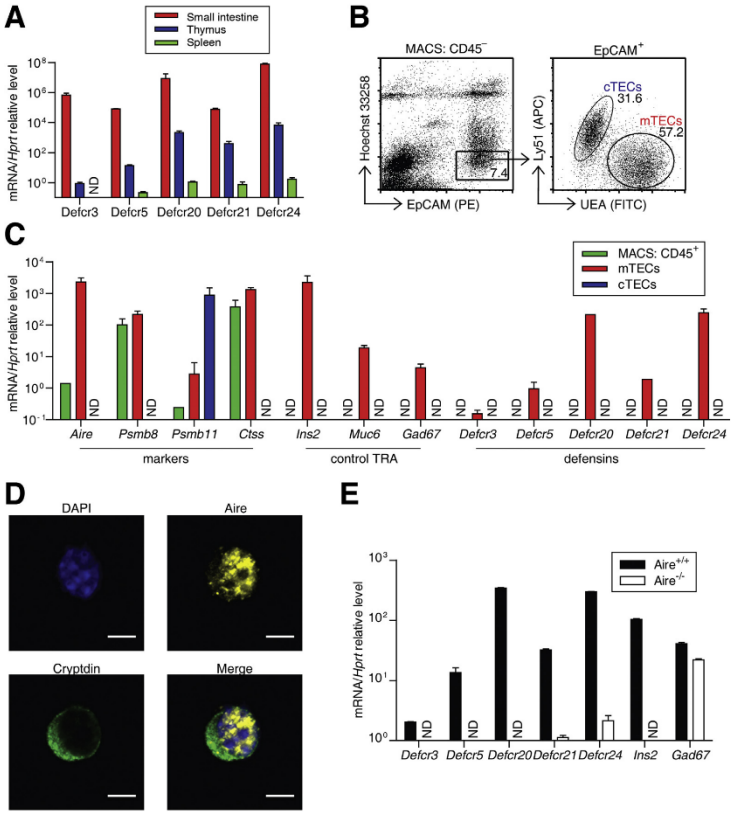


**Figure 3.** Thymic cryptdins are produced exclusively by murine mTECs. (*A*) Comparative quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of cryptdin expression levels in the small intestine, thymus, and spleen. Data represent mean gene expression level (± SD) in organs isolated from 3 animals, each sample was assessed in triplicate. (*B*) Sorting strategy of thymic epithelial cells: CD45$^-$ EpCAM$^+$ thymic fraction (*left panel*) were separated into mTECs (UEA-1$^+$ Ly51$^{+/-}$) and cTECs (UEA-1$^{+/-}$ Ly51$^+$) (*right panel*). (*C*) The expression level of lineage-restricted markers (*Aire*, *Psmb8*, *Psmb11*, and *Ctss*), selected TRA genes (*Ins2*, *Muc6*, *Gad67*), and 5 cryptdins (*Defcr3*, *5*, *20*, *21*, and *24*) in MACS-enriched CD45$^+$ cells, mTECs, and cTECs) assessed by qRT-PCR analysis. Data shows the mean gene expression level (± SD) in denominated cells isolated and pooled from 4 animals and represents 3 independent experiments, each assessed in triplicate. ND, not detected. (*D*) Confocal immunofluorescence of mouse mTECs co-stained with antibodies specific for Aire and newly generated antibody against cryptdins marked as ED5 (Supplementary Figure 6). Nuclei are stained by 4′,6-diamidino-2-phenylindole (*blue*). Scale bar = 5 $\mu$m. One representative image is shown. (*E*) The expression of the 5 most abundant cryptdins in mTECs derived from WT and Aire $^{-/-}$ animals. Data represent the mean gene expression level (± SD) in mTECs isolated and pooled from 4 animals. Data are from 3 independent experiments, each was assessed in triplicate.

### *Cryptdin-Specific T Cells Mediate Intestinal Pathology in Nude Recipient Mice*

Next, we assessed the pathogenic relevance of cryptdin-specific T cells in vivo. T cells isolated from pLNs and mLNs of cryptdin-immunized WT or Aire$^{-/-}$ mice were re-stimulated in vitro with cryptdin-pulsed syngenic BMDCs. Subsequently, these T cells were adoptively transferred into athymic mice (Nude) that were examined 8 weeks later for signs of gut-related autoimmunity. As illustrated in Figure 4*B*, mLNs of Aire$^{-/-}$→Nude, but not Aire$^{+/+}$→Nude, were dramatically enlarged and their cellularity was increased. Concomitantly, we observed a mild increase in the frequency of CD8$^+$ T cells in both pLNs and mLNs, while the frequency of CD4$^+$ T cells remained comparable (Figure 4*C*). In addition, both mLNs and pLNs from Aire$^{-/-}$→Nude contained a significantly increased number of activated, CD69-positive

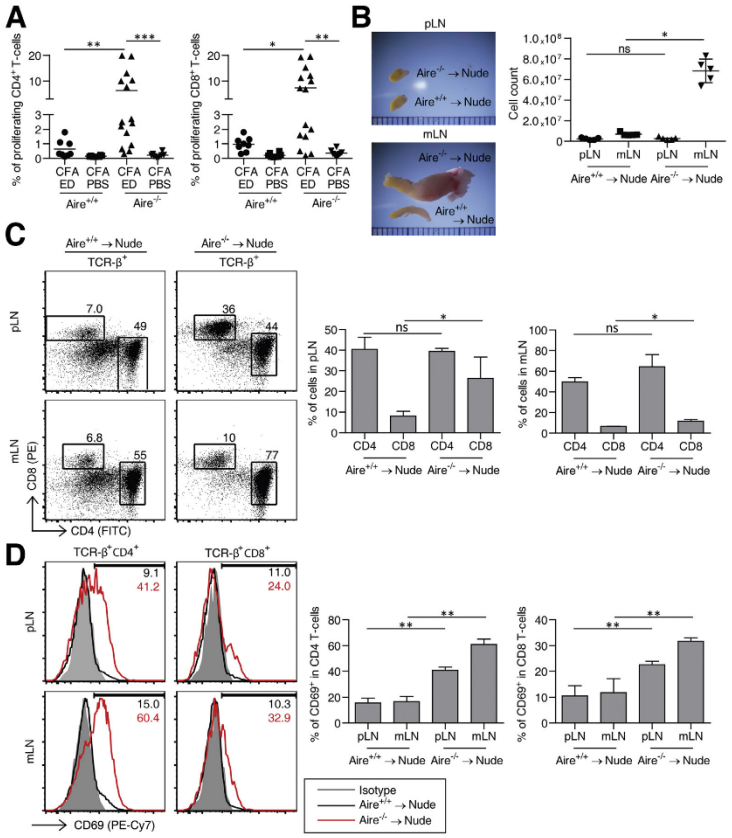


**Figure 4.** Establishment of nude mouse model of T-cell–mediated cryptdin-specific autoimmunity. (*A*) Visualization of cryptdin-specific T cells. T cells from LNs of Aire deficient ($Aire^{-/-}$) or WT ($Aire^{+/+}$) mice immunized with a mixture of Complete Freund's adjuvant with cryptdins (CFA–ED) or CFA with phosphate-buffered saline were labeled with proliferation dye and co-cultured with cryptdin-pulsed bone marrow-derived dendritic cells. The proliferation of T cells was measured by FACS. *Graphs* show the quantification of cryptdin-specific $CD4^+$ and $CD8^+$ T-cell responses. Statistical analysis was performed using 2-tailed Mann-Whitney test, $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$. (*B*) The expansion of cryptdin-specific T cells in lymph nodes of nude mice. T cells from immunized $Aire^{-/-}$ or $Aire^{-/+}$ mice were transferred into nude mice recipients ($Aire^{-/-} \rightarrow$ Nude or $Aire^{+/+} \rightarrow$ Nude, respectively). *Images* illustrate the size of pLNs and mLNs. The *graph* shows the total cell count for pLN and mLN from each group of animals. Data are presented as mean ± SD, n = 5. Statistical analysis was performed using 2-tailed Mann-Whitney test, $^*P < .05$; NS, not significant. (*C*) An increased frequency of $CD8^+$ T cells in both pLNs and mLNs of $Aire^{-/-} \rightarrow$ Nude mice enumerated by FACS. Representative dot-plots are shown. The *bar graphs* show the statistical analysis of this experiment as mean ± SD, n = 5, performed using 2-tailed Student's *t* test, $^*P < .05$, ns, not significant. (*D*) LNs contained the increased number of activated $CD4^+$ and $CD8^+$ T cells. Histogram overlays represent the level of expression of the activation marker CD69 on $CD4^+$ and $CD8^+$ T cells derived from the pLN (*upper panel*) and mLN (*bottom panel*) of $Aire^{-/-} \rightarrow$ Nude (*red line*) and control $Aire^{+/+} \rightarrow$ Nude mice (*black line*), on the background of isotype control staining (*gray line*). Representative histograms are shown. The *bar graphs* show the statistical analysis of this experiment as mean ± SD, n = 5, performed using 2-tailed Student *t* test, $^{**}P < .01$.

$CD4^+$ and $CD8^+$ T cells in comparison with $Aire^{+/+} \rightarrow$ Nude (Figure 4*D*).

Microscopic data and FACS quantification performed on the duodenum of $Aire^{-/-} \rightarrow$ Nude mice showed an approximately 4-fold increase in T cells infiltrating the gut (Figure 5*A* and *B*). Consistent with the presence of self-reactive T cells capable of targeting cryptdin-expressing cells (Supplementary Figure 7), the number of PCs in these mice was markedly reduced (Figure 5*C* and *D*). Immunofluorescent examinations indicated that the destruction of PCs

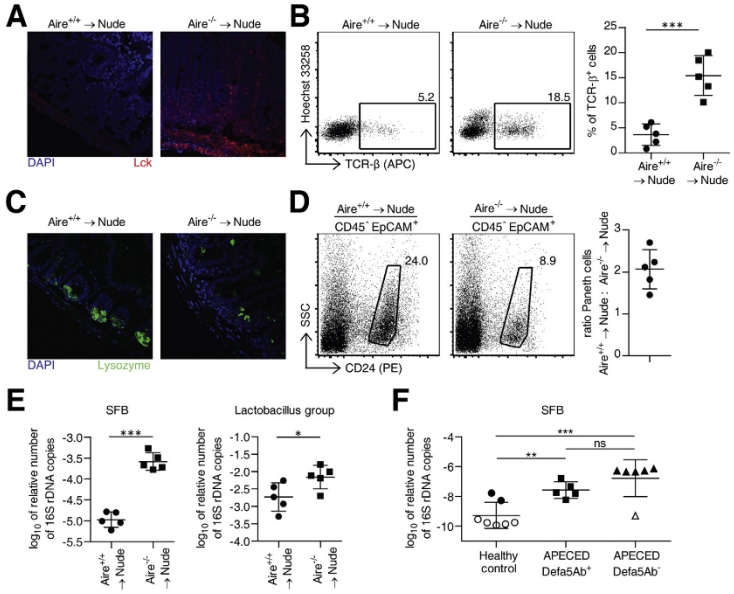


**Figure 5.** Cryptdin-specific T cells mediate intestinal autoimmunity. (*A*) Comparative anti-Lck immunostaining of small intestinal sections from Aire$^{+/-}$→Nude (*left panel*) and Aire$^{-/-}$→Nude mice (*right panel*). Representative images are shown. (*B*) The comparative analysis of frequency of gut-infiltrating T cells. T-cell receptor $\beta^+$ T cells from Aire$^{-/-}$→Nude and control Aire$^{+/-}$→Nude mice (n = 5) were quantified by FACS analysis. Data are shown as mean ± SD, ***$P < .001$, 1-tailed Student *t* test. (*C*) The loss of PCs visualized by lysozyme staining (*green*) on small intestinal sections from Aire$^{+/+}$→Nude (*left panel*) and Aire$^{-/-}$→Nude mice (*right panel*). Representative images are shown. (*D*) FACS analysis revealed the reduced number of CD24$^{+}$ PCs in Aire$^{-/-}$→Nude in comparison to control Aire$^{+/-}$→Nude mice (*2 left dot plot panels*). The *graph* depicts the relative fold-decrease of PCs in Aire$^{-/-}$→Nude mice. Data are presented as mean ± SD. (*E*) Changes in the microbiome composition in the gut of Aire$^{-/-}$→Nude mice in comparison to Aire$^{+/-}$→Nude controls determined by 16S quantitative reverse transcription polymerase chain reaction analysis. The relative enrichment of SFB bacteria and the Lactobacillus group are shown. Data are presented as mean ± SD, *$P < .05$; ***$P < .001$, 2-tailed Student *t* test. (*F*) The enrichment of SFB bacteria was also observed in stool samples from DEFA5-seronegative (Defa5Ab$^{-}$, n = 6) as well as seropositive (Defa5Ab$^{+}$, n = 5) APECED patients in comparison with the healthy control group (n = 7). Data are presented as mean ± SD, **$P < .01$; ***$P < .001$, ns, not significant, 2-tailed Student *t* test. The *open circles* and *triangle* represent samples below the sensitivity threshold of quantitative polymerase chain reaction assay for SFB (see Materials and Methods).

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was cell-specific, as their diminution had no apparent impact on the overall intestinal architecture (Figure 5*A* and *C*). The involvement in this destruction process of cryptdin-specific antibodies could be largely excluded as the serum from both Aire$^{+/+}$→Nude and Aire$^{-/-}$→Nude failed to show any apparent crossreactivity with PCs (Supplementary Figure 8). The very same sera showed no reactivity toward EECs (Supplementary Figure 9*A*), whose distribution, morphology, and numbers in both Aire$^{+/+}$→Nude and Aire$^{-/-}$→Nude mice remained largely comparable (Supplementary Figure 9*B*).

As PCs are the exclusive source of cryptdins, which are involved in the regulation of gut microflora, we examined the composition of microbiota in Aire$^{-/-}$→Nude and Aire$^{+/+}$→Nude mice. Of 7 prevalent bacterial groups tested, segmented filamentous bacteria (SFB) and the lactobacillus group showed significant changes in their abundance (Figure 5*E*). It is important to stress that Aire$^{-/-}$ mice, which served as a source of cryptdin-specific T cells for adoptive transfers (Figure 4*A*), also exhibited significant, albeit less dramatic enrichment in SFB 8 weeks after immunization with cryptdins (Supplementary Figure 10). The shared genetic basis of gut autoimmunity observed in Aire$^{-/-}$ mice and APECED patients led us to examine the human gut microflora as well. A comparative analysis of the intestinal microbiome of APECED patients and healthy controls also showed significant enrichment of SFB in enteric α-defensin–seropositive and seronegative patients, with a more dramatic increase in the latter group (Figure 5*F*).

Because SFB are potent inducers of intestinal Th17 cells,[29] we next examined the polarization of CD4$^{+}$ T cells toward inflammation-inducing Th17 phenotype. The results demonstrated an 8- to 10-fold increase in the frequency of interleukin (IL)17A producing CD4$^{+}$ T cells in pLN and mLN

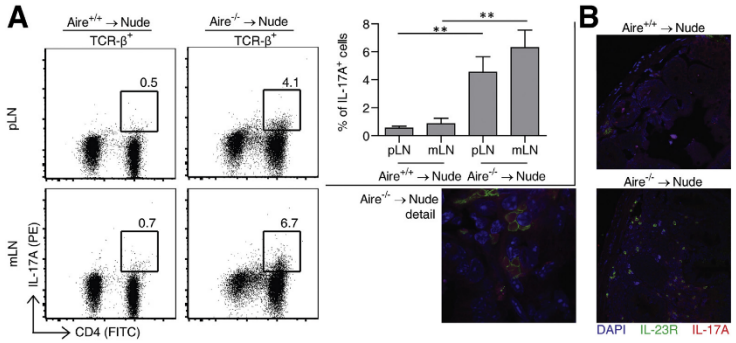


**Figure 6.** Cryptdin-specific T cells mediate inflammation-inducing Th17 phenotype. (*A*) The frequency of IL17A$^+$ CD4$^+$ T cells was determined by FACS analysis of T-cell receptor $\beta^+$ T cells from pLN (*upper panel*) and mLN (*bottom panel*) of Aire$^{-/-}$→Nude (n = 5) and control Aire$^{+/-}$→Nude mice (n = 5). The *bar graph* represents mean ± SD, $^{**}P < .01$, 2-tailed Student *t* test. (*B*) Representative images visualize the presence of pathogenic T cells co-expressing IL23 receptor (*green*) and IL17A (*red*) in the gut of Aire$^{-/-}$→Nude mice.

from Aire$^{-/-}$→Nude mice in comparison with controls (Figure 6*A*). Recently, it was reported that induction of autoimmune pathology by Th17 T cells in mice also requires the elevated co-expression of the IL23 receptor.[30] As illustrated in Figure 6*B*, immunofluorescence analysis of IL17A and IL23 receptor co-stained intestine sections showed a detectable presence of these cells in Aire$^{-/-}$→Nude but not in Aire$^{+/+}$→Nude mice. Collectively, these rodent data support the notion that self-reactive cryptdin-specific T cells are capable of infiltrating the small intestine and initiating PC-related pathologic changes in comparison with those observed in the gut of APECED patients.

## Discussion

Despite the clinical manifestation of intestinal symptoms in a relatively large proportion of APECED patients,[9,31,32] there are only a few reports related to their underlying molecular causes. So far, the absence of EECs[33] accompanied by the detection of antibodies against tryptophan hydroxylase and histidine decarboxylase produced by these cells, has been the only abnormality found in the intestines of some APECED patients.[23,24] This study identifies enteric α-defensins and their cellular source, PCs, as additional autoimmune targets in AIRE-deficient humans and mice.

We provide evidence that cryptdins are expressed by mTECs under the control of Aire. These data are consistent with previous reports that show that the *Defcr5* and *Defcr2-rs* gene expression were down-regulated in mTECs derived from mice bearing the *G228W* dominant-negative variant of Aire[25] and Aire$^{-/-}$,[3] respectively. Our results also demonstrate that several cryptdin transcripts belong to a rare class of genes whose expression in mTECs is fully dependent on Aire.[1,25]

Considering enteric α-defensins as gut-derived self-antigens, the critical step would be their presentation to, and activation of, cognate self-reactive T cells. Due to observed commonalities between Aire$^{-/-}$ mice and patients with APECED in their failure to tolerize enteric α-defensin–specific T cells, in Supplementary Figure 11, we propose a model depicting the possible steps leading to such a scenario. Specifically, bacteria-bound enteric α-defensins are picked up and presented by intestinal M cells and/or dendritic cells. After their activation, enteric α-defensin–specific CD8$^+$ T cells attack PCs and diminish their overall number. Such an outcome was observed in the entire cohort of nude mice transferred with enteric α- defensin–specific T cells and also in 6 of 10 available archival intestinal biopsies from APECED patients.

Another novel finding was the change in the composition of intestinal microbiota, especially the enrichment of SFB. These gram-positive commensal bacteria exhibit a striking ability to directly adhere to epithelial cells and induce Th17 responses.[21] Notably, while the transgenic production of human DEFA5 by mouse PCs[20] led to decreased levels of SFB,[21] diminished production of cryptdins increased both the level of SFB and the intestinal Th17 T-cell subset.[21] Similarly, de novo colonization of several mice models with SFB resulted in the robust development of the Th17 subset[29,34] and intestine autoimmunity.[35,36] These data have demonstrated a causal link among the production of cryptdins, SFB levels, and Th17-driven pro-inflammatory responses in a mouse model. Our data are also consistent with the conclusion of a previous study that the presence of SFB positively affects the abundance of Lactobacillus.[37]

Importantly, although the presence of SFB in infants and in a fraction of adult stool samples has been reported recently, their role in the regulation of human intestinal Th17 immune homeostasis is currently unknown.[37] To the best of our knowledge, this is the first report documenting the enrichment of SFB under intestinal autoimmune conditions in humans. In this context, the SFB status could be used as a clinical marker of intestinal cellular autoimmunity in APECED. However, the caveat of bluntly applying the described link among the cryptdin production, SFB levels,

and Th17 responses to humans, is that, due to the presence of neutralizing anti-IL17F antibodies, the APECED patients suffer from a significant loss of Th17 cells.[38] Although this could explain a striking difference in high and low levels of cellular infiltrates in mouse model and APECED patients, respectively, the number and contribution of intestine-residing Th17 cells to gut autoimmunity at an early stage of disease, has not yet been analyzed.

Whether the presence of DEFA5 autoantibodies in APECED is linked to any pathologic function is currently unknown. However, we could not find any evidence for their involvement in the destruction of PCs in Aire$^{-/-}$→Nude mice (Supplementary Figure 8). Similarly, no direct evidence for the adverse role of autoantibodies in Aire$^{-/-}$ mice has been found so far.[39] The association between the occurrence of DEFA5 antibodies and the incidence of diarrhea found among Finnish/Czech patients can be simply related to the presence of enteric α-defensin–reactive T cells in the periphery (Supplementary Figure 5). It is unclear why Sardinian patients failed to show a similar association. The combination of 2 distinct and prevailing AIRE mutations, R257X and R139X, in Finnish/Czech and Sardinian populations, respectively, with other genetic and environmental factors (such as HLA, diet, and climate) likely accounts for this discrepancy. The precedence for the nearly similarly different manifestation of APECED symptoms between Finnish and Sardinian patients, notably the absence of autoimmune hypothyroidism in the latter, has been recognized previously.[32] A multicenter longitudinal study of a large group of patients is needed to address this issue.

Because of the failure of central tolerance in APECED patients, the periphery contains self-reactive T-cell clones that can simultaneously target numerous intestinal components. This notion is supported by the following lines of evidence: sera from APECED patients lacking EECs cross-reacted with Paneth and Goblet cells[23] and DEFA5-seropositive patients or those lacking PCs display a deteriorated number of EECs (Table 1); DEFA5-seropositive and PC-lacking patients showed high cross-reactivity with many other, yet unidentified intestinal autoantigens (Figure 2*B* and Supplementary Figure 3*A*); intestine-related disease progresses through stages with an increasing number of self-reactivities, culminating in the probably concurrent loss or severe diminishment of PCs and EECs (Supplementary Figure 4, Table 1). Given this gradually developing nature of multi-targeted intestine autoimmunity concomitantly affecting distinct type of cells, it is seemingly difficult to establish the cellular basis for its variable manifestations, such as diarrhea, constipation, malabsorption, and abdominal pain.[11] However, our data provide an initial clue linking at least 2 of the symptoms mentioned to specific autoimmune cellular targets. First, we found a significant association between DEFA5 seropositivity and the manifestation of diarrhea among Finnish/Czech patients. Importantly, all 4 biopsies from DEFA5-seropositive patients revealed decreased or no PCs, one of them with a normal number of EECs. Second, we have recently shown that the tryptophan hydroxylase and decarboxylase seropositivity, 2 enzymes responsible for the production of the main product of EECs, serotonin, as well as the lack of serotonin-expressing EECs, significantly correlated with constipation in APECED patients.[40]

The complexities of gastrointestinal symptoms in APECED patients represents a combinatorial effect of multi-targeted autoimmune attack that includes minimal PCs and EECs, without obvious suspect of the initial autoimmune target. Although our data provide full support for such a scenario, the key point of this report is the destruction of PCs by immunologic mechanism, whereby self-reactive enteric α-defensin–recognizing T cells drive the process of initiation of PC destruction, leading to intestinal microbiome dysregulation and presumably to enhanced Th17 responses, which further amplify inflammatory autoimmunity in the intestine. Although it is impossible to evaluate this scenario in humans, our mouse data suggest that, in experimentally controlled conditions, autoimmunity toward PCs can be uncoupled from autoimmunity, which targets EECs.

Together these findings provide evidence that enteric α-defensins are clinically important self-antigens in gut-related autoimmunity in APECED patients. They also establish a mechanistic link between the disruption of central tolerance to enteric α-defensins and the onset of intestinal autoimmunity. In addition, the novel mouse model of gut-related symptoms provides a future experimental framework for clarifying the cellular and molecular basis of intestinal autoimmune processes in APECED patients. Importantly, these findings also warrant further prospective studies testing the concept that prevention strategies targeting the composition of intestinal microbiota can attenuate inflammation-propelled gastrointestinal dysbiosis and symptoms in APECED patients.



## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.05.009.

## References

1. Mathis D, Benoist C. Aire. Annu Rev Immunol 2009; 27:287–312.
2. Klein L, Kyewski B, Allen PM, et al. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nat Rev Immunol 2014;14:377–391.
3. Anderson MS, Venanzi ES, Klein L, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science 2002;298:1395–1401.
4. Liston A, Lesage S, Wilson J, et al. Aire regulates negative selection of organ-specific T cells. Nat Immunol 2003;4:350–354.
5. Aschenbrenner K, D'Cruz LM, Vollmann EH, et al. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. Nat Immunol 2007;8:351–358.
6. **Nagamine K, Peterson P, Scott HS**, et al. Positional cloning of the APECED gene. Nat Genet 1997; 17:393–398.

7. Consortium F-GA. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet 1997; 17:399–403.
8. Kisand K, Peterson P. Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy: known and novel aspects of the syndrome. Ann N Y Acad Sci 2011; 1246:77–91.
9. Perheentupa J. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Clin Endocrinol Metab 2006;91:2843–2850.
10. Ahonen P, Myllarniemi S, Sipila I, et al. Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. N Engl J Med 1990;322:1829–1836.
11. Kluger N, Jokinen M, Krohn K, et al. Gastrointestinal manifestations in APECED syndrome. J Clin Gastroenterol 2013;47:112–120.
12. Clevers HC, Bevins CL. Paneth cells: maestros of the small intestinal crypts. Annu Rev Physiol 2013; 75:289–311.
13. Bevins CL. The Paneth cell and the innate immune response. Curr Opin Gastroenterol 2004;20:572–580.
14. Selsted ME, Ouellette AJ. Mammalian defensins in the antimicrobial immune response. Nat Immunol 2005; 6:551–557.
15. **Amid C, Rehaume LM**, Brown KL, et al. Manual annotation and analysis of the defensin gene cluster in the C57BL/6J mouse reference genome. BMC Genomics 2009;10:606.
16. Shanahan MT, Tanabe H, Ouellette AJ. Strain-specific polymorphisms in Paneth cell alpha-defensins of C57BL/6 mice and evidence of vestigial myeloid alpha-defensin pseudogenes. Infect Immun 2011;79:459–473.
17. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. J Biol Chem 1992;267:23216–23225.
18. Jones DE, Bevins CL. Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel. FEBS Lett 1993;315:187–192.
19. Wilson CL, Ouellette AJ, Satchell DP, et al. Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 1999;286:113–117.
20. Salzman NH, Ghosh D, Huttner KM, et al. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. Nature 2003; 422:522–526.
21. Salzman NH, Hung K, Haribhai D, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol 2010;11:76–83.
22. Soderbergh A, Myhre AG, Ekwall O, et al. Prevalence and clinical associations of 10 defined autoantibodies in autoimmune polyendocrine syndrome type I. J Clin Endocrinol Metab 2004;89:557–562.
23. Ekwall O, Hedstrand H, Grimelius L, et al. Identification of tryptophan hydroxylase as an intestinal autoantigen. Lancet 1998;352:279–283.
24. Skoldberg F, Portela-Gomes GM, Grimelius L, et al. Histidine decarboxylase, a pyridoxal phosphate-dependent enzyme, is an autoantigen of gastric enterochromaffin-like cells. J Clin Endocrinol Metab 2003;88:1445–1452.
25. Su MA, Giang K, Zumer K, et al. Mechanisms of an autoimmunity syndrome in mice caused by a dominant mutation in Aire. J Clin Invest 2008;118:1712–1726.
26. Lewin K. The Paneth cell in disease. Gut 1969; 10:804–811.
27. Betterle C, Dal Pra C, Mantero F, et al. Autoimmune adrenal insufficiency and autoimmune polyendocrine syndromes: autoantibodies, autoantigens, and their applicability in diagnosis and disease prediction. Endocr Rev 2002;23:327–364.
28. Vogel A, Strassburg CP, Obermayer-Straub P, et al. The genetic background of autoimmune polyendocrino pathy-candidiasis-ectodermal dystrophy and its autoimmune disease components. J Mol Med (Berl) 2002; 80:201–211.
29. **Ivanov II, Atarashi K**, Manel N, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell 2009;139:485–498.
30. **Lee Y, Awasthi A**, Yosef N, et al. Induction and molecular signature of pathogenic TH17 cells. Nat Immunol 2012;13:991–999.
31. Husebye ES, Perheentupa J, Rautemaa R, et al. Clinical manifestations and management of patients with autoimmune polyendocrine syndrome type I. J Intern Med 2009;265:514–529.
32. Meloni A, Willcox N, Meager A, et al. Autoimmune polyendocrine syndrome type 1: an extensive longitudinal study in Sardinian patients. J Clin Endocrinol Metab 2012;97:1114–1124.
33. Posovszky C, Lahr G, von Schnurbein J, et al. Loss of enteroendocrine cells in autoimmune-polyendocrine-candidiasis-ectodermal-dystrophy (APECED) syndrome with gastrointestinal dysfunction. J Clin Endocrinol Metab 2012;97:E292–E300.
34. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity 2009;31:677–689.
35. **Lee YK, Menezes JS**, Umesaki Y, et al. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 2010;108(Suppl 1):4615–4622.
36. Wu HJ, Ivanov II, Darce J, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 2010;32:815–827.
37. Yin Y, Wang Y, Zhu L, et al. Comparative analysis of the distribution of segmented filamentous bacteria in humans, mice and chickens. ISME J 2013; 7:615–621.
38. **Kisand K, Boe Wolff AS**, Podkrajsek KT, et al. Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines. J Exp Med 2010;207:299–308.
39. Gavanescu I, Kessler B, Ploegh H, et al. Loss of Aire-dependent thymic expression of a peripheral tissue antigen renders it a target of autoimmunity. Proc Natl Acad Sci U S A 2007;104:4583–4587.

40. Kluger N, Jokinen M, Lintulahti A, et al. Gastrointestinal immunity against tryptophan hydroxylase-1, aromatic L-amino-acid decarboxylase, AIE-75, villin and Paneth cells in APECED. Clin Immunol 2015;158:212–220.

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**Conflicts of interest**
The authors disclose no conflicts.

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## 3. AIRE-EXPRESSING ILC3-LIKE CELLS IN THE LYMPH NODE DISPLAY POTENT APC FEATURES

While the function and regulation of AIRE in the mechanisms of thymic central tolerance are well understood, its expression, function, and cellular source in the immune periphery are still poorly defined and controversial. The initial experiments based on the fate-mapping and *Aire* mRNA detection revealed its presence in embryonic tissues and secondary lymphoid as well as non-lymphoid organs (Adamson et al., 2004; Halonen et al., 2001; Heino et al., 1999; Nishikawa et al., 2010; Poliani et al., 2010; Schaller et al., 2008). On the other hand, using AIRE specific monoclonal antibody it was suggested that the presence of *Aire* transcripts does not correlate with actual protein expression and that AIRE protein-positive cells could be found only in the thymic tissue (Hubert et al., 2008). The presence of AIRE-expressing cells in the secondary lymphoid organs (lymph nodes and spleen) was confirmed by the generation of *Aire* reporter transgenic mice that led to the identification of eTACs (Gardner et al., 2008). These cells were described as $CD11c^+$ $EpCAM^+$ DC-like population residing in the border of T-cell and B-cell zone in the lymph nodes that can anergize or clonally deleted self-reactive $CD8^+$ or $CD4^+$ peripheral T-cells (Gardner et al., 2008; Gardner et al., 2013). Recently, the presence of $CCR7^+CD127^+$ DC-like population of eTACs was also described in human tonsils (Fergusson et al., 2018). Nevertheless, the origin of eTACs remains still controversial since several publications also described these cells as $CD45^-$ LNSCs (Fletcher et al., 2010; Gardner et al., 2008).

In the presented study, compared to others, we have identified three phenotypically very distinct populations of hematopoietic cells that express transgenic GFP driven by activation of *Aire* promotor (*Aire-GFP* reporter mice) or *Aire* mRNA. However, the presence of AIRE protein was exclusively found only in $CD11c^-EpCAM^-$ population. Using multicolor flow cytometry, RNA sequencing, and microscopy, we identified these cells as ILC3-like population. $AIRE^+$ ILC3-like cells are characterized as RORγ-dependent, recombination activating gene 2 (RAG2) independent population expressing high levels of MHCII and co-stimulatory molecules. Functionally, these cells were shown to be very potent in presenting endogenous antigens to neo-self specific T-cells and their

subsequent deletion. Interestingly the comparison of AIRE$^{+}$ ILC3-like cells transcriptome between *Aire*$^{-/-}$ and *Aire*$^{+/+}$ mice revealed only very mild changes in TRAs expression, suggesting that AIRE controls the distinct transcriptional program in these cells compared to mTECs (Yamano et al., 2019).

Author's contribution: Protein expression profile analysis of AIRE$^{+}$ ILC3-like cells by flow cytometry, enumeration of AIRE$^{+}$ cells in newborn mice, verification of genes from RNA sequencing by quantitative PCR, flow cytometry analysis of expression of co-stimulatory molecules on AIRE$^{+}$ ILC3-like cells.

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BRIEF DEFINITIVE REPORT

# Aire-expressing ILC3-like cells in the lymph node display potent APC features

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**The autoimmune regulator (Aire) serves an essential function for T cell tolerance by promoting the "promiscuous" expression of tissue antigens in thymic epithelial cells. Aire is also detected in rare cells in peripheral lymphoid organs, but the identity of these cells is poorly understood. Here, we report that Aire protein–expressing cells in lymph nodes exhibit typical group 3 innate lymphoid cell (ILC3) characteristics such as lymphoid morphology, absence of "classical" hematopoietic lineage markers, and dependence on RORγt. Aire$^+$ cells are more frequent among lineage-negative RORγt$^+$ cells of peripheral lymph nodes as compared with mucosa-draining lymph nodes, display a unique Aire-dependent transcriptional signature, express high surface levels of MHCII and costimulatory molecules, and efficiently present an endogenously expressed model antigen to CD4$^+$ T cells. These findings define a novel type of ILC3-like cells with potent APC features, suggesting that these cells serve a function in the control of T cell responses.**

## Introduction

The autoimmune regulator (Aire)'s crucial function in the promotion of promiscuous gene expression in medullary thymic epithelial cells (mTECs) is well established. mTECs express thousands of tissue-restricted antigens (TRAs) and present these on MHCI and II (Kyewski and Klein, 2006; Mathis and Benoist, 2007; Peterson et al., 2008; Klein et al., 2014). Aire deficiency strongly diminishes TRA expression in mTECs, offering an explanation how Aire mutations cause the human autoimmune polyendocrine syndrome type 1 (Husebye et al., 2018) and similar autoimmune manifestations in mice (Anderson et al., 2002; Ramsey et al., 2002).

Defective tolerance in Aire$^{-/-}$ mice also includes antigens whose expression in mTECs is Aire independent (Anderson et al., 2005; Kuroda et al., 2005; Hubert et al., 2011). This suggests that Aire coordinates mTEC functions beyond promiscuous gene expression such as mTEC differentiation (Yano et al., 2008; Nishikawa et al., 2010), cytokine and chemokine production (Yano et al., 2008; Laan et al., 2009; Lei et al., 2011; Fujikado et al., 2016), or antigen handling and presentation (Anderson et al., 2005; Hubert et al., 2011). Furthermore, it remains open how two key disease manifestations in autoimmune polyendocrine syndrome type 1 patients, candidiasis and ectodermal dystrophy, can be reconciled with Aire serving its functions exclusively in mTECs.

Fate-mapping revealed Aire expression outside mTECs during embryonic development (Nishikawa et al., 2010). In adult mice, Aire mRNA can be detected in secondary lymphoid organs and also in nonimmune cell types, but there is some controversy as to how well Aire transcripts correlate with actual protein expression (Heino et al., 2000; Halonen et al., 2001; Adamson et al., 2004; Hubert et al., 2008; Schaller et al., 2008; Gardner et al., 2009; Fletcher et al., 2010; Poliani et al., 2010). Aire reporter mice have been instrumental in the identification of a unique cell subset referred to as extrathymic Aire-expressing cells (eTACs), hematopoietic APCs that morphologically resemble dendritic cells (DCs; Gardner et al., 2008, 2013). Aire-expressing DCs have recently also been described in human tonsils (Fergusson et al., 2019).

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Here, we aimed to clarify the identity of Aire-expressing cells in lymph nodes. We identified three phenotypically distinct subsets of hematopoietic cells that expressed endogenous Aire mRNA, including the previously described EpCAM$^+$CD11c$^+$ eTACs. However, Aire protein was exclusively found in an EpCAM$^-$CD11c$^-$ innate lymphoid cell (ILC) 3–like cell type with potent APC features.

## Results and discussion

We confirmed in two independent mouse strains that Aire-reporter expression in LNs was confined to MHCII$^+$ cells (Gardner et al., 2013). Surprisingly, Aire-reporter$^+$ cells not only contained cells with the reported EpCAM$^+$CD11c$^+$ "eTAC" phenotype, but also similar proportions of EpCAM$^-$CD11c$^+$ and EpCAM$^-$CD11c$^-$ cells (Figs. 1 A and S1 A). Endogenous Aire mRNA was highest in EpCAM$^-$CD11c$^-$ cells (Figs. 1 B and S1 B). Aire-protein was detectable by intracellular staining (ICS) in 10–20% of Aire-reporter$^+$EpCAM$^-$CD11c$^-$ cells, but not in the other two subsets (Figs. 1 C and S1 C). Aire was localized in nuclear dots, akin to its subcellular distribution in mTECs (Fig. 1 D).

Independent of reporter systems, Aire$^+$ LN cells were detectable by ICS, and these cells were uniformly CD45$^+$EpCAM$^-$CD11c$^-$ (Fig. 1 E). Aire-ICS$^+$ cells were found at similar frequencies in various peripheral (p)LNs, yet were less frequent in mesenteric LNs (Fig. S1 D). Their abundance was very low in newborns and reached a transient maximum in 2-wk-old mice (Fig. 1 F). Bone marrow (BM) chimeras confirmed their hematopoietic origin (Fig. 1 G).

Aire$^+$ LN cells did not express common lineage-specific surface molecules (Fig. 2 A) and contained negligible mRNA levels of signature genes specific for T or B cells, DCs, macrophages, or granulocytes (Fig. 2 B). However, they expressed genes characteristic for ILCs such as *Id2*, *Kit*, *Il7r*, *Itga4*, and *Itgb7* (Fig. 2 C; Robinette et al., 2015; Seehus et al., 2015). Of the transcription factors (TFs) that regulate the development and function of distinct ILC subsets (Spits et al., 2013), Aire$^+$EpCAM$^-$CD11c$^-$ LN cells expressed the group 3 ILC signature TF RORγt, but not or only weakly T-bet, Eomesodermin, GATA3, and RORα (Fig. 2, C and D).

Aire$^+$EpCAM$^-$CD11c$^-$ LN cells also displayed other ILC hallmarks. First, they had a typical lymphoid morphology, albeit being slightly larger than "bulk" Lin$^-$Rorγt$^+$ ILC3s from LNs, whereas Aire-reporter$^+$EpCAM$^+$CD11c$^+$ "eTACs," as previously described, resembled DCs (Fig. 2 E). Second, they were independent of *Rag* genes (Figs. 2 F and S2 A). Third, and most significantly, *Rorc*$^{-/-}$ precursors failed to give rise to Aire$^+$ LN cells in mixed [*Rorc*$^{-/-}$: *Rorc*$^{+/+}$] → *WT* BM-chimeras, indicating that these cells, like ILC3s (Eberl et al., 2004; Luci et al., 2009; Satoh-Takayama et al., 2009), intrinsically depended on RORγt for their development (Fig. 2 G). Given these commonalities between Aire$^+$ LN cells and ILC3s, we asked whether a "classical" cytometric ILC3 gating strategy including "larger" cells would comprise Aire$^+$ cells. Indeed, around 15% of Lin$^-$CD45$^+$RORγt$^+$ pLN cells were Aire-ICS$^+$ (Figs. 2 H and S2 B).

Group 3 ILCs can be subclassified based on anatomical distribution, ontogeny, and surface phenotype (Spits et al., 2013; Montaldo et al., 2015). CCR6$^+$NKp46$^-$ lymphoid tissue-inducer (LTi) cells arise embryonically and predominantly reside in secondary lymphoid tissues. Postnatally emerging ILC3s can be subdivided into CCR6$^+$NKp46$^-$ LTi-like cells and CCR6$^-$ ILC3s. LTi-like cells are the predominant population in LNs, whereas CCR6$^-$ ILC3s are most abundant in mucosal tissues. To assess how Lin$^-$RORγt$^+$Aire$^+$ cells fit into these categories, we compared their phenotype and ontogeny to Lin$^-$RORγt$^+$Aire$^-$ canonical ILC3s from pLNs. RORγt levels were similar in both cell types (Fig. S2 C). Aire$^+$ cells were also strikingly similar to canonical pLN ILC3s with regard to expression of the TF Id2, surface expression of c-Kit and CCR6, and absence of NKp46 (Fig. 2 J). However, despite sharing the CCR6$^+$NKp46$^-$ phenotype of canonical LN-ILC3s, Aire$^+$ cells were homogeneously negative for IL7Rα, CD90, and CD4, whereas expression of these molecules was heterogeneous on CCR6$^+$NKp46$^-$Aire$^-$ ILC3s (Fig. 2 J). Aire$^+$ LN cells were very rare at birth, peaked at the time of weaning, and slightly declined thereafter, whereas canonical ILC3s were abundant in neonates and decreased with age (Jones et al., 2018; Figs. 1 F and 2 K). Thus, Aire$^+$ LN cells resembled LTi-like ILC3s, yet displayed distinctive features, and from here on will be referred to as Aire$^+$ ILC3-like cells.

Aire, besides its role in TRA expression, has been suggested to control the development and/or survival of mTECs (Gray et al., 2007; Hikosaka et al., 2008; Wang et al., 2012). To address whether a lack of Aire in cells that otherwise express it would perturb their differentiation, we generated mixed [*Aire*$^{-/-}$ Aire-reporter: *Aire*$^{+/+}$ Aire-reporter] → *WT* BM-chimeras. Aire-reporter$^+$ cells that had emerged from either *Aire*$^{-/-}$ or *Aire*$^{+/+}$ precursors were present at equal frequencies. Thus, Aire is unlikely to be a developmental or homeostatic regulator of Aire$^+$ ILC3-like cells (Fig. 3 A).

Aire expression in mTECs is orchestrated by NF-κB signals that emanate from receptors of the TNF superfamily (Akiyama et al., 2012) and converge on an essential enhancer-element (CNS1) in the *Aire* gene (Haljasorg et al., 2015; LaFlam et al., 2015). Aire$^+$ LN cells were absent in *Aire-CNS1*$^{-/-}$ mice, indicating that the gene-proximal requirements for Aire expression in Aire$^+$ ILC3-like cells resemble those in mTECs (Fig. 3 B). MHCII-dependent cognate interactions of mTECs and thymic B cells with developing CD4 T cells provide a crucial platform for Aire-inducing Rank or CD40 signals (Rossi et al., 2007; Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008; Roberts et al., 2012; Yamano et al., 2015). We asked whether a similar scenario applies to Aire$^+$ LN cells. In mixed [*MHCII*$^{-/-}$: *MHCII*$^{+/+}$] → *WT* BM-chimeras, MHCII-deficient precursor cells efficiently gave rise to Aire$^+$ LN cells (Fig. 3 C). Moreover, Aire$^+$ LN cells were similarly abundant in *Rag2*$^{-/-}$ mice and *WT* controls (Fig. 3 D). Thus, in contrast to what has been established for mTECs and thymic B cells, Aire$^+$ LN cells are independent of cross-talk with T cells, indicating that an "innate program" governs their cellular identity.

Given the critical role of Aire's NF-κB response element in Aire$^+$ LN cells, it was likely that extrinsic TNF receptor family signals of "nonadaptive" origin were crucial for their differentiation. Aire$^+$ LN cells emerged with equal efficacy from either *CD40*$^{-/-}$ or *CD40*$^{+/+}$ precursors (Fig. 3 E). By contrast, Rank

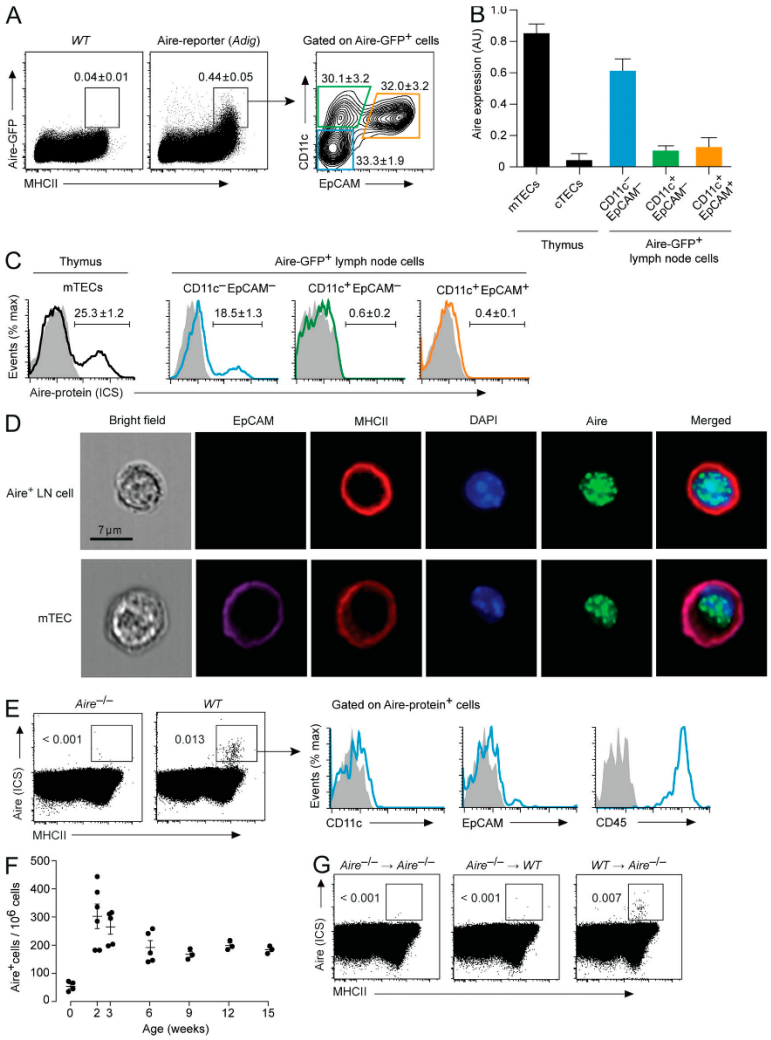


Figure 1. **Phenotype of Aire-expressing cells in LNs. (A)** Expression of GFP and MHCII in LN cells from *Adig* Aire-reporter mice and *WT* controls and staining for CD11c and EpCAM on gated Aire-GFP$^+$MHCII$^+$ cells (representative of $n \geq 4$ each). **(B)** Aire mRNA in medullary and cortical thymic epithelial cells (mTECs and cTECs, respectively) and in Aire-GFP$^+$ LN cells sorted according to expression of CD11c and EpCAM. Data are mean values ± SEM of triplicates. AU, arbitrary units. **(C)** ICS for Aire protein in subsets of Aire-GFP$^+$ LN cells. The average frequency ± SEM of Aire-ICS$^+$ cells is indicated ($n = 4$). **(D)** Nuclear localization of Aire protein and surface marker expression in Aire-expressing LN cells or mTECs visualized by imaging flow cytometry. **(E)** ICS for Aire protein and surface expression of MHCII in total LN cells from *WT* and *Aire*$^{-/-}$ mice. Histograms on the right show CD11c, EpCAM, and CD45 on gated Aire$^+$MHCII$^+$ cells (representative of $n \geq 5$). **(F)** Number of Aire$^+$ LN cells per $10^6$ total pLN cells in mice of the indicated age ($n \geq 3$ each; 0 wk = 4 d old). **(G)** Aire and MHCII expression in total LN cells from *Aire*$^{-/-}$ → *Aire*$^{-/-}$, *Aire*$^{-/-}$ → *WT* or *WT* → *Aire*$^{-/-}$ BM chimeras (representative of $n \geq 4$ each).

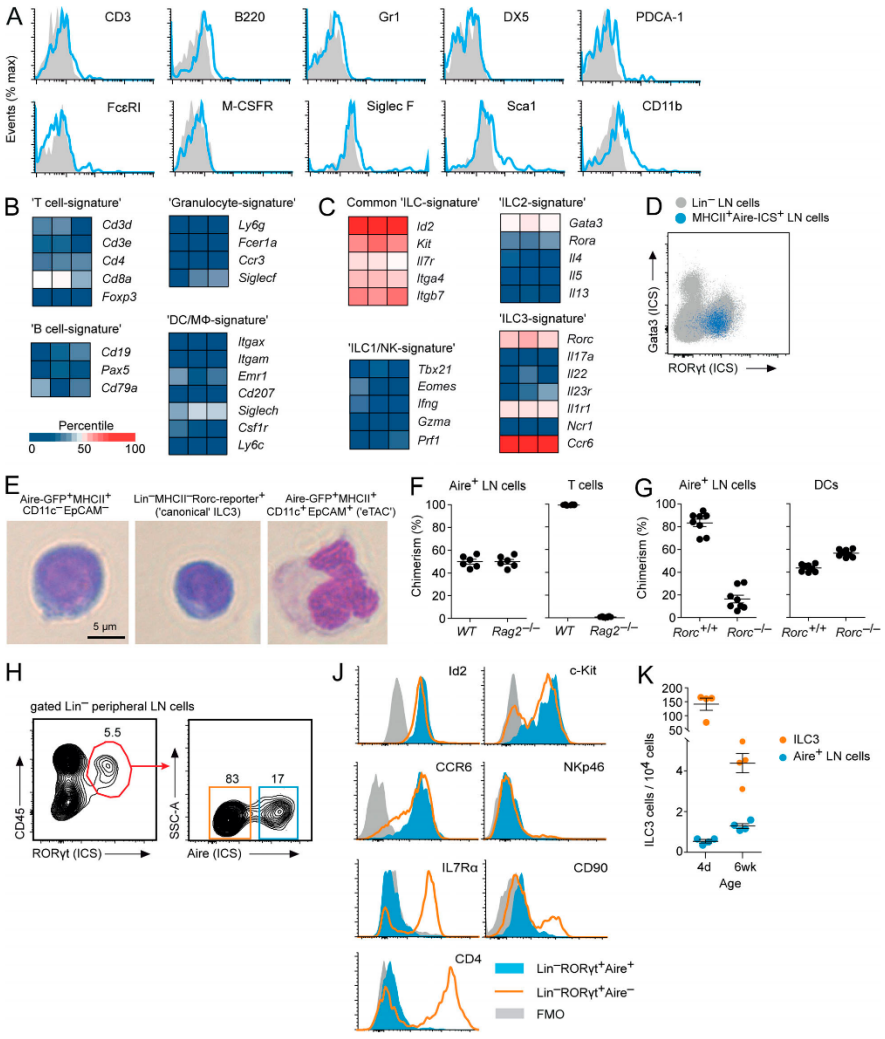


Figure 2. **Aire⁺ LN cells display ILC3 characteristics. (A)** Expression of hematopoietic lineage markers on Aire-GFP⁺MHCII⁺CD11c⁻EpCAM⁻ LN cells from *Adig* mice (representative of $n \geq 4$). **(B)** RNA expression of hematopoietic lineage-specific signature genes in Aire⁺ LN cells. **(C)** RNA expression of ILC signature genes in enriched Aire⁺ LN cells. **(D)** ICS for RORγt and Gata3 in MHCII⁺Aire⁺ LN cells (blue dots) back-gated on total Lin⁻ (CD3, CD19, B220, Gr-1, CD11c, and CD11b) LN cells (gray dots; representative of $n = 3$). **(E)** Morphology of representative Aire-GFP⁺MHCII⁺CD11c⁻EpCAM⁻ LN cells, "canonical" LN ILC3s (Lin⁻MHCII⁻RORγt⁺IL7Rα⁺), and eTACs (Aire-GFP⁺MHCII⁺EpCAM⁺ CD11c⁺). **(F)** Contribution of *WT* and *Rag2*$^{-/-}$ precursor cells to Aire-ICS⁺ LN cells or T cells in 1:1 mixed [*Rag2*$^{-/-}$ : *WT*] → *WT* BM-chimeras ($n = 6$). **(G)** Contribution of *Rorc*$^{+/+}$ and *Rorc*$^{-/-}$ precursor cells to Aire-ICS⁺ cells in LN or spleen or to DCs in 1:1 mixed [*Rorc*$^{+/+}$: *Rorc*$^{-/-}$] → *WT* BM-chimeras ($n = 7$). **(H)** Gating strategy for Lin⁻ (Lin1: CD3, CD19, B220, Gr-1; Lin 2: CD11c and CD11b) RORγt⁺ LN ILC3s and expression of Aire by ICS. **(J)** Intracellular staining for Id2 protein and surface expression of c-kit, CCR6, NKp46, IL7Rα, CD90, and CD4 in Aire⁺ (filled blue histogram) and Aire⁻ (open orange histogram) Lin⁻RORγt⁺ cells (representative of $n = 3$). FMO, fluorescence minus one. **(K)** Number of "canonical" LN ILC3s (orange) and Aire⁺ LN cells (blue) in 4-d- and 6-wk-old animals ($n = 4$). Data are mean ± SEM.

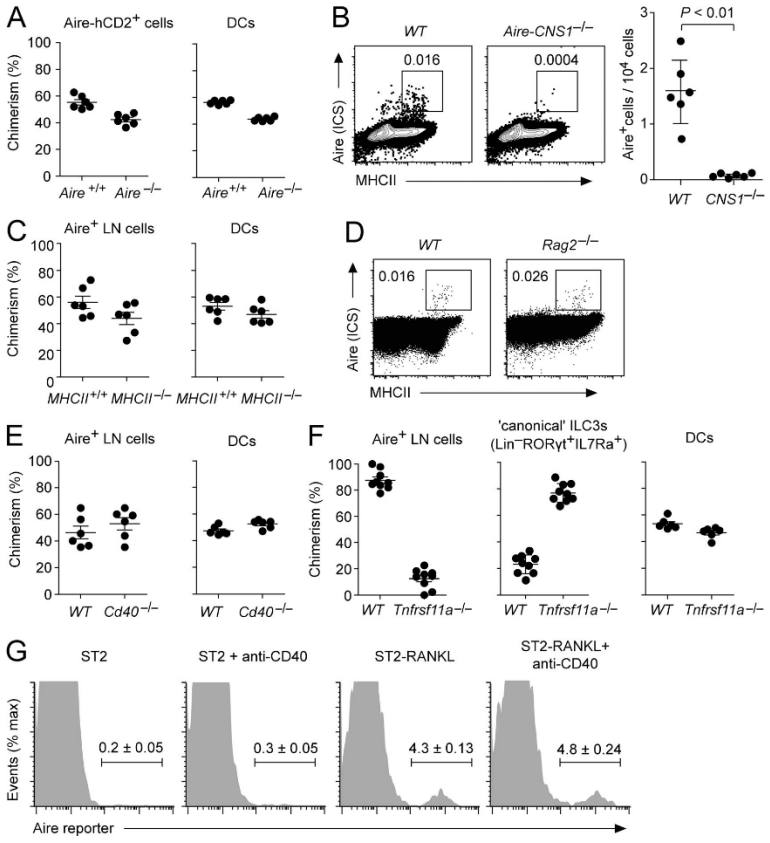


Figure 3. **Molecular and cellular requirements for Aire expression in Aire⁺ ILC3-like cells. (A)** Contribution of *Aire*$^{+/+}$ and *Aire*$^{-/-}$ precursor cells to Aire-reporter⁺ LN cells or DCs in 1:1 mixed [*Aire*$^{+/+}$ *Aire-HCO*: *Aire*$^{-/-}$ *Aire*$^{+/+}$ *Aire-HCO*] → *WT* BM-chimeras ($n$ = 6). **(B)** Aire (ICS) and MHCII in LN cells from *WT* controls and *Aire-CNS1*$^{-/-}$ mice and number of Aire-ICS⁺MHCII⁺ cells per $10^4$ LN cells ($n$ = 6 each). **(C)** Contribution of *MHCII*$^{+/+}$ and *MHCII*$^{-/-}$ precursor cells to Aire⁺ LN cells or DCs in 1:1 mixed [*MHCII*$^{+/+}$: *MHCII*$^{-/-}$] → *WT* BM-chimeras ($n$ = 6). **(D)** Aire (ICS) and MHCII in LN cells from *Rag2*$^{-/-}$ mice and *WT* controls ($n \geq 3$ each). **(E)** Contribution of *Cd40*$^{+/+}$ and *Cd40*$^{-/-}$ precursor cells to Aire⁺ LN cells or DCs in 1:1 mixed [*Cd40*$^{+/+}$: *Cd40*$^{-/-}$] → *WT* BM-chimeras ($n$ = 6). **(F)** Contribution of *Tnfrsf11a*$^{+/+}$ and *Tnfrsf11a*$^{-/-}$ precursor cells to Aire⁺ LN cells, "canonical" ILC3s (Lin⁻RORγt⁺IL7Rα⁺), or DCs in 1:1 mixed [*Tnfrsf11a*$^{+/+}$: *Tnfrsf11a*$^{-/-}$] → *WT* fetal liver chimeras ($n$ = 9). **(G)** Aire-reporter expression in "canonical" IL7Rα⁺ LN ILC3s after in vitro culture with or without agonistic anti–CD40 antibody or with ST2-RankL cells (representative of $n$ = 3 each). Data are mean ± SEM. Student's $t$ test was used to calculate P values.

deficiency resulted in a severe cell-intrinsic defect of hematopoietic precursors to contribute to Aire⁺ LN cells in mixed chimeras (Fig. 3 F). Intriguingly, concomitant to being virtually absent from Aire⁺ ILC3-like cells, Rank-deficient cells were strongly over-represented among canonical IL7Rα⁺ ILC3s. A possible explanation for this was that LN ILC3s under steady-state conditions differentiate into Aire⁺ ILC3-like cells upon Rank stimulation, yet accumulate when their capacity to signal via Rank is genetically ablated. In support of such a precursor/progeny relationship, in vitro culture of canonical LN ILC3s with Rank-ligand–expressing stromal cells induced Aire expression in a fraction of cells (Fig. 3 G).

RNA sequencing (RNA-seq) of Aire-reporter⁺ LN cells from WT and Aire-deficient mice revealed 707 differentially expressed transcripts (Fig. 4 A). Of these, 334 were Aire-induced, and 373 were repressed (Tables S1 and S2). Quantitative PCR analysis of selected Aire-induced or Aire-repressed genes confirmed their Aire-regulated expression (Fig. 4 B). Among Aire-induced transcripts, only 60 were classified as TRAs (Sansom et al., 2014; Fig. 4 A). Thus, in contrast to what is known for

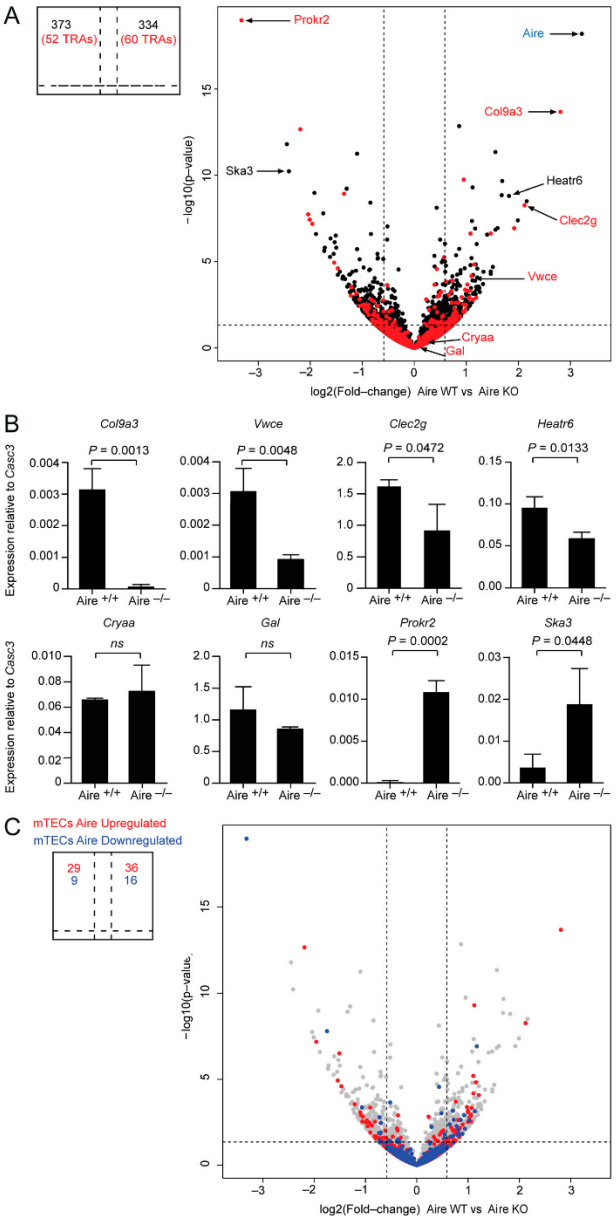


Figure 4. **Aire orchestrates a distinct genetic program in Aire⁺ ILC3-like cells. (A)** RNA-seq data from Lin⁻MHCIIʰⁱCD80⁺ cells from *Aire*$^{+/+}$ and *Aire*$^{-/-}$ mice. Genes encoding for TRAs are colored in red. Fold-change cutoff: 1.5; P value: 0.05 (indicated by a dashed line). **(B)** Quantitative PCR analysis of mRNA expression of selected differentially expressed genes. Data are mean ± SD of three independent experiments. ns, not significant. **(C)** Comparison of Aire-dependent gene expression in Aire⁺ LN cells and mTECs. Genes that are up-regulated (depicted in red; 1,732 genes) or down-regulated (depicted in blue; 423 genes) by Aire in mTECs by at least 1.5-fold were projected onto the volcano plot shown in A. Student's *t* test was used to calculate P values.

mTECs (Anderson et al., 2002; Kyewski and Klein, 2006), the Aire-induced transcriptome of Aire$^+$ LN cells is not biased toward TRAs. Interestingly, Aire-regulated genes in Aire$^+$ LN cells and mTECs were largely nonoverlapping, that is, only 90 of 707 Aire-regulated transcripts in Aire$^+$ LN cells were also affected by Aire deficiency in mTECs. Moreover, many of these genes displayed opposing effects in mTECs or Aire$^+$ LN cells (Fig. 4 C). Thus, Aire controls a distinct transcriptional program in Aire$^+$ ILC3-like cells.

Aire$^+$ LN cells had high surface levels of MHCII and strongly expressed mRNAs encoding for costimulatory or coinhibitory molecules (Fig. 5 A). To relate this to canonical LN ILC3s, we directly compared the surface phenotype of Aire$^+$IL7Rα$^-$ or Aire$^-$IL7Rα$^+$ Lin$^-$CD45$^+$RORγt$^+$ LN cells (Figs. 5 B and S3 A). Aire$^+$ ILC3-like pLN cells had homogeneously high surface levels of MHCII, CD80, CD86, CD40, and Icos-L and detectable amounts of PD-L1. By contrast, with the exception of CD86, these molecules were substantially lower or absent on Aire$^-$IL7Rα$^+$ ILC3s (Fig. 5 C). We confirmed and extended these data through gating on MHCII$^{hi}$IL7Rα$^-$ or MHCII$^{lo/int}$IL7Rα$^+$ cells among Lin$^-$CD45$^+$RORγt$^+$ cells, which clearly separated Aire$^+$ ILC3-like cells and Aire$^-$ canonical ILC3s (Fig. S3, A and B). Consistent with our previous observations, MHCII$^{hi}$IL7Rα$^-$ Aire$^+$ ILC3-like cells were far more abundant in pLNs as compared with mesenteric LNs (Fig. S3, A–C), were slightly lower for CD45 (Fig. S3 D), and had much higher surface levels of APC-associated molecules as compared with MHCII$^{lo/int}$IL7Rα$^+$ cells (Fig. S3 E). Moreover, MHCII$^{hi}$IL7Rα$^-$ Aire$^+$ ILC3-like cells were negative for CD4, CD90, and CD25, whereas canonical ILC3s were heterogeneous for CD4 and CD90 and homogeneously positive for CD25 (Fig. S3 F).

Given the potent APC features of Aire$^+$ ILC3-like cells, we asked whether a model antigen expressed by these cells was visible to CD4 T cells. Influenza hemagglutinin (HA)–specific CD4 T cells and polyclonal "reference" T cells were adoptively transferred into *Aire-HCO* mice, which express HA from a bacterial artificial chromosome–transgenic *Aire* gene (Hinterberger et al., 2010). 14 d after transfer, HA-specific CD4 T cells were strongly diminished in *Aire-HCO* recipients but not in *WT* controls (Fig. 5 D), indicating that peripheral antigen encounter resulted in the deletion of these cells. Similar results were obtained when *Aire-HCO* → *WT* BM-chimeras were used as recipients, indicating that deletion of TCR-HA cells resulted from HA expression in the hematopoietic compartment (Fig. 5 E). To directly assess presentation of endogenously expressed HA, Aire$^+$ ILC3-like cells from *Aire-HCO* mice were cultured with HA-specific CD4 T cell hybridoma cells carrying a GFP IL-2 reporter (Aschenbrenner et al., 2007). For comparison, we performed these assays also with mTECs and EpCAM$^+$CD11c$^+$ eTACs. Aire$^+$ ILC3-like cells presented HA with similar efficacy as mTECs. By contrast, no direct presentation of HA was measurable with eTACs, despite similar expression of the Aire-reporter (Figs. 5 F and S1 A). When loaded with exogenous HA-peptide, all three cell types elicited similar responses, excluding that differential expression of MHCII accounted for these differences (Fig. 5 F).

In sum, our data suggest that Aire protein–expressing cells in mouse pLNs may represent a hitherto unknown ILC3 subset. In distinction from the majority of Lin$^-$RORγt$^+$ LN ILC3s, they do not express the IL7Rα, which, besides the absence of Aire$^+$ ILC3s from the intestine and their low frequency in mesenteric LNs, may explain why Aire expression has not been noticed in transcriptomic analyses of ILC subsets (Robinette et al., 2015; Gury-BenAri et al., 2016). It will be interesting to clarify the potential precursor progeny relationship between canonical ILC3s and Aire$^+$ LN cells and how the Rank pathway is involved. It was recently shown that Rank signaling negatively regulates the abundance and effector functions of intestinal ILC3s (Bando et al., 2018). Together with our findings, this suggests that both in pLNs as well as in the intestine, ILC3s receive tonic Rank stimulation, yet may differ with regards to the ensuing biological response.

Aire$^+$ ILC3-like cells display potent APC features, consistent with accruing evidence that ILC3s can orchestrate CD4 T cell responses. Selective ablation of MHCII molecules on RORγt$^+$ cells resulted in impaired tolerance to commensals and intestinal inflammation (Hepworth et al., 2013, 2015). Other reports linked MHCII deficiency in RORγt$^+$ cells to impaired T and B cell responses to "foreign" antigens (von Burg et al., 2014). How this relates to Aire$^+$ ILC3-like cells and under which circumstances antigen recognition on these cells may result in tolerance versus immunity remains to be seen. Along these lines, it will be interesting to see whether Aire expression in Aire$^+$ ILC3-like cells is of true physiological significance, as these cells do not recapitulate bona fide "promiscuous" TRA expression as it is seen in mTECs. It remains possible that Aire influences features of Aire$^+$ LN cells that are unrelated to TRA expression.

## Materials and methods

### Mice

Adig (Gardner et al., 2008), Aire-HCO (Hinterberger et al., 2010), Aire$^{-/-}$ (Ramsey et al., 2002), Aire-CNS1$^{-/-}$ (Haljasorg et al., 2015), TCR-HA (Kirberg et al., 1994), Cd40$^{-/-}$ (Kawabe et al., 1994), Rag2$^{-/-}$ (Hao and Rajewsky, 2001), Rorc$^{-/-}$ (Eberl et al., 2004) Rorc-EGFP (Lochner et al., 2008), and Tnfrsf11a$^{-/-}$ (Li et al., 2000) mice have been described previously. Aire$^{-/-}$ and Adig mice were backcrossed onto a BALB/c background for ≥10 generations. Animal studies were approved by local authorities (Regierung von Oberbayern Az. 02-17-193 and Ethical Committee of the Czech Academy of Sciences).

### Preparation of LN single-cell suspension

Unless stated otherwise, experiments were performed with pooled pLNs (axillary, brachial, inguinal, and cervical). LNs were pierced with a needle and enzymatically digested with 0.1 mg/ml Dispase (Gibco) in RPMI. After incubation at 37°C for 10 min, the supernatant was replenished, and cell suspensions were homogenized by gently pipetting up and down. To stop the digestion, the supernatant was adjusted to 3% FCS and 2 mM EDTA and gently spun down (4°C, 300 *g*, 5 min). The procedure was repeated until all clumping was removed. In the experiment shown in Fig. S3, LNs were digested in the presence of 0.5 U/ml Liberase (Roche). The cell suspensions were then resuspended in PBS containing 3% FCS and 2 mM EDTA and used for further analysis.

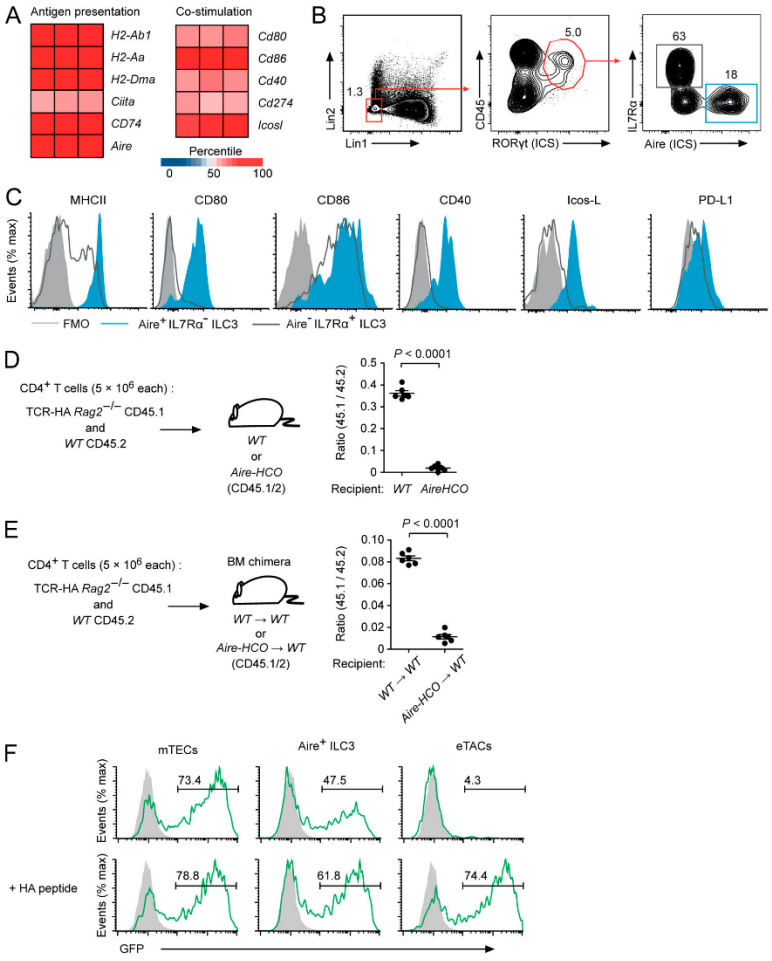


Figure 5. **Aire⁺ ILC3-like cells display potent APC features and directly present endogenous antigen on MHCII. (A)** RNA expression of genes involved in antigen presentation in Aire⁺ ILC3s. **(B)** Frequency of Aire⁺IL7Rα⁻ and Aire⁻IL7Rα⁺ cells among Lin⁻RORγt-ICS⁺CD45⁺ cells in pLNs (Lin1: CD3, CD19, B220, Gr-1; Lin 2: CD11c and CD11b). **(C)** Surface expression of MHCII and costimulatory molecules on Aire⁺IL7Rα⁻ (filled blue) or Aire⁻IL7Rα⁺ (open black) Lin⁻RORγt-ICS⁺CD45⁺ pLN cells (representative of $n \geq 3$). **(D)** Peripheral deletion of HA-specific CD4 T cells upon adoptive transfer into *Aire-HCO* mice ($n = 6$ each). **(E)** Peripheral deletion of HA-specific CD4 T cells upon adoptive transfer into [*Aire-HCO* → *WT*] BM chimeric mice ($n = 6$ each). **(F)** GFP expression in HA-specific CD4⁺ NFAT-GFP-reporter hybridoma cells after 16 h co-culture with mTECs, Aire⁺ ILC3-like cells (Aire-hCD2⁺MHCII⁺CD11c⁻EpCAM⁻), or eTACS (Aire-hCD2⁺MHCII⁺CD11c⁺EpCAM⁺) from Aire–HCO mice without (upper row) or with (lower row) exogenous HA-peptide. Filled gray histograms are from A5 cells that were cultured alone (representative of three experimental replicates). Data are mean ± SEM. Student's *t* test was used to calculate P values.

### Flow cytometry

Single-cell suspensions of digested LNs were surface stained according to standard procedures. For intracellular Aire or RORγt staining, cells were fixed and permeabilized using reagents from the Foxp3 staining kit (eBioscience) and stained with anti-Aire mAb 5H12 (eBioscience) conjugated to Alexa Fluor 660, Alexa Fluor 488, or FITC- or PE-conjugated RORγt mAb B2D (eBioscience). Cells were analyzed or sorted using a BD FACSCANTOII or LSRII flow cytometer or BD FACSAriaFusion or BD Influx cell sorters.

### Imaging flow cytometry

LN cells were prepared by enzymatic digestion. Thymic stroma cells were prepared by enzymatic digestion and density fractionation. Cells were stained for surface markers, fixed and permeabilized, and stained with Alexa Fluor 488–conjugated anti-Aire mAb 5H12 (eBioscience). DAPI was added immediately before analysis. Images were acquired using the ImageStream imaging flow cytometer (Amnis), and data were analyzed with the Ideas 6.0 software.

### In vitro stimulation of ILC3s

LN cells from Aire-HCO mice were prepared by enzymatic digestion. B cells and T cells were depleted with biotin-conjugated anti-B220, anti-TCRβ, and anti-biotin MicroBeads (Miltenyi Biotech). Lineage-negative (CD3, CD11c, and CD19), hCD2-negative, IL7Rα$^+$MHCII$^+$-positive cells were sorted. Sorted cells ($4 \times 10^4$) were cultured for 3 d together with $2 \times 10^4$ irradiated (3,000 rad) ST2 or ST2-RankL (Nutt et al., 1999) mouse BM stroma cells in flat-bottom 96-well plates. Where indicated, agonistic anti-CD40 mAb (10 µg/ml; FGK45; Bio X Cell) was added.

### RNA-seq

Total mRNA was extracted from Lin$^-$CD45$^+$MHCII$^{high}$CD80$^+$ cells using the RNeasy Plus Microkit (Qiagen). The quality of the isolated RNA was controlled by Bioanalyzer 2100 electrophoresis (Agilent). RNA-seq libraries were prepared by the European Molecular Biology Laboratory Genomics core facility in Heidelberg, Germany using a NEXTflex rapid illumina RNA-Seq library prep kit (Bioo Scientific) after polyA enrichment with NEXTflex Poly(A) Beads (Bioo Scientific), starting with ∼1–5 pg of total RNA. Sequencing was performed on an Illumina NextSeq 500 sequencer (Illumina). Low quality reads and adaptor sequences were trimmed out using cutadapt. Reads mapping to ribosomal RNA were filtered out using SortMeRNA. Preprocessed mRNA reads were mapped to the mouse genome (BALB/cJ) from Ensembl version 86 (Mus_musculus_balbcj.BALB_cJ_v1.dna_sm.toplevel.fa) using GSNAP version 2017–02-25. Gene annotations were downloaded from Ensembl (Mus_musculus_balbcj.BALB_cJ_v1.86.gff3). DESeq2 version 1.14.1 was used for feature counting, data normalization, and comparison of the different groups. Differentially expressed genes were selected based on Storey's q-value < 0.05 and at least 1.5-fold change in transcription activity. For the construction of heat maps, fragments per kilobase of transcript per million mapped reads–normalized counts were used. The raw sequencing data were deposited at the ArrayExpress database under accession no. E-MTAB-7088.

### Quantitative PCR

Total mRNA was extracted from cells using the RNeasy Plus Microkit (Qiagen). Reverse transcription was performed using random primers (Thermo Fisher Scientific) and RevertAid reverse transcription (Thermo Fisher Scientific). Gene expression was determined by quantitative PCR reaction using Sybr green and LC480-II cycler (Roche) and quantified using the relative quantification method (Pfaffl, 2001).

### Antigen-presentation assay

$10^4$ HA-TCR hybridoma cells (A5 cells) were co-cultured with $2 \times 10^3$ APCs in 200 µl IMDM supplemented with 1% FCS. After 17 h, cells were harvested, and GFP expression of A5 cells was measured by flow cytometry.

### BM and fetal liver chimeras

BM was depleted of B and T cells using biotinylated mAbs to B220 and TCRβ and streptavidin magnetic activated cell sorting beads (Miltenyi Biotech). Recipient mice were irradiated with $2 \times 450$ rad and reconstituted with $1 \times 10^7$ BM cells. For the generation of mixed chimeras, congenically marked BM or fetal liver cells from Rag2$^{-/-}$ (CD45.1$^{+/+}$), MHCII$^{-/-}$, RORc$^{-/-}$, CD40$^{-/-}$, or Tnfrsf11a$^{-/-}$ mice (CD45.2$^{+/+}$), and BM or fetal liver cells from WT mice of matching congenic genotype, were mixed at a ratio of 1:1 ($5 \times 10^6$ each) and intravenously injected into lethally irradiated recipient mice (CD45.1$^{+/-}$CD45.2$^{+/-}$).

### Adoptive T cell transfer

Single cell suspensions of LN cells from HA-TCR-RAG2$^{-/-}$ (CD45.1) and WT mice (CD45.2) were prepared. LN T cells from WT mice were enriched with biotin-conjugated anti-CD4 and streptavidin MicroBeads (Miltenyi Biotech) according to standard procedures. LN T cells were mixed ($5 \times 10^6$) at a ratio of 1:1 and i.v. injected into recipient mice (CD45.1$^{+/-}$ CD45.2$^{+/-}$). After 14 d, the LNs were collected and single cell suspensions were analyzed by flow cytometry.

### Statistical analysis

Unless indicated otherwise, statistical significance was assessed using the two-tailed Student's *t* test.

### Online supplemental material

Fig. S1 shows the phenotype of Aire-expressing LN cells. Fig. S2 shows gating strategies. Fig. S3 shows that MHCII$^{high}$IL7Rα$^-$ ILC3-like cells express Aire$^-$ and costimulatory molecules. Table S1 shows Aire-induced genes in Aire$^+$ ILC3-like cells. Table S2 shows Aire-repressed genes in Aire$^+$ ILC3-like cells.

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## References

Adamson, K.A., S.H. Pearce, J.R. Lamb, J.R. Seckl, and S.E. Howie. 2004. A comparative study of mRNA and protein expression of the autoimmune regulator gene (Aire) in embryonic and adult murine tissues. *J. Pathol.* 202:180–187. https://doi.org/10.1002/path.1493

Akiyama, T., Y. Shimo, H. Yanai, J. Qin, D. Ohshima, Y. Maruyama, Y. Asaumi, J. Kitazawa, H. Takayanagi, J.M. Penninger, et al. 2008. The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity.* 29:423–437. https://doi.org/10.1016/j.immuni.2008.06.015

Akiyama, T., M. Shinzawa, and N. Akiyama. 2012. TNF receptor family signaling in the development and functions of medullary thymic epithelial cells. *Front. Immunol.* 3:278. https://doi.org/10.3389/fimmu.2012.00278

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

Anderson, M.S., E.S. Venanzi, Z. Chen, S.P. Berzins, C. Benoist, and D. Mathis. 2005. The cellular mechanism of Aire control of T cell tolerance. *Immunity.* 23:227–239. https://doi.org/10.1016/j.immuni.2005.07.005

Aschenbrenner, K., L.M. D'Cruz, E.H. Vollmann, M. Hinterberger, J. Emmerich, L.K. Swee, A. Rolink, and L. Klein. 2007. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat. Immunol.* 8:351–358. https://doi.org/10.1038/ni1444

Bando, J.K., S. Gilfillan, C. Song, K.G. McDonald, S.C. Huang, R.D. Newberry, Y. Kobayashi, D.S.J. Allan, J.R. Carlyle, M. Cella, and M. Colonna. 2018. The Tumor Necrosis Factor Superfamily Member RANKL Suppresses Effector Cytokine Production in Group 3 Innate Lymphoid Cells. *Immunity.* 48:1208–1219.e4. https://doi.org/10.1016/j.immuni.2018.04.012

Eberl, G., S. Marmon, M.J. Sunshine, P.D. Rennert, Y. Choi, and D.R. Littman. 2004. An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat. Immunol.* 5:64–73. https://doi.org/10.1038/ni1022

Fergusson, J.R., M.D. Morgan, M. Bruchard, L. Huitema, B.A. Heesters, V. van Unen, J.P. van Hamburg, N.N. van der Wel, D. Picavet, F. Koning, et al. 2019. Maturing Human CD127+ CCR7+ PDL1+ Dendritic Cells Express AIRE in the Absence of Tissue Restricted Antigens. *Front. Immunol.* 9:2902. https://doi.org/10.3389/fimmu.2018.02902

Fletcher, A.L., V. Lukacs-Kornek, E.D. Reynoso, S.E. Pinner, A. Bellemare-Pelletier, M.S. Curry, A.R. Collier, R.L. Boyd, and S.J. Turley. 2010. Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *J. Exp. Med.* 207:689–697. https://doi.org/10.1084/jem.20092642

Fujikado, N., A.O. Mann, K. Bansal, K.R. Romito, E.M.N. Ferre, S.D. Rosenzweig, M.S. Lionakis, C. Benoist, and D. Mathis. 2016. Aire Inhibits the Generation of a Perinatal Population of Interleukin-17A-Producing γδ T Cells to Promote Immunologic Tolerance. *Immunity.* 45:999–1012. https://doi.org/10.1016/j.immuni.2016.10.023

Gardner, J.M., J.J. Devoss, R.S. Friedman, D.J. Wong, Y.X. Tan, X. Zhou, K.P. Johannes, M.A. Su, H.Y. Chang, M.F. Krummel, and M.S. Anderson. 2008. Deletional tolerance mediated by extrathymic Aire-expressing cells. *Science.* 321:843–847. https://doi.org/10.1126/science.1159407

Gardner, J.M., A.L. Fletcher, M.S. Anderson, and S.J. Turley. 2009. AIRE in the thymus and beyond. *Curr. Opin. Immunol.* 21:582–589. https://doi.org/10.1016/j.coi.2009.08.007

Gardner, J.M., T.C. Metzger, E.J. McMahon, B.B. Au-Yeung, A.K. Krawisz, W. Lu, J.D. Price, K.P. Johannes, A.T. Satpathy, K.M. Murphy, et al. 2013. Extrathymic Aire-expressing cells are a distinct bone marrow-derived population that induce functional inactivation of CD4+ T cells. *Immunity.* 39:560–572. https://doi.org/10.1016/j.immuni.2013.08.005

Gray, D., J. Abramson, C. Benoist, and D. Mathis. 2007. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J. Exp. Med.* 204:2521–2528. https://doi.org/10.1084/jem.20070795

Gury-BenAri, M., C.A. Thaiss, N. Serafini, D.R. Winter, A. Giladi, D. Lara-Astiaso, M. Levy, T.M. Salame, A. Weiner, E. David, et al. 2016. The Spectrum and Regulatory Landscape of Intestinal Innate Lymphoid Cells Are Shaped by the Microbiome. *Cell.* 166:1231–1246.e13. https://doi.org/10.1016/j.cell.2016.07.043

Haljasorg, U., R. Bichele, M. Saare, M. Guha, J. Maslovskaja, K. Kõnd, A. Remm, M. Pihlap, L. Tomson, K. Kisand, et al. 2015. A highly conserved NF-κB-responsive enhancer is critical for thymic expression of Aire in mice. *Eur. J. Immunol.* 45:3246–3256. https://doi.org/10.1002/eji.201545928

Halonen, M., M. Pelto-Huikko, P. Eskelin, L. Peltonen, I. Ulmanen, and M. Kolmer. 2001. Subcellular location and expression pattern of autoimmune regulator (Aire), the mouse orthologue for human gene defective in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED). *J. Histochem. Cytochem.* 49:197–208. https://doi.org/10.1177/002215540104900207

Hao, Z., and K. Rajewsky. 2001. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *J. Exp. Med.* 194:1151–1164. https://doi.org/10.1084/jem.194.8.1151

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

Hepworth, M.R., L.A. Monticelli, T.C. Fung, C.G. Ziegler, S. Grunberg, R. Sinha, A.R. Mantegazza, H.L. Ma, A. Crawford, J.M. Angelosanto, et al. 2013. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. *Nature.* 498:113–117. https://doi.org/10.1038/nature12240

Hepworth, M.R., T.C. Fung, S.H. Masur, J.R. Kelsen, F.M. McConnell, J. Dubrot, D.R. Withers, S. Hugues, M.A. Farrar, W. Reith, et al. 2015. Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4+ T cells. *Science.* 348:1031–1035. https://doi.org/10.1126/science.aaa4812

Hikosaka, Y., T. Nitta, I. Ohigashi, K. Yano, N. Ishimaru, Y. Hayashi, M. Matsumoto, K. Matsuo, J.M. Penninger, H. Takayanagi, et al. 2008. The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. *Immunity.* 29:438–450. https://doi.org/10.1016/j.immuni.2008.06.018

Hinterberger, M., M. Aichinger, O. Prazeres da Costa, D. Voehringer, R. Hoffmann, and L. Klein. 2010. Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. *Nat. Immunol.* 11:512–519. https://doi.org/10.1038/ni.1874

Hubert, F.X., S.A. Kinkel, K.E. Webster, P. Cannon, P.E. Crewther, A.I. Proietto, L. Wu, W.R. Heath, and H.S. Scott. 2008. A specific anti-Aire antibody reveals aire expression is restricted to medullary thymic epithelial cells and not expressed in periphery. *J. Immunol.* 180:3824–3832. https://doi.org/10.4049/jimmunol.180.6.3824

Hubert, F.X., S.A. Kinkel, G.M. Davey, B. Phipson, S.N. Mueller, A. Liston, A.I. Proietto, P.Z. Cannon, S. Forehan, G.K. Smyth, et al. 2011. Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood.* 118:2462–2472. https://doi.org/10.1182/blood-2010-06-286393

Husebye, E.S., M.S. Anderson, and O. Kämpe. 2018. Autoimmune Polyendocrine Syndromes. *N. Engl. J. Med.* 378:2543–2544. https://doi.org/10.1056/NEJMra1713301

Irla, M., S. Hugues, J. Gill, T. Nitta, Y. Hikosaka, I.R. Williams, F.X. Hubert, H.S. Scott, Y. Takahama, G.A. Holländer, and W. Reith. 2008. Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity. *Immunity.* 29:451–463. https://doi.org/10.1016/j.immuni.2008.08.007

Jones, R., E.J. Cosway, C. Willis, A.J. White, W.E. Jenkinson, H.J. Fehling, G. Anderson, and D.R. Withers. 2018. Dynamic changes in intrathymic ILC populations during murine neonatal development. *Eur. J. Immunol.* 48: 1481–1491. https://doi.org/10.1002/eji.201847511

Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity.* 1:167–178. https://doi.org/10.1016/1074-7613(94)90095-7

Kirberg, J., A. Baron, S. Jakob, A. Rolink, K. Karjalainen, and H. von Boehmer. 1994. Thymic selection of CD8+ single positive cells with a class II major histocompatibility complex-restricted receptor. *J. Exp. Med.* 180:25–34. https://doi.org/10.1084/jem.180.1.25

Klein, L., B. Kyewski, P.M. Allen, and K.A. Hogquist. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat. Rev. Immunol.* 14:377–391. https://doi.org/10.1038/nri3667

Kuroda, N., T. Mitani, N. Takeda, N. Ishimaru, R. Arakaki, Y. Hayashi, Y. Bando, K. Izumi, T. Takahashi, T. Nomura, et al. 2005. Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. *J. Immunol.* 174:1862–1870. https://doi.org/10.4049/jimmunol.174.4.1862

Kyewski, B., and L. Klein. 2006. A central role for central tolerance. *Annu. Rev. Immunol.* 24:571–606. https://doi.org/10.1146/annurev.immunol.23.021704.115601

Laan, M., K. Kisand, V. Kont, K. Möll, L. Tserel, H.S. Scott, and P. Peterson. 2009. Autoimmune regulator deficiency results in decreased expression of CCR4 and CCR7 ligands and in delayed migration of CD4+ thymocytes. *J. Immunol.* 183:7682–7691. https://doi.org/10.4049/jimmunol.0804133

LaFlam, T.N., G. Seumois, C.N. Miller, W. Lwin, K.J. Fasano, M. Waterfield, I. Proekt, P. Vijayanand, and M.S. Anderson. 2015. Identification of a novel cis-regulatory element essential for immune tolerance. *J. Exp. Med.* 212:1993–2002. https://doi.org/10.1084/jem.20151069

Lei, Y., A.M. Ripen, N. Ishimaru, I. Ohigashi, T. Nagasawa, L.T. Jeker, M.R. Bösl, G.A. Holländer, Y. Hayashi, R.W. Malefyt, et al. 2011. Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. *J. Exp. Med.* 208:383–394. https://doi.org/10.1084/jem.20102327

Li, J., I. Sarosi, X.Q. Yan, S. Morony, C. Capparelli, H.L. Tan, S. McCabe, R. Elliott, S. Scully, G. Van, et al. 2000. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl. Acad. Sci. USA.* 97:1566–1571. https://doi.org/10.1073/pnas.97.4.1566

Lochner, M., L. Peduto, M. Cherrier, S. Sawa, F. Langa, R. Varona, D. Riethmacher, M. Si-Tahar, J.P. Di Santo, and G. Eberl. 2008. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *J. Exp. Med.* 205:1381–1393. https://doi.org/10.1084/jem.20080034

Luci, C., A. Reynders, I.I. Ivanov, C. Cognet, L. Chiche, L. Chasson, J. Hardwigsen, E. Anguiano, J. Banchereau, D. Chaussabel, et al. 2009. Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat. Immunol.* 10:75–82. https://doi.org/10.1038/ni.1681

Mathis, D., and C. Benoist. 2007. A decade of AIRE. *Nat. Rev. Immunol.* 7: 645–650. https://doi.org/10.1038/nri2136

Montaldo, E., K. Juelke, and C. Romagnani. 2015. Group 3 innate lymphoid cells (ILC3s): Origin, differentiation, and plasticity in humans and mice. *Eur. J. Immunol.* 45:2171–2182. https://doi.org/10.1002/eji.201545598

Nishikawa, Y., F. Hirota, M. Yano, H. Kitajima, J. Miyazaki, H. Kawamoto, Y. Mouri, and M. Matsumoto. 2010. Biphasic Aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation. *J. Exp. Med.* 207:963–971. https://doi.org/10.1084/jem.20092144

Nutt, S.L., B. Heavey, A.G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature.* 401:556–562. https://doi.org/10.1038/44076

Peterson, P., T. Org, and A. Rebane. 2008. Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. *Nat. Rev. Immunol.* 8: 948–957. https://doi.org/10.1038/nri2450

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. https://doi.org/10.1093/nar/29.9.e45

Poliani, P.L., K. Kisand, V. Marrella, M. Ravanini, L.D. Notarangelo, A. Villa, P. Peterson, and F. Facchetti. 2010. Human peripheral lymphoid tissues contain autoimmune regulator-expressing dendritic cells. *Am. J. Pathol.* 176:1104–1112. https://doi.org/10.2353/ajpath.2010.090956

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

Roberts, N.A., A.J. White, W.E. Jenkinson, G. Turchinovich, K. Nakamura, D. R. Withers, F.M. McConnell, G.E. Desanti, C. Benezech, S.M. Parnell, et al. 2012. Rank signaling links the development of invariant γδ T cell progenitors and Aire(+) medullary epithelium. *Immunity.* 36:427–437. https://doi.org/10.1016/j.immuni.2012.01.016

Robinette, M.L., A. Fuchs, V.S. Cortez, J.S. Lee, Y. Wang, S.K. Durum, S. Gilfillan, and M. Colonna. Immunological Genome Consortium. 2015. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nat. Immunol.* 16:306–317. https://doi.org/10.1038/ni.3094

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

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

Satoh-Takayama, N., L. Dumoutier, S. Lesjean-Pottier, V.S. Ribeiro, O. Mandelboim, J.C. Renauld, C.A. Vosshenrich, and J.P. Di Santo. 2009. The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. *J. Immunol.* 183:6579–6587. https://doi.org/10.4049/jimmunol.0901935

Schaller, C.E., C.L. Wang, G. Beck-Engeser, L. Goss, H.S. Scott, M.S. Anderson, and M. Wabl. 2008. Expression of Aire and the early wave of apoptosis in spermatogenesis. *J. Immunol.* 180:1338–1343. https://doi.org/10.4049/jimmunol.180.3.1338

Seehus, C.R., P. Aliahmad, B. de la Torre, I.D. Iliev, L. Spurka, V.A. Funari, and J. Kaye. 2015. The development of innate lymphoid cells requires TOX-dependent generation of a common innate lymphoid cell progenitor. *Nat. Immunol.* 16:599–608. https://doi.org/10.1038/ni.3168

Spits, H., D. Artis, M. Colonna, A. Diefenbach, J.P. Di Santo, G. Eberl, S. Koyasu, R.M. Locksley, A.N. McKenzie, R.E. Mebius, et al. 2013. Innate lymphoid cells--a proposal for uniform nomenclature. *Nat. Rev. Immunol.* 13:145–149. https://doi.org/10.1038/nri3365

von Burg, N., S. Chappaz, A. Baerenwaldt, E. Horvath, S. Bose Dasgupta, D. Ashok, J. Pieters, F. Tacchini-Cottier, A. Rolink, H. Acha-Orbea, and D. Finke. 2014. Activated group 3 innate lymphoid cells promote T-cell-mediated immune responses. *Proc. Natl. Acad. Sci. USA.* 111:12835–12840. https://doi.org/10.1073/pnas.1406908111

Wang, X., M. Laan, R. Bichele, K. Kisand, H.S. Scott, and P. Peterson. 2012. Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens. *Front. Immunol.* 3:19. https://doi.org/10.3389/fimmu.2012.00019

Yamano, T., J. Nedjic, M. Hinterberger, M. Steinert, S. Koser, S. Pinto, N. Gerdes, E. Lutgens, N. Ishimaru, M. Busslinger, et al. 2015. Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction. *Immunity.* 42:1048–1061. https://doi.org/10.1016/j.immuni.2015.05.013

Yano, M., N. Kuroda, H. Han, M. Meguro-Horike, Y. Nishikawa, H. Kiyonari, K. Maemura, Y. Yanagawa, K. Obata, S. Takahashi, et al. 2008. Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. *J. Exp. Med.* 205: 2827–2838. https://doi.org/10.1084/jem.20080046

## 4. A NOVEL CONDITIONAL *AIRE* ALLELE ENABLES CELL-SPECIFIC ABLATION OF THE IMMUNE TOLERANCE REGULATOR AIRE

Apart from the critical role of AIRE in mechanisms of central tolerance, AIRE was also shown to be expressed in various ontogenically very distinct cell lineages. Specifically, by the population of thymic B-cell, which after interaction with thymocytes undergo intrathymic licensing leading to the upregulation of AIRE, MHCII, and CD80 and which seem to be important for promoting the tolerance to B-cell related antigens (Yamano et al., 2015). Also, the rare population of hematopoietic cells expressing AIRE was described in lymph nodes, spleen, and tonsils (Gardner et al., 2008; Gardner et al., 2009; Gardner et al., 2013). Although this population was originally attributed to DC-lineage, they rather represent a specific population of ILC3-like cells that are capable to clonally delete self-reactive T-cell in the immune periphery (Yamano et al., 2019). The AIRE was also found to be expressed at the pregastrulation stage of mouse development and by the oocytes and cells associated with spermatogenesis (Gu et al., 2017; Nishikawa et al., 2010; Schaller et al., 2008). In general, the function of AIRE in all of those cell-types seems to be important for the regulation of different cell-specific transcriptional programs than described in mTECs. To study the exact function of AIRE in those cell-types, the genetic system that enables its cell or tissue-specific genetic ablation is needed.

Here, we generated the transgenic mouse in which the exons 6 and 7 of the *Aire* gene are flanked by LoxP sites. The function of this newly generated $Aire^{fl/fl}$ model was verified by crossing with $Foxn1^{Cre}$ and $Vav1^{Cre}$ mouse models, that enables the specific depletion of AIRE in TECs and all hematopoietic cells (containing thymic B-cells), respectively. Interestingly the fertility of those AIRE depleted mice models was not affected. This suggests that infertility of $Aire^{-/-}$ mice is not caused by the AIRE depletion in TECs or hematopoietic cells, such as thymic B-cell or $AIRE^{+}$ ILC3-like cells (Dobeš et al., 2018). The $Aire^{fl/fl}$ mice are now available at the Jackson Laboratories (Stock # 031409).

Author's contribution: Verification of exon 6 and 7 excisions by PCR, FACS analysis of AIRE expression in B-cells, and mTECs in $Aire^{-/-}$ and $Aire^{+/+}$ mice, quantitative PCR detection of *Aire* mRNA in several tissues.

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# Letter to the Editor

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## A novel conditional *Aire* allele enables cell-specific ablation of the immune tolerance regulator Aire

Central immune tolerance is achieved by the presentation of self-antigens to developing thymocytes. Several types of antigen presenting cells are involved in this process [1], but only medullary thymic epithelial cells (mTECs) exhibit the unique capacity to autonomously express and present around 75% of host proteins. Of this pool, the ectopic expression of ~3000 genes, including many tissue restricted antigens (TRAs), are driven by the autoimmune regulator (Aire) [2]. Aire deficiency was shown to decrease the expression of Aire-dependent TRAs in mTECs, leading to the escape of self-reactive T cells, potentially promoting multi-organ autoimmunity in both mice [3] and humans (autoimmune polyglandular syndrome type 1 – APS1) [4]. Apart from its critical role in the establishment of immune tolerance, Aire is also expressed in ontogenetically distinct cell lineages, where it serves other functions. Notably, a recent study demonstrated that immature B cells can be intrathymically licensed by interaction with cognate $CD4^+$ thymocytes and acquire the expression of Aire, MHCII and CD80 which enables them to promote self-tolerance [5]. A rare population of extrathymic Aire-expressing cells was described in lymph nodes and spleen. This subset expresses a set of Aire-regulated TRAs and is capable of anergizing self-reactive T cells in the immune periphery [6]. Aire expression was also detected at the pregastrulation stage, but Aire's role in embryonic development remains elusive [7]. Finally, oocytes and spermatogenic cells also express Aire, but its biological function in reproductive organs awaits further clarification [7, 8].

To enable cell type-specific ablation of the *Aire* gene, we generated transgenic mice with a LoxP-flanked exon 6 and exon 7 of the *Aire* locus (Supporting Information Fig. 1A). These exons encode Aire's SAND domain which through the interaction with ATF7ip-MBD1 complex mediates Aire targeting to loci encoding TRAs [9]. Moreover, a number of *AIRE* mutations were annotated in this domain, including those which are prevalent in Finnish and Sardinian APS1 patients [4].

The position of LoxP sites in $Aire^{fl/fl}$ mice was confirmed by PCR followed by restrictase digestion (Supporting Information Fig. 1B). Ultimately, we verified the susceptibility of the $Aire^{fl/fl}$ allele to Cre-mediated deletion in vivo by crossing $Aire^{fl/fl}$ mice with the Vav1-Cre strain in which Cre expression is restricted to hematopoietic lineages. The activity of Cre-recombinase led to the excision of *Aire* exons 6 and 7, demonstrating full functionality of LoxP sites (Supporting Information Fig. 1C).

To determine the impact of Aire ablation specifically in mTECs, $Aire^{fl/fl}$ animals were crossed with a Foxn1-Cre strain in which Cre expression is restricted to the thymic epithelium. There are two major mTEC populations: (i) $MHCII^{high}$ mTECs ($mTECs^{high}$) which express Aire protein together with high levels of TRAs, essential features required for negative selection; and (ii) $MHCII^{low}$ mTECs ($mTECs^{low}$) which contain precursors of $mTECs^{high}$ [3]. Intracellular staining of $mTECs^{high}$ from $Foxn1\text{-}Cre^{+}Aire^{fl/fl}$ animals with antibodies specific for the C-terminal part of Aire revealed its complete absence (Fig. 1A). This suggests that the Cre-mediated ablation of *Aire's* SAND domain leads to protein truncation and/or Aire transcript instability and ultimately to undetectable levels of Aire protein.

Previous reports have described an increased frequency of $mTECs^{high}$ at the expense of $mTECs^{low}$ in $Aire^{-/-}$ mice [3]. In accordance with this notion, we observed ~35% increase in $mTEC^{high}$ populations in both $Aire^{-/-}$ as well as $Foxn1\text{-}Cre^{+}Aire^{fl/fl}$ animals (Fig. 1A and B). Comparative expression analysis of Aire-dependent (*Ins2* – insulin 2 and *Defa24* – enteric alpha-defensin24) and Aire-independent TRAs (*Crp* – C-reactive protein and *Gad67* – Glutamate decarboxylase 1) [3] revealed the complete absence of the former, but not the latter, in $Aire^{-/-}$ and $Foxn1\text{-}Cre^{+}Aire^{fl/fl}$ compared to their pattern of expression in relevant control mice (Fig. 1C).

Last, the ability of the $Aire^{fl/fl}$ allele to undergo cell-specific ablation in distinct Aire-expressing subsets residing in the same organ was determined. Specifically, the thymus accommodates both Aire-expressing mTECs [3] and Aire-expressing B cells [5], hence Foxn1-Cre and Vav1-Cre drivers were used to independently ablate Aire expression in these two cell lineages, respectively. $mTEC^{hi}$, but not thymic B-cells, from $Foxn1\text{-}Cre^{+}Aire^{fl/fl}$ animals lacked Aire protein (Fig. 2A and B).

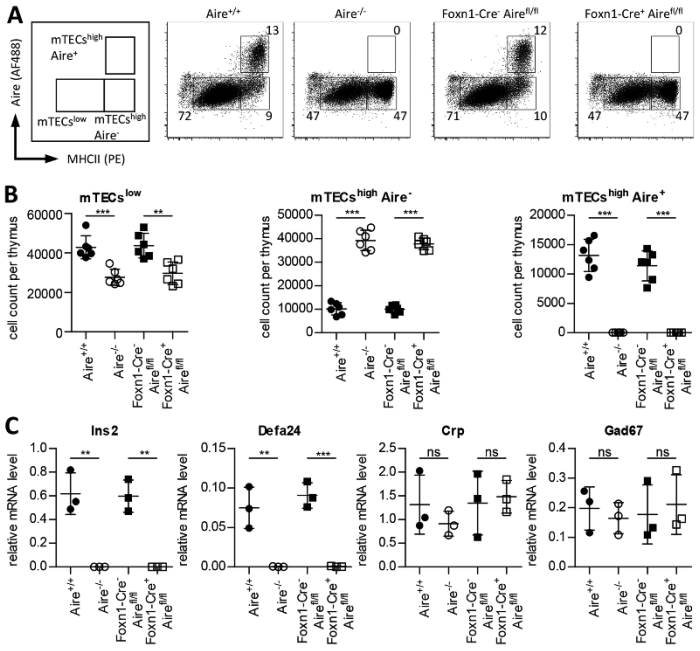


**Figure 1.** Comparative analysis of the mTEC compartment and its TRA expression between Aire$^{-/-}$ and Foxn1-Cre$^{+}$Aire$^{fl/fl}$ strains. (A) Representative FACS analysis of mTECs; one thymus of the indicated genotype per experiment, six independent experiments (per each genotype). TECs were pre-gated as CD45$^{-}$ EpCAM$^{+}$ cells (see Supporting Information Figure 2A for details). (B) Statistical analysis of data shown in (A). Scatterplots show the total number of detected cells of indicated phenotype per thymus ($n = 6$). (C) Expression of indicated TRAs by FACS-sorted mTECs$^{high}$ determined by qRT PCR. Two thymi were pooled and sorted together per experiment, three independent experiments. Data in (B) and (C) is presented as mean ± SD; 2-tailed Student's *t*-test, $^{***}p<0.001$, $^{**}p<0.01$, ns–not significant.

Reciprocally, thymic B cells, but not mTEC$^{hi}$, from Vav1-Cre$^{+}$Aire$^{fl/fl}$ animals were negative for Aire (Fig. 2A and B). This data demonstrates that the Aire$^{fl/fl}$ allele can be ablated in Cre-driver-dependent cell-specific manner.

Interestingly, in contrast to Aire$^{-/-}$ animals which are infertile and thus must be bred as heterozygotes [3], the fertility of Foxn1-Cre$^{+}$Aire$^{fl/fl}$ homozygote animals (Fig. 2C) and Vav1-Cre$^{+}$ Aire$^{fl/fl}$ males or females looks normal (Supporting Information Fig. 4A). Consistently, while Aire expression in testes in Aire$^{-/-}$ mice is abrogated (Supporting Information Fig.4B), in Foxn1-Cre$^{+}$Aire$^{fl/fl}$ and Vav1-Cre$^{+}$Aire$^{fl/fl}$ animals it remains unperturbed (Supporting Information Fig.4C). This suggests that Aire expression outside of the thymic stroma and hematopoietic system underpins the infertility of Aire$^{-/-}$ mice. Together, our data illustrate that the Aire$^{fl/fl}$ mouse strain is a suitable model to scrutinize cell-specific function of Aire in distinct biological processes. The Aire$^{fl/fl}$ strain will be available to the research community from the Jackson Laboratories under the Stock #031409.

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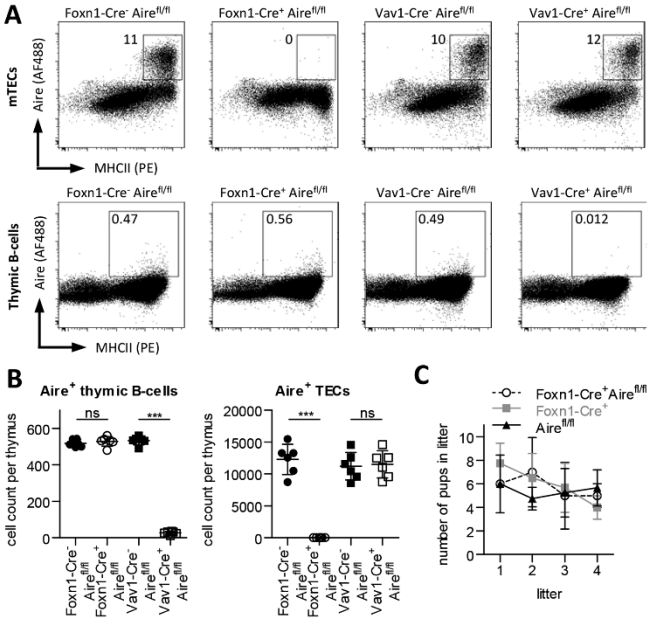


**Figure 2.** Lineage-specific Aire ablation. (A) Representative FACS analysis of Aire expression in mTECs and thymic B-cells from Foxn1-Cre$^{+}$Aire$^{fl/fl}$ and Vav1-Cre$^{+}$Aire$^{fl/fl}$ animals; one thymus of indicated genotype per experiment, six independent experiments (per each genotype). For gating strategy see Supporting Information Fig. 2. For comparison with analogous FACS data from Aire $^{-/-}$ animals, see Supporting Information Fig. 3. (B) Statistical analysis of FACS data shown in (A). Scatterplots show the total number of detected cells of indicated phenotype per thymus ($n = 6$). (C) Foxn1-Cre$^{-}$Aire$^{fl/fl}$ mice are fertile. The graph shows the average number of pups at each of four successive litters from four independent experiments ($n = 4$). Data in (B) and (C) is presented as mean ± SD; two-tailed Student's $t$-test, $^{***}p<0.001$, $^{**}p<0.01$. No significant differences in fertility were observed between indicated genotypes.

versity in Prague. All experiments performed on animals were approved by the local ethical committee under the accreditation no. 98/2016. We apologize to colleagues whose work could not be cited owing to space limitation.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

## References

1 **Klein, L. et al.**, *Nat. Rev. Immunol.* 2014. **14**: 377–391.

2 **Danan-Gotthold, M. et al.**, *Genome Biology* 2016. **17**: 219–231.

3 **Anderson, M. S. et al.**, *Science* 2002. **298**: 1395–1401.

4 **Abramson, J. et al.**, *Immunol. Rev.* 2016. **271**: 127–140.

5 **Yamano, T. et al.**, *Immunity* 2015. **42**: 1048–1061.

6 **Gardner, J. M. et al.**, *Immunity* 2013. **39**: 560–572.

7 **Nishikawa, Y. et al.**, *J. Exp. Med.* 2010. **207**: 963–971.

8 **Schaller, C. E. et al.**, *J. Immunol.* 2008. **180**: 1338–1343.

9 **Waterfield, M. et al.**, *Nat. Immunol.* 2014. **15**: 258–265.

*Abbreviations:* **Aire**: Autoimmune regulator · **APS1**: autoimmune polyglandular syndrome type 1 · **DC**: dendritic cell · **mTEC**: medullary thymic epithelial cell · **TCR**: T cell receptor · **TRA**: tissue restricted antigen

Keywords: Aire · Conditional knockout · Immune tolerance · Medullary thymic epithelial cells

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# DISCUSSION AND CONCLUSIONS

The novelty of the presented thesis pertains to several discoveries that describe the function, origin, and regulation of AIRE-expressing cells in immune tolerance. We found that mTECs$^{high}$ express a battery of TLRs whose signaling is wired to the production of chemokines and genes associated with post-AIRE mTECs development. We subsequently discovered that receptors for these chemokines are predominantly expressed by the newly described population of thymic moDCs whose recruitment to the thymic medulla and subsequent CAT is regulated by mTEC-intrinsic TLR9 signaling. In association with this, we showed that TLR signaling in mTECs is essential for the development of Tregs since its abrogation led to the worsening of colon inflammation in the mouse model of experimental colitis. We also found that enteric α-defensins, expressed in the crypts of the small intestine by PCs, are also expressed by mTECs in AIRE-dependent manner. We described that loss of its thymic expression in some of the APS-1 patients led to the destruction of PCs and the presence of defensin-specific autoantibodies in sera of these patients which was associated with severe pathologies in gut tissue. Using *Aire*$^{-/-}$ mouse model we found the presence of defensin-specific T-cells in lymphoid organs and demonstrated that the adoptive transfer of these T-cells into the athymic mice resulted in a loss of PCs, microbial dysbiosis and induction of pathologic Th17 response in the gut tissue. Apart from the discoveries in the regulation of AIRE$^{+}$ mTECs in the thymus we also characterized a novel population of AIRE$^{+}$ ILC3-like cells that resides in the lymph nodes and are able to efficiently present the model endogenous antigens to CD4$^{+}$ T-cells. We described that the differentiation of those AIRE protein-expressing cells from "conventional" ILC3s is fully dependent on RANKL/NF-κB signaling and that the function of AIRE in these cells in not specifically wired to the promiscuous TRAs expression. Lastly, our previous discoveries lead us to the development of a novel *Aire*$^{fl/fl}$ mouse model that enables the cell-specific depletion of AIRE and thus to study its role in different anatomical locations and tissues.

All of these findings, not only describe the mechanisms of a novel regulatory pathway for AIRE-expressing cells in thymus and lymph nodes but also open a plethora of new questions, the solution of which would significantly advance our knowledge in the

understanding of interrelated mechanisms establishing, maintaining and enforcing immune tolerance. I present and discuss some of these questions below.

First, what type of natural thymic-localized ligand could serve as a trigger of TLR9 signaling in mTECs? Since the TLRs were postulated to sense both microbial-derived and endogenous molecules, the determination of the origin of the ligand would be essential for answering this question (Kawai and Akira, 2010). Previously it was demonstrated that MyD88$^{-/-}$Aire$^{-/-}$ double knockout mice developed more severe sights of autoimmunity than Aire$^{-/-}$, suggesting the role of TLR-signaling in tolerance induction. On the other hand, neither the enhancement of TLR/MyD88 signaling by intraperitoneal injection of TLRs ligands nor the diminishment of microbial-associated ligands in mice from germ-free (GF) conditions changed the severity of AIRE-dependent autoimmune disease (Gray et al., 2007b). Recently, it was also described that the development of tTregs was not altered in mice from either GF or "dirty" conditions (Owen et al., 2019). This was corroborated by our study showing that the lack of microbial-derived TLR ligands in GF mice did not affect the expression of MyD88-associated chemokines and cytokines in mTECs$^{high}$ (Vobořil et al., 2020). Together these results indicate that the signals which trigger TLR responses in mTECs are likely of intrathymic endogenous origin and not derived from a microbiota. In association with this, we can only speculate what is the nature of the thymic-derived endogenous ligand. Since the TLR9 was shown to sense DNA released from necrotic or apoptotic cells or HMGB1 that specifically binds DNA (Tian et al., 2007) and the thymus during the process of negative selection generates millions of apoptotic T-cells (von Boehmer et al., 1989), we have hypothesized that ligands released from clonally deleted thymocytes could be the trigger of TLR/MyD88 responses in mTECs. This would suggest that negatively selected self-reactive T-cells have the ability to stimulate TLR/MyD88 signaling pathway on mTECs and thus drive their differentiation into post-AIRE cells that subsequently increase the attraction of moDCs into the thymic medulla. This hypothesis was partially proofed by the fact, that differentiation of AIRE$^{+}$ mTECs and migration of moDCs into the thymus is abrogated in *RAG2*$^{-/-}$*OT-II* mice (our unpublished observation), where almost no clonal deletion occurs (Breed et al., 2019). This hypothesis would then suggests that TLR signaling in mTECs form a positive feedback loop, that regulates the processes of T-cell selection by the clonal deletion of

self-reactive T-cells. Nevertheless, further experiments are needed to prove this hypothesis.

Second, are the post-AIRE mTECs the major subtype responsible for the transfer of antigen to the thymic population of moDCs? It has been postulated that $AIRE^+$ mTECs further differentiate into the population of post-AIRE cells (Metzger et al., 2013; Yano et al., 2008). Although it was shown that differentiation of those cells comes after the development of $AIRE^+$ mTECs and is mainly regulated by the LTβR pathway, the exact regulatory mechanisms are still poorly defined (White et al., 2010). As described above, we have suggested that TLR/MyD88 signaling which senses the thymic endogenous ligands could also regulate the development of post-AIRE cells. Moreover, we have shown that this correlates with the enhanced production of chemokines that attract increased numbers of moDCs in the thymic medulla (Vobořil et al., 2020). To this point, we can only speculate whether such processes are regulated directly by post-AIRE cells. Interestingly, the terminal differentiation of $AIRE^+$ mTECs is very similar to the development of skin keratinocytes and is accompanied by upregulation of several corneocytes-associated molecules such as KRT1, KRT10, IVL, desmogleins, and clusterins (Bornstein et al., 2018; Metzger et al., 2013; Miller et al., 2018; Wang et al., 2012). Similar to corneocytes, the post-AIRE mTECs also lose the nuclei and differentiate into Hassall's corpuscles (see Figure 3) (Wang et al., 2012). This suggests that the process of cornification is used by $AIRE^+$ mTECs as an alternative route to cell death (Michel et al., 2017). Moreover, the differentiation into post-AIRE cells is also accompanied by the downregulation of MHCII and AIRE and significant upregulation of chemokines attracting neutrophils, moDCs, or macrophages to the proximity of Hassall's corpuscles (Metzger et al., 2013; Wang et al., 2019b). This, together with the fact that these cells still express a reasonable amount of TRAs, predispose them for the transfer of those antigens to thymic populations of DCs (Kadouri et al., 2020). In support of this hypothesis, it was recently described that the CAT form mTECs to thymic DCs occurs mostly by the scavenging of apoptotic bodies by myeloid cells (Perry et al., 2018), suggesting that terminally differentiated pre-apoptotic post-AIRE cells could be the major source of these bodies. Since the expression of scavenger receptors is enriched in thymic moDCs compared to other DC-like subsets (Vobořil et al., 2020), the antigen transfer most likely

occurs between post-AIRE cells and moDCs. On the other hand, the development of a model system that enables the specific targeting of post-AIRE cells would be essential for the final elucidation of such a hypothesis.

Third, what is the function of the newly discovered population of thymic moDC in the context of T-cell clonal deletion and/or Tregs generation? This seems to be a quite complicated question since there is no suitable mouse model that would enable the very specific targeting of monocyte-derived cells in general, and in the thymic tissue, in particular. Since due to the phenotypic similarity the moDCs are often co-clustered with the SIRPα$^{+}$ cDC2 subset, the specific function of this cell subset is also attributed to cDC2 (Guilliams et al., 2014). Both moDCs and cDC2 were showed to originate extrathymically and then migrate to the thymic medullary region (Li et al., 2009). So far, the only described factors driving the migration of cDC2 into the thymus are the ligands of CCR2 (Baba et al., 2009). Interestingly, the CCR2 was shown to be expressed mostly by circulating monocytes (Croxford et al., 2015) and in the thymus by the population of moDCs (Vobořil et al., 2020). This suggests that only a partial depletion of SIRPα$^{+}$ cDC2 cells from the thymus of *Ccr2*$^{-/-}$ mice is caused rather by the reduction of moDCs than conventional cDC2. Since the *Ccr2*$^{-/-}$ mice show impaired negative selection against blood-borne antigens, the function of moDCs is likely attributed to the T-cells selection of those antigens (Baba et al., 2009; Bonasio et al., 2006). Also, the phenotypically very similar population of CX3CR1$^{+}$ moDCs resides in mucosal tissues where they, by sampling of intestinal antigens, mediate tolerance to food or microbiota-derived antigens (Hadis et al., 2011; Kim et al., 2018; Niess et al., 2005). Thus, one can speculate that together with pDCs, thymic moDCs could transfer intestinal antigens to the thymus and subsequently induce central tolerance to them (Hadeiba et al., 2012). As suggested above, due to the increased phagocytic and endocytic activity, the thymic moDCs are also very potent in CAT from mTECs. We also demonstrated that the influx of these cells into the thymic medulla correlates with the enhanced generation of tTregs (Vobořil et al., 2020). This is in agreement with the fact that neither the depletion of cDC1 nor pDCs affected the number of AIRE-dependent MJ23 TCR-specific T-cells. In contrast, the generation of these T-cells was diminished in mice lacking MHCII molecules on all thymic DCs (*CD11c*$^{Cre}$*MHCII*$^{fl/fl}$ mice), suggesting the role of moDCs and/or cDC2 in this process

(Leventhal et al., 2016). We also found that moDCs, recruited in increased numbers after TLR9 intrathymic stimulation, were of the MHCII$^{lo/int}$ phenotype (Vobořil et al., 2020), which was recently associated with the enhanced potential to generate Tregs (Hu et al., 2017; Klein et al., 2019). To describe the involvement of moDCs in the selection of CD4$^+$ T-cells, the models enabling the depletion of MHCII expression specifically in this cell subset is needed.

The others attached publications that deal more specifically with the function of AIRE in the thymus and periphery, also raised several questions. First, what are the potential mechanisms of PCs destruction in *Aire*$^{-/-}$ models? In our experimental system, both CD4$^+$ and CD8$^+$ T-cell infiltrates were detected in increased numbers in the mLN of athymic mice injected by T-cells isolated from *Aire*$^{-/-}$ model. In addition, CD8$^+$ T-cells isolated from *Aire*$^{-/-}$ mice had the capacity to increase the rate of apoptosis of PC in *in vitro* co-cultivation experiment. This suggests that activated CD8$^+$ T-cells are responsible for PC destruction in seropositive APS-1 patients and in *Aire*$^{-/-}$ defensin-specific mouse model (Dobeš et al., 2015). This hypothesis could be supported by the fact, that high levels of IFNγ, the cytokine produced mainly by CD8$^+$ T-cells or Th1 cells, promote the degranulation and partial depletion of PCs (Farin et al., 2014; Raetz et al., 2013). Also, the Th1 differentiation and subsequent production of IFNγ was shown to be regulated by microbiota composition (Al Nabhani et al., 2019). Thus, we can speculate that defensin-specific CD8$^+$ T-cells mediate the first round of attack leading to a partial depletion of PCs with overall decreased production of their antimicrobial peptides and the onset of microbial dysbiosis, which induces the Th1 generation and IFNγ overproduction which ultimately results in PCs diminishment. This hypothesis is strengthened by the fact that segmented filamentous bacteria (SFB) that are enriched in *Aire*$^{-/-}$ models (Dobeš et al., 2015) can induce IFNγ expression (Gaboriau-Routhiau et al., 2009). Together, this suggests that both CD8$^+$ and CD4$^+$ (Th1) T-cells contributed to the observed devastating AIRE-dependent phenotype of PCs.

Second, what is the function of defensin-specific Tregs in AIRE-dependent intestinal autoimmunity? As described in introductory chapters the presentation of AIRE-dependent TRAs by mTECs is mostly attributed to the generation of Tregs (Hassler et al., 2019;

Malhotra et al., 2016). Also, it was described that in the absence of AIRE the T-cell clones usually restricted to Treg lineage can be found in a repertoire of Tconv cells (Malchow et al., 2016). Thus we can hypothesize that Tregs specific to enteric α-defensins are in the case of *Aire*$^{-/-}$ mice "converted" to self-reactive effector T-cells. This would then suggest that the inflammatory phenotype caused by the adoptive transfer of defensin-specific T-cells isolated from *Aire*$^{-/-}$, compared to *Aire*$^{+/+}$ mice, could be further amplified by the absence of Tregs (Dobeš et al., 2015). The generation of tetramers specific to particular enteric α-defensins or development of transgenic mouse strain with enteric α-defensin-specific TCR would enable us to elucidate this question.

Lastly, what is the role of AIRE in AIRE$^{+}$ ILC3-like cells and other peripheral AIRE-expressing cells? With the discovery of eTACs it has been shown that AIRE regulates the production of the same set of TRAs as in mTECs. However, the gene expression overlap between these cell-types was negligible, suggesting that eTACs specifically promote tolerance to different self-antigens then those presented in the thymus (Gardner et al., 2008). On the other hand, using the bulk RNA sequencing of AIRE$^{+}$ ILC3-like cells isolated either from *Aire*$^{+/+}$ or *Aire*$^{-/-}$, it was described that their AIRE-induced transcriptome is not biased towards TRAs (Yamano et al., 2019). This was also corroborated by the recent publication which showed no TRAs enrichment in AIRE$^{+}$ DC-like cells isolated from human tonsils (Fergusson et al., 2018). As described in the literature overview chapter, AIRE also coordinates other functions of mTECs beyond TRAs production such as expression of cytokines, chemokines, or regulation of antigen handling and presentation (Anderson et al., 2005; Fujikado et al., 2016; Hubert et al., 2011). Since the AIRE$^{+}$ ILC3-like cells express higher surface levels of MHCII and co-stimulatory molecules compared to conventional ILC3s, we can speculate that AIRE is specifically associated with the production of these molecules (Yamano et al., 2019). Also, the expression of several effector cytokines by mTECs and ILC3-like cells (our unpublished observation) were shown to be regulated by AIRE (Fujikado et al., 2016). This suggests that similar to the mucosal tissue-associated ILC3s (Hepworth et al., 2015; Hepworth et al., 2013), AIRE$^{+}$ ILC3-like cells could be essential for the tolerogenic modulation of T-cells response in the site of inflammation and that the expression of its effector molecules is regulated by AIRE. The crossing of our newly generated *Aire*$^{fl/fl}$

mouse (Dobeš et al., 2018) with the specific drivers of Cre recombinase-containing models (*Rorc*$^{Cre}$ mice for AIRE depletion in ILC3-like cells) would significantly help in elucidation of the role of AIRE in different cell-types and anatomical locations.

Taken together, the presented thesis describes several observations that significantly extend the knowledge of the processes of central and peripheral immune tolerance and suggests the generation of new murine models that could help the scientist to elucidate numerous unanswered questions.

# REFERENCES


Abramson, J., and Anderson, G. (2017). Thymic Epithelial Cells. Annu Rev Immunol *35*, 85-118.

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

Abramson, J., and Husebye, E.S. (2016). Autoimmune regulator and self-tolerance - molecular and clinical aspects. Immunol Rev *271*, 127-140.

Abreu, M.T. (2010). Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol *10*, 131-144.

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

Akashi-Takamura, S., and Miyake, K. (2008). TLR accessory molecules. Curr Opin Immunol *20*, 420-425.

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*, 248-251.

Akiyama, T., Shimo, Y., Yanai, H., Qin, J., Ohshima, D., Maruyama, Y., Asaumi, Y., Kitazawa, J., Takayanagi, H., Penninger, J.M.*, et al.* (2008). The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. Immunity *29*, 423-437.

Al Nabhani, Z., Dulauroy, S., Marques, R., Cousu, C., Al Bounny, S., Déjardin, F., Sparwasser, T., Bérard, M., Cerf-Bensussan, N., and Eberl, G. (2019). A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. Immunity *50*, 1276-1288.e1275.

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

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

Andonegui, G., Zhou, H., Bullard, D., Kelly, M.M., Mullaly, S.C., McDonald, B., Long, E.M., Robbins, S.M., and Kubes, P. (2009). Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. J Clin Invest *119*, 1921-1930.

Apostolou, I., Sarukhan, A., Klein, L., and von Boehmer, H. (2002). Origin of regulatory T cells with known specificity for antigen. Nat Immunol *3*, 756-763.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P., Liu, H., Cross, J.R., Pfeffer, K., Coffer, P.J.*, et al.* (2013). Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature *504*, 451-455.

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

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y.*, et al.* (2011). Induction of colonic regulatory T cells by indigenous Clostridium species. Science *331*, 337-341.

Atibalentja, D.F., Byersdorfer, C.A., and Unanue, E.R. (2009). Thymus-blood protein interactions are highly effective in negative selection and regulatory T cell induction. J Immunol *183*, 7909-7918.

Atibalentja, D.F., Murphy, K.M., and Unanue, E.R. (2011). Functional redundancy between thymic CD8α+ and Sirpα+ conventional dendritic cells in presentation of blood-derived lysozyme by MHC class II proteins. J Immunol *186*, 1421-1431.

Ayabe, T., Satchell, D.P., Wilson, C.L., Parks, W.C., Selsted, M.E., and Ouellette, A.J. (2000). Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol *1*, 113-118.

Baba, T., Nakamoto, Y., and Mukaida, N. (2009). Crucial contribution of thymic Sirp alpha+ conventional dendritic cells to central tolerance against blood-borne antigens in a CCR2-dependent manner. J Immunol *183*, 3053-3063.

Baker, B.S., Ovigne, J.M., Powles, A.V., Corcoran, S., and Fry, L. (2003). Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. Br J Dermatol *148*, 670-679.

Balounová, J., Šplíchalová, I., Dobešová, M., Kolář, M., Fišer, K., Procházka, J., Sedlacek, R., Jurisicova, A., Sung, H.K., Kořínek, V.*, et al.* (2019). Toll-like receptor 2 expression on c-kit. Nat Commun *10*, 5176.

Balounová, J., Vavrochová, T., Benešová, M., Ballek, O., Kolář, M., and Filipp, D. (2014). Toll-like receptors expressed on embryonic macrophages couple inflammatory signals to iron metabolism during early ontogenesis. Eur J Immunol *44*, 1491-1502.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barbalat, R., Lau, L., Locksley, R.M., and Barton, G.M. (2009). Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. Nat Immunol *10*, 1200-1207.

Barton, G.M., Kagan, J.C., and Medzhitov, R. (2006). Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol *7*, 49-56.

Beg, A.A. (2002). Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol *23*, 509-512.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet *27*, 20-21.

Benson, M.J., Pino-Lagos, K., Rosemblatt, M., and Noelle, R.J. (2007). All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med *204*, 1765-1774.

Bernasconi, P., Barberis, M., Baggi, F., Passerini, L., Cannone, M., Arnoldi, E., Novellino, L., Cornelio, F., and Mantegazza, R. (2005). Increased toll-like receptor 4 expression in thymus of myasthenic patients with thymitis and thymic involution. Am J Pathol *167*, 129-139.

Beutler, B. (2004). Innate immunity: an overview. Mol Immunol *40*, 845-859.

Bhandoola, A., von Boehmer, H., Petrie, H.T., and Zúñiga-Pflücker, J.C. (2007). Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from. Immunity *26*, 678-689.

Bhoj, V.G., and Chen, Z.J. (2009). Ubiquitylation in innate and adaptive immunity. Nature *458*, 430-437.

Bianchi, T., Pincus, L.B., Wurbel, M.A., Rich, B.E., Kupper, T.S., Fuhlbrigge, R.C., and Boes, M. (2009). Maintenance of peripheral tolerance through controlled tissue homing of antigen-specific T cells in K14-mOVA mice. J Immunol *182*, 4665-4674.

Blackburn, C.C., Augustine, C.L., Li, R., Harvey, R.P., Malin, M.A., Boyd, R.L., Miller, J.F., and Morahan, G. (1996). The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. Proc Natl Acad Sci U S A *93*, 5742-5746.

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. J Exp Med *198*, 757-769.

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

Bonito, A.J., Aloman, C., Fiel, M.I., Danzl, N.M., Cha, S., Weinstein, E.G., Jeong, S., Choi, Y., Walsh, M.C., and Alexandropoulos, K. (2013). Medullary thymic epithelial cell depletion leads to autoimmune hepatitis. J Clin Invest *123*, 3510-3524.

Bornstein, C., Nevo, S., Giladi, A., Kadouri, N., Pouzolles, M., Gerbe, F., David, E., Machado, A., Chuprin, A., Tóth, B.*, et al.* (2018). Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells. Nature *559*, 622-626.

Botos, I., Segal, D.M., and Davies, D.R. (2011). The structural biology of Toll-like receptors. Structure *19*, 447-459.

Breed, E.R., Watanabe, M., and Hogquist, K.A. (2019). Measuring Thymic Clonal Deletion at the Population Level. J Immunol *202*, 3226-3233.

Brennecke, P., Reyes, A., Pinto, S., Rattay, K., Nguyen, M., Küchler, 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*, 933-941.

Brown, C.C., Gudjonson, H., Pritykin, Y., Deep, D., Lavallée, V.P., Mendoza, A., Fromme, R., Mazutis, L., Ariyan, C., Leslie, C.*, et al.* (2019). Transcriptional Basis of Mouse and Human Dendritic Cell Heterogeneity. Cell *179*, 846-863.e824.

Buono, M., Facchini, R., Matsuoka, S., Thongjuea, S., Waithe, D., Luis, T.C., Giustacchini, A., Besmer, P., Mead, A.J., Jacobsen, S.E.*, et al.* (2016). A dynamic niche provides Kit ligand in a stage-specific manner to the earliest thymocyte progenitors. Nat Cell Biol *18*, 157-167.

Burberry, A., Zeng, M.Y., Ding, L., Wicks, I., Inohara, N., Morrison, S.J., and Núñez, G. (2014). Infection mobilizes hematopoietic stem cells through cooperative NOD-like receptor and Toll-like receptor signaling. Cell Host Microbe *15*, 779-791.

Calderón, L., and Boehm, T. (2012). Synergistic, context-dependent, and hierarchical functions of epithelial components in thymic microenvironments. Cell *149*, 159-172.

Cario, E., and Podolsky, D.K. (2000). Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun *68*, 7010-7017.

Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. J Exp Med *204*, 1653-1664.

Cavalcante, P., Barzago, C., Baggi, F., Antozzi, C., Maggi, L., Mantegazza, R., and Bernasconi, P. (2018). Toll-like receptors 7 and 9 in myasthenia gravis thymus: amplifiers of autoimmunity? Ann N Y Acad Sci *1413*, 11-24.

Cavalcante, P., Galbardi, B., Franzi, S., Marcuzzo, S., Barzago, C., Bonanno, S., Camera, G., Maggi, L., Kapetis, D., Andreetta, F.*, et al.* (2016). Increased expression of Toll-like receptors 7 and 9 in myasthenia gravis thymus characterized by active Epstein-Barr virus infection. Immunobiology *221*, 516-527.

Cebula, A., Seweryn, M., Rempala, G.A., Pabla, S.S., McIndoe, R.A., Denning, T.L., Bry, L., Kraj, P., Kisielow, P., and Ignatowicz, L. (2013). Thymus-derived regulatory T cells contribute to tolerance to commensal microbiota. Nature *497*, 258-262.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., and Colonna, M. (1999). Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med *5*, 919-923.

Cisse, B., Caton, M.L., Lehner, M., Maeda, T., Scheu, S., Locksley, R., Holmberg, D., Zweier, C., den Hollander, N.S., Kant, S.G.*, et al.* (2008). Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. Cell *135*, 37-48.

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

Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S., and Vignali, D.A. (2007). The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature *450*, 566-569.

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

Coombes, J.L., Siddiqui, K.R., Arancibia-Cárcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med *204*, 1757-1764.

Cooper, G.S., Bynum, M.L., and Somers, E.C. (2009). Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. J Autoimmun *33*, 197-207.

Cosway, E.J., Lucas, B., James, K.D., Parnell, S.M., Carvalho-Gaspar, M., White, A.J., Tumanov, A.V., Jenkinson, W.E., and Anderson, G. (2017). Redefining thymus medulla specialization for central tolerance. J Exp Med *214*, 3183-3195.

Cosway, E.J., Ohigashi, I., Schauble, K., Parnell, S.M., Jenkinson, W.E., Luther, S., Takahama, Y., and Anderson, G. (2018). Formation of the Intrathymic Dendritic Cell Pool Requires CCL21-Mediated Recruitment of CCR7. J Immunol *201*, 516-523.

Cowan, J.E., Baik, S., McCarthy, N.I., Parnell, S.M., White, A.J., Jenkinson, W.E., and Anderson, G. (2018). Aire controls the recirculation of murine Foxp3. Eur J Immunol *48*, 844-854.

Croxford, A.L., Lanzinger, M., Hartmann, F.J., Schreiner, B., Mair, F., Pelczar, P., Clausen, B.E., Jung, S., Greter, M., and Becher, B. (2015). The Cytokine GM-CSF Drives the Inflammatory Signature of CCR2+ Monocytes and Licenses Autoimmunity. Immunity *43*, 502-514.

D'Amico, A., and Wu, L. (2003). The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. J Exp Med *198*, 293-303.

Danan-Gotthold, M., Guyon, C., Giraud, M., Levanon, E.Y., and Abramson, J. (2016). Extensive RNA editing and splicing increase immune self-representation diversity in medullary thymic epithelial cells. Genome Biol *17*, 219.

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

den Haan, J.M., Lehar, S.M., and Bevan, M.J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med *192*, 1685-1696.

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

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

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

Detanico, T., Heiser, R.A., Aviszus, K., Bonorino, C., and Wysocki, L.J. (2011). Self-tolerance checkpoints in CD4 T cells specific for a peptide derived from the B cell antigen receptor. J Immunol *187*, 82-91.

Dhalla, F., Baran-Gale, J., Maio, S., Chappell, L., Holländer, G.A., and Ponting, C.P. (2020). Biologically indeterminate yet ordered promiscuous gene expression in single medullary thymic epithelial cells. EMBO J *39*, e101828.

Dobeš, J., Edenhofer, F., Vobořil, M., Brabec, T., Dobešová, M., Čepková, A., Klein, L., Rajewsky, K., and Filipp, D. (2018). A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire. Eur J Immunol *48*, 546-548.

Dobeš, J., Neuwirth, A., Dobešová, M., Vobořil, M., Balounová, J., Ballek, O., Lebl, J., Meloni, A., Krohn, K., Kluger, N*., et al.* (2015). Gastrointestinal Autoimmunity Associated With Loss of Central Tolerance to Enteric α-Defensins. Gastroenterology *149*, 139-150.

Dress, R.J., Dutertre, C.A., Giladi, A., Schlitzer, A., Low, I., Shadan, N.B., Tay, A., Lum, J., Kairi, M.F.B.M., Hwang, Y.Y., *et al.* (2019). Plasmacytoid dendritic cells develop from Ly6D. Nat Immunol *20*, 852-864.

Dubrot, J., Duraes, F.V., Harlé, G., Schlaeppi, A., Brighouse, D., Madelon, N., Göpfert, C., Stokar-Regenscheit, N., Acha-Orbea, H., Reith, W., *et al.* (2018). Absence of MHC-II expression by lymph node stromal cells results in autoimmunity. Life Sci Alliance *1*, e201800164.

Dubrot, J., Duraes, F.V., Potin, L., Capotosti, F., Brighouse, D., Suter, T., LeibundGut-Landmann, S., Garbi, N., Reith, W., Swartz, M.A., *et al.* (2014). Lymph node stromal cells acquire peptide-MHCII complexes from dendritic cells and induce antigen-specific CD4+ T cell tolerance. J Exp Med *211*, 1153-1166.

Dudziak, D., Kamphorst, A.O., Heidkamp, G.F., Buchholz, V.R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H.W., Park, C.G., *et al.* (2007). Differential antigen processing by dendritic cell subsets in vivo. Science *315*, 107-111.

Durai, V., and Murphy, K.M. (2016). Functions of Murine Dendritic Cells. Immunity *45*, 719-736.

Eberl, G. (2010). A new vision of immunity: homeostasis of the superorganism. Mucosal Immunol *3*, 450-460.

Ekwall, O., Hedstrand, H., Grimelius, L., Haavik, J., Perheentupa, J., Gustafsson, J., Husebye, E., Kämpe, O., and Rorsman, F. (1998). Identification of tryptophan hydroxylase as an intestinal autoantigen. Lancet *352*, 279-283.

Esashi, E., Sekiguchi, T., Ito, H., Koyasu, S., and Miyajima, A. (2003). Cutting Edge: A possible role for CD4+ thymic macrophages as professional scavengers of apoptotic thymocytes. J Immunol *171*, 2773-2777.

Farin, H.F., Karthaus, W.R., Kujala, P., Rakhshandehroo, M., Schwank, G., Vries, R.G., Kalkhoven, E., Nieuwenhuis, E.E., and Clevers, H. (2014). Paneth cell extrusion and release of antimicrobial products is directly controlled by immune cell-derived IFN-γ. J Exp Med *211*, 1393-1405.

Farr, A.G., Dooley, J.L., and Erickson, M. (2002). Organization of thymic medullary epithelial heterogeneity: implications for mechanisms of epithelial differentiation. Immunol Rev *189*, 20-27.

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

Fergusson, J.R., Morgan, M.D., Bruchard, M., Huitema, L., Heesters, B.A., van Unen, V., van Hamburg, J.P., van der Wel, N.N., Picavet, D., Koning, F., *et al.* (2018). Maturing Human CD127+ CCR7+ PDL1+ Dendritic Cells Express AIRE in the Absence of Tissue Restricted Antigens. Front Immunol *9*, 2902.

Filipp, D., Brabec, T., Vobořil, M., and Dobeš, J. (2018). Enteric α-defensins on the verge of intestinal immune tolerance and inflammation. Semin Cell Dev Biol.

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

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

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol *4*, 330-336.

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

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

Fujikado, N., Mann, A.O., Bansal, K., Romito, K.R., Ferre, E.M.N., Rosenzweig, S.D., Lionakis, M.S., Benoist, C., and Mathis, D. (2016). Aire Inhibits the Generation of a Perinatal Population of Interleukin-17A-Producing γδ T Cells to Promote Immunologic Tolerance. Immunity *45*, 999-1012.

Fukata, M., Chen, A., Vamadevan, A.S., Cohen, J., Breglio, K., Krishnareddy, S., Hsu, D., Xu, R., Harpaz, N., Dannenberg, A.J.*, et al.* (2007). Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterology *133*, 1869-1881.

Gaboriau-Routhiau, V., Rakotobe, S., Lécuyer, E., Mulder, I., Lan, A., Bridonneau, C., Rochet, V., Pisi, A., De Paepe, M., Brandi, G.*, et al.* (2009). The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity *31*, 677-689.

Gallegos, A.M., and Bevan, M.J. (2004). Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J Exp Med *200*, 1039-1049.

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

Gardner, J.M., Fletcher, A.L., Anderson, M.S., and Turley, S.J. (2009). AIRE in the thymus and beyond. Curr Opin Immunol *21*, 582-589.

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

Gerbe, F., Legraverend, C., and Jay, P. (2012). The intestinal epithelium tuft cells: specification and function. Cell Mol Life Sci *69*, 2907-2917.

Ghosh, H.S., Cisse, B., Bunin, A., Lewis, K.L., and Reizis, B. (2010). Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. Immunity *33*, 905-916.

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

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

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. Eur J Immunol *39*, 956-964.

Gossens, K., Naus, S., Corbel, S.Y., Lin, S., Rossi, F.M., Kast, J., and Ziltener, H.J. (2009). Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. J Exp Med *206*, 761-778.

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

Gray, D.H., Gavanescu, I., Benoist, C., and Mathis, D. (2007b). Danger-free autoimmune disease in Aire-deficient mice. Proc Natl Acad Sci U S A *104*, 18193-18198.

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

Gu, B., Lambert, J.P., Cockburn, K., Gingras, A.C., and Rossant, J. (2017). AIRE is a critical spindle-associated protein in embryonic stem cells. Elife *6*.

Guerau-de-Arellano, M., Martinic, M., Benoist, C., and Mathis, D. (2009). Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. J Exp Med *206*, 1245-1252.

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. J Biol Chem *292*, 6542-6554.

Guilliams, M., Dutertre, C.A., Scott, C.L., McGovern, N., Sichien, D., Chakarov, S., Van Gassen, S., Chen, J., Poidinger, M., De Prijck, S.*, et al.* (2016). Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. Immunity *45*, 669-684.

Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B.U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. Nat Rev Immunol *14*, 571-578.

Haas, T., Metzger, J., Schmitz, F., Heit, A., Müller, T., Latz, E., and Wagner, H. (2008). The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. Immunity *28*, 315-323.

Hacker, C., Kirsch, R.D., Ju, X.S., Hieronymus, T., Gust, T.C., Kuhl, C., Jorgas, T., Kurz, S.M., Rose-John, S., Yokota, Y.*, et al.* (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. Nat Immunol *4*, 380-386.

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

Hadis, U., Wahl, B., Schulz, O., Hardtke-Wolenski, M., Schippers, A., Wagner, N., Müller, W., Sparwasser, T., Förster, R., and Pabst, O. (2011). Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. Immunity *34*, 237-246.

Haljasorg, U., Bichele, R., Saare, M., Guha, M., Maslovskaja, J., Kond, K., Remm, A., Pihlap, M., Tomson, L., Kisand, K.*, et al.* (2015). A highly conserved NF-kappaB-responsive enhancer is critical for thymic expression of Aire in mice. Eur J Immunol *45*, 3246-3256.

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

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. Nat Immunol *8*, 304-311.

Hassler, T., Urmann, E., Teschner, S., Federle, C., Dileepan, T., Schober, K., Jenkins, M.K., Busch, D.H., Hinterberger, M., and Klein, L. (2019). Inventories of naive and tolerant mouse CD4 T cell repertoires reveal a hierarchy of deleted and diverted T cell receptors. Proc Natl Acad Sci U S A *116*, 18537-18543.

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

Hedstrand, H., Ekwall, O., Haavik, J., Landgren, E., Betterle, C., Perheentupa, J., Gustafsson, J., Husebye, E., Rorsman, F., and Kämpe, O. (2000). Identification of tyrosine hydroxylase as an autoantigen in autoimmune polyendocrine syndrome type I. Biochem Biophys Res Commun *267*, 456-461.

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

Hemmers, S., Schizas, M., Azizi, E., Dikiy, S., Zhong, Y., Feng, Y., Altan-Bonnet, G., and Rudensky, A.Y. (2019). IL-2 production by self-reactive CD4 thymocytes scales regulatory T cell generation in the thymus. J Exp Med *216*, 2466-2478.

Hepworth, M.R., Fung, T.C., Masur, S.H., Kelsen, J.R., McConnell, F.M., Dubrot, J., Withers, D.R., Hugues, S., Farrar, M.A., Reith, W.*, et al.* (2015). Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells. Science *348*, 1031-1035.

Hepworth, M.R., Monticelli, L.A., Fung, T.C., Ziegler, C.G., Grunberg, S., Sinha, R., Mantegazza, A.R., Ma, H.L., Crawford, A., Angelosanto, J.M.*, et al.* (2013). Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature *498*, 113-117.

Herbin, O., Bonito, A.J., Jeong, S., Weinstein, E.G., Rahman, A.H., Xiong, H., Merad, M., and Alexandropoulos, K. (2016). Medullary thymic epithelial cells and CD8α. J Autoimmun *75*, 141-149.

Hettinger, J., Richards, D.M., Hansson, J., Barra, M.M., Joschko, A.C., Krijgsveld, J., and Feuerer, M. (2013). Origin of monocytes and macrophages in a committed progenitor. Nat Immunol *14*, 821-830.

Hikosaka, Y., Nitta, T., Ohigashi, I., Yano, K., Ishimaru, N., Hayashi, Y., Matsumoto, M., Matsuo, K., Penninger, J.M., Takayanagi, H.*, et al.* (2008). The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. Immunity *29*, 438-450.

Hildner, K., Edelson, B.T., Purtha, W.E., Diamond, M., Matsushita, H., Kohyama, M., Calderon, B., Schraml, B.U., Unanue, E.R., Diamond, M.S.*, et al.* (2008). Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science *322*, 1097-1100.

Hinterberger, M., Aichinger, M., Prazeres da Costa, O., Voehringer, D., Hoffmann, R., and Klein, L. (2010). Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. Nat Immunol *11*, 512-519.

Hodgkin, P.D. (2018). Modifying clonal selection theory with a probabilistic cell. Immunol Rev *285*, 249-262.

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

Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17-27.

Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A.*, et al.* (2005). Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med *11*, 263-270.

Hou, B., Reizis, B., and DeFranco, A.L. (2008). Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity *29*, 272-282.

Hozumi, K., Mailhos, C., Negishi, N., Hirano, K., Yahata, T., Ando, K., Zuklys, S., Holländer, G.A., Shima, D.T., and Habu, S. (2008). Delta-like 4 is indispensable in thymic environment specific for T cell development. J Exp Med *205*, 2507-2513.

Hu, Z., Lancaster, J.N., Sasiponganan, C., and Ehrlich, L.I. (2015). CCR4 promotes medullary entry and thymocyte-dendritic cell interactions required for central tolerance. J Exp Med *212*, 1947-1965.

Hu, Z., Li, Y., Van Nieuwenhuijze, A., Selden, H.J., Jarrett, A.M., Sorace, A.G., Yankeelov, T.E., Liston, A., and Ehrlich, L.I.R. (2017). CCR7 Modulates the Generation of Thymic Regulatory T Cells by Altering the Composition of the Thymic Dendritic Cell Compartment. Cell Rep *21*, 168-180.

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

Huang, H.B., Xiang, Q.H., Wu, H., Ansari, A.R., Wen, L., Ge, X.H., Wang, J.X., Peng, K.M., and Liu, H.Z. (2014). TLR4 is constitutively expressed in chick thymic epithelial cells. Vet Immunol Immunopathol *158*, 182-188.

Hubert, F.X., Kinkel, S.A., Crewther, P.E., Cannon, P.Z., Webster, K.E., Link, M., Uibo, R., O'Bryan, M.K., Meager, A., Forehan, S.P.*, et al.* (2009). Aire-deficient C57BL/6 mice mimicking the common human 13-base pair deletion mutation present with only a mild autoimmune phenotype. J Immunol *182*, 3902-3918.

Hubert, F.X., Kinkel, S.A., Davey, G.M., Phipson, B., Mueller, S.N., Liston, A., Proietto, A.I., Cannon, P.Z., Forehan, S., Smyth, G.K.*, et al.* (2011). Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. Blood *118*, 2462-2472.

Hubert, F.X., Kinkel, S.A., Webster, K.E., Cannon, P., Crewther, P.E., Proeitto, A.I., Wu, L., Heath, W.R., and Scott, H.S. (2008). A specific anti-Aire antibody reveals aire expression is restricted to medullary thymic epithelial cells and not expressed in periphery. J Immunol *180*, 3824-3832.

Huoh, Y.S., Wu, B., Park, S., Yang, D., Bansal, K., Greenwald, E., Wong, W.P., Mathis, D., and Hur, S. (2020). Dual functions of Aire CARD multimerization in the transcriptional regulation of T cell tolerance. Nat Commun *11*, 1625.

Husebye, E.S., Anderson, M.S., and Kämpe, O. (2018). Autoimmune Polyendocrine Syndromes. N Engl J Med *378*, 2543-2544.

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

Chaudhry, A., Samstein, R.M., Treuting, P., Liang, Y., Pils, M.C., Heinrich, J.M., Jack, R.S., Wunderlich, F.T., Brüning, J.C., Müller, W.*, et al.* (2011). Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity *34*, 566-578.

Chen, E.L.Y., Thompson, P.K., and Zúñiga-Pflücker, J.C. (2019). RBPJ-dependent Notch signaling initiates the T cell program in a subset of thymus-seeding progenitors. Nat Immunol *20*, 1456-1468.

Chen, K., Zhao, B.S., and He, C. (2016). Nucleic Acid Modifications in Regulation of Gene Expression. Cell Chem Biol *23*, 74-85.

Chen, Z.J. (2012). Ubiquitination in signaling to and activation of IKK. Immunol Rev *246*, 95-106.

Choe, J., Kelker, M.S., and Wilson, I.A. (2005). Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science *309*, 581-585.

Chuprin, A., Avin, A., Goldfarb, Y., Herzig, Y., Levi, B., Jacob, A., Sela, A., Katz, S., Grossman, M., Guyon, C.*, et al.* (2015). The deacetylase Sirt1 is an essential regulator of Aire-mediated induction of central immunological tolerance. Nat Immunol *16*, 737-745.

Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C., and Song, S.Y. (2004). Retinoic acid imprints gut-homing specificity on T cells. Immunity *21*, 527-538.

Jameson, S.C., Hogquist, K.A., and Bevan, M.J. (1994). Specificity and flexibility in thymic selection. Nature *369*, 750-752.

Janeway, C.A., and Medzhitov, R. (2002). Innate immune recognition. Annu Rev Immunol *20*, 197-216.

Jang, M.H., Sougawa, N., Tanaka, T., Hirata, T., Hiroi, T., Tohya, K., Guo, Z., Umemoto, E., Ebisuno, Y., Yang, B.G.*, et al.* (2006). CCR7 is critically important for migration of dendritic cells in intestinal lamina propria to mesenteric lymph nodes. J Immunol *176*, 803-810.

Jenkinson, W.E., Rossi, S.W., Parnell, S.M., Agace, W.W., Takahama, Y., Jenkinson, E.J., and Anderson, G. (2007). Chemokine receptor expression defines heterogeneity in the earliest thymic migrants. Eur J Immunol *37*, 2090-2096.

Jiang, D., Liang, J., Fan, J., Yu, S., Chen, S., Luo, Y., Prestwich, G.D., Mascarenhas, M.M., Garg, H.G., Quinn, D.A., *et al.* (2005a). Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med *11*, 1173-1179.

Jiang, W., Anderson, M.S., Bronson, R., Mathis, D., and Benoist, C. (2005b). Modifier loci condition autoimmunity provoked by Aire deficiency. J Exp Med *202*, 805-815.

Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., and Li, X. (2002). Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Mol Cell Biol *22*, 7158-7167.

Jin, M.S., Kim, S.E., Heo, J.Y., Lee, M.E., Kim, H.M., Paik, S.G., Lee, H., and Lee, J.O. (2007). Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell *130*, 1071-1082.

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 self-peptide. Nat Immunol *2*, 301-306.

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

Kadouri, N., Nevo, S., Goldfarb, Y., and Abramson, J. (2020). Thymic epithelial cell heterogeneity: TEC by TEC. Nat Rev Immunol *20*, 239-253.

Kajiura, F., Sun, S., Nomura, T., Izumi, K., Ueno, T., Bando, Y., Kuroda, N., Han, H., Li, Y., Matsushima, A., *et al.* (2004). NF-kappa B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner. J Immunol *172*, 2067-2075.

Kalekar, L.A., Schmiel, S.E., Nandiwada, S.L., Lam, W.Y., Barsness, L.O., Zhang, N., Stritesky, G.L., Malhotra, D., Pauken, K.E., Linehan, J.L., *et al.* (2016). CD4(+) T cell anergy prevents autoimmunity and generates regulatory T cell precursors. Nat Immunol *17*, 304-314.

Kang, J.Y., Nan, X., Jin, M.S., Youn, S.J., Ryu, Y.H., Mah, S., Han, S.H., Lee, H., Paik, S.G., and Lee, J.O. (2009). Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity *31*, 873-884.

Kappler, J.W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. Cell *49*, 273-280.

Kawagoe, T., Sato, S., Matsushita, K., Kato, H., Matsui, K., Kumagai, Y., Saitoh, T., Kawai, T., Takeuchi, O., and Akira, S. (2008). Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. Nat Immunol *9*, 684-691.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol *11*, 373-384.

Kawasaki, T., and Kawai, T. (2014). Toll-like receptor signaling pathways. Front Immunol *5*, 461.

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. J Exp Med *211*, 761-768.

Kim, H.M., Park, B.S., Kim, J.I., Kim, S.E., Lee, J., Oh, S.C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O.J., *et al.* (2007a). Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. Cell *130*, 906-917.

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

Kim, M., Galan, C., Hill, A.A., Wu, W.J., Fehlner-Peach, H., Song, H.W., Schady, D., Bettini, M.L., Simpson, K.W., Longman, R.S., *et al.* (2018). Critical Role for the Microbiota in CX. Immunity *49*, 151-163.e155.

Kim, S., Takahashi, H., Lin, W.W., Descargues, P., Grivennikov, S., Kim, Y., Luo, J.L., and Karin, M. (2009). Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. Nature *457*, 102-106.

Kim, Y.M., Brinkmann, M.M., Paquet, M.E., and Ploegh, H.L. (2008). UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. Nature *452*, 234-238.

Kinoshita, D., Hirota, F., Kaisho, T., Kasai, M., Izumi, K., Bando, Y., Mouri, Y., Matsushima, A., Niki, S., Han, H., *et al.* (2006). Essential role of IkappaB kinase alpha in thymic organogenesis required for the establishment of self-tolerance. J Immunol *176*, 3995-4002.

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

Klein, L. (2009). Dead man walking: how thymocytes scan the medulla. Nat Immunol *10*, 809-811.

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

Klein, L., and Jovanovic, K. (2011). Regulatory T cell lineage commitment in the thymus. Semin Immunol *23*, 401-409.

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. J Exp Med *188*, 5-16.

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

Klein, L., Robey, E.A., and Hsieh, C.S. (2019). Central CD4. Nat Rev Immunol *19*, 7-18.

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

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., *et al.* (2008). Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity. Proc Natl Acad Sci U S A *105*, 15878-15883.

Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., Manley, N.R., Duarte, A., Macdonald, H.R., and Radtke, F. (2008). Delta-like 4 is the essential,

nonredundant ligand for Notch1 during thymic T cell lineage commitment. J Exp Med *205*, 2515-2523.

Kollewe, C., Mackensen, A.C., Neumann, D., Knop, J., Cao, P., Li, S., Wesche, H., and Martin, M.U. (2004). Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. J Biol Chem *279*, 5227-5236.

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

Kroger, C.J., Spidale, N.A., Wang, B., and Tisch, R. (2017). Thymic Dendritic Cell Subsets Display Distinct Efficiencies and Mechanisms of Intercellular MHC Transfer. J Immunol *198*, 249-256.

Kumamoto, Y., Linehan, M., Weinstein, J.S., Laidlaw, B.J., Craft, J.E., and Iwasaki, A. (2013). CD301b$^{+}$ dermal dendritic cells drive T helper 2 cell-mediated immunity. Immunity *39*, 733-743.

Kunkel, E.J., Campbell, J.J., Haraldsen, G., Pan, J., Boisvert, J., Roberts, A.I., Ebert, E.C., Vierra, M.A., Goodman, S.B., Genovese, M.C.*, et al.* (2000). Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. J Exp Med *192*, 761-768.

Kurd, N.S., Lutes, L.K., Yoon, J., Chan, S.W., Dzhagalov, I.L., Hoover, A.R., and Robey, E.A. (2019). A role for phagocytosis in inducing cell death during thymocyte negative selection. Elife *8*.

Kwan, J., and Killeen, N. (2004). CCR7 directs the migration of thymocytes into the thymic medulla. J Immunol *172*, 3999-4007.

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

Laan, M., Kisand, K., Kont, V., Moll, 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. J Immunol *183*, 7682-7691.

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. J Exp Med *212*, 1993-2002.

Lancaster, J.N., Li, Y., and Ehrlich, L.I.R. (2018). Chemokine-Mediated Choreography of Thymocyte Development and Selection. Trends Immunol *39*, 86-98.

Lancaster, J.N., Thyagarajan, H.M., Srinivasan, J., Li, Y., Hu, Z., and Ehrlich, L.I.R. (2019). Live-cell imaging reveals the relative contributions of antigen-presenting cell subsets to thymic central tolerance. Nat Commun *10*, 2220.

Lande, R., Gregorio, J., Facchinetti, V., Chatterjee, B., Wang, Y.H., Homey, B., Cao, W., Su, B., Nestle, F.O., Zal, T.*, et al.* (2007). Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature *449*, 564-569.

Lathrop, S.K., Bloom, S.M., Rao, S.M., Nutsch, K., Lio, C.W., Santacruz, N., Peterson, D.A., Stappenbeck, T.S., and Hsieh, C.S. (2011). Peripheral education of the immune system by colonic commensal microbiota. Nature *478*, 250-254.

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 *37*, 475-486.

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

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 *43*, 896-908.

Lei, Y., Ripen, A.M., Ishimaru, N., Ohigashi, I., Nagasawa, T., Jeker, L.T., Bosl, M.R., Hollander, G.A., Hayashi, Y., Malefyt Rde, W.*, et al.* (2011). Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development. J Exp Med *208*, 383-394.

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.*, et al.* (2016). Dendritic Cells Coordinate the Development and Homeostasis of Organ-Specific Regulatory T Cells. Immunity *44*, 847-859.

Lewis, K.L., Caton, M.L., Bogunovic, M., Greter, M., Grajkowska, L.T., Ng, D., Klinakis, A., Charo, I.F., Jung, S., Gommerman, J.L.*, et al.* (2011). Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. Immunity *35*, 780-791.

Li, J., Park, J., Foss, D., and Goldschneider, I. (2009). Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. J Exp Med *206*, 607-622.

Li, M.O., Sanjabi, S., and Flavell, R.A. (2006). Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. Immunity *25*, 455-471.

Lin, J., Yang, L., Silva, H.M., Trzeciak, A., Choi, Y., Schwab, S.R., Dustin, M.L., and Lafaille, J.J. (2016). Increased generation of Foxp3(+) regulatory T cells by manipulating antigen presentation in the thymus. Nat Commun *7*, 10562.

Lin, S.C., Lo, Y.C., and Wu, H. (2010). Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. Nature *465*, 885-890.

Lind, E.F., Prockop, S.E., Porritt, H.E., and Petrie, H.T. (2001). Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. J Exp Med *194*, 127-134.

Lio, C.W., and Hsieh, C.S. (2008). A two-step process for thymic regulatory T cell development. Immunity *28*, 100-111.

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

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

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

Liu, K., Victora, G.D., Schwickert, T.A., Guermonprez, P., Meredith, M.M., Yao, K., Chu, F.F., Randolph, G.J., Rudensky, A.Y., and Nussenzweig, M. (2009). In vivo analysis of dendritic cell development and homeostasis. Science *324*, 392-397.

Lkhagvasuren, E., Sakata, M., Ohigashi, I., and Takahama, Y. (2013). Lymphotoxin β receptor regulates the development of CCL21-expressing subset of postnatal medullary thymic epithelial cells. J Immunol *190*, 5110-5117.

Lu, F.T., Yang, W., Wang, Y.H., Ma, H.D., Tang, W., Yang, J.B., Li, L., Ansari, A.A., and Lian, Z.X. (2015). Thymic B cells promote thymus-derived regulatory T cell development and proliferation. J Autoimmun *61*, 62-72.

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. Nat Immunol *15*, 638-645.

MacNabb, B.W., Kline, D.E., Albright, A.R., Chen, X., Leventhal, D.S., Savage, P.A., and Kline, J. (2019). Negligible Role for Deletion Mediated by cDC1 in CD8. J Immunol *202*, 2628-2635.

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

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.*, et al.* (2016). Tolerance is established in polyclonal CD4(+) T cells by distinct mechanisms, according to self-peptide expression patterns. Nat Immunol *17*, 187-195.

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 *44*, 1102-1113.

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

Mancuso, G., Gambuzza, M., Midiri, A., Biondo, C., Papasergi, S., Akira, S., Teti, G., and Beninati, C. (2009). Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. Nat Immunol *10*, 587-594.

Manley, N.R., and Condie, B.G. (2010). Transcriptional regulation of thymus organogenesis and thymic epithelial cell differentiation. Prog Mol Biol Transl Sci *92*, 103-120.

Marie, J.C., Liggitt, D., and Rudensky, A.Y. (2006). Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity *25*, 441-454.

Mariño, E., Richards, J.L., McLeod, K.H., Stanley, D., Yap, Y.A., Knight, J., McKenzie, C., Kranich, J., Oliveira, A.C., Rossello, F.J.*, et al.* (2017). Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. Nat Immunol *18*, 552-562.

Marson, A., Housley, W.J., and Hafler, D.A. (2015). Genetic basis of autoimmunity. J Clin Invest *125*, 2234-2241.

Mashayekhi, M., Sandau, M.M., Dunay, I.R., Frickel, E.M., Khan, A., Goldszmid, R.S., Sher, A., Ploegh, H.L., Murphy, T.L., Sibley, L.D.*, et al.* (2011). CD8α(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. Immunity *35*, 249-259.

McCaughtry, T.M., Baldwin, T.A., Wilken, M.S., and Hogquist, K.A. (2008). Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. J Exp Med *205*, 2575-2584.

Means, T.K., Latz, E., Hayashi, F., Murali, M.R., Golenbock, D.T., and Luster, A.D. (2005). Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest *115*, 407-417.

Means, T.K., and Luster, A.D. (2005). Toll-like receptor activation in the pathogenesis of systemic lupus erythematosus. Ann N Y Acad Sci *1062*, 242-251.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Meredith, M., Zemmour, D., Mathis, D., and Benoist, C. (2015). Aire controls gene expression in the thymic epithelium with ordered stochasticity. Nat Immunol *16*, 942-949.

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

Meyer, S., Woodward, M., Hertel, C., Vlaicu, P., Haque, Y., Kärner, J., Macagno, A., Onuoha, S.C., Fishman, D., Peterson, H.*, et al.* (2016). AIRE-Deficient Patients Harbor Unique High-Affinity Disease-Ameliorating Autoantibodies. Cell *166*, 582-595.

Michel, C., Miller, C.N., Küchler, R., Brors, B., Anderson, M.S., Kyewski, B., and Pinto, S. (2017). Revisiting the Road Map of Medullary Thymic Epithelial Cell Differentiation. J Immunol *199*, 3488-3503.

Miller, C.N., Proekt, I., von Moltke, J., Wells, K.L., Rajpurkar, A.R., Wang, H., Rattay, K., Khan, I.S., Metzger, T.C., Pollack, J.L.*, et al.* (2018). Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development. Nature *559*, 627-631.

Millet, V., Naquet, P., and Guinamard, R.R. (2008). Intercellular MHC transfer between thymic epithelial and dendritic cells. Eur J Immunol *38*, 1257-1263.

Miragaia, R.J., Zhang, X., Gomes, T., Svensson, V., Ilicic, T., Henriksson, J., Kar, G., and Lönnberg, T. (2018). Single-cell RNA-sequencing resolves self-antigen expression during mTEC development. Sci Rep *8*, 685.

Misslitz, A., Pabst, O., Hintzen, G., Ohl, L., Kremmer, E., Petrie, H.T., and Förster, R. (2004). Thymic T cell development and progenitor localization depend on CCR7. J Exp Med *200*, 481-491.

Modigliani, Y., Thomas-Vaslin, V., Bandeira, A., Coltey, M., Le Douarin, N.M., Coutinho, A., and Salaün, J. (1995). Lymphocytes selected in allogeneic thymic epithelium mediate dominant tolerance toward tissue grafts of the thymic epithelium haplotype. Proc Natl Acad Sci U S A *92*, 7555-7559.

Mortha, A., Chudnovskiy, A., Hashimoto, D., Bogunovic, M., Spencer, S.P., Belkaid, Y., and Merad, M. (2014). Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. Science *343*, 1249288.

Mouri, Y., Ueda, Y., Yamano, T., Matsumoto, M., Tsuneyama, K., and Kinashi, T. (2017). Mode of Tolerance Induction and Requirement for Aire Are Governed by the Cell Types That Express Self-Antigen and Those That Present Antigen. J Immunol *199*, 3959-3971.

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

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*, 1349-1353.

Nadafi, R., Gago de Graça, C., Keuning, E.D., Koning, J.J., de Kivit, S., Konijn, T., Henri, S., Borst, J., Reijmers, R.M., van Baarsen, L.G.M.*, et al.* (2020). Lymph Node Stromal Cells Generate Antigen-Specific Regulatory T Cells and Control Autoreactive T and B Cell Responses. Cell Rep *30*, 4110-4123.e4114.

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

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

Nakagawa, Y., Ohigashi, I., Nitta, T., Sakata, M., Tanaka, K., Murata, S., Kanagawa, O., and Takahama, Y. (2012). Thymic nurse cells provide microenvironment for secondary T cell receptor α rearrangement in cortical thymocytes. Proc Natl Acad Sci U S A *109*, 20572-20577.

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

Nehls, M., Kyewski, B., Messerle, M., Waldschütz, R., Schüddekopf, K., Smith, A.J., and Boehm, T. (1996). Two genetically separable steps in the differentiation of thymic epithelium. Science *272*, 886-889.

Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G.*, et al.* (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science *307*, 254-258.

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

Nishikawa, Y., Hirota, F., Yano, M., Kitajima, H., Miyazaki, J., Kawamoto, H., Mouri, Y., and Matsumoto, M. (2010). Biphasic Aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation. J Exp Med *207*, 963-971.

Nutsch, K.M., and Hsieh, C.S. (2012). T cell tolerance and immunity to commensal bacteria. Curr Opin Immunol *24*, 385-391.

Oftedal, B.E., Hellesen, A., Erichsen, M.M., Bratland, E., Vardi, A., Perheentupa, J., Kemp, E.H., Fiskerstrand, T., Viken, M.K., Weetman, A.P.*, et al.* (2015). Dominant Mutations in the

Autoimmune Regulator AIRE Are Associated with Common Organ-Specific Autoimmune Diseases. Immunity *42*, 1185-1196.

Oganesyan, G., Saha, S.K., Guo, B., He, J.Q., Shahangian, A., Zarnegar, B., Perry, A., and Cheng, G. (2006). Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature *439*, 208-211.

Ohnmacht, C., Park, J.H., Cording, S., Wing, J.B., Atarashi, K., Obata, Y., Gaboriau-Routhiau, V., Marques, R., Dulauroy, S., Fedoseeva, M.*, et al.* (2015). MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORγt$^{+}$ T cells. Science *349*, 989-993.

Ohnmacht, C., Pullner, A., King, S.B., 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. J Exp Med *206*, 549-559.

Onder, L., Nindl, V., Scandella, E., Chai, Q., Cheng, H.W., Caviezel-Firner, S., Novkovic, M., Bomze, D., Maier, R., Mair, F.*, et al.* (2015). Alternative NF-κB signaling regulates mTEC differentiation from podoplanin-expressing precursors in the cortico-medullary junction. Eur J Immunol *45*, 2218-2231.

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

Ouyang, W., Beckett, O., Ma, Q., and Li, M.O. (2010). Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development. Immunity *32*, 642-653.

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

Owen, D.L., Mahmud, S.A., Sjaastad, L.E., Williams, J.B., Spanier, J.A., Simeonov, D.R., Ruscher, R., Huang, W., Proekt, I., Miller, C.N.*, et al.* (2019). Thymic regulatory T cells arise via two distinct developmental programs. Nat Immunol *20*, 195-205.

Owen, D.L., Mahmud, S.A., Vang, K.B., Kelly, R.M., Blazar, B.R., Smith, K.A., and Farrar, M.A. (2018). Identification of Cellular Sources of IL-2 Needed for Regulatory T Cell Development and Homeostasis. J Immunol *200*, 3926-3933.

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

Park, B.S., Song, D.H., Kim, H.M., Choi, B.S., Lee, H., and Lee, J.O. (2009). The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature *458*, 1191-1195.

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

Perheentupa, J. (2006). Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. J Clin Endocrinol Metab *91*, 2843-2850.

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

Perry, J.S., and Hsieh, C.S. (2016). Development of T-cell tolerance utilizes both cell-autonomous and cooperative presentation of self-antigen. Immunol Rev *271*, 141-155.

Perry, J.S.A., Lio, C.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 *41*, 414-426.

Perry, J.S.A., Russler-Germain, E.V., Zhou, Y.W., Purtha, W., Cooper, M.L., Choi, J., Schroeder, M.A., Salazar, V., Egawa, T., Lee, B.C.*, et al.* (2018). Transfer of Cell-Surface Antigens by Scavenger Receptor CD36 Promotes Thymic Regulatory T Cell Receptor Repertoire Development and Allo-tolerance. Immunity *48*, 1271.

Petrie, H.T., and Zúñiga-Pflücker, J.C. (2007). Zoned out: functional mapping of stromal signaling microenvironments in the thymus. Annu Rev Immunol *25*, 649-679.

Pitkänen, J., Doucas, V., Sternsdorf, T., Nakajima, T., Aratani, S., Jensen, K., Will, H., Vähämurto, P., Ollila, J., Vihinen, M.*, et al.* (2000). The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein. J Biol Chem *275*, 16802-16809.

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

Plotkin, J., Prockop, S.E., Lepique, A., and Petrie, H.T. (2003). Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. J Immunol *171*, 4521-4527.

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

Posovszky, C., Lahr, G., von Schnurbein, J., Buderus, S., Findeisen, A., Schröder, C., Schütz, C., Schulz, A., Debatin, K.M., Wabitsch, M.*, et al.* (2012). Loss of enteroendocrine cells in autoimmune-polyendocrine-candidiasis-ectodermal-dystrophy (APECED) syndrome with gastrointestinal dysfunction. J Clin Endocrinol Metab *97*, E292-300.

Price, A.E., Shamardani, K., Lugo, K.A., Deguine, J., Roberts, A.W., Lee, B.L., and Barton, G.M. (2018). A Map of Toll-like Receptor Expression in the Intestinal Epithelium Reveals Distinct Spatial, Cell Type-Specific, and Temporal Patterns. Immunity *49*, 560-575.e566.

Proietto, A.I., van Dommelen, S., Zhou, P., Rizzitelli, A., D'Amico, A., Steptoe, R.J., Naik, S.H., Lahoud, M.H., Liu, Y., Zheng, P.*, et al.* (2008). Dendritic cells in the thymus contribute to T-regulatory cell induction. Proc Natl Acad Sci U S A *105*, 19869-19874.

Qiu, J., Guo, X., Chen, Z.M., He, L., Sonnenberg, G.F., Artis, D., Fu, Y.X., and Zhou, L. (2013). Group 3 innate lymphoid cells inhibit T-cell-mediated intestinal inflammation through aryl hydrocarbon receptor signaling and regulation of microflora. Immunity *39*, 386-399.

Raetz, M., Hwang, S.H., Wilhelm, C.L., Kirkland, D., Benson, A., Sturge, C.R., Mirpuri, J., Vaishnava, S., Hou, B., Defranco, A.L.*, et al.* (2013). Parasite-induced TH1 cells and intestinal dysbiosis cooperate in IFN-γ-dependent elimination of Paneth cells. Nat Immunol *14*, 136-142.

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*, 397-409.

Rezende, R.M., and Weiner, H.L. (2017). History and mechanisms of oral tolerance. Semin Immunol *30*, 3-11.

Ribeiro, A.R., Rodrigues, P.M., Meireles, C., Di Santo, J.P., and Alves, N.L. (2013). Thymocyte selection regulates the homeostasis of IL-7-expressing thymic cortical epithelial cells in vivo. J Immunol *191*, 1200-1209.

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. J Autoimmun *81*, 56-67.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., and Bazan, J.F. (1998). A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A *95*, 588-593.

Rodewald, H.R. (2008). Thymus organogenesis. Annu Rev Immunol *26*, 355-388.

Rodrigues, P.F., Alberti-Servera, L., Eremin, A., Grajales-Reyes, G.E., Ivanek, R., and Tussiwand, R. (2018). Distinct progenitor lineages contribute to the heterogeneity of plasmacytoid dendritic cells. Nat Immunol *19*, 711-722.

Ross, J.O., Melichar, H.J., Au-Yeung, B.B., Herzmark, P., Weiss, A., and Robey, E.A. (2014). Distinct phases in the positive selection of CD8+ T cells distinguished by intrathymic migration and T-cell receptor signaling patterns. Proc Natl Acad Sci U S A *111*, E2550-2558.

Rossi, S.W., Chidgey, A.P., Parnell, S.M., Jenkinson, W.E., Scott, H.S., Boyd, R.L., Jenkinson, E.J., and Anderson, G. (2007a). Redefining epithelial progenitor potential in the developing thymus. Eur J Immunol *37*, 2411-2418.

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*, 988-991.

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.*, et al.* (2007b). RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. J Exp Med *204*, 1267-1272.

Roth, D.B. (2014). V(D)J Recombination: Mechanism, Errors, and Fidelity. Microbiol Spectr *2*.

Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R.*, et al.* (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity *28*, 546-558.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol *155*, 1151-1164.

Sanos, S.L., Nowak, J., Fallet, M., and Bajenoff, M. (2011). Stromal cell networks regulate thymocyte migration and dendritic cell behavior in the thymus. J Immunol *186*, 2835-2841.

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 Res *24*, 1918-1931.

Sefik, E., Geva-Zatorsky, N., Oh, S., Konnikova, L., Zemmour, D., McGuire, A.M., Burzyn, D., Ortiz-Lopez, A., Lobera, M., Yang, J.*, et al.* (2015). MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORγ$^{+}$ regulatory T cells. Science *349*, 993-997.

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 *41*, 753-761.

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 *319*, 822-825.

Schaefer, L., Babelova, A., Kiss, E., Hausser, H.J., Baliova, M., Krzyzankova, M., Marsche, G., Young, M.F., Mihalik, D., Götte, M.*, et al.* (2005). The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J Clin Invest *115*, 2223-2233.

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. J Immunol *180*, 1338-1343.

Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H.C., Belardelli, F., and Gabriele, L. (2002). ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. J Exp Med *196*, 1415-1425.

Schlitzer, A., Sivakamasundari, V., Chen, J., Sumatoh, H.R., Schreuder, J., Lum, J., Malleret, B., Zhang, S., Larbi, A., Zolezzi, F.*, et al.* (2015). Identification of cDC1- and cDC2-committed DC progenitors reveals early lineage priming at the common DC progenitor stage in the bone marrow. Nat Immunol *16*, 718-728.

Schneider, C., O'Leary, C.E., and Locksley, R.M. (2019). Regulation of immune responses by tuft cells. Nat Rev Immunol *19*, 584-593.

Schulz, O., Jaensson, E., Persson, E.K., Liu, X., Worbs, T., Agace, W.W., and Pabst, O. (2009). Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med *206*, 3101-3114.

Skogberg, G., Telemo, E., and Ekwall, O. (2015). Exosomes in the Thymus: Antigen Transfer and Vesicles. Front Immunol *6*, 366.

Sköldberg, F., Portela-Gomes, G.M., Grimelius, L., Nilsson, G., Perheentupa, J., Betterle, C., Husebye, E.S., Gustafsson, J., Rönnblom, A., Rorsman, F.*, et al.* (2003). Histidine decarboxylase, a pyridoxal phosphate-dependent enzyme, is an autoantigen of gastric enterochromaffin-like cells. J Clin Endocrinol Metab *88*, 1445-1452.

Söderbergh, A., Myhre, A.G., Ekwall, O., Gebre-Medhin, G., Hedstrand, H., Landgren, E., Miettinen, A., Eskelin, P., Halonen, M., Tuomi, T.*, et al.* (2004). Prevalence and clinical associations of 10 defined autoantibodies in autoimmune polyendocrine syndrome type I. J Clin Endocrinol Metab *89*, 557-562.

Song, X., Sun, X., Oh, S.F., Wu, M., Zhang, Y., Zheng, W., Geva-Zatorsky, N., Jupp, R., Mathis, D., Benoist, C.*, et al.* (2020). Microbial bile acid metabolites modulate gut RORγ. Nature *577*, 410-415.

Stadinski, B.D., Blevins, S.J., Spidale, N.A., Duke, B.R., Huseby, P.G., Stern, L.J., and Huseby, E.S. (2019). A temporal thymic selection switch and ligand binding kinetics constrain neonatal Foxp3. Nat Immunol *20*, 1046-1058.

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

Stritesky, G.L., Xing, Y., Erickson, J.R., Kalekar, L.A., Wang, X., Mueller, D.L., Jameson, S.C., and Hogquist, K.A. (2013). Murine thymic selection quantified using a unique method to capture deleted T cells. Proc Natl Acad Sci U S A *110*, 4679-4684.

Sun, C.M., Hall, J.A., Blank, R.B., Bouladoux, N., Oukka, M., Mora, J.R., and Belkaid, Y. (2007). Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med *204*, 1775-1785.

Suzuki, S., Honma, K., Matsuyama, T., Suzuki, K., Toriyama, K., Akitoyo, I., Yamamoto, K., Suematsu, T., Nakamura, M., Yui, K.*, et al.* (2004). Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. Proc Natl Acad Sci U S A *101*, 8981-8986.

Swiecki, M., and Colonna, M. (2015). The multifaceted biology of plasmacytoid dendritic cells. Nat Rev Immunol *15*, 471-485.

Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J.*, et al.* (2004). Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc Natl Acad Sci U S A *101*, 3516-3521.

Tai, X., Cowan, M., Feigenbaum, L., and Singer, A. (2005). CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat Immunol *6*, 152-162.

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 *163*, 975-987.

Takahama, Y., Ohigashi, I., Baik, S., and Anderson, G. (2017). Generation of diversity in thymic epithelial cells. Nat Rev Immunol *17*, 295-305.

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

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. Proc Natl Acad Sci U S A *109*, 7847-7852.

Thiault, N., Darrigues, J., Adoue, V., Gros, M., Binet, B., Perals, C., Leobon, B., Fazilleau, N., Joffre, O.P., Robey, E.A.*, et al.* (2015). Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors. Nat Immunol *16*, 628-634.

Tian, J., Avalos, A.M., Mao, S.Y., Chen, B., Senthil, K., Wu, H., Parroche, P., Drabic, S., Golenbock, D., Sirois, C.*, et al.* (2007). Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nat Immunol *8*, 487-496.

Trampont, P.C., Tosello-Trampont, A.C., Shen, Y., Duley, A.K., Sutherland, A.E., Bender, T.P., Littman, D.R., and Ravichandran, K.S. (2010). CXCR4 acts as a costimulator during thymic beta-selection. Nat Immunol *11*, 162-170.

Tsan, M.F., and Gao, B. (2009). Heat shock proteins and immune system. J Leukoc Biol *85*, 905-910.

Uematsu, S., Fujimoto, K., Jang, M.H., Yang, B.G., Jung, Y.J., Nishiyama, M., Sato, S., Tsujimura, T., Yamamoto, M., Yokota, Y.*, et al.* (2008). Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol *9*, 769-776.

Ueno, T., Saito, F., Gray, D.H., 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. J Exp Med *200*, 493-505.

Vaishnava, S., Behrendt, C.L., Ismail, A.S., Eckmann, L., and Hooper, L.V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc Natl Acad Sci U S A *105*, 20858-20863.

van Delft, M.A., Huitema, L.F., and Tas, S.W. (2015). The contribution of NF-κB signalling to immune regulation and tolerance. Eur J Clin Invest *45*, 529-539.

Viglianti, G.A., Lau, C.M., Hanley, T.M., Miko, B.A., Shlomchik, M.J., and Marshak-Rothstein, A. (2003). Activation of autoreactive B cells by CpG dsDNA. Immunity *19*, 837-847.

Villaseñor, J., Besse, W., Benoist, C., and Mathis, D. (2008). Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated. Proc Natl Acad Sci U S A *105*, 15854-15859.

Vobořil, M., Brabec, T., Dobeš, J., Šplíchalová, I., Březina, J., Čepková, A., Dobešová, M., Aidarova, A., Kubovčiak, J., Tsyklauri, O.*, et al.* (2020). Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation. Nat Commun *11*, 2361.

Vollmer, J., Tluk, S., Schmitz, C., Hamm, S., Jurk, M., Forsbach, A., Akira, S., Kelly, K.M., Reeves, W.H., Bauer, S.*, et al.* (2005). Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. J Exp Med *202*, 1575-1585.

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

von Boehmer, H., Teh, H.S., and Kisielow, P. (1989). The thymus selects the useful, neglects the useless and destroys the harmful. Immunol Today *10*, 57-61.

Wang, H., Breed, E.R., Lee, Y.J., Qian, L.J., Jameson, S.C., and Hogquist, K.A. (2019a). Myeloid cells activate iNKT cells to produce IL-4 in the thymic medulla. Proc Natl Acad Sci U S A *116*, 22262-22268.

Wang, J., Sekai, M., Matsui, T., Fujii, Y., Matsumoto, M., Takeuchi, O., Minato, N., and Hamazaki, Y. (2019b). Hassall's corpuscles with cellular-senescence features maintain IFNα production through neutrophils and pDC activation in the thymus. Int Immunol *31*, 127-139.

Wang, X., Laan, M., Bichele, R., Kisand, K., Scott, H.S., and Peterson, P. (2012). Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens. Front Immunol *3*, 19.

Waskow, C., Liu, K., Darrasse-Jèze, G., Guermonprez, P., Ginhoux, F., Merad, M., Shengelia, T., Yao, K., and Nussenzweig, M. (2008). The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. Nat Immunol *9*, 676-683.

Watanabe, N., Wang, Y.H., Lee, H.K., Ito, T., Cao, W., and Liu, Y.J. (2005). Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. Nature *436*, 1181-1185.

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

Weist, B.M., Kurd, N., Boussier, J., Chan, S.W., and Robey, E.A. (2015). Thymic regulatory T cell niche size is dictated by limiting IL-2 from antigen-bearing dendritic cells and feedback competition. Nat Immunol *16*, 635-641.

Werlen, G., Hausmann, B., Naeher, D., and Palmer, E. (2003). Signaling life and death in the thymus: timing is everything. Science *299*, 1859-1863.

White, A.J., Nakamura, K., Jenkinson, W.E., Saini, M., Sinclair, C., Seddon, B., Narendran, P., Pfeffer, K., Nitta, T., Takahama, Y.*, et al.* (2010). Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells. J Immunol *185*, 4769-4776.

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

Wohn, C., Le Guen, V., Voluzan, O., Fiore, F., Henri, S., and Malissen, B. (2020). Absence of MHC class II on cDC1 dendritic cells triggers fatal autoimmunity to a cross-presented self-antigen. Sci Immunol *5*.

Worbs, T., Bode, U., Yan, S., Hoffmann, M.W., Hintzen, G., Bernhardt, G., Förster, R., and Pabst, O. (2006). Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. J Exp Med *203*, 519-527.

Wu, L., D'Amico, A., Winkel, K.D., Suter, M., Lo, D., and Shortman, K. (1998). RelB is essential for the development of myeloid-related CD8alpha- dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells. Immunity *9*, 839-847.

Wuest, S.C., Edwan, J.H., Martin, J.F., Han, S., Perry, J.S., Cartagena, C.M., Matsuura, E., Maric, D., Waldmann, T.A., and Bielekova, B. (2011). A role for interleukin-2 trans-presentation in dendritic cell-mediated T cell activation in humans, as revealed by daclizumab therapy. Nat Med *17*, 604-609.

Yamano, T., Dobeš, J., Vobořil, M., Steinert, M., Brabec, T., Ziętara, N., Dobešová, M., Ohnmacht, C., Laan, M., Peterson, P.*, et al.* (2019). Aire-expressing ILC3-like cells in the lymph node display potent APC features. J Exp Med.

Yamano, T., Nedjic, J., Hinterberger, M., Steinert, M., Koser, S., Pinto, S., Gerdes, N., Lutgens, E., Ishimaru, N., Busslinger, M.*, et al.* (2015). Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction. Immunity *42*, 1048-1061.

Yang, H., and Tracey, K.J. (2010). Targeting HMGB1 in inflammation. Biochim Biophys Acta *1799*, 149-156.

Yang, S., Fujikado, N., Kolodin, D., Benoist, C., and Mathis, D. (2015). Immune tolerance. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance. Science *348*, 589-594.

Yano, M., Kuroda, N., Han, H., Meguro-Horike, M., Nishikawa, Y., Kiyonari, H., Maemura, K., Yanagawa, Y., Obata, K., Takahashi, S.*, et al.* (2008). Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance. J Exp Med *205*, 2827-2838.

Yarovinsky, F., Zhang, D., Andersen, J.F., Bannenberg, G.L., Serhan, C.N., Hayden, M.S., Hieny, S., Sutterwala, F.S., Flavell, R.A., Ghosh, S.*, et al.* (2005). TLR11 activation of dendritic cells by a protozoan profilin-like protein. Science *308*, 1626-1629.

Yoshida, H., Bansal, K., Schaefer, U., Chapman, T., Rioja, I., Proekt, I., Anderson, M.S., Prinjha, R.K., Tarakhovsky, A., Benoist, C.*, et al.* (2015). Brd4 bridges the transcriptional regulators, Aire and P-TEFb, to promote elongation of peripheral-tissue antigen transcripts in thymic stromal cells. Proc Natl Acad Sci U S A *112*, E4448-4457.

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

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

Zlotoff, D.A., Sambandam, A., Logan, T.D., Bell, J.J., Schwarz, B.A., and Bhandoola, A. (2010). CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. Blood *115*, 1897-1905.

Žuklys, S., Handel, A., Zhanybekova, S., Govani, F., Keller, M., Maio, S., Mayer, C.E., Teh, H.Y., Hafen, K., Gallone, G.*, et al.* (2016). Foxn1 regulates key target genes essential for T cell development in postnatal thymic epithelial cells. Nat Immunol *17*, 1206-1215.