Charles University

Faculty of Science

Study programme: Immunology
Branch of study: D-IMUN

Mgr. Jiří Březina

Cooperative antigen transfer in establishment of central immune tolerance

Úloha antigenního transferu v ustanovení centrální imunitní tolerance

Ph.D. Thesis

Supervisor: RNDr. Dominik Filipp, CSc.

Prague, 2024

Declaration:

I hereby declare that this Ph.D. thesis is a presentation of my original research and that I have cited all relevant literary sources. I also declare that neither this work nor any substantial part of it has been used in the past for the awarding of any academic degree or diploma. I would like to state that in this thesis, DeepL Write AI was used to improve English grammar.

Prague, 27.10.2024

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu. Chtěl bych uvést, že syntaxe anglického jazyka práce byla vylepšena pomocí DeepL Write AI.

V Praze, 27.10.2024

Podpis (Signature):

Acknowledgements:

It has been 10 years since I started my studies at the Faculty of Science Charles University. I am grateful for the opportunity to be a part of this institution and I would like to sincerely thank all my colleagues and friends I have met and worked with during my 10-year journey. First of all, I would like to thank my supervisor, Dr. Dominik Filipp, for giving me the opportunity to work in his lab on my Ph.D. Also, I cannot forget his great support during my undergraduate and graduate studies when I was not completely convinced that I wanted to be a scientist and he gave me space and time to figure it out. I would like to express my sincere gratitude to my former colleagues, Matouš Vobořil and Tomáš Brabec, for their invaluable guidance. I am indebted to them and to Dominik Filipp for my professional development. I cannot forget my current colleagues Ondřej Ballek, David Machač, Vojtěch Sýkora, Valerie Tahtahová, Kristína Jančovičová, and Jasper Manning who are my friends and team players, and I consider myself lucky to be surrounded by such a great group of people. Special thanks to Jasper Manning for the daily English chats, which help me a lot to improve my language skills. I would also like to highlight the work of the Czech Young Immunologists (CYI) group, of which I am a member and thanks to which I have met many friends and colleagues. I am also thankful to Prof. Graham Anderson's lab for giving me the opportunity to experience science at the University of Birmingham. I would like to thank Dr. Jan Dobeš for his valuable advice during my Ph.D. studies. I would like to thank the staff of the facilities at the Institute of Molecular Genetics for their contribution to my work. I am especially indebted to Zdeněk Cimburek, Dr. Matyáš Šíma, and Dr. Michal Kolář. I would also like to acknowledge the labs of Dr. Jan Dobeš, Dr. Meritxell Alberich Jorda, Dr. Ondřej Štěpánek, and Prof. Jan Černý for our long-term colaboration. Outside of the scientific world, I would like to thank my parents and Veronika Březinová for their support throughout my studies at the Faculty of Science and for their belief that this was the right path for me. Last but not least, I would like to thank my partner Kristýna Jelínková for her support, for giving me a much-needed break from the lab, and for the many memorable moments we shared while working on our Ph.D. theses.

Abstract:

The clonal repertoire of T cells naturally recognizes antigens of self and non-self origin. To prevent immune responses against self or harmless non-self antigens, leading to autoimmunity, food allergy, inflammatory bowel disease, etc., the T cell repertoire must be kept in check by presenting these antigens via antigen-presenting cells. This phenomenon, known as immune tolerance, applies to T cells that undergo development in the thymus and to which self-antigens are being presented (central tolerance) as well as to those that leave the thymus and enter the secondary lymphoid organs and peripheral tissues to which e.g. microbial or food antigens are being presented (peripheral tolerance). With respect to central tolerance, the cooperation between thymic DCs and medullary thymic epithelial cells (mTECs) in self-antigen presentation has been described as critical. While mTECs produce and present self-antigens in an autonomous manner, DCs acquire them from mTECs by cooperative antigen transfer (CAT). Importantly, upon strong recognition of self-antigen, mTECs and DCs induce clonal deletion of a particular self-reactive T cell and upon self-antigen recognition of intermediate strenght, they convert it into a T regulatory cell (Treg). The analysis of CAT was the main focus of my Ph.D. studies. I participated in a study showing that TLR signaling in mTECs leads to enhanced migration of monocyte-derived DCs into the thymus and their increased capacity in CAT. This in turn led to the enhancement of Treg selection. Furthermore, we analyzed the preferential pairing between subsets of mTECs and DCs with respect to CAT and established the conventional DC type 1 (DC1) lineage as the most specialized for this process. Finally, we searched for molecular determinants of CAT in the DC1 lineage and found Claudin 1 as its determinant as well as the homeostatic DC1 maturation driver. In addition, we demonstrated that Claudin 1 expression by thymic DC1s is critical for clonal deletion and Treg selection. The presented thesis provides an overview of antigen presentation in the thymus. Antigen presentation in the context of intestinal immune tolerance is also briefly reviewed, as this topic was, even though only marginally, addressed during my Ph.D. studies and in many aspects it is analogous to and connected with the processes of central tolerance.

Abstrakt (CZ):

T buněčný repertoár je klonální a rozpoznává, jak tělu vlastní, tak cizí antigeny. Aby nedocházelo k imunitní odpovědi vůči tělu vlastním antigenům a neškodným antigenům cizího původu, což může vést například k rozvoji autoimunit, potravinové alergie, či nespecifických střevních zánětů, jsou tyto antigeny předkládány T buňkám antigen-prezentujícími buňkami. Tento fenomén nazýváme imunitní tolerancí a týká se již vyvíjejících se T lymfocytů v brzlíku prostřednictvím prezentace tělu vlastních antigenů (centrální tolerance) a posléze i T buněk, které opustily brzlík do imunitní periferie, jimž jsou předkládány například mikrobiální, či potravní antigeny (periferní tolerance). Ustanovení centrální tolerance je závislé na kooperaci mezi dendritickými buňkami a medulárními epiteliálními buňkami brzlíku (mTEC buňky). Zatímco mTEC buňky prezentují tělu vlastní antigeny, které si samy vyprodukovaly, dendritické buňky získávají tyto antigeny od mTEC buněk skrz tzv. kooperativní antigenní transfer. V případě silného rozpoznání tělu vlastního antigenu, dendritické i mTEC buňky indukují klonální deleci auto reaktivních T buněk, v případě středně silného rozpoznání je přeměňují na T regulační buňky. Studium antigenního transferu bylo hlavním cílem mého doktorského studia. Podílel jsem se na publikaci, která ukázala, že mTEC buňky signalizují přes Toll-like receptory, a že jejich aktivace vede k posílení migrace dendritických buněk derivovaných z monocytů do brzlíku. Rovněž jsme v tomto kontextu zaznamenali posílený antigenní transfer do těchto dendritických buněk a pravděpodobně v následku toho jsme pozorovali zvýšenou produkci T regulačních buněk. Dále jsme analyzovali preferenční párování jednotlivých populací dendritických a mTEC buněk v rámci antigenního transferu a podařilo se nám zjistit, že klasické dendritické buňky typu 1 jsou nejvíce specializované pro tento proces. Rozhodli jsme se tedy hledat molekulární determinanty antigenního transferu právě v těchto buňkách a jakožto důležitou molekulu pro tento proces jsme objevili Claudin 1, který se také ukázal důležitý pro homeostatickou maturaci dendritických buněk. Rovněž se nám podařilo detekovat, že Claudin 1 je podstatnou molekulou pro indukci klonální delece i generování T regulačních buněk. Předložená práce je aktuálním literárním přehledem antigenní prezentace v brzlíku. Ve zkratce jsem však shrnul i antigenní prezentaci v kontextu střevní imunitní tolerance, protože jsem se tomuto tématu v rámci mého doktorského studia okrajově věnoval a vnímám ji v mnoha ohledech jako analogickou a úzce spojenou s tolerancí centrální.

Abbreviations:

aDC1/2	activated conventional dendritic cell type 1/2
Aire	Autoimmune regulator
APC	antigen-presenting cell
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS-1/2	Autoimmune polyglandular syndrome type 1/2
BM	bone marrow
bulk-seq	bulk RNA sequencing
CARD	caspase recruitment domain
CAT	cooperative antigen transfer
CBP	CREB-binding protein
CMJ	cortico-medullary junction
cTECs	cortical thymic epithelial cells
CTV	CellTrace Violet
DCs	dendritic cells
DC1/2	conventional dendritic cell type 1/2
Defa6	enteric α-defensin 6
DN1-4	double negative T cell stage 1-4
DP	double positive T cell
DSS	dextran sulfate sodium
E	embryonic day
EAE	experimental autoimmune encephalomyelitis
EpCAM	epithelial cell adhesion molecule
GAPs	goblet cell-associated antigen passages
GF	germ free
HEL	hen-egg lysozyme
H3K4me0	unmethylated histones 3 lysine 4
IECs	intestinal epithelial cells
IELs	intraepithelial lymphocytes
IFN	interferon
ILC3	innate lymphoid cells type 3
IL17Ra	IL17 receptor
IRBP	interphotoreceptor retinoid binding protein
ISG	interferon-stimulated gene
MC	mimetic cell
MHCI/II	major histocompatibility complex class I/II

MLN	mesenteric lymph node
moDC	monocyte-derived dendritic cell
mOVA	membrane-bound ovalbumin
mTECs	medullary thymic epithelial cells
ODN	CpG oligonucleotides
OVA	ovalbumin
PCs	Paneth cells
pDC	plasmacytoid dendritic cell
PHD 1/2	plant homeodomain 1/2
PLP	proteolipid protein
pMHC	peptide-major histocompatibility complex
PRR	pattern-recognition receptor
PSA	prostate-specific antigen
pTreg	peripheral T regulatory cell
RA	retinoic acid
RIP	rat insulin promoter
RTEs	recent thymic emigrants
scRNAseq	single-cell RNA sequencing
SFB	segmented filamentous bacteria
SI-LP	small intestine lamina propria
Sirt1	Sirtuin 1
SM	semimature single positive T cell
SP	single positive T cell
TAC TECs	transit-amplifying thymic epithelial cells
TCR	T cell receptor
TECs	thymic epithelial cells
TEDs	trans-epithelial dendrites
TF	transcription factor
T_H17	conventional Rorγ^+ $\alpha\beta$ T cell
TLR	Toll-like receptor
TRA	tissue-restricted antigen
Treg	T regulatory cell
TSP	thymic-seeding progenitors
TSS	transcription start sites
tTreg	thymus-derived T regulatory cell

Table of contents:

A. INTRODUCTION

To adapt to high mutational rate of pathogens or even our own genomes and protect our bodies against danger inflicted by infection, cancer, etc., immune system of jawed vertebrates evolved αβ T lymphocytes, hereafter referred to simply as T cells. Based on the contemporary mathematical models, human T cell repertoire reveals ~$1x10^{10}$ clones among total number of ~$3x10^{11}$ T cells (Jenkins *et al.*, 2010; Lythe *et al.*, 2016). Hypothetically, this means that almost every T cell possesses unique T cell receptor (TCR) that recognizes specific array of antigens to cover as many sources of potential threats as possible. Nevertheless, the stochastic nature of VDJ recombination during which αβTCRs are assembled leads to the generation of TCR variants which recognize non-self, altered-self and unfortunately also self-antigens. Therefore, activation of T cells after recognition of their cognate antigen has to be tightly controlled, since in the case of self-antigen recognition, it might manifest in immune response against self-tissues and autoimmunity. Throughout 1970s, *Ralph M. Steinman and colleagues* described cell type specialized for antigen sampling and subsequent presentation to T cells which they termed **dendritic cells (DCs)** according to their dendritic shape resembling neurons (Steinman and Cohn, 1973). It became clear in the following years that DCs present antigens to TCRs of T cells in a form of peptide-major histocompatibility complexes (pMHC) and express costimulatory molecules such as CD80 or CD86, the engagement of which by CD28 expressed on T cells was found necessary for T cell activation. More than two decades after the discovery of DCs, Toll-like receptors (TLR) were found to be expressed by DCs which through the binding of molecules of pathogen or danger-associated origin promote immunogenic DC maturation. During this process DCs enhance their antigen presentation capacity, upregulate costimulatory molecules and increase expression of cytokines. Thus, it is the DCs which can distinguish between harmful and harmless using TLRs and other receptors collectively referred to as pattern-recognition receptors (PRR) to guide T cell activation based on the presence of pathogens or any other danger (Cabeza-Cabrerizo *et al.*, 2021; Bosteels and Janssens, 2024).

Protection against unwanted activation of T cells is, however, established on several levels collectively termed immune tolerance. In fact, the entry level, **central tolerance**, is established in the thymus during T cell development, before newly generated T cells enter the immune periphery, and protects vertebrates mostly against the onset of autoimmunity (Klein *et al.*, 2014). Once T cells enter the body, there are mechanisms of **peripheral tolerance** which further reinforce tolerance to self and govern equally important tolerance to microbiota or other harmless, non-self antigens such as food antigens (Miranda-Waldetario and Curotto de Lafaille, 2024). In the end of 1980s, several research groups studying central tolerance noticed that thymus resembles “patchwork quilt” in regards of antigens expressed in this tissue (Linsk *et al.*, 1989). Specifically, thousands of tissue-restricted antigens (TRA) which are outside of the thymus expressed by single or limited number of tissues such as enteric defensins, which are in the immune periphery expressed by Paneth cells, can be found in the thymic medulla, where they are produced by **medullary thymic epithelial cells (mTECs)** (Derbinski *et al.*, 2001; Dobeš *et al.*, 2015). Thus, thymic medulla serves as almost complete “library” of self-antigens encompassing TRAs as well as ubiquitous self-antigens which

are by the definition expressed across the body. Given that mTECs are potent antigen-presenting cells (APC), they display self-antigens on their pMHCs to test self-reactivity of T cell clones. In general, in the case of strong recognition of self-antigen by a T cell clone, this clone is eliminated through the process of clonal deletion. If the affinity/avidity of such recognition is of intermediate strength, T cell clone can be converted into T regulatory cell (Treg) which after its exit from the thymus suppresses autoimmune responses mediated by self-reactive T cells which escaped the central tolerance (Klein *et al.*, 2014). Hereby, central tolerance contributes indirectly to establishment of peripheral tolerance, since Tregs represent its major cellular components (Dikiy and Rudensky, 2023). Arguably, the most valuable APCs of the thymus are DCs which, along with mTECs, can induce clonal deletion and agonist selection. However, compared to mTECs, thymic DC use very distinct mechanisms to achieve this goal. To establish immune tolerance, DCs can carry self-antigens to the thymus which were sampled in the immune periphery (Bonasio *et al.*, 2006). Nevertheless, abundant subset of thymic DCs referred to as conventional DCs type 1 (DC1) develops intrathymically, is thymus resident and therefore could not sample antigens outside of the thymus (Cosway *et al.*, 2018; Breed *et al.*, 2022). Surprisingly, thymic DCs and especially DC1s were found highly positive for TRAs even though their production of these antigens is limited (Perry *et al.*, 2014, 2018). In fact, since the discovery of thymic DCs thirty years ago (Humblet, Rudensky and Kyewski, 1994) it has been demonstrated that thymic DCs acquire TRAs from mTECs via process referred to as **cooperative antigen transfer (CAT)** and indirectly present them to T cells to establish central tolerance (Perry and Hsieh, 2016; Kadouri *et al.*, 2020).

Research in the field of central tolerance, and CAT in particular, was the major focus of my Ph.D. studies. Recently, many publications which focused on central and peripheral tolerance or cancer have described the maturation of DCs without the presence of any pathogen or danger-associated signals referred to as homeostatic DC maturation (Maier *et al.*, 2020; Park *et al.*, 2020; Breed *et al.*, 2022; Bosteels *et al.*, 2023; Ashby *et al.*, 2024; Bosteels and Janssens, 2024). In our laboratory, I participated as the first author in two studies which unraveled a close relationship between CAT and homeostatic DC maturation (Vobořil *et al.*, 2022; Březina *et al.*, 2024). In addition, I became the first author of a review article which summarized these findings in the context of current knowledge (Březina, Vobořil and Filipp, 2022). Further, I participated as a co-author in a study demonstrating changes in thymus biology after TLR sensing by mTECs (Vobořil *et al.*, 2020). Finally, outside of the thesis focus, I also contributed to two studies focused on the peripheral tolerance to intestinal microbiota and an interplay between immune cells and intestinal epithelial cells (IECs) (Brabec *et al.*, 2023, 2024).

In the section (B) of this thesis, I have summarized the current state of knowledge in the field of central tolerance, followed by a brief overview of antigen uptake and presentation with respect to intestinal tolerance. Then I have highlighted the main aims of my Ph.D. studies (section C) and the results of immune tolerance-related projects I have participated in (section D). The following section, the Discussion and

Conclusions (E), places our research and its results in a broader context of immune tolerance. I would like to note that if not stated otherwise, all the studies I am referring to were conducted using mouse models.

B. CURRENT STATE OF KNOWLEDGE

The thymus is a primary lymphoid organ and the only site of T cell development where the mechanisms of tolerance are applied to developing T cells prior to their entry into the immune periphery, an immunological term for the extrathymic anatomical space. Therefore, tolerance mechanisms applied to T cells within the thymus are referred to as central tolerance, while those applied outside of the thymus are referred to as peripheral tolerance. The thymus consists of two morphologically and functionally distinct areas: the outer area, called the cortex, and the inner area, called the medulla, which are separated by a vascularized cortico-medullary junction (CMJ). Both the cortex and medulla contain their unique thymic epithelial cells (TECs), referred to as the cortical thymic epithelial cells (cTECs) and mTECs, respectively. TECs are derived from the endoderm via a common bipotent progenitor (see **BOX 1** for further reading on TEC ontogeny) and represent the major cell subset in shaping the T cell repertoire within both thymic compartments. While cTECs ensure the formation of a T cell repertoire capable of recognizing pMHCs regardless of the origin of antigens they display, mTECs purge self-reactive clones from this repertoire (Abramson and Anderson, 2017). Remarkably, the mTEC population achieve the establishment of tolerance to TRAs via its cooperation with thymic DC subpopulations (Perry and Hsieh, 2016; Kadouri *et al.*, 2020).

BOX 1. TEC ontogeny. In mouse embryos thymus begins to develop around the embryonic day (E) 11, when the master regulator of TECs, TF Foxn1, starts to be expressed in thymic anlage. Foxn1 is indispensable for TEC development and maintenance and in fact many here discussed genes crucial for TEC function (*Dll4*, *Psmb11*, *Prss16* etc.) are direct targets of Foxn1 (Han and Zúñiga-Pflücker, 2021). The expression of Foxn1 in the thymus is TEC-specific and therefore it can be used as optimal Cre driver for studying this population (Gordon *et al.*, 2007). In the embryo, cTECs and mTECs originate from common bipotent progenitor which, however, displays the expression of cTEC markers such as β5t. Therefore, there is a consensus that by default, embryonic bipotent progenitors give rise to cTECs. It is presumed that generation of mTECs from these progenitors is mediated via unipotent Claudin 3 and 4^+ mTEC progenitor intermediate which gives rise to mTECs upon sensing Lymphotoxin β and at later stages RANKL and CD40L all provided by developing T cells (Abramson and Anderson, 2017).

Strikingly, as soon as at E 11.5, thymus is seeded with T cell progenitors. Hence, in the embryo, TECs and T cells develop in parallel and it has been repeatedly shown that their lymphostromal interactions enhance each other's development (Abramson and Anderson, 2017). By the same token, T cell development during embryonic and perinatal period is critical for establishment of functional and self-tolerant repertoire in the postnatal period of life, e.g. by the formation of unique perinatal Tregs (Yang *et al.*, 2015). mTECs reveal high turnover rate and during postnatal life they have to be continually replenished (Gray, Abramson, *et al.*, 2007). Although it became clear that postnatal TEC pool is maintained by distinct progenitors from that in the embryo, their phenotype and bi/unipotency are still matter of debate. However, there is evidence provided by several studies that postnatal mTEC pool is maintained by $Pdpn^+$ TEC progenitors localized to CMJ and therefore termed junctional TECs (Kadouri *et al.*, 2020). It is of note that recently described embryonic $K19^+CD9^+$ mTEC progenitors were shown to sufficiently maintain mTEC pool into the adulthood (Lucas *et al.*, 2023). Strikingly, thymic DC pool is formed postnatally *(Li et al.*, 2009; Cosway *et al.*, 2018). Given that CAT is mediated via acquisition of mTEC apoptotic bodies (Perry *et al.*, 2018), dying mTECs serve as an ideal substrate for DCs to promote central tolerance.

1. Development of functional T cell repertoire

Precursors of developing T cells rise in the bone marrow (BM) and they enter the thymus through CMJ in a multipotent stage referred to as thymic-seeding progenitors (TSP) (Luis *et al.*, 2016; Krueger, 2018).

Critical role in TSP entry play cTECs which attract them via production of chemokines CCL25 and CXCL12 into the thymic cortex (Zlotoff *et al.*, 2010; Calderón and Boehm, 2011). There, cTECs reveal complex "maze-shaped" network in order to manage interactions with the mass of developing T cells, despite their limited cellularity (~9 x 10^5 cTECs in 5 weeks old female mouse) (Hirakawa *et al.*, 2018; Sakata, Ohigashi and Takahama, 2018; Venables *et al.*, 2019). In this microenvironment, cTECs provide T cell precursors with critical factors that promote their development through four distinct $CD4^-CD8^-$ double negative T cell stages (DN1-DN4) which undergo VDJ recombination of TCRβ (DN3) to form $CD4^+CD8^+$ double positive (DP) stage (via immature single positive $CD8^+$ intermediate). DP T cells perform TCRα re-arrangement to accomplish second half of VDJ recombination, thus, are marked by expression of fully assembled αβTCR on their surface (Han and Zúñiga-Pflücker, 2021). The first progeny of TSPs in the thymus are Kit^+ early thymic progenitors that form limited founder subset of DN1 T cells. Importantly, cTECs in concert with CMJ endothelial cells are the major producers of Kit-ligand which is critical for survival and proliferation of early T cell developmental stages, in particular DN1 and DN2 (Buono *et al.*, 2016). Another crucial factor for T cell development that is in the thymus expressed exclusively by TECs in the proximity of CMJ is IL7 (Alves *et al.*, 2009). This cytokine is critical for development of all DN stages in which it first activates anti-apoptotic Bcl-2 pathway (DN2) and later at the transition between DN3 and DP stage promotes proliferation, differentiation and delays TCRα recombination to be executed in DP stage (Boudil *et al.*, 2015). It is of note that the decisive T cell lineage commitment is governed already at early DN stages through notch signaling, in particular via notch ligand DLL4 that is expressed by cTECs (Hozumi *et al.*, 2008; Koch *et al.*, 2008). Strikingly, delivery of DLL4 and IL7 to T cells is regulated via positive feedback, since lymphostromal interactions of T cells and TECs potentiate their expression (Fiorini *et al.*, 2008; Alves *et al.*, 2010).

cTECs exhibit a unique ability to present peptides of self-origin in the context of either major histocompatibility complex class I (MHCI) or class II (MHCII) molecules to test functionality of αβTCRs in the process referred to as positive selection (Klein *et al.*, 2014). In fact, TECs show high rate of constitutive macroautophagy which enables them an unconventional loading of endogenous self-antigens onto MHCII molecules (Nedjic *et al.*, 2008). On top of this, cTECs express unique proteases that process antigens which are then bound to MHCs. Specifically, in the case of MHCI antigen-processing, cTECs utilize thymoproteasome that is assembled from β5t subunit encoded by *Psmb11* gene (Murata *et al.*, 2007; Nitta *et al.*, 2010). MHCII loading in cTECs is on the other hand governed by Cathepsin L and thymus-specific serine protease encoded by *Ctsl* and *Prss16* gene, respectively (Honey *et al.*, 2002; Gommeaux *et al.*, 2009). It is presumed that cTEC-specific proteases create unique peptidome which could not be find anywhere else in the body and which is recognized by DP T cells with low affinity to ensure optimal calibration of TCR signaling during positive selection (Santori *et al.*, 2002; Lo *et al.*, 2009; Sasaki *et al.*, 2015; Takada *et al.*, 2015). Here it is important to note that presentation of ubiquitous self-antigens or TRAs by cTECs is unwanted, because it might lead to preferential positive selection of self-reactive T cell clones

(Klein *et al.*, 2014). In addition, along with antigen-processing, β5t was recently shown to regulate overall gene expression landscape of cTECs, although there has been a debate regarding credibility of this finding (Apavaloaei, Laverdure and Perreault, 2021; Ohigashi and Takahama, 2021).

The recognition of pMHCI and pMHCII during positive selection provides T cells with survival signal and decides whether T cells trigger Runx3 transcription factor (TF) and become single positive (SP) $CD8^+$ cytotoxic T cells in the case of pMHCI recognition or they deviate into SP $CD4^+$ helper T cells by triggering expression of TF Th-POK through binding of pMHCII (Teh *et al.*, 1988; Kaye *et al.*, 1989; He *et al.*, 2005; Setoguchi *et al.*, 2008; Luckey *et al.*, 2014). Interestingly, activation of Runx3 transcriptional program was shown to require more sustained TCR signaling than Th-POK program (Kurd and Robey, 2016). In line with this, recent approach utilized Cite-seq to fatemap positively selected T cells and found that there is a first wave of TCR signaling during which emerging helper and cytotoxic T cells share CD4 molecular fate and it is the second wave of TCR signaling which digresses the development of cytotoxic T cells from their helper counterparts by supporting CD8 molecular fate (Steier *et al.*, 2023).

Importantly, positive selection does not rely on a costimulation (Jenkinson *et al.*, 1994). This is in marked contrast with further discussed clonal deletion and Treg selection during which costimulation is a necessary step (Watanabe *et al.*, 2020). Importantly, during the transition between DP and SP stage, T cells become $CCR7^+$ (Ueno *et al.*, 2004). Specialized subset of mTECs which strongly expresses CCL21 (CCR7 ligand) then mobilizes positively-selected T cells to migrate into the thymic medulla to accomplish their development (Lkhagvasuren *et al.*, 2013; Kozai *et al.*, 2017). In addition, there are additional, no less important chemokine pathways such as CCR4-CCL17/22 that navigate T cells from thymic cortex to the medulla (Lancaster, Li and Ehrlich, 2018). Interestingly, only less than 10% of DP T cells are positively selected and enter the medulla, while the rest of them are unable to recognize pMHCs and die by neglect in the cortex (von Boehmer, Teh and Kisielow, 1989; Krueger, Ziętara and Łyszkiewicz, 2017), where they are immediately scavenged by macrophages (Zhou *et al.*, 2022).

2. Development of self-tolerant T cell repertoire

Processes of central tolerance applied to "post-positive selection" T cells rely on the presentation of self-antigens mainly by mTECs and thymic DCs. TRA-specific T cell repertoire in the thymic medulla is largely tolerized due to the ectopic expression of TRAs in mTECs, the process referred to as promiscuous gene expression. The main driver of ectopic expression is Autoimmune regulator (Aire) which through an unconventional transcriptional mechanism, described bellow in more detail, drives the gene expression of thousands of TRAs (Abramson and Goldfarb, 2016; Kadouri *et al.*, 2020). The relevance of Aire for establishment of self-tolerant T cell repertoire and beyond is demonstrated by various organ-specific autoimmune manifestations which accompany its mutations in mice and humans (see **BOX 2** for further reading on manifestations of Aire mutations) (Bruserud *et al.*, 2016).

BOX 2. Mutations of Autoimmune regulator. *AIRE* gene was cloned in 1997 as a gene whose recessive mutations are responsible for monogenic autoimmune polyglandular syndrome type 1 (APS-1), also reffered to as APECED (Aaltonen *et al.*, 1997; Nagamine *et al.*, 1997). More than a hundred of recessive mutations of *AIRE* gene has been described to cause APS-1. The patients who are diagnosed from APS-1 usually exhibit at least two out of three following manifestations: i) autoimmune disruption of adrenal cortex (reffered to as Addison's disease), ii) autoimmunity of parathyroid gland and iii) chronic mucocutaneous candidiasis. These manifestations are often accompanied with additional organ-specific autoimmunity, in particular, gastrointestinal autoimmunity, thyroid or ovarian insufficiency, etc. (Bruserud *et al.*, 2016). It has been found recently that susceptibility of APS-1 patients to *Candida albicans* infection is caused by Aire deficiency in innate lymphoid cells type 3-like cells that reside outside of the thymus in secondary lymphoid organs and require Aire for controlling immune reactions against yeast (Dobeš *et al.*, 2022). Interestingly, characteristic trait of APS-1 is the presence of organ specific autoantibodies and especially anti-type I IFN antibodies whose increased titer is projected into higher susceptibility of APS-1 patients to viral infections including covid-19 (Bastard *et al.*, 2021) and protection against type 1 diabetes, which is less frequent in patients possesing higher titers of these autoantibodies (Meyer *et al.*, 2016). Prevalence of APS-1 in global population is rare with higher frequency within populations of north-eastern Europe, Sardinians or Iranian jews (Bruserud *et al.*, 2016).

Remarkably, there are dominant negative mutations of *AIRE* gene, some of which reveal incomplete penetrance, whose prevalence in the global population is frequent (~1 mutation per 1000 individuals). These mutations are responsible for non-classical APS-1 that reveals, in general, milder and more variable clinical manifestations reminiscent of polygenic APS-2. For example, pernicious anemia caused by autoimmune gastritis, vitiligo or type 1 diabetes are more prevalent in dominant negative mutations patients (Oftedal *et al.*, 2015; Goldfarb *et al.*, 2021). Recent study by *Abramson and Husebye labs* elucidated phenotype of particular dominant negative mutations of *AIRE* using mouse models mimicking natural human mutations. In fact, there were huge differences between the phenotypes. While C311Y mutation of PHD 1 domain revealed relatively severe phenotype manifesting in ovarian insufficiency and similar loss of Aire-dependent TRA expression as in *Aire*$^{-/-}$ mice, highly prevalent mutation of PHD 1 domain, V301M, did not develop autoimmunity and did not act in mice as the dominant negative mutation. On the other hand, C446G mutation of PHD 2 domain originally described as recessive in human, revealed traits of dominant negative mutation in mice. Thus, depending on the nature of the particular *Aire* mutation, an individual may have mild autoimmune symptoms or severe manifestations reminiscent of those of classical APS-1 patients or *Aire*$^{-/-}$ mice (Goldfarb *et al.*, 2021).

2.1. Medullary thymic epithelial cells

A routine use of single-cell RNA sequencing (scRNAseq) in the last decade yielded robust knowledge in regards to the phenotypic and functional heterogeneity of mTEC subsets (Bornstein *et al.*, 2018; Baran-Gale *et al.*, 2020; Dhalla *et al.*, 2020; Park *et al.*, 2020; Wells *et al.*, 2020; Bautista *et al.*, 2021; Michelson *et al.*, 2022; Givony *et al.*, 2023). The original view concerning mTEC heterogeneity which dates back to the beginning of this millennium was that mTECs^{LO}, named after their low expression of MHCII and costimulatory molecules, are immature precursors of mTECs^{HI} which are well equipped for antigen presentation and exhibit a strong ectopic expression of TRAs (Gäbler, Arnold and Kyewski, 2007). Thus, this original linear model of mTEC development proposed a single mTEC developmental pathway, where mTEC progenitors (see BOX 1) give rise to the immature mTEC^{LO} intermediate which in turn give rise to mature mTECs^{HI}.

Nevertheless, the data from scRNAseq analyses advocate for much more complex branching of mTECs during their development (Park *et al.*, 2020; Wells *et al.*, 2020; Bautista *et al.*, 2021). For example, they unanimously suggest that majority of mTECs^{LO} are mature CCL21^{+} mTECs, which do not give rise to or do not rise from mTECs^{HI}. In fact, both CCL21^{+} mTECs and mTECs^{HI} are considered to originate from transit-amplifying TECs, the progeny of mTEC progenitors termed TAC-TECs (Wells *et al.*, 2020). It is of note that CCL21^{+} mTECs^{LO} reveal only faint ectopic expression of TRAs and are specialized to attract

developing T cells into the thymic medulla (Lkhagvasuren *et al.*, 2013; Danan-Gotthold *et al.*, 2016; Kozai *et al.*, 2017). Recently, it was found that cooperation between CCL21^{+} mTECs^{LO} and Aire^{+} mTECs^{HI} is fundamental for the establishment of tolerance, since mice possessing *Ccl21*$^{-/-}$*Aire*$^{-/-}$ thymi revealed more severe autoimmune phenotype than *Aire*$^{-/-}$ mice (Ushio *et al.*, 2024). Within the mTEC pool, the Aire gene is expressed solely by mTECs^{HI} and endows them with the ability to present TRAs, the core process underlying establishment of effective central tolerance (Anderson *et al.*, 2002).

In early studies, mTECs^{HI} were considered as terminally differentiated mature cells with short lifespan (Gray, Abramson, *et al.*, 2007), presumably due to the formation of double strand breaks of their DNA required for Aire-driven ectopic expression (Abramson *et al.*, 2010; Bansal *et al.*, 2017; Guha *et al.*, 2017). However, it was shown recently that a significant portion of mTECs^{HI} can further differentiate into an array of mTEC subsets which mimic various epithelial cell types from the immune periphery, and thus referred to as mimetic cells or as often named in the older literature, the post-Aire mTECs (White *et al.*, 2010; Metzger *et al.*, 2013; Bornstein *et al.*, 2018; Michelson *et al.*, 2022; Givony *et al.*, 2023). Surprisingly, mimetic cell subsets are only partially dependent on the expression of Aire in mTECs^{HI}, rendering their ontogeny unclear (Miller *et al.*, 2018; Michelson *et al.*, 2022).

2.1.1.Ectopic expression of TRAs in mTECs^{HI}

While most of the murine peripheral tissues express around 65% of protein-coding genome, whole population of mTECs^{HI} expresses up to 85% of genes (Danan-Gotthold *et al.*, 2016). In fact, there are ~6500 genes encoding TRAs, majority of which are expressed ectopically by mTECs^{HI}. Interestingly, only around ~40% of TRAs in mTECs are regulated by Aire and the regulation of expression of the remaining 60% is incompletely understood (Sansom *et al.*, 2014). During the last decade, several studies suggested alternative regulators of ectopic expression, in particular serine/threonine protein kinase Hipk2 or zinc finger TF Fezf2, where only the latter is not an interacting partner of Aire and the TRA repertoire which it governs is non-overlapping with that of Aire (Rattay *et al.*, 2015; Takaba *et al.*, 2015). Recently, two comprehensive studies demonstrating zinc finger TFs Ikaros and Insm1 as the master regulators of mTECs showed their contribution to the expression of some of the Aire-dependent TRAs, Fezf2-dependent TRAs as well as genes encoding TRAs which are independent of both Aire and Fezf2 (Sin *et al.*, 2023; Tao *et al.*, 2023). Hence, along with Aire which triggers TRA transcription by acting on chromatin (Abramson and Goldfarb, 2016), the ectopic expression of many TRAs is likely regulated by a combinatorial action of several zinc finger TFs as well.

The ectopic expression of TRAs in mTECs^{HI} follows distinct rules than the physiological expression of TRAs in the immune periphery. First, TRA expression by mTECs can be both biallelic and monoalelic, whereas peripheral TRA expression is strictly bialelic (Villaseñor *et al.*, 2008). Second, mTECs^{HI} reveal high rate of RNA editing and alternative splicing, thereby covering many variants of particular proteins that can be found in the immune periphery (Keane, Ceredig and Seoighe, 2015; Danan-Gotthold *et al.*, 2016).

Third, it operates on a purpose of "ordered stochasticity" (Meredith *et al.*, 2015). In particular, the term "stochasticity" refers to the fact that while mTECs^{HI} express almost complete TRA repertoire of the body on the population level, each TRA is produced only by 1-3% of mTECs (Derbinski *et al.*, 2008; Sansom *et al.*, 2014), resulting in a characteristic mosaic pattern of TRA expression (Brennecke *et al.*, 2015). The term "ordered" then softens the previous one by referring to the fact that it was shown that there are co-expression groups of TRAs in individual mTECs (Pinto *et al.*, 2013; Brennecke *et al.*, 2015; Meredith *et al.*, 2015; Rattay *et al.*, 2016; Dhalla *et al.*, 2020). Strikingly, mTECs^{HI} do not co-express TRAs according to their expression pattern in the peripheral tissues, i.e. some mTECs^{HI} would express pancreatic whereas other would express intestinal TRAs. Rather, TRAs are co-expressed based on their inter- and intra-chromosomal clustering in the genome and regardless of whether they are Aire-dependent or -independent (Brennecke *et al.*, 2015). In line with these observations, mTECs^{HI} express TRAs independently of TFs which guide their expression in peripheral tissues. For example, when the expression of TF Pdx1 was absent from mTECs, the expression of its target TRA genes by mTECs as well as tolerance to their products such as insulin was retained (Villaseñor *et al.*, 2008; Danso-Abeam *et al.*, 2013). Interestingly, the groups of coexpressed TRAs seem to differ between individual mice, further corroborating stochastic character of the ectopic expression (Meredith *et al.*, 2015).

In contrast to the Aire-independent ectopic expression which is the subject of current research, transcription of Aire-dependent genes has been studied in detail for more than 25 years. Functional Aire protein comprises four domains in each monomer and is assembled into homodimeric or homotetrameric protein complexes (Kumar *et al.*, 2001). These domains are caspase recruitment domain (CARD) essential for homooligomerization of Aire protein, SAND domain and two plant homeodomains (PHD 1 and 2) (Abramson and Goldfarb, 2016). While SAND and PHD 1 domains are responsible for the recognition of TRA-encoding genes (Koh *et al.*, 2008; Org *et al.*, 2008; Waterfield *et al.*, 2014), PHD 2 domain serves as a docking interface for the molecules which interact with Aire (Yang *et al.*, 2013). Aire reveals two nuclear localization signals that mediate its active import by karyopherins through the nuclear pore into the nucleus (Abramson and Goldfarb, 2016). There, Aire is strongly bound to a nuclear matrix (cytoskeletal structure) which enables the formation of chromatin loops and localizes via its CARD domain to "speckled-shaped" areas called nuclear bodies (Akiyoshi *et al.*, 2004; Tao *et al.*, 2006). In fact, Aire was recently shown to promote ectopic expression via binding to super enhancers and bringing them into the proximity of Aire-dependent genes by transcriptional chromatin looping (Bansal *et al.*, 2017, 2021). This suggests that TRA co-expression groups are established among genes which are brought to the proximity of a particular super enhancer.

Unlike classical TFs, Aire does not regulate transcription via binding to consensus sequences. In fact, mTECs^{HI} can express genes which encode Aire-dependent TRAs which localize to gene loci persisting in a non-acessible heterochromatin state. Therefore, Aire recognizes its target genes based on the presence of a combination of silent chromatin marks (Abramson and Goldfarb, 2016). Namely, Aire was shown to

utilize its PHD 1 domain to directly bind to unmethylated histones 3 lysine 4 (H3K4me0) in the promoter regions of the Aire-dependent genes (Koh *et al.*, 2008; Org *et al.*, 2008). In addition, Aire-dependent genes were shown to be marked by the absence of active chromatin marks H3K4me3 or acetylated histones 3 and by the presence of repressive marks H3K27me3 and H3K9me3 or methylated CpG islands (Org *et al.*, 2009; Sansom *et al.*, 2014; Handel *et al.*, 2018; Koh *et al.*, 2018). Interestingly, Aire recognizes methylated CpG islands indirectly via interaction of its SAND domain with MBD1 and ATF7ip proteins, which are repressors of gene expression (Waterfield *et al.*, 2014). In line with this, Aire-dependent genes associate with gene silencing polycomb proteins (Sansom *et al.*, 2014). Thus, Aire targets its genes based on the combination of markers of repressed chromatin.

Recently, a novel mechanism of how Aire targets its genes was postulated, connecting Aire-dependent gene recognition with the formation of DNA double strand breaks. In particular, promoter regions of Aire-induced genes were found enriched with Z-DNA conformation and it was demonstrated that at these sites, DNA is prone to double strand breaks (Fang *et al.*, 2024). Indeed, opening a silenced chromatin via the formation of DNA double strand breaks by Aire-mediated recruitment of topoisomerases and DNA damage response proteins is the key factor in promoting Aire-dependent ectopic expression (Abramson *et al.*, 2010; Bansal *et al.*, 2017; Guha *et al.*, 2017). Altogether, Aire recruits to the transcription start sites (TSS) of Aire-dependent genes more than 50 partner molecules (Abramson *et al.*, 2010; Giraud *et al.*, 2014). These include, beyond the above mentioned, also proteins involved in transcriptional elongation. It is of note that RNA polymerases II are physically present at TSS of silent genes, however, they are not operational unless they interact with the transcription elongation factors (Giraud *et al.*, 2012). Indeed, Aire promotes the ectopic gene expression via recruitment of elongation factor p-TEFb to TSS (Oven *et al.*, 2007; Giraud *et al.*, 2014; Yoshida *et al.*, 2015) and subsequent release of stalled polymerases (Giraud *et al.*, 2012).

In addition, during the transcription, Aire recruits molecules participating in RNA editing and alternative splicing, hereby rendering templates for distinct variants of particular TRAs (Abramson *et al.*, 2010; Giraud *et al.*, 2014; Keane, Ceredig and Seoighe, 2015; Danan-Gotthold *et al.*, 2016). However, recent studies have shown that the role of Aire in alternative splicing of TRA transcripts is limited. Instead, alternative splicing in mTECs^{HI} is governed by splicing factors such as RBFOX family proteins or Raver 2, and is more active with respect to Aire-independent TRA transcripts (St-Pierre *et al.*, 2015; Jansen *et al.*, 2021; Carter *et al.*, 2022; Padonou *et al.*, 2022). Thus, mTECs^{HI} reveal a profound but selective alternative splicing that does not correspond to the number of spliced variants present in the immune periphery. These findings may explain why C57BL/6 mice are resistant to experimental autoimmune encephalomyelitis (EAE) while SJL mice are susceptible to this disease. The splice variant encompassing the major T cell epitope of the Aire-dependent TRA proteolipid protein (PLP) recognized in EAE on the C57BL/6 background is produced by mTECs, while the splice variant comprising the EAE-related PLP epitope on the SJL background is not (Klein *et al.*, 2000).

The expression of Aire, similarly to the whole developmental program of mTECs^{HI}, is dependent on NF-κB signaling. Aire expression is controlled through cis-regulatory element CNS 1 which binds NF-κB TFs p65 and Relb and whose genetic ablation results in a similar phenotype as is possessed by *Aire*$^{-/-}$ mice (Haljasorg *et al.*, 2015; LaFlam *et al.*, 2015). Other TFs including Irf4, Irf8, Tbx21 or Tcf7 were reported to participate in the transcription of Aire as well (Herzig *et al.*, 2016). Once Aire is translated into functional protein, its activation and deactivation is regulated by deacetylase Sirtuin 1 (Sirt1) and acetylase CREB-binding protein (CBP), respectively. In fact, mTEC-restricted *Sirt1*$^{-/-}$ mice recapitulate the phenotype of *Aire*$^{-/-}$ mice, suggesting that deacetylation of acetyl residues of Aire between nuclear localization signal and SAND domain generated by CBP is essential for Aire-induced ectopic expression (Chuprin *et al.*, 2015). Interestingly, CBP was discovered as the first partner molecule of Aire, whose putative role is the acetylation of Aire-dependent genes, facilitating their expression (Pitkänen *et al.*, 2000, 2005; Akiyoshi *et al.*, 2004). Recently, another acetyltransferase, Kat7, was found to be critical for operational Aire-dependent ectopic expression by increasing chromatin accessibility at the loci of TRA-encoding genes (Heinlein *et al.*, 2022). Thus, in regards to the ectopic expression, acetylation fine tunes the accessibility of Aire-dependent genes as well as Aire protein itself.

2.1.2. Thymic mimetic cells

More than a hundred years before the discovery that individual TRAs are expressed throughout the thymic medulla, scientists observed that the thymus, whose function was unknown at that time, serves as a residency to cell types that one would only find in a specific peripheral tissue, such as keratinocytes in the skin. Nevertheless, these original findings were overshadowed for a period of several decades by the discovery of mTECs, Aire and ectopic expression (Michelson and Mathis, 2022). While the research of Aire biology was at its peak, a few studies recapitulated the 19th century observations in the thymus by finding cell types typical for lungs, thyroid and parathyroid gland or skin in the thymus using microscopy (Dooley, Erickson and Farr, 2005, 2009; White *et al.*, 2010). Importantly, during the last six years, with the expansion of scRNAseq, single cell ATAC seq, CHIP seq and other high-throughput tools, these observations were confirmed (Bornstein *et al.*, 2018; Baran-Gale *et al.*, 2020; Dhalla *et al.*, 2020; Wells *et al.*, 2020; Bautista *et al.*, 2021; Michelson *et al.*, 2022; Givony *et al.*, 2023). In the seminal study by *Michelson and colleagues*, these cells were ultimately named as mimetic cells (MC), referring to their "mimicry" of epithelial cell types (Michelson *et al.*, 2022).

With the exception of myoid MCs (mimicking skeletal muscle cells), all the MC subsets described using high-throughput methods are the progeny of mTECs^{HI}. These MCs mimic predominantly cell types from the skin, lungs and gut. Specifically, these are: i) keratinocyte MCs, also referred to as Post-Aire mTECs, forming cornficicated islets in the thymic medulla called Hassal's corpuscles, ii) tuft MCs, iii) enterocyte/hepatocyte MCs and iv) their progeny, microfold MCs, v) neuroendocrine MCs, vi) ciliated MCs, vii) ionocyte MCs, viii) secretory MCs and ix) basal MCs resembling the basal cells of the skin and

lung epithelia which are putative progenitors of keratinocyte, secretory and ionocyte MCs. In addition, it seems that the MC compartment may also contain other cell types: x) parathyroid and xi) thyroid MCs, which however have not been re-detected by high-throughput methods and therefore their existence is questionable (Michelson and Mathis, 2022). Thus, the pedigree of mTECs is highly complex, considering that mTECs^{HI} give rise to several MC subsets, some of which further differentiate into a transcriptomically versatile progeny (**Figure 1**).

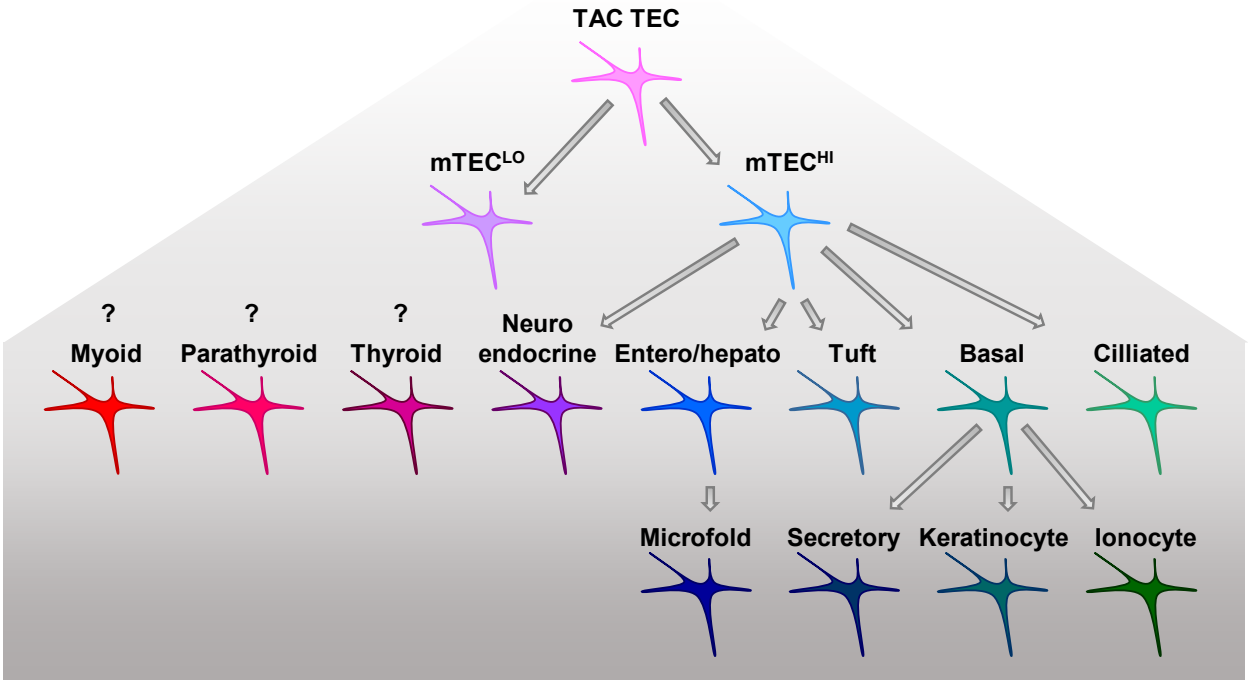

Figure 1. Postnatal mTEC development. Progenitors of mTECs (see BOX 1) give rise to immature, transit-amplifying TAC TECs which develop into either CCL21^{+} mTECs^{LO} or Aire^{+} mTECs^{HI}. The latter give rise to several MC progeny. Of these, Enterocyte/hepatocyte MCs further differentiate into Microfold MCs. Basal MCs, also derived from mTECs^{HI}, are putative precursors of Keratinocyte, Secretory, and Ionocyte MCs. The development of Myoid MCs is independent of mTECs^{HI} and Aire, however, its developmental pathway is otherwise unknown. The trajectory of Thyroid and Parathyroid MCs development has not been studied.

MCs are defined as cells which share lineage-defining TFs, gene expression profile and chromatin accessibility with particular epithelial, extrathymic cell type, while retaining mTEC-lineage specific traits (Michelson and Mathis, 2022). In fact, they keep the mTEC gene expression profile to a very high degree, since the principal component analyses performed separately on several MC subsets (microfold, enterocyte/hepatocyte, myoid and ciliated MCs) revealed that each of these subsets was more similar to other mTECs than to their peripheral counterparts (Michelson *et al.*, 2022, 2023). However, tuft MCs were found to be genetically more similar to intestinal tuft cells than to other mTECs (Bornstein *et al.*, 2018). Despite being decendants of mTECs^{HI}, MCs do not express Aire, and their development was demonstrated to rely predominantly on lineage defining TFs, as their genetic ablation led to the complete loss of particular MC subsets (Bornstein *et al.*, 2018; Miller *et al.*, 2018; Michelson *et al.*, 2022, 2023; Givony *et al.*, 2023;

Tao *et al.*, 2023). However, Aire as well as zinc-finger TFs Fezf2 and Ikaros, were shown to regulate relative percentage composition of MCs. While Fezf2 deletion led to a significant drop within tuft MCs (Lammers *et al.*, 2023; Ushio *et al.*, 2024), Ikaros deletion was translated into a pronounced increase of tuft and myoid MCs and reduction of other MC subsets (Sin *et al.*, 2023). Interestingly, *Aire*$^{-/-}$ mice showed decreased cellularity of majority of MC subsets, with ciliated and enterocyte/hepatocyte MCs being the most affected (Michelson *et al.*, 2022). This may be related to the fact that the expression of many TRAs was found to be only enhanced by, and not fully dependent on, Aire (Sansom *et al.*, 2014).

Similar to mTECs^{HI}, MCs express high levels of TRAs, however, using different regulatory principles. Critical determinants of TRA expression in MCs are individual lineage-defining TFs. In marked contrast to Aire, lineage-defining TFs are sequence specific, therefore, TRA expression in MCs is supposed to be non-random, following standard gene expression rules (Bornstein *et al.*, 2018; Miller *et al.*, 2018; Michelson *et al.*, 2022; Givony *et al.*, 2023). Thus, in marked contrast to mTECs^{HI} and their stochasticity in ectopic expression of TRAs, MCs represent a cell-type-specific and thus predictable source of TRAs in the thymus, in which physiologically related TRAs, that are characteristic of particular peripheral cell type, are co-expressed. So far, not much is known about the direct impact of TRA expression by MCs on the establishment of central tolerance. It is noteworthy that production of the model self-antigen YFP specifically in myoid or ciliated MCs resulted in clonal deletion of YFP-restricted T cells, but failed to divert these T cells into Tregs (Michelson *et al.*, 2022). Furthermore, thymus-specific ablation of tuft and neuroendocrine MCs resulted in the generation of autoantibodies against the tuft cell-derived cytokine IL25 and the neuroendocrine cell-enriched gastric fundus, respectively (Miller *et al.*, 2018; Givony *et al.*, 2023; Tao *et al.*, 2023). However, the lineage-defining TF of neuroendocrine MCs, Insm1, whose TEC-specific deletion ablated neuroendocrine MCs, also emerged as a master regulator of the ectopic expression of TRAs in mTECs^{HI}, and given this, it is unclear what underlies the autoimmune potential of neuroendocrine MC ablated mice (Tao *et al.*, 2023). Consistent with this skepticism, the same mice show lymphocytic infiltrates in the pancreas, salivary glands, lungs and kidney (Tao *et al.*, 2023), the organs whose autoimmunity is typically associated with Aire-driven ectopic expression deficiency (Jiang *et al.*, 2005).

The nature of gene expression in MCs is also related to the fact that they mimic the morphology and function of peripheral cell types. For example, keratinocyte MC-formed Hassal's corpuscles look like small patches of skin inside the thymus to which thymic DCs or even tuft MCs tend to localize and which are pronounced especially in the human thymus, where they serve as a hub for the selection of Tregs (Watanabe *et al.*, 2005; Miller *et al.*, 2018). Microfold MCs harbor thymic B cells in their lymphocyte pockets, like their “doppelgangers”, the M cells of Peyer's patches, to ensure IgA production in the thymus (Givony *et al.*, 2023). Similar to their peripheral counterparts, tuft MCs produce IL25, the cytokine that orchestrates type 2 immune responses in the gut, which regulates the development of invariant NKT cells and the cellularity of type 2 innate lymphoid cells in the thymus (Bornstein *et al.*, 2018; Miller *et al.*, 2018). Neuroendocrine MCs delay thymic involution by producing ghrelin into their granules (Givony *et al.*, 2023). Thus, MCs

not only serve as another source of TRAs, but also form a local microenvironment important for the thymus physiology. On top of that, the production of IL25, IgA, or ghrelin likely serves a dual role, as fragments of these molecules are presented along with other TRAs to developing T cells to establish central tolerance.

In summary, mTECs serve as a library of peripheral self through the production of thousands of TRAs that is highly dependent on Aire and well established ectopic expression in mTECs^{HI}. However, recent research changed the entire view of TRA generation in the thymus, by discovering array of mTEC subtypes which mimic peripheral cells not only in their gene expression profiles, but also in their morphology and even function. In fact, it was shown that on a population level, MCs form majority of mTECs, and for example, tuft MCs alone almost equal mTECs^{HI} in their numbers (Bornstein *et al.*, 2018; Miller *et al.*, 2018; Michelson *et al.*, 2022; Givony *et al.*, 2023). Considering the fact that ~60% of TRAs are expressed in the thymus independently of Aire (Sansom *et al.*, 2014), these could be largely those expressed by MCs in an Aire-independent fashion. Therefore, the establishment of tolerance to TRAs is much more complex than previously thought, involving a "division of labor" between cells that produce TRAs with ordered stochasticity and, equally important, cells with coordinated TRA expression.

2.2. Thymic dendritic cells

While the primary role of mTECs is to establish tolerance to TRAs, thymic DCs cover a much broader spectrum of antigens to which they tolerize the T cell repertoire. This is largely due to the marked heterogeneity of thymic DCs. In fact, there are four major lineages of thymic DCs: (i) DC1 lineage and (ii) DC type 2 (DC2) lineage, and these two DC lineages are collectively referred to as conventional or classical, (iii) monocyte-derived DC (moDC) lineage, and (iv) plasmacytoid DC (pDC) lineage (**Figure 2**). These differ greatly in their molecular makeup (scavengers, antigen-presentation associated proteins, cytokines, chemokine receptors, etc.) and therefore are predestined to sample distinct antigens and present them in different molecular contexts (Hadeiba *et al.*, 2012; Perry *et al.*, 2018; Vollmann *et al.*, 2021; Zegarra-Ruiz *et al.*, 2021; Breed *et al.*, 2022; Ashby *et al.*, 2024). Given the scope of my Ph.D. studies, I will focus this chapter on reviewing direct and indirect TRA presentation, as well as CAT which is a prerequisite for the latter. In addition, I will describe the heterogeneity of thymic DCs with a focus on the homeostatic maturation of conventional DCs.

2.2.1. Cooperation between mTECs and thymic DCs in TRA presentation

mTECs^{HI} and substantial proportion of MCs are APCs with robust capacity to present TRA fragments to developing T cells (Michelson *et al.*, 2022). This presentation is termed direct, as mTECs present endogenously produced TRAs both on MHCI and, unconventionally, on MHCII molecules (Nedjic *et al.*, 2008; Aichinger *et al.*, 2013). Direct presentation contributes to both clonal deletion and Treg selection as shown by studies based on transgenic T cells specific to Ovalbumin (OVA) that mimicked TRA (Gallegos and Bevan, 2004; Mouri *et al.*, 2017; Lancaster *et al.*, 2019), or elegant miRNA approaches in which the MHCII transactivator Ciita was specifically silenced in the Aire-dependent lineage of mTECs, leading to

their knockdown in MHCII expression (Hinterberger *et al.*, 2010; Perry *et al.*, 2014). On the other hand, these studies unanimously demonstrated that in the absence of MHCII on BM-derived APCs, and in particular on thymic DCs, direct presentation is unable to fully establish tolerance against TRAs. A closer comparison between the T cell repertoires of mice with MHCII knockdown in mTECs and chimeras with MHCII deficient BM revealed that mTECs contribute to clonal deletion of a smaller pool of T cell clones compared to thymic DCs (Perry *et al.*, 2014). This finding is logical because DCs present, in addition to TRAs, a broad spectrum of non-TRA antigens (see chapter 2.2.3), the recognition of which preferentially results in clonal deletion (Legoux *et al.*, 2015; Malhotra *et al.*, 2016).

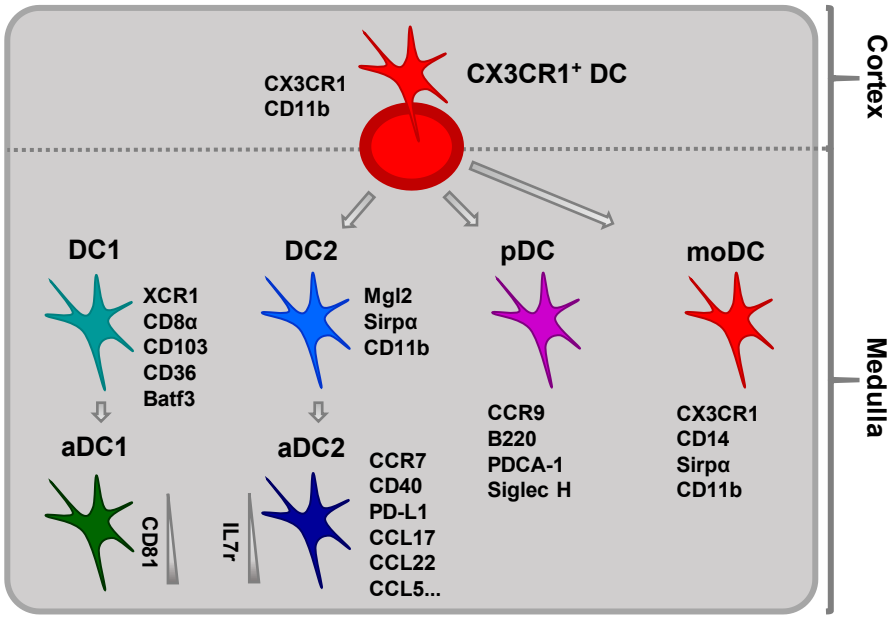

Figure 2. Thymic DC subsets. DC2s, pDCs, moDCs and CX3CR1⁺ DCs are migratory and enter the thymus via the bloodstream. CX3CR1⁺ DCs have been reported to form protrusions in the endotel, through which they acquire blood-borne antigens. Thus, they reside in the CMJ in permanent contact with the vasculature. Although the distinction between DC2s and moDCs is challenging and requires combinatoric analysis of the expression of their markers, CX3CR1⁺ DCs are most likely moDCs, which were showed to also reside in the thymic medulla (Vobořil *et al.*, 2020) along with DC1s, DC2s, and pDCs. DC1s rise intrathymically and, together with DC2s, undergo homeostatic maturation into aDC1 and aDC2, respectively. A hallmark of homeostatic DC maturation is the loss of expression of lineage-specific marker genes, resulting in phenotypic convergence of aDC1 and aDC2. However, we have established CD81 and Il7r as suitable markers for aDC1 and aDC2, respectively, as their expression gradually increases with maturation (Březina *et al.*, 2024). The molecules enlisted are selected markers of individual thymic DC lineages.

Importantly, clonal deletion of many TRA-specific CD4 T cell clones showed its complete dependence on indirect presentation of mTEC-derived TRAs by thymic DCs (TRAs were not produced by thymic DCs, hence the name indirect) (Gallegos and Bevan, 2004; Perry *et al.*, 2014, 2018; Mouri *et al.*, 2017), including clones specific for known TRA targets: (i) PLP ((Koble and Kyewski, 2009; Hassler *et al.*, 2019) and (ii) interphotoreceptor retinoid binding protein (IRBP) (Taniguchi *et al.*, 2012). In contrast, clonal deletion of TRA-specific CD8 T cells, although shown to physically interact with pMHCIs on thymic DCs using two-

photon microscopy (Lancaster *et al.*, 2019), was demonstrated to be dependent on direct TRA presentation by mTECs (Gallegos and Bevan, 2004; Hubert *et al.*, 2011). This was further supported by the surprising finding that ablation of the DC1 lineage, which is specialized for antigen cross-presentation and CD8 T cells "cross-tolerance" in the immune periphery (Belz *et al.*, 2002; Liu *et al.*, 2002), had no effect on clonal deletion of CD8 T cells in the thymus (MacNabb *et al.*, 2019). Notably, *Kurd and colleagues* suggested another function for DCs in clonal deletion of CD8 T cells. They proposed that DCs phagocytose CD8 T cells immediately after their clonal deletion, thus ensuring that they do not survive the induction of apoptosis and will not escape to the immune periphery (Kurd *et al.*, 2020).

It is noteworthy that, in contrast to clonal deletion, Treg selection is preferentialy induced by recognition of TRA-derived peptides within the pMHCs. There is a consensus that a lower affinity and/or avidity of TCR-pMHC interaction, compared to that which is required for clonal deletion, determines whether Treg selection is favored over the clonal deletion (Jordan *et al*., 2001; Hinterberger *et al*., 2010; Klein, Robey and Hsieh, 2019; Ashby *et al*., 2024). Individual TRAs are sparsely expressed throughout the thymic medulla, therefore, their recognition in the context of pMHC has a rather intermediate avidity and hence can lead to Treg selection. However, the choice between clonal deletion and Treg selection is influenced by several factors, primarily by the availability of IL2, modes of costimulation, and other factors (Klein, Robey and Hsieh, 2019). In particular, Tregs themselves play an important role in agonist Treg selection, as they recirculate back to the thymus and compete with Treg precursors for IL2, forming a negative feedback loop (Thiault *et al*., 2015).

In any case, while mice carrying MHCII knockdown in mTECs showed an increased frequency of Tregs in the thymus, their Treg clonal repertoire shrank compared to WT controls, suggesting that although the lower avidity forced by MHCII deficiency increased Treg selection, the reduction in MHCII simultaneously affected the spectrum of TRAs presented and overall restricted the repertoire of selected Tregs (Hinterberger *et al.*, 2010; Perry *et al.*, 2014). Strikingly, thymic DCs contributed to Treg selection via indirect TRA presentation to the level comparable to a direct presentation by mTECs. While DCs induced Tregs from clones with more common TCRs, mTECs were more specialized for the rare TCRs, therefore, there were many Treg clones selected in a non-overlapping manner by mTECs and DCs (Perry *et al*., 2014), including naturally occurring MJ23 Tregs specific to prostate specific antigen (PSA), which completely relied on indirect presentation (Leventhal *et al*., 2016). Thus, DCs present TRAs in a different context than mTECs, probably due to their antigen processing machinery, which is different from that of mTECs, and hence they not only enhance TRA presentation but also broaden the scope of central tolerance (**Figure 3**).

To assemble pMHCs, antigens to be presented must be chopped up and loaded onto MHC molecules in the form of short peptides. When presented on the surface of an APC, it is the pMHCs and not the whole antigens that are recognized by TCRs (Neefjes *et al*., 2011). Therefore, different segments of each TRA may be recognized by different T cell clones. Moreover, given that mTECs, DCs, and even their individual

subsets differ in antigen processing machinery, levels of MHC expression, and the overall context of antigen presentation, some segments of a given TRA will be presented directly by mTECs, while others will be preferentially presented indirectly via thymic DCs and some segments will not be presented at all (Perry *et al*., 2014; Legoux *et al*., 2015; Klein, Robey and Hsieh, 2019). This was nicely demonstrated in a study that followed the clonal deletion of two natural T cell clones specific for the retinal TRA, IRBP. While the deletion of the first clone was completely dependent on Aire and indirect presentation, the second clone was simply not deleted, presumably because its cognate segment of IRBP was not presented in the thymus (Taniguchi *et al.*, 2012). Thus, as also shown by more recent studies (Legoux *et al.*, 2015; Malhotra *et al.*, 2016; Hassler *et al.*, 2019; Hemmers *et al.*, 2019), clonal deletion, also referred to as recessive tolerance, is inherently incomplete, therefore, it is crucial to have a proper Treg selection, also referred to as dominant tolerance, that fills the gaps in central tolerance through the suppressive function of Tregs in the immune periphery (Dikiy and Rudensky, 2023). Consistent with this notion, it has been shown that when Aire is abrogated, the clones that should be undergoing Treg selection and not clonal deletion are those that contribute the most to autoimmunity (Malchow *et al.*, 2016). Recent study corroborated these findings by showing that mice with conditional knockout of the NF-κB inducing kinase in mTECs, which is critical for their development and function, exhibited autoimmune manifestations early in life, and these were demonstrated to be caused by the failure to generate thymic Tregs rather than a clonal deletion deficiency (Haftmann *et al.*, 2021).

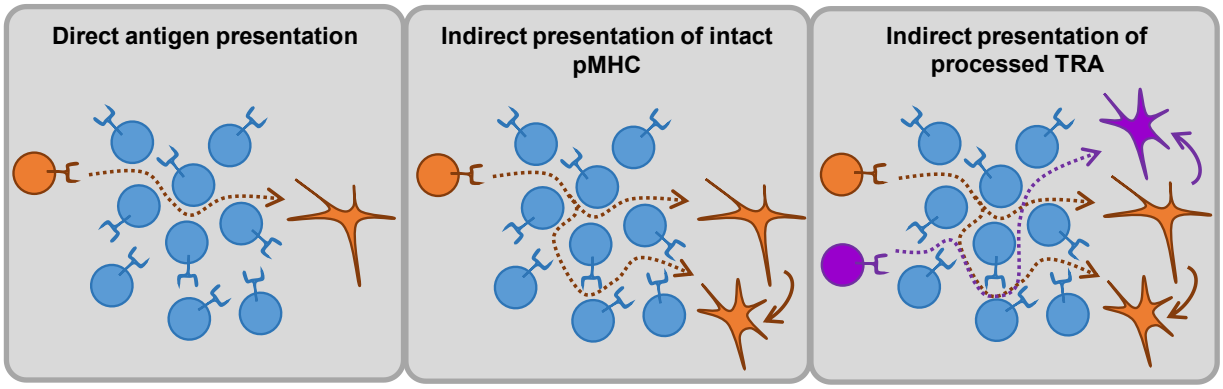

Figure 3. The significance of indirect presentation. (Left panel) Cognate pMHC of the self-reactive T cell (orange) is presented only directly by mTEC (orange cell with protrusions). Therefore, there is less routes (only one of the possible is demarcated) the self-reactive T cell can take to encounter its cognate pMHC. (Middle panel) Intact pMHC, to which the self-reactive T cell is specific, is transferred from mTEC to DC by CAT. As a result, there are more routes for the self-reactive T cell to encounter its cognate pMHC, since it is presented both directly and indirectly by mTEC and DC, respectively. (Right panel) In addition to the transfer of intact pMHC by the orange DC, another DC (purple) acquired, processed and loaded an mTEC-derived antigen onto its MHC molecules. The presented segments of this antigen are different from those presented by orange APCs. Therefore, the purple DC selects self-reactive T cell (purple) that carries TCR of a different specificity than the orange one that is selected by the orange APCs from the previous panels. Overall, CAT enhances antigen presentation and expands the repertoire of self-reactive T cell clones which is subjected to central tolerance selection processes. Adopted from (Březina, Vobořil and Filipp, 2022).

Using the model TRA hen-egg lysozyme (HEL) expressed under either insulin or thyroglobulin promoter, it was shown that the TRA source also determines whether central tolerance mechanisms rely on direct or indirect presentation. While the absence of MHCII on BM APCs abolished clonal deletion in the case of insulin-driven HEL expression, it did not alter clonal deletion and surprisingly enhanced Treg selection in the case of thyroglobulin-driven HEL expression (Yap *et al.*, 2018). Similarly, when membrane-bound OVA (mOVA) was expressed under the rat insulin promoter (RIP) or Aire promoter, its clonal deletion depended on indirect presentation or was completely independent of it, respectively, while Treg selection depended on indirect presentation in both scenarios (Mouri *et al.*, 2017). This could be explained by the fact that in the RIP mOVA system, mOVA is predominantly expressed by mTECs with low expression of MHCII, therefore, to ensure that mOVA presentation is sufficient for clonal deletion, mOVA must be transferred to more potent APCs, whereas the Aire promoter drives mOVA expression mainly in $mTECs^{HI}$, which are robust APCs (Mouri *et al.*, 2017). Many MC subsets downregulate their antigen-presenting capacity while expressing a variety of TRAs (Baran-Gale *et al.*, 2020; Michelson *et al.*, 2022) and also chemokines that attract thymic DCs (Hubert *et al.*, 2011; Lei *et al.*, 2011; Morimoto *et al.*, 2018), and this may also be the case of the RIP mOVA system. Therefore, MCs may serve as a "warehouse" of TRAs used for indirect presentation by thymic DCs. In addition, several studies comparing the establishment of central tolerance to mOVA and soluble OVA, both mimicking TRAs, revealed substantial differences in the modes of central tolerance depending on the cellular localization of OVA (Hubert *et al*., 2011; Lancaster *et al*., 2019). This phenomenon is most likely related to the accessibility of the particular antigen to individual presentation pathways and antigen transfer to DCs.

Taken together, these results clearly show that the contribution of direct and indirect presentation to the establishment of central tolerance is cooperative, where one mode relies on and influences the other. They also point to the fact that it is not only the nature of the antigen, but also the context in which these antigens are processed, fragmented and displayed that predetermines whether it is preferentially presented directly or indirectly, and crucially, whether its recognition leads to clonal deletion or Treg selection.

2.2.2. Cooperative antigen transfer

Peripheral DCs act as the body's sentinels, since their primary role is to sample the surrounding antigens and then present them to T cells to induce either an immune response or tolerance (Cabeza-Cabrerizo *et al.*, 2021; Bosteels and Janssens, 2024). Similarly, thymic DCs act as sentinels of the body by indirectly presenting TRAs to purge the T cell repertoire of self-reactive clones. However, as with peripheral DCs, the antigens used for presentation by thymic DCs to test the self-reactivity of T cell clones must first be harvested from the environment. Thus, cooperative antigen transfer (CAT) is simply a thymus-specific code name for antigen sampling, referring to the cooperative nature of tolerance establishment to TRAs between mTECs and thymic DCs (Herbin *et al.*, 2016; Perry and Hsieh, 2016; Morimoto *et al.*, 2023).

CAT has been repeatedly described as a unidirectional antigen transfer from mTECs to thymic DCs, that relies entirely on their mutual contact (Millet, Naquet and Guinamard, 2008; Kroger *et al.*, 2017; Perry *et al*., 2018). Results from several independent studies demonstrated that CAT does not represent a regular endocytosis/phagocytosis, as thymic DCs, when cocultured with mTECs, completely outcompete their peripheral counterparts in this assay (Koble and Kyewski, 2009; Kroger *et al*., 2017). Thus, thymic DCs must express molecular determinants of CAT that predispose them to perform this process much more efficiently than non-thymic DCs. In fact, recent study demonstrated that thymic DC phenotype is dictated by thymic microenvironment, since splenic DCs introduced into reaggregated thymic organ cultures reflected thymic DC phenotype after several days in these reaggregated thymi (Herppich, Beckstette and Huehn, 2022).

Theoretically, CAT can be performed via several cell contact-dependent pathways: (i) trogocytosis, (ii) engulfment of mTECs or their apoptotic bodies, and (iii) gap junctions (Koble and Kyewski, 2009). All these possible mechanisms rely on the attraction and adhesion of DCs to mTECs. Therefore, one of the critical determinants of CAT is Aire, as it regulates the production of a number of chemokines that attract thymic DC1 (XCL1), DC2 (CCL2, CCL8, CCL12), and moDC (CCL2, CX3CL1) to the the proximity of mTECs (Hubert *et al*., 2011; Lei *et al*., 2011). In addition, *Aire*$^{-/-}$ mice reveal high, ectopic expression of Ctla-4 which dampens CAT and blocks CD80/86 molecules on DCs (Morimoto *et al*., 2022). Thus, Aire regulates CAT on several distinct levels. Once in the close contact, a candidate determinant driving adhesion of conventional DCs to mTECs is the epithelial cell adhesion molecule (EpCAM). Since EpCAM is the most highly expressed in CAT-experienced DCs and is also a marker of mTECs, there was a consensus that EpCAM is acquired by thymic DCs from mTECs (Koble and Kyewski, 2009). However, more recent study (Kroger *et al*., 2017) and our unpublished data showed that thymic DCs produce their own EpCAM, likely in order to promote homotypic, adhesive interaction with mTEC-derived EpCAM to facilitate CAT.

In the study that discovered CAT, *Humblet and colleagues* demonstrated that mTEC-derived pMHCIIs are transferred to DCs (Humblet, Rudensky and Kyewski, 1994), a finding that was later published elsewhere (Millet, Naquet and Guinamard, 2008; Kroger *et al*., 2017; Perry *et al*., 2018). Since these pMHCs were found intact after their transfer to DCs, a consensus emerged that CAT is mediated by the process of trogocytosis. During trogocytosis, entire segments of plasma membranes, including the membrane-bound proteins, are exchanged between the two interacting cells (Schriek and Villadangos, 2023). Theoretically, trogocytosis is the most rapid mechanism of CAT because it allows indirect presentation of preserved mTEC-derived pMHCs immediately after CAT. However, this mechanism of transfer skips the processing of transferred antigens by DCs, thus, it does not expand the repertoire of molecular fragments presented (Miyake *et al*., 2017). Importantly, a direct evidence for the involvement of trogocytosis in CAT is lacking. However, since trogocytosis has been shown to be dependent on PI3K signaling, and its blockade does not affect CAT to the DC2 lineage (in contrast to the DC1 or pDC lineage), one can assume that DC2s acquire

mTEC-derived antigens in a trogocytosis-independent manner. Interestingly, although pMHC molecules are localized to lipid rafts, disruption of these membrane structures by two different chemicals, methyl β-cyclodextrin and nystatin, did not affect pMHC transfer (Kroger *et al*., 2017).

While the contribution of trogocytosis to CAT remains enigmatic, CAT mediated by engulfment of mTECs or their apoptotic bodies is a much better understood process that has been already visualized using confocal microscopy (Morimoto *et al.*, 2023). Due to the massive formation of DNA double-strand breaks in mTECs caused by the action of Aire and its partners, the mTEC population frequently undergoes apoptosis (Gray, Abramson, *et al*., 2007). In fact, as of today, the scavenger receptor CD36 is the only verified molecular determinant of CAT which drives the engulfment of apoptotic mTECs by DCs (Perry *et al*., 2018). However, while it has been documented that CD36 is specifically expressed by the DC1 lineage in the thymus, reaches its highest expression at the time when DC1s become CAT-experienced (Bosteels *et al*., 2023; Morimoto *et al*., 2023), and *Cd36*$^{-/-}$ mice displayed autoimmunity, CD36 deficiency only moderately affected CAT (Perry *et al*., 2018). Furthermore, it only affected CAT of membrane-bound antigens (Perry *et al*., 2018). Since CAT mediated by mTEC engulfment also involves cytosolic antigens (Koble and Kyewski, 2009), this finding suggests that CD36 is only one among other molecules that drive CAT. In fact, *Perry and colleagues* identified that CD36 performs CAT in concert with "find me" signals that are broadcasted to DC1s through their expression of purinergic receptor P2Y14 (Perry *et al*., 2018). One of the possible candidate determinants of CAT is another scavenger receptor, Tim-4. While *Kurd and colleagues* interpreted the impaired central tolerance in *Timd4*$^{-/-}$ mice as the failure of DCs to properly engulf clonally deleted T cells which then escaped to the immune periphery, the authors did not consider the possibility that they observed, similar to the scenario in the *Cd36*$^{-/-}$ mice, the defects in CAT and subsequent insufficiency in the indirect antigen presentation (Kurd *et al*., 2020).

Although it has not been analyzed in the published studies, according to our unpublished scRNAseq data and open access scRNAseq data library (Park *et al*., 2020) (https://app.cellatlas.io/thymus-development/dataset/11/scatterplot), mTECs and cells from thymic DC1 lineage express several gap junction-forming connexin family members, through which they might exchange molecules weighing up to 1.8 kDa (Neijssen *et al.*, 2005). Since these cells are known to reside in permanent contact (Lei *et al.*, 2011; Park *et al*., 2020), we cannot exclude the possibility that gap junctions contribute to the antigen transfer, as originally hypothesized (Koble and Kyewski, 2009).

Taken together, it seems that CAT involves several, perhaps many molecular determinants, the combination of which determines the mechanisms by which a specific thymic DC subset is able to acquire TRAs. To a large extent, the use of a particular mechanism determines the context of indirect presentation itself. Thus, the outcome of central tolerance is fine-tuned by different modes of CAT, the utility of which is affected by mechanisms influencing how antigens are processed and loaded onto MHC molecules, or whether they are transferred as intact pMHC molecules.

2.2.3. Heterogeneity of thymic DCs

So far, we have discussed central tolerance only from the perspective of TRAs. In fact, all thymic DCs are capable of acquiring and presenting antigens from mTECs, including TRAs, via CAT, although with different efficacy (Perry and Hsieh, 2016; Kroger *et al*., 2017; Vobořil *et al*., 2022). However, the majority of self-antigens presented by thymic DCs are ubiquitously expressed or blood-borne antigens. In conjunction with the avidity model (chapter 2.2.1), since these self-antigens are presented in the thymus within pMHCs in abundance, their recognition was shown to lead to the clonal deletion (Legoux *et al*., 2015; Malhotra *et al*., 2016; Klein, Robey and Hsieh, 2019). Surprisingly, clonal deletion of these T cells does not occur preferentially in the thymic medulla. As mentioned in chapter 1, positively selected T cells undergo a transition from DP to SP stage, and during this transition they upregulate chemokine receptors that guide their migration from the cortex, across the CMJ to the thymic medulla. However, it takes about a day before DP T cell fully commits to CD4 T cell lineage and in the case of the CD8 T cell lineage, this transition takes up to four days (Saini *et al*., 2010; Melichar *et al*., 2013; Kurd and Robey, 2016). Thus, when a T cell arrives in the vascularized region of CMJ, it does so with the highest probability in DP stage which was documented to be a stage prone to apoptosis triggered by strong TCR signaling (Kishimoto and Sprent, 1997). Up to this point, DP T cells interacted with cTECs and obtained only weak TCR inputs, since cTECs present low affinity peptides and could not provide strong costimulation as discussed in the chapter 1. Once in the CMJ, the antigen presentation context dramatically changes due to presence of thymic DCs (Ladi *et al*., 2008; Melichar *et al*., 2013) which can provide a strong costimulation necessary for clonal deletion (Watanabe *et al*., 2020). These DCs induce clonal deletion of DP T cells that is at least as frequent as clonal deletion of SP T cells in the medulla (McCaughtry *et al*., 2008; Sinclair *et al*., 2013; Stritesky *et al*., 2013).

DCs which localize to CMJ are either of DC2 or moDC lineage origin and reside in the proximity of blood vessels through which they transport the ubiquitously expressed and blood-borne antigens into the thymus (Bonasio *et al.*, 2006; Baba, Nakamoto and Mukaida, 2009; Li *et al*., 2009; Atibalentja, Murphy and Unanue, 2011; Vollmann *et al*., 2021). In general, clonal deletion in CMJ serves as a first deletional wave that leads to the apoptosis of T cells specific primarily to antigens other than TRAs. It is of note that majority of thymic DCs localizes to the thymic medulla, where they govern the second wave of clonal deletion as well as Treg selection through the acquisition of mTEC-derived antigens, including TRAs (Perry *et al*., 2014; Hu *et al*., 2015; Ardouin *et al*., 2016; Leventhal *et al*., 2016; Lancaster *et al*., 2019; Park *et al*., 2020). Consistent with the two separate waves of clonal deletion, it has recently been found that CCR4 and CCR7-guided migration towards the medulla drives clonal deletion of T cells within hours and several days after their positive selection, respectively (Li *et al*., 2023). Thus, the developmental stage as well as the cortex/medulla localization of such T cells is completely different at these distant time points.

Importantly, the mechanism of blood-borne antigen transfer into the thymus has been described lately. There are CX3CR1$^+$ DCs that home to the interface between thymic tissue and endothelium in a CX3CL1 chemokine-dependent manner (Vollmann *et al*., 2021). This homing is permanent, as the depletion of DCs from the blood by clodronate did not affect the transfer of blood-borne antigens into the thymus (Atibalentja, Murphy and Unanue, 2011), whereas constitutive ablation of the CX3CR1-CX3CL1 axis abolished it and, in addition, resulted in defective clonal deletion of blood-borne antigen-specific CD8 T cells (Vollmann *et al*., 2021). Using microscopy, it has been described that CX3CR1$^+$ DCs form protrusions through which they acquire antigens from the blood (Vollmann *et al*., 2021). *Givony and colleagues* proposed yet another pathway of blood-borne antigen uptake in which microfold MCs form these protrusions and, like their intestinal counterparts, M cells, deliver these antigens through transcytosis to CX3CR1$^+$ DCs which have been described to interact with microfold MCs (Givony *et al*., 2023).

Recently, *Zegarra-Ruiz and colleagues* published an article that may change the whole perspective of which antigens can be presented in the thymus and how tolerance to them is established. They showed that when mice are colonized with segmented filamentous bacteria (SFB) (Schnupf *et al*., 2017) during weaning, a period that is critical for the natural microbial colonization of the intesines and the development of the immune system, during which mice are deprived of access to their mother's milk (Al Nabhani *et al*., 2019), CX3CR1$^+$ DCs carry SFB antigens into the thymus. Strikingly, when this happens, the population of SFB-specific T cells is not deleted in the thymus with higher efficacy, as one would expect, but surprisingly increases in number. Unfortunately, T cell repertoire of such mice led to a significant worsening of experimental colitis compared to that induced by a control repertoire derived from mice not colonized with SFB. Since experiments with colonization with another commensal bacterial species, E. coli, showed comparable results, this study suggests that dysbiosis during weaning impairs central tolerance, which is projected into an adult T cell repertoire that is incompletely tolerant to commensal microbiota (Zegarra-Ruiz *et al.*, 2021).

It is noteworthy that pDCs were shown to migrate from the intestine to the thymus, this migration was found to be dependent on microbial sensing, and in the absence of it, the cellularity of invariant T cells as well as innate lymphoid cells in the thymus was reduced (Ennamorati *et al*., 2020). Thus, there are several subsets of DCs in the thymus that shape lymphocyte repertoire based on their interaction with intestinal microbiota. Moreover, OVA-pulsed pDCs injected into the bloodstream deleted thymic OTII cells, suggesting that they form another DC subset along with DC2s/moDCs that deliver peripheral antigens to the thymus and establish recessive tolerance to them. Indeed, when mice were injected subcutaneously with fluorescent beads of a single color in the left paw and differently colored beads in the right paw, most of the pDCs in the thymus were found carrying both colors, suggesting that pDCs migrate vigorously within the periphery and from the periphery to the thymus (Hadeiba *et al*., 2012).

Thymic DC1s principally differ from other thymic DC subsets, since they develop intrathymically from their precursors and hence are thymus resident (Cosway *et al*., 2018; Breed *et al*., 2022). In marked contrast with e.g. CX3CR1^{+} DCs they are inefficient in acquisition of blood-borne antigens and overall endocytic activity analyzed *in vitro* (Baba, Nakamoto and Mukaida, 2009; Atibalentja, Murphy and Unanue, 2011; Kroger, Wang and Tisch, 2016). Perhaps because thymic DC1s do not have access to antigens in the immune periphery, they are specialized for the acquisition of TRAs from mTECs. Indeed, when antigen transfer from mTECs was tracked using GFP expressed under the Aire promoter, more than 80% of CAT-experienced DCs belonged to the DC1 lineage (Perry *et al*., 2018), suggesting that DC1s outcompete their DC counterparts in the transfer of TRAs. Since Treg selection is highly dependent on the presentation of TRAs, as discussed above, one would expect DC1s to be specialized for the selection of Tregs. Although there are publications favoring the contribution of DC1s to Treg selection over DC2s (Perry *et al*., 2014, 2018), it is apparent that DC1s do not cover the full spectrum of TRA-specific Treg clones, as DC2s are thought to select, for example, PSA-specific MJ23 Tregs (Leventhal *et al*., 2016). There are even publications that suggest that thymic DC1s select Tregs only to a limited extent and that DC2s are superior in this respect (Proietto *et al*., 2008; Hu *et al.*, 2017). It should be noted, however, that these findings are based on *in vitro* experiments and that both DCs and T cells can act differently in the 3D structure of the thymus. A recent study has shed light on this issue by finding Mgl2^{+} DC2s that are enriched in the thymus and whose presence there is dependent on type 2 cytokine signaling. Using *Mgl2^{DTR}* mice, the authors demonstrated that in the absence of Mgl2^{+} DC2s, Treg selection is unaffected, while conventional T cells showed decreased cleaved caspase 3 expression and a much broader TCR repertoire compared to WT controls, suggesting defects in clonal deletion (Breed *et al.*, 2022).

In sum, of the four thymic DC lineages, only DC1s develop in the thymus and preferentially cooperate with mTECs to establish tolerance to TRAs. DC2s, moDCs, and pDCs are migratory and transfer ubiquitous, blood-borne, and non-self innocuous antigens, such as microbial peptides, across the CMJ into the thymus to establish tolerance to them. Among the conventional DCs, DC1s are thought to prefer Treg selection, whereas DC2s prefer clonal deletion. It is noteworthy that in the older literature, the distinction between DC2 and moDC lineages was unclear, mainly because of the use of Sirpα or CD11b as DC2 lineage-specific markers, despite their expression across the moDC lineage (Guilliams *et al.*, 2014; Ginhoux, Guilliams and Merad, 2022) (Figure 2). In any case, recent studies have shown that blood-borne (Vollmann *et al*., 2021) or microbiota-derived (Zegarra-Ruiz *et al*., 2021) antigens are delivered to the thymus by CX3CR1^{+} DCs, the expression profile of which corresponds to that of moDCs. In the case of the thymic DC2 lineage, Mgl2 (in combination with other markers) was proven as its reliable marker (Breed *et al*., 2022).

2.2.4.Homeostatic maturation of thymic conventional DCs

In their seminal study, *Ardouin and colleagues* demonstrated that portion of thymic DC1s reveals an activated phenotype at steady state that is highly similar to that of peripheral DC1s activated via TLR

stimulation, e.g. TLR3 via Poly(I:C), thus undergoing immunogenic maturation (Ardouin *et al.*, 2016). However, in marked contrast to the immunogenic maturation, thymic DCs got mature in the absence of MyD88, TRIF, MAVS and type I interferon (IFN) signaling and even in germ free (GF) mice. These steady-state, homeostatic mature DCs were reported to have very similar phenotypes in the thymus, spleen, and lymph nodes (Ardouin *et al.*, 2016; Oh *et al*., 2018), however, their percentage within conventional DC lineages was reported to be significantly higher in the thymus than in the steady-state spleen (Oh *et al*., 2018). Moreover, lineage tracing and sequencing experiments confirmed that homeostatic maturation is a general phenomenon among thymic conventional DCs and encompasses intrathymic maturation of DC1 into activated DCs type 1 (aDC1) and DC2 into aDC2 (Ardouin *et al.*, 2016; Oh *et al.*, 2018; Park *et al.*, 2020; Breed *et al.*, 2022; Ashby *et al.*, 2024). Apart from immature conventional DCs, aDCs do not cycle, express the chemokine receptor CCR7, which serves as a reliable marker for their analysis, and upregulate whole set of genes (Ardouin *et al*., 2016; Breed *et al.*, 2022) the protein products of which are crucial players in clonal deletion (e.g. PD-L1, CCL17, CCL22) (Hu *et al*., 2015; May *et al*., 2024) or Treg selection (e.g. CD40, CD70) (Coquet *et al*., 2013; Oh *et al*., 2018).

Importantly, tracking CAT in aDC1s revealed that they are more experienced in this process than immature DC1s, and *in vitro* proliferation assays showed that aDC1s also outperform their counterparts in antigen presentation (Ardouin *et al.*, 2016). In fact, according to a recent study focused on splenic DCs, it is the late immature DC1 stage that is the most efficient at capturing apoptotic cells (Bosteels *et al*., 2023). Relevant to thymic research, *Bosteels and colleagues* have shown that scavenging of apoptotic bodies, specifically cholesterol uptake and its subsequent efflux, drives homeostatic maturation of splenic DC1. LXR-β, the master regulator of cholesterol efflux, was found to be a critical determinant of such a maturation (see also Figure 5 in the section E). Importantly, a similar mechanism has been described in the tumor, where DC1s give rise to aDC1s in a scavenger receptor Axl-dependent manner after ingestion of apoptotic tumor cells (Maier *et al*., 2020), in the lungs where $CD103^+$ DC1s got mature via scavenging of apoptotic cells in a scavenger receptor Mertk-dependent manner (Silva-Sanchez *et al.*, 2023) and in the intestine where $CD103^+$ DCs were shown to mature in a similar fashion after the uptake of apoptotic IECs (Cummings *et al*., 2016). Therefore, it seems that the uptake of apoptotic cells may be a common mechanism of DC1 maturation and hence CAT, with its determinant scavenger CD36 (Perry *et al.*, 2018), may be its driver in the thymus (see also the section E). Interestingly, splenic DC2 maturation occurs independently of apoptotic cell acquisition (Bosteels *et al.*, 2023), suggesting that each DC lineage has its own set of determinants that control its maturation.

Recent articles have provided evidence for DC lineage-specific determinants of homeostatic maturation in the thymus. Interestingly, small subsets of $mTECs^{HI}$ were found to express type I and III IFNs in a PRR-independent and Aire-dependent manner, to which many thymic APCs were found to respond (Ashby *et al*., 2024). While *Ifnar*$^{-/-}$ mice, defective in type I IFN signaling, showed unaltered aDC1 frequency, consistent with a previous study (Ardouin *et al*., 2016), aDC1s were shown to be reduced by half in *Ifnar*$^{-}$

$^{-/-}$*Ifnlr*$^{-/-}$ mice, suggesting that type III IFN signaling is a critical determinant of their maturation. Such a reduction was not observed in the spleen, suggesting that this is a thymus-specific mechanism of DC1 maturation. Importantly, *Ifnar*$^{-/-}$*Ifnlr*$^{-/-}$ mice showed a reduced frequency of thymic Tregs as well as reduced diversity of their TCR repertoire. Since the same mice also exhibited increased thymic frequency of Mgl2^{+} DC2s, cleaved caspase 3^{+} conventional CD4 T cells and decreased conventional TCR diversity compared to the WT (Ashby *et al*., 2024), this data corroborated earlier data-driven hypothesis that the DC2 lineage is specialized for clonal deletion (Breed *et al.*, 2022), while suggesting that aDC1 may be critical for Treg selection. Consistent with this, aDC1 were visualized to colocalize with and express chemokines/chemokine receptors that attract them to both mTECs^{HI} and Tregs (Park *et al.*, 2020), pointing to the fact that aDC1s first interact with mTECs^{HI} to perform CAT, and then utilize acquired TRAs to generate Tregs.

Notably, type III IFNs are also involved in CD8 T cell tolerance, since they are responsible for very high MHCI expression by mTECs, which when abrogated in *Ifnlr*$^{-/-}$ mice leads to its breakdown and autoimmune manifestations as soon as at 9 months of age (Benhammadi *et al.*, 2020). It is striking that homeostatic maturation of thymic Mgl2^{+} DC2s was shown to depend on type 2 cytokines IL4 and IL13 (Breed *et al*., 2022). Thus, distinct cytokine environment favors either DC1 or DC2 maturation and this presumably has an impact on favoring Treg selection over clonal deletion or vice versa. It is noteworthy that *Ashby and colleagues* hypothesized that the reason for type III IFN-driven DC1 maturation is the upregulation and subsequent presentation of IFN-stimulated genes (ISG) by aDC1s to which they may establish central tolerance. In fact, several recent studies have opened up the previously unexplored field of central tolerance to inflammation-related self-antigens, hence, I showcased their findings in the **BOX 3**.

Finally, there are several determinants of homeostatic maturation that are shared between thymic DC1 and DC2. The experiments with *Rag1*$^{-/-}$ and *TCRα*$^{-/-}$ mice showed that in the absence of mature thymocytes, maturation of thymic DCs is almost absent. In fact, it turned out, that it is the cognate interaction between pMHCII and TCR that contributes to homeostatic DC maturation as mice in which DCs do not carry MHCII or mice in which DCs do not present OVA (*OTIIxRag1*$^{-/-}$ mice that possess monoclonal, OVA-specific T cell repertoire) showed reduced aDCs frequency. Although to a lesser extent, CD40-CD40L costimulation also contributed to homeostatic DC maturation. Surprisingly, mice with *β2m*$^{-/-}$ BM exhibited normal aDC frequencies, suggesting that CD8-pMHCI interactions do not lead to aDC generation (Oh *et al*., 2018).

3. T cell entry into the immune periphery

From its entry into the thymus as TSP to its exit as either conventional T cell or thymus-derived Treg (tTreg), developing T cells spend in the thymus about a month (Krueger, Ziętara and Łyszkiewicz, 2017) during which they interact with many APCs and antigens (**Figure 4**). Using *Rag2*^{GFP} mice (Boursalian *et al*., 2004), in which GFP is produced during TCR rearrangements so that its expression is strictly terminated in DP T cells, and tracking the GFP half-time, the post-positive selection T cell development was put into

the temporal context. Strikingly, developing T cells were found to spend 4-5 days at maximum in the medulla (McCaughtry, Wilken and Hogquist, 2007), which is just about one-seventh of their thymic residence time. During this short time-course, SP T cells develop from $CD69^+$ immature/semimature (SM) stage that is prone to apoptosis upon strong TCR signals to $CD69^-CD62L^+S1P1^+$ mature SP T cells. These T cells resist apoptosis, instead they proliferate after strong TCR signaling and egress from the thymus (Kishimoto and Sprent, 1997; Hogquist *et al*., 2015; James, Jenkinson and Anderson, 2018). Thus, given that only the SM stage is highly susceptible to apoptosis, the time window for clonal deletion in the medulla is just about 2 days (Hogquist *et al*., 2015).

BOX 3. Central tolerance to inflammation-related self-antigens. About a decade ago, several studies reported that there is a population of thymic B cells that are very well equipped to present antigens and thus induce clonal deletion and Treg selection (Perera *et al*., 2013; Walters *et al*., 2014). A comprehensive study by *Yamano and colleagues* then demonstrated that B cells undergo intrathymic licensing dependent on CD40-CD40L costimulation during cognate interaction with developing CD4 T cells. This resulted in increased antigen presenting capacity of thymic B cells, their expansion and class switching, and interestingly, upregulation of Aire expression. Although licensed B cells were shown to establish tolerance to endogenous self-peptides, the role of Aire-driven TRA expression in this process seemed unlikely since TRA expression by thymic B cells was found to be limited (Yamano *et al*., 2015). Recent studies have shed light on the role of thymic B cells in central tolerance. It has been shown that B cell licensing in the thymus, apart from CD40-CD40L interaction, depends on type III IFN-sensing and that in the absence of either B cells or the type III IFN receptor IFNLR1 on B cells, Treg frequencies and Treg TCR repertoire in the thymus were reduced (Martinez *et al*., 2023). The authors hypothesized that the endogenous self-peptides upon which thymic B cells select Tregs are ISGs and antibodies (immunoglobulins) (Rudensky, Mazel and Yurin, 1990). Indeed, using an experimental model in which ISG expression in the thymus was dampened, the same research group demonstrated that, in contrast to the T cell repertoire of *Aire*$^{-/-}$ mice, which expands rapidly after introduction into WT mice, the T cell repertoire of *Ifnb1*$^{-/-}$ mice does so only in the presence of an inflammatory stimulus (Ashby *et al*., 2024). Thus, it is suggested that thymic B cells establish CD4 T cell tolerance to inflammation-related self-antigens.

Importantly, another recent study described CD8 T cell tolerance to these antigens. The authors showed that there is a population of innate-like T cells in the thymus expressing *Gzmb*, *Ifng*, *Il4*, *Il13*, *Il17a*, etc. Using experimental systems based on *IL4*^{GFP} mice, the authors showed that solely innate-like T cells were sufficient to establish CD8 tolerance to this neo-self inflammatory antigen, and that they did so directly through the interaction of their pMHCI with CD8 TCRs. Furthermore, it was shown that reducing the level of another model antigen, IL17a-GFP, resulted in the elimination of T_H17 cells in the immune periphery. Similarly, expression of IFNg only by hematopoietic cells was sufficient to establish tolerance to IFNg in an IFNg immunization experiment (You *et al*., 2024). Thus, these mechanisms protect the body against the onset of autoimmunity during infection or injury when titers of inflammatory self-antigens are high and, at the same time, pathogen- or danger-associated signals switch the overall state of the immune system from tolerogenic to immunogenic.

Critical determinant that governs T cell exit from the thymus, the expression of which is triggered via only partially understood mechanism in mature SP T cells (Yuan *et al*., 2024), is TF KLF2 (Carlson *et al*., 2006). This protein upregulates the expression of S1P1, which is the receptor sensing the gradient of sphingosine-1-phosphate that is increasing towards the vasculature within CMJ and thus enables T cell egress to the bloodstream, and CD62L which is crucial for the subsequent T cell extravasation from the bloodstream into lymph nodes (Matloubian *et al*., 2004; Bai *et al*., 2007), where newly generated T cells home (Boursalian *et al*., 2004). Moreover, CD69 expressed by SM T cells was shown to repress S1P1 (Matloubian *et al*., 2004), hereby preventing premature T cell exit before their potential clonal deletion/Treg selection. In any case, even if T cells already express S1P1, it is ensured (by an incompletely understood mechanism likened to a "conveyor belt") that only the most mature of them leave the thymus predominantly (James *et al*.,

2018). It is of note that S1P1-independent T cell egress that is dependent on type 2 cytokine crosstalk between mTECs and invariant NKT cells was described as well (White *et al.*, 2017).

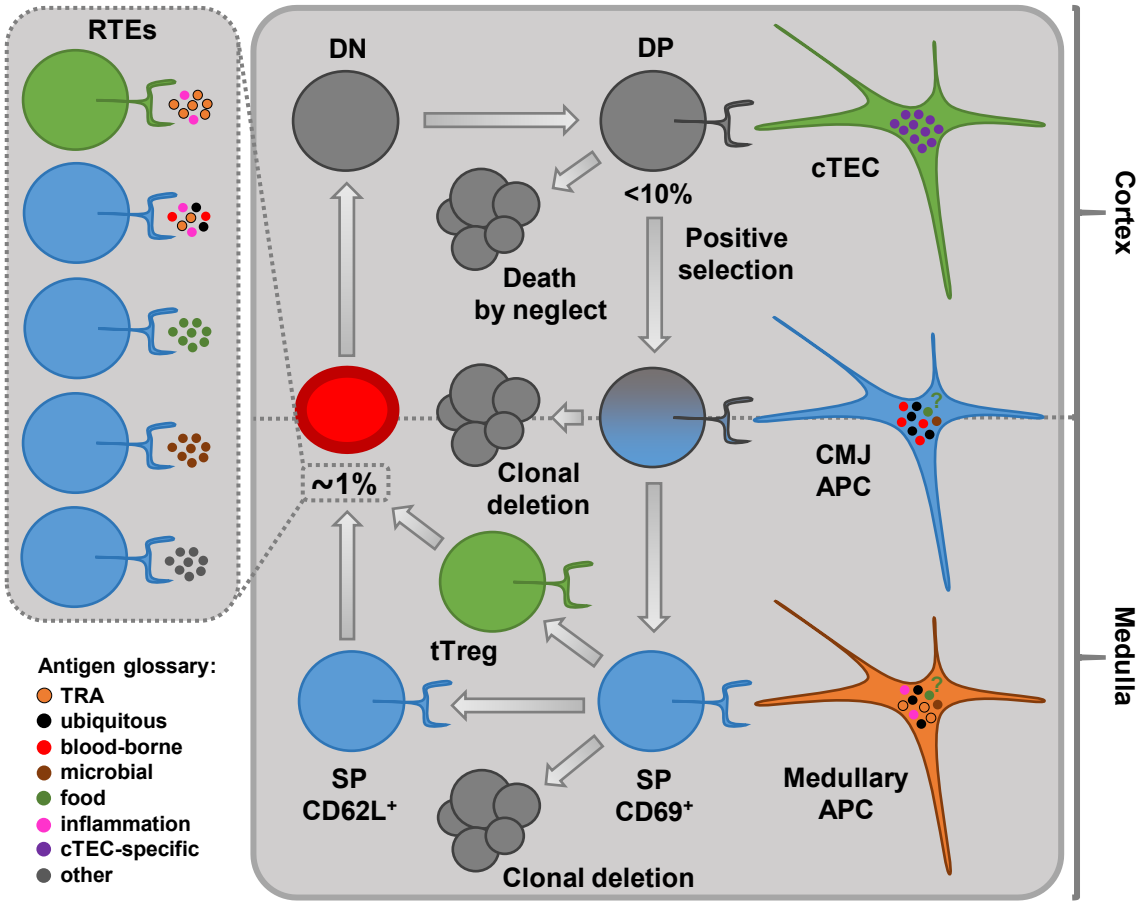

Figure 4. Distribution of antigen presentation in the thymus. T cell precursors enter the thymus via the bloodstream, commit to the T cell lineage as DN stage and develop into DP stage with fully assembled αβTCR, <10% of which undergo positive selection through the interaction with cTEC pMHCs loaded with unique, cTEC-specific antigens. The remainder of the DP T cells die by neglect in the cortex. Positively selected T cells migrate towards the medulla across the CMJ, where they undergo first wave of clonal deletion through the recognition of blood-borne, ubiquitous and possibly microbial or food antigen-loaded pMHCs. During this migration they also undergo the transition from the DP to the SP CD69$^+$ stage, in which they are highly prone to apoptosis induced by strong TCR signaling. Those T cells that survive the first wave of clonal deletion enter the medulla where they interact with many APCs which present TRA, ubiquitous, inflammation-related, and microbial or possibly food antigen-loaded pMHCs. Through this presentation, SP CD69$^+$ T cells undergo a second wave of clonal deletion, agonist Treg selection to form thymic derived Tregs (tTregs) or they mature into the SP CD62L$^+$ stage, which leaves the thymus together with tTregs. These constitute <1% of the developing T cells if we count the DP stage as an input. T cells that leave the thymus are referred to as recent thymic emigrants (RTEs) and consist of tTregs specific for TRAs or inflammation-related antigens, self-reactive conventional T cells that have escaped the clonal deletion, RTEs specific for food antigens or microbiota and RTEs specific for other antigens, including those associated with pathogens or danger to which the immune response is desired.

The last outstanding question in regards to central tolerance that I would like to comment on is the composition of the T cell repertoire entering the body. There is about 2x10^8 DP T cells whose numbers shrank to 2x10^7 SP T cells after the positive selection and it is estimated that only 1% of T cells leaves the

thymus if we count DP T cells as an input (Krueger, Ziętara and Łyszkiewicz, 2017). These T cells are composed of self and non-self-reactive clones. While the first group of clones is formed by tTregs and minor population of self-reactive conventional T cells which escaped either clonal deletion or Treg selection (Malhotra *et al.*, 2016; Hemmers *et al.*, 2019), the second, non-self-reactive group, is not fully tolerant to our body as well since it encompasses conventional T cell clones specific to microbiota, food, etc. Importantly, T cells that egress from the thymus: "recent thymic emigrants" (RTEs) are yet phenotypically and functionally different from naïve T cells (Cunningham, Helm and Fink, 2018). It is of note that RTEs maturation into fully competent naïve T cells takes about three weeks in lymph nodes during which they are prone once again to clonal deletion, deviation into peripheral Tregs (pTregs) or another mechanism of peripheral tolerance that depends on the absence of sufficient costimulation, anergy (Thangavelu *et al*., 2011; Bhaumik *et al.*, 2013; Paiva *et al.*, 2013; Friesen, Ji and Fink, 2016), leaving window of opportunity for tolerization of clones specific to self, microbial or food peptides, through the peripheral tolerance mechanisms (Miranda-Waldetario and Curotto de Lafaille, 2024). Finally, in several previous instances, I have discussed invariant NKT cells. It is important to highlight that in the thymus several invariant T cell lineages originate, exhibiting a distinct developmental trajectory from that of αβ T cells. In addition to invariant NKT cells, this category of cells encompasses γδ T cells, mucosal associated invariant T cells and CD8αα intraepithelial lymphocytes (IELs) (Ashby and Hogquist, 2024). It is noteworthy that IELs, like Tregs, can also be induced from conventional αβ T cells in the immune periphery (Cheroutre, Lambolez and Mucida, 2011).

4. Analogy between thymic and intestinal mechanisms of tolerance induction

As will be comented further, the main objective of my Ph.D. work was to investigate the Claudin 1-dependent mechanism of CAT, the mechanism that is analogous to that described in the intestine (Rescigno *et al*., 2001). Moreover, out of the scope of my Ph.D. thesis aims, I participated in two studies related to the intestinal peripheral tolerance that I also included into this thesis (Brabec *et al*., 2023, 2024). Therefore, I would like to conclude the section (B) by briefly describing the highlights of mechanisms of antigen uptake and tolerogenic presentation in the intestinal milieu, emphasizing the analogy between the induction of tolerance in the thymus and intestine. The intestinal tolerogenic machinery is more complex than that in the thymus and varies tremendously between colon and small intestine as well as between individual segments of the latter, from proximal duodenum to distal ileum (Mowat and Agace, 2014). Moreover, the establishment of tolerance in the gut is related to the phenomenon of oral tolerance, which describes that antigens orally introduced into the system are generally tolerated and that this tolerance spreads to other mucosal tissues, leading, for example, to the suppression of allergies (Mucida *et al*., 2005). However, in simple terms, both microbial and food peptides can cross the intestinal epithelium where they are taken up by APCs which are located either in direct contact with the epithelium or in its proximal mucosal tissue, the lamina propria. These APCs then migrate to the draining mesenteric lymph nodes (MLN) to exert

tolerogenic functions that are subsequently projected back into the intestinal lamina propria to establish peripheral tolerance (Miranda-Waldetario and Curotto de Lafaille, 2024).

4.1. Tolerance to microbial antigens

Although DCs are key players in peripheral tolerance, experiments with *Clec9a*^{Cre}*Iab*$^{Fl/Fl}$ and *Batf3*$^{-/-}$ (DC1 lineage) or *CD11c*^{Cre}*Irf4*$^{Fl/Fl}$ and *huLangerin*^{DTA} (DC2 lineage) mice have demonstrated their dispensability for inducing tolerance to the intestinal microbiota (Kedmi *et al.*, 2022; Lyu *et al.*, 2022). Instead, two seminal studies by *Hepworth and colleagues* suggested that Rorγ^{+} APCs of lymphoid origin do so (Hepworth *et al.*, 2013, 2015). Specifically, they showed that fraction of innate lymphoid cells type 3 (ILC3) in MLN expresses high levels of MHCII and other molecules critical for antigen processing and presentation. In fact, these cells were shown to engulf, process and present antigens to T cells. When MHCII expression in these cells was specifically abrogated using *Rorc*^{Cre}*Iab*$^{Fl/Fl}$ mice, it led to an inflammatory T cell response against commensal microbiota that could be rescued by the introduction of antibiotics (Hepworth *et al*., 2013). Using *CBir1* transgenic mice in which T cells are specific for the common clostridial flagelin (Lodes *et al*., 2004), it was demonstrated that MHCII^{+} ILC3s directly induce clonal deletion of microbiota-specific T cells and when the MHCII was expressed only by them, it was still sufficient to prevent inflammation against commensal microbiota. Interestingly, as in mTECs, MHCII expression in ILC3s was found independent on IFNγ, instead it is controlled by the transactivator Ciita (Hepworth *et al*., 2015).

Remarkably, the majority of Tregs in the colon are Rorγ^{+} pTregs that do not reside outside of the intestinal area and emerge during the weaning in response to colonization of the intestines by specific bacteria such as *Clostridia* and other bacteria from the *Firmicutes* and several other phylums (Ohnmacht *et al*., 2015; Sefik *et al*., 2015; Al Nabhani *et al*., 2019). Notably, SFB that are referred to here in the context of weaning and central tolerance (Zegarra-Ruiz *et al*., 2021) do not induce Rorγ^{+} pTregs (Sefik *et al*., 2015). Strikingly, Rorγ^{+} pTregs which act as gatekeepers of the microbiota tolerance in the colon are induced by MHCII^{+} ILC3s in MLN (Akagbosu *et al*., 2022; Kedmi *et al*., 2022; Lyu *et al*., 2022). It has been shown that in the absence of microbial antigen presentation by these APCs, microbiota-specific conventional Rorγ^{+} T cells (T_H17 cells) expand and conduct inflammatory response against the microbiota. In fact, all three mentioned studies unanimously demonstrated that MHCII^{+} ILC3s express integrins Itgav and Itgb8 which through forming of avβ8 complex process TGFβ to its active form. Since avβ8 complex was shown as critical determinant of food-antigen specific pTreg generation by conventional DCs (Worthington *et al*., 2011), it is assumed that MHCII^{+} ILC3s are endowed to generate microbiota-specific pTregs in a similar fashion. In addition, it was found that the presentation of microbial antigens by MHCII^{+} ILC3s alone is sufficient to establish the homeostatic balance between Rorγ^{+} T_H17 cells and Rorγ^{+} pTregs in favor of the latter (Kedmi *et al*., 2022). However, it should be noted that TGFβ is required for the induction of both pTregs and T_H17 cells, in the latter case together with IL6 (Veldhoen *et al*., 2006). Thus, it is likely that MHCII^{+} ILC3s fine-

tune the balance in favor of pTreg generation by some other as yet unknown mechanism. For example, retinoic acid (RA) production or cooperation with conventional DCs, which were shown to produce RA (Coombes *et al*., 2007), could be the case, as RA inhibits IL6 and thus can attenuate the entire T_H17 program in favor of pTreg generation (Mucida *et al*., 2007).

Notably, Aire$^+$ ILC3s, discussed here in the context of APECED (Yamano *et al.*, 2019) (BOX 2), proved to be dispensable for the induction of Rorγ$^+$ pTregs in all the three studies (Akagbosu *et al*., 2022; Kedmi *et al.*, 2022; Lyu *et al*., 2022). However, these publications did not agree on whether MHCII$^+$ ILC3s are ILCs per se or whether they are a new cell type, termed in one of the publications as Thetis cells, that develop neither from ILCs nor DCs. Since CCR7 expression by MHCII$^+$ ILC3s has been shown to be critical for their ability to generate pTregs (Kedmi *et al*., 2022), it is suggested that they take up microbial antigens in the intestinal epithelium and migrate to the MLN to exert their functions (Mackley *et al.*, 2015). However, details of the mechanism of antigen uptake by MHCII$^+$ ILC3s are still elusive. In addition, the presentation of commensal microbiota antigens by MHCII$^+$ ILC3s also influences B cell class switching in MLN, thereby reducing the levels of anti-commensal microbiota IgA in the intestinal mucosa and preventing dysbiosis (Melo-Gonzalez *et al*., 2019). Consistent with MHCII$^+$ ILC3 functions and given that these APCs were shown to be present in human intestine (Hepworth *et al*., 2013, 2015), MHCII$^+$ ILC3s were examined in Crohn's disease patients where they were found in significantly reduced numbers compared to the healthy controls (Hepworth *et al*., 2015; Lyu *et al*., 2022).

4.2. Tolerance to food antigens

Whereas tolerance to microbial antigens primarily involves the generation of Rorγ$^+$ pTregs in the colon-draining MLN, from which they migrate to the colon lamina propria to perform their effector functions (Miranda-Waldetario and Curotto de Lafaille, 2024), food-antigen specific pTregs localize predominantly to small intestine, are Rorγ$^-$ and their generation takes place in the small intestine-draining MLNs (Worbs *et al*., 2006; Benson *et al*., 2007; Sun *et al*., 2007; Hadis *et al*., 2011; Kim *et al*., 2016). Crucially, it was shown that food antigens are sampled in the small intestine by conventional CD103$^+$ DCs which deliver these antigens to the MLN in a CCR7-dependent manner (Worbs *et al.*, 2006). Once in the MLN, CD103$^+$ DCs generate food antigen-specific pTregs via several mechanisms. First, they produce TGFβ (Coombes *et al*., 2007; Sun *et al*., 2007) and, similar to MHCII$^+$ ILC3s, avβ8 integrin complexes that process TGFβ into its active form (Worthington *et al*., 2011). Second, they specifically express the enzyme retinaldehyde dehydrogenase 2, which is responsible for the production of RA, which is a critical cofactor in pTreg generation and, once they are generated, also directs pTreg homing back to the small intestinal lamina propria (Benson *et al*., 2007; Coombes *et al*., 2007; Mucida *et al*., 2007; Sun *et al*., 2007). Third, CD103$^+$ DCs induce food antigen-specific pTregs via expression of another enzyme indoleamine 2,3-dioxygenase also known as IDO (Matteoli *et al*., 2010), which catabolizes tryptophan to kynurenine that is critical developmental factor of Tregs (Mezrich *et al*., 2010).

It was shown that phenotype of splenic conventional DCs can be reprogrammed by IECs to correspond to that of the intestinal CD103$^+$ DCs, and thus preconditioned to generate pTregs, for example via IECs-derived TGFβ and RA (Iliev *et al.*, 2009). Here I would like to recall the parallel mechanism regarding central tolerance that mTECs can also reprogram splenic DCs to the thymic phenotype, which is essential for the generation of tTregs (Herppich, Beckstette and Huehn, 2022). Thus, it is a more general phenomenon that interactions between dendritic and epithelial cells can fine-tune Treg generation. The tolerogenic phenotype of DCs is also supported by their interaction with mucus that is produced by intestinal goblet cells (Rivera *et al*., 2022). Remarkably, goblet cells were shown to be instrumental in traficiking of food antigens from the intestinal lumen to CD103$^+$ DCs by forming goblet cell-associated antigen passages (GAPs) (McDole *et al*., 2012; Kulkarni *et al*., 2020). Using *in vivo* microscopy, CD103$^+$ DCs were shown to directly interact with GAPs and take up soluble OVA. These DCs were sorted and cocultured with OTI cells to test whether they presented the engulfed OVA, and while they readily induced OTI proliferation under normal circumstances, they failed to do so when GAPs were absent from the gut in a goblet cell-deficient mouse model (McDole *et al*., 2012).

A recent study demonstrated a direct link between GAPs, dietary antigen tolerance and pTreg generation using several GAP-deficient mouse models in which pTreg generation was disrupted and these mice failed to develop oral tolerance to OVA in an immunization challenge (Kulkarni *et al*., 2020). Notably, in this study, CX3CR1$^+$ APCs, possibly macrophages, also acquired antigens through GAPs. Interestingly, both macrophages and CD103$^+$ DCs have been shown to express many members of the connexin family that form gap junctions (Mazzini *et al*., 2014). *Mazzini and colleagues* examined the proliferation of OTII cells cocultured with DCs and macrophages isolated from the small intestine of connexin 43-deficient *Gja1*$^{-/-}$ mice fed with OVA. While DCs isolated from WT mice readily induced proliferation of OTII cells, macrophages failed to do so. However, the situation completely reversed when macrophages and DCs were connexin 43-deficient, rendering only macrophages capable to induce proliferation of OTII cells. Therefore, it was proposed that it is the macrophages that take up dietary antigens, however, they pass them on to CD103$^+$ DCs through gap junctions to establish tolerance. Notably, a more recent study showed that macrophages are dispensable for tolerance to food antigens and that CD103$^+$ DCs belonging to the DC1 lineage are critical (Esterházy *et al*., 2016). Along these lines, the data from the unpublished preprint (Canesso *et al*., 2022) suggests that oral tolerance is generated by cells that are phenotypically very similar to mature thymic and splenic DC1s (Bosteels and Janssens, 2024). Put simply, it seems that cells of aDC1 phenotype may induce both self and food antigen-specific Tregs.

4.3. DCs form trans-epithelial dendrites to snorkel in the epithelium

Although GAPs represent the only physiological route which is tightly linked to the establishment of food tolerance, there are several other routes of antigen uptake in the intestine that are associated with immunogenic responses against bacteria such as transcytosis of bacterial antigens through the M cells

(Mabbott *et al*., 2013), with still ill defined role in peripheral tolerance. This is also the case of another microbial antigen uptake pathway that DCs perform at the apical epithelium of villi in the small intestine by forming trans-epithelial dendrites (TEDs) (Rescigno *et al*., 2001; Niess *et al*., 2005; Chieppa *et al*., 2006; Farache *et al*., 2013). The founder of this topic, *Paola Ricciardi-Castagnoli*, described in her recently published commentary the rationale behind the discovery of TEDs. At that time, it was known that *Salmonella typhimurium* was only transferred into the body through M cells if it expressed invasion proteins, but *Salmonella* lacking these proteins were still detectable in the spleen after oral ingestion, so there had to be another, M cell-independent mechanism of uptake. Her research group hypothesized that perhaps these non-invasive bacteria could be taken up directly by DCs, which at that time were visualized to form protrusions into epithelial layers (Ricciardi-Castagnoli, 2020).

Indeed, intestinal DCs have been shown to express many tight junction proteins, including Claudin 1 or Occludin, to "snorkel" between IECs interconnected by tight junction proteins without violating the barrier integrity of these junctions (Rescigno *et al.*, 2001). Using TEDs, DCs were shown to engulf various bacteria, including non-invasive *Salmonella typhimurium*. Importantly, while infection and specifically TLR-sensing by IECs (e.g., TLR9-CpG engagement) enhanced DC recruitment and subsequent TED formation leading to a protective immune response, antibiotic-treated mice exhibited reduced TED formation compared to the steady-state controls (Rescigno *et al*., 2001; Niess *et al*., 2005; Chieppa *et al*., 2006; Farache *et al*., 2013). Accordingly, antibiotic treatment has been shown to reduce the expression of tight junction proteins by intestinal DCs, which likely contributes to the described TED reduction (Rescigno *et al*., 2001). TED formation was shown to depend also on the expression of chemokine receptor CX3CR1 (Niess *et al*., 2005), in a manner similar to the formation of CX3CR1-dependent trans-endothelial protrusions by thymic DCs (Vollmann *et al.*, 2021). Since it is possible that the same DCs in the thymus also present antigens of the commensal microbiota (SFB- or E. coli-derived) (Zegarra-Ruiz *et al*., 2021), this could be the mechanism of their uptake in the intestine. In line with this, TEDs were visualized even at steady state when they sampled commensal microbiota and soluble antigens (Rescigno *et al*., 2001; Farache *et al.*, 2013), leaving a window for a possible tolerogenic function of TEDs with respect to both microbial and food peptides.

While the original studies did not pay attention to trace the origin of TED-forming cells, more recent follow-up established these as $CD103^+$ conventional DCs (Farache *et al*., 2013). As already discussed, these DCs were shown to peform antigen uptake via GAPs (Kulkarni *et al.*, 2020), but what has not been mentioned above is that *Kulkarni and colleagues* have questioned the existence of TEDs under physiological conditions, suggesting that, unlike GAPs, they are an artifact of tissue processing. Crucially, $CD103^+$ and/or $CD207^+$ (Langerin) DCs were shown to acquire antigens by forming TED-like protrusions in other epithelial organs such as the skin or lungs (Sung *et al*., 2006; Kubo *et al.*, 2009), suggesting that TED formation is a common process rather than an artifact associated with tissue isolation. In any case, the thymus is also an epithelial organ because it contains mTECs (and cTECs), but in marked contrast to

intestinal, skin, or lung epithelium, mTECs do not form a bilayer that serves a barrier function, but rather a network that I would compare to a sea sponge through which T cells are being "filtered" (Lancaster *et al*., 2019). Importantly, mTECs^{HI} also express tight junction proteins such as Claudin 3 that connect and perhaps immobilize these cells within this network (Hamazaki *et al*., 2007; Dhalla *et al*., 2020), likely forming medullary segments bounded by tight junction barriers, as proposed by electron and confocal microscopy (Ichimiya and Kojima, 2006). Microscopic images also revealed that Claudin1^{+} DCs home to this network (Ichimiya and Kojima, 2006; Sanos *et al*., 2011). Strikingly, within tight junctions, Claudin 1 was shown to be a specific binding partner of Claudin 3 (Furuse, Sasaki and Tsukita, 1999; Daugherty *et al.*, 2007) which predicts the formation of a tightly interconnected cellular network between Claudin 3^{+} mTECs^{HI} and Claudin 1^{+} DC1s.

While working on the main topic of my Ph.D. thesis, the molecular determinants of CAT, it turned out that Claudin 1 expressed by thymic CD103^{+}CD207^{+} DC1s drives CAT (likely from Claudin 3^{+} mTECs). Mechanistically, it seems that the Claudin 1 expression enables DC1s to acquire TRAs from mTEC network and to drive their conversion into CCR7^{+} aDC1s that contribute to clonal deletion and TRA-specific Treg selection via indirect presentation. Thus, we predict that DC1s can snorkel into the mTEC network via the expression of Claudin 1, to exert their tolerogenic functions (see also Figure 6 in the section E).

C. THESIS AIMS

My Ph.D. studies extended my master's thesis work, which focused on the role of CX3CR1$^+$ DCs in CAT and induction of central tolerance. This focus represented a continuation of a previous project run in our laboratory during which we found that TLR signaling in mTECs enhances the recruitment of CX3CR1$^+$ DCs to the thymus and their capacity in CAT (Vobořil *et al.*, 2020). I mainly contributed to the revision of the publication describing this work which was titled *"Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation"*. Our aim was:

To analyze impact of TLR sensing by mTECs on CAT and central tolerance

Rationale: While the expression of TLRs in the thymus has been reported, its impact on thymic biology was unclear. To this end, we analyzed the outcomes of TLR sensing by mTECs.

Working on this aim helped us to finalize the main framework of my Ph.D. thesis, since while working on this project and analyzing our very first scRNAseq, we discovered aDC subset of thymic DCs. My task in this context was to translate this knowledge into a newly designed flow cytometry gating strategy. Achieving this goal, we were able to analyze the aDC subsets in much more detail and reveal new phenotypical and functional relationships among these subsets as well as their role in CAT. Thus, the particular aims of my Ph.D. studies were:

To analyze preferential pairing between mTEC and thymic DC subsets in CAT

Rationale: The routine use of scRNAseq has unraveled the complexity of the mTEC and thymic DC pool, revealing many previously overlooked populations, including aDCs. We hypothesized that since each mTEC/DC subset has its specific molecular makeup, some subsets may be more compatible with respect to CAT and would prefer their interactions with only specific subsets of mTECs, while there may be subsets that do not cooperate to perform CAT at all. In our view, these preferences could be critical as they may determine the immunological outcome of central tolerance.

This aim and results of this study were described in the publication *"A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer"*.

To find molecular determinants of CAT in thymic DC1 lineage

Rationale: *In vitro* cocultivation experiments of DCs with mTECs, described in several publications cited herein, have shown that thymic DCs largely outcompete peripheral, e.g. splenic, DCs in CAT and thus must express some molecular determinants that account for their capacity to efficiently perform this process. Furthermore, the results that addressed our previous aims showed that the thymic DC1 lineage, and in particular aDC1, is by far the most efficient in CAT. To understand why aDC1s excel in CAT, we searched for molecular determinants of CAT in the thymic DC1 lineage.

Results of this study are summarized in a manuscript *"Claudin 1 on dendritic cells type 1 regulates central tolerance via antigen transfer"*, which has been already submitted for publication.

D. RESULTS

Here I provide a list of studies that address the thesis aims and are related to the core of the thesis:

Vobořil M, Brabec T, Dobeš J, Šplíchalová I, **Březina J**, Čepková A, Dobešová M, Aidarova A, Kubovčiak J, Tsyklauri O, Štěpánek O, Beneš V, Sedláček R, Klein L, Kolář M, and Filipp D | *Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation* | Nature Communications (2020) 11:1–16 | doi: 10.1038/s41467-020-16081-3 | IF_{2020}=14.9

Vobořil M*, **Březina J***, Brabec T, Dobeš J, Ballek O, Dobešová M, Manning J, Blumberg RS, and Filipp D | *A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer* | Elife (2022) 11 | doi: 10.7554/ELIFE.71578 | *= equal contribution | IF_{2022}=7.7

Březina J, Brabec T, Vobořil M, Machač D, Jančovičová K, Sýkora V, Ballek O, Dobeš J, Manning J, Dobešová M, Čepková A, Tahtahová V, Kubovčiak J, Kolář M, Kašpárek P, Sedláček R, Tsukita S, Malissen B, Anderson G, and Filipp D | *Claudin 1 on dendritic cells type 1 regulates central tolerance via antigen transfer* | manuscript submitted for publication

Here is a review directly related to the thesis aims and core of the thesis:

Březina J*, Vobořil M*, and Filipp D | *Mechanisms of Direct and Indirect Presentation of Self-Antigens in the Thymus* | Frontiers in Immunology (2022) 13:1–13 | doi: 10.3389/fimmu.2022.926625 | *= equal contribution | IF_{2022}=7.3

Here are studies that are outside of the main thesis scope, however, focused on the immune tolerance:

Brabec T, Vobořil M, Schierová D, Valter E, Šplíchalová I, Dobeš J, **Březina J**, Dobešová M, Aidarova A, Jakubec M, Manning J, Blumberg RS, Waisman A, Kolář M, Kubovčiak J, Šrůtková D, Hudcovic T, Schwarzer M, Froňková E, Pinkasová T, Jabandžiev P, and Filipp D | *IL-17-driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum* | Mucosal Immunology (2023) 16:373–385 | doi: 10.1016/j.mucimm.2023.01.005 | IF_{2023}=7.9

Brabec T, Schwarzer M, Kováčová K, Dobešová M, Schierová D, **Březina J**, Pacáková I, Šrůtková D, Ben-Nun O, Goldfarb Y, Šplíchalová I, Kolář M, Abramson J, Filipp D, and Dobeš J | *Segmented filamentous bacteria-induced epithelial MHCII regulates cognate $CD4^+$ IELs and epithelial turnover* | Journal of Experimental Medicine (2024) 221 (1): e20230194 | doi: 10.1084/jem.20230194 | IF_{2023}=12.6

Please note that these articles and their supplementary information are included in a separate appendix to this thesis. They are numbered according to their order in the Results section.

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

By definition, TLRs are receptors that sense signals associated with pathogens or danger, such as nucleic acids, polysaccharides, or even our own "mislocalized" proteins, and thus, as discussed in the sections (A) and (B), their activation is usually associated with the induction of an immune response (Kawai *et al.*, 2024). Unexpectedly, we detected the expression of TLR2, 3, 4 and 9 in mTECs, one of the major cell types that induce immune tolerance. To investigate the relevance of TLR signaling in mTECs, we generated an mTEC-specific knockout of the common TLR signaling component MyD88, the *Foxn1*^{Cre}*MyD88*$^{Fl/Fl}$ mouse model, and found that MyD88-deficient mTECs exhibit reduced expression of chemokines that attract myeloid cells and DCs in particular. The fact that mTECs from GF mice did not show a reduction in these chemokines suggested that the ligand that triggers TLR signaling in mTECs is endogenous. We further focused on TLR9, as mTECs^{HI} showed the highest expression of this TLR, and challenged *Foxn1*^{Cre}*MyD88*$^{Fl/Fl}$ mice and WT littermate controls with intrathymic injections of CpG oligonucleotides (ODN), which are TLR9 ligands. While knockout mTECs were unaffected by ODN introduction, WT mTECs upregulated genes associated with keratinocyte MCs and the expression of DC-attracting chemokines. Therefore, we decided to investigate the thymic DC pool using scRNAseq and found a previously unrecognized population of Sirpα^{+}CD14^{+}CX3CR1^{+} moDCs that was significantly reduced in *Foxn1*^{Cre}*MyD88*$^{Fl/Fl}$ mice and highly increased in ODN-stimulated WT mice. Furthermore, ODN stimulation also enhanced CAT to these moDCs. Given the profound role of DCs in agonist Treg selection (chapter 2.2.1), we examined Treg frequencies in the thymus of *Foxn1*^{Cre}*MyD88*$^{Fl/Fl}$ and ODN-stimulated WT mice. As expected, we detected decreased and increased tTreg frequency in these mice, respectively, which, in turn, correlated with increased and decreased thymic moDC frequencies and their capacity to participate in CAT. In fact, *Itgax*^{Cre}*Iab*$^{Fl/Fl}$ mice, in which DCs do not express MHCII, treated with ODNs failed to recapitulate the increased tTreg frequency which we observed in ODN-stimulated WT, further confirming that TLR-driven Treg generation is mediated via indirect presentation by moDCs. In line with this, using *in vitro* cocultivation system we demonstrated that moDCs are capable to present mTEC-derived antigens to T cells and induce their proliferation. Finally, consistent with the fact that we observed a lower CD25 expression by Tregs from *Foxn1*^{Cre}*MyD88*$^{Fl/Fl}$ mice, we confirmed their diminished capacity to suppress T cell proliferation *in vitro* as well as *in vivo* using a diabetes mouse model. We also tested the whole CD4^{+} T cell autoimmune repertoire of *Foxn1*^{Cre}*MyD88*$^{Fl/Fl}$ mice in a colitis model in which we observed a much higher colitis severity which was comparable to that of the CD4^{+} T cell repertoire without Tregs that we used as a positive control.

Contribution: In this study, I performed in vitro approach to show moDC capacity in the presentation of mTEC-derived antigen. I also conducted other in vitro assays, participated in experiments with GF mice and I helped my colleagues with some of the other relevant experiments.

A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer

The introduction of scRNAseq into thymic biology studies unearthed the heterogeneity of TRA-expressing mTECs (mTECs^{HI}, keratinocyte MCs, tuft MCs) (Bornstein *et al.*, 2018) and showed that there are DC subsets, DC1s, aDCs (at that time we called them cDC1a (Vobořil *et al.*, 2020)), and moDCs in particular, which seemed to be molecularly predisposed to CAT via interaction with specific mTEC subsets (DC1s and aDCs with mTECs^{HI}, moDCs with keratinocyte MCs) (Vobořil *et al.*, 2020). Therefore, we hypothesized that there might be a preferential pairing between individual subsets of mTECs and DCs with respect to CAT. We tested this prediction using newly established flow cytometry panels to assess the mTEC and DC heterogeneity and several transgenic mouse models which allowed us to trace the transfer of fluorescent proteins during CAT. Specifically, we followed the transfer of TdTOMATO in three different mouse models of CAT: (i) *Foxn1*^{Cre}*R26*^{TdTOMATO}, in which TdTOMATO was ubiquitously expressed among TECs, (ii) *Csnb*^{Cre}*R26*^{TdTOMATO}, in which mTECs^{HI} and their progeny expressed TdTOMATO and (iii) *Defa6*^{iCre}*R26*^{TdTOMATO}, in which TdTOMATO mimicked the expression of a single Aire-dependent TRA, α-defensin 6 (Defa 6). Since only a fraction of all mTECs expresses TRAs (chapter 2.1), most TdTOMATO^{+} TECs in the *Foxn1*^{Cre} system did not express high levels of TRAs, whereas TdTOMATO^{+} mTECs that did express high levels of TRAs were more abundant in the *Csnb*^{Cre} system and found predominant in the *Defa6*^{iCre} system. By analyzing the relative contribution of thymic DC subsets to TdTOMATO transfer in each system and using linear regression to correlate it with the relative production of TdTOMATO by mTEC subsets in each system, we obtained mathematical model of preferential pairing. Consistent with our hypothesis, DC1s and aDCs preferred, with respect to CAT, pairing with mTECs^{HI}. The latter also preferred "pre-post-Aire mTECs", which from today's perspective might correspond at least partially to the keratinocyte MCs, while tuft MCs were preferred only by the aDC1 subset. Importantly, the capacity of aDC1s to perform CAT was by far the strongest in all the systems we analyzed, with the most visible efficiency when TdTOMATO availability was very limited (*Defa6*^{iCre} system). Overall, we found aDC1 to be the most specialized subset for CAT. Contrary to our hypothesis, moDCs did not prefer keratinocyte MCs in CAT, in fact they did not prefer any mTEC subset. Therefore, we used *Foxn1*^{Cre}*Confetti*$^{Brainbow2.1}$ mice, in which TECs are either RFP^{+} or YFP^{+} in a strictly non-overlapping manner, to see how moDCs can manage the repetitive transfer from multiple mTECs. Strikingly, moDCs excelled at multiple CAT. Given the promiscuity of moDCs in CAT, we also tested DC to DC transfer using a mixed BM chimera in which half of the DCs were WT and the other half expressed TdTOMATO. We found that DC to DC transfer was quite common among all DC subsets, but as we expected, moDCs also dominated this mode of antigen transfer.

Contribution: My contribution to this study, which I co-designed, was major. Together with my colleague Matouš Vobořil, with whom I share the first co-authorship of this paper, we performed most of the experiments equally. We also wrote the first draft of the manuscript and I was heavily involved in the experimental work on revisions of this paper.

Claudin 1 on dendritic cells type 1 regulates central tolerance via antigen transfer

Several independent studies demonstrated that thymic DCs outcompete splenic DCs in CAT when cocultured with mTECs (Koble and Kyewski, 2009; Kroger *et al.*, 2017), and thus they must express molecular determinants that predispose them to perform better in this process. To analyze which genes are upregulated in thymic DCs, we reanalyzed publicly available datasets comparing thymic and splenic DCs (Elpek *et al.*, 2011; Ardouin *et al.*, 2016), and strikingly, in both of these datasets one of the most upregulated genes was *Cldn1*, which encodes tight junction protein Claudin 1 (data not shown). At that time, we also obtained the data that thymic mature DC1s, the aDC1 subset, outcompete other thymic DCs in CAT (Vobořil *et al.*, 2022). To further pursue the search for molecular determinants of CAT, we sorted CAT-experienced (TdTOMATO^{+}) and -inexperienced (TdTOMATO^{-}) myeloid cells from thymi of *Foxn1*^{Cre}*R26*^{TdTOMATO} mice with the idea that upregulated genes in CAT-experienced cells encode the candidate determinants. We focused on the DC1 lineage because of its specialization for CAT (chapter 2.2) (Vobořil *et al.*, 2022) and to our surprise, *Cldn1* was among the top ten upregulated genes in CAT-experienced DC1s, along with *Itgae* (CD103) and *Cd207* (Langerin) (chapter 4.3). Better resolution of scRNAseq than in the previous work (Vobořil *et al.*, 2020) revealed that aDC1s consist of two states: aDC1a and aDC1b. By combining fluorescent reporter and BrdU *in vivo* lineage tracing approaches, we found that DC1s homestatically mature into early mature aDC1a, part of which fully matures into aDC1b, while losing expression of DC1 lineage-specific markers such as XCR1 (chapter 2.2.4). Therefore, aDC1b were overlooked in previous studies. Notably, we detected a very high expression of Claudin 1 ligand, Claudin 3 (chapter 4.3), in Defa6^{+} mTECs. To test Claudin 1 as a molecular determinant of CAT we generated its conditional knockout in DC1 lineage, *XCR1*^{iCre}*Cldn1*$^{Fl/Fl}$ mice, and generated mixed BM chimeras in which *Defa6*^{iCre}*R26*^{TdTOMATO} mice were recipients receiving BM with Claudin 1-sufficient and -deficient DC1 lineage cells in a 1:1 ratio. Importantly, CAT to aDC1b was significantly affected, demonstrating that Claudin 1 acts as a CAT determinant. Unexpectedly, we observed a pronounced decrease in the frequency of both Claudin 1-deficient aDC1a and aDC1b compared to the controls, suggesting that Claudin 1 is also a determinant of DC1 homeostatic maturation (chapter 2.2.4 and section E). In addition, we directly demonstrated that antigen presentation on DC1-derived cells is essential for proper Treg selection (chapter 2.2.3). Taken together, we hypothesized that Claudin 1 deficiency might lead to a breakdown of central tolerance. Therefore, we developed the *Defa6*^{iCre}*R26*$^{TdT\text{-}OVA}$ mouse model, which allowed us to analyze clonal deletion and Treg selection of OTII cells in mixed BM chimeras. Using this novel system, we demonstrated that both clonal deletion and Treg selection are impaired when Claudin 1 is absent from the DC1 lineage, underscoring the critical role of CAT and aDC1s in the establishment of central tolerance.

Contribution: This was my main Ph.D. project. I co-designed and performed majority of the experiments and co-designed the R26$^{TdT\text{-}OVA}$ *mouse model. I also wrote the first draft of the manuscript. However, I would like to highlight the contribution of Tomáš Brabec, who came up with a number of innovative solutions and performed accurate analysis of scRNAseq, which was essential for the discovery of aDC1b.*

Mechanisms of direct and indirect presentation of self-antigens in the thymus

In this review article we summarized knowledge regarding direct and indirect TRA presentation (chapter 2.2.1) and additional modes of antigen presentation in the thymus (chapter 2.2.3) and put it into the context of the last decade's discoveries regarding APC heterogeneity. We also included a chapter about the mechanisms of CAT and its possible molecular determinants (chapter 2.2.2).

Contribution: I participated in this review article as an co-author together with Matouš Vobořil and Dominik Filipp. Specifically, I wrote the chapters on indirect antigen presentation and CAT and prepared figures.

IL-17-driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum

Defa 6, whose encoding gene is routinely used in our central tolerance studies as a driver of TdTOMATO expression mimicking Aire-dependent TRA (Vobořil *et al.*, 2022; Březina *et al.*, 2024), is one of the antimicrobial peptides from the family of α-defensins that are specifically produced by a subset of IECs called Paneth cells (PCs) to control the composition of the intestinal microbiota (Bevins and Salzman, 2011; Dobeš *et al.*, 2015). The importance of α-defensins in preventing dysbiosis can be demonstrated in patients with Crohn's disease, where their reduced production contributes to the pathogenesis of this disease (Wehkamp *et al.*, 2004, 2005; Simms *et al.*, 2008). Importantly, the major cytokine that controls α-defensin production is IL17 expressed by T_H17 cells (Liang *et al.*, 2006), but its direct effect on PCs has not been studied. In this study, we showed that PCs respond to IL17 treatment because they express IL17 receptor (IL17Ra). Therefore, we generated the *Defa6*^{iCre}*IL-17ra*$^{Fl/Fl}$ mouse model, which serves as a conditional knockout of IL17Ra in these cells, and analyzed its effect on PCs using bulk sequencing (bulk-seq). We found that a number of genes encoding antimicrobial peptides were downregulated. In fact, we observed a pattern in this downregulation, as mainly the genes encoding ileal-specific α-defensins such as *Defa20*, *21* or *22* were downregulated. Therefore, we focused on the ileum (the distal part of small intestine), where we detected decreased number of PCs in the *Defa6*^{iCre}*IL-17ra*$^{Fl/Fl}$ model. Furthermore, we performed bulk-seq of ileal tissue from *Defa6*^{iCre}*IL-17ra*$^{Fl/Fl}$ mice and observed upregulation of genes associated with inflammation. However, there was no apparent pathology of the ileum in these conditional knockouts. Thus, we challenged *Defa6*^{iCre}*IL-17ra*$^{Fl/Fl}$ mice and their littermate WT controls with dextran sulfate sodium (DSS) induced colitis and while both experimental groups developed colitis as we expected, only the knockouts developed ileitis. Since we detected dysbiosis in the ileums of *Defa6*^{iCre}*IL-17ra*$^{Fl/Fl}$ mice, we attempted to transfer their ileal microbiota to an experimental model based on the *IL-10*$^{-/-}$ mouse which is susceptible to induction of inflammation by dysbiotic microbiota. While mice colonized with WT microbiota survived throughout the experiment, mice receiving conditional knockout microbiota died after 6-7 days. We repeated this experiment to analyze ileal pathology and confirmed it in the same experimental group of mice that died in the previous experiment. To see if our findings might have clinical implications in humans, we analyzed pediatric patients recently diagnosed with Crohn's disease. We found that there are some patients with PC numbers similar to healthy controls, but there was a second group of patients who had severly reduced numbers of PCs, which correlated with lower serum levels of IL17a than in patients with normal PC numbers and, importantly, significantly worse histological score of their disease. In conclusion, our study suggests that reduced numbers of PCs or their α-defensin products in the absence of IL17-sensing by PCs results in microbial dysbiosis in mice or humans who are therefore prone to ileitis.

Contribution: I had only minor experimental and intellectual contribution to this study. I critically read the manuscript and suggested some changes and analyses to improve its quality during the revision process.

Segmented filamentous bacteria-induced epithelial MHCII regulates cognate CD4$^+$ IELs and epithelial turnover

While most commensal microbionts are prevented from direct contact with IECs, e.g. by α-defensins, SFBs form projections through which they establish permanent contact with IECs (Ladinsky *et al.*, 2019). In fact, SFBs are known to induce MHCII expression in IECs (Goto *et al.*, 2014), however, the significance of this and how such expression is regulated has not been addressed. Interestingly, SFBs were shown to induce the expression of IFNγ (type II IFN), which often acts as an up-regulator of MHCII expression (Gaboriau-Routhiau *et al.*, 2009). Here we introduced IFNγ-blocking antibodies into SFB-colonized mice and found that MHCII was absent from IECs, suggesting that IFNγ indeed drives their MHCII upregulation. To search for the source of IFNγ, we performed scRNAseq of small intestinal lamina propria (SI-LP) from SFB-colonized and control mice and selected αβ T, NK and NKT cells as candidate sources. While the absence of NK/NKT cells did not affect MHCII on IECs, the absence of T cells in *Rag1^{KO}* mice had a major effect in this regard. Conversely, using a mouse model in which either NK/NKT cells or T cells expressed IFNγ, only T cells were sufficient to induce MHCII$^+$ IECs. Thus, T cells are the effector cells producing IFNγ in response to SFB colonization. DC1s were shown to govern T_H1 response against *Cryptosporidium*, which also protrudes into IECs, characterized by the IFNγ up-regulation (Russler-Germain *et al.*, 2021). Therefore, we depleted the DC1 lineage using *XCR1^{iCre}DTA* mice and detected a 95% reduction of MHCII on IECs from SFB-colonized mice. In addition, these mice had almost no IFNγ expression in αβ T cells from SI-LP. Since MHCII depletion in the DC1 lineage from *XCR1^{iCre}Iab$^{Fl/Fl}$* mice also led to an almost complete loss of MHCII$^+$ IECs, it is the DC1s that sense SFB and upregulate IFNγ through antigen presentation to CD4$^+$ T cells. Importantly, in addition to high IFNγ expression, SI-LP CD4$^+$ T cells from SFB-colonized mice upregulated *Gzma* and *Gzmb*, which encode granzymes, suggesting that SFB-stimulated upregulation of MHCII$^+$ IECs may induce CD4$^+$ IELs, which, in contrast to conventional CD4$^+$ αβ T cells, are cytotoxic (Cheroutre, Lambolez and Mucida, 2011). Therefore, we performed scRNAseq of intraepithelial cells and found an increase in CD4$^+$ IELs in SFB-colonized mice with increased expression of *Gzmb*. By monocolonizing GF mice with SFB, we demonstrated that SFB directly regulates the expansion of induced CD4$^+$ IELs. We hypothesized that MHCII$^+$ IECs control the conversion of conventional CD4$^+$ αβ T cells into induced, cytotoxic CD4$^+$ IELs through the presentation of SFB antigens, and we confirmed this hypothesis using fate mapping of SFB-specific CD4$^+$ T cells. Given the cytotoxic function of IELs, we had the bold idea that they regulate the turnover of IECs. Indeed, we demonstrated increased proliferation of the intestinal epithelium after SFB colonization and, conversely, decreased proliferation when IECs were unable to present antigens to CD4$^+$ T cells. Finally, we showed that the addition of SFB-specific IELs to small intestinal organoids resulted in increased enterocyte differentiation.

Contribution: I irradiated mice in the BM chimera experiments and given my experience with the XCR1^{iCre} model I helped to co-design the experiments with DC1 depletion/MHCII deficiency. I also helped with some of the other experiments and critically read the manuscript.

E. DISCUSSION AND CONCLUSIONS

In summary, our studies provide a crucial contribution to the understanding of the relevance and mechanisms of CAT and indirect presentation. They elucidate the intricate division of labor between different subsets of thymic DCs in the establishment of central tolerance. They also thoroughly characterized the phenotype, ontogeny, and function of aDCs and consolidated the role of the DC1 lineage in tolerance to TRAs. In addition, they opened the topic of thymic moDCs and outlined their possible role in central tolerance. Finally, they provided insights into how epithelial cells respond to immunoregulatory molecules, making them an important component of the immune system and tolerance. Since each of the studies described in the section (D) contains its own discussion, I have decided to give more room to specific topics that I consider interesting and relevant to our research within the framework of central tolerance, which were omitted in our publications due to space limitation or their research has recently advanced.

Several principal findings of our research indicate that thymus establishes tonic inflammation to maintain its homeostasis. In fact, we have shown that mTECs express a number of TLRs and that their signalling outcomes remain unchanged in the absence of microbiota or after systemic introduction of ODNs (Vobořil *et al*., 2020). In conjunction with our findings, it was shown that while the severity of autoimmunity in *MyD88*$^{-/-}$*Aire*$^{-/-}$ mice is more pronounced than in *Aire*$^{-/-}$ mice, and thus TLR signalling has a protective effect in the context of Aire deficiency-driven autoimmunity, general reduction of TLR signaling using a GF background or a systemic introduction of TLR ligands via intraperitoneal injections did not alter the phenotype of *MyD88*$^{-/-}$*Aire*$^{-/-}$ mice (Gray, Gavanescu, *et al*., 2007). Thus, collectively, it can be inferred that it is very likely that mTECs sense endogenous TLR ligands of thymic origin. While the source and nature of such a TLR ligand remains elusive, recent perspective by *Laan and colleagues* has shed light on this issue by finding that human post-Aire mTECs that form Hassal's corpuscles, currently referred to as keratinocyte MCs, are characterized by upregulation of the pro-inflammatory protein complex S100a8/S100a9, which activates TLR4 (Laan *et al.*, 2021). This, together with our observation that TLR signalling in mTECs activates genes such as *Ivl*, which underpins the phenotype of keratinocyte MCs (Vobořil *et al*., 2020), suggests that Hassal's corpuscles may serve as a tonic inflammation hub that is automatically maintained by a positive feedback loop. We have also shown that in response to TLR signalling, mTECs upregulate a number of chemokines, and in the absence of this, not only the moDCs discussed above, but also pDCs showed a reduced frequency in the thymus (Vobořil *et al*., 2020). In this context, the recent publication suggested that keratinocyte MCs attract pDCs to Hassal's corpuscles where they stimulate them to produce type I IFNs as a result of cytokine and TLR ligands sensing (J. Wang *et al*., 2019).

Interestingly, out of all murine tissues, thymus exhibits the strongest constitutive expression of type I IFNs (Lienenklaus *et al*., 2009) and it has been recently described as a hub of type III IFN signaling (Benhammadi *et al*., 2020; Martinez *et al*., 2023; Ashby *et al*., 2024). The majority of type I and III IFNs is produced by

mTECs^{HI} in an Aire-dependent manner (Ashby *et al*., 2024). Given the Aire-dependency, one can assume that mTECs express IFNs to establish their tolerance. Indeed, APECED patients suffer from type I IFN autoantibodies (Meyer *et al*., 2016) and thus they are more prone to severe manifestations of viral diseases as it was confirmed during the covid-19 pandemics (Bastard *et al*., 2021). In any case, type I and III IFNs produced in the thymus are functional and play a pivotal role in the establishment of tonic inflammation. While they act directly on mTECs, endowing them with very high MHCI levels to efficiently induce clonal deletion (Benhammadi *et al*., 2020), recent studies have described that they drive also the maturation of B cells, macrophages and DC1s and are in general essential for agonist Treg selection (Martinez *et al*., 2023; Ashby *et al*., 2024). Here, it is important to note that another critical component of tonic inflammation in the thymus is the high steady-state expression of type 2 cytokines (White *et al*., 2017; Breed *et al*., 2022), which is also important for thymic tissue regeneration in perturbed conditions after an acute injury (Cosway *et al*., 2022, 2023; Nevo *et al*., 2024). Interestingly, type 2 cytokine, IL4, is expressed in the thymus in a similar fashion as type I/III IFNs, so that it is presented to T cells to establish its tolerance (You *et al*., 2024). IL4 also plays a critical role in the maturation of thymic APCs, Mgl2^{+} DC2s in particular, hereby reinforcing the clonal deletion (Breed *et al*., 2022). The major producers of IL4 in the steady state thymus are invariant NKT cells (White *et al*., 2017; H. Wang *et al*., 2019), however, tuft MCs instruct this production through their IL25 expression (Miller *et al*., 2018; Lucas *et al*., 2020). Given that tuft MCs tend to colocalize with keratinocyte MCs into the Hassal's corpuscles (Miller *et al*., 2018), and that type I and III IFNs are produced by small, non-overlapping fractions of mTECs^{HI} (Ashby *et al*., 2024), I personally prefer the idea that tonic inflammation in the thymus is compartmentalized into local hubs rather than being formed in the gradients across the whole medulla.

While tonic inflammation driven by either type III IFNs or type 2 cytokines favours intrathymic homeostatic maturation of DC1s and DC2s, respectively (Breed *et al*., 2022; Ashby *et al*., 2024), our results point to yet another determinant of strictly DC1s maturation, namely CAT itself (Březina *et al*., 2024). In the experiments in which we simulated the transfer of the Aire-dependent TRA Defa6 using *Defa6*^{iCre}*R26*^{TdTOMATO} mice, XCR1^{+} and XCR1^{-} aDCs were virtually the only cells positive for mTEC-derived TdTOMATO (Vobořil *et al*., 2022). In the case of the latter, we assumed that these cells were of DC2 lineage origin, as they did not express XCR1. However, our recent thymic DC1 lineage tracing approaches revealed that these CAT-experienced XCR1^{-} aDCs belonged to the DC1 lineage, in particular into the aDC1b subset, which we found to lack DC1 lineage-specific markers, including XCR1 (Březina *et al*., 2024). Thus, in the *Defa6*^{iCre}*R26*^{TdTOMATO} model, aDC1s represented the absolute majority of CAT-experienced cells. We wondered why we detected such a low frequency of CAT-experienced immature DC1s (Vobořil *et al*., 2022; Březina *et al*., 2024) which we (Březina *et al*., 2024) and others (Ardouin *et al*., 2016; Perry *et al*., 2018) found to be well equipped to perform CAT, and hypothesized that CAT-experienced DC1s undergo a homeostatic maturation in response to CAT, rendering aDC1s as the predominant CAT-experienced cells.

In support of this hypothesis, in our scRNAseq of thymic DCs from the *Foxn1*^{Cre}*R26*^{TdTOMATO} mice, we found that CAT-inexperienced cells were 5-7 times less frequent among aDC1s than among DC1s, meaning that CAT-inexperienced aDC1s were almost completely absent from the dataset (Březina *et al*., 2024). But do aDC1s still acquire antigens after becoming mature? In fact, the rapid downregulation of antigen sampling in the early phase of DC maturation is well documented in the immune periphery (Bosteels and Janssens, 2024), however, there is a consensus (Ardouin *et al*., 2016; Oh *et al*., 2018) which is consistent with our unpublished data, that aDC1s retain their high capacity to perform CAT, at least in their early mature stage. Here, I would like to mention that Claudin 1 deficiency, unexpectedly, perturbed CAT only in the case of late mature aDC1b, in which the Claudin 1 expression is very low (Březina *et al*., 2024). Given the high dynamics of maturation observed within the thymic DC1 lineage using BrdU tracing, it is plausible that the aDC1b precursors (aDC1a) may be the primary affected cells in our experimental systems. However, this effect is manifested only later, at the aDC1b stage. In line with this, aDC1a subset expresses the highest level of Claudin 1 within the DC1 lineage (Březina *et al*., 2024).

As it turned out, our hypothesis that CAT controls the homeostatic maturation of DC1s has recently been investigated in detail in the spleen (Bosteels *et al*., 2023). In this study, the uptake of CellTrace Violet (CTV)-labelled apoptotic cells by immature DC1s was tracked *in vivo* (12 hours after their introduction), and strikingly, the frequency of mature cells increased dramatically among CTV^{+} DC1s. Remarkably, it was shown that blocking endocytosis *in vivo* reduced the frequency of mature DC1s by half, whereas such a hindrance of endocytosis did not affect mature DC2s frequency, suggesting that maturation of the DC2 lineage is independent of apoptotic cell engulfment. Interestingly, introduction of the TLR3 ligand Poly(I:C) into this system triggered immunogenic maturation of DC1s independently of endocytosis. The authors not only suggested that homeostatic DC1 maturation is linked to changes in cholesterol metabolism after apoptotic cell uptake and pointed to the LXR-β as a driver of homeostatic DC1 maturation (chapter 2.2.4). They delved further into this issue by proposing that LXR-β represses the expression of ISGs and thus the gene expression programme induced by IFNs. Moreover, several studies reviewed in *Bosteels and Janssens, 2024* showed that IRF3 signalling, e.g. induced by TLR3 activation, represses LXR-β. In this context, DC1s that matured after ingestion of poly(I:C)-loaded lipid nanoparticles neither upregulated LXR-β nor effluxed the ingested cholesterol, in contrast to those that took up empty lipid nanoparticles (Bosteels *et al*., 2023). Taken together, it has been proposed that cholesterol metabolism limits immunogenic maturation by preventing the IFN expression programme, and vice versa, that immunogenic maturation signaling represses LXR-β expression and thus shuts down cholesterol metabolism (**Figure 5**).

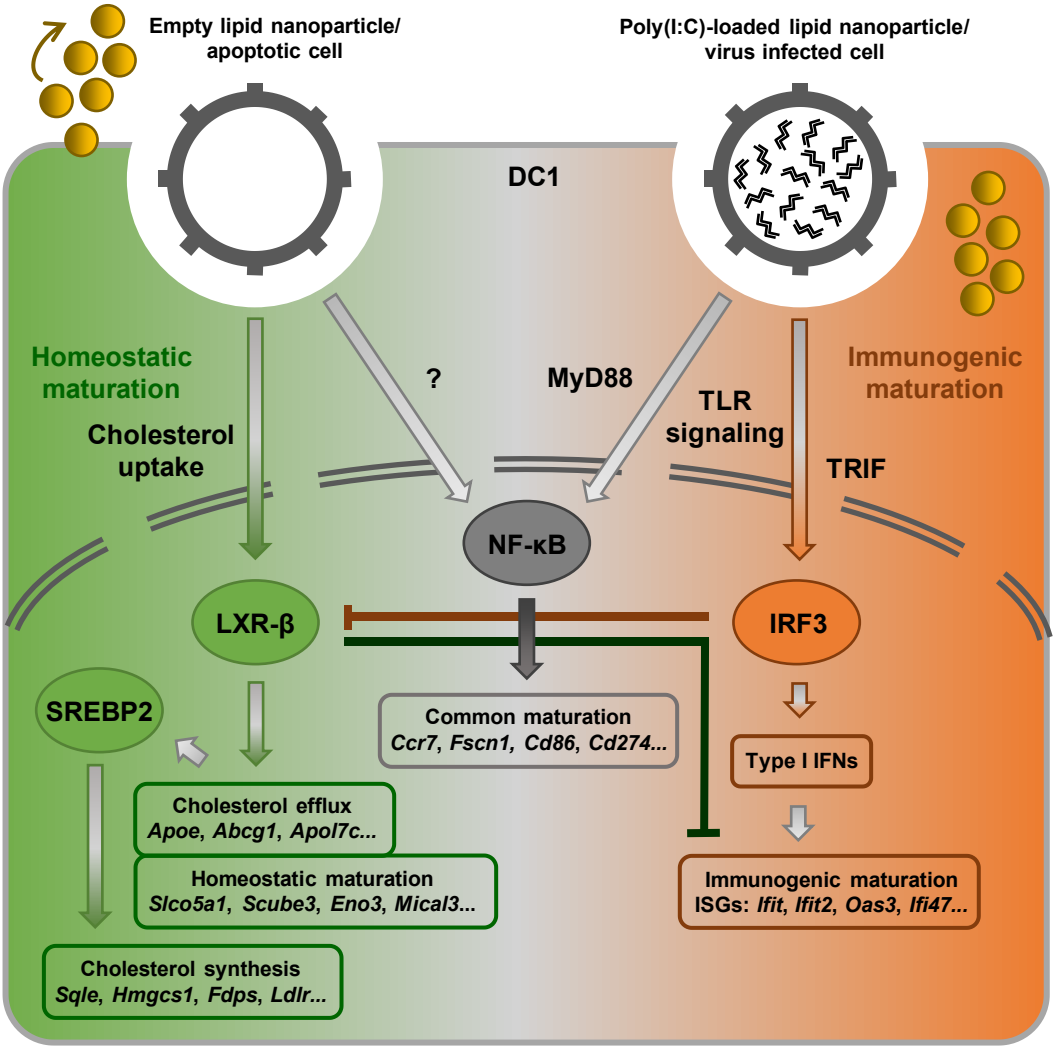

Figure 5. Cholesterol metabolism promotes homeostatic DC1 maturation. Homeostatic DC1 maturation is triggered by engulfment of apoptotic cells or empty lipid nanoparticles in an experimental model and is MyD88 and TRIF-independent. Immunogenic maturation, on the other hand, is dependent on the MyD88/TRIF pathway as it is initiated upon activation of PRRs, such as TLR3, by ingestion of Poly(I:C)-loaded lipid nanoparticles. Both maturation modes converge on the NF-κB signaling pathway by inducing genes such as *Ccr7* or *Cd274*. Therefore, it is hypothesized that the majority of gene expression changes upon DC1 maturation are shared between homeostatic and immunogenic maturation. The unique determinant of homeostatic DC1 maturation is the TF LXR-β, which is triggered by cholesterol uptake and activates the gene expression machinery that causes the subsequent cholesterol efflux (yellow droplets exiting the DC1). Somewhat unexpectedly, homeostatic DC1 maturation is also associated with cholesterol synthesis, which is induced by the TF SREBP2 in response to cholesterol efflux. Other hallmark genes of homeostatic maturation such as *Slco5a1m*, *Scube3*, *Eno3* or *Mical3* are also thought to be upregulated by the actions of LXR-β. Importantly, immunogenic DC1 maturation is characterized by the retention of cholesterol. It is suggested that IRF3, which is activated by TLR3/TRIF signaling, represses LXR-β in immunogenic mature DC1, as has been demonstrated in macrophages, leading to cholesterol retention. The IRF3 pathway also drives the expression of type I IFNs, which subsequently induce the expression of ISGs, whose persistent expression characterizes immunogenic maturation. Notably, there is also a transient upregulation of ISGs that accompanies the early phase of homeostatic DC1 maturation, which is supressed by LXR-β. Importantly, DC-restricted ablation of LXR-β led to the breakdown of peripheral tolerance under non-immunogenic conditions. Thus, the immunogenic maturation setting is dominant and must be actively repressed by LXR-β during homeostasis. Adopted from (Bosteels and Janssens, 2024).

But how does this scenario apply to the thymus, which has the highest constitutive expression of type I and III IFNs, and where DC1 maturation has recently been shown to be largely dependent on type III IFNs? In fact, ISG expression is in the context of immunogenic maturation believed to be triggered through the TLR activation (Bosteels and Janssens, 2024) and type III IFNs were shown to drive homeostatic DC maturation irrespective of TLRs and other inflammatory signals (Ashby *et al*., 2024). Importantly, even homeostatic mature splenic DC1s have been found to upregulate ISGs, but for a limited period of time shortly after apoptotic cell engulfment (Bosteels *et al*., 2023). So we can speculate that in the thymus, type III IFNs might act as homeostatic maturation "cofactors" in its initial phase. However, I can also imagine that, given the inflammatory state of the thymic microenvironment, apoptotic cell engulfment and type III IFN signalling may to some extent represent non-overlapping pathways of thymic DC1 maturation based on a local level of tonic inflammation.

Cholesterol metabolism-dependent DC1 maturation was found to be characterized by the upregulation of genes such as *Abcg1*, *Apoe*, *Apol7c* or *Nr1h2*, which encodes the TF LXR-β (Bosteels *et al*., 2023). In our study, we observed that CAT-experienced immature DC1s upregulate these genes, and the more mature they become, the higher the expression of these genes they reveal (Březina *et al*., 2024). It is noteworthy that among the top ten upregulated genes in the CAT-experienced DC1s over their -inexperienced counterparts, were genes such as *Itgae*, *Cd207* and *Dnase1l3*, all of which were previously shown to be involved in apoptotic cell uptake (Qiu *et al*., 2009; Sisirak *et al*., 2016; Bosteels *et al*., 2023). Accordingly, *Itgae*-encoded CD103, which is a critical integrin for epithelial homing, marked DC1s entering homeostatic maturation induced by apoptotic cell uptake in spleen, lungs and intestine (Cummings *et al*., 2016; Bosteels *et al*., 2023; Silva-Sanchez *et al.*, 2023). Moreover, splenic $CD103^+$ DC1s with the highest capacity to engulf apoptotic cells expressed the highest levels of CD36 (Bosteels *et al*., 2023), a receptor through which thymic DC1s scavenge mTEC apoptotic bodies (Perry *et al*., 2018). Putting all these pieces together, I suggest that cholesterol metabolism associated with engulfment of apoptotic mTECs (CAT) drives thymic homeostatic DC1 maturation. Strikingly, CD103 and Langerin (encoded by *Cd207*) were also shown to mark DCs which snorkel through the epithelium by forming TEDs in Claudin 1-dependent manner (chapter 4.3). Therefore, these findings may indicate why Claudin 1 is a driver of both CAT and homeostatic maturation (Březina *et al*., 2024). I presume that due to the Claudin 1 expression, DC1s can snorkel through the mTEC epithelium to place themselves in the optimal position for CAT, and the subsequent uptake of lipids from apoptotic bodies may trigger their maturation.

Intriguingly, our data obtained from the $Foxn1^{Cre}Confetti^{Brainbow2.1}$ mice suggests that aDC1s are also capable to perform CAT independently of mTEC endocytosis (Vobořil *et al*., 2022). This model allows for the tracking of the transfer of cytosolic RFP and YFP from non-overlapping mTEC populations, as described in the section (D), as well as the transfer of membrane-bound CFP. Imagestream technology was employed to visualize the fate of the transferred fluorescent proteins. We detected RFP and YFP to be present exclusively within the intracellular vesicles of aDC1s, indicating that they had been engulfed from

dying mTECs. However, in the majority of cases of CFP transfer, the CFP signal was not present within the vesicles. Instead, it was detected just on the plasma membranes of aDC1s. This observation suggests that membrane CFP transfer may be mediated by trogocytosis, whose role in CAT has been suggested but not yet confirmed (chapter 2.2.2). If this is true, cells of the DC1 lineage could indirectly present TRAs that they have processed themselves after their engulfment, as well as intact, transferred pMHCs that were loaded with TRAs by the antigen processing machinery of mTECs (see the Figure 3), theoretically making the "TRA ligandome" of aDC1s even broader than that of mTECs on a population level.

For more than a decade there has been a debate about the contribution of individual DC lineages to Treg selection in the thymus (chapter 2.2.3). It was shown that ablation of the DC1 lineage in *Batf3*$^{-/-}$ mice did not decrease the frequency of Tregs in the thymus, as would be expected, but instead slightly increased it (Leventhal *et al*., 2016). Accordingly, *Ccr7*$^{-/-}$ mice showed an increased frequency of Tregs in the thymus, when DC1s suffered from accelerated apoptosis (Hu *et al*., 2017). Thus, there was a consensus that the DC1 lineage has a rather negative impact on Treg selection. However, a closer look at what happens to the TCR repertoire of Tregs in *Batf3*$^{-/-}$ mice revealed that many Treg clones were missing when DC1s were absent (Perry *et al*., 2014, 2018). Accordingly, when all DCs were deleted in *Itgax*^{Cre}*R26*^{DTA} mice, the frequency of thymic Tregs was unchanged compared to the WT control (Ohnmacht *et al.*, 2009), but their TCR repertoire was found to be largely incomplete (Perry *et al.*, 2014). In marked contrast, when MHCII was deleted on all DCs in *Itgax*^{Cre}*Iab*$^{Fl/Fl}$ mice, Treg frequency in the thymus was reduced by almost half compared to the WT control (Leventhal *et al.*, 2016). This suggests a critical contribution of DCs to Treg selection. In line with this, we recently detected a significantly reduced frequency of tTregs and their CD25^{+} precursors when MHCII was deleted exclusively in the DC1 lineage using *XCR1*^{iCre}*Iab*$^{Fl/Fl}$ mice, demonstrating that this lineage is indeed critical for Treg generation (Březina *et al*., 2024). Moreover, since the reduction in Treg selection was not that pronounced as in the case of *Itgax*^{Cre}*Iab*$^{Fl/Fl}$ mice (Leventhal *et al*., 2016), our data suggests that other DC lineages can also induce Tregs.

Taken together, there is a large discrepancy between the results obtained by DC ablation and MHCII-deficient DCs approaches. We propose that this unexpected phenomenon is related to the positioning of APCs in the medulla. We presume that when MHCII-deficient DCs are present in their niche, they physically block other APCs to get into the correct position where Tregs could be generated. On the other hand, when DCs are deleted, their niche could be repopulated by other APCs (Bennett and Clausen, 2007) that can substitute for them in Treg generation, e.g. DC2 lineage was proposed to substitute for DC1 in *Batf3*$^{-/-}$ mice (Leventhal *et al.*, 2016). We hypothesized that this substitution could be imperfect since each APC subset presents different antigens in different molecular contexts (Březina, Vobořil and Filipp, 2022), leading to changes in the composition of the Treg repertoire or even a slight increase of Treg frequency, as in the case of *Batf3*$^{-/-}$ mice. Therefore, we suggest that constitutive ablation of individual DC lineages is not an ideal option to study the contribution of each particular DC lineage or, even worse, of the remaining DC lineages to Treg selection.

Given that TLR-sensing in mTECs contributed on one hand to an increased intrathymic migration of moDCs and on the other hand to reinforced Treg selection, we were the first to propose that moDCs induce Tregs in the thymus (Vobořil *et al*., 2020). In chapter 2, I discussed that to drive Treg selection, the avidity of pMHCII interaction with the TCR must be intermediate, which is typically the case with TRA presentation. Thus, if moDCs are generating Tregs, it is likely that they are doing so through the presentation of TRA fragments. Indeed, we have demonstrated that specifically moDCs have an increased capacity in CAT when mice are intrathymically injected with ODNs (Vobořil *et al*., 2020), which lends further support for this notion. However, we recently found that in marked contrast to cells of the DC1 lineage, which we have shown to prefer CAT from mTECs, expressing plethora of TRAs (Vobořil *et al*., 2022) and present TRAs to select Tregs (Březina *et al*., 2024), moDCs do not show such a preference (Vobořil *et al*., 2022). Importantly, it is likely that our moDCs overlap with CX3CR1^{+} DCs recently described in the context of tolerance to blood-borne antigens and clonal deletion (Vollmann *et al*., 2021) or transfer of microbial antigens to the thymus (Zegarra-Ruiz *et al*., 2021) (chapter 2.2.3). Thus, it seems that moDCs can exert multiple distinct tasks within the framework of cental tolerance.

I can imagine that under certain conditions, such as the activation of TLRs in mTECs, increased migration of moDCs into the thymus may lead to an enhanced presentation of microbial peptides and subsequent generation of microbiota-specific Tregs, which I believe is a closely watched but still elusive topic. Given the specialization of moDCs to acquire antigens from other thymic DCs (Vobořil *et al*., 2022) and the fact that mature DCs are prone to apoptosis (Bosteels and Janssens, 2024), I can also envision moDCs engulfing dying aDC1s, thereby recycling once transferred TRAs.

At this point, I would like to mention that although we were able to outline with a high accuracy which DC subsets have preferences within the CAT (DC1 lineage) and which do not (moDCs), we only included three of the TRA-producing mTEC subsets in our study (mTECs^{HI}, keratinocyte MCs, and tuft MCs) (Vobořil *et al.*, 2022). However, based on recent articles, the heterogeneity of MCs has been extended to at least twelve subsets (see the Figure 1). Therefore, it would be very interesting to project this heterogeneity in the context of CAT. I think this would be relatively easy to do using the *Csnb*^{Cre}*R26*^{TdTOMATO} mice, which has been recently shown to express TdTOMATO in virtually all MC subsets (Givony *et al*., 2023), crossed with mouse knockouts of individual MC lineage-defining TFs (Bornstein *et al*., 2018; Michelson *et al*., 2022; Givony *et al*., 2023) (chapter 2.1.2) and then analyzing if or to what extent the transfer of TdTOMATO to DCs was affected. In sum, this experiment should render information about the contribution of individual MC subsets to CAT.

The overarching topic of our studies listed in the section (D) is the mutual communication between immune and epithelial cells, rendering the latter a vital component of the immune system. It is remarkable that we often see parallels between the thymus and the intestine in this regard. Hence, I reviewed those associated with antigen sampling and presentation in the chapter 4. To conclude, however, I would like to elaborate

on the parallel that relates to the formation of TEDs, as it permits direct interaction between IECs and DC1s, thereby enabling DC1s to become integrated into the epithelium (chapter 4.3). Unlike IECs, mTECs do not form a barrier, but rather a network of individual mTEC islands (Rodewald *et al*., 2001) that act as a "porous filter", allowing continuous interaction with developing T cells on a large surface (Lancaster *et al*., 2019). My idea of this filter is largely based on two-photon imaging of thymic slices published by *Lauren Ehrlich's lab* (https://ehrlichlab.squarespace.com/movies), showing how developing T cells vigorously migrate through individual pores of the filter created by the mTEC network. We hypothesize that it is the immersion of DC1s into this network through Claudin 1 expression in a manner similar to TED formation that allows them to become a part of this filter (Ichimiya and Kojima, 2006; Sanos *et al*., 2011). Based on our observations (Březina *et al*., 2024) and data of others (Lei *et al*., 2011; Park *et al*., 2020), we believe that it is the positioning of DC1 lineage cells within the mTEC network that confers their strong tolerogenic properties (**Figure 6**). The last thing that I would like to mention is that it has been shown repeatedly that when TLR signaling is triggered, for example the one ascending from TLR9, TED formation is greatly enhanced (chapter 4.3). Therefore, I speculate that tonic inflammation in the thymus, and TLR-sensing by mTECs in particular, may enhance the integration of DC1s into the mTEC network, connecting together the results of our two separate studies (Vobořil *et al.*, 2020; Březina *et al.*, 2024).

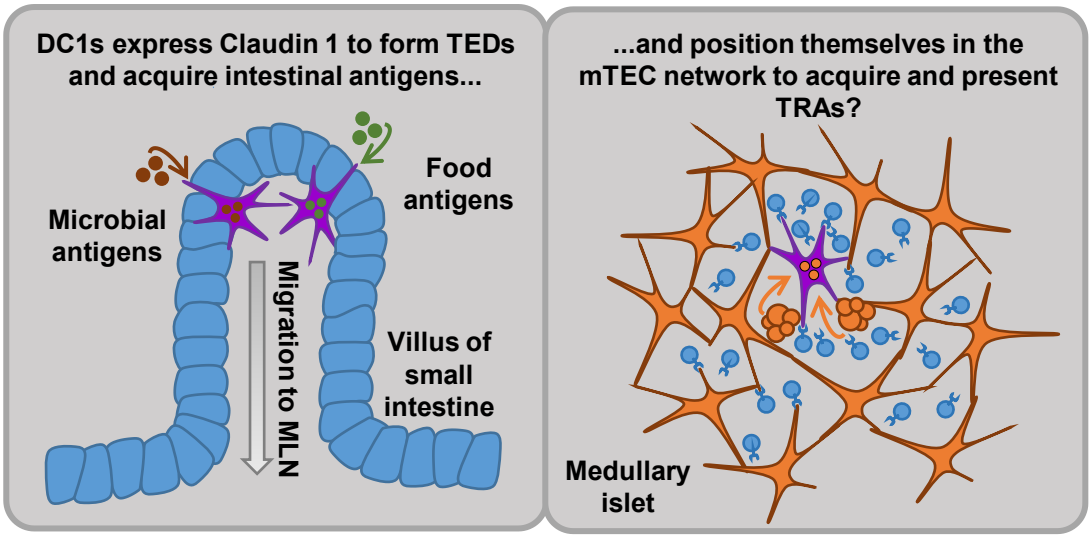

Figure 6. Model of Claudin 1 role in the central tolerance induction. (Left panel) Intestinal DC1s (purple cells) were demonstrated to form protrusions, at the apical part of villi in the small intestine, through the formation of trans-epithelial dendrites (TEDs). Critical for TED formation were shown tight junction proteins including Claudin 1 that these DCs express. TEDs enabled DC1s to immerse into the intestinal epithelial layer without affecting its integrity and to acquire microbial or food antigens from the gut lumen. To present antigens acquired through TEDs to T cells, DC1s migrate to MLN. (Right panel) The individual mTECs (orange cells) are interconnected into a network by forming Claudin 3-enriched tight junctions, among other means. Importantly, as the DC1s in the intestine, thymic DC1s are marked by the expression of Claudin 1 which is a ligand of Claudin 3. Here we propose a model in which Claudin 1 drives the immersion of DC1s into the mTEC network, facilitating uptake of TRAs (orange arrows) from dying mTECs (orange blebs) and subsequent homeostatic maturation that endows DC1s with a strong capacity to induce clonal deletion and Treg selection (blue T cells interacting with CAT-experienced purple DC).

F. REFERENCES

Aaltonen, J. *et al.* (1997) 'An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains.', *Nature genetics*, 17, pp. 399–403. doi: 10.1016/j.cell.2009.12.030.

Abramson, J. *et al.* (2010) 'Aire's Partners in the Molecular Control of Immunological Tolerance', *Cell*, 140(1), pp. 123–135. doi: 10.1016/j.cell.2009.12.030.

Abramson, J. and Anderson, G. (2017) 'Thymic Epithelial Cells', *Annual Review of Immunology*, 35, pp. 85–118. doi: 10.1146/annurev-immunol. **Review**

Abramson, J. and Goldfarb, Y. (2016) 'AIRE: From promiscuous molecular partnerships to promiscuous gene expression', *European Journal of Immunology*, 46(1), pp. 22–33. doi: 10.1002/eji.201545792. **Review**

Aichinger, M. *et al.* (2013) 'Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance.', *The Journal of experimental medicine*, 210(2), pp. 287–300. doi: 10.1084/jem.20122149.

Akagbosu, B. *et al.* (2022) 'Novel antigen-presenting cell imparts Treg-dependent tolerance to gut microbiota', *Nature*, 610(7933), pp. 752–760. doi: 10.1038/s41586-022-05309-5.

Akiyoshi, H. *et al.* (2004) 'Subcellular expression of autoimmune regulator is organized in a spatiotemporal manner', *Journal of Biological Chemistry*, 279(32), pp. 33984–33991. doi: 10.1074/jbc.M400702200.

Alves, N. L. *et al.* (2009) 'Characterization of the thymic IL-7 niche in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*, 106(5), pp. 1512–7. doi: 10.1073/pnas.0809559106.

Alves, N. L. *et al.* (2010) 'Cutting Edge: A Thymocyte-Thymic Epithelial Cell Cross-Talk Dynamically Regulates Intrathymic IL-7 Expression In Vivo', *The Journal of Immunology*, 184(11), pp. 5949–5953. doi: 10.4049/JIMMUNOL.1000601.

Anderson, M. S. *et al.* (2002) 'Projection of an immunological self shadow within the thymus by the Aire protein', *Science*, 298(5597), pp. 1395–1401. doi: 10.1126/science.1075958.

Apavaloaei, A., Laverdure, J. P. and Perreault, C. (2021) 'PSMB11 regulates gene expression in cortical thymic epithelial cells', *Cell Reports*, 36(10), p. 109546. doi: 10.1016/j.celrep.2021.109546.

Ardouin, L. *et al.* (2016) 'Broad and Largely Concordant Molecular Changes Characterize Tolerogenic and Immunogenic Dendritic Cell Maturation in Thymus and Periphery', *Immunity*, 45, pp. 305–318. doi: 10.1016/j.immuni.2016.07.019.

Ashby, K. M. *et al.* (2024) 'Sterile production of interferons in the thymus affects T cell repertoire selection', *Science Immunology*, 9(July), pp. 1–15. doi: 10.1126/sciimmunol.adp1139.

Ashby, K. M. and Hogquist, K. A. (2024) 'A guide to thymic selection of T cells', *Nature Reviews Immunology*, 24(2), pp. 103–117. doi: 10.1038/s41577-023-00911-8. **Review**

Atibalentja, D. F., Murphy, K. M. and Unanue, E. R. (2011) 'Functional Redundancy between Thymic CD8+ and Sirpa+ Conventional Dendritic Cells in Presentation of Blood-Derived Lysozyme by MHC Class II Proteins', *The Journal of Immunology*, 186(3), pp. 1421–1431. doi: 10.4049/jimmunol.1002587.

Baba, T., Nakamoto, Y. and Mukaida, N. (2009) 'Crucial Contribution of Thymic Sirp + Conventional Dendritic Cells to Central Tolerance against Blood-Borne Antigens in a CCR2-Dependent Manner', *The Journal of Immunology*, 183(5), pp. 3053–3063. doi: 10.4049/jimmunol.0900438.

Bai, A. *et al.* (2007) 'Krüppel-Like Factor 2 Controls T Cell Trafficking by Activating L-Selectin (CD62L) and Sphingosine-1-Phosphate Receptor 1 Transcription', *The Journal of Immunology*, 178(12), pp. 7632–7639. doi: 10.4049/JIMMUNOL.178.12.7632.

Bansal, K. *et al.* (2017) 'The transcriptional regulator Aire binds to and activates super-enhancers', *Nature Immunology*, 18(3), pp. 263–273. doi: 10.1038/ni.3675.

Bansal, K. *et al.* (2021) 'Aire regulates chromatin looping by evicting CTCF from domain boundaries and favoring accumulation of cohesin on superenhancers', *Proceedings of the National Academy of Sciences of the United States of America*, 118(38). doi: 10.1073/pnas.2110991118.

Baran-Gale, J. *et al.* (2020) 'Ageing compromises mouse thymus function and remodels epithelial cell differentiation', *eLife*, 9, pp. 1–27. doi: 10.1101/2020.03.02.973008.

Bastard, P. *et al.* (2021) 'Preexisting autoantibodies to type I IFNs underlie critical COVID-19 pneumonia in patients with APS-1', *Journal of Experimental Medicine*, 218(7). doi: 10.1084/JEM.20210554.

Bautista, J. L. *et al.* (2021) 'Single-cell transcriptional profiling of human thymic stroma uncovers novel cellular heterogeneity in the thymic medulla', *Nature Communications*, 12(1). doi: 10.1038/s41467-021-21346-6.

Belz, G. T. *et al.* (2002) 'The CD8α+ dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens', *Journal of Experimental Medicine*, 196(8), pp. 1099–1104. doi: 10.1084/jem.20020861.

Benhammadi, M. *et al.* (2020) 'IFN-λ Enhances Constitutive Expression of MHC Class I Molecules on Thymic Epithelial Cells', *The Journal of Immunology*, 205(5), pp. 1268–1280. doi: 10.4049/jimmunol.2000225.

Bennett, C. L. and Clausen, B. E. (2007) 'DC ablation in mice: promises, pitfalls, and challenges', *Trends in Immunology*, 28(12), pp. 525–531. doi: 10.1016/j.it.2007.08.011. **Review**

Benson, M. J. *et al.* (2007) 'All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation', *Journal of Experimental Medicine*, 204(8), pp. 1765–1774. doi: 10.1084/jem.20070719.

Bevins, C. L. and Salzman, N. H. (2011) 'Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis', *Nature Reviews Microbiology 2011 9:5*, 9(5), pp. 356–368. doi: 10.1038/NRMICRO2546. **Review**

Bhaumik, S. *et al.* (2013) 'Retinoic Acid Hypersensitivity Promotes Peripheral Tolerance in Recent Thymic Emigrants', *The Journal of Immunology*, 190(6), pp. 2603–2613. doi: 10.4049/JIMMUNOL.1200852.

von Boehmer, H., Teh, H. S. and Kisielow, P. (1989) 'The thymus selects the useful, neglects the useless and destroys the harmful', *Immunology today*, 10(2), pp. 6–10. doi: 10.1016/0167-5699(89)90307-1. **Review**

Bonasio, R. *et al.* (2006) 'Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus.', *Nature immunology*, 7(10), pp. 1092–100. doi: 10.1038/ni1385.

Bornstein, C. *et al.* (2018) 'Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells', *Nature*, 559(7715), pp. 622–626. doi: 10.1038/s41586-018-0346-1.

Bosteels, V. *et al.* (2023) 'LXR signaling controls homeostatic dendritic cell maturation', *Science Immunology*, 8(83). doi: 10.1126/sciimmunol.add3955.

Bosteels, V. and Janssens, S. (2024) 'Striking a balance: new perspectives on homeostatic dendritic cell maturation', *Nature reviews. Immunology*. doi: 10.1038/S41577-024-01079-5. **Review**

Boudil, A. *et al.* (2015) 'IL-7 coordinates proliferation, differentiation and Tcra recombination during thymocyte b-selection', *Nature immunology*, 16. doi: 10.1038/ni.3122.

Boursalian, T. E. *et al.* (2004) 'Continued maturation of thymic emigrants in the periphery', *Nature Immunology*, 5(4), pp. 418–425. doi: 10.1038/ni1049.

Brabec, T. *et al.* (2023) 'IL-17-driven induction of Paneth cell antimicrobial functions protects the host from microbiota dysbiosis and inflammation in the ileum', *Mucosal Immunology*, 16(4), pp. 373–385. doi: 10.1016/j.mucimm.2023.01.005.

Brabec, T. *et al.* (2024) 'Segmented filamentous bacteria-induced epithelial MHCII regulates cognate CD4+ IELs and epithelial turnover', *The Journal of experimental medicine*, 221(1). doi: 10.1084/jem.20230194.

Breed, E. R. *et al.* (2022) 'Type 2 cytokines in the thymus activate Sirpα+ dendritic cells to promote clonal deletion', *Nature Immunology*, 23(7), pp. 1042–1051. doi: 10.1038/s41590-022-01218-x.

Brennecke, P. *et al.* (2015) 'Single-cell transcriptome analysis reveals coordinated ectopic gene-expression patterns in medullary thymic epithelial cells', *Nature Immunology*, 16(9), pp. 933–941. doi: 10.1038/ni.3246\rhttp://www.nature.com/ni/journal/v16/n9/abs/ni.3246.html#supplementary-information.

Březina, J. *et al.* (2024) 'Claudin 1 on dendritic cells type 1 regulates central tolerance via antigen transfer', *Manuscript submitted for publication*.

Březina, J., Vobořil, M. and Filipp, D. (2022) 'Mechanisms of Direct and Indirect Presentation of Self-Antigens in the Thymus', *Frontiers in Immunology*, 13(June), pp. 1–13. doi: 10.3389/fimmu.2022.926625. **Review**

Bruserud, Ø. *et al.* (2016) 'AIRE -mutations and autoimmune disease', *Current Opinion in Immunology*, 43, pp. 8–15. doi: 10.1016/j.coi.2016.07.003. **Review**

Buono, M. *et al.* (2016) 'A dynamic niche provides Kit ligand in a stage-specific manner to the earliest thymocyte progenitors', *Nature cell biology*, 18(2), pp. 157–167. doi: 10.1038/ncb3299.

Cabeza-Cabrerizo, M. *et al.* (2021) 'Dendritic Cells Revisited', *Annual Review of Immunology*, 39, pp. 131–166. doi: 10.1146/annurev-immunol-061020-053707. **Review**

Calderón, L. and Boehm, T. (2011) 'Three chemokine receptors cooperatively regulate homing of hematopoietic progenitors to the embryonic mouse thymus', *Proceedings of the National Academy of Sciences of the United States of America*, 108(18), pp. 7517–7522. doi: 10.1073/pnas.1016428108.

Canesso, M. C. C. *et al.* (2022) 'Identification of dendritic cell-T cell interactions driving immune responses to food', *bioRxiv*, p. 2022.10.26.513772. doi: 10.1101/2022.10.26.513772. **Preprint**

Carlson, C. M. *et al.* (2006) 'Kruppel-like factor 2 regulates thymocyte and T-cell migration', *Nature*, 442(7100), pp. 299–302. doi: 10.1038/nature04882.

Carter, J. A. *et al.* (2022) 'Transcriptomic diversity in human medullary thymic epithelial cells', *Nature Communications*, 13(1), pp. 1–15. doi: 10.1038/s41467-022-31750-1.

Cheroutre, H., Lambolez, F. and Mucida, D. (2011) 'The light and dark sides of intestinal intraepithelial lymphocytes', *Nature Reviews Immunology 2011 11:7*, 11(7), pp. 445–456. doi: 10.1038/nri3007. **Review**

Chieppa, M. *et al.* (2006) 'Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement', *Journal of Experimental Medicine*, 203(13), pp. 2841–2852. doi: 10.1084/jem.20061884.

Chuprin, A. *et al.* (2015) 'The deacetylase Sirt1 is an essential regulator of Aire-mediated induction of central immunological tolerance.', *Nature immunology*, 16(7), pp. 737–745. doi: 10.1038/ni.3194.

Coombes, J. L. *et al.* (2007) 'A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β -and retinoic acid-dependent mechanism', *Journal of Experimental Medicine*, 204(8), pp. 1757–1764. doi: 10.1084/jem.20070590.

Coquet, J. M. *et al.* (2013) 'Epithelial and dendritic cells in the thymic medulla promote CD4+ Foxp3+ regulatory T cell development via the CD27 – CD70 pathway', *Journal of Experimental Medicine*, 210(4), pp. 715–728. doi: 10.1084/jem.20112061.

Cosway, E. J. *et al.* (2018) 'Formation of the Intrathymic Dendritic Cell Pool Requires CCL21-Mediated Recruitment of CCR7+ Progenitors to the Thymus', *Journal of Immunology*, 201, pp. 516–523. doi: 10.4049/jimmunol.1800348.

Cosway, E. J. *et al.* (2022) 'Eosinophils are an essential element of a type 2 immune axis that controls thymus regeneration', *Science Immunology*, 7(69). doi: 10.1126/SCIIMMUNOL.ABN3286.

Cosway, E. J. *et al.* (2023) 'The alarmin IL33 orchestrates type 2 immune-mediated control of thymus regeneration', *Nature Communications 2023 14:1*, 14(1), pp. 1–11. doi: 10.1038/s41467-023-43072-x.

Cummings, R. J. *et al.* (2016) 'Different tissue phagocytes sample apoptotic cells to direct distinct homeostasis programs', *Nature*, 539(7630), pp. 565–569. doi: 10.1038/nature20138.

Cunningham, C. A., Helm, E. Y. and Fink, P. J. (2018) 'Reinterpreting recent thymic emigrant function: defective or adaptive?', *Current Opinion in Immunology*, 51, pp. 1–6. doi: 10.1016/j.coi.2017.12.006. **Review**

Danan-Gotthold, M. *et al.* (2016) 'Extensive RNA editing and splicing increase immune self-representation diversity in medullary thymic epithelial cells', *Genome Biology*, 17(219), pp. 1–13. doi: 10.1186/s13059-016-1079-9.

Danso-Abeam, D. *et al.* (2013) 'Aire mediates thymic expression and tolerance of pancreatic antigens via an unconventional transcriptional mechanism', *European Journal of Immunology*, 43, pp. 75–84. doi: 10.1002/eji.201242761.

Daugherty, B. L. *et al.* (2007) 'Regulation of heterotypic claudin compatibility', *Journal of Biological Chemistry*, 282(41), pp. 30005–30013. doi: 10.1074/jbc.M703547200.

Derbinski, J. *et al.* (2001) 'Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self.', *Nature immunology*, 2(11), pp. 1032–9. doi: 10.1038/ni723.

Derbinski, J. *et al.* (2008) 'Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism.', *Proceedings of the National Academy of Sciences of the United*

States of America, 105(2), pp. 657–62. doi: 10.1073/pnas.0707486105.

Dhalla, F. *et al.* (2020) 'Biologically indeterminate yet ordered promiscuous gene expression in single medullary thymic epithelial cells', *The EMBO Journal*, 39(1). doi: 10.15252/EMBJ.2019101828/SUPPL_FILE/EMBJ2019101828-SUP-0004-TABLEEV3.XLSX.

Dikiy, S. and Rudensky, A. Y. (2023) 'Principles of regulatory T cell function', *Immunity*, 56(2), pp. 240–255. doi: 10.1016/j.immuni.2023.01.004. **Review**

Dobeš, J. *et al.* (2015) 'Gastrointestinal Autoimmunity Associated With Loss of Central Tolerance to Enteric α-Defensins', *Gastroenterology*, 149(1), pp. 139–150. doi: 10.1053/j.gastro.2015.05.009.

Dobeš, J. *et al.* (2022) 'Extrathymic expression of Aire controls the response to Candida albicans', *Nature Immunology*, 23, pp. 1098–1108. doi: 10.1038/s41590-022-01247-6.

Dooley, J., Erickson, M. and Farr, A. G. (2005) 'An Organized Medullary Epithelial Structure in the Normal Thymus Expresses Molecules of Respiratory Epithelium and Resembles the Epithelial Thymic Rudiment of Nude Mice', *The Journal of Immunology*, 175(7), pp. 4331–4337. doi: 10.4049/jimmunol.175.7.4331.

Dooley, J., Erickson, M. and Farr, A. G. (2009) 'Lessons from Thymic Epithelial Heterogeneity: FoxN1 and Tissue-Restricted Gene Expression by Extrathymic, Endodermally Derived Epithelium', *The Journal of Immunology*, 183(8), pp. 5042–5049. doi: 10.4049/jimmunol.0901371.

Elpek, K. G. *et al.* (2011) 'Lymphoid organ-resident dendritic cells exhibit unique transcriptional fingerprints based on subset and site', *PLoS ONE*, 6(8). doi: 10.1371/journal.pone.0023921.

Ennamorati, M. *et al.* (2020) 'Intestinal microbes influence development of thymic lymphocytes in early life', *Proceedings of the National Academy of Sciences of the United States of America*, 117(5), pp. 2570–2578. doi: 10.1073/pnas.1915047117.

Esterházy, D. *et al.* (2016) 'Classical dendritic cells are required for dietary antigen-mediated induction of peripheral T reg cells and tolerance', *Nature Immunology*, 17(5), pp. 545–555. doi: 10.1038/ni.3408.

Fang, Y. *et al.* (2024) 'AIRE relies on Z-DNA to flag gene targets for thymic T cell tolerization', *Nature*, 628, pp. 400–407. doi: 10.1038/s41586-024-07169-7.

Farache, J. *et al.* (2013) 'Luminal Bacteria Recruit CD103+ Dendritic Cells into the Intestinal Epithelium to Sample Bacterial Antigens for Presentation', *Immunity*, 38(3), pp. 581–595. doi: 10.1016/j.immuni.2013.01.009.

Fiorini, E. *et al.* (2008) 'Cutting Edge: Thymic Crosstalk Regulates Delta-Like 4 Expression on Cortical Epithelial Cells', *The Journal of Immunology*, 181(12), pp. 8199–8203. doi: 10.4049/JIMMUNOL.181.12.8199.

Friesen, T. J., Ji, Q. and Fink, P. J. (2016) 'Recent thymic emigrants are tolerized in the absence of inflammation', *Journal of Experimental Medicine*, 213(6), pp. 913–920. doi: 10.1084/JEM.20151990.

Furuse, M., Sasaki, H. and Tsukita, S. (1999) 'Manner of Interaction of Heterogeneous Claudin Species Within and Between Tight Junction Strands', *Journal of Cell Biology*, 147(4), pp. 891–903. doi: 10.1083/jcb.147.4.891.

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

Gaboriau-Routhiau, V. *et al.* (2009) 'The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses', *Immunity*, 31(4), pp. 677–689. doi: 10.1016/J.IMMUNI.2009.08.020.

Gallegos, A. M. and Bevan, M. J. (2004) 'Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation.', *The Journal of Experimental Medicine*, 200(8), pp. 1039–49. doi: 10.1084/jem.20041457.

Ginhoux, F., Guilliams, M. and Merad, M. (2022) 'Expanding dendritic cell nomenclature in the single-cell era', *Nature Reviews Immunology*, 22(2), pp. 67–68. doi: 10.1038/s41577-022-00675-7. **Review**

Giraud, M. *et al.* (2012) 'Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells', *Proceedings of the National Academy of Sciences*, 109(2), pp. 535–540. doi: 10.1073/pnas.1119351109.

Giraud, M. *et al.* (2014) 'An RNAi screen for Aire cofactors reveals a role for Hnrnpl in polymerase release and Aire-activated ectopic transcription.', *Proceedings of the National Academy of Sciences of the United States of America*, 111(4), pp. 1491–6. doi: 10.1073/pnas.1323535111.

Givony, T. *et al.* (2023) 'Thymic mimetic cells function beyond self-tolerance', *Nature*, 622, pp. 164–172. doi: 10.1038/s41586-023-06512-8.

Goldfarb, Y. *et al.* (2021) 'Mechanistic dissection of dominant AIRE mutations in mouse models reveals AIRE autoregulation', *Journal of Experimental Medicine*, 218(11). doi: 10.1084/jem.20201076.

Gommeaux, J. *et al.* (2009) 'Thymus-specific serine protease regulates positive selection of a subset of CD4+ thymocytes', *European Journal of Immunology*, 39(4), pp. 956–964. doi: 10.1002/eji.200839175.

Gordon, J. *et al.* (2007) 'Specific expression of lacZ and cre recombinase in fetal thymic epithelial cells by multiplex gene targeting at the Foxn1 locus', *BMC Developmental Biology*, 7(69). doi: 10.1186/1471-213X-7-69.

Goto, Y. *et al.* (2014) 'Segmented filamentous bacteria antigens presented by intestinal dendritic cells

drive mucosal Th17 cell differentiation', *Immunity*, 40(4), pp. 594–607. doi: 10.1016/j.immuni.2014.03.005.

Gray, D., Gavanescu, I., *et al.* (2007) 'Danger-free autoimmune disease in Aire-deficient mice', *Proceedings of the National Academy of Sciences of the United States of America*, 104(46), pp. 18193–18198. doi: 10.1073/pnas.0709160104.

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

Guha, M. *et al.* (2017) 'DNA breaks and chromatin structural changes enhance the transcription of Autoimmune Regulator target genes', *Journal of Biological Chemistry*, (3), p. jbc.M116.764704. doi: 10.1074/jbc.M116.764704.

Guilliams, M. *et al.* (2014) 'Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny', *Nature Reviews Immunology*, 14(8), pp. 571–578. doi: 10.1038/nri3712. **Review**

Hadeiba, H. *et al.* (2012) 'Plasmacytoid Dendritic Cells Transport Peripheral Antigens to the Thymus to Promote Central Tolerance', *Immunity*, 36(3), pp. 438–450. doi: 10.1016/j.immuni.2012.01.017.

Hadis, U. *et al.* (2011) 'Intestinal Tolerance Requires Gut Homing and Expansion of FoxP3+ Regulatory T Cells in the Lamina Propria', *Immunity*, 34(2), pp. 237–246. doi: 10.1016/j.immuni.2011.01.016.

Haftmann, C. *et al.* (2021) 'Protection against autoimmunity is driven by thymic epithelial cell–mediated regulation of Treg development', *Science Immunology*, 6(65). doi: 10.1126/sciimmunol.abf3111.

Haljasorg, U. *et al.* (2015) 'A highly conserved NFkB responsive enhancer is critical for thymic expression of Aire in mice', *European Journal of Immunology*, 45(12), pp. 3246–3256. doi: 10.1002/eji.201545928.

Hamazaki, Y. *et al.* (2007) 'Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin.', *Nature immunology*, 8(3), pp. 304–311. doi: 10.1038/ni1438.

Han, J. and Zúñiga-Pflücker, J. C. (2021) 'A 2020 View of Thymus Stromal Cells in T Cell Development', *The Journal of Immunology*, 206(2), pp. 249–256. doi: 10.4049/JIMMUNOL.2000889. **Review**

Handel, A. E. *et al.* (2018) 'Comprehensively Profiling the Chromatin Architecture of Tissue Restricted Antigen Expression in Thymic Epithelial Cells Over Development', *Frontiers in Immunology*, 9(September), pp. 1–10. doi: 10.3389/fimmu.2018.02120.

Hassler, T. *et al.* (2019) 'Inventories of naive and tolerant mouse CD4 T cell repertoires reveal a hierarchy of deleted and diverted T cell receptors', *Proceedings of the National Academy of Sciences of*

the United States of America, 116(37), pp. 18537–18543. doi: 10.1073/pnas.1907615116.

He, Xiao *et al.* (2005) 'The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment.', *Nature*, 433(7028), pp. 826–33. doi: 10.1038/nature03338.

Heinlein, M. *et al.* (2022) 'The acetyltransferase KAT7 is required for thymic epithelial cell expansion, expression of AIRE target genes, and thymic tolerance', *Science immunology*, 7(67), p. eabb6032. doi: 10.1126/sciimmunol.abb6032.

Hemmers, S. *et al.* (2019) 'IL-2 production by self-reactive CD4 thymocytes scales regulatory T cell generation in the thymus', *Journal of Experimental Medicine*, 216(11), pp. 2466–2478. doi: 10.1084/jem.20190993.

Hepworth, M. R. *et al.* (2013) 'Innate lymphoid cells regulate CD4 + T-cell responses to intestinal commensal bacteria', *Nature*, 498(7452), pp. 113–117. doi: 10.1038/nature12240.

Hepworth, M. R. *et al.* (2015) 'Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4+ T cells', *Science*, 348(6238), pp. 1031–1035. doi: 10.1126/science.aaa4812.

Herbin, O. *et al.* (2016) 'Medullary thymic epithelial cells and CD8α+dendritic cells coordinately regulate central tolerance but CD8α+cells are dispensable for thymic regulatory T cell production', *Journal of Autoimmunity*, 75, pp. 141–149. doi: 10.1016/j.jaut.2016.08.002.

Herppich, S., Beckstette, M. and Huehn, J. (2022) 'The thymic microenvironment gradually modulates the phenotype of thymus-homing peripheral conventional dendritic cells', *Immunity, Inflammation and Disease*, 10(2), pp. 175–188. doi: 10.1002/IID3.559.

Herzig, Y. *et al.* (2016) 'Transcriptional programs that control expression of the autoimmune regulator gene Aire', *Nature Immunology*, 18(December), pp. 1–14. doi: 10.1038/ni.3638.

Hinterberger, M. *et al.* (2010) 'Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance.', *Nature immunology*, 11(6), pp. 512–519. doi: 10.1038/ni.1874.

Hirakawa, M. *et al.* (2018) 'Fundamental parameters of the developing thymic epithelium in the mouse', *Scientific Reports*, 8(11095). doi: 10.1038/s41598-018-29460-0.

Hogquist, K. A. *et al.* (2015) 'T Cell Adolescence: Maturation Events Beyond Positive Selection', *The Journal of Immunology*, 195(4), pp. 1351–1357. doi: 10.4049/JIMMUNOL.1501050. **Review**

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

Hozumi, K. *et al.* (2008) 'Delta-like 4 is indispensable in thymic environment specific for T cell

development.', *The Journal of experimental medicine*, 205(11), pp. 2507–13. doi: 10.1084/jem.20080134.

Hu, Z. *et al.* (2015) 'CCR4 promotes medullary entry and thymocyte–dendritic cell interactions required for central tolerance', *The Journal of Experimental Medicine*, 212(11), pp. 1947–1965. doi: 10.1084/jem.20150178.

Hu, Z. *et al.* (2017) 'CCR7 Modulates the Generation of Thymic Regulatory T Cells by Altering the Composition of the Thymic Dendritic Cell Compartment', *Cell Reports*, 21(1), pp. 168–180. doi: 10.1016/j.celrep.2017.09.016.

Hubert, F.-X. *et al.* (2011) 'Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance', *Blood*, 118(9), pp. 2462–2472. doi: 10.1182/blood-2010-06-286393.An.

Humblet, C., Rudensky, A. Y. and Kyewski, B. (1994) 'Presentation and intercellular transfer of self antigen within the thymic microenvironment: Expression of the Eα peptide-l-Abcomplex by isolated thymic stromal cells', *International Immunology*, 6(12), pp. 1949–1958. doi: 10.1093/intimm/6.12.1949.

Ichimiya, S. and Kojima, T. (2006) 'Cellular networks of human thymic medullary stromas coordinated by p53-related transcription factors', *Journal of Histochemistry and Cytochemistry*, 54(11), pp. 1277–1289. doi: 10.1369/jhc.6A7028.2006.

Iliev, I. D. *et al.* (2009) 'Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning', *Mucosal Immunology*, 2(4), pp. 340–350. doi: 10.1038/mi.2009.13.

James, K. D. *et al.* (2018) 'Endothelial cells act as gatekeepers for LTβRdependent thymocyte emigration', *Journal of Experimental Medicine*, 215(12), pp. 2984–2993. doi: 10.1084/jem.20181345.

James, K. D., Jenkinson, W. E. and Anderson, G. (2018) 'T-cell egress from the thymus: Should I stay or should I go?', *Journal of Leukocyte Biology*, 104(2), pp. 275–284. doi: 10.1002/JLB.1MR1217-496R. **Review**

Jansen, K. *et al.* (2021) 'RBFOX splicing factors contribute to a broad but selective recapitulation of peripheral tissue splicing patterns in the thymus', *Genome Research*, 31(11), pp. 2022–2034. doi: 10.1101/gr.275245.121.

Jenkins, M. K. *et al.* (2010) 'On the Composition of the Preimmune Repertoire of T Cells Specific for Peptide–Major Histocompatibility Complex Ligands', *Annual Review of Immunology*, 28, pp. 275–294. doi: 10.1146/ANNUREV-IMMUNOL-030409-101253. **Review**

Jenkinson, E. J. *et al.* (1994) 'Positive Selection By Purified Mhc Class II+ Thymic Epithelial Cells in Vitro: Costimulatory Signals Mediated By B7 are Not Involved', *Developmental Immunology*, 3(4), pp. 265–271. doi: 10.1155/1994/75434.

Jiang, W. *et al.* (2005) 'Modifier loci condition autoimmunity provoked by Aire deficiency.', *The Journal of experimental medicine*, 202(6), pp. 805–15. doi: 10.1084/jem.20050693.

Jordan, M. S. *et al.* (2001) 'Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist selfpeptide.', *Nature immunology*, 2(4), pp. 301–306. doi: 10.1038/86302.

Kadouri, N. *et al.* (2020) 'Thymic epithelial cell heterogeneity: TEC by TEC', *Nature Reviews Immunology*, 20(4), pp. 239–253. doi: 10.1038/s41577-019-0238-0. **Review**

Kawai, T. *et al.* (2024) 'Decoding Toll-like receptors: Recent insights and perspectives in innate immunity', *Immunity*, 57(4), pp. 649–673. doi: 10.1016/j.immuni.2024.03.004. **Review**

Kaye, J. *et al.* (1989) 'Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor', *Nature 1989 341:6244*, 341(6244), pp. 746–749. doi: 10.1038/341746a0.

Keane, P., Ceredig, R. and Seoighe, C. (2015) 'Promiscuous mRNA splicing under the control of AIRE in medullary thymic epithelial cells', *Bioinformatics*, 31(7), pp. 986–990. doi: 10.1093/bioinformatics/btu785.

Kedmi, R. *et al.* (2022) 'A RORγt+ cell instructs gut microbiota-specific Treg cell differentiation', *Nature*, 610(7933), pp. 737–743. doi: 10.1038/s41586-022-05089-y.

Kim, K. S. *et al.* (2016) 'Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine', *Science*, 351(6275), pp. 858–863. doi: 10.1126/science.aac5560.

Kishimoto, H. and Sprent, J. (1997) 'Negative selection in the thymus includes semimature T cells.', *The Journal of experimental medicine*, 185(2), pp. 263–71. doi: 10.1084/jem.185.2.263.

Klein, L. *et al.* (2000) 'Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells', *Nature Medicine 2000 6:1*, 6(1), pp. 56–61. doi: 10.1038/71540.

Klein, L. *et al.* (2014) 'Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see).', *Nature Reviews Immunology*, 14(6), pp. 377–91. doi: 10.1038/nri3667. **Review**

Klein, L., Robey, E. A. and Hsieh, C. (2019) 'Central CD4+ T cell tolerance: deletion versus regulatory T cell differentiation', *Nature Reviews Immunology*, 19, pp. 7–18. doi: 10.1038/s41577-018-0083-6. **Review**

Koble, C. and Kyewski, B. (2009) 'The thymic medulla: a unique microenvironment for intercellular self-antigen transfer.', *The Journal of experimental medicine*, 206(7), pp. 1505–13. doi: 10.1084/jem.20082449.

Koch, U. *et al.* (2008) 'Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell

lineage commitment.', *The Journal of experimental medicine*, 205(11), pp. 2515–2523. doi: 10.1084/jem.20080829.

Koh, A. S. *et al.* (2008) 'Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(41), pp. 15878–83. doi: 10.1073/pnas.0808470105.

Koh, A. S. *et al.* (2018) 'Rapid chromatin repression by Aire provides precise control of immune tolerance', *Nature Immunology*, 19(February), pp. 162–171. doi: 10.1038/s41590-017-0032-8.

Kozai, M. *et al.* (2017) 'Essential role of CCL21 in establishment of central self- tolerance in T cells', *Journal of Experimental Medicine*, 214(7), pp. 1925–1935. doi: https://doi.org/10.1084/jem.20161864.

Kroger, C. J. *et al.* (2017) 'Thymic Dendritic Cell Subsets Display Distinct Efficiencies and Mechanisms of Intercellular MHC Transfer', *The Journal of Immunology*, 198(1), pp. 249–256. doi: 10.4049/jimmunol.1601516.

Kroger, C. J., Wang, B. and Tisch, R. (2016) 'Temporal increase in thymocyte negative selection parallels enhanced thymic SIRPα+ DC function', *European Journal of Immunology*, 46, pp. 2352–2362. doi: 10.1002/eji.201646354.

Krueger, A. (2018) 'Thymus Colonization: Who, How, How Many?', *Archivum Immunologiae et Therapiae Experimentalis*, 66(2), pp. 81–88. doi: 10.1007/S00005-017-0503-5/FIGURES/2. **Review**

Krueger, A., Ziętara, N. and Łyszkiewicz, M. (2017) 'T Cell Development by the Numbers', *Trends in Immunology*, 38(2), pp. 128–139. doi: 10.1016/j.it.2016.10.007. **Review**

Kubo, A. *et al.* (2009) 'External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers', *Journal of Experimental Medicine*, 206(13), pp. 2937–2946. doi: 10.1084/jem.20091527.

Kulkarni, D. H. *et al.* (2020) 'Goblet cell associated antigen passages support the induction and maintenance of oral tolerance', *Mucosal Immunology*, 13(2), pp. 271–282. doi: 10.1038/s41385-019-0240-7.

Kumar, P. G. *et al.* (2001) 'The Autoimmune Regulator (AIRE) Is a DNA-binding Protein', *Journal of Biological Chemistry*, 276(44), pp. 41357–41364. doi: 10.1074/jbc.M104898200.

Kurd, N. and Robey, E. A. (2016) 'T-cell selection in the thymus: A spatial and temporal perspective', *Immunological Reviews*, 271(1), pp. 114–126. doi: 10.1111/imr.12398. **Review**

Kurd, N. S. *et al.* (2020) 'A role for phagocytosis in inducing cell death during thymocyte negative selection', *eLife*, 9, pp. 1–18. doi: 10.7554/eLife.56027.

Laan, M. *et al.* (2021) 'Post-Aire Medullary Thymic Epithelial Cells and Hassall's Corpuscles as Inducers of Tonic Pro-Inflammatory Microenvironment', *Frontiers in Immunology*, 12(April), pp. 1–9. doi: 10.3389/fimmu.2021.635569. **Review with original data**

Ladi, E. *et al.* (2008) 'Thymocyte-Dendritic Cell Interactions near Sources of CCR7 Ligands in the Thymic Cortex', *Journal of Immunology*, 181, pp. 7014–7023. doi: 10.3791/652.

Ladinsky, M. S. *et al.* (2019) 'Endocytosis of commensal antigens by intestinal epithelial cells regulates mucosal T cell homeostasis', *Science*, 363(6431). doi: 10.1126/SCIENCE.AAT4042/SUPPL_FILE/AAT4042S3.MOV.

LaFlam, T. N. *et al.* (2015) 'Identification of a novel cis-regulatory element essential for immune tolerance', *Journal of Experimental Medicine*, 212, pp. 1993–2002. doi: 10.1084/jem.20151069.

Lammers, S. *et al.* (2023) 'Ehf and Fezf2 regulate late medullary thymic epithelial cell and thymic tuft cell development', *Frontiers in Immunology*, 14(February), pp. 1–18. doi: 10.3389/fimmu.2023.1277365.

Lancaster, J. N. *et al.* (2019) 'Live-cell imaging reveals the relative contributions of antigen-presenting cell subsets to thymic central tolerance', *Nature Communications*, 10(1). doi: 10.1038/s41467-019-09727-4.

Lancaster, J. N., Li, Y. and Ehrlich, L. I. R. (2018) 'Chemokine-Mediated Choreography of Thymocyte Development and Selection', *Trends in Immunology*, 39(2), pp. 86–98. doi: 10.1016/j.it.2017.10.007. **Review**

Legoux, F. P. *et al.* (2015) 'CD4+ T Cell Tolerance to Tissue-Restricted Self Antigens Is Mediated by Antigen-Specific Regulatory T Cells Rather Than Deletion', *Immunity*, 43(5), pp. 896–908. doi: 10.1016/j.immuni.2015.10.011.

Lei, Y. *et al.* (2011) 'Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development.', *The Journal of experimental medicine*, 208(2), pp. 383–394. doi: 10.1084/jem.20102327.

Leventhal, D. S. *et al.* (2016) 'Dendritic Cells Coordinate the Development and Homeostasis of Organ-Specific Regulatory T Cells', *Immunity*, 44(4), pp. 847–859. doi: 10.1016/j.immuni.2016.01.025.

Li, J. *et al.* (2009) 'Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus', *The Journal of Experimental Medicine*, 206(3), pp. 607–622. doi: 10.1084/jem.20082232.

Li, Y. *et al.* (2023) 'CCR4 and CCR7 differentially regulate thymocyte localization with distinct outcomes for central tolerance', *eLife*, 12, pp. 1–30. doi: 10.7554/eLife.80443.

Liang, S. C. *et al.* (2006) 'Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively

enhance expression of antimicrobial peptides', *Journal of Experimental Medicine*, 203(10), pp. 2271–2279. doi: 10.1084/JEM.20061308.

Lienenklaus, S. *et al.* (2009) 'Novel Reporter Mouse Reveals Constitutive and Inflammatory Expression of IFN-β In Vivo', *The Journal of Immunology*, 183(5), pp. 3229–3236. doi: 10.4049/jimmunol.0804277.

Linsk, R. *et al.* (1989) 'Are Tissues a patch quilt of ectopic gene expression?', *Science*, 246, p. 261. doi: 10.1126/science.2799388. **Commentary**

Liu, K. *et al.* (2002) 'Immune tolerance after delivery of dying cells to dendritic cells in situ', *Journal of Experimental Medicine*, 196(8), pp. 1091–1097. doi: 10.1084/jem.20021215.

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

Lo, W.-L. *et al.* (2009) 'An endogenous peptide positively selects and augments the activation and survival of peripheral CD4+ T cells.', *Nature immunology*, 10(11), pp. 1155–61. doi: 10.1038/ni.1796.

Lodes, M. J. *et al.* (2004) 'Bacterial flagellin is a dominant antigen in Crohn disease', *Journal of Clinical Investigation*, 113(9), pp. 1296–1306. doi: 10.1172/JCI200420295.

Lucas, B. *et al.* (2020) 'Diversity in medullary thymic epithelial cells controls the activity and availability of iNKT cells', *Nature Communications*, 11(1), pp. 1–14. doi: 10.1038/s41467-020-16041-x.

Lucas, B. *et al.* (2023) 'Embryonic keratin19+ progenitors generate multiple functionally distinct progeny to maintain epithelial diversity in the adult thymus medulla', *Nature Communications*, 14(1), pp. 1–14. doi: 10.1038/s41467-023-37589-4.

Luckey, M. a *et al.* (2014) 'The transcription factor ThPOK suppresses Runx3 and imposes CD4(+) lineage fate by inducing the SOCS suppressors of cytokine signaling.', *Nature immunology*, 15(7), pp. 638–45. doi: 10.1038/ni.2917.

Luis, T. C. *et al.* (2016) 'Initial seeding of the embryonic thymus by immune-restricted lympho-myeloid progenitors', *Nature Immunology 2016 17:12*, 17(12), pp. 1424–1435. doi: 10.1038/ni.3576.

Lythe, G. *et al.* (2016) 'How many TCR clonotypes does a body maintain?', *The Journal of Theoretical Biology*, 389, pp. 214–224. doi: 10.1016/j.jtbi.2015.10.016. **Review**

Lyu, M. *et al.* (2022) 'ILC3s select microbiota-specific regulatory T cells to establish tolerance in the gut', *Nature*, 610(7933), pp. 744–751. doi: 10.1038/s41586-022-05141-x.

Mabbott, N. A. *et al.* (2013) 'Microfold (M) cells: Important immunosurveillance posts in the intestinal epithelium', *Mucosal Immunology*, 6(4), pp. 666–677. doi: 10.1038/mi.2013.30. **Review**

Mackley, E. C. *et al.* (2015) 'CCR7-dependent trafficking of RORγ+ ILCs creates a unique microenvironment within mucosal draining lymph nodes', *Nature Communications*, 6. doi: 10.1038/ncomms6862.

MacNabb, B. W. *et al.* (2019) 'Negligible Role for Deletion Mediated by cDC1 in CD8+ T Cell Tolerance', *Journal of Immunology*, 202, pp. 2628–2635. doi: 10.4049/jimmunol.1801621.

Maier, B. *et al.* (2020) 'A conserved dendritic-cell regulatory program limits antitumour immunity', *Nature*, 580(7802), pp. 257–262. doi: 10.1038/s41586-020-2134-y.

Malchow, S. *et al.* (2016) 'Aire Enforces Immune Tolerance by Directing Autoreactive T Cells into the Regulatory T Cell Lineage', *Immunity*, 44(5), pp. 1102–1113. doi: 10.1016/j.immuni.2016.02.009.

Malhotra, D. *et al.* (2016) 'Tolerance is established in polyclonal CD4+ T cells by distinct mechanisms, according to self-peptide expression patterns', *Nature Immunology*, 17(2), pp. 187–95. doi: 10.1038/ni.3327.

Martinez, R. J. *et al.* (2023) 'Type III interferon drives thymic B cell activation and regulatory T cell generation', *Proceedings of the National Academy of Sciences of the United States of America*, 120(9). doi: 10.1073/PNAS.2220120120/-/DCSUPPLEMENTAL.

Matloubian, M. *et al.* (2004) 'Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1', *Nature*, 427, pp. 355–360. doi: 10.1038/nature02284.

Matteoli, G. *et al.* (2010) 'Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction', *Gut*, 59(5), pp. 595–604. doi: 10.1136/gut.2009.185108.

May, J. F. *et al.* (2024) 'Establishment of CD8+ T Cell Thymic Central Tolerance to Tissue-Restricted Antigen Requires PD-1', *The Journal of Immunology*, 212(2), pp. 271–283. doi: 10.4049/JIMMUNOL.2200775.

Mazzini, E. *et al.* (2014) 'Oral Tolerance Can Be Established via Gap Junction Transfer of Fed Antigens from CX3CR1+ Macrophages to CD103+ Dendritic Cells', *Immunity*, 40(2), pp. 248–261. doi: 10.1016/j.immuni.2013.12.012.

McCaughtry, T. M. *et al.* (2008) 'Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla', *Journal of Experimental Medicine*, 205(11), pp. 2575–2584. doi: 10.1084/jem.20080866.

McCaughtry, T. M., Wilken, M. S. and Hogquist, K. a (2007) 'Thymic emigration revisited.', *The Journal of experimental medicine*, 204(11), pp. 2513–2520. doi: 10.1084/jem.20070601.

McDole, J. R. *et al.* (2012) 'Goblet cells deliver luminal antigen to CD103 + dendritic cells in the small

intestine', *Nature*, 483(7389), pp. 345–349. doi: 10.1038/nature10863.

Melichar, H. J. *et al.* (2013) 'Distinct temporal patterns of T cell receptor signaling during positive versus negative selection in situ', *Science Signaling*, 6(297), pp. 1–15. doi: 10.1126/scisignal.2004400].

Melo-Gonzalez, F. *et al.* (2019) 'Antigen-presenting ILC3 regulate T cell-dependent IgA responses to colonic mucosal bacteria', *Journal of Experimental Medicine*, 216(4), pp. 728–742. doi: 10.1084/jem.20180871.

Meredith, M. *et al.* (2015) 'Aire controls gene expression in the thymic epithelium with ordered stochasticity', *Nature Immunology*, 16(9), pp. 942–949. doi: 10.1038/ni.3247.

Metzger, T. C. *et al.* (2013) 'Lineage Tracing and Cell Ablation Identify a Post-Aire-Expressing Thymic Epithelial Cell Population', *Cell Reports*, 5(1), pp. 166–179. doi: 10.1016/j.celrep.2013.08.038.

Meyer, S. *et al.* (2016) 'AIRE-Deficient Patients Harbor Unique High-Affinity Disease-Ameliorating Autoantibodies', *Cell*, 166, pp. 582–595. doi: 10.1016/j.cell.2016.06.024.

Mezrich, J. D. *et al.* (2010) 'An Interaction between Kynurenine and the Aryl Hydrocarbon Receptor Can Generate Regulatory T Cells', *The Journal of Immunology*, 185(6), pp. 3190–3198. doi: 10.4049/jimmunol.0903670.

Michelson, D. A. *et al.* (2022) 'Thymic epithelial cells co-opt lineage-defining transcription factors to eliminate autoreactive T cells', *Cell*, 185, pp. 1–17. doi: 10.1016/j.cell.2022.05.018.

Michelson, D. A. *et al.* (2023) 'Hnf4 activates mimetic-cell enhancers to recapitulate gut and liver development within the thymus', *The Journal of experimental medicine*, 220(10). doi: 10.1084/jem.20230461.

Michelson, D. A. and Mathis, D. (2022) 'Thymic mimetic cells: tolerogenic masqueraders', *Trends in Immunology*, 43(10), pp. 782–791. doi: 10.1016/j.it.2022.07.010. **Review**

Miller, C. N. *et al.* (2018) 'Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development', *Nature*, 559, pp. 627–631. doi: 10.1038/s41586-018-0345-2.

Millet, V., Naquet, P. and Guinamard, R. R. (2008) 'Intercellular MHC transfer between thymic epithelial and dendritic cells', *European Journal of Immunology*, 38(5), pp. 1257–1263. doi: 10.1002/eji.200737982.

Miranda-Waldetario, M. C. G. and Curotto de Lafaille, M. A. (2024) 'Oral tolerance to dietary antigens and Foxp3+ regulatory T cells', *Immunological Reviews*, 326, pp. 8–16. doi: 10.1111/imr.13370. **Review**

Miyake, K. *et al.* (2017) 'Trogocytosis of peptide-MHC class II complexes from dendritic cells confers antigen-presenting ability on basophils', *Proceedings of the National Academy of Sciences of the United*

States of America, 114(5), pp. 1111–1116. doi: 10.1073/PNAS.1615973114.

Morimoto, J. *et al.* (2018) 'Aire Controls in Trans the Production of Medullary Thymic Epithelial Cells Expressing Ly-6C/Ly-6G', *Journal of Immunology*, 201(11), pp. 3244–3257. doi: 10.4049/jimmunol.1800950.

Morimoto, J. *et al.* (2022) 'Aire suppresses CTLA-4 expression from the thymic stroma to control autoimmunity', *Cell Reports*, 38(7), p. 110384. doi: 10.1016/J.CELREP.2022.110384.

Morimoto, J. *et al.* (2023) 'Cooperative but Distinct Role of Medullary Thymic Epithelial Cells and Dendritic Cells in the Production of Regulatory T Cells in the Thymus', *The Journal of Immunology*, 210(11), pp. 1653–1666. doi: 10.4049/jimmunol.2200780.

Mouri, Y. *et al.* (2017) 'Mode of Tolerance Induction and Requirement for Aire Are Governed by the Cell Types That Express Self-Antigen and Those That Present Antigen', *The Journal of Immunology*, 199(12), pp. 3959–3971. doi: 10.4049/jimmunol.1700892.

Mowat, A. M. and Agace, W. W. (2014) 'Regional specialization within the intestinal immune system', *Nature Reviews Immunology*, 14(10), pp. 667–685. doi: 10.1038/nri3738. **Review**

Mucida, D. *et al.* (2005) 'Oral tolerance in the absence of naturally occurring Tregs', *Journal of Clinical Investigation*, 115(7), pp. 1923–1933. doi: 10.1172/JCI24487.

Mucida, D. *et al.* (2007) 'Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid', *Science*, 317(5835), pp. 256–260. doi: 10.1126/SCIENCE.1145697/SUPPL_FILE/PAPV2.PDF.

Murata, S. *et al.* (2007) 'Regulation of CD8+ T cell development by thymus-specific proteasomes', *Science*, 316, pp. 1349–1353. doi: 10.1126/science.1141915.

Al Nabhani, Z. *et al.* (2019) 'A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult', *Immunity*, 50(5), pp. 1276-1288.e5. doi: 10.1016/j.immuni.2019.02.014.

Nagamine, K. *et al.* (1997) 'Positional cloning of the APECED gene.', *Nature genetics*, 17, pp. 393–398. doi: 10.1038/ng1297-393.

Nedjic, J. *et al.* (2008) 'Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance.', *Nature*, 455(7211), pp. 396–400. doi: 10.1038/nature07208.

Neefjes, J. *et al.* (2011) 'Towards a systems understanding of MHC class i and MHC class II antigen presentation', *Nature Reviews Immunology*, 11(12), pp. 823–836. doi: 10.1038/nri3084. **Review**

Neijssen, J. *et al.* (2005) 'Cross-presentation by intercellular peptide transfer through gap junctions', *Nature*, 434(7029), pp. 83–88. doi: 10.1038/NATURE03290.

Nevo, S. *et al.* (2024) 'Tuft cells and fibroblasts promote thymus regeneration through ILC2-mediated type 2 immune response', *Science Immunology*, 9(91). doi: 10.1126/SCIIMMUNOL.ABQ6930/SUPPL_FILE/SCIIMMUNOL.ABQ6930_MDAR_REPRODUCIBILITY_CHECKLIST.PDF.

Niess, J. H. *et al.* (2005) 'CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance', *Science*, 307(5707), pp. 254–258. doi: 10.1126/science.1102901.

Nitta, T. *et al.* (2010) 'Thymoproteasome Shapes Immunocompetent Repertoire of CD8+ T Cells', *Immunity*, 32(1), pp. 29–40. doi: 10.1016/j.immuni.2009.10.009.

Oftedal, B. E. *et al.* (2015) 'Dominant Mutations in the Autoimmune Regulator AIRE Are Associated with Common Organ-Specific Autoimmune Diseases', *Immunity*, 42(6), pp. 1185–1196. doi: 10.1016/j.immuni.2015.04.021.

Oh, J. *et al.* (2018) ' CD40 Mediates Maturation of Thymic Dendritic Cells Driven by Self-Reactive CD4 + Thymocytes and Supports Development of Natural Regulatory T Cells ', *The Journal of Immunology*, 200(4), pp. 1399–1412. doi: 10.4049/jimmunol.1700768.

Ohigashi, I. and Takahama, Y. (2021) 'Specific impact of β5t on proteasome subunit composition in cortical thymic epithelial cells', *Cell Reports*, 36(10), p. 109657. doi: 10.1016/j.celrep.2021.109657.

Ohnmacht, C. *et al.* (2009) 'Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity.', *The Journal of experimental medicine*, 206(3), pp. 549–59. doi: 10.1084/jem.20082394.

Ohnmacht, C. *et al.* (2015) 'The microbiota regulates type 2 immunity through RORγt+ T cells', *Science*, 349(6251), pp. 989–993. doi: 10.1126/science.aac4263.

Org, T. *et al.* (2008) 'The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression.', *EMBO reports*, 9(4), pp. 370–6. doi: 10.1038/sj.embor.2008.11.

Org, T. *et al.* (2009) 'AIRE activated tissue specific genes have histone modifications associated with inactive chromatin', *Human Molecular Genetics*, 18(24), pp. 4699–4710. doi: 10.1093/hmg/ddp433.

Oven, I. *et al.* (2007) 'AIRE recruits P-TEFb for transcriptional elongation of target genes in medullary thymic epithelial cells.', *Molecular and cellular biology*, 27(24), pp. 8815–23. doi: 10.1128/MCB.01085-07.

Padonou, F. *et al.* (2022) 'Aire-dependent transcripts escape Raver2-induced splice-event inclusion in the thymic epithelium', *EMBO reports*, 23(3), pp. 1–16. doi: 10.15252/embr.202153576.

Paiva, R. S. *et al.* (2013) 'Recent thymic emigrants are the preferential precursors of regulatory T cells differentiated in the periphery', *Proceedings of the National Academy of Sciences of the United States of*

America, 110(16), pp. 6494–6499. doi: 10.1073/PNAS.1221955110.

Park, J. E. *et al.* (2020) 'A cell atlas of human thymic development defines T cell repertoire formation', *Science*, 367(6480). doi: 10.1126/science.aay3224.

Perera, J. *et al.* (2013) 'Autoreactive thymic B cells are efficient antigen-presenting cells of cognate self-antigens for T cell negative selection.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(42), pp. 17011–6. doi: 10.1073/pnas.1313001110.

Perry, J. S. A. *et al.* (2014) 'Distinct contributions of Aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus', *Immunity*, 41(3), pp. 414–426. doi: 10.1016/j.immuni.2014.08.007.

Perry, J. S. A. *et al.* (2018) 'Transfer of Cell-Surface Antigens by Scavenger Receptor CD36 Promotes Thymic Regulatory T Cell Receptor Repertoire Development and Allotolerance', *Immunity*, 48(5), pp. 923-936.e4. doi: 10.1016/j.immuni.2018.04.007.

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

Pinto, S. *et al.* (2013) 'Overlapping gene coexpression patterns in human medullary thymic epithelial cells generate self-antigen diversity.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(37), pp. E3497-505. doi: 10.1073/pnas.1308311110.

Pitkänen, J. *et al.* (2000) 'The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein', *Journal of Biological Chemistry*, 275(22), pp. 16802–16809. doi: 10.1074/jbc.M908944199.

Pitkänen, J. *et al.* (2005) 'Cooperative activation of transcription by autoimmune regulator AIRE and CBP', *Biochemical and Biophysical Research Communications*, 333(3), pp. 944–953. doi: 10.1016/j.bbrc.2005.05.187.

Proietto, A. I. *et al.* (2008) 'Dendritic cells in the thymus contribute to T-regulatory cell induction', *Proceedings of the National Academy of Sciences*, 105(50), pp. 19869–19874. doi: 10.1073/pnas.0810268105.

Qiu, C.-H. *et al.* (2009) 'Novel Subset of CD8α+ Dendritic Cells Localized in the Marginal Zone Is Responsible for Tolerance to Cell-Associated Antigens', *The Journal of Immunology*, 182(7), pp. 4127–4136. doi: 10.4049/jimmunol.0803364.

Rattay, K. *et al.* (2015) 'Homeodomain-Interacting Protein Kinase 2, a Novel Autoimmune Regulator Interaction Partner, Modulates Promiscuous Gene Expression in Medullary Thymic Epithelial Cells', *The*

Journal of Immunology, 194(3), pp. 921–928. doi: 10.4049/jimmunol.1402694.

Rattay, K. *et al.* (2016) 'Evolutionary conserved gene co-expression drives generation of self-antigen diversity in medullary thymic epithelial cells', *Journal of Autoimmunity*, 67, pp. 65–75. doi: 10.1016/j.jaut.2015.10.001.

Rescigno, M. *et al.* (2001) 'Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria.', *Nature Immunology*, 2(4), pp. 361–7. doi: 10.1038/86373.

Ricciardi-Castagnoli, P. (2020) 'Sensing the gut microbiota', *Nature Immunology*, 21(7), pp. 704–705. doi: 10.1038/s41590-020-0645-1. **Commentary**

Rivera, C. A. *et al.* (2022) 'Epithelial colonization by gut dendritic cells promotes their functional diversification', *Immunity*, 55(1), pp. 129-144.e8. doi: 10.1016/j.immuni.2021.11.008.

Rodewald, H. R. *et al.* (2001) 'Thymus medulla consisting of epithelial islets each derived from a single progenitor.', *Nature*, 414(6865), pp. 763–768. doi: 10.1038/414763a.

Rudensky, A. Y., Mazel, S. M. and Yurin, V. L. (1990) 'Presentation of endogenous immunoglobulin determinant to immunoglobulin-recognizing T cell clones by the thymic cells', *European Journal of Immunology*, 20(10), pp. 2235–2239. doi: 10.1002/eji.1830201012.

Russler-Germain, E. V. *et al.* (2021) 'Commensal Cryptosporidium colonization elicits a cDC1-dependent Th1 response that promotes intestinal homeostasis and limits other infections', *Immunity*, 54(11), pp. 2547-2564.e7. doi: 10.1016/J.IMMUNI.2021.10.002.

Saini, M. *et al.* (2010) 'Regulation of Zap70 expression during thymocyte development enables temporal separation of CD4 and CD8 repertoire selection at different signaling thresholds', *Science Signaling*, 3(114), pp. 1–13. doi: 10.1126/scisignal.2000702.

Sakata, M., Ohigashi, I. and Takahama, Y. (2018) 'Cellularity of Thymic Epithelial Cells in the Postnatal Mouse', *Journal of Immunology*, 200, pp. 1382–1388. doi: 10.4049/jimmunol.1701235.

Sanos, S. L. *et al.* (2011) 'Stromal Cell Networks Regulate Thymocyte Migration and Dendritic Cell Behavior in the Thymus', *Journal of Immunology*, 186, pp. 2835–2841. doi: 10.4049/jimmunol.1003563.

Sansom, S. N. *et al.* (2014) 'Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia', *Genome Research*, 24(12), pp. 1918–1931. doi: 10.1101/gr.171645.113.

Santori, F. R. *et al.* (2002) 'Rare, structurally homologous self-peptides promote thymocyte positive selection', *Immunity*, 17(2), pp. 131–142. doi: 10.1016/S1074-7613(02)00361-8.

Sasaki, K. *et al.* (2015) 'Thymoproteasomes produce unique peptide motifs for positive selection of

CD8(+) T cells.', *Nature communications*, 6(May), p. 7484. doi: 10.1038/ncomms8484.

Schnupf, P. *et al.* (2017) 'Segmented filamentous bacteria, Th17 inducers and helpers in a hostile world', *Current Opinion in Microbiology*, 35, pp. 100–109. doi: 10.1016/j.mib.2017.03.004. **Review**

Schriek, P. and Villadangos, J. A. (2023) 'Trogocytosis and cross-dressing in antigen presentation', *Current Opinion in Immunology*, 83, p. 102331. doi: 10.1016/j.coi.2023.102331. **Review**

Sefik, E. *et al.* (2015) 'Individual intestinal symbionts induce a distinct population of RORγ+ regulatory T cells', *Science*, 349(6251), pp. 993–997. doi: 10.1126/science.aaa9420.

Setoguchi, R. *et al.* (2008) 'Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development.', *Science*, 319(5864), pp. 822–825. doi: 10.1126/science.1151844.

Silva-Sanchez, A. *et al.* (2023) 'Activation of regulatory dendritic cells by Mertk coincides with a temporal wave of apoptosis in neonatal lungs', *Science Immunology*, 8(84), pp. 1–17. doi: 10.1126/sciimmunol.adc9081.

Simms, L. A. *et al.* (2008) 'Reduced α-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease', *Gut*, 57(7), pp. 903–910. doi: 10.1136/gut.2007.142588.

Sin, J. H. *et al.* (2023) 'Ikaros is a principal regulator of Aire+ mTEC homeostasis, thymic mimetic cell diversity, and central tolerance', *Science immunology*, 8(88), p. eabq3109. doi: 10.1126/sciimmunol.abq3109.

Sinclair, C. *et al.* (2013) 'Asymmetric thymocyte death underlies the CD4:CD8 T-cell ratio in the adaptive immune system', *Proceedings of the National Academy of Sciences of the United States of America*, 110(31), pp. E2905–E2914. doi: 10.1073/PNAS.1304859110/SUPPL_FILE/PNAS.201304859SI.PDF.

Sisirak, V. *et al.* (2016) 'Digestion of Chromatin in Apoptotic Cell Microparticles Prevents Autoimmunity', *Cell*, 166(1), pp. 88–101. doi: 10.1016/j.cell.2016.05.034.

St-Pierre, C. *et al.* (2015) 'Differential Features of AIRE-Induced and AIRE-Independent Promiscuous Gene Expression in Thymic Epithelial Cells.', *Journal of Immunology*, 195(2), pp. 498–506. doi: 10.4049/jimmunol.1500558.

Steier, Z. *et al.* (2023) 'Single-cell multiomic analysis of thymocyte development reveals drivers of CD4+ T cell and CD8+ T cell lineage commitment', *Nature Immunology*, 24(9), pp. 1579–1590. doi: 10.1038/s41590-023-01584-0.

Steinman, R. M. and Cohn, Z. A. (1973) 'Identification of novel cell type in peripheral lymphoid organs of mice I. Morphology, Quantitation, Tissue distribution', *Journal of Experimental Medicine*, 137, pp. 1142–1162. doi: 10.1084/jem.137.5.1142.

Stritesky, G. L. *et al.* (2013) 'Murine thymic selection quantified using a unique method to capture deleted T cells', *Proceedings of the National Academy of Sciences*, 110(12), pp. 4679–4684. doi: 10.1073/pnas.1217532110.

Sun, C. M. *et al.* (2007) 'Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid', *Journal of Experimental Medicine*, 204(8), pp. 1775–1785. doi: 10.1084/jem.20070602.

Sung, S.-S. J. *et al.* (2006) 'A Major Lung CD103 (αE)-β7 Integrin-Positive Epithelial Dendritic Cell Population Expressing Langerin and Tight Junction Proteins', *The Journal of Immunology*, 176(4), pp. 2161–2172. doi: 10.4049/JIMMUNOL.176.4.2161.

Takaba, H. *et al.* (2015) 'Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance', *Cell*, 163(4), pp. 975–987. doi: 10.1016/j.cell.2015.10.013.

Takada, K. *et al.* (2015) 'TCR affinity for thymoproteasome-dependent positively selecting peptides conditions antigen responsiveness in CD8 + T cells', *Nature immunology*, 16(10), pp. 1069–1077. doi: 10.1038/ni.3237.

Taniguchi, R. T. *et al.* (2012) 'Detection of an autoreactive T-cell population within the polyclonal repertoire that undergoes distinct autoimmune regulator (Aire)-mediated selection.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(20), pp. 7847–52. doi: 10.1073/pnas.1120607109.

Tao, W. *et al.* (2023) 'Insm1 regulates mTEC development and immune tolerance', *Cellular and Molecular Immunology*, 20(12), pp. 1472–1486. doi: 10.1038/s41423-023-01102-0.

Tao, Y. *et al.* (2006) 'AIRE recruits multiple transcriptional components to specific genomic regions through tethering to nuclear matrix', *Molecular Immunology*, 43(4), pp. 335–345. doi: 10.1016/j.molimm.2005.02.018.

Teh, H. S. *et al.* (1988) 'Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells', *Nature*, 335(6187), pp. 229–233. doi: 10.1038/335229A0.

Thangavelu, G. *et al.* (2011) 'Programmed death-1 is required for systemic self-tolerance in newly generated T cells during the establishment of immune homeostasis', *Journal of Autoimmunity*, 36(3–4), pp. 301–312. doi: 10.1016/J.JAUT.2011.02.009.

Thiault, N. *et al.* (2015) 'Peripheral regulatory T lymphocytes recirculating to the thymus suppress the development of their precursors', *Nature immunology*, 16(6), pp. 628–634. doi: 10.1038/ni.3150.

Ueno, T. *et al.* (2004) 'CCR7 signals are essential for cortex-medulla migration of developing

thymocytes', *The Journal of experimental medicine*, 200(4), pp. 493–505. doi: 10.1084/jem.20040643.

Ushio, A. *et al.* (2024) 'Functionally diverse thymic medullary epithelial cells interplay to direct central tolerance', *Cell Reports*, 43(4), p. 114072. doi: 10.1016/j.celrep.2024.114072.

Veldhoen, M. *et al.* (2006) 'TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells', *Immunity*, 24(2), pp. 179–189. doi: 10.1016/j.immuni.2006.01.001.

Venables, T. *et al.* (2019) 'Dynamic changes in epithelial cell morphology control thymic organ size during atrophy and regeneration', *Nature Communications*, 10(1), pp. 1–15. doi: 10.1038/s41467-019-11879-2.

Villaseñor, J. *et al.* (2008) 'Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated', *Proc Natl Acad Sci U S A*, 105(41), pp. 15854–15859. doi: 10.1073/pnas.0808069105.

Vobořil, M. *et al.* (2020) 'Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation', *Nature Communications*, 11(1), pp. 1–16. doi: 10.1038/s41467-020-16081-3.

Vobořil, M. *et al.* (2022) 'A model of preferential pairing between epithelial and dendritic cells in thymic antigen transfer', *eLife*, 11. doi: 10.7554/ELIFE.71578.

Vollmann, E. H. *et al.* (2021) 'Specialized transendothelial dendritic cells mediate thymic T-cell selection against blood-borne macromolecules', *Nature Communications*, 12(1). doi: 10.1038/s41467-021-26446-x.

Walters, S. N. *et al.* (2014) 'A role for intrathymic B cells in the generation of natural regulatory T cells.', *Journal of Immunology*, 193(1), pp. 170–6. doi: 10.4049/jimmunol.1302519.

Wang, H. *et al.* (2019) 'Myeloid cells activate iNKT cells to produce IL-4 in the thymic medulla', *Proceedings of the National Academy of Sciences of the United States of America*, 116(44), pp. 22262–22268. doi: 10.1073/PNAS.1910412116/SUPPL_FILE/PNAS.1910412116.SAPP.PDF.

Wang, J. *et al.* (2019) 'Hassall ' s corpuscles with cellular-senescence features maintain IFN α production through neutrophils and pDC activation in the thymus', *International Immunology*, 31(3), pp. 127–139. doi: 10.1093/intimm/dxy073.

Watanabe, M. *et al.* (2020) 'B7-CD28 co-stimulation modulates central tolerance via thymic clonal deletion and Treg generation through distinct mechanisms', *Nature Communications*, 11(1), pp. 1–14. doi: 10.1038/s41467-020-20070-x.

Watanabe, N. *et al.* (2005) 'Hassall ' s corpuscles instruct dendritic cells to induce CD4+ CD25+

regulatory T cells in human thymus', *Nature*, 436(August), pp. 1181–1185. doi: 10.1038/nature03886.

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

Wehkamp, J. *et al.* (2004) 'NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal α-defensin expression', *Gut*, 53(11), pp. 1658 - 1664. doi: 10.1136/gut.2003.032805.

Wehkamp, J. *et al.* (2005) 'Reduced Paneth cell α-defensins in ileal Crohn's disease', *Proceedings of the National Academy of Sciences of the United States of America*, 102(50), pp. 18129–18134. doi: 10.1073/PNAS.0505256102/SUPPL_FILE/05256FIG5.PDF.

Wells, K. L. *et al.* (2020) 'Combined transient ablation and single cell rna sequencing reveals the development of medullary thymic epithelial cells', *eLife*, 9, pp. 1–80. doi: 10.7554/eLife.60188.

White, A. J. *et al.* (2010) 'Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells', *J Immunol*, 185(8), pp. 4769–4776. doi: 10.4049/jimmunol.1002151.

White, A. J. *et al.* (2017) 'A type 2 cytokine axis for thymus emigration', *Journal of Experimental Medicine*, 214(8), pp. 2205–2216. doi: 10.1084/jem.20170271.

Worbs, T. *et al.* (2006) 'Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells', *Journal of Experimental Medicine*, 203(3), pp. 519–527. doi: 10.1084/jem.20052016.

Worthington, J. J. *et al.* (2011) 'Intestinal dendritic cells specialize to activate transforming growth factor-β and induce Foxp3+ regulatory T cells via integrin αvβ8', *Gastroenterology*, 141(5), pp. 1802–1812. doi: 10.1053/j.gastro.2011.06.057.

Yamano, T. *et al.* (2015) 'Thymic B Cells Are Licensed to Present Self Antigens for Central T Cell Tolerance Induction', *Immunity*, 42(6), pp. 1048–1061. doi: 10.1016/j.immuni.2015.05.013.

Yamano, T. *et al.* (2019) 'Aire-expressing ILC3-like cells in the lymph node display potent APC features', *Journal of Experimental Medicine*, 216(5), pp. 1027–1037. doi: https://doi.org/10.1084/jem.20181430.

Yang, S. *et al.* (2013) 'Aire's plant homeodomain(PHD)-2 is critical for induction of immunological tolerance', *Proceedings of the National Academy of Sciences of the United States of America*, 110(5), pp. 1833–1838. doi: 10.1073/pnas.1222023110.

Yang, S. *et al.* (2015) 'Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance', *Science*, 348(6234), pp. 589–594. doi: 10.1126/science.aaa7017.

Yap, J. Y. *et al.* (2018) 'Indirect presentation in the thymus limits naive and regulatory T-cell differentiation by promoting deletion of self-reactive thymocytes', *Immunology*, 154(3), pp. 522–532. doi: 10.1111/imm.12904.

Yoshida, H. *et al.* (2015) 'Brd4 bridges the transcriptional regulators, Aire and P-TEFb, to promote elongation of peripheral-tissue antigen transcripts in thymic stromal cells.', *Proceedings of the National Academy of Sciences of the United States of America*, 112(32), pp. E4448-57. doi: 10.1073/pnas.1512081112.

You, Y. *et al.* (2024) 'Direct presentation of inflammation-associated self-antigens by thymic innate-like T cells induces elimination of autoreactive CD8+ thymocytes', *Nature Immunology*, 25, pp. 1367–1382. doi: 10.1038/s41590-024-01899-6.

Yuan, N. *et al.* (2024) 'Med1 controls thymic T-cell migration into lymph node through enhancer-based Foxo1-Klf2 transcription program', *European Journal of Immunology*, p. 2350887. doi: 10.1002/EJI.202350887.

Zegarra-Ruiz, D. F. *et al.* (2021) 'Thymic development of gut-microbiota-specific T cells', *Nature*, 594, p. 413. doi: 10.1038/s41586-021-03531-1.

Zhou, T. A. *et al.* (2022) 'Thymic macrophages consist of two populations with distinct localization and origin', *eLife*, 11, pp. 1–35. doi: 10.7554/eLife.75148.

Zlotoff, D. A. *et al.* (2010) 'CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus', *Blood*, 115(10), pp. 1897–1906. doi: 10.1182/blood-2009-08-237784.The.