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Heterogeneity of antigen-presenting cells in the thymus and its relevance for the establishment of central tolerance

Heterogenita antigen-prezentujících buněk v thymu a její význam v ustanovení centrální tolerance

BACHELOR´S THESIS

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Prohlášení:

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

V Praze, 02.01.2023 Vojtěch Sýkora

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Abstract:

The crucial function of the thymus is the establishment of central tolerance. In this process, developing T-cells are tested for their self-reactivity, since self-reactive T-cells might cause the autoimmunity if they would escape from the thymus to the periphery. Many thymic antigen-presenting cells are essential for establishment of central tolerance. Their role is to present self-antigens to the developing T-cells. Such presentation is capable to reveal the self-reactive potential of T-cells which can be then directly removed or deviated into suppressive T-regulatory cells. In the last several years, a high level of heterogeneity has been described among the thymic antigen-presenting cells and the molecular mechanisms that govern their functions towards enforcement of tolerance began to be uncovered.

This thesis summarises recent knowledge in the field of heterogeneity of the thymic antigen-presenting cells and its relevance for establishment of the central tolerance, with the major focus on conventional dendritic cells and post-AIRE medullary thymic epithelial cells. This thesis also outlines recent advances in understanding of functional mechanisms and regulations of maturation of the antigen-presenting cells.

Key words:

thymus, central tolerance, dendritic cell, medullary thymic epithelial cell, heterogeneity

Abstrakt:

Zásadní funkcí brzlíku je ustanovení centrální tolerance. V tomto procesu je testována autoreaktivita vyvíjejících se T-buněk. Autoreaktivní T-buňky mohou totiž způsobit autioimunitu v případě, že uniknou z brzlíku do periferie. Pro ustanovení centrální tolerance je důležité velké množství thymických antigen-prezentujících buněk, které prezentují antigeny vyvíjejícím se T-buňkám. Tato prezentace umožňuje rozpoznat autoreaktivní potenciál těchto T-buněk, které mohou být následně odstraněny nebo přeměněny na supresivní T-regulační buňky. V posledních několika letech bylo zjištěno, že tyto antigen-prezentující buňky jsou výrazně heterogenní a začíná docházet také k objevům molekulárních mechanizmů, které stojí za funkcí těchto buněk v ustanovení centrální tolerance.

Tato práce shrnuje současné znalosti v oblasti heterogenity thymických antigen-prezentujících buněk a její význam v ustanovení centrální tolerance. Především se zaměřuje na konvenční dendritické buňky a post-AIRE medulární thymické epiteliální buňky. Popisuje také současné znalosti funkčních mechanizmů a regulace maturace thymických antigen-prezentujících buněk.

Klíčová slova:

brzlík, centrální tolerance, dendritická buňka, medulární epiteliální buňka, heterogenita

List of abbreviations:

*s	"s" in the end of abbreviation stands for plural
*^{lo}	Low level (MHCII^{lo} = low level of MHCII)
*^{hi}	High level (MHCII^{hi} = high level of MHCII)
aDC1	Activated cDC1
aDC2	Activated cDC2
aDC3	Activated dendritic cell type 3
AIRE	Autoimmune regulator
BM	Bone marrow
CAT	Cooperative antigen transfer
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
cDC1	Conventional dendritic cell type 1
cDC2	Conventional dendritic cell type 2
COX-2	Cyclooxygenase 2
cTEC	Cortical thymic epithelial cell
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DN	Double negative
DP	Double positive
DP thymocyte	Double positive CD4+ CD8+ thymocyte
dsDNA breaks	double strand DNA breaks
EpCAM	epithelial cell adhesion molecule
FEZF2	FEZ family zinc finger 2
FLT3	Fms-related receptor tyrosine kinase 3
FOXN1	Forkhead box protein N1
HCs	Hassall´s corpuscles
ILC	Innate lymphoid cell
iNKT	Invariant natural killer T-cell
IVL	Involucrin
KO	Knockout
KRT	Keratin
LN	Lymph node
LTβR	Lymphotoxin beta receptor
MHCI	Major histocompatibility complex I
MHCII	Major histocompatibility complex II
moDC	Monocyte-derived dendritic cell

mregDC	Mature DC enriched in immunoregulatory molecules
mTEC	Medullary thymic epithelial cell
NFkB	Nuclear factor kappa B
NIK	NFkB inducing kinase
pAg	Peripheral antigen
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell
pMHCI or II	Peptide MHCI or MHCII
PRR	Pattern recognition receptor
RANK	Receptor activator of NfKB
RANKL	RANK ligand
SASPase	Retroviral-like 1 aspartic peptidase
scRNA-seq	Single cell RNA sequencing
Sirpα	Signal-regulatory protein alfa
SP	Single positive
SP thymocyte	Single positive CD4+ or CD8+ thymocyte or both
Tc	Cytotoxic T-cell
TEC	Thymic epithelial cell (mTEC/cTEC)
TGFβ	Transforming growth factor beta
Th	Helper T-cell
thymocyte	Developing T-cell found in thymus
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRA	Tissue restricted antigen
Treg	Regulatory T-cell
TSLP	Thymic stroma lymphopoietin
TSLPR	TSLP receptor
UEA1	Ulex europeaues agglutinin 1
uAg	Ubiquitously expressed antigen
XCR1	C sub-family chemokine receptor 1

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A. Introduction

Thymus is an indispensable immune organ, although it is sometimes overlooked. Multiorgan autoimmune diseases emerge, if the thymic function is disrupted, because this primary lymphoid organ of vertebrates is responsible for the development of both functional as well as self-tolerant repertoire of T-cells. T-cells, due to their clonality, are capable to recognize plethora of peptides referred to as antigens e.g. those which in the context of immune system are mostly derived from pathogens or tumours. Nevertheless, a considerable portion of T-cells recognize antigens from self-tissues and these so called "self-reactive" T-cells have to be dealt with in the thymus to avoid onset of the autoimmunity in the periphery. Therefore, these processes collectively referred to as "central tolerance" represents a set of thymic mechanisms that prevent our immune system from attacking our own body. In the last decade, significant advances in the field of central tolerance were achieved via discovery of novel cell types and cell physiological states that play a role in the thymic function. In this context, this thesis is focused on the antigen-presenting cells in the thymus, especially on the thymic epithelial cells (TEC) and the dendritic cells (DC). The aim is to summarize their newly described heterogeneity and based on the current knowledge, to propose their potential functions in T-cell selection processes. Emphasis is also put on the specific maturation states of those antigen-presenting cells, specifically the activated conventional dendritic cells type 1 (cDC1) and activated conventional dendritic cells type 2 (cDC2), whose function in central tolerance could be indispensable, although it has not been studied broadly yet. It is also important to note that this thesis is focused exclusively on the selection processes associated with the intrathymic development of αβ T-cells, although other T-cell types also develop in the thymus.

B. Thymus and central tolerance

Mammals have a complex immune system, capable to mount a response against pathogens, tumour cells or other dangerous entities. This is achieved through generation of a huge repertoire of immune lymphocyte receptors which can bind to specific epitopes present on these entities (Parkin and Cohen, 2001). One of these receptors are T-cell receptors (TCR), present on αβ T-lymphocytes, which are generated via rearrangement of specific parts of DNA encoding particular segments of TCRs. This mechanism is called VDJ recombination and is able to generate TCRs with specificity to almost every molecule they can encounter (Lai et al., 1989; Roth, 2015). Downside of the VDJ recombination, due to its random nature, is the generation of completely unfunctional TCRs and self-reactive TCRs that recognize molecules of the host and could promote autoimmunity if not controlled (Palmer, 2003). The T-cell clones bearing those problematic TCRs are effectively removed by a set of distinct mechanisms. First, during "positive selection" the developing T-cells (thymocytes) bearing unfunctional TCR commit apoptosis (death by neglect) and the rest of thymocytes continue in development. Secondly, during "negative selection" the thymocytes bearing self-reactive TCR are either deleted (recessive tolerance) or deviated into T-regulatory (Treg) cells which subsequently dampen the immune responses (dominant tolerance) (Klein et al., 2014; Liu, 2006). If some self-reactive T cells escape into the immune periphery at physiological circumstances, it is the Treg subset which supresses their autoimmune reactivity (Kim et al., 2006; Kurd et al., 2019).

B.1 T-cell development and selection in thymic cortex

T-cell progenitors, which migrate to the thymus from bone marrow (Cosway et al., 2018) begin their thymic journey as DN ($CD4^-$ $CD8^-$ double negative) cells and enter the thymic cortex, where they perform VDJ recombination (Kyewski and Klein, 2006; Palmer, 2003). Subsequently, they progress into DP ($CD4^+$ $CD8^+$ double positive) stage and are subjected to positive selection, which is performed by cognate interactions of their TCR and unique peptide-Major histocompatibility complex I or II (pMHCI or pMHCII) molecules, displayed on the surface of cortical thymic epithelial (cTEC) cells. The cTECs bear unique antigen processing and presenting machinery, which enables them to present a unique epitope of antigens to facilitate the positive selection. Thymocytes unable to bind for having unfunctional TCR will die by neglect. The thymocytes that successfully passed through the positive selection progress into the SP ($CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single positive) stage and migrate into the medulla. While still in the cortex, SP thymocytes can engage TCR-pMHC interactions with cortical dendritic cells that display ubiquitously expressed antigens (uAg) found in all cell

types in the body. The thymocytes which have too strong interaction with the dendritic cells are self-reactive and are removed by negative selection (Anderson et al., 2002; Klein et al., 2019; McCaughtry et al., 2008). Problem is that in addition to uAgs, there are antigens, which are expressed only in specific cell types and tissues, such as the insulin or caseinβ. Thus, the selection using only uAgs would be incomplete and might lead to severe autoimmunity (Anderson et al., 2002). The solution to this problem is provided by the existence of a special type of APCs, the medullary thymic epithelial cells (mTEC) in the thymic medulla, which have the unique ability to express tissue-restricted antigens (TRA). These TRAs, such as insulin or caseinβ, are defined as proteins which are expressed in immune periphery by a single or limited number of tissues (Klein et al., 2014; Vobořil et al., 2022).

B.2 Antigens displayed in the thymic medulla

The expression of many of these TRAs in mTECs is regulted by a specific transcription factor, Autoimmune regulator (AIRE) (Anderson et al., 2002; Derbinski et al., 2016; Wells et al., 2020). The AIRE interacts with multiple DNA processing factors and promotes TRAs expression by activating epigenetically silenced chromatin. This is achieved for example by formation of double strand breaks inside DNA of mTECs (Abramson et al., 2010; Abramson and Husebye, 2016). AIRE regulates expression of approximately 4000 TRA genes (Anderson et al., 2002; Sansom et al., 2014). Novel studies suggested a model, in which AIRE facilitates the expression in "ordered stochasticity" meaning, that there are multiple TRA clusters, that are typically expressed together, but they have no connection in function or DNA position. They are likely clustered together in 3D structure of chromatin and thus expressed together (Derbinski et al., 2008; Meredith et al., 2015). On the contrary, there are very recent studies that suggested a presence of so-called mimetic cells (*section C.1.3.5*) that possess an ordered expression of TRAs and highly resemble a specific types of differentiated peripheral cells (Abramson et al., 2022; Michelson et al., 2022; Michelson and Mathis, 2022). AIRE deficiency leads in both mice and human to the development of multiorgan autoimmunity (Anderson et al., 2002; Nagamine et al., 1997). In mice, the severity of autoimmune manifestations is dependent on a mouse strain, with C57BL/6 mice presenting only mild autoimmune symptoms, while BALB/c and especially NOD strains develop severe multiorgan autoimmunity (Jiang et al., 2005). In human, recessive mutations in AIRE gene lead to development of rare multiorgan autoimmune syndrome called Autoimmune Polyendocrinopathy Candidiasis Epidermal Dystrophy (APECED). This syndrome manifests itself as Addisson´s disease, hypoparathyroidsm, candidiasis, and autoimmunity of other organs including gut, skin, eye, testes or ovary (Kisand and Peterson, 2015). More recently, AIRE dominant mutations were identified, which lead to milder manifestations, and are much more frequent in human population than the recessive ones (Oftedal et al., 2015). Aside from the AIRE, there are other transcription factors that facilitate TRA expression, for example Fezf2, but they are not as well characterized as the AIRE (Takaba et al., 2015).

Furthermore, peripheral antigens, for example from the intestine are transported into the medulla by dendritic cells (DC) (Hadeiba et al., 2012; Spidale et al., 2014; Zegarra-Ruiz et al., 2021) and both the mTECs and DCs in the medulla also express and present the uAgs. The TRAs, pAgs and uAgs are subsequently presented on medullary APCs, mainly on the mTECs and DCs via their MHCI and MHCII molecules (Hinterberger et al., 2010; Klein et al., 2014; Perry et al., 2014). Despite the fact that DCs do not express AIRE, they are able to present the TRAs since they acquire them from the mTECs via the mechanism of cooperative antigen transfer (CAT) (*section C.2*) (Lancaster et al., 2019; Vobořil et al., 2022). Aside from mTECs and DCs, the antigens are presented also by other, minor thymic APCs, for example thymic B-cells and macrophages (Perera et al., 2016, 2013; Yamano et al., 2015; Zhou et al., 2022).

B.3 Medullary part of the thymocyte selection

Thymocytes migrate through the medulla, where they engage their TCRs with pMHCI or II molecules on the APCs and gauge the intensity of signal generated by these interactions (Klein et al., 2019, 2014). The signal intensity is determined by two factors. First, it is the strength of one given interaction, which is determined by the level of TCR self-reactivity. The higher the self-reactivity, the more intense signal is received during one TCR-pMHC interaction. Second, it is the quantity, i.e. multiplicity of interactions which the cell with its TCR assembly cumulatively performs. The more interactions, the stronger the signal. The quantity of

interactions is determined by the type and presentation pattern of the antigen recognized by thymocyte. Since uAgs are expressed and presented by all APCs, whereas TRAs are expressed and presented only by a limited set of APCs, overall uAgs provide much stronger signal than TRAs (Derbinski et al., 2008; Legoux et al., 2015; Malhotra et al., 2016). The coincidence also plays a role in this process. Thymocytes rapidly scan the medulla in a semi-random manner (Ehrlich et al., 2009; Le Borgne et al., 2009), thus one cell might perform more, while other cells might perform less interactions, leading to a stronger versus fainter signal, respectively, despite having similar TCR specificity. This provides a stochastic element to the selection (Klein et al., 2019, 2014). These factors which affect the signal strength received, additively determine the fate of the selected thymocytes (*Figure 1*). The cells that receive minimal signal (considered as they are non-self-reactive) differentiate into SP T-cells. The cells that receive too strong signal, undergo negative selection and are deleted for being highly self-reactive. The cells being in between these two are deviated into Treg cells. In addition, there are other signals generated by costimulation and/or soluble factors that further influence the fate of thymocyte, especially the conversion into the Treg lineage or its deletion. These factors are for example the cytokine IL-2, that promotes Treg development, CD28 – CD80 and CD86 interactions which are needed for effective thymocyte selection and transforming growth factor-β (TGFβ) which potentiates the Treg development (Klein et al., 2019). It is also of note, that some cryptic antigens, which are only minimally present in the thymus, can be also tolerated via their ignorance (Malhotra et al., 2016). The T-cells and Tregs that completed selection and survived, finally leave the thymus and exit into periphery (Klein et al., 2014; Malhotra et al., 2016). The model of thymocyte selection presented here is depicted in *Figure 2*.

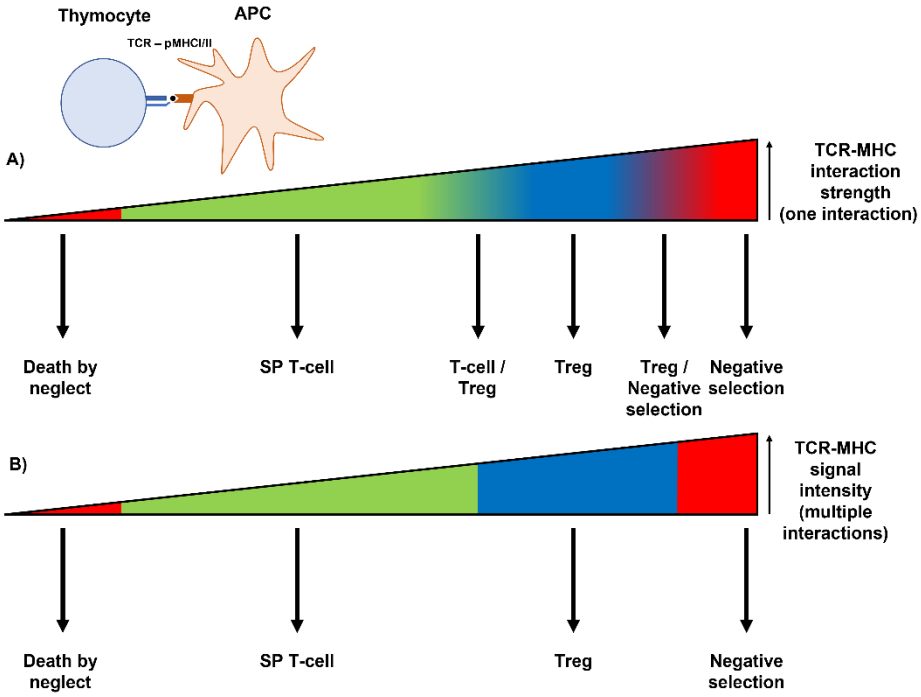

Figure 1: Signal strength model of thymocyte selection

(A) *Self-reactivity of TCR defines the strength of TCR-pMHCI or II interaction. Only strength of the interaction defines the fate of thymocyte imprecisely. The blurred zones are accounted to the stochasticity in a fate of thymocyte with given level of self-reactivity, caused by semi-random movement in the medulla.*
(B) *The fate of thymocyte is determined by the signal intensity, summarized from all TCR-MHCI or II interactions performed by the cell. More abundant or strong interactions lead to strong signal, less abundant or weak interactions lead to weak signal. No signal lead to death by neglect, weak signal leads to mature T-cell deviation, stronger signal leads to Treg generation and too high signal leads to death by negative selection. Thus, the fate of thymocyte is dependent on its self-reactivity but also on the type of antigen to which particular T-cell is specific.* *Adopted from Klein et al. 2019.*

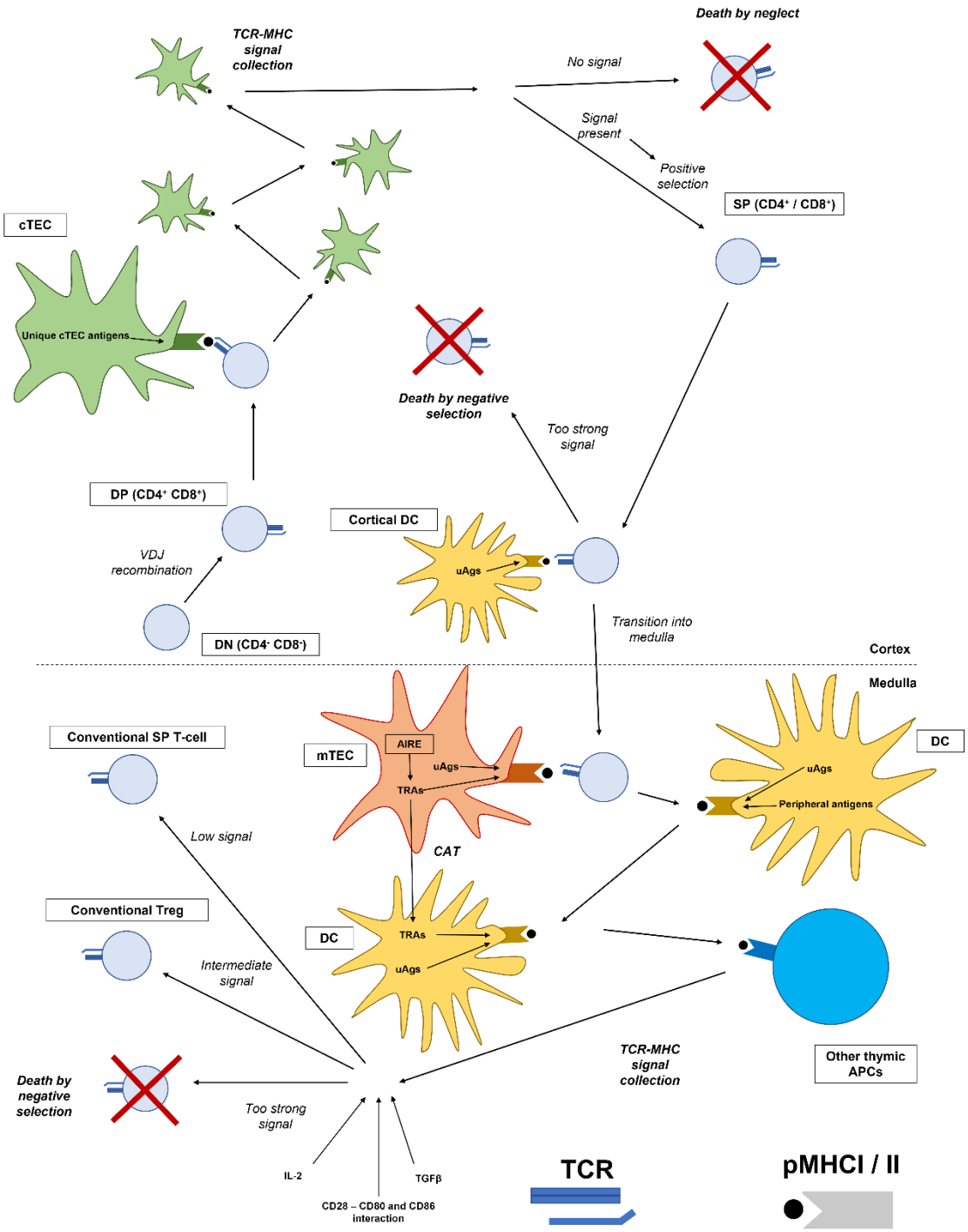

Figure 2: Model of thymocyte selection

Thymocytes are selected via TCR – MHCI or II interaction with multiple APCs. Signalling intensity of the interaction determines the fate of thymocyte. The thymocytes with unfunctional TCR die by neglect, thymocytes with highly self-reactive TCR are eliminated by negative selection. The rest of thymocytes mature into SP T-cells or Tregs and leave the thymus to the periphery.

Adopted from Klein et al. 2014.

C. Heterogeneity of thymic antigen-presenting cells (APCs)

In the last decade, several studies showed, that thymic populations are more heterogenous than it was previously proposed (Ardouin et al., 2016; Metzger et al., 2013). Especially the use of single-cell RNA sequencing (scRNA-seq) led to breakthrough discoveries in unraveling of the heterogeneity of thymic APCs and distinct contributions to their thymic function (Baran-Gale et al., 2020; Bautista et al., 2021; Bornstein et al., 2018; Breed et al., 2022; Dhalla et al., 2020; Kernfeld et al., 2018; Michelson et al., 2022; Park et al., 2020; Vobořil et al., 2020; Wells et al., 2020). The scRNA-seq approach enables to acquire a complete messenger RNA (mRNA) transcription profile of every single cell in a given sample (sorted cells or whole organ suspension). These profiles can be subsequently analysed in order to sort the cells into groups according to the similarities and/or differences in their gene expression, to compare the expression of one cell type across tissues, to reveal new markers for known populations or to find new cell subsets, or activation states (Papalexi and Satija, 2017). Considering these new advances in the field of thymus physiology, the aim of this chapter is to describe the most important thymic APCs and their potential function in establishment of central tolerance.

C.1 Heterogeneity of thymic epithelial cells (TECs)

Thymic epithelial cells consist of two major subsets: cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC) (Klein et al., 2014). Both of these populations are highly heterogenous (Bautista et al., 2021). While other thymic APCs are of hematopoietic origin and therefore are $CD45^+$, TECs are of stromal origin and can be described as $CD45^-$ $EpCAM^+$ cells that express various combinations of keratins (KRT) (Bautista et al., 2021; Wells et al., 2020).

C.1.1.1. Immature TECs

The population to start with are immature TECs. The presence of this population has been suggested by multiple studies in both human and mouse thymus (Bautista et al., 2021; Wells et al., 2020). These cells express markers such as CLDN3, CLDN4, KRT5, KRT8 (Hamazaki et al., 2007; Kernfeld et al., 2018; Nusser et al., 2022). Since these cells express general markers of TECs, such as FOXN1, PAX9, SIX1 and EpCAM, but no markers of other TEC subsets with more specialized functions, it has been suggested, that they are precursors of TEC populations. Specifically, it has been suggested that the immature TECs are start point of three differentiation pathways of TECs: first pathway leading to the development of cTEC population, second leading to $mTECs^{hi}$ and mimetic cells and the third leading to $mTECs^{lo}$ (Bautista et al., 2021; Kernfeld et al., 2018; Nusser et al., 2022). All the populations will be further discussed (*section C.1.2, C.1.3*.). Importantly, the immature TECs are the only TEC population which actively proliferates based on the expression of genes associated with cell cycle and mki67 which is a proliferation marker of precursor cells (Wells et al., 2020). These progenitors are considered to consist of two subsets, one being a bipotent embryonal progenitor, having characteristic expression of $PSMB11^{hi}$, and the second, being bipotent postnatal progenitor, expressing only low levels of PSMB11 and being closest to so called "Intertypical TECs" known from scRNA-seq analyses (Baran-Gale et al., 2020; Nusser et al., 2022). Importantly, scRNA-seq experiments on human thymus showed, that these precursors also express LTβR and CD40 receptors, which are crucial for nuclear factor kappa B (NFkB) signalling (Bautista et al., 2021). The NFkB signalling facilitates the development of TECs and is highly dependent on thymocytes, since they provide ligands for these receptors. This together creates a loop in which thymocytes control development of TECs and TECs control development of thymocytes (Akiyama et al., 2012; Nitta et al., 2011). This topic will be further discussed, but here, it is important to mention that observations provided by *Bautista et al.* show that this mechanism plays a role already on the level of TEC precursors. This was further confirmed by a recent study, which showed that self-reactive $CD4^+$ thymocytes interact with TEC progenitors where they induce transcription factors, regulate the downstream TEC populations and enhance their selection capabilities (for example by enhancing expression of TRAs) (Lopes et al., 2022).

Nevertheless, the exact developmental trajectories and definition of the precursor population remains to be established, because all the studies agree on the presence of some precursors, but details differ in each study. This indicates that the precursor population might be more complex in respect to its heterogeneity.

C.1.2. Cortical thymic epithelial cells (cTECs)

As their name implies, the cTECs are present in the thymic cortex. These cells bear the typical expression markers of TEC lineage, being $CD45^-$ $EpCAM^+$ $PAX9^+$ $SIX1^+$ and $FOXN1^+$ (Bautista et al., 2021; Park et al., 2020). They can be distinguished from their medullary counterparts (mTECs) by the expression of LY51 and ulex europeaues agglutinin 1 (UEA1) markers, since cTECs are $LY51^+$ $UEA1^-$, whereas mTECs are $LY51^-$ $UEA1^+$ (Bornstein et al., 2018; Morimoto et al., 2018). They are marked also by PRSS16, PSMB11, LY75, ACKR4, CD83 and Tbata expression (Bornstein et al., 2018; Gao et al., 2022; Kernfeld et al., 2018).

As previously mentioned (*section B.1*), the cTECs are equipped with a specific antigen processing and presenting machinery. They express unique lysosomal protease cathepsin L1 (Nakagawa et al., 1998), specific thymic serine protease TSSP (Gommeaux et al., 2009) and unconventional subunit of proteasome: β5t (PSMB11) (Florea et al., 2010; Ohigashi et al., 2021). Furthermore, similar to mTECs, they are capable of macroautophagy (Nedjic et al., 2009). Together, this enables cTECs to facilitate positive selection by presenting unique peptides (ligandome), that are not present anywhere else in the body, thus they do not bias the selection towards self-reactivity (Klein et al., 2014; Sasaki et al., 2015).

Although the level of heterogeneity of cTECs was for a long time unknown, recent study showed, that they comprise two subpopulations. First, $CXCL12^+$ $Foxn1^+$ cells, which are normal cTECs with their respective functions. Second, $CXCL12^-$ $Foxn1^-$ cells, whose development is controlled by DN thymocytes and which lose the typical cTEC gene signature due to the loss of Foxn1, which suggests a different function than positive selection (White et al., 2022). Nevertheless, a deeper insight into the cTEC heterogeneity and function is expected to come soon.

C.1.3. Medullary thymic epithelial cells (mTECs)

The other group of TECs, generated from the TEC precursors are mTECs, which are present in thymic medulla. In the same manner as cTECs, they have markers typical for TEC lineage, being $CD45^-$ $EpCAM^+$ $PAX9^+$ $SIX1^+$ and $FOXN1^+$ cells (Bautista et al., 2021; Park et al., 2020), but are $LY51^-$ $UEA1^+$ (Bornstein et al., 2018; Morimoto et al., 2018). They are also well known to express KRT5 and KRT14 (Nusser et al., 2022). This population is highly heterogenous and can be divided into $mTEC^{lo}$, $mTEC^{hi}$, post-AIRE mTEC, tuft-cell mTEC and mimetic cell subsets. Together, these populations play an indispensable role in the establishment of central tolerance.

C.1.3.1. $mTEC^{lo}$ (mTEC I)

These cells are also referred to as mTEC I (Bornstein et al., 2018; Park et al., 2020) and can be distinguished by their high expression of CCL21, accompanied with low expression of CD80, MHCII and no expression of AIRE (Bautista et al., 2021; Lkhagvasuren et al., 2013; Lucas et al., 2020). They also express ITGA6, LY6A and CD104 (Bornstein et al., 2018; Lucas et al., 2020; Wells et al., 2020). Their low expression of CD80 and MHCII was previously explained in the way that $mTECs^{lo}$ are precursors of the CD80 and MHCII high mTEC subsets. However, it has been recently shown, that although they descend from the TEC progenitor, the $mTECs^{lo}$ are mature cells, that do not further proliferate or differentiate, thus they are not progenitors of $mTECs^{hi}$ (Wells et al., 2020). The low expression of MHCII and CD80 indicates, that antigen presentation is not their main function. The crucial importance of $mTECs^{lo}$ relies on their high expression of CCL21. Given that positive selection induces in SP thymocytes the expression of CCR7, the receptor for CCL19 and CCL21 chemokines, the main function of $mTECs^{lo}$ is the recruitment of SP thymocytes into the medulla via CCL21-CCR7 axis (Bautista et al., 2021; Lkhagvasuren et al., 2013; Ueno et al., 2004). They also positively regulate intrathymic and peripheral availability of iNKT1 and iNKT17 cells by transpresentation of IL-15. (Lucas et al., 2020). The NFkB-dependent development of $mTECs^{lo}$ is predominantly regulated by LTβ signalling through their LTβR (Lkhagvasuren et al., 2013; Lucas et al.,

2020). In the human thymus, mTECs^{lo} also express high levels of CCL21, thus their function is likely similar to those in mice. They were also detected to express LTβR, CD40 and TNFR which are crucial to sense activators of NFkB signalling (Bautista et al., 2021).

C.1.3.2. mTEC^{hi} (mTEC II)

Also called as mTEC II (Bornstein et al., 2018; Park et al., 2020), these cells, aside from the typical TEC markers, express high levels of CD80, MHCII and other molecules promoting antigen presentation (Bornstein et al., 2018), which distinguishes them from mTECs^{lo}. They also express, SPIB, CD70 and CLDN4 (Bautista et al., 2021; Bornstein et al., 2018; Park et al., 2020), but most importantly, they are marked by high levels of AIRE and FEZF2, the regulators of TRA transcription (Bautista et al., 2021; Bornstein et al., 2018; Kernfeld et al., 2018). Altogether, expression of these molecules enables them to perform their main thymic functions, which are the production of TRAs, and presentation of pMHCs loaded with TRA fragments to the thymocytes (Sansom et al., 2014; Takaba et al., 2015). Original studies claimed that just approximately half of mTECs^{hi} expresses AIRE (Derbinski et al., 2008, 2005). Nevertheless, novel studies showed that AIRE is expressed both on mRNA or protein level in the vast majority of mTECs^{hi} (Bornstein et al., 2018; Dobeš et al., 2018). However, since the expression of AIRE in some mTECs^{hi} is faint, it implicates that these cells might be in a transitional state, either mTEC^{hi} precursors or "pre-post-AIRE" cells. The mTECs^{hi} are crucial for both negative selection and Treg generation of self-reactive thymocytes. Importantly, it has been shown that selection via antigens expressed as TRAs predominantly leads to Treg generation (Aschenbrenner et al., 2007; Legoux et al., 2015; Malhotra et al., 2016). This is in agreement with the signal intensity model of negative selection (*section B*), given that one distinct TRA is typically expressed in only about 1-3% of cells (Cloosen et al., 2007). Further evidence for mTECs^{hi} being indispensable for thymocyte selection comes from AIRE knockout (KO) mice. The result of ablated AIRE expression is that T-cell clones which are normally (in WT mice) converted into Treg lineage become conventional T-cells. This effect is caused by the lack of presentation of AIRE-dependent TRAs which would otherwise direct the T-cell clones towards Tregs. Since mTECs^{hi} are the major AIRE and TRAs expressing cells in the thymus, it seems this outcome is caused specifically by AIRE deficiency in mTECs^{hi} cells (Malchow et al., 2016). Nevertheless, the recognition of TRAs can also lead to negative selection if the cognate interaction is too strong or if the cognate antigen is uAg (Liston et al., 2003; Malhotra et al., 2016).

Development of mTECs^{hi} in both humans and mice, completely relies on NFkB signals through the receptors from TNF family, LTβR, CD40 and especially RANK receptors (Bautista et al., 2021; Bornstein et al., 2018; Kernfeld et al., 2018). In the absence of such signals, mTECs^{hi} die, leading to the disruption of medullary microenvironment and morphology (Akiyama et al., 2008; Boehm et al., 2003; Cowan et al., 2013). Accordingly, the expression of AIRE in mTECs^{hi} is also NFkB-dependent (Haljasorg et al., 2015; LaFlam et al., 2015). Thus, in the absence of NFkB sensing, thymus exhibits a completely abolished generation of Tregs, but strikingly, it keeps the clonal deletion mechanism operational (Cowan et al., 2013). It is of note that another crucial receptor from TNFR family CD70, which is expressed by mTECs^{hi}, drives Treg generation by engaging CD27 on developing thymocytes (Coquet et al., 2013). It is also important to mention that if mTECs^{hi} are absent, post-AIRE mTECs (*section C.1.3.3*) (Metzger et al., 2013), tuft-cell mTECs (*section C.1.3.4*) (Miller et al., 2018) or mimetic cells (*section C.1.3.5*) (Michelson et al., 2022) are absent as well, since they all, at least partially, rely on the expression of AIRE and develop from mTECs^{hi}.

C.1.3.3. post-AIRE mTEC (mTEC III)

The original point of view on mTEC^{hi} subset was that they form the only subset that is in a mature state. The consensus was that they display a rapid turnover and their cellular pool is quickly replenished by newly developing cells from immature stages (Gray et al., 2007). Nevertheless, about a decade ago, several studies showed that at least part of mTECs^{hi} survives and further matures into terminally differentiated state, unable of further differentiation or proliferation, that was named the post-AIRE mTECs (Metzger et al., 2013; White et al., 2010). In more recent studies, they were renamed as mTEC III (Bautista et al., 2021; Bornstein et al., 2018; Park et al., 2020). Importantly, the use of scRNA-seq in recent years unravelled the complexity and functional significance of post-AIRE mTECs and therefore they are further discussed below.

C.1.3.3.1. Properties of post-AIRE mTECs

The post-AIRE mTECs are the most mature, terminally differentiated population of mTECs (Laan et al., 2021). They were firstly described in 2010 by *White et al.* as involucrin (IVL) positive cells. These cells were localized within thymic medulla, where they (in case of mice) were organised into structures, resembling "Hassall´s corpuscles" of humans (White et al., 2010). Since then, several studies further confirmed their presence in the murine Hassall´s corpuscle-like structures. Importantly, these structures closely resemble epidermal epithelium (Farr et al., 2002; Metzger et al., 2013; Wang et al., 2012; White et al., 2010; Yano et al., 2008). The post-AIRE mTECs can be clearly distinguished from mTECs^{hi}, since mTECs^{hi} are CD80^{hi} MHCII^{hi} and do not express KRT10 nor IVL, whereas post-AIRE mTECs are CD80^{lo} MHCII^{lo} KRT10^{+} IVL^{+} (White et al., 2010), also express LY6D and CLDN4 markers (Bautista et al., 2021; Bornstein et al., 2018; Miller et al., 2018; Park et al., 2020) and antimicrobial peptides such as Defensins, Lipocalin2 and S100 proteins (J. Wang et al., 2019). As previously mentioned, these cells are generated intrathymically from AIRE^{+} mTECs^{hi} population (Metzger et al., 2013) which descends from AIRE^{-} immature TEC precursor population (Metzger et al., 2013; Wells et al., 2020). Despite their origin, the post-AIRE mTECs do not express AIRE anymore and that is why they are called "post-AIRE" (J. Wang et al., 2019).

In accordance with their presence in Hassal´s corpuscles, the post-AIRE mTECs closely resemble keratinocytes from the skin (Wang et al., 2012). Both the keratinocytes and post-AIRE mTECs express IVL (J. Wang et al., 2019; White et al., 2010; Yano et al., 2008) and the KRT10, which is also a marker of terminal epithelial differentiation in the skin (Jetten, 1990). Furthermore, retroviral-like 1 aspartic peptidase (SASPase), an enzyme which is highly present in skin keratinocytes where it processes the profilaggrin to filaggrin and plays a role in maintaining the texture and hydration of skin and wrinkles (Matsui et al., 2011, 2006), is also highly expressed in the post-AIRE mTECs (J. Wang et al., 2019). Moreover, the post-AIRE mTECs show multiple features of cellular senescence (J. Wang et al., 2019), lose their nuclei and specifically express desmogleins (Wang et al., 2012), which further confirms, that they are indeed the terminally differentiated stage of mTECs.

The intrathymical half-life of post-AIRE mTECs is around two weeks (Nishikawa et al., 2014) and they can be detected in the thymus already at the day 5 after birth, subsequent to the AIRE^{+} mTECs^{hi}, which are readily detected at the day 0 after birth. This order is logical since the mTECs^{hi} are precursors of the post-AIRE mTECs (White et al., 2010).

C.1.3.3.2. Regulation of development of post-AIRE mTECs

As previously described (*section C.1.3.2*), the NFkB signalling plays a crucial role in the regulation and establishment of mTECs^{hi} and is also required for proper development of their progeny, among them the post-AIRE mTECs. The post-AIRE mTECs also express receptors for the NFkB signalling, although not in high levels, but it indicates that they can be to some extent controlled by this pathway (Bautista et al., 2021; Bornstein et al., 2018; Kernfeld et al., 2018). For example, the LTβR was reported to be especially important for the development of post-AIRE mTECs with LTα provided as a ligand by thymocytes. Furthermore, the signalling through LTβR was suggested to be dispensable for development of mTECs^{hi} (White et al., 2010). Finally, it was also shown, that the NFkB signalling occurs already in the TEC precursors, thus the pathway accompanies the entire developmental path from TEC precursor to mTEC^{hi} and subsequently leads to the senescent post-AIRE mTECs. This highlights the pivotal role of NFkB signalling in the regulation of development of thymic mTECs (Bautista et al., 2021; Lopes et al., 2022).

As mentioned previously (*section C.1.3.2*) the NFkB signalling directly promotes AIRE expression in mTECs^{hi} (Haljasorg et al., 2015; LaFlam et al., 2015). In addition, it has been demonstrated that AIRE expression is also needed for the mTECs to efficiently become the post-AIRE mTECs (Nishikawa et al., 2014; Wang et al., 2012; Yano et al., 2008). The AIRE actions in mTECs drastically interfere with the integrity of DNA, causing dsDNA breaks (Abramson et al., 2010; Abramson and Husebye, 2016) and subsequently cell stress. This might physiologically activate some toll-like receptors (TLR) whose activation was shown to promote development of post-AIRE mTECs (Vobořil et al., 2020). Furthermore, the intense stress could also contribute to the senescent phenotype of post-AIRE mTECs (J. Wang et al., 2019). For the

post-AIRE mTECs and mTECs^{hi} being strongly stressed argues also the fact, that high activity of p53 was detected in mTECs^{hi} and especially in post-AIRE mTECs (Bautista et al., 2021; Rodrigues et al., 2017). Thus, the AIRE expression that promotes the expression of TRAs might also cause high levels of stress among the mTECs^{hi}, which subsequently activate TLRs and by that, promote the development of post-AIRE mTECs. The development of post-AIRE mTECs is depicted in *Figure 3*.

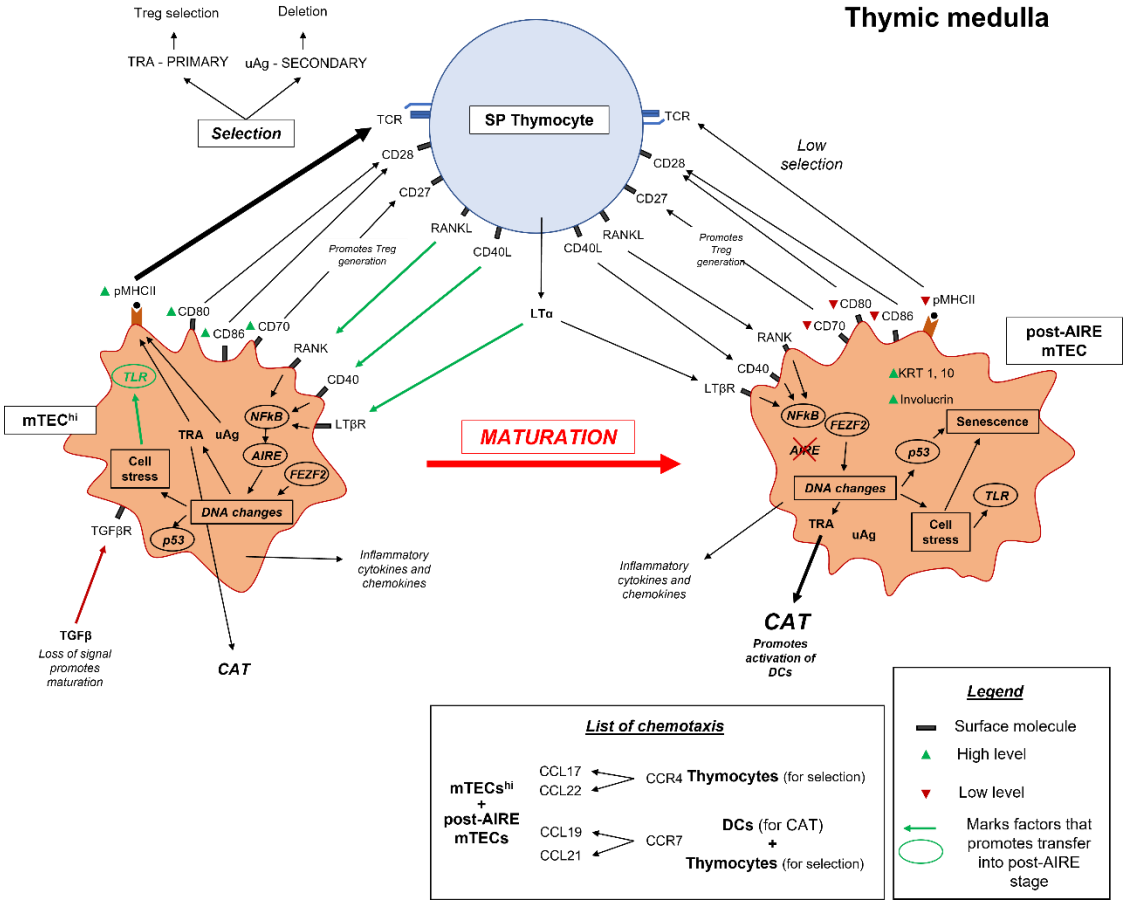

Figure 3: Maturation of mTECs^{hi} into Post AIRE mTECs

This figure is a summary of the contemporary model of maturation of mTECs^{hi} into post-AIRE mTECs in the context of the thymic heterogeneity and crosstalk with other cell types, especially the thymocytes in this case. The figure depicts the factors that promote the maturation of post-AIRE mTECs and the functional relevance of the mTECs^{hi} and post-AIRE mTECs in the central tolerance, mainly the thymocyte selection and CAT. Importantly, the NFkB signalling in the post-AIRE mTECs rather maintains their state than promotes their maturation.

C.1.3.3.3. Functional significance of post-AIRE mTECs

The post-AIRE mTECs have two major roles in the thymic function. First, they produce cytokines that play a role in establishment of thymic-sterile inflammatory microenvironment. Second, they represent an important cellular component of the thymic APC machinery.

Upon TLR stimulation, the mTECs^{hi} were found to express a vast array of chemokines (Cxcl1, 2, 3, 5 and Ccl 3, 5, 20) and cytokines (TNFalfa, IL-6, IL-12, CSF2) and other molecules (CD40) that are related to the inflammation and recruitment of neutrophils and myeloid cells (Belperio et al., 2002; Griffith et al., 2014; Vobořil et al., 2020). Since mTECs^{hi} with active TLR signalling progress into post-AIRE stage, the production of the above-mentioned chemokines and cytokines position them somewhere in between these two differentiation stages (Vobořil et al., 2020). In comparison with mTEC^{hi}, in post-AIRE mTECs, CCL21, CCL17 and XCL1 expression is decreased. In contrast, post-AIRE mTECs express IL-7, CCL19, CCL22, TSLP, RANK and LTβR at the same levels as mTECs^{hi} and the expression of CD40 is lower than in mTECs^{hi}.

The mTECs^{hi} have the highest expression of MHCII and CD80 of all mTECs. But during the maturation process, the MHCII is downregulated and terminally differentiated post-AIRE mTECs are MHCII^{lo} and also downregulate other molecules such as CD80 and CD86 (Metzger et al., 2013; J. Wang et al., 2019). Given the CD80, CD86 and MHCII are needed to facilitate the selection of thymocytes (Leventhal et al., 2016; Watanabe et al., 2020) this suggests that the post-AIRE mTECs do not function as the primary APCs. Furthermore, although the post-AIRE mTECs do not express AIRE anymore and retain only residual expression of FEZF2 (Bautista et al., 2021), they still retain TRAs (J. Wang et al., 2019), and even in higher levels than mTECs^{hi} (Baran-Gale et al., 2020). This is accounted by the fact, that AIRE expression precedes and regulates the TRAs expression but is not required to maintain it, because their DNA is irreversibly altered and damaged by AIRE (Baran-Gale et al., 2020; Metzger et al., 2013; Wells et al., 2020). The most probable explanation of the discrepancy that post-AIRE mTECs downregulate their capabilities as APCs, but still express TRAs is that they serve as a reservoir of TRAs and making it available to DCs. DCs thus can acquire these TRAs from post-AIRE mTECs via the mechanisms of CAT (*section C.2*) (Vobořil et al., 2022, 2020).

C.1.3.4. Thymic tuft-cell mTECs (mTEC IV)

Closely resembling their peripheral counterparts, the thymic tuft cells express genes associated with taste transduction pathway, such as GNAT3, PLCG2, PLCB, TRPM5, CHAT and DCLK1. They express KRT8 and KRT10, that are also expressed by peripheral tuft cells and also share the specific morphology of tuft cells (*Figure 4*) (Gerbe and Jay, 2016; Miller et al., 2018).

Furthermore, the thymic tuft cells are dependent on transcription factor Pou2f3 in the same way as the intestinal tuft cells (Bornstein et al., 2018; Miller et al., 2018). Aside from Pou2f3, recent study also showed epigenetic modificator Sirt-6 to be important for tuft cell development (Zhang et al., 2022). The development and function of thymic tuft cells is also regulated by NFkB pathway, especially by LTβR signalling (Lucas et al., 2020) and to some point they are also dependent on AIRE expression, given some part of the tuft cells pass through AIRE^{+} stage and AIRE KO mice have disorganised tuft cell compartment. Key factor here could be HIPK2 which binds to the AIRE and promotes differentiation of thymic tuft cells (Miller et al., 2018). In human thymi, tuft cells were shown to associate with Hassall´s corpuscles and transcriptionally were even closer to the tuft cells in murine thymus then to the tuft cells present in human respiratory tract (Bautista et al., 2021; Miller et al., 2018).

Although they were reported to be ontogenetically closer to the mucosal tuft cells than the other mTECs (Bornstein et al., 2018), they are still accounted to the mTECs, for being CD45^{-} EpCAM^{+} and FOXN1^{+}, which suggests that they are deviated from the TEC lineage (Bautista et al., 2021; Miller et al., 2018; Park et al., 2020). They are also called as mTEC IV (Bornstein et al., 2018; Park et al., 2020). Importantly, they do not express CD104 and CCL21, which separates them from mTEC^{lo} (Lucas et al., 2020). They express chemosensory receptors from TAS2R family, that are not expressed by intestinal tuft cells as well as previously mentioned GNAT3 (Miller et al., 2018). They also express TRPM5, SOX9, low levels of CD80 and MHCII (Bornstein et al., 2018; Lucas et al., 2020; Miller et al., 2018). The latter two suggest that they might have some capabilities in thymocyte selection. Indeed, it was shown that via the MHCII, the thymic tuft cells contribute to antigen presentation and tolerizing of CD4^{+} thymocytes against their endogenous antigens such as IL-25 (Miller et al., 2018).

The primary thymic function of tuft cells is connected to the IL-25 production, because they are the only source of IL-25 in thymus and by that, they negatively regulate intrathymic ILC2 cells which express IL-25R (Bornstein et al., 2018; Lucas et al., 2020). Furthermore, through the IL-25, they positively regulate development and availability of iNKT2 cells in the thymus and also their availability in the periphery. The iNKT2 cells subsequently facilitate IL-4 production to establish the specific type 2 inflammatory environment in the thymus (Lucas et al., 2020; Miller et al., 2018). The IL-4 was further shown to be important in establishment of balance between thymic conventional dendritic cells type 1 (cDC1) (*section C.3.1.1*) and conventional dendritic cells type 2 (cDC2) (*section C.3.1.2*) (Lack of iNKT cells cause higher presence of cDC2s) and also in thymus without iNKT cells the activation of DCs is lowered (*section D*) (Lucas et al., 2020).

C.1.3.5. Thymic mimetic cells

Historically, there were reports describing the intrathymic presence of cells that closely resembled cells from the periphery (Farr and Rudensky, 1998), however, the function and origin of these cells for a long time remained elusive. Recent studies, using scRNA-seq detected in human thymus mTECs ($EpCAM^+$, $cytokeratin^+$, $CD45^-$), that resemble specific type of peripheral cells, such as neuroendocrine cells (BEX1, NEUROD1, NEUROG1, CHGA), Schwann cells (MPZ, SOX10, MBP, S100A1, myelin), ionocytes (FOXI1, ASCL3, CFTR, CLCNKB) or muscle cells (MYOD1, MYOG, DES) (Bautista et al., 2021; Dhalla et al., 2020; Park et al., 2020), but these studies were focused on classical mTECs. Using also scRNA-seq approach, the latest study focused on these specific subsets, confirmed that indeed there are cells that, based on their transcription signature as well as morphology, are strongly reminiscent of peripheral cells. Furthermore, these cells have expression of lineage-defining transcription factors and gene expression corresponding to the specific types of peripheral cells. However, these cells retain their mTEC characteristics. Thus, because they are mTECs, that mimic peripheral cells, they were called "Mimetic cells" (Michelson et al., 2022). Inside mouse thymus, they discovered a large heterogeneity of the mimetic cells: SpiB and Sox8-dependent microfold mTECs, that resemble gut M cells, Foxj1-dependent ciliated cells, Ckm-dependent Myog expressing muscle cells, Foxi1 and Foxi2 expressing ionocytes, Foxa1, 2 and 3-dependent neuroendocrine cells, goblet cells, keratinocytes and more, all depicted in *Figure 4* (Michelson et al., 2022). Presence of mimetic cells is crucially dependent of the respective lineage-defining transcription factors. If these factors are absent, the mimetic cells are almost completely missing. AIRE is also important, but surprisingly not absolutely critical. In AIRE KO mice, the mimetic cells were reduced but not absent, thus AIRE enhances the presence of mimetic cells. Also, most of the mimetic cells (except muscle and tuft cells) were detected to be downstream of AIRE expression, thus they can be accounted into post-AIRE subpopulation (Michelson et al., 2022).

Importantly, according to this brand-new definition of mimetic cells, thymic tuft-cells (mTEC IV) (*section C.1.3.4, Figure 4*), which were discovered in 2018, should form one of the mimetic cells subpopulations since they express genes typical for their peripheral counterparts, have a similar morphology and are dependent on a tuft cell lineage-defining transcription factor Pou2f3 (Bornstein et al., 2018; Michelson et al., 2022; Miller et al., 2018). Furthermore, based on the same logic, I suggest that the post-AIRE mTECs should also represent one of the mimetic cell subsets because they are mTECs, they are also highly similar to the skin keratinocytes based on both their gene expression and morphology (*section C.1.3.3, Figure 4*). It is also indicated by the study which discovered the presence of the mimetic cells (Michelson et al., 2022).

Regarding function of mimetic cells, they are obviously important in tolerizing thymocyte pool in a similar way as $AIRE^+$ mTECs (Michelson et al., 2022). Although function of thymic tuft-cells and post-AIRE mTECs was unravelled at least partially, the exact function of the other thymic mimetic cells beyond mimicking peripheral cell types awaits resolution. Tuft cells have been shown to play a crucial role in establishing of thymic microenvironment by expression of IL-25 (Bornstein et al., 2018; Lucas et al., 2020; Miller et al., 2018), which indicates, that the other mimetic cells could also serve similar functions. For example, microfold mTECs were shown to express Ccl6, Ccl9 and Tnfrsf11b (Michelson et al., 2022) and also in their absence, thymic B-cells were slightly impaired (Michelson et al., 2022). But these indicative results need further experimental investigation, focused on specific mimetic cell-subsets.

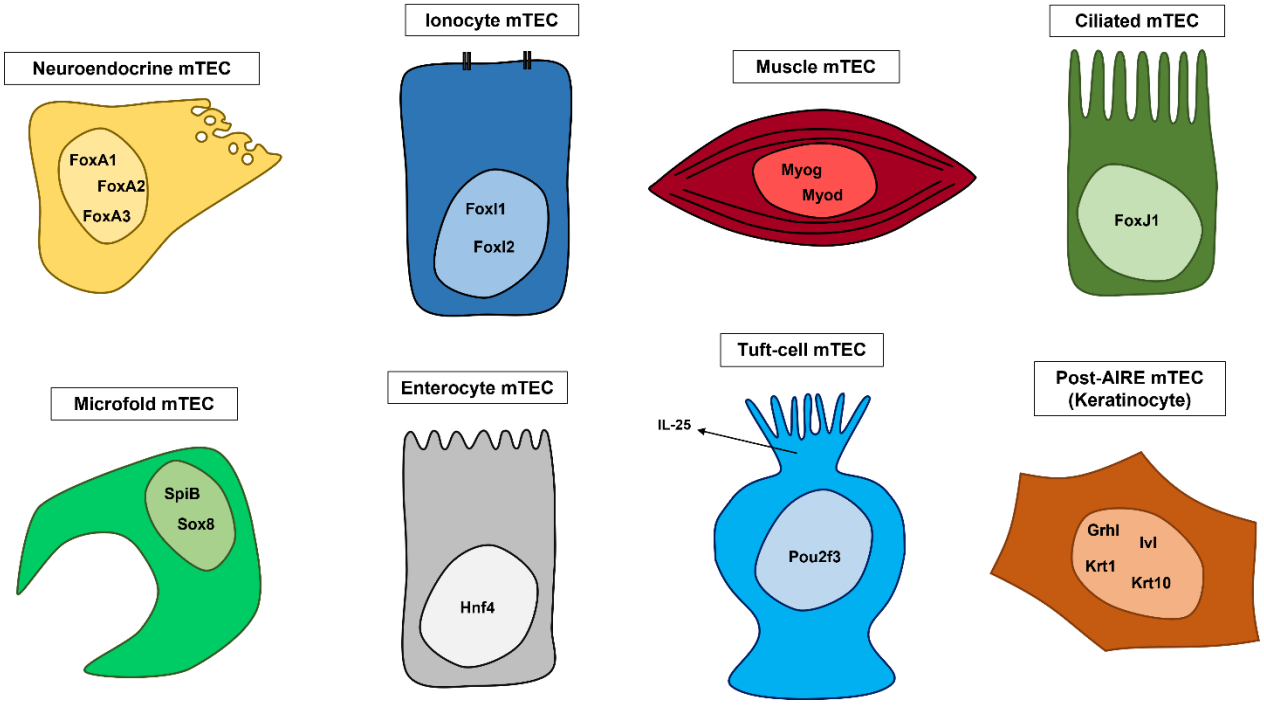

Figure 4: List of currently known mimetic cells

Depiction of currently known mimetic cells and their distinguishing markers, including tuft-cell mTECs and post-AIRE mTECs which should also form mimetic cell subsets. Adopted from Michelson et.al. 2022.

C.1.4. Summary of the TEC heterogeneity

Taken together, the use of scRNA-seq in the past decade, gave us a novel insight into the thymic TEC subset by revealing new cell populations and enabled us to study their potential functions and mutual relationships. The whole TEC compartment probably descends of the newly described TEC precursors. Also, the cTECs are more heterogenous than it was previously thought although further understanding of their heterogeneity and functions of the subpopulations remain to be assessed. The use of scRNA-seq also led to discovery of new subpopulations of mTECs as well as to proposal of their functions and regulational relations. Importantly the mTECs^{lo} whose main function is to recruit thymocytes into the medulla were shown not to be the progenitors of mTECs^{hi}. The mTECs^{hi}, the key TRA producers and critically important antigen presentators were found to give rise to the post-AIRE mTECs which also express TRAs but are poor in antigen presentation. They serve mainly as a TRA reservoir for the other thymic APCs and also contribute to the specific thymic sterile-inflammatory microenvironment. The thymic tuft cells were also shown to play some role in the establishment of thymic microenvironment. Importantly, the thymocytes are essential for the regulation of development of thymic mTEC populations with NFkB pathway being the main driver of this functional relationship. mTECs and thymocytes together create a genuine functional loop, in which thymic environment capable of selective processes is fully established only if there are thymocytes that need to be selected (*Figure 3*).

Very interesting and promising new step in understanding of thymic environment is the recent discovery of mimetic cells, which puts the whole mTEC subset into a completely new perspective. While mimetic cells transcriptionally resemble peripheral cells by expressing tissue specific transcription factors which drive their cell type specific differentiation and expression of the respective TRAs, AIRE in mTECs^{hi} drives TRA expression in a semi-stochastic fashion. Even more striking is the fact, that the discovery of whole range of mimetic cells suggests, that the post-AIRE mTECs and tuft-cell mTECs could be in fact a part of the mimetic subset. The intriguing similarity of these subpopulations with peripheral cells as well as other properties such as AIRE semi-dependence is very puzzling. Thus, despite new discoveries, the whole image of the mTECs, their heterogeneity and especially their regulatory mechanisms and functions are still incomplete understood and thus warrant further discoveries.

C.2 Cooperation of thymic mTECs and DCs

Although mTECs play a significant role in αβ SP thymocyte selection, they strongly cooperate with thymic dendritic cells (DC). It has been shown by many studies, that both populations participate in negative selection and Treg selection and that each subset has its own part of thymocyte clonal poplulation for selection and that these parts do not overlap very much (Hinterberger et al., 2010; Lancaster et al., 2019; Ohnmacht et al., 2009; Perry et al., 2014). There are also hypotheses that propose reasons, why this cooperation is needed and why only mTECs do not suffice to facilitate the selection. First, it was the idea, that there is too many thymocytes and not enough TECs to present antigens to all of them, which results in the need for another APC, namely DCs (Klein, 2009). But this is probably not the main case, because this idea was based on cytometric data (Gray et al., 2008), where the cellularity of mTECs was highly underestimated due to cell loses during tissue preparation. Cell counting on thymic microscopic sections revealed that the disparity in numbers between the thymocytes and mTECs is not as high as it was previously thought (*Table 1*) and thus sufficient for selection (Sakata et al., 2018). On the other hand, one might suggest that a typical tissue restricted antigen (TRA) is expressed in only about 1-3% of mTECs at the same time (Cloosen et al., 2007) and thus there still might not be enough mTECs expressing the given TRA to sufficiently facilitate the presentation. But it is estimated, that around 200-500 mTECs is enough to fully cover the need for presentation of a given TRA to facilitate selection (Abramson and Anderson, 2017). Therefore, there is enough mTECs to fully facilitate the TRA expression and presentation, given the *Sakata et al., 2018* detected around $1{,}1*10^6$ mTECs to be present in a thymus of which 1% is 11000 cells.

Cell type	Count (approximately)	Cell type	Count (approximately)
cTECs	$9*10^5$	mTECs	$1{,}1*10^6$
DP thymocytes	$3{,}7*10^8$	CD4 thymocytes	$5{,}5*10^7$
Ratio cTECs : DP thymocytes		Ratio mTECs : CD4 thymocytes	
1 : 411		1 : 50	

Table 1: Ratios of TECs : thymocytes, based on microscopic counting

Cell numbers of whole thymus in young adult mouse and ratios of TECs : thymocytes that are selected via the TECs. Data from the (Sakata et al., 2018). *The cytometric studies proposed ratios about tenfold higher (approximately 1:3700 and 1:200 of cTECs : DP thymocytes and mTECs : CD4 thymocytes respectively)* (Gray et al., 2008).

Second and more recent proposal comes from the idea, that DCs posess a different antigen processing and presenting machinery from mTECs, thus when the same antigen is presented on mTEC and on DC, the selection epitope (pMHC complex) will be different. This results in a wider scope of epitopes that thymocytes can scan and enables more efficient and strict selection (Březina et al., 2022; Perry and Hsieh, 2016).

The crucial part of this cooperation is the ability of DCs to facilitate selection against TRAs, despite they do not express AIRE nor TRAs as mTECs. DCs can acquire TRAs from mTECs via mechanism called "Cooperative antigen transfer" (CAT) (Gallegos and Bevan, 2004; Perry and Hsieh, 2016; Vobořil et al., 2022). According to current knowledge, CAT can be facilitated by three distinct, cell contact dependent mechanisms. The first and the most understood mechanism is phagocytosis of whole mTECs or their apoptotic bodies by DCs. In this way, the DCs acquire all proteins from the mTEC, including both uAgs and TRAs. For example, conventional dendritic cells type 1 (cDC1) (*section C.3.1.1*) population can phagocyte apoptotic bodies of post-AIRE mTECs (Perry et al., 2018). The phagocytosis is performed by scavenger receptors, such as CD36, or possibly TIM-4 and AXL (Kurd et al., 2019; Perry et al., 2018; Vobořil et al., 2022). The second mechanism is trogocytosis, which enables a cell to pick-up a part of cell membrane from another cell and integrate it into itself, including intact proteins from the membrane (Dopfer et al., 2011). By this, the DCs can acquire the whole pMHCI and pMHCII complexes from mTECs (Kroger et al., 2017; Millet et al., 2008). But most DC subsets are poor in facilitating trogocytosis. Only activated DCs (aDC) (*section D*) were shown to be efficient (Vobořil et al., 2022). The last possible mechanism is usage of cell-cell gap junctions, which in immune periphery enable APCs to acquire small molecules from surrounding

cells (Koble and Kyewski, 2009; Neijssen et al., 2005). Apparently, this is also possible in the thymus since mTECs and DCs reside in contact with each other (Lei et al., 2011) but the experimental evidence for this mechanism being operational in the thymus is so far elusive. It has been repeatedly shown that CAT and subsequent indirect presentation of TRAs by DCs is required for the proper function of DCs in central tolerance (Gallegos and Bevan, 2004; Leventhal et al., 2016; Perry et al., 2018, 2014). As will be discussed in the next chapter, thymic DCs reveal a high degree of heterogeneity (*section C.3*) (Park et al., 2020; Vobořil et al., 2020). The recent study by *Vobořil et al.* put the DC heterogeneity into the context of CAT and showed that despite all DC subsets can participate in this process, they dramatically differ in the efficiency of CAT. Furthermore, they proposed, that specific DC subsets preferentially acquire antigens from distinct subsets of TECs, which enables us to better understand, how the whole mechanism is orchestrated given the high heterogeneity of thymic APCs (Vobořil et al., 2022). The preferential pairing is depicted in *Table 2*.

	cTEC	**mTEC^{hi}**	**post-AIRE mTEC**	**mTEC^{low}**	**tuft-cell mTEC**
Antigen acquired	*uAg*	*TRA*	*TRA*	*uAg*	*uAg*
cDC1		+			
cDC2	+			+	
aDC1		+	+		+
aDC2		+	+		
pDC			+	+	
moDC					

Table 2: Model of preferential pairing between the thymic DCs and mTECs

The (+) marks the preferential acquisition of antigens during the CAT from the specific subsets of TECs (top line) by the specific subsets of DCs (left column). Based on the data from (Vobořil et al., 2022). The specific subsets of DCs are further described in subsequent chapters: conventional dendritic cells type 1 (cDC1) (*section C.3.1.1*), conventional dendritic cells type 2 (cDC2) (*section C.3.1.2*), activated dendritic cells type 1 and 2 (aDC1 and 2) (*section D*), plasmacytoid dendritic cells (pDC) (*section C.3.2*) and monocyte-derived dendritic cells (moDC) (*section C.3.3*).

C.3 Heterogeneity of thymic dendritic cells (DCs)

Thymic DCs represent numerous, motile APC populations the heterogeneity of which in the context of antigen presentation is even more complex than population of mTECs (Březina et al., 2022). These cells are CD11c^{+} CD45^{+} and comprise the conventional DCs (cDC), plasmacytoid DCs (pDC) and monocyte-derived DCs (moDC) (Breed et al., 2022; Guilliams et al., 2016; Kernfeld et al., 2018; Oh et al., 2018; Vobořil et al., 2020). Generally, these DC subsets are responsible for establishment of thymic microenvironment and enlargement of the scope of presented antigens for thymocyte selection.

C.3.1. Conventional dendritic cells (cDCs)

Conventional dendritic cells are the major, and historically mostly studied among thymic DCs (Wu and Shortman, 2005). They consist of conventional DC type1 (cDC1) and conventional DC type 2 (cDC2) populations and can be characterized as CD11c^{+} CD45^{+} CD26^{+} MHCII^{+} cells (Guilliams et al., 2016; Park et al., 2020; Vobořil et al., 2020), that can be distinguished from the other thymic DCs by their expression of IRF-8 and IRF-4, given the cDC1s are IRF8^{hi} IRF4^{lo} and cDC2s are IRF8^{lo} IRF4^{hi} and the other subsets are mostly IRF8^{int} IRF4^{int} (Guilliams et al., 2016). But crucially, apart from other DCs, development of the conventional DCs relies on the engagement of fms-related receptor tyrosine kinase 3 (Flt3) by its ligand. Hence, the Flt3 is an accurate marker of conventional DCs (Waskow et al., 2008). Another specific marker of cDC lineage is the transcription factor Zbtb46 (Satpathy et al., 2012).

C.3.1.1. cDC1

The cDC1 population is present in the thymic medulla and is considered to originate intrathymically from precursors which migrate into the thymus in CCR7-dependent manner (Cosway et al., 2018; Lei et al., 2011; Li et al., 2009). Aside from the expression of cDC markers, cDC1 can be characterized as $XCR1^{+}$ $Sirp\alpha^{-}$ $CD8^{+}$ $CD11b^{-}$ $IRF8^{hi}$ $IRF4^{lo}$ cells (Guilliams et al., 2016; Lei et al., 2011; Oh et al., 2018; Park et al., 2020; Vobořil et al., 2020). They also express Clec9a and are dependent on a transcription factor Batf3 (Guilliams et al., 2016; Hildner et al., 2008; Park et al., 2020). The cDC1s were proposed to be the main population responsible for acquisition of TRAs from mTECs via CAT, their presentation and very efficient Treg generation (Perry et al., 2014; Vobořil et al., 2022). Nevertheless, they are also capable of efficient negative selection mainly via presentation of uAgs (Oh et al., 2018; Vobořil et al., 2022; Herbin et al., 2016). It is of note that cDC1s are the only DC population with known driver of CAT, the scavenger receptor CD36, through which cDC1 acquire mTEC apoptotic bodies (Perry et al., 2018). This is further strengthened by the finding that $mTECs^{hi}$ express XCL1 which enables them to recruit cDC1s to their proximity (Lei et al., 2011), since CAT is a cell contact-dependent process (Koble and Kyewski, 2009; Millet et al., 2008). In line with their high capacity of CAT, cDC1s are known to be highly efficient in cross-presentation in the immune periphery (Schulz and Reis E Sousa, 2002). Therefore, the finding that cDC1 are dispensable for the negative selection of $CD8^{+}$ thymocytes was highly unexpected (MacNabb et al., 2019). Strikingly, in contrast to other DCs, cDC1 capacity of repetitive CAT, i.e. transfer of TRA from more than one cellular source of mTEC or other DCs, is limited (Vobořil et al., 2022). This suggests that thymic cDC1 likely reside in a specific medullar niche, where the fluctuation of cells with which cDC1 interact, is low. Since cDC1s localize to proximity of $mTECs^{hi}$, it is possible that single cDC1 remains in a long-term contact with single $mTEC^{hi}$, and hypothetically remains there until particular mTEC matures into post-AIRE stage. Despite undoubtable significance of cDC1 in central tolerance, it is becoming clear that many characteristics assigned to them are actually attributes of their activated form. This brand-new topic will be discussed further (*section D*).

C.3.1.2. cDC2

Population of cDC2 is predominantly present in the thymic medulla and cortico-medullary junction and is considered to be of extrathymic origin (Hadeiba et al., 2012; Li et al., 2009; Zegarra-Ruiz et al., 2021). In the same manner as cDC1s, they express general markers of cDC lineage and can be described as $XCR1^{-}$ $Sirp\alpha^{+}$ $CD8^{-}$ $CD11b^{+}$ $IRF8^{lo}$ $IRF4^{hi}$ cells (Guilliams et al., 2016; Park et al., 2020; Vobořil et al., 2020). They also express CLEC10a, MGL2, CD209a and are dependent on transcription factor *Irf4* (Guilliams et al., 2016; Park et al., 2020; Vobořil et al., 2020). Concerning the primary thymic function of cDC2 population, they were reported to be able to transport antigens from the blood and from the periphery into the thymus (Bonasio et al., 2006; Hadeiba et al., 2012; Li et al., 2009). Recently, a specific subset of cDC2s was identified, which uses CX3CR1 to connect to the endothelia of thymic microvessels and which is specialised in acquisition of blood-borne antigens. This subset is called "Transendothelial DCs" (Breed et al., 2022; Vollmann et al., 2021). The $CX3CR1^{+}$ cDC2s can also transport the antigens from intestinal microbiota and present them intrathymically (Zegarra-Ruiz et al., 2021). In accordance with their capability to present non-TRA antigens such as blood-borne and microbiota antigens, the cDC2s were reported to establish tolerance, primarily by negative selection (Breed et al., 2022; Lancaster et al., 2019). What is even more important, the antigens transported by cDC2 from the periphery include self as well as foreign antigens (Bonasio et al., 2006; Zegarra-Ruiz et al., 2021). Thus, cDC2s might have the crucial and nonredundant role in establishment of tolerance against food and microbial antigens inside the thymus. Nevertheless, some studies claim that cDC2 are also very efficient in generation of Tregs (Hu et al., 2017; Leventhal et al., 2016; Proietto et al., 2008). Accordingly, cDC2s are able to perform CAT and present mTEC-derived TRAs (Vobořil et al., 2022), even though their capacity of CAT is limited, especially if the particular transferred antigen is expressed in a non-ubiquitous manner (Vobořil et al., 2022). Thus, the functional contributions of cDC1s and cDC2s might be little bit blurred as shown by *Hsieh´s* and *Savage´s* research groups that were not able to agree which of the cDC1 or cDC2 subsets is the one crucial for Treg selection (Leventhal et al., 2016; Perry et al., 2018, 2014). Finally, as well as cDC1s, thymic population of cDC2s comprises cells which differ dramatically in their activation status (Oh et al., 2018; Park et al., 2020). Therefore, the exact dissection of which functions can

be attributed to cDC2s and which to their activated form still awaits resolution, and the currently known "pieces of puzzle" will be discussed below (*section D*).

C.3.2. Plasmacytoid dendritic cells (pDCs)

Localised predominantly in the cortico-medullary junction (Hadeiba et al., 2012), the pDCs can be characterized as $B220^+$ $LY6c^+$ $CD11b^-$ cells, that are typically IRF-8^{int} and IRF-4^{int} (Dress et al., 2019; Guilliams et al., 2016; Vobořil et al., 2020). They also express IL3RA, CLEC4C, BST2, CCR9, SinglecH and LY6d and only partially require transcription factor IRF-4 (Dress et al., 2019; Guilliams et al., 2016; Park et al., 2020; Vobořil et al., 2020). In a similar way as the cDC2s, pDCs were reported to be a migratory subset that transfers to the thymus from the periphery (Li et al., 2009). The migration is facilitated by their CCR9 receptor, which recognizes CCL25, produced by mTECs. Similarly, as the cDC2s, they are able to transport peripheral antigens into the thymus and promote tolerance towards them, especially by negative selection of cognate thymocytes (Hadeiba et al., 2012; Li et al., 2009). On the other hand, in RIP-OVA-OTI/II model, the pDCs were not detected to take part in thymocyte deletion (Lancaster et al., 2019). It is of note, that it has been shown, that TLR stimulation of pDCs prevents them from migration to the thymus, hereby preventing establishment of tolerance against pathogens under inflammatory conditions. Similar mechanism will probably be also present in cDC2s (Hadeiba et al., 2012). On the other hand, TLR2 sensing of commensal microbiota was reported to be needed for pDCs to be able to migrate into the thymus (Ennamorati et al., 2020). The focus of pDCs on presentation of antigens acquired in the immune periphery is also highlighted by the fact that pDCs are highly inefficient in CAT (Vobořil et al., 2022).

The pDCs also contribute to the thymic microenvironment. For example, they participate in the regulation loop, which begins with post-AIRE mTECs, that recruit and activate neutrophils from the bloodstream via CXCL5-CXCR2 axis. The neutrophils subsequently produce IL-23, which stimulates pDCs via IL-23 receptors to constitutively produce IFN-α which promotes maturation of SP thymocytes (Persson et al., 2003; J. Wang et al., 2019). In the context of this loop, it is of note that since the generation of post-AIRE mTECs is AIRE-dependent (Yano et al., 2008), the formation of the thymic microenvironment is also to some extent dependent on AIRE. Therefore, the damage to the thymic function, caused by AIRE deficiency can be attributed not only to the loss of TRA expression but also to disruption of thymic microenvironment (J. Wang et al., 2019).

Importantly, the thymic pDCs were not significantly altered by the absence of thymocytes, thus their regulational pathways might be different from the other DCs (Spidale et al., 2014). It was further shown that some part of pDCs can also arise intrathymically, since it was reported, that they can be generated from one of thymocyte progenitors (Lavaert et al., 2020). This further confirms, that although they are considered as DCs, they are developmentally distinct, since they are generated from $Ly6d^+$ $CD2^+$ lymphoid progenitor and not from myeloid progenitor as the other DCs (Dress et al., 2019; Lavaert et al., 2020). Nevertheless, some studies propose their development from the myeloid progenitors (Rodrigues et al., 2018). In human thymus, pDCs were reported to facilitate Treg generation, and to produce CCL-22 and CCL-17 which both attract SP thymocytes and recirculating Tregs to the medulla (Hanabuchi et al., 2010; Klein et al., 2019; Martín-Gayo et al., 2010).

C.3.3. Monocyte-derived dendritic cells (moDCs)

These cells are present in thymic medulla and phenotypically are very close to the cDC2 lineage. They are $Sirp\alpha^+$ $MGL2^+$ and $CD14^+$ whereas cDC2s are $Sirp\alpha^+$ $MGL2^+$ and $CD14^-$ (Vobořil et al., 2022). With cDC lineage, they also share expression of ITGAX, ITGAM, IRF-4 and IRF-8 (Guilliams et al., 2016; Vobořil et al., 2020). On the other hand, they share expression markers with macrophages, such as LYZ2, MERTK, MAFB and LY6C (Vobořil et al., 2020). They can be distinguished by specific expression of CD14, FCGR1, and CSF2R, they also do not express CD135, which is one of markers for DCs (Guilliams et al., 2016; Hettinger et al., 2013; Vobořil et al., 2020). They were shown to be capable of CAT, however, while having no preferential pairing partners among TEC subsets (*Table 2*), moDCs are capable to acquire antigens from multiple mTECs and also being the most potent subset in performing CAT from other DC subsets. The

moDCs can efficiently present the acquired TRAs to thymocytes and have a role in thymocyte selection, especially in Treg generation (Vobořil et al., 2022, 2020). The CAT is probably enabled by physiological stimulation of TLRs in the mTECs (mainly mTECs^{hi} and post-AIRE mTECs), which subsequently produce chemokines such as CCL19 and CCL21 that recruit moDCs to their proximity. Furthermore, it was shown that in MyD88-deficient mice which is lacking TLR signaling only in TECs, the cellularity of moDCs was diminished. In opposite situation, when WT thymi were stimulated with CpG (TLR9 ligand) the number of thymic moDCs were dramatically increased. Therefore, the size of moDCs compartment in the thymus is regulated by TLR signalling in mTECs (Vobořil et al., 2020).

C.3.4. Summary of dendritic cells heterogeneity

The highly heterogenous population of dendritic cells plays an indispensable role in the thymic function. DCs play a pivotal role in thymocyte selection by presenting their endogenous antigens, peripheral antigens or antigens acquired from other cells, especially mTECs, by CAT and thus they significantly widen the scope of antigen presentation in the thymus. Although their roles can to some extent overlap, the cDC1s primarily facilitate selection via endogenous and CAT-acquired antigens, favouring the Treg generation above negative selection. The cDC2s with pDCs are crucially important in transporting peripheral antigens into the thymus and facilitate selection against them predominantly by deletion while also being potent in facilitating selection via endogenous and CAT-acquired antigens. The moDCs are also potent in antigen presentation, favouring Treg generation while also being efficient in CAT an especially the CAT with other DCs and multiple mTECs. The thymic DCs also contribute to the establishment of specific thymic low-sterile-inflammatory microenvironment by producing various cytokines and chemokines. Importantly, there is increasing evidence that specific activated state of cDC1s and cDC2s play a crucial role in the thymic function, although their presence was frequently overlooked and thus exact dissection of functions between the activated and non-activated forms still awaits its resolution.

C.4 Other thymic antigen-presenting cells

In addition to the two major thymic APC populations, TECs and DCs, there are other, minor APC populations, that are not as much explored, but still contribute to establishment of central tolerance.

C.4.1. Thymic B-cells

The thymic medulla is populated by a specific CD45$^+$ CD19$^+$ B220$^+$ B-cell population (Perera et al., 2016, 2013). These cells originate from intrathymic precursors, although they can also migrate to thymus from the periphery (Perera et al., 2013; Yamano et al., 2015). The B-cells were detected to express high levels of MHCII, CD80, which indicates, that they have a role in antigen presentation and selection of thymocytes (Lancaster et al., 2019; Yamano et al., 2015). And indeed, depletion of thymic B-cells results in diminished deletion of CD4 thymocytes, thus the B-cells play a role, especially in negative selection (Lancaster et al., 2019). Most probably, they establish tolerance against their endogenous antigens, which are highly abundant during infection, when B-cells proliferate and thus must be efficiently tolerated (Lancaster et al., 2019; Yamano et al., 2015). Furthermore, they were suggested to eliminate thymocytes that have TCR reactive to their BCR, which might result in inadequate help provided to B-cells by their self-reactivity (Munthe et al., 2005). On the other hand, they are also able to use their BCR, to capture antigens from their surroundings and promote tolerance to the intrathymic soluble antigens. Thus, their BCRs are at least to some extent self-reactive (Perera et al., 2016, 2013). It was also shown that their function is potentiated by CD40-CD40L interactions with self-reactive thymocytes, which creates a functional loop similar to that described for mTECs and thymocytes (*section C.1.3.3*). This interaction also resembles “help” provided by helper T-cells (Th) in the periphery, and it was shown that it results in class-switching of the intrathymic B-cells (Perera et al., 2016; Yamano et al., 2015). Furthermore, the B-cells were also detected to express AIRE (Yamano et al., 2015), which suggests their involvement in inducing tolerance to TRAs, but this has not been examined thoroughly, therefore, the primary role of thymic B-cells remains in establishing tolerance to their endogenous antigens.

C.4.2. Thymic macrophages

Representing about 0,1 % of thymic cells, the thymic macrophages can be generally described as $MHCII^+$ $LYZ2^+$ $SPIC^+$ $CD64^+$ $F4/80^+$ $MERTK^+$ $CD11b^{lo}$ cells, that are typically IRF-4^{int} IRF-8^{int} and also intrathymically express CD11c which is not a typical marker for macrophages (Guilliams et al., 2016; Kernfeld et al., 2018; Zhou et al., 2022). They can be divided into two populations. The first is $TIM4^+$ $CX3CR1^-$ population, which is present in thymic cortex. This population is predominantly of embryonic origin and is maintained by in-situ proliferation, although, their numbers decrease with age. The second is $TIM4^-$ $CX3CR1^+$, which is present in medulla and in cortico-medullary junction and is derived from adult hematopoietic cells (Zhou et al., 2022). Concerning the function of thymic macrophages, they are most closely related to the spleen red pulp macrophages and Kupfer cells and they express phagocytic receptors such as TIM4, CD51 and AXL (Zhou et al., 2022). They were shown to highly express proteins of pathways connected with antigen presentation which suggests that they might be involved in antigen presentation and tolerizing of thymocytes. However, in the RIP-OVA-OTI/II model, macrophages were not detected to take part in thymocyte deletion. On the other hand, in vitro experiments showed, that OVA-loaded thymic macrophages were able to efficiently induce proliferation of OT-II T-cells, whereas peritoneal and other tissue resident macrophages were not. This suggests that they are indeed antigen-presenting cells although not as potent as thymic DCs (Lancaster et al., 2019; Zhou et al., 2022). Still, the major proposed function of thymic macrophages is scavenging of apoptotic thymocytes, since the macrophages were shown to be the main thymic population, responsible for clearing of apoptotic cells (Zhou et al., 2022).

D. Homeostatic activation of thymic dendritic cells

This chapter takes a closer look on the cDC1s and cDC2s, that were previously presented in the scope of thymic heterogeneity. Here, the specific maturation which both of these cell populations undergo will be discussed. Importantly, except distinct thymic APC populations, use of scRNA-seq unravelled that within cDC1s and cDC2s, particular cells differ transcriptionally, since they exhibit activated/mature status. Since the activated subsets of cDCs are perfectly equipped on a molecular level for participation in T-cell selection processes, scRNA-seq era opened brand new research field which is of great importance in understanding the central tolerance. Although the "mature" stage of cDCs, which is from this point onward referred to as activated DCs (aDC), was discovered in the thymus already in 2016 (Ardouin et al., 2016), the relevance of this subset in the thymus was not fully appreciated until recently. All the studies that described aDCs clearly showed, that they have important functions and thus should be seriously taken into account. First of all, it is important to explain, what exactly is the "Homeostatic activation" and how it is connected with already well-described immunologic activation of DCs in the periphery.

DCs are present throughout the body and serve a plethora of functions that are crucial for survival of the organism. The "activation" of these cells plays a key role. *Cabeza-Cabrerizo et al.* defined the activation as a process which enables the DCs to convey information to other cells and thus enabling to facilitate stimulation of adaptive and innate immune response, cytokine production, infection detection, cell-to-cell communication and also peripheral and central tolerance (Cabeza-Cabrerizo et al., 2021; Guilliams et al., 2016; Reis E Sousa, 2006; Steinman, 1991). Several studies called this process as a "maturation" although using this term can bring about confusion with DC-poiesis, implying that it represents the next step in development of DCs from bone marrow. However, "mature" DC is a completely developed cell in a steady-state that is ready to facilitate its functions. "Activated" DC, on the other hand, is a cell-state of already mature DC which throughout the process of activation undergoes changes in gene expression which lead to enhanced expression of chemokines, checkpoint molecules, molecules of antigen-presenting machinery etc., and this process is dependent on antigen recognition and surrounding microenvironment and might not be temporarily subsequent to the "maturation". Thus, using a term "activation" for the aforementioned process should be preferred (Cabeza-Cabrerizo et al., 2021; Ginhoux et al., 2022).

In the periphery, DCs can be activated in two fundamental ways. The first is "classical" immunogenic activation in which DCs are activated via interactions of their pattern recognition receptor (PRRs), for example TLRs, with their ligands, the pathogen associated molecular patterns (PAMP) (Spörri and Reis E

Sousa, 2005), cytokines (Kaka et al., 2008), cell to cell contacts (Sadeghlar et al., 2021), or danger-associated molecular patterns (DAMP) (Laursen et al., 2018), to facilitate immunostimulatory response against pathogens. The second form of activation is "Homeostatic activation", which is independent on pathogens and biases DCs towards tolerogenic and immunomodulatory functions (Ardouin et al., 2016; Cabeza-Cabrerizo et al., 2021; Oh et al., 2018). *Ardouin et al.* performed a genome-wide transcriptomic analysis of DCs from the periphery and from the thymus. They showed that DC subsets from all tissues tested comprise both activated and non-activated DCs, thus DCs can be activated under sterile conditions, independently of inflammation. Hierarchical clustering of the analysed cells linked DC subsets across tissues according to their expression pattern. The homeostatically activated DCs clustered together, irrespective of tissue or DC subtype. Non-activated DCs clustered together in a same manner. Thymic aDCs clustered together with aDCs from other tissues. A large number of genes (around 300) were upregulated or downregulated upon maturation of DCs in all tissues tested and the patterns of gene upregulation or downregulation were greatly overlapping across tissues. These experiments showed that homeostatic activation of DCs is a widespread phenomenon and that it is clearly independent of infection and that the peripheral homeostatic activation is similar to the thymic homeostatic activation (closest to the activation in Lymph nodes). Similar results were also obtained by a microarray analysis of aDCs from LNs and from thymus performed by *Oh et al* (Oh et al., 2018).

Furthermore, *Ardouin et al*. performed a transcriptomic analysis of thymic homeostatically activated DCs and immunogenically activated DCs (by introduction of TLR ligands) and found, that homeostatically maturated DCs are significantly different from the immature DCs and even more different from the immunologically maturated DCs. The unique expression pattern for immunogenic activation included genes for viral DNA sensing, antiviral responses, IFN-I and IFNγ signalling genes. Also, the homeostatic activation had its specific genes, for example genes for cholesterol biosynthesis. On the other hand, both the activation subprograms have a broad convergence in gene expression. Over 300 genes are expressed upon activation, irrespectively whether it is immunological or homeostatic, and the activated cells share many common features. For example, both the immunogenically and homeostatically activated DCs share characteristic expression of molecules such as cytokines (Il-15, Il-12β), chemokines (Ccl5), cytokine receptors (Il-15Rα), chemokine receptors (Ccr7), TNF superfamily receptors (Ox40, Cd40, Rank), molecules associated with NFkB signalling (*RelB*) and especially high expression of molecules associated with antigen presentation (MhcII, Cd80, Cd86, Cd83, Cd274) (Ardouin et al., 2016). These molecules can therefore be understood as general markers of DCs activation. Another common feature of all activated DCs is the mitotic arrest and stopping their cell cycle. (Ardouin et al., 2016; Oh et al., 2018). Taken together, these studies showed, that thymic and peripheral homeostatically activated DCs have a lot in common and that immunogenic and homeostatic activation also share many common features, although they are two distinct mechanisms.

The specific pattern of homeostatic activation is slightly different in each tissue (Ardouin et al., 2016; Oh et al., 2018). This difference is not surprising, given the fact, that DCs are capable of a complex sensing of signals and factors from their surroundings and that the microenvironment is different in each tissue. There are different cell types, interactions, cytokines, antigens and other signals, that impact DCs and thus causing the homeostatic activation and subsequently biasing the different expression pattern in DCs (Cabeza-Cabrerizo et al., 2021; Jonuleit et al., 1997; Mellman and Steinman, 2001). It is also good to mention that the DCs are activated in a progressive manner. The homeostatic activation is rather a process of subsequential expression changes than just one event. Accordingly, the aDC1 and aDC2 are more similar than their non-activated precursors. It is due to the inactivation of many genes that are responsible for functional and phenotypic discrimination of cDC1 and cDC2 during homeostatic activation (Ardouin et al., 2016). Moreover, in human thymus, DC subset called aDC3 was described which likely comprises activated cells from both cDC lineages. It has been proposed that aDC3s represent terminally activated stage of DCs marked by complete loss of the expression of lineage-defining markers (Park et al., 2020).

D.1.1. Thymic microenvironment that inflicts activation of dendritic cells

Thymic dendritic cells are heterogenous. But from all the populations described, only cDC1 and cDC2 were confirmed to undergo intrathymic homeostatic activation (Ardouin et al., 2016; Breed et al., 2022; Park et al., 2020). In a normal thymus, around 40% of cDC1 and around 50% of cDC2 cells are in activated state, remaining cells of the respective populations are non-activated (Oh et al., 2018). This high level of activation suggests that the activation process plays an important role in thymic function. The thymic aDC subset, as previously mentioned, can be generally described as $CD11c^{hi\text{-}int}$ $MHCII^{hi}$, $CD80^{hi}$, $CD86^{hi}$, $CD40^{hi}$ and $CCR7^{hi}$ cells. Since aDCs emerge from $CD11c^{hi}$ $XCR1^{+}$ cDC1s (Ardouin et al., 2016; Park et al., 2020) and from $CD11c^{hi}$ $Sirp\alpha^{+}$ cDC2s (Breed et al., 2022; Park et al., 2020), the aDC subset can be further divided into $CD11c^{hi\text{-}int}$ $MHCII^{hi}$ $CD80^{hi}$ $CD86^{hi}$ $CD40^{hi}$ $CCR7^{hi}$ $XCR1^{lo}$ aDC1 subset and $CD11c^{hi\text{-}int}$ $MHCII^{hi}$ $CD80^{hi}$ $CD86^{hi}$ $CD40^{hi}$ $CCR7^{hi}$ $Sirp\alpha^{lo}$ aDC2 subset. It is important to note, that during the activation process, the cDC1s and cDC2s downregulate their lineage-defining molecules but to an extent which still enables their discrimination (Breed et al., 2022; Park et al., 2020). Furthermore, the markers of thymic DCs activation are very similar for aDC1 and aDC2, thus the regulation properties and functions of thymic aDCs will be probably also similar (Ardouin et al., 2016; Park et al., 2020).

As previously mentioned, the homeostatically activated DCs where discovered in the thymus by *Ardouin et al.* in 2016. Since their discovery, the factors that promote the activation of thymic cDCs and their thymic function has been studied. It has been shown that thymic microenvironment plays a key role in the homeostatic activation of thymic DCs, but the whole picture, showing the exact signals that promote DCs activation, exact molecular mechanisms of the activation, properties of activated cells and thymic function of aDCs is not yet complete. The factors of thymic microenvironment, that have direct impact on thymic DCs activation can be divided into three groups: cell-to-cell contacts, soluble molecules and antigen transport related factors (Ardouin et al., 2016)

D.1.1.1. Factors of cell-to-cell contacts

Inside thymus, DCs can contact multiple cell types, especially mTECs from which they acquire antigens via CAT, they also contact other DCs to share the antigens they acquired (Vobořil et al., 2022) and finally, they contact thymocytes to promote their selection (Klein et al., 2014). It is intuitive to suggest, that some of these interactions could provide signals for their activation.

Oh et al. studied the impacts of thymocytes on aDCs generation in the thymus. Using RAG-1 and TCRα knockouts, where development of thymocytes is impaired, they found, that when thymocytes are depleted from the thymus, generation of aDCs is strongly reduced (aDC1s were reduced by about 80%, aDC2s by about 60%). They found that $CD4^{+}$ SP thymocytes were the primary needed population, because in mice deficient for $CD4^{+}$ SP thymocytes, there was a significant reduction of aDCs and that MHCII molecule present on the DCs is needed for the activation. Furthermore, in $RAG^{-/-}$ OT-II mice, where all $CD4^{+}$ SP thymocytes have TCR reactive for OVA that is not by default present in mice, there was significant reduction of aDCs (same percentage as in $RAG^{-/-}$ model). In $RAG^{+/-}$ OT-II mice where some part of $CD4^{+}$ SP thymocytes is self-reactive there was higher number of aDCs but still not matching numbers of WT mice. Finally, when OVA was injected intravenously into $RAG^{-/-}$ OT-II mice the number of aDCs was mostly restored, but still did not match the WT state (Oh et al., 2018). The numbers of DCs producing IL-12 were also markedly decreased in thymus lacking TCR interactions, further supporting the need of thymocyte for activation of DCs (Spidale et al., 2014).

By the similar set of experiments, only with OT-I mice where OVA is recognized by $CD8^{+}$ SP thymocytes they showed, that cognate interactions with $CD8^{+}$ SP thymocytes are not needed for the activation in WT state but can also lead to some extent of DCs activation, suggesting some functional redundancy of $CD8^{+}$ and $CD4^{+}$ SP thymocytes in this mechanism. Furthermore, the activation after OVA injection in this model was stronger in cDC1 subset than in the cDC2. This can be explained by the fact that cDC1s are better in cross presentation than cDC2s (Schulz and Reis E Sousa, 2002).

These observations showed, that DCs presenting antigens via MHCII are activated predominantly by cognate interactions with self-reactive $CD4^{+}$ thymocytes. Nevertheless, OVA injection into the $RAG^{-/-}$ OT-II mice

does not fully restore aDC pool and when thymocytes are depleted from the thymus, aDCs are still present, thus this interaction with thymocytes is not the only activator of thymic DCs. These results were also confirmed by *Ardouin et al* (Ardouin et al., 2016; Oh et al., 2018). It is also good to keep in mind, that these interactions are separated from the periphery and that the intrathymic activation is not a result of peripheral immune responses, although the inflammation in the periphery can affect the thymus (Spidale et al., 2014).

Furthermore, *Oh et al.* found, that in mixed BM chimeras, where WT mice were reconstituted by mix of CD40 sufficient and deficient BM, there was significantly lower number of aDCs in DCs population derived from CD40 deficient BM, showing CD40 molecule as a critical part of the mechanism. But again, aDC2 were lowered by about 40% and aDC1 by about 30%, thus this is not the only mechanism of activation. These results were confirmed in vitro with OT-II $CD4^+$ thymocytes, where $CD40^+$ OVA-loaded DCs were activated more efficiently than their $CD40^-$ counterparts. Interestingly, when this experiment was performed with OT-I $CD8^+$ thymocytes, the activation level of DCs was the same, irrespective whether they were $CD40^+$ or CD40, indicating the presence of other activation mechanisms. Similar results were also obtained by *Spidale et al.* They found that activation of thymic DCs is dependent on feedback either from $CD8^+$ thymocytes or $CD4^+$ thymocytes via TCR-antigen-specific recognition and that CD40 is crucial for facilitating the activation via interaction with $CD4^+$ thymocytes. Finally, *Oh et al.* showed, that CD40 potentiates Treg development because when CD40 deficient DCs, loaded with OVA, where cocultured with OT-II $CD4^+$ thymocytes, the Treg generation was significantly decreased when compared with CD40 sufficient DCs (Oh et al., 2018; Spidale et al., 2014).

The significance of the CD40 costimulation was further explored recently. In peripheral cancer models, cognate interactions of cDC1 cells with $CD4^+$ T-cells, lead to expression of multiple genes, among them COX-2, Bcl2l1 and CD70, which is one of the markers of DC activation (Park et al., 2020; Wu et al., 2022). The CD70 is recognized by CD27 on $CD8^+$ T-cells and this axis plays a crucial role in facilitation of antigen-specific $CD8^+$ T-cells proliferation and activation. Nevertheless, in cancer models, CD40 is a crucial for tumour rejection, whereas CD70 plays only a partial role in the rejection. Similar effects were observed with COX-2 (cyclooxygenase-2) expression in cDC1s. The COX-2 enhances proliferation of cognate $CD8^+$ T-cells and only partially contributes to tumour rejection. Bcl2l1 expression in cDC1 enhances survival of cDC1 during antigen presentation (Wu et al., 2022). Although these results were obtained in tumour models and were studied only in cDC1s, the implication for the thymus would perfectly explain the potential mechanism behind the CD40 signalling, given the fact, that CD70 on aDC1s is recognized by CD27 on thymocytes and plays a crucial role in thymic Treg generation by inhibition of Treg apoptosis caused by cognate interactions (Coquet et al., 2013).

Taken together, cognate interactions with self-reactive $CD4^+$ thymocytes are needed for thymic activation of both cDC1s and cDC2s, although it is not the only mechanism that facilitates the activation. The $CD8^+$ thymocytes does not seem to play a crucial role in this mechanism. The key molecule that facilitates the activation of both cDC1s and cDC2s is CD40, although it is not the only activator, and the exact molecular mechanism of the activation is unknown, although it is probably related to the NFkB signalling. In the case of the aDC1s, the CD40 activation promotes expression of Bcl2l1 which enhances the survival of aDC1s, COX-2 that enhances the stimulation of thymocytes and especially CD70, which is recognized by CD27 on thymocytes, protects them from apoptosis and potentiates the Treg development, supporting the predominant role of aDC1s in Treg generation. It is also important to note that the factors of phagocytosis and CAT, which will be discussed subsequently, are also facilitated by cell-to-cell contacts, but hence these mechanisms were found to play a pivotal role in homeostatic activation of thymic DCs, they were placed into separate chapter.

D.1.1.2. Factors of antigen-transport related activation

Peripheral dendritic cells can be homeostatically activated by recognition of tumour apoptotic cells. Though, it is not clear whether it is caused only by tumour apoptotic cells or apoptotic cells in general, but the recognition of apoptotic tumour cells leads to activation of cDC1 and partially cDC2 cells into mregDCs, which closely resemble classical aDCs and the most probably are the same subtype as aDCs according to the markers they express. Scavenger receptor AXL might play a key role in facilitating such activation (Ginhoux et al., 2022; Maier et al., 2020). This form of activation has not yet been experimentally proven in the thymus

but given the AIRE^{+} mTECs can to some extent resemble cancer cells, since the DNA of mTECs is highly stressed by the actions of AIRE and the consequence of such stress is their rapid turnover (Abramson et al., 2010; Abramson and Husebye, 2016) or generation of slowly dying post-AIRE cells, thus in the thymus, phagocytosis of apoptotic mTECs during CAT might drive homeostatic activation of DCs in the same manner as in tumours (Březina et al., 2022).

Signs of the activation subsequent to CAT-related antigen uptake can be also seen in a paper from *Vobořil et al.* where they found aDC1s being the most effective cells performing CAT and aDC2s being the second most effective cells. Both the aDC subsets preferentially acquire TRAs from AIRE^{+} mTECs^{hi} and post-AIRE mTECs and aDC1 can be detected in the proximity of the AIRE-expressing mTECs. The cDC1s are known to be attracted to their proximity via XCL1-XCR1 axis (Lei et al., 2011; Park et al., 2020; Vobořil et al., 2022). But a "snapshot" of aDCs directly performing CAT has never been observed, because experiments performed by *Vobořil et al.* only focused on model TRA antigens being present inside DCs after CAT (Vobořil et al., 2022) thus, the TRAs can be acquired by non-activated DCs but observed after their activation. Furthermore, given the fact, that DCs upon immunogenic activation cease their phagocytic activity and focus rather on antigen presentation and costimulation (Reis E Sousa, 2006; Shin et al., 2006), the model in which DC acquire antigens from mTEC and subsequently undergo maturation is likely, but still, these mechanisms have not been proven experimentally.

D.1.1.3. Factors of soluble molecules

As mentioned before, the peripheral immunogenic activation of DCs is influenced by a plethora of cytokines and other soluble molecules. Homeostatic activation of thymic DCs is influenced in a similar way by the specific thymic cytokine microenvironment. It has been shown, that innate immune signalling does not influence the activation of thymic DCs, because in Myd88, TRIF and MAVS knockouts, the levels of DCs activation where comparable to those in WT mice (Oh et al. 2018). TSLP is produced by post-AIRE mTECs and was suggested to play an important role in activation of DCs, since it was reported to be able to promote activation of thymic DCs in vitro (Watanabe et al. 2005). And the TSLP role in activating of DCs was also shown in the periphery, where TSLP was able to induce to some extent the peripheral DC activation. The TSLP also served as a potent survival factor for the DCs. On the other hand, the DCs were shown to be potent T-cell stimulators that nudges immune responses towards Th2, thus suggesting that this activation might be rather immunogenic than homeostatic (Ebner et al. 2007). The importance of TSLP for DC activation is still matter of debate, because in mice deficient for TSLPR the amounts of homeostatically activated DCs were the same as in WT mice (Oh et al. 2018).

Despite the fact, that aDC2 subset is poorly understood, it was found that type 2 cytokines are essential for their activation. Absence of IL-4 in the thymus caused decrease of aDC2s by about 50% and the same results were observed when IL-13 was absent. If both IL-4 and IL-13 are missing, the aDC2 subset is completely missing from the thymus and same results are obtained in IL-4Rα (receptor for IL-4 and IL-13) knockouts (Breed et al., 2022). It was previously mentioned (*section C.1.3.4*) that the type 2 cytokines are in steady-state thymus produced by iNKT2 cells that are under control of tuft cell mTECs by IL-25 (Lucas et al., 2020; Miller et al., 2018) and beside the cDC2 activation, the cytokines play also a role in thymic regeneration via CCL11-CCR3-dependent recruitment of eosinophils (Cosway et al., 2022; H. Wang et al., 2019). The role of this axis was further confirmed by specific depletion of thymic iNKT cells which caused significant reduction of aDC2 population (Breed et al., 2022).

Furthermore, it has been shown that the expression pattern of aDCs includes a lot of IFN-I activated genes (Ardouin et al., 2016). And since the type I interferons (IFN-I) are produced in steady-state thymus by thymic epithelial cells (Lienenklaus et al., 2009), it suggests that the IFN-I might play a role in promotion of homeostatic activation of thymic DCs. Nevertheless, in the mice lacking IFN-I receptors, there was no significant change in the levels of DCs activation at least in cDC1 subset. Expression of only two genes was impacted by the lack of IFN-I signalling, thus suggesting that the IFN-I rather potentiate the activation than promote it (Ardouin et al., 2016).

It is also good to mention, that some studies suggest, that the homeostatic maturation of DCs can be also facilitated by the loss of a signal(s) that are required for maintaining their immature state, for example the TGFβ was shown to keep DCs in the non-activated state, nevertheless these data were acquired in epidermal Langerhans cells (Kel et al., 2010).

D.1.2. Functional properties of thymic activated dendritic cells

Regarding functions of thymic DCs, not many studies took into account the presence of aDCs in the thymus, since they were discovered in 2016 and were often taken as a part of cDCs, thus some characteristics, that are unique to aDCs, might be attributed to the cDC1 and cDC2 (Ardouin et al., 2016). The aDCs express many markers, that have been previously discussed, such as the MHCII, CD80 and CD86 serving for antigen presentation and costimulation (Watanabe et al., 2005). CD40 promotes the activation of cDCs and further promotes the Treg selection by activation of expression of CD70 (which is also a marker of aDCs), COX-2 and Bcl2l (Coquet et al., 2013; Park et al., 2020; Wu et al., 2022). Aside from that, the aDCs also express many other molecules that predestine them to be a key subset in facilitating the central tolerance (Park et al., 2020). They express for example CCL17 and CCL22, which are recognized by CCR4 on CD4 thymocytes, which results in attraction of the thymocytes and Tregs to the proximity of aDCs to undergo selection. They also express PD-L1 and PD-L2 which can participate during deletion of self-reactive thymocytes (Oh et al., 2018). The aDCs were also detected to express AIRE, which might suggest that they can produce TRAs themselves, but the function of AIRE in aDCs remains controversial (Park et al., 2020). There is a possibility that AIRE mRNA might be transferred from mTECs as a by-product of CAT, because mRNA encoding AIRE-independent TRA Casein beta was also detected in aDCs (Ardouin et al., 2016; Park et al., 2020).

The crucial part of the thymic cDCs function is undoubtedly the selection of thymocytes, illustrated by the findings, that the deletion of cDCs results in impaired T-cell selection (especially in negative selection) and results in autoimmunity (Ohnmacht et al., 2009). By the same token, the selection of Tregs is highly dependent on DCs and cannot be mediated only by mTECs (Leventhal et al., 2016; Perry et al., 2018, 2014). Both the cDC1 and cDC2 populations undergo intrathymic homeostatic activation, thus their populations consist of both activated and non-activated cells (Oh et al., 2018). But it has been shown that both activated and non-activated DCs can acquire and process antigens and facilitate, to some extent, activation of T-cells in vitro (Veeraswamy et al., 2003). Similar results were also obtained using OVA antigen and OT-II $CD4^+$ T-cells (Oh et al., 2018). On the other hand, when the CCR7, CD80 and CD86 molecules are deleted from the DCs, the thymic Treg generation is significantly impaired (Leventhal et al., 2016). This observation is supported by finding, that DCs are able to instruct self-reactive thymocytes to differentiate into $Foxp3^+$ Tregs via antigen presentation and CD80, CD86 costimulation of CD28 on thymocytes (Watanabe et al., 2020). Given that high expression of these molecules is present in aDCs (Ardouin et al., 2016), it strongly suggest that aDCs play a critical role in Treg selection. Furthermore, the aDCs were proven to be the most positive cells for TRAs acquired from mTECs via the CAT and since TRAs are needed for thymocyte selection, it provides further support for the key role of aDCs in this process (Vobořil et al., 2022). Finally, aDCs were shown to be better in stimulation of T-cells because non-activated DCs were able to stimulate T-cells only in short period of time after antigen encounter for having a “weak memory”. This was caused by the fact, that non-activated DCs produce MHCII molecules, but their turnover is fast and thus cannot be stimulative for a long time. On the other hand, the aDCs stop the turnover and accumulate MHCII on their surface to potentiate their antigen-presenting capacity (Cella et al., 1997). This effect is achieved by high level of ubiquitination of MHCII molecules in non-activated DCs which results in their endocytosis and low numbers on the cellular membrane and accumulation inside late endosomes and lysosomes. When the DCs are activated, they stop ubiquitination of their MHCII, which results in accumulation of pMHCII complexes on their surface (Shin et al., 2006). These results indicate, that aDCs are the primary cDC subset, responsible for Treg generation and negative selection of thymocytes, although they can by to some extent substituted by their non-activated counterparts. Nevertheless, further experimental evidence will be required to confirm these indications.

Thymic cDC1s are believed to originate in the thymus and being resident in the medulla (Lei et al., 2011; Spidale et al., 2014), which implicates, that their function will probably not be connected with the peripheral antigens as it is in the case of cDC2s and that they are more biased towards selection via TRAs. The specific

functional role of aDC1s was assessed by *Ardouin et al.* by showing, that cDC1s is able to create a long protrusions on their cellular membrane, suggesting enhanced antigen-presenting capacity. They also showed, that only the activated $XCR1^+$ aDC1s were able to cross-present (on MHCI) surface OVA antigen that was produced by mTECs. This also shows that given their cross-presenting activity, the aDC1s might be important in tolerizing $CD8^+$ thymocytes via TRAs. Furthermore, they claimed, that aDCs in thymus are important in antigen presentation. Only $XCR1^+$ $CCR7^+$ aDC1 and not $CCR7^-$ non-activated cDC1 cells were able to acquire and present a selected intracellular peptide, that was produced only in mTECs (in this model resembling TRA peptide) (Ardouin et al., 2016). Nevertheless, these results can be questioned since most of the DCs are able to acquire TRAs from mTECs, although the aDC1s were shown to be the most potent cells in CAT (Vobořil et al., 2022). Study from *Leventhal et al.* used prostate-specific MJ23 thymocytes that in the WT develop into prostate antigen-specific Tregs, to assess the roles of cDC1 and cDC2 subsets on Treg selection. Because both cDC1s and cDC2s were able to induce Treg generation in vitro, it has been suggested, that thymic DC subsets have a redundant role in Treg selection. Furthermore, Batf3 deficiency did not have any effect on the Treg generation, thus implying, that that cDC1s do not play an indispensable role in the Treg selection. Also, thymic pDCs were found dispensable for Treg selection in mice (Leventhal et al., 2016).

Unlike cDC1s, the cDC2s originate in the periphery and are able to acquire peripheral antigens from the microvessels or from the peripheral tissues and facilitate their transport into the thymus (Baba et al., 2009; Lei et al., 2011; Spidale et al., 2014; Zegarra-Ruiz et al., 2021). They migrate to the thymus via P-selectin, VLA-4 - VCAM-1 axis and upon the migration, they upregulate MHCII and CD86 and produce long protrusions of their cytoplasmic membrane, suggesting their enhanced antigen-presenting capacity (Bonasio et al., 2006). They acquire peripheral and blood-borne antigens and present them inside the thymus (Baba et al., 2009; Vollmann et al., 2021). Importantly, immunologically activated cDC2s (after exposure to LPS) were unable to enter thymus. This mechanism is probably protecting the host from establishing tolerance to pathogens (Bonasio et al., 2006). However, the cDC2s are also able to acquire TRAs from mTECs via the CAT (Vobořil et al., 2022). The function of aDC2s in general is even less explored than aDC1s, due to current lack of apropriate models targeting cDC2 lineage specifically (Breed et al., 2022). But it has been shown, that in the thymus, there exists a population of cDC2s that is $CD301b^+$ which is considered to be the activated state of cDC2s. Around 30-60% of thymic cDC2s are in this state. This subset resides mainly in medulla and is not migratory and stays in the thymus. The $CD301b^+$ population mediates central tolerance by clonal deletion. Using transgenic mice where GFP was expressed only in $CD301b^+$ cDC2 population, *Breed et al.* showed that in this system GFP-specific CD4 T-cells were efficiently deleted and there was no significant impact on Treg generation. Furthermore, when the $CD301b^+$ population was deleted from the thymus by DTR-diphtheria toxin treatment, the deletion of thymocytes in medulla was significantly lower and Treg generation was not affected. Suggesting that $CD301b^+$ population has a nonredundant role in thymocyte deletion in thymic medulla (Breed et al., 2022). This conclusion was also confirmed by the finding that the circulating cDC2 present antigens which they capture in the periphery or from the blood stream and facilitate mainly clonal deletion of thymocytes (Bonasio et al., 2006).

Thus, even though the roles of thymic aDC subsets can be overlapping, it can be concluded that aDC2s are supposed to be responsible primarily for negative selection of cognate thymocytes towards antigens from periphery and from blood, given the antigens were obtained before their homeostatic activation. The primary function of aDC1s is, most likely, Treg generation, given the Treg generation is clearly function of cDCs in the thymus (Leventhal et al., 2016; Watanabe et al., 2005) and that it is not facilitated by aDC2s.

D.1.3. Summary of homeostatic activation of thymic cDCs

Taken together, activation of DCs can be facilitated by two distinct mechanisms, immunogenic and homeostatic and although they share many features, they are not the same. The inductors of homeostatic activation in the thymus and in other tissues remain to be exactly defined, but they are connected with cellular signalling (CD40) or cytokine signaling (IL-4) or can be facilitated by phagocytosis of apoptotic cells. The aDCs inside thymus mediate thymocyte selection. Although their exact roles are uncertain, the evidence we currently have suggests that aDC1s have a dominant role in CAT and Treg selection and that aDC2s primarily facilitate negative selection of cognate thymocytes towards antigens that were collected from the bloodstream

and from the periphery. However, since there are also studies that indicate opposite functions, the commonalities as well as differences between the functions of aDC1s and aDC2s needs further refinement and further studies. In conclusion, it seems that cDC1s and cDC2s and their respective activated forms, have redundant and non-redundant roles, and that one subset could be a better performer of some physiological function(s) than the other one. However, it does not mean that they cannot compensate each other and thus the attempt to attribute a very exact or "primary" function(s) to each subset might not be an ideal approach. The activation of thymic cDCs is also depicted and summarised in *Figure 5*.

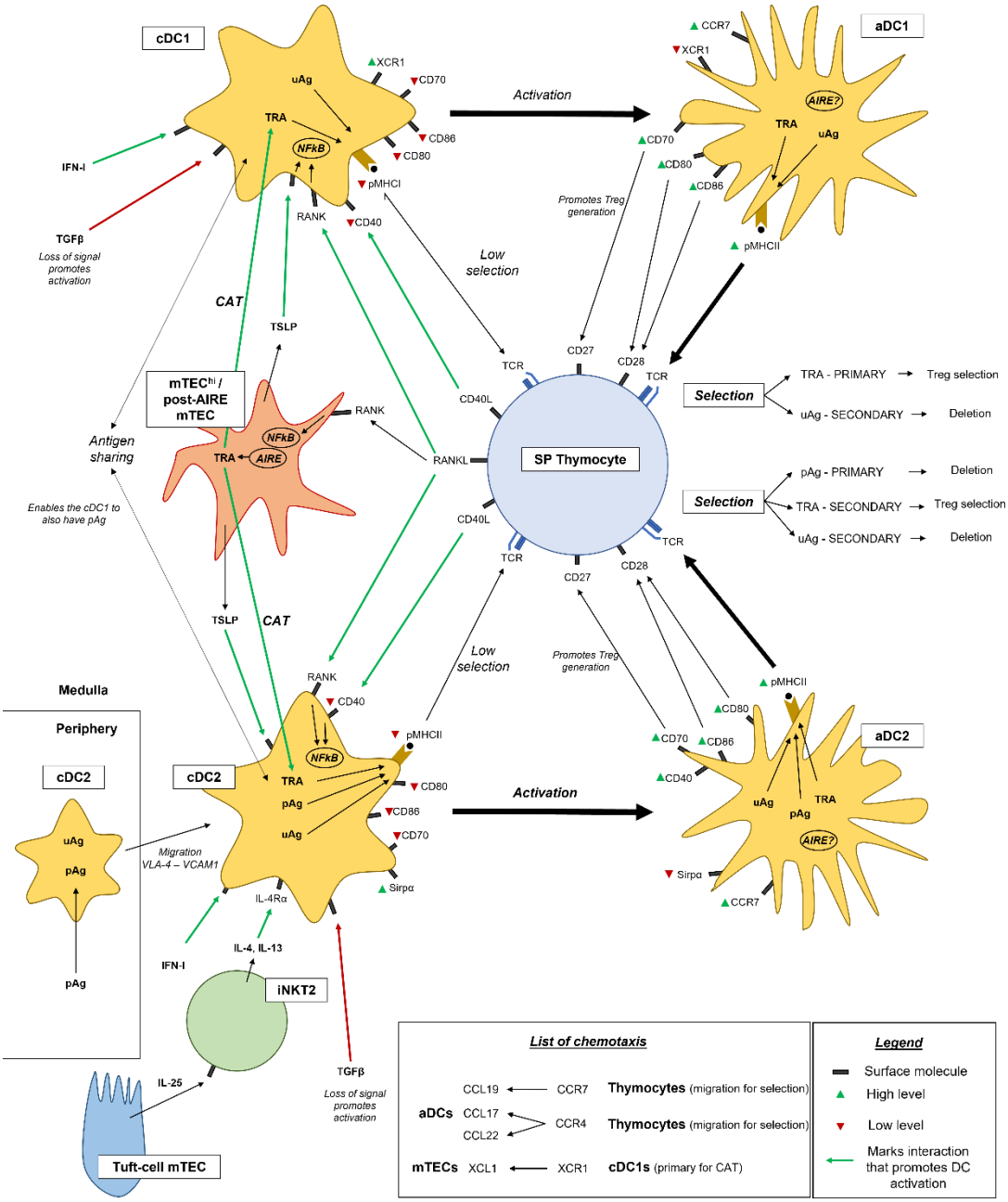

Figure 5: Homeostatic activation of thymic dendritic cells

The figure summarises the contemporary model of homeostatic activation of thymic DCs in the context of the heterogeneity of thymic cells and the complex crosstalk between these cells, especially thymocytes, mTECs and other DCs. Factors, that promote activation of the DCs are depicted here in the context of antigen presentation and subsequent thymocyte selection, that is promoted by the aDC1s and aDC2s.

E. Summary and discussion

In the last decade, a multitude of novel discoveries brought to us many insights into the cellular functions and molecular mechanisms that drive the function of the thymus in the context of establishment of central tolerance. The invention of scRNA-seq approach caused a major breakthrough in this rapidly developing field of research. It enabled us to assess and reveal an unexpectedly broad heterogeneity of the thymic cell subsets, to discover a set of new markers for identification of these subsets, to trace their developmental trajectories and to propose their functional properties, interdependence and coordinated mode of actions. In the light of their heterogeneity, the features our model of the thymic antigen-presenting machinery which underpins the establishment of central tolerance has become much more accurate and complex. The downside of this progress, however, can be, that a large amount of information which is produced by the scRNA-seq can cause confusion, since discrepancies between various studies in respect of newly identified markers of distinct cell subsets as well as the nomenclature of the novel cell subsets have not been fully settled.

For example, in case of DCs, a novel nomenclature was recently proposed by *Ginhoux et al.* They pointed out the importance of distinguishing between cell types and cell states, thus the aDC1 and aDC2 are activated cell states of cell types cDC1 and cDC2. Aside from the cDC1 and cDC2, defined by expression of XCR1 and Sirpα respectively, the authors also defined the DC3 cell type, which can be considered as a part of cDC2, but is definitely a different cell type, since it ontogenetically arises from a different pathway (Ginhoux et al., 2022). According to the surface and gene expression markers, the DC3 subset is the closest to the human and mouse moDCs, since they are also Sirpα$^+$ CD14$^+$ and undergo different development than cDC2. I therefore assume, that it is very likely that the moDCs and DC3 are the same cell subset (Ginhoux et al., 2022; Villar and Segura, 2020; Vobořil et al., 2020). They also proposed that a recently identified mregDC subset which can be found in cancers (Maier et al., 2020), as a cell state of cDCs. The mregDC cell state arises upon uptake of cell-associated antigens, toll-like receptor or cytokine stimulation and is characterized by downregulation of cDC1 and cDC2 markers while upregulating CD80, CD86, CD40, CD83, CD70, CCR7 and MHCII. The mregDCs are migratory and high in expression of many immunoregulatory genes such as CCL5, CCL22, PD-L1 or FAS (Ginhoux et al., 2022; Maier et al., 2020). With these characteristics, the mregDCs are highly similar to the homeostatically activated aDCs (Ardouin et al., 2016; Park et al., 2020) and are probably the same cells although they can have some transcriptional or surface differences, which can be attributed to slightly different environment that affects their maturation (thymus versus tumour). Thus, according to the novel nomenclature, the moDCs and aDCs should be referred to as DC3 and mregDCs, respectively (Ginhoux et al., 2022). However, since the novel nomenclature has not yet been officially revised, this thesis prefers the use the established "older" nomenclature. It is expected that the nomenclature of DC cell subsets will be reviewed and classification improved in a very near future.

The single cell RNA sequencing analysis (scRNA-seq) also enabled us to better determine the expression pattern of already established as well as new markers among all identified cell subsets. It is currently well-established fact, that the mTECs are highly controlled by NFkB signalling pathway and that the primary exogenous cellular drivers of their development are thymocytes (*section C.1.3*). Interestingly, according to the scRNA-seq and other data, the thymic aDCs also express significant levels of the receptors and factors involved in NFkB signalling, such as CD40, RANK or Relb (Ardouin et al., 2016; Oh et al., 2018; Park et al., 2020; Vobořil et al., 2020). Although this has not yet been studied, it suggests that the thymic DCs and their activation status might be regulated by the NFkB signalling in the similar way as the mTECs, and thus it is quite possible that they are also regulated by the thymocytes. Therefore, both the mTECs and DCs, which are crucially important for the selection of the thymocytes would by fully established only if there are the thymocytes that need to be selected. The study performed by *Oh et al.* also strongly supports this possibility (Oh et al., 2018). These mechanisms would also explain the involution of thymus which occurs during ageing due to lowered production of new T-cells and also the autoimmune manifestations that accompanies the BM transplantations (Alawam et al., 2021; Baran-Gale et al., 2020). The scRNA-seq analysis also led to a recent discovery of mimetic cells in the thymus, that, same as the AIRE$^+$ mTECs, express TRAs, but in a manner that resembles the expression profile of *bona-fide* peripheral cells (Michelson et al., 2022). It is important to note, that this pattern of expression contradicts the previously well-established models of ordered stochasticity of TRA expression facilitated by AIRE (Derbinski et al., 2008; Meredith et al., 2015). On the

other hand, it demonstrates that establishment of central tolerance can rely on several independent but complementary mechanisms which are operational in the thymus. Specifically, the semi-stochastic expression occurs predominantly in the mTECs^{hi} (*section C.1.3.2*), whereas the mimetic cells are part of the post-AIRE population, and the logical expression of TRAs is facilitated by both AIRE and tissue-specific transcription factors (*section C.1.3.5*). Thus, these two distinct mechanisms are not counterplaying each other, rather they together widen the scope of central tolerance by presenting TRAs in semi-random manner in AIRE^{+} mTECs and also in manner which resembles real peripheral cells in mimetic cells.

The other important topic which was thoroughly discussed in this thesis is the homeostatic activation of the thymic cDCs (*section D*). Despite the fact that this mechanism was frequently overlooked, the accumulating evidence of their importance is starting to bring more focus into the study of aDCs. Furthermore, discovery of the homeostatic activation showed, that many functions of DCs can be attributed to their specific activation state, which parallels a well-known immunological activation (Cabeza-Cabrerizo et al., 2021). Therefore, it is important to distinguish between these two forms of activation. Nevertheless, both the homeostatic and immunogenic activation have a lot in common, which suggests, that they are rather than completely distinct mechanisms, two branches of one general capacity of DCs to get activated. One is specialised to fight infection, the other to establish tolerogenic functions of DCs. The main distinguishing factor is the environment, which promotes the activation, because the DCs are potent in complex interpretation of the signals from their surroundings (Ardouin et al., 2016). It is also important to mention that most of the studies of the thymic homeostatic activation were mainly focused on the cDC1 subset, because there is currently lack of proper models for the study of cDC2s. Nevertheless, a novel study from *Breed et al.* brought a new insight into the aDC2s by examining the CD301b^{+} subset, which they claimed to be most likely the activated subset of aDC2s (Breed et al., 2022). But there is a struggle to precisely define the CD301b^{+} cDC2s, since the Mgl2-DTR mouse model used in this study only imperfectly targets cDC2 lineage (CD301b^{+} is encoded by Mgl2 gene). First, the CD301b molecule could be just a marker of activated cDC2, such as CCR7. However, it is unlikely since the CD301b marker is present on a portion of both phenotypically activated and non-activated cDC2 cells. Nevertheless, CD301b^{+} cells express several markers of DC activation such as molecules involved in antigen processing and presentation, for example MHCII. However, and importantly, a typical activation marker CCR7 is missing from most of the CD301b^{+} subset. The CCR7 is present only in part of CD301b^{+} cells and the cells that have high amounts of CCR7 (and thus are considered activated) have lowered amount of CD301b compared to other cells from CD301b subset. Nevertheless, *Breed et al.* concludes, that the CD301b^{+} is not a subpopulation but, in the context of the nomenclature published by *Ginhoux et al.* (see above), it represents the activated cell-state of cDC2 (Breed et al., 2022; Ginhoux et al., 2022).

I suggest that this confusing position of CD301b^{+} subset could be explained if we consider the CD301b not as the marker of cDC2 activation state, but rather a marker of a transition from non-activated to activated state. In this hypothetical model, when cDC2 receives the signal for activation, expression of CD301b is induced, continues to increase during the activation process, and then gradually decreases when DC is fully maturated, as shown in *Figure 6*.

Furthermore, if we model the CD301b^{+} as a molecule, that is crucial for the transition from non-activated to activated state, then I predict that the DT treatment, which was used by *Breed et al.* to deplete CD301b^{+} cells from the thymus, would result not just in the deletion CD301b^{+} subset, but in the elimination or severe diminishment of entire aDC2 subset which descends from CD301b^{+} subset. Therefore, the results obtained by *Breed et al.* can be applied not only to CD301b^{+} but to all aDC2 cells suggesting that aDC2s have the key role in deleting thymocytes in thymic medulla (Breed et al., 2022).

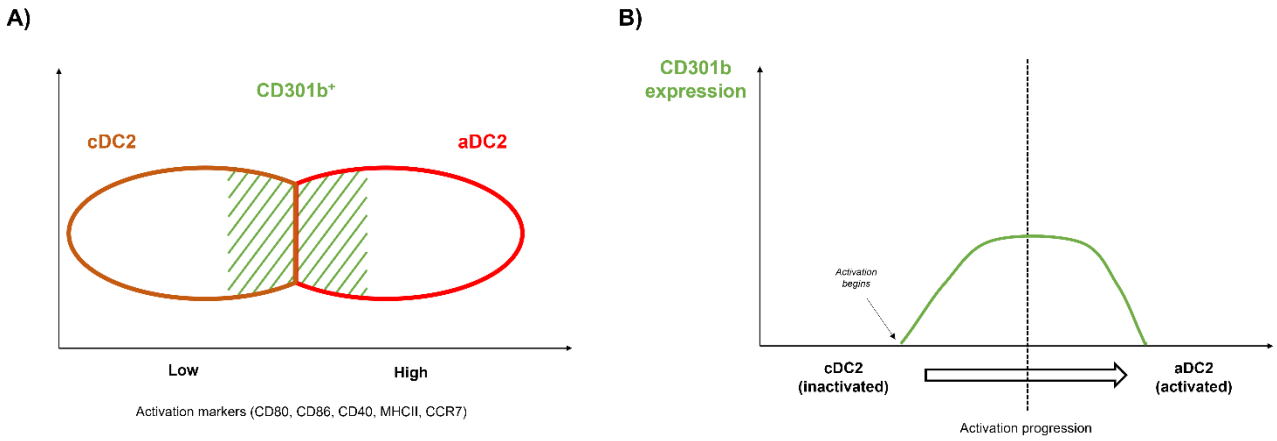

Figure 6: The hypothetical model of CD301b as a marker of transition from non-activated to activated state of cDC2

A) Possible kinetics of appearance of CD301b⁺ subset among cDC2 population during the activation.
B) Possible CD301b⁺ expression during the transition of cDC2 cell from non-activated to activated state.

In conclusion, the near future will certainly increase our knowledge in respect of the regulation and molecular mechanisms in understanding of the importance of the homeostatic activation, since the homeostatically activated DCs are not present only in the thymus, but also in many other tissues (Ardouin et al., 2016). Thanks to the scRNA-seq methods, the understanding of the cellular heterogeneity of the thymus becomes possible along with deeper comprehension of the complexity of whole thymic antigen-presenting machinery. Another possible approach apart from focusing on a general mechanism of central tolerance is to study how exactly the tolerance is established in the case of distinct antigens. This would enable us to construct the representation of central tolerance by combining specific pathways of selection for each antigen. Deeper understanding of processes which underlie the central tolerance will ultimately unable us to design new therapeutical approaches to manipulate the whole immune system towards the desired outputs. Such approach might provide the way for balancing between self-defence and self-destruction by enhancing the tolerance to antigens that should be tolerated and thus to prevent autoimmunity-related diseases, and on the other side to block the tolerance to antigens that should not be tolerated, mainly the cancer-related antigens to promote immune elimination of malignant tissues. The basic research in the field of the thymic function requires a tremendous effort and investment, which will begin to pay off in the future. In this sense, it is realistic to expect that new treatments will allow us to manipulate our immune system on the level which has never been seen before.

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