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Funkce CX3CR1^{+} migratorních dendritických buněk v mechanismech centrální tolerance

CX3CR1^{+} migratory dendritic cells in the mechanisms of central tolerance

DIPLOMOVÁ PRÁCE

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V Praze, 11.8.2019 Jiří Březina

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Abstract:

Display of thousands of self-antigens in the thymus is fundamental for the establishment of central tolerance as its failure can lead to the development of autoimmunity. Medullary thymic epithelial cells (mTECs) and thymic dendritic cells (DCs) constitute essential populations of antigen presenting cells (APCs) which present these self-antigens to developing T cells. While mTECs produce and present antigens in self-autonomous manner, DCs can hijack mTEC-derived antigens by the process of cooperative antigen transfer (CAT). It is well found that CAT is essential for working central tolerance, however, the overall heterogeneity of thymic APCs participating in CAT remains unclear. Using transgenic mouse models and multicolor flow cytometry analysis, we determined that APCs involved in CAT are exclusively of $CD11c^+$ phenotype. Within these cells, we identified previously unrecognized $CX3CR1^+$ subset of migratory DCs (mDCs) exhibiting monocyte/macrophage markers. These $CX3CR1^+$ mDCs are more efficient in CAT than their $CX3CR1^-$ counterparts and reveal robust antigen presenting properties with the capability to present CAT-acquired antigen. Genetic ablation of $CX3CR1^+$ mDCs resulted in increased cellularity of $CD8^+$ and $CD4^+$ thymocytes, indicating importance of this mDC subset for negative selection of self-reactive T cell clones. In addition, for the very first time, we visualized CAT *in vitro* by using fluorescence microscopy. While further work is required to formally prove the role of $CX3CR1^+$ mDCs in thymic T cell selection processes, our work, in a broad sense, provides a comprehensive analysis of the contribution of distinct subsets of thymic cells to CAT and shows an experimental platform for the assessment of functional relevance of various smaller DC subsets in establishment of central immune tolerance.

Key words:

central tolerance, mTEC, dendritic cell, cooperative antigen transfer, CX3CR1, transgenic mouse

Abstrakt:

Produkce mnoha tělu vlastních antigenů v thymu je zcela nezbytná pro ustanovení centrální tolerance, neboť její selhání může vést k rozvoji autoimunity. Tyto antigeny jsou prezentovány vyvíjejícím se T lymfocytům medulárními epiteliálními buňkami (mTECs) a dendritickými buňkami (DCs), které tvoří hlavní populace antigen-prezentujících buněk (APCs) v thymu. Zatímco mTECs prezentují antigeny, které samy produkují, DCs získávají tyto antigeny od mTECs procesem kooperativního antigenního transferu (CAT). Přestože zásadní funkce CAT v mechanismech centrální tolerance je známá, celková heterogenita APC buněk v thymu účastnících se tohoto procesu je nejasná. Díky využití transgenních myších modelů a pokročilé průtokové cytometrie jsme objevili, že APCs, které získávají antigeny od mTECs, jsou striktně CD11c pozitivní. V rámci populace DCs v thymu jsme identifikovali dříve nerozpoznanou $CX3CR1^{+}$ subpopulaci migratorních DCs (mDCs), která se vyznačuje expresí molekulárních markerů monocytů a makrofágů. Tyto $CX3CR1^{+}$ mDCs jsou více efektivní v získávání antigenů procesem CAT než $CX3CR1^{-}$ populace a díky velice dobrým antigen-prezentujícím schopnostem dokáží tyto antigeny prezentovat T lymfocytům. Genetické odstranění $CX3CR1^{+}$ mDCs vedlo ke zvýšení počtu $CD8^{+}$ a $CD4^{+}$ thymocytů, což naznačuje, že tyto buňky hrají roli v negativní selekci auto-reaktivních klonů T lymfocytů. Dále se nám jako prvním, s využitím fluorescenční mikroskopie, podařilo vizualizovat CAT *in vitro*. Přestože pro objasnění role $CX3CR1^{+}$ mDCs v selekčních procesech T lymfocytů je třeba dalších experimentů, naše práce přináší obsáhlou analýzu role různých populací buněk thymu v rámci CAT a poukazuje na možnou experimentální cestu pro určení funkčního významu menších subpopulací thymických DCs v ustanovení centrální tolerance.

Klíčová slova:

centrální tolerance, mTEC, dendritická buňka, antigenní transfer, CX3CR1, transgenní myš

Abbreviations:

Aire	Autoimmune regulator
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APC(s)	Antigen presenting cell(s)
BM	Bone marrow
BM APC(s)	BM-derived APC(s)
CAT	Cooperative antigen transfer
CBP	Creb-binding protein
cDC	Conventional dendritic cell
CDP	Common DC progenitor
CLP	Common lymphoid progenitor
CMJ	Cortico-medullary junction
cMoP	Common monocyte progenitor
cTEC(s)	Cortical TEC(s)
DC(s)	Dendritic cell(s)
DN	Double negative
DNA-PK	DNA protein kinase
DP	Double positive
DTA	Diphtheria toxin
DTR	Diphtheria toxin receptor
FACS	Fluorescent Activated Cell Sorting
FELASA	Federation of European Laboratory Animal Science Associations
FLT3L	FMS-like tyrosine kinase 3 ligand
FMO	Fluorescence minus one
Foxn1	Forkhead box protein N1
H3K4me0	Unmethylated histone H3 lysine 4
H3K4me3	Trimethylated histone H3 lysine 4
H3K27me3	Trimethylated histone H3 lysine 27
HA	Influenza hemagglutinin
HEL	Hen egg lysozyme
HSC	Hematopoietic stem cell
ILC(s)	Innate lymphoid cell(s)
IMG	Institute of Molecular Genetics of the ASCR, v.v.i.
KI	Knock in
KO	Knock out
MARS	Massively-parallel scRNA-seq

MC(s)	Monocyte-derived cell(s)
M-CSF	Macrophage-colony stimulating factor
mDC(s)	Migratory dendritic cell(s)
MDP	Monocyte/macrophage-DC progenitor
MHC	Major histocompatibility complex
mOVA	Membrane-bound OVA
mTEC(s)	Medullary TEC(s)
NHEJ	Non-homologous end joining
NOD	Non-obese diabetic (mouse)
OT-I	OVA-restricted $CD8^+$ T cells
OT-II	OVA-restricted $CD4^+$ T cells
OVA	Ovalbumin
pDC(s)	Plasmacytoid dendritic cell(s)
PGE	Promiscuous gene expression
PI3K	Phosphoinositide 3-kinase
pMHC(s)	MHC-peptide complex(es)
RIP	Rat insulin promotor
RTE	Recent thymic emigrant
S1P	Sphingosine-1-phosphate
S1P1	Sphingosine-1-phosphate receptor 1
scRNA-seq	Single-cell RNA sequencing
Sirt1	Sirtuin 1
sOVA	Secreted OVA
SP	Single positive
SPF	Specific-pathogen-free
TCR	T cell receptor
tDC(s)	Thymic-derived DC(s)
TEC(s)	Thymic epithelial cell(s)
TF	Transcription factor
Thymocytes	Developing T cells
TLR	Toll-like receptor
TRA(s)	Tissue-restricted antigen(s)
Treg(s)	T regulatory cell(s)
TSP	Thymic seeding progenitor
tTreg(s)	Thymic-derived Treg(s)
T1D	Diabetes mellitus type 1
WT	Wild type

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A. INTRODUCTION

T cells represent essential cell type of immune system which is evolutionary adapted to deal with a high mutational rate of pathogens. Such capability is endowed by the process of VDJ recombination which guides the generation of T cell receptors (TCRs) and on the level of an individual organism establishes a vast T cell repertoire. However, as the VDJ recombination process occurs stochastically, T cell repertoire, to a large extent, also comprises cell clones expressing TCRs specific to self. Since the occurrence of self-reactive T cells in the body constitutes a potential threat for autoimmune manifestations, the development of T cells in the thymus must be accompanied by several mechanisms which severely limit self-reactive T cell repertoire, collectively referred to as **central tolerance**. These mechanisms are based on the presentation of a collection of self-antigens from various tissues to developing T cells and subsequent negative selection or deviation to T regulatory cells (Tregs) of those which engage such antigens. Thymus accommodates various antigen-presenting cell (APC) subsets, including functionally unique medullary thymic epithelial cells (mTECs) and several distinct subtypes of thymic dendritic cells (DCs) which underpin the above-mentioned mechanisms of central tolerance. While the former, are capable to produce and directly display self-antigens to developing T cells, the latter can present them indirectly, i.e. after their acquisition either from mTECs in the thymus or elsewhere in the body. Importantly, both modes of antigen presentation are fundamental for establishment of tolerance, since the absence of mTECs as well as DCs results in the manifestation of severe autoimmunity in mice (Akiyama *et al.*, 2008; Ohnmacht *et al.*, 2009).

The contribution of mTECs and DCs to central tolerance is functionally coupled by their participation in **cooperative antigen transfer (CAT)**. Basically, during such process, self-antigens generated by mTECs are transferred over to thymic DCs which further process and present them (indirect presentation). There is accumulated evidence that CAT is essential for establishment of central tolerance (Perry and Hsieh, 2016). However, still largely unexplored field is the investigation of other, less defined cells, on top of thymic DCs, B cells or macrophages, whose contribution to central tolerance was already described. Potentially, so far unrecognized or unappreciated subset of antigen presenting cells might be also engaged in CAT. This seems to be a hunting task as the composition of thymus homing cells is incompletely understood and the list of such cell subsets is ever expanding, especially due to the exploration of state-of-the-art method, single-cell RNA sequencing (scRNA-seq) (Bornstein *et al.*, 2018; Kernfeld *et al.*, 2018). The identification of novel cell subsets involved in CAT and their potential function forms the major objective of this thesis.

B. CURRENT STATE OF KNOWLEDGE

1. Cell composition of the thymus

Thymus is a specialized primary lymphoid organ of jawed vertebrates where the maturation and the development of T cells occur. In mice and human, the thymus is morphologically composed of two lobes

and consist of three functionally and histologically distinct areas: the *cortex* (the outer part), *medulla* (the inner part) and a largely vascularized interface called the *cortico-medullary junction* (CMJ) (Rodewald, 2008). In general, the thymus accommodates various cell types of stromal ($CD45^-$) and hematopoietic ($CD45^+$) origin. The hematopoietic fraction constitutes mostly of αβ T cells which are present as the continuum of their various developing stages (thymocytes), as well as invariant T cells and APCs, the latter including thymic DCs, B cells and macrophages. On the other hand, the thymic stromal compartment consists of fibroblasts, endothelial cells and thymic epithelial cells (TECs) (Rodewald, 2008; Kernfeld *et al.*, 2018). Importantly, although all stromal cell subsets, due to their production of various molecules, are important for thymocyte maturation, only TECs act as unique and functionally highly specialized APCs which directly, or in collaboration with other hematopoietic APCs, drive the development of functional and self-tolerant repertoire of αβ T cells (further referred to as T cells) (Anderson *et al.*, 1997; Klein *et al.*, 2014; Shi *et al.*, 2016).

2. *Thymic epithelial cells*

2.1. Foxn1: master regulator of thymic epithelial cells

The development of stromal thymic compartment displays several distinct features. Whereas fibroblasts and endothelial cells originate from neural crest mesenchyme (Rodewald, 2008), TECs in the murine thymus are derived from the third pharyngeal pouch, which is of endodermal origin (Gordon *et al.*, 2004). The development of TECs is completely dependent on autonomous expression of transcription factor (TF) Foxn1 (Forkhead box protein N1), whose spontaneous loss-of-function mutations lead to a "nude mice" phenotype (Blackburn *et al.*, 1996). These mice are characterized by hair-loss, undeveloped and dysfunctional thymus and nearly complete absence of T cells. The human Foxn1 mutation leads to the development of rare Guarino-Pignata syndrome whose manifestations include congenital alopecia, nail dystrophy and severe combined immunodeficiency (Gallo *et al.*, 2017).

The accumulated evidence demonstrated that Foxn1 is indispensable in TECs ontogenesis (Vaidya, Briones Leon and Blackburn, 2016) as it functions as the master-regulator of their development, survival and physiology. Specifically, Foxn1 is constantly and continually expressed in TECs and, as shown in the experiments with artificially decreased Foxn1 expression in the postnatal thymus, it serves as their pro-survival factor and the regulator of thymic involution (Chen, Xiao and Manley, 2009). Second, it directly influences the ability of TECs to guide thymocytes development since it controls the expression of essential molecules for this process such as notch ligand DLL4, chemokines CCL25 or CXCL12, β5t subunit of thymoproteasome or thymus-specific serine protease, whose function is explained in the following chapters (Calderón and Boehm, 2012; Žuklys *et al.*, 2016). Finally, Foxn1 in the thymus is exclusively expressed by TECs and their progenitors and therefore can be utilized as an experimental TEC-specific driver by using the Cre-lox systems (Gordon *et al.*, 2007).

2.2. Subpopulations of thymic epithelial cells

TECs could be sub-divided according to the thymic microenvironment where they localize: the cortical (cTECs) and medullary TECs (mTECs). Even though cTECs and mTECs arise from the same bipotent TEC progenitors, their functions are distinct (Rossi *et al.*, 2006). In general, cTECs guide the early thymocytes development and mediate positive selection of those thymocytes which productively engage the major histocompatibility complex (MHC) molecules (Germain, 2002) expressed on cTECs. The function of cTECs will be further discussed in a later chapter. In contrast, mTECs, by presenting self-peptides in the context of their MHC molecules (pMHC), interact with more developed thymocytes and negatively select those which strongly recognize these pMHC complexes, thus providing the cellular platform for establishment of central tolerance (Liston *et al.*, 2003). Remarkable and quite unique ability of mTECs is the production and presentation of tissue-restricted antigens (TRAs) which, outside of the thymus, are expressed only by one or few other peripheral tissues (Klein *et al.*, 2014). Such "promiscuous gene expression" (PGE) of many, but not all TRAs, is driven by the Autoimmune regulator (Aire) (Anderson *et al.*, 2002).

Recently, a novel method called massively-parallel scRNA-seq (MARS) has been developed, which enables to study the gene expression profile in thousands individual cells from a specific tissue by virtue of their single-cell flow cytometry sorting into 384 well plate (Jaitin *et al.*, 2014). By using MARS, the heterogeneity of TECs was recently assessed and its results clarified that TECs are composed of at least five different sub-populations from which only one equals to cTECs and the rest corresponds to different subsets of mTECs (I-IV) (Bornstein *et al.*, 2018).

2.2.1. Heterogeneity of medullary thymic epithelial cells

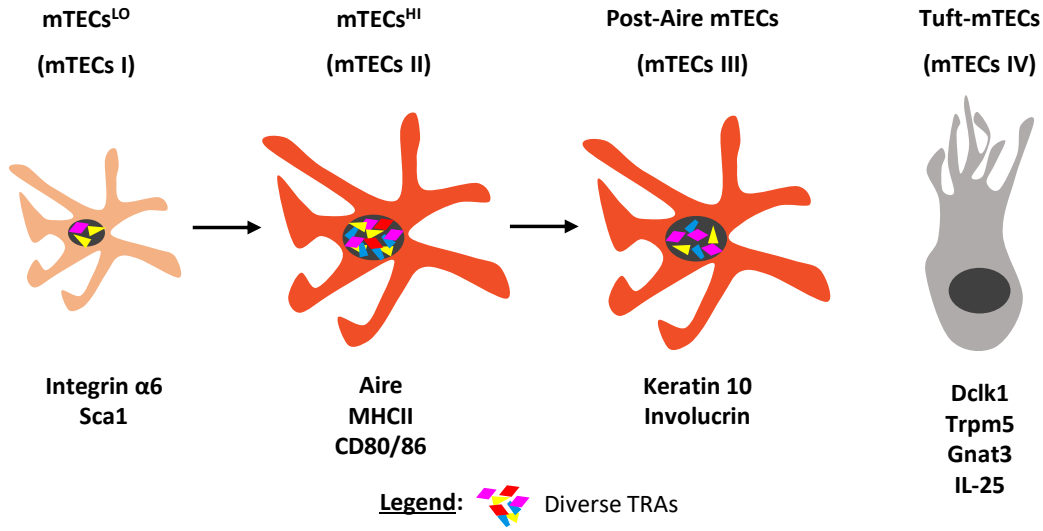
Traditionally, mTECs are subdivided according to the expression of CD80, MHCII, Aire and TRAs to immature mTECs^{LO} (CD80^{LO} MHCII^{LO}) subset which gives rise to mature mTECs^{HI} (CD80^{HI} MHCII^{HI}) subset, exhibiting better antigen-presenting properties and exclusive expression of Aire along with more robust PGE (Derbinski *et al.*, 2005). When compared to data using MARS approach, mTECs^{LO} and mTECs^{HI} overlap with mTECs I and mTECs II, respectively (Bornstein *et al.*, 2018).

mTECs I are defined according to the expression of integrin α6 and Sca1 (Bornstein *et al.*, 2018). It is assumed that these cells display stem cell potential and function as precursors of mTECs^{HI} which they dynamically replenish (Gray *et al.*, 2006; Wong *et al.*, 2014). Similar precursors are also detected in the embryonic thymus, where they give rise to mTECs^{HI}, even though they display distinct markers such as claudin 3 and 4 (Sekai, Hamazaki and Minato, 2014). Importantly, to the mTECs^{LO} subset belong also highly specialized mature mTECs which by the production of the chemokine CCL21 attract thymocytes from the cortex to the medulla (Lkhagvasuren *et al.*, 2013).

mTECs^{HI} were historically considered as terminally differentiated cells with relatively short lifespan about

three days (Gray *et al.*, 2007). Several studies however reported that at least a fraction of mTECs^{HI} downregulate the expression of MHCII, CD80, Aire and TRAs and trigger the expression of involucrin or keratin 10, the markers of terminally differentiated epithelium (White *et al.*, 2010; Metzger *et al.*, 2013). These senescent, so called "post-Aire" mTECs, are descendants of mTECs^{HI} and might be crucial for establishment of central tolerance by transferring TRAs to other thymic APCs which can present them to thymocytes within the realm of medulla (Metzger *et al.*, 2013). In line with this notion, post-Aire mTECs show enhanced production of various chemokines and adhesion molecules potentially facilitating their interactions with thymocytes or APCs (Morimoto *et al.*, 2018). In the MARS results, post-Aire mTECs were detected as mTECs III (Bornstein *et al.*, 2018).

Approximately 10% of mTECs detected by *Bornstein et.al. 2018* displayed so far unrecognized phenotype, marked by the absence of the Foxn1 expression. The development of these cells, referred to as mTECs IV, depends on TF Pou2f3 expressed also in the intestinal tuft cells. Indeed, mTECs IV highly express cytokine IL-25, doublecortin-like kinase 1 (Dclk1) and genes associated with canonical taste transduction pathway such as Trpm5 or Gnat3 which all belong to tuft cell signature genes. The function of "tuft-mTECs" remains enigmatic. While they lack promiscuous gene expression, they possess antigen presenting capacity and thus can interact with thymocytes. It has been proposed that tuft-mTECs, analogously to intestinal tuft cells, regulate the function of thymic innate lymphoid cells (ILC) type 2 through IL-25 (Bornstein *et al.*, 2018). Tuft-mTECs were also discovered by different approaches in another independent study, the results of which imply that their production of IL-25 upregulates the production of TH2 cytokines by NKT cells (Miller *et al.*, 2018). However, the physiological importance of such regulatory network in the thymus needs to be elucidated. Basic information about heterogeneity of mTEC subsets is shown in **Scheme 1**.



Scheme 1. mTECs heterogeneity: *mTECs are subdivided into four subsets. mTECs^{LO} are integrin α6^{+} Sca1^{+} immature precursors. mTECs^{HI} are defined by the expression of Aire, MHCII, CD80/86 and display a robust antigen presenting properties. Post-Aire mTECs are terminally differentiated descendants of mTECs^{HI}, marked by involucrin or keratin 10. Tuft-mTECs consist developmentally unrelated subset which is dependent on Pou2f3 and produces IL-25. Black arrows represent developmental relationship. Amount of colored geometric shapes (TRAs) represents level of PGE.*

2.3. Promiscuous gene expression of tissue-restricted antigens

As described in the previous chapter, the heterogeneity of mTECs concerns not only their phenotype but also their function. Specifically, $mTECs^{HI}$ (mTECs II) were shown to be critical cells imposing the mechanisms of central tolerance, namely the negative selection of self-reactive thymocytes or their deviation into Tregs (Klein *et al.*, 2014). These processes are highly dependent on the presentation of plethora of self-antigens on MHC molecules to thymocytes. To test the self-reactivity of newly generated TCRs, mTECs together with ubiquitous antigens produce and present thousands of TRAs by the process of PGE (Derbinski *et al.*, 2001). Notably, mTECs express around 19000 genes which represent almost 85% of the murine protein coding genome (Danan-Gotthold *et al.*, 2016). Out of these, the expression of more than 3000 genes, which represent mainly TRAs, was shown to be regulated by Aire (Sansom *et al.*, 2014; Danan-Gotthold *et al.*, 2016).

2.3.1. Autoimmune regulator and APECED

So far, Aire is the only well described regulator of PGE and is highly expressed by approximately a half of the $mTECs^{HI}$ (Derbinski *et al.*, 2005). Human AIRE was discovered in 1997 as a gene the mutation of which causes a severe multiorgan autoimmune syndrome called Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (Nagamine *et al.*, 1997; The Finnish-German APECED consortium, 1997). APECED is routinely diagnosed by the manifestation of autoimmunity to endocrine organs such as Addison´s disease or autoimmune hypoparathyroidism, usually accompanied by the systemic candida albicans infection. In most cases, however, the patients show a combination of additional symptoms including autoinflammation of the intestine, vitiligo and/or diabetes mellitus type 1 (T1D) to mention just a few (Kisand and Peterson, 2015). Counterintuitively, while APECED was classified as a monogenic autosomal recessive disease, according to the recent study, dominant mutations of AIRE gene, specifically in its PHD1 domain, are relatively frequent in human populations (1:1000), with manifestations of milder symptoms which are distinct from those observed in the "classical" APECED subset (Oftedal *et al.*, 2015). Although, most of the symptoms in APECED patients are T cell mediated, their sera contain excessive titers of high affinity neutralizing autoantibodies against the array of cytokines, such as IFNα and IL-17. Since these cytokines are critical humoral factors associated with the development of T1D and protection against candida albicans infection, respectively, presence of autoantibodies specific to them can dramatically block the disease in the first case or promote the disease in the second case (Kisand *et al.*, 2010; Puel *et al.*, 2010; Meyer *et al.*, 2016).

To study APECED, several Aire knock out (KO) mouse models have been constructed, which, as expected, showed symptoms of multiorgan autoimmunity (Anderson *et al.*, 2002; Jiang *et al.*, 2005; Hubert *et al.*, 2009). However, the phenotype of Aire KO mice varies and differs tremendously among strains. While C57BL/6 mice manifest only a mild phenotype with frequent autoimmunity to pancreas, salivary glands and retina only, BALB/c mice suffer from a more severe phenotype accompanied, in addition, with stomach

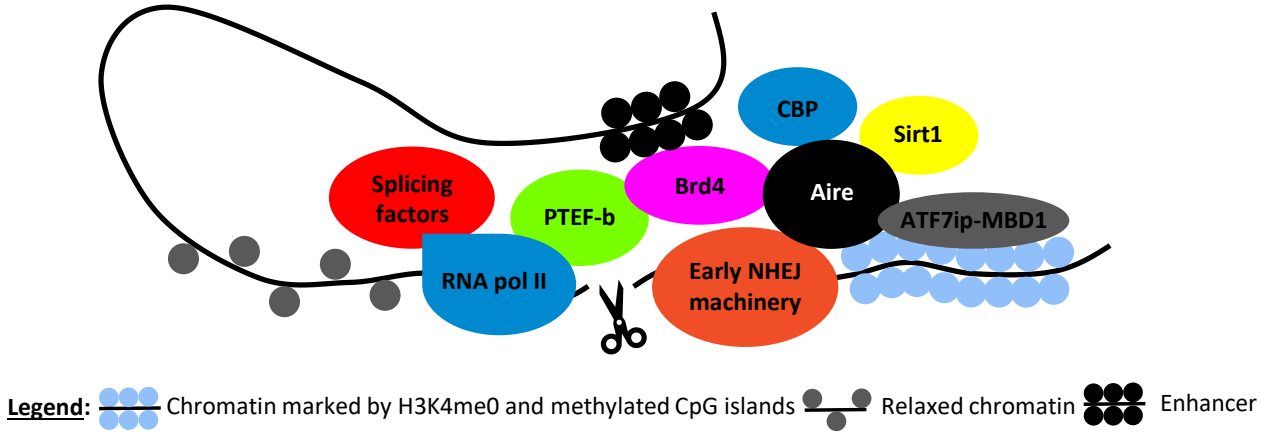
and liver autoimmunity. Lastly, non-obese diabetic (NOD) mouse strain exhibits the strongest autoimmune phenotype, with autoimmunity to all organs tested and with shortened lifespan to approximately 15-20 weeks (Jiang *et al.*, 2005).

2.3.2.Aire-dependent mechanisms of promiscuous gene expression

Aire is responsible for the expression of more than 3000 TRAs encoded by genes with very distinct regulatory elements. Thus, it is postulated that Aire operates not as a classical TF, but rather activates the expression of target genes which are characteristically epigenetically marked and localized into silenced chromatin. Aire protein consists of several functional domains, namely the SAND, CARD, PHD 1 and PHD 2 domains (Abramson and Goldfarb, 2016). The recognition of silenced chromatin is mediated by Aire's PHD 1 domain which targets the unmethylated histone H3 lysine 4 (H3K4me0) in the promotor regions of Aire-dependent TRA genes (Koh *et al.*, 2008). Moreover, these promotors lack a trimethylated histone H3 lysine 4 (H3K4me3), but contain a trimethylated histone H3 lysine 27 (H3K27me3) and are enriched for methylated CpG islands, indicative of other mechanisms responsible for the selectivity of Aire (Sansom *et al.*, 2014; Waterfield *et al.*, 2014). Indeed, SAND domain interacts with ATF7ip-MBD1 repressor complexes which bind CpG islands in the promotors of Aire regulated genes. This interaction was suggested to be essential for the function of Aire, since MBD1 KO mice shows a multiorgan autoimmunity (Waterfield *et al.*, 2014). In addition, Aire interacts with more than fifty "partner" molecules whose function is mainly related with the transport of Aire to the nucleus and its effect on chromatin remodeling, transcription and pre-mRNA processing (Abramson *et al.*, 2010; Bansal *et al.*, 2017).

The transactivating properties of Aire were found to highly depend on its combinatorial acetylation status. Specifically, Aire is activated by deacetylase Sirtuin 1 (Sirt1) whose expression largely overlaps with that of Aire and whose conditional KO in mTECs abrogates Aire function and leads to Aire KO-like autoimmunity (Chuprin *et al.*, 2015). On the other hand, Aire is also positively regulated by its acetylation. It has been shown that the Creb-binding protein (CBP), one of the Aire's interacting partners (Pitkänen *et al.*, 2000), acetylates SAND domain of Aire, hereby increases its stability and regulates its selectivity for particular TRA genes (Saare *et al.*, 2012).

Fundamental role in activation of PGE play particularly the molecules of early non-homologous end joining (NHEJ) machinery, such as DNA protein kinase (DNA-PK), PARP1 and Topoisomerase 2 (Abramson *et al.*, 2010). These molecules are recruited by Aire to the promotor regions of TRA genes where they cooperate in relaxation of chromatin by introduction of DNA double strand breaks (Abramson *et al.*, 2010; Guha *et al.*, 2017). This promotes the access of general TFs and RNA II polymerases to the promotors of Aire-dependent genes, including another partner of Aire, the elongation factor P-TEFb (Oven *et al.*, 2007). Aire and P-TEFb directly activate the transcription of TRA genes by unleashing RNA II polymerases from the transcriptional start sites (Giraud *et al.*, 2012). Current view on Aire-regulated PGE is summarized in **Scheme 2**.



Scheme 2. Model of Aire-regulated PGE: *Transactivating properties of Aire are enhanced by CBP and Sirt1. Hereby activated Aire engages Aire-dependent genes by its binding to H3K4me0 marked chromatin and by its interaction with ATF7ip-MBD1 complex which recognizes methylated CpG islands. After that, Aire recruits Early NHEJ machinery which relaxes chromatin of Aire-dependent genes by forming DNA double-strand brakes (visualized by scissors). That enables Aire to unleash RNA pol II by recruiting PTEF-b and activate transcription of Aire-dependent genes. Interaction between Aire and PTEF-b is mediated by Brd4 which further supports transcription by its binding to enhancers. Aire recruits also splicing factors which process the pre-mRNA products of Aire-regulated transcription. Adopted from* (Abramson and Goldfarb, 2016).

2.3.3. Basic aspects of promiscuous gene expression

Although the expression of many TRAs is Aire-dependent, remaining TRAs produced by mTECs undergo Aire-independent PGE whose regulation is still incompletely understood (Derbinski *et al.*, 2005). It has been reported that production of some Aire-independent TRAs is driven by TF Fezf2 whose expression is not restricted only to mTECs^{HI} and the depletion of which leads to the development of multiorgan autoimmunity (Takaba *et al.*, 2015). PGE of Aire-independent TRAs was also found to be affected by molecules that form multimolecular complexes with Aire, such as TF Hipk2 or Brg1, whose deficiency in mTECs leads to the development of even more severe autoimmunity (Rattay *et al.*, 2015; Koh *et al.*, 2018).

In general, there are some features of PGE that are similar for Aire-dependent and Aire-independent TRAs. PGE of each TRA is stochastic (highly variable between mTECs), transient (TRA repertoire of each mTEC changes over time) and much weaker than its expression in peripheral tissues (Venanzi *et al.*, 2008; Pinto *et al.*, 2013; Brennecke *et al.*, 2015). TRAs whose genes are positionally adjacent on chromosomes tend to be co-expressed (Rattay *et al.*, 2016). As indicated by the studies of the casein β locus whereby the casein β gene itself is an Aire-independent TRA, PGE within the clusters involves both Aire-dependent and Aire-independent genes (Derbinski *et al.*, 2008; Tykocinski *et al.*, 2010). Recently, it has been found that multimolecular complexes which regulate PGE concentrate into accessible parts of chromatin named super-enhancers (Bansal *et al.*, 2017). It is assumed that those TRAs which are in proximity to super-enhancer form the PGE co-expression cluster.

As a result of all the above described regulatory circuits, each mTEC^{HI} expresses at given time point only 1-3% of Aire-dependent and around 9% of Aire-independent TRAs (Derbinski *et al.*, 2008; Sansom *et al.*,

2014). Since, mTECs constitute a relatively rare cell subset with numbers reaching around 100000 cells per thymus of two week old mice, stochastic and sparse occurrence of each TRA in mTECs might limit the protective capacity of central tolerance (Klein, 2009).

Even though Aire and Aire-dependent TRAs are in the thymus expressed primarily by mTECs, they are also produced by thymic B cells. Nevertheless, the TRA repertoire of thymic B cells is limited with only minor contribution to central tolerance (Yamano *et al.*, 2015). Outside the thymus, Aire is expressed in testes, ovary and peripheral lymphoid tissues (Heino *et al.*, 2000). Although the expression of Aire in human tonsils and mouse lymph nodes was attributed to DCs (Gardner *et al.*, 2013; Fergusson *et al.*, 2019) and ILC type 3 (Yamano *et al.*, 2019), the proper function of Aire in those cells remains enigmatic, as their transcriptome is not enriched for TRAs.

Taken together, among all TEC subtypes, only mTECs^{HI} are the major producers of TRAs and are highly potent in their presentation in the context of MHCII molecules to developing thymocytes. The expression of TRAs by those cells is predominantly regulated by protein Aire whose relevance can be demonstrated by the development of multiorgan autoimmunity in APECED patients or in Aire KO mice. Due to the stochastic expression of either Aire-dependent or independent TRAs, each individual mTEC reveals unique set of TRAs, the production of which, due to limited cellularity of mTECs, can be presented only to a limited number of developing thymocytes. Importantly, and as highlighted in following sections, such restriction is compensated by the spreading of TRAs on other types of APCs, specifically thymic DCs.

3. T cell development

As described in previous chapters, all TEC subtypes contribute to the development of functional and self-tolerant repertoire of T cells. To fulfill this function, TECs produce series of diverse signals that fine-tune the migration and development of thymocytes. It is well established that these signals are spatially and temporarily highly ordered in respect of thymic compartmentalization where they act on successive developmental stage of thymocytes.

3.1. Early T cell development and positive selection

Thymic seeding progenitors (TSPs) arise within the bone marrow (BM) and are navigated to migrate through the bloodstream towards the ligands of their CCR7 (CCL19 and CCL21) and CCR9 (CCL25) receptors expressed in the thymus which they enter via a highly vascularized CMJ (Lind *et al.*, 2001; Zlotoff *et al.*, 2010). After entering the thymus, TSPs upregulate chemokine receptor CXCR4, which through its ligand CXCL12, produced by cTECs, drives their migration into the cortical part of the thymus (Plotkin *et al.*, 2003; Trampont *et al.*, 2010). cTECs were also shown to be the major producers of DLL4 and IL-7 (Moore *et al.*, 1993; Hozumi *et al.*, 2008), the essential molecules required for T cell commitment, which drive the development of TSPs into four stages of double-negative (DN1-DN4) thymocytes (named after the lack of CD8 and CD4 co-receptors) (Ceredig and Rolink, 2002). In line with this, DN thymocytes (DN2-

DN4) undergo complete T-cell lineage specification and begin to rearrange their TCRβ chain genes by the process of VDJ recombination, after which they start to express a unique variant of TCR on their surface (Roth, 2014). Afterwards, DN4 thymocytes activate the expression of CD8 and CD4 co-receptors, become double-positive (DP) and migrate back to the CMJ, where they rearrange their TCRα chain genes. An important role in this phase of thymocyte development was assigned to the GTPase-activating protein GIT2 which blocks CXCL12 signaling and enables "slow and random walk" of DP thymocytes near the CMJ (Phee *et al.*, 2010).

DP thymocytes possessing TCRs with the low affinity to pMHCs presented by cTECs are positively selected (Klein *et al.*, 2014). Such recognition also results in the differentiation of DP thymocytes into the single positive (SP) $CD8^+$ cytotoxic or $CD4^+$ helper lineage based on the MHCI or MHCII engagement together with the presence of specific TFs Runx3 or Th-POK (Setoguchi *et al.*, 2008; Luckey *et al.*, 2014). However, it is of note that the vast majority of DP thymocytes (~90%) possesses a TCR that is incapable to engage pMHCs and therefore they die by neglect (von Boehmer, Teh and Kisielow, 1989).

To drive the process of positive selection, cTECs produce and present, in the context of MHC molecules, a unique set of self-peptides (Xing, Jameson and Hogquist, 2013; Sasaki *et al.*, 2015). These peptides are products of cTEC-specific proteolytic machinery which consist of specific β5t subunit of "thymoproteasome" (involved in MHCI loading) (Murata *et al.*, 2007) and two important lysosomal proteases, the cathepsin L and thymus-specific serine protease (involved in MHCII loading) (Nakagawa *et al.*, 1998; Bowlus *et al.*, 1999). Although the context of peptide presentation during positive selection is incompletely understood, the evidence shows that defects in these unique proteases result in reduced numbers, repertoire and functionality of T cells (Nakagawa *et al.*, 1998; Bowlus *et al.*, 1999; Nitta *et al.*, 2010).

3.2. Cortex to medulla migration of thymocytes

After the positive selection, SP thymocytes relocate to thymic medulla (Ueno *et al.*, 2004). This intrathymic repositioning is fundamental for the establishment of self-tolerant repertoire of T cells, since its prevention results in the premature egress of SP thymocytes from the cortex to immune periphery and subsequently to the development of severe autoimmunity (Kurobe *et al.*, 2006; Hu *et al.*, 2015). Among all molecules involved in the cortex/medulla relocation, the chemokine receptors CCR7, CCR4 and EBI2, expressed by thymocytes, are particularly important (Lancaster, Li and Ehrlich, 2018). Specifically CCL21, the ligand of CCR7, is highly expressed by $mTECs^{LO}$ and is considered as the main medullary-chemoattractant of SP thymocytes (Lkhagvasuren *et al.*, 2013; Kozai *et al.*, 2017). This relies on the fact that both semimature $CD62L^{LO}$ $CD69^{HI}$ as well as mature $CD62L^{HI}$ $CD69^{LO}$ SP thymocytes express CCR7 and are responsive to CCL21 (Kurobe *et al.*, 2006). In contrast, CCR4-dependent chemoattraction is restricted only to $CD4^+$ semimature SP thymocytes and, unexpectedly, to $CCR7^-$ $CD69^+$ DP thymocytes which just underwent the positive selection. This alternative pathway relies on the expression of CCL17 and CCL22, the ligands of

CCR4, by medullary DCs (Hu *et al.*, 2015). Finally, 7α25-OHC, the ligand of EBI2, is also expressed by $mTECs^{LO}$ and drives the migration of $CD4^+$ SP thymocytes from the cortex to medulla. (Ki *et al.*, 2017).

3.3. Mechanisms of central tolerance in the thymic medulla

Given that VDJ recombination is a stochastic process, many thymocytes acquire a functional TCR recognizing self-peptides. In order to remove these potentially harmful thymocytes, mTECs present predominantly self-peptides on MHCI or, due to the constitutive macroautophagy, also on MHCII molecules (Aichinger *et al.*, 2013). It is well established that high affinity interactions of thymocytes with such peptides induce their apoptosis by the process of negative selection (Liston *et al.*, 2003). However, slightly weaker interactions lead to the deviation of self-reactive thymocytes into Tregs (Aschenbrenner *et al.*, 2007). Since ~80% of Tregs originate in the thymus, thymic Treg selection is of great importance in the mechanisms of peripheral immune tolerance (Thornton *et al.*, 2010; Weiss *et al.*, 2012). According to recent reports, fundamental parameters in a decision making process between negative and Treg selection are the affinity of antigen engagement, abundance of presented antigen, thymic millieu, auxiliary costimulation and soluble factors available during interaction (Klein, Robey and Hsieh, 2019).

3.3.1.Negative selection of self-reactive thymocytes

Negative selection of self-reactive thymocytes was originally discovered, using the hen egg lysozyme (HEL) neo-self-antigen expressed under the rat-insulin promotor (RIP). This system mimics the expression of TRA, since RIP is active only in pancreatic β cells, kidney, testes and mTECs, where its activity is dependent on protein Aire. By crossing this model with TCR-HEL transgenic system, most of the HEL specific T-cells were deleted by the process of negative selection (Liston *et al.*, 2003). Analogous results were obtained by using RIP-mOVA mouse model, where membrane-bound ovalbumin (mOVA) is expressed under RIP and introduction of OVA specific T cells leads to their deletion (Anderson *et al.*, 2005). As those TCR transgenic models possess an abnormally high affinity to neo-self-antigens, these T cells are prone to negative selection (Koehli *et al.*, 2014).

On the other hand, using the MHC-tetramer experimental system which enables to assess the polyclonal T cell repertoire and therefore natural TCR affinities (lower than in the above described cases), it has been shown that mTECs establish tolerance against TRAs not only by negative selection (Taniguchi *et al.*, 2012) but preferentially by the selection of Tregs which requires slightly weaker interactions than the negative selection (Legoux *et al.*, 2015; Malhotra *et al.*, 2016). Except for the affinity, it is assumed that important role in the negative/Treg selection decision plays the avidity of TCR/pMHC interactions. It has been found that abundantly expressed ubiquitous antigens are usually negatively selected due to the high-avidity interactions whereas TRAs which preferentially promote low avidity interactions are often converted to Tregs (Legoux *et al.*, 2015; Malhotra *et al.*, 2016).

3.3.2. Deviation of self-reactive thymocytes into T regulatory cells

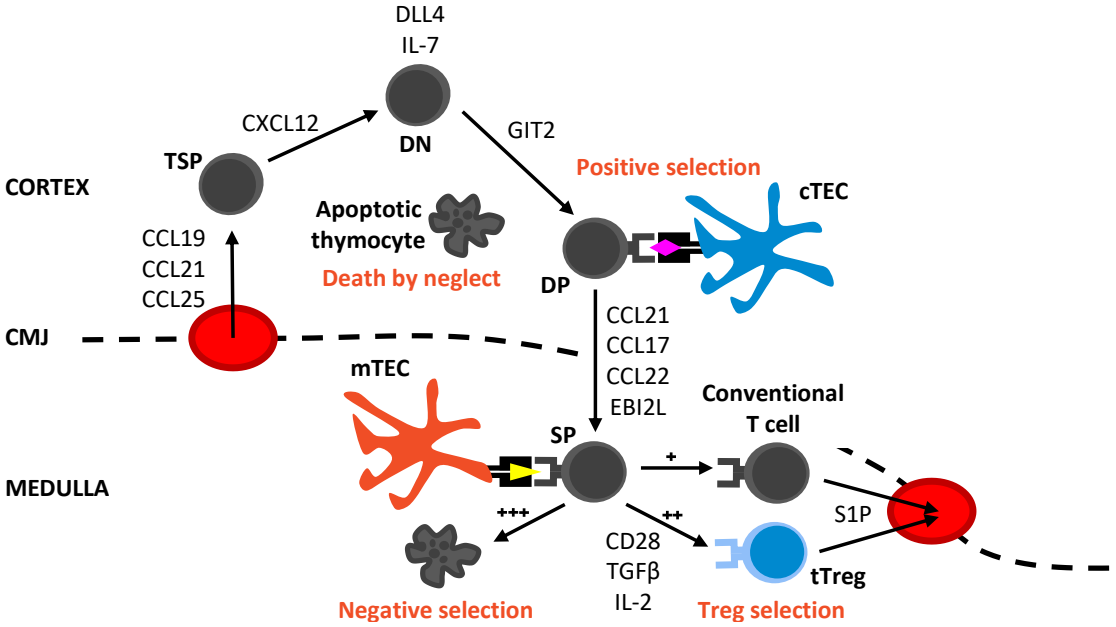
Apart from negative selection, the generation of thymic-derived Tregs (tTregs) is restricted only to medullary microenvironment (Cowan *et al.*, 2013). Using Aire-HA (influenza hemagglutinin) mice, an Aire-driven neo-self-antigen model, and TCR-HA T cell transgenic system, it was shown that about 25% of HA-specific T cells was deviated into tTregs. As the expression of HA depends on the presence of Aire, the $Aire^{+}$ $mTECs^{HI}$ were described as a crucial population for tTregs selection (Aschenbrenner *et al.*, 2007). This phenomenon was corroborated by a more physiological study which identified a natural Aire-dependent TRA: prostate-specific antigen MJ23, the presentation of which induces tTreg selection (Malchow *et al.*, 2013). The direct role of Aire in the mechanisms of tTregs generation was described by the TCR sequencing experiments, where those T cell clones which are destined to be tTregs are in Aire KO mice converted to self-reactive conventional T cells (Malchow *et al.*, 2016). On the other hand, Aire was shown to be required only for the generation of tTregs during the perinatal period and not in the adult thymus (Yang *et al.*, 2015; Stadinski *et al.*, 2019).

Proper development of tTregs is also highly dependent on provision of the "second and third" signal represented by the CD28/CD80 or CD86 costimulation and TGFβ and IL-2 cytokine signaling, respectively (Josefowicz, Lu and Rudensky, 2012). Costimulatory signals were shown to be essential for the expression of TF Foxp3 (Tai *et al.*, 2005), the master regulator of Treg development (Fontenot, Gavin and Rudensky, 2003). TGFβ signaling was shown to act as a "molecular switch" between negative and Treg selection, since it blocks their deletion by supporting of anti-apoptotic signals (Ouyang *et al.*, 2010). Nevertheless, direct role of TGFβ in tTreg commitment (e.g Foxp3 expression) is still controversial (Zheng *et al.*, 2010; Ouyang *et al.*, 2010; Konkel *et al.*, 2014). On the other hand, the cytokines of γ-chain family, specifically IL-2, was shown to be absolutely crucial for tTregs development (Vang *et al.*, 2008; Tai *et al.*, 2013).

In general, tTreg development proceeds through two steps: first, TCR signaling triggers the expression of CD25, a high-affinity IL-2 receptor and, second, direct binding of IL-2, together with CD28 signaling, activate Foxp3 expression (Lio and Hsieh, 2008). Recently, it was demonstrated that mature $CD25^{+}$ $Foxp3^{+}$ tTregs originate not only from $CD25^{+}$ $Foxp3^{-}$ precursors, as previously thought, but also from $CD25^{-}$ $Foxp3^{LO}$ cells. Although the development of both precursors requires certain levels of IL-2, the differentiation of $CD25^{-}$ $Foxp3^{LO}$ is highly dependent on IL-4 and IL-25, the cytokines regulated by tuft-mTECs (Owen *et al.*, 2019).

By using RAG2-GFP mouse model in which only newly generated T cells exhibit GFP expression, it has been shown that mature Tregs which already lost GFP expression re-emigrate back to the thymus from the periphery. Here, their presence slows down the generation of new tTregs, as they selectively compete for IL-2 (Thiault *et al.*, 2015). Importantly, migration of these cells into the thymus is governed Aire-dependent chemokine CCL20, whose receptor, CCR6, is highly expressed by GFP^{-} Tregs but not by the newly generated ones (Cowan *et al.*, 2018).

In general, thymocytes spend around 5 days in the thymic medulla, during which they intensively interact with pMHC complexes on mTECs (Mccaughtry, Wilken and Hogquist, 2007). After they complete selection processes, thymocytes upregulate the expression of Sphingosine-1-phosphate receptor 1 (S1P1) and enter the bloodstream in CMJ. These T-cells are then called "recent thymic emigrants" (RTE) (Matloubian *et al.*, 2004). Nevertheless, it was recently shown, that RTE can exit the thymus by Sphingosine-1-phosphate- (S1P) independent egress, which depends on TH2 cytokines produced by NKT cells (White *et al.*, 2017). Hypothetically, tuft-mTECs might be involved in this process via already mentioned production of IL-25 (Miller *et al.*, 2018). As the summary, the crucial steps of T cell development are highlighted in **Scheme 3**.



Scheme 3. Overview of T cell development: *TSP migrate into the thymus according to the gradient of chemokines CCL19, CCL21 and CCL25. They enter the thymus from CMJ blood vessels, translocate into the cortex by the chemokine CXCL12 and under the influence of DLL4 and IL7 transform into DN thymocytes. Those, after their T cell commitment, return into proximity of CMJ by the expression of GIT2 where they differentiate into DP thymocytes and undergo positive selection by the recognition of pMHCs on cTECs. After the positive selection, thymocytes migrate into the medulla along the gradient of chemokines CCL21, CCL17, CCL22 and EBI2L (ligand of EBI2) where they, as SP thymocytes, interact with pMHCs on mTECs. High affinity interactions of thymocyte's TCRs (+++) with these pMHCs lead to their negative selection, while their low affinity recognition (+) result in their maturation into conventional T cells. The intermediate affinity recognition (++) leads into the deviation of thymocytes into tTregs which further requires CD28 costimulation and local availability of IL-2 and TGFβ. Conventional T cells and tTregs leave the thymus via CMJ blood vessels along the S1P chemokine gradient. Adopted from* (Lancaster, Li and Ehrlich, 2018).

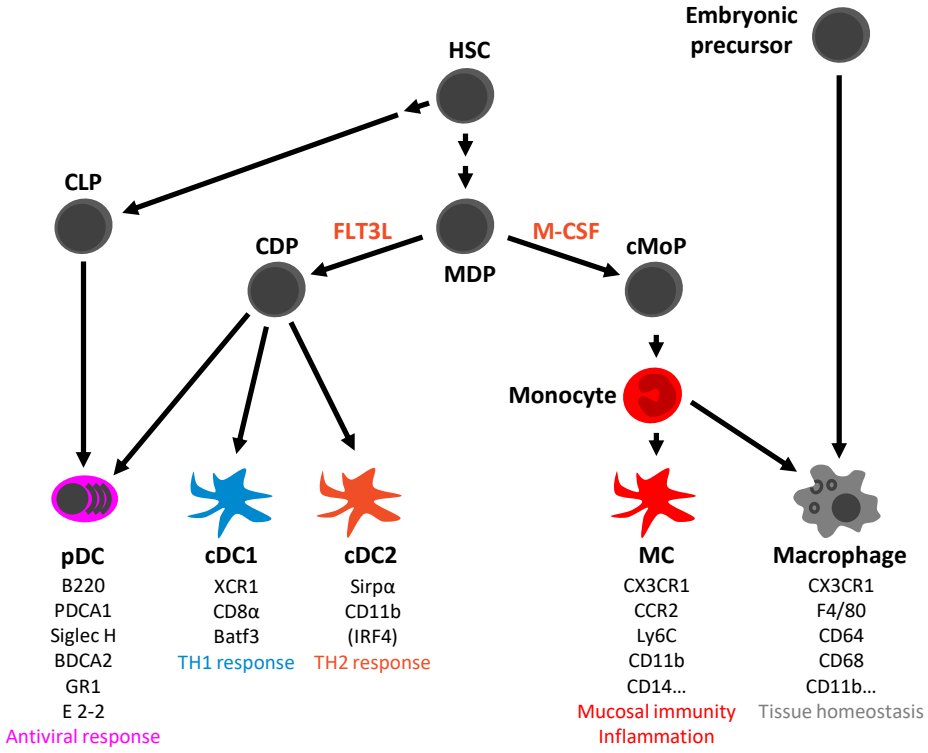
4. The role of dendritic cells in the mechanisms of central tolerance

So far, the mechanisms of central tolerance were discussed in the context of mTEC/thymocyte interactions. However, thymus also accommodates other types of hematopoietic BM-derived APCs (BM APCs) which together with mTECs contribute to the establishment of central tolerance (Perry and Hsieh, 2016). Specifically, thymic Aire-expressing B cells were shown to be able to present endogenous antigens to

developing thymocytes and negatively select self-reactive clones or convert them into tTregs (Perera *et al.*, 2013; Walters *et al.*, 2014; Yamano *et al.*, 2015). Also, a potential role in negative selection can be exerted by thymic macrophages, which were shown to delete monoclonal repertoire of mOVA-specific thymocytes *in vitro* (Guerri *et al.*, 2013). Nevertheless, the best described BM APCs, whose direct cooperation with mTECs is essential for the functional central tolerance (Perry *et al.*, 2014) and the depletion of which results in a severe multiorgan autoimmunity, are thymic DCs (Ohnmacht *et al.*, 2009). Since DCs are highly heterogeneous, the following sub-chapters will elucidate the differences among DC subsets, with specific emphasis on their phenotype and function in the thymus.

4.1. Heterogeneity of dendritic cells

Classification of DCs, which is summarized in the context of other mononuclear phagocytes in **Scheme 4**, is based on their ontogeny, phenotype, function and localization (Guilliams *et al.*, 2014).



Scheme 4. Development of dendritic cell subsets, monocytes and macrophages: *This scheme represents the overview of DCs development, compared to that of other mononuclear phagocytes. The cytokines that are critical for differentiation into indicated lineages are displayed in orange. Major molecular markers and functions of differentiated cell types are depicted. Note that due to extensive heterogeneity, only selected markers of MCs and macrophages are shown. HSC stands for hematopoietic stem cell. Adopted from* (Eisenbarth, 2019).

DCs share origin with monocytes and macrophages in monocyte/macrophage-DC progenitor (MDP) which gives rise to "DC-specific" common DC precursor (CDP) (Liu *et al.*, 2009). Differentiation of DCs from CDP is completely dependent on a cytokine FMS-like tyrosine kinase 3 ligand (FLT3L) (Waskow *et al.*, 2008). DCs consist of two subsets of conventional DCs (cDC1 and cDC2) and plasmacytoid DCs (pDCs) (Guilliams *et al.*, 2014). Nevertheless, the origin of pDCs is still controversial, since $Ly6D^{+}$ pDC precursors

originate either from common lymphoid progenitor (CLP) (Dress *et al.*, 2019) or both CLP and CDP (Rodrigues *et al.*, 2018).

Development of cDC1 is completely dependent on TF Batf3, since Batf3 KO mice display severe reduction of these cells. Phenotypically, cDC1 are defined by the surface expression of XCR1 and CD8α. These cells are considered to participate mainly in cytotoxic immunity and TH1 responses, as they are very potent in cross-presentation and production of cytokine IL-12 (Hildner *et al.*, 2008; Savina *et al.*, 2009; Edelson *et al.*, 2010; Mashayekhi *et al.*, 2011; Bachem *et al.*, 2012). *Vice versa*, cDC2, expressing CD11b and Sirpα, were shown to be more effective in MHCII presentation and thus, they are involved in the activation of $CD4^+$ T cells and play a role especially in TH2 responses (Dudziak *et al.*, 2007; Gao *et al.*, 2013; Calabro *et al.*, 2016). In contrast to cDC1, a specific TF which would define cDC2 lineage is so far missing. Nevertheless, IRF4 is used instead to mark cDC2, even though it is evenly involved in the development of cDC1 (Tussiwand *et al.*, 2012). Consistent with being essential for responses to viral infection, pDCs express high levels of type I interferons and cross-present viral antigens on MHCI molecules (Colonna, Trinchieri and Liu, 2004; Di Pucchio *et al.*, 2008). In general, pDCs are characterized by their dependence on TF E2-2 and expression of B220, PDCA1, Siglec-H, GR1 and BDCA2 markers (Colonna, Trinchieri and Liu, 2004; Cisse *et al.*, 2008). Since the expression of BDCA2 is largely restricted to the pDC-lineage, the BDCA2-DTR (diphtheria toxin receptor) mouse can be used as a suitable model for their depletion (Swiecki *et al.*, 2010).

Due to their analogous function, common origin in MDP and sharing markers with cDC2 (i.e. CD11c, MHCII, CD11b etc.), differentiated monocytes, further referred to as monocyte-derived cells (MCs), are often inaccurately incorporated among DC subsets (Satpathy *et al.*, 2012). However, monocytes, MCs and some macrophages differentiate from a common monocyte progenitor (cMoP) and this process highly depends on macrophage-colony stimulating factor (M-CSF) but not FLT3L (Hettinger *et al.*, 2013) (**Scheme 4**). Therefore, MCs can´t be considered as DCs "per se" (Guilliams *et al.*, 2014). Based on expression levels of chemokine receptor CX3CR1 and Ly6C, monocytes can be divided into immature $CX3CR1^{LO}$ $Ly6C^+$ and mature $CX3CR1^{HI}$ $Ly6C^–$ subset (Geissmann, Jung and Littman, 2003; Yona *et al.*, 2013). Specifically, MCs originate from the former, especially in mucosal tissues (Varol *et al.*, 2007), where they, by direct sampling of luminal antigens, mediate antibacterial and antifungal responses as well as the tolerance against commensal microbiota and food antigens. Importantly, such mucosal MCs can be phenotypically distinguished from cDC2 by the expression of CX3CR1 (Niess *et al.*, 2005; Varol *et al.*, 2009; Diehl *et al.*, 2013; Kim *et al.*, 2018; Leonardi *et al.*, 2018). Nevertheless, it is important to note, that MCs are ill-defined cell subset which comprises also functionally and phenotypically distinct populations from $CX3CR1^+$ MCs, such as $Ly6C^+$ $CCR2^+$ population which is proposed to be fundamental for inflammatory responses against intracellular bacteria (Serbina *et al.*, 2003).

Lineage tracing experiments performed by introduction of CX3CR1-Cre (constitutive expression) and

CX3CR1-Cre^{ER} (inducible expression by tamoxifen) mouse models unraveled that, except monocytes and MCs, CX3CR1 is highly expressed by MDPs, intestinal macrophages and also by embryonic-derived tissue resident macrophages such as microglia or Kupfer cells (Yona *et al.*, 2013). It is of note that distinction between mucosal MCs and intestinal macrophages is vaguely defined and such MCs are commonly referred to as CD11c^{+} macrophages (Gross, Salame and Jung, 2015).

In conclusion, among the all CD11c^{+} DC-like populations, we can distinguish two types of cDCs (cDC1 and 2), pDCs and MCs. The phenotype and function of these subpopulations was shown to be highly tissue-specific and readily modified by their microenvironment.

4.2. Thymic dendritic cells

Thymic DCs form roughly 0,5 % of all thymic cells (Wu and Shortman, 2005). The majority of them is localized in the medullary region, where they together with mTECs form a complex APC-networks (Sanos *et al.*, 2011). However, a fraction of DCs occupies also thymic cortex, specifically its perivascular regions adjacent to CMJ (Ladi *et al.*, 2008).

Thymus comprises all classical DC subsets defined in the previous chapter (Hadeiba and Butcher, 2013). While pDCs and cDC2 develop extrathymically and recirculate between the thymus and periphery, ("thymic") cDC1 originate and reside merely in the thymus (Li *et al.*, 2009). In this regard, cDC1 will be further referred to as the thymic-derived DCs (tDCs) and cDC2 as migratory DCs (mDCs) (Hadeiba and Butcher, 2013). The thymic and extrathymic origin of DC subsets strongly correlates with the nature of antigens which they present for thymocytes selection. Due to the complexity of thymic DC subpopulations, the precise role of certain DC subset in the mechanisms of central tolerance will be discussed in the following chapters.

4.2.1. Plasmacytoid dendritic cells

As described in previous section, pDCs develop in the immune periphery, migrate to the thymus and present peripheral antigens to the developing thymocytes (Li *et al.*, 2009). Injecting the ovalbumin (OVA) pulsed pDCs into the bloodstream, resulted in the migration of these cells into the thymus and in subsequent negative selection of OVA specific thymocytes (Bonasio *et al.*, 2006; Hadeiba *et al.*, 2012). The migration of pDCs to the thymic medulla is driven by CCL25/CCR9 chemokine pathway. To avoid presentation of antigens the tolerance to which is undesirable, e.g. pathogenic antigens, pDCs which underwent immunogenic maturation lose the expression of CCR9 and cannot migrate to the thymus (Hadeiba *et al.*, 2012). In contrast to their role in negative selection, there is no evidence that murine pDCs mediate the deviation of self-reactive thymocytes into tTregs. Nevertheless, experiments from human thymus showed that pDCs might participate in such process by interacting with Hassall´s corpuscles (Watanabe *et al.*, 2005; Hanabuchi *et al.*, 2010), microscopic structures in the murine thymus formed by post-Aire mTECs (Wang *et al.*, 2019).

4.2.2. Thymic-derived dendritic cells

As the medulla-residing mature $XCR1^+$ tDCs are generated in the thymus, they predominantly present thymic-derived antigens. Specifically, tDCs can acquire antigens produced by mTECs through the process of CAT (Perry and Hsieh, 2016). To achieve close localization to mTECs, tDCs express chemokine receptor XCR1 and migrate along the gradient of its ligand, XCL1, which is expressed by $mTECs^{HI}$ in Aire-dependent manner. The functional importance of this chemoattraction is manifested by decreased numbers of tTregs in XCL1 KO mice (Lei *et al.*, 2011). Along with XCL1, the medullary localization of tDCs is also influenced by CCR7. tDCs develop from $CCR7^+$ precursors which migrate into the medulla according to the gradient of CCL21 (Cosway *et al.*, 2018). These progenitors give rise to $CCR7^-$ immature tDCs which under the influence of still unknown stimulus undergo homeostatic maturation and become $CCR7^+$ tDCs. Therefore, CCR7 KO mice has significantly reduced numbers of tDCs (Ardouin *et al.*, 2016). In line with above mentioned importance of tDCs for Treg selection, it is postulated that mTECs and tDCs communicate with self-reactive T cells through the CD70/CD27 engagement which rescues thymocytes from the mitochondria-mediated apoptotic pathway and thus divert their development into tTregs (Coquet *et al.*, 2013). Nevertheless, a recent study argues that although the cooperation between mTECs and tDCs is important for establishment of central tolerance via deletional tolerance of self-reactive thymocytes, tDCs are dispensable for the generation of tTregs (Herbin *et al.*, 2016).

4.2.3. Migratory dendritic cells

Unlike tDCs, $Sirp\alpha^+$ mDCs are of extrathymic origin and, similar to pDCs, can carry peripheral blood-borne antigens into the thymus to which they mediate negative selection (Li *et al.*, 2009; Atibalentja, Murphy and Unanue, 2011). Intrathymic migration of mDCs is dependent on chemokine receptor CCR2, as the CCR2 KO mice show selective diminishment of these cells in the thymus (Baba, Nakamoto and Mukaida, 2009). The CCR2-dependent recruitment of mDCs is modified by the interactions between mTECs and thymocytes, since in their absence, mTEC's production of CCR2 ligands (CCL2, CCL8 and CCL12) is increased, which in turn, attracts more mDCs into the thymus to enhance negative selection (Lopes *et al.*, 2018). The majority of mDCs localize to thymic medulla, and due to their peripheral origin, are detectable around vascular regions (Hu *et al.*, 2015). Other report showed that mDCs accumulate also in the perivascular regions of the cortex (Baba, Nakamoto and Mukaida, 2009). Thus, CCR2 signaling is important for migration of peripheral mDCs into the thymus, but not for their final intrathymic position (Klein *et al.*, 2014). As mentioned above, mDCs also express ligands for chemokine receptor CCR4 (CCL17 and CCL22) and not only attract $CCR4^+$ "post-positive selection" thymocytes into the medulla, but also mediate their negative selection. The importance of CCR4 chemokine pathway in deletional tolerance has been clearly demonstrated by development of autoimmunity in CCR4 KO mice (Hu *et al.*, 2015).

In addition to negative selection, mDCs, in contrast to pDCs and tDCs, are very potent in the deviation of

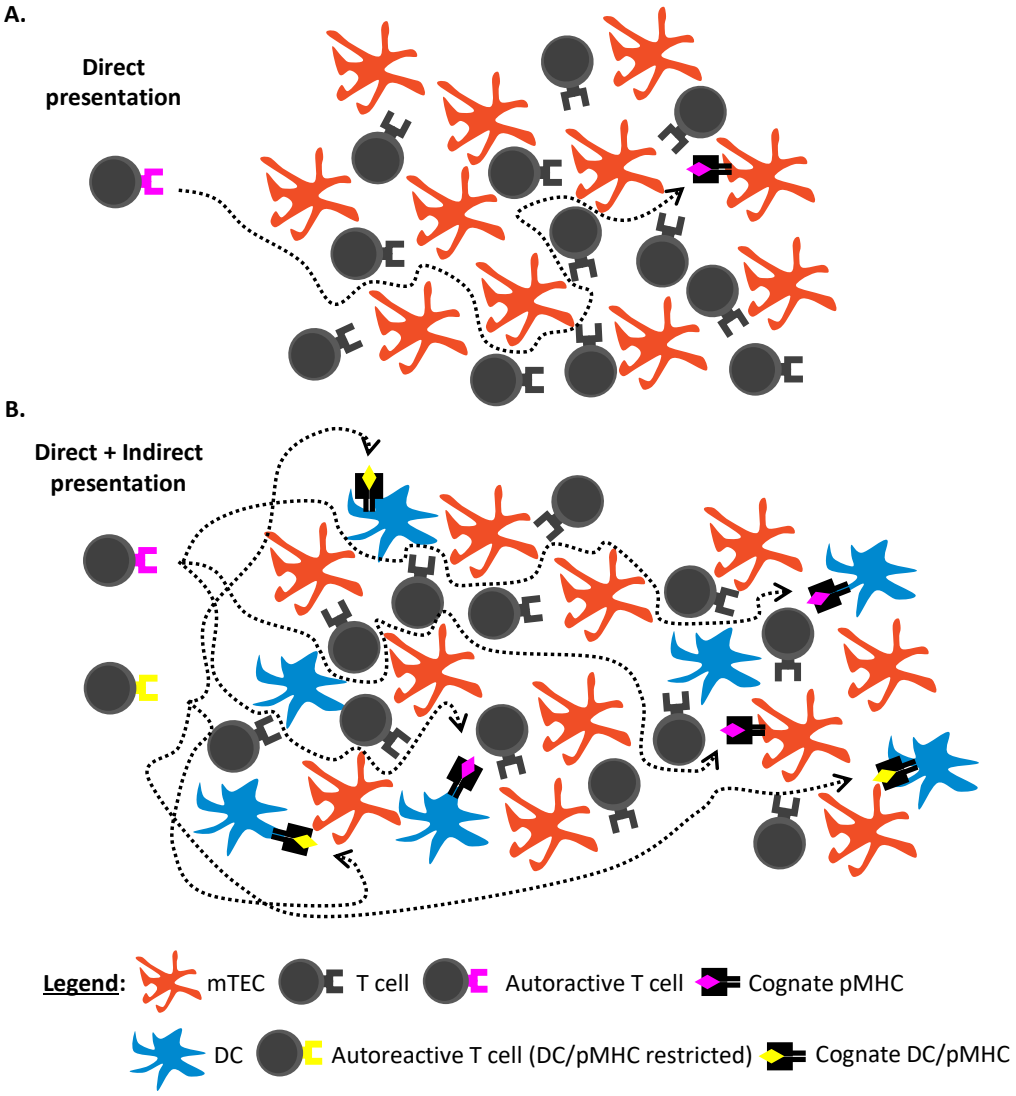
self-reactive thymocytes into tTregs (Proietto *et al.*, 2008). This has been recently demonstrated by showing that the selection of MJ23 Tregs (TCR specific to prostate antigen) which is in general dependent on the presence of DCs in the thymus was unperturbed in the absence of either tDCs (using Batf3 KO mice) nor pDCs (using BDCA2-DTR tg mice) (Leventhal *et al.*, 2016). Also, the sequencing of TCRα repertoire of tTregs from Batf3-sufficient and Batf3 KO mice revealed only negligible differences in their polyclonal repertoire, thus supporting the principal role of mDCs in tTregs generation (Leventhal *et al.*, 2016). Involvement of mDCs in the mechanisms of Tregs selection was further demonstrated by the observation that the cellularity of Tregs is highly elevated in Batf3 KO or CCR7 KO mice, where the selective depletion of tDCs leads to proportionally increased numbers of mDCs (Leventhal *et al.*, 2016; Hu *et al.*, 2017). This compensatory effect is likely caused by immigration of peripheral mDCs into the thymus, filling up the empty thymic niche after tDCs depletion. Importantly, newly immigrating mDCs display $MHCII^{LO}$ phenotype which is assumed to predispose them to be highly efficient cellular mediator in generation of tTregs (Hu *et al.*, 2017). Thus, mDCs exhibit previously unrecognized heterogeneity and can be classified into $MHCII^{LO}$ and $MHCII^{HI}$ subsets, whereby the former is crucial for the deviation of self-reactive thymocytes into tTregs.

Taken together, thymus accommodates all three major subsets of DCs, which due to their distinct origin and localization fulfill different roles in the mechanisms of central tolerance. Although the nature of antigens presented by a given subtype of thymic DCs is arguably different, all these DCs localize predominantly in the thymic medulla and possess the ability to cooperate with mTECs. This cooperation predisposes these cells to acquire mTEC-derived antigens and present them to developing thymocytes to enforce the thymic tolerance.

5. Cooperative antigen transfer in the thymus

As described previously, mTECs as a whole population, express thousands of TRAs. However, on a single cell level, each mTEC presents a distinct set of TRAs that constitutes only around 1-3% of entire TRA pool. This fact, together with very limited number of mTECs in the thymus, represents the bottleneck of mTEC-mediated mechanisms of central tolerance (Derbinski *et al.*, 2008; Klein, 2009). To overcome such limitation, TRAs are presented to developing thymocytes not only by mTECs, but also indirectly by thymic DCs, via the process of CAT. It is currently established that tDCs as well as mDCs, participate in CAT and are capable of indirect presentation of TRAs (Leventhal *et al.*, 2016; Kroger *et al.*, 2017). CAT not only increases the number of APCs presenting TRAs but also enables their presentation in the different cellular context, i.e. the surface of thymic DCs. It has been shown that array of TRA-specific thymocytes is deviated into Tregs merely by DCs, since they likely possess distinct antigen-processing machinery from mTECs and therefore can present differently processed versions of TRA-derived pMHCs (Perry *et al.*, 2014). Besides, DCs, in contrast to mTECs, are motile cells capable of rapid migration throughout the medulla which can significantly enhance presentation efficiency of central tolerance (Perry and Hsieh, 2016). The

reinforcement of central tolerance by indirect presentation is highlighted in **Scheme 5**.



Scheme 5. Model of direct and indirect antigen presentation: A. *Direct presentation of mTEC-derived antigen. Specific antigen is presented by very limited number of APCs and developing thymocyte is forced to perform several interactions to find its cognate pMHC molecule.* **B**. *Direct and indirect presentation of mTEC-derived antigen. Specific antigen is presented by higher number of APCs (mTECs and DCs) and number of TCR/pMHC interactions is reduced. Indirect presentation also enables to present antigens that are not processed by mTECs itself (yellow color). Arrows represent potential routes which lead to successful pMHC recognition by each self-reactive thymocyte.*

5.1. Indirect presentation of tissue-restricted antigens

The process of indirect presentation was discovered by using already mentioned RIP mOVA mouse model, where mOVA mimics TRA, since it is expressed primarily by mTECs in the thymus. MHCI and MHCII-deficient BM cells which comprised OVA-restricted $CD8^+$ (OT-I) and $CD4^+$ (OT-II) T cells, respectively, were transferred into lethally irradiated RIP mOVA mice. In this setting, mTECs expressed both MHCI and MHCII, whereas BM APCs were deficient either in MHCI or MHCII. It was found that negative selection of OT-I thymocytes was unaffected by MHCI deficiency on BM APCs, even though BM APCs

were capable of mOVA cross-presentation. In marked contrast, the negative selection of OT-II thymocytes was shown to be highly dependent on MHCII presentation by BM APCs (Gallegos and Bevan, 2004). Hence, indirect presentation was suggested to play a significant role in negative selection of $CD4^+$ thymocytes. In contrast, using two photon microscopy on *ex vivo* thymic slices from RIP mOVA mice, it was shown that most of $CD8^+$ OT-I cells were activated by BM APCs through the process of indirect presentation, while the activation of $CD4^+$ OT-II cells was dependent on both direct and indirect presentation. Contrary to RIP mOVA mice (membrane bound OVA), the RIP OVA^{HI} mice that produce secreted form of OVA (sOVA) under RIP, showed much higher activation of $CD4^+$ OT-II cells by BM APCs than $CD8^+$ OT-I cells (Lancaster *et al.*, 2019). Taken together, an intra/extracellular localization of OVA predicates its predominant indirect presentation on MHCI or MHCII molecules, which is likely caused by a distinct CAT mechanism or acquisition by different APC subsets, and proposes that in case of polyclonal repertoire, indirect presentation plays a primary role in the deletion of $CD4^+$ thymocytes.

The comparison of RIP-mOVA system with newly generated Aire-OVA knock in (Aire-OVA-KI) mice, where OVA is translated together with Aire, shows major differences in OT-I/OT-II thymocytes selection. When mOVA was expressed under RIP, the selection of OT-II thymocytes was found to be completely dependent on the indirect presentation by BM APCs. In the case of Aire-OVA-KI, the selection was restricted to direct presentation by mTECs (Mouri *et al.*, 2017). The discrepancy between these models likely relies on the fact that mOVA is expressed predominantly by $mTECs^{LO}$ in RIP mOVA system (Mouri *et al.*, 2017; Lancaster *et al.*, 2019). Since $mTECs^{LO}$ are poor APCs, their direct presentation of mOVA is presumably insufficient to induce proper negative/Treg selection of OT-II thymocytes, without support of DCs. On the other hand the expression of OVA in Aire-OVA-KI mice is restricted only to $mTECs^{HI}$ population, whose presentation capacity seems to be sufficient for OT-II selection (Mouri *et al.*, 2017). This hypothesis also supports previously published data, where reduced expression of MHCII (CIITA knock-down mice) specifically on mTECs leads to the impaired selection of OVA-specific thymocytes, regardless of DCs depletion (Hinterberger *et al.*, 2010). Contrary to this observation, the indirect presentation was also shown to be highly dependent on Aire, since it upregulates the expression of several chemokines which attract DCs to the vicinity of Aire-expressing $mTECs^{HI}$ (Hubert *et al.*, 2011; Mouri *et al.*, 2017).

To distinguish the contribution of indirect and direct presentation to the mechanisms of central tolerance under more physiological conditions, the TCRα repertoire in BM chimeras with partial or full deficiency of MHCII on mTECs and BM APCs, respectively, was sequenced. It has been found that TCR specificities sensitive to indirect presentation are mostly non-overlapping with those engaging mTECs. Furthermore, BM APCs were found to be crucial not only for negative selection but mainly for the generation of tTregs, as most of tTreg-TCRs were dependent on MHCII presentation by BM APCs. Moreover, a vast array of TCR specificities which were either negatively selected or deviated into tTregs by BM APCs turned out to be dependent on Aire, which points to the importance of indirect presentation for the selection of Aire-

dependent TRAs (Perry *et al.*, 2014). The requirement for indirect presentation of Aire-dependent TRAs was also documented by studies, where the selection of TCRs specific to interphotoreceptor retinoid binding protein (Taniguchi *et al.*, 2012) or prostate-specific antigen MJ23 (Leventhal *et al.*, 2016) was dependent on DCs. Finally, except the altered TCR repertoire, the abrogation of antigen-presenting properties in BM APCs leads also to decreased numbers of Tregs, further corroborating the importance of BM APCs for Treg selection (Leventhal *et al.*, 2016).

5.2. The mechanisms of cooperative antigen transfer

CAT is operational in the thymus in one direction only, where mTECs being the donors, and thymic DCs the acceptors of antigens (Millet, Naquet and Guinamard, 2008; Koble and Kyewski, 2009). The fact, that analogous process in reverse order occurs also in lymph nodes (from DCs to stromal cells) infers that the local microenvironment somehow conditions DCs whether to handover or acquire antigen (Dubrot *et al.*, 2014). In addition, by using *in vitro* co-cultivation assays, CAT was found significantly less efficient in the case of splenic compared to thymic DCs (Koble and Kyewski, 2009; Kroger *et al.*, 2017).

The process of CAT can be achieved by several mechanisms, which are either cell contact-dependent or independent. In consideration of the latter, it has been demonstrated that human mTECs can secrete exosomes containing TRAs (Skogberg *et al.*, 2015), such mechanism was not confirmed in mouse studies (Millet, Naquet and Guinamard, 2008; Kroger *et al.*, 2017; Perry *et al.*, 2018). Thus, it seems that CAT is achieved mainly by cell contact-dependent mechanisms such as the endocytosis of apoptotic bodies or trogocytosis, i.e. the exchange of portion of plasma membrane between two cells (Koble and Kyewski, 2009).

Trogocytosis is proposed to participate in CAT of MHCII molecules, since these surface proteins remain intact after their transfer from mTECs to DCs (Millet, Naquet and Guinamard, 2008; Koble and Kyewski, 2009). However, while MHCII molecules reside predominantly in lipid rafts, these membrane structures are not required for CAT to occur (Kroger *et al.*, 2017). Interestingly, mDCs, in contrast to other thymic DC subsets, do not require phosphoinositide 3-kinase (PI3K) signaling to accomplish CAT of MHCII molecules (Kroger *et al.*, 2017). Since trogocytosis was found to be dependent on PI3K signaling in T cells (Martínez-Martín *et al.*, 2011), it seems that MHCII CAT to mDCs is independent of trogocytosis. Rather, mDCs employ endocytosis, in which they are much more efficient than other DCs (Baba, Nakamoto and Mukaida, 2009).

To test whether also intracellular antigens are subjected to CAT, *Koble and Kyewski* constructed Foxn1-eGFP mouse model, in which only TECs, and no other thymic cells produce the cytosolic eGFP protein. In this model, couple of observations argued for efficient uptake of cytosolic eGFP by thymic DCs likely via endocytosis of mTEC apoptotic bodies: (i) the level of eGFP positivity in thymic DCs was much lower than that of mTECs and (ii) those DCs which acquired eGFP, frequently revealed also positivity for EpCAM, a typical TECs marker, suggesting that these molecules were co-transferred. It is of note that $eGFP^+$ $EpCAM^+$

DCs further expressed high levels of MHCII and costimulatory molecules, and were of $CD8\alpha^{+}$, $CD11b^{+}$ and $CD103^{HI}$ phenotype, suggesting that they comprised both tDCs and mDCs (Koble and Kyewski, 2009).

So far, together with Aire, which regulates the recruitment of DCs to the vicinity of mTECs (Hubert *et al.*, 2011), the only other molecule known to facilitate CAT is the scavenger receptor CD36. This molecule is among all thymic DCs expressed exclusively by tDCs and endows them to endocytose mTEC apoptotic bodies (Perry *et al.*, 2018). This might be a very frequent event since a proportion of mTECs die rapidly within two or three days (Gray *et al.*, 2007). However, the fact that only surface but not intracellular antigens were found to be transferred by the CD36-dependent pathway, questions this conclusion. Despite this conundrum, *Perry et.al. 2018* demonstrated that both negative and Treg selection of some TCR specificities requires CD36. By the same token, many TCRs whose negative/Treg selection relies on tDCs were not affected in CD36 KO mice. This strongly argues that tDCs possess additional mechanisms by which they acquire mTEC-derived antigens (Perry *et al.*, 2018).

5.3. Thymic dendritic cell subsets and their participation in cooperative antigen transfer

To discern the contribution of thymic DCs versus other thymic BM APCs to indirect presentation, CD11c-Cre-Rosa26-DTA mouse model was used, in which DCs are depleted by diphtheria toxin A (DTA). When several specificities of self-reactive thymocytes, whose deviation to Treg development is dependent on the presentation of TRAs by BM APCs, were intrathymically injected into DC-depleted mouse, such deviation was completely abrogated (Perry *et al.*, 2014). In addition, the importance of DCs for Treg selection was tested also for polyclonal T cell repertoire. When MHCII expression on DCs was genetically ablated, the frequency of polyclonal Tregs was reduced almost twice compared to the control (Leventhal *et al.*, 2016). Thus, these results provide strong evidence that the thymic selection of tTregs is driven by indirect presentation of mTEC's antigens by DCs. Moreover, the recent analysis of *ex vivo* RIP mOVA/RIP OVA^{HI} thymic slices suggested that thymic DC-mediated indirect presentation is also indispensable for deletional tolerance (Lancaster *et al.*, 2019).

Importantly, using *in vitro* co-cultivation assays with mTECs, it has been shown that tDCs, mDCs as well as pDCs are capable to acquire mTEC-derived MHC molecules. While pDCs were weak in such acquisition, tDCs and to lower extent also mDCs, were efficient in their acquisition and presentation when measured by the activation of T cells (Kroger *et al.*, 2017). By introduction of above mentioned Treg-destined specificities of self-reactive thymocytes into the thymus of Batf3 KO (lack of tDCs) and BDCA2-DTR mice (lack of pDCs), it has been found that Treg selection of half of eight tested and none out of four tested thymocyte specificities, respectively, were negatively affected by such deficiencies. This suggests that both tDCs and mDCs participate in CAT and subsequent indirect presentation of TRAs which leads to Treg selection (Perry *et al.*, 2014). On the other hand, using BM chimera experiment, it was shown that tDCs are much more efficient in CAT of MHCII molecules than mDCs (Perry *et al.*, 2014). This is in line with the above discussed study analyzing *in vitro* MHCII transfer (Kroger *et al.*, 2017). It was also

demonstrated that CAT of MHCII is restricted only to the mature $CCR7^+$ population of tDCs, which were also more efficient in acquiring of mTEC-derived mOVA than immature $CCR7^-$ cells (Ardouin *et al.*, 2016).

As discussed above, mDCs are the major population of thymic DCs which contribute to the generation of Aire-dependent MJ23 tTregs (Leventhal *et al.*, 2016). However, tDCs and mDCs were both found to promote MJ23 tTreg development *in vitro*. Hence, presumably, tDCs and mDCs reciprocally substitute each other in Treg selection when one of these subsets is missing such as tDCs in Batf3 KO mice (Leventhal *et al.*, 2016). Sequencing TCRα repertoire of Tregs in Batf3-sufficient and deficient mice showed that only 12% of Treg specificities is strictly tDC-dependent (Leventhal *et al.*, 2016; Perry *et al.*, 2018) indicating only a minor role for tDCs in tTreg development. Yet, such minor abrogated selection of specific Treg clones in Batf3 KO mice is sufficient to trigger autoinflammatory reaction (Perry *et al.*, 2018). In contrast, it has been shown that CAT in $mTEC^{HI}$-restricted Aire-GFP mouse model (Gardner *et al.*, 2008) occurs mainly to tDCs and with only very limited scope to mDCs (Perry *et al.*, 2018). This discrepancy is likely caused by the fact that tDCs and mDCs interact with mTECs at their distinct developmental stages: tDCs with $mTECs^{HI}$ (Lei *et al.*, 2011; Perry *et al.*, 2018) using CD36 to acquire mTEC-derived antigens (Perry *et al.*, 2018), and mDCs presumably with $mTECs^{LO}$ or post-Aire mTECs via efficient endocytosis (Baba, Nakamoto and Mukaida, 2009; Morimoto *et al*., 2018; Lancaster *et al*., 2019).

Thus, as discussed above, it is the context of TRA production which determines whether it's presentation via CAT is mediated via tDCs or mDCs, and/or directly by mTECs. There are still several crucial questions that remain to be answered: (i) what is the nature of the mechanisms mediating CAT via tDCs or mDCs; (ii) do tDCs and mDCs interact with identical or distinct mTEC subsets and how the mechanism of CAT differs when these DC subsets engage distinct mTEC subsets; (iii) how homo/heterogenous are tDC and mDC subsets functionally and phenotypically; and (iv) what are molecular and/or cellular factors that determine the negative or Treg selection.

Recently, our research group obtained experimental evidence showing that Toll-like receptor (TLR) 9 signaling regulates CAT. Specifically, TLR9 signaling in mTECs triggers the expression of several Aire-independent chemokines. As a result, mDCs rapidly migrate and enrich to medulla and exhibit an enhanced CAT-related potency. At the same time, the number of tDCs in the thymus decreases and the cellularity of Tregs increases. Reversibly, mice with ablated TLR signaling in mTECs suffer from decreased numbers of Tregs. An analogous impact of increased/decreased numbers of mDCs/tDCs on Treg selection has been already reported by two independent studies (Leventhal *et al.*, 2016; Hu *et al.*, 2017). Importantly, such immigrating mDCs were marked by a low MHCII expression (Hu *et al.*, 2017). In addition, using scRNA-seq, we showed that mDCs which migrate to the thymus under TLR9-stimulatory conditions expressed high levels of CX3CR1, CD14, lysozyme 2 and apolipoprotein E, signature genes of monocytes/macrophage lineage.

Extending these studies further, the involvement of monocyte/macrophage markers-expressing mDCs in

CAT, their indirect antigen presentation and their potential function in central tolerance mechanisms, which weren´t specifically studied so far, were experimentally tested in this thesis.

C. MATERIALS AND METHODS

1. Mouse models

All experimental mouse models used in the thesis were bred at the animal facility of the Institute of Molecular Genetics of the ASCR (IMG) under specific-pathogen-free conditions (SPF) set by the Federation of European Laboratory Animal Science Associations (FELASA). Experimental protocols were approved by the Ministry of Agriculture of the Czech Republic and the ethical committee of the Institute of Molecular Genetics. Usually, 4-8 weeks old animals were used. Foxn1-Cre (Gordon et al., 2007) and Rosa26-tdTomato (Madisen et al., 2010) mice were used in the majority of the experiments. CX3CR1-Cre (Yona et al., 2013) and Rosa26-DTA (Voehringer, Liang and Locksley, 2008) mice were used to deplete $CX3CR1^+$ cells. All these models were on C57BL/6J genetic background and were purchased from Jackson Laboratories. mTECs from *A*ire-HA mice (Aschenbrenner et al., 2007) were used for antigen presentation assay as they express HA antigen under the promotor of Aire. This mouse model is on BALB/c genetic background and was kindly provided to us by Dr. Ludger Klein, Institute for Immunology, Ludwig Maximilian University of Munich. MHCII-eGFP knock in mice on C57BL/6J genetic background (Boes et al., 2002), used for visualization of DCs via fluorescent microscopy, were kindly provided by Prof. Jan Černý, Department of Cell Biology, Faculty of Science, Charles University in Prague. The littermates were used as controls, except the case of Foxn1-Cre Rosa26-tdTomato mice, where Foxn1-Cre mice were used.

2. Isolation of thymic antigen-presenting cells

To isolate thymic APCs, the entire thymus was cut into small pieces and treated with Dispase II (Gibco) at concentration 0.1 mg/ml dissolved in RPMI medium (Sigma-Aldrich). To homogenize the tissue, sample was several times smoothly pipetted up and down. After 10 min incubation in thermo-shaker at 37 °C, the supernatant was collected and replaced with fresh Dispase II solution. The procedure was repeated 5-6 times to digest the whole thymus tissue. Enzymatic reaction was stopped using 3% FCS (Sigma-Aldrich) with 2mM EDTA (Gibco) and the sample was spun down (4°C, 300g, 10 minutes). After the final digestion, cells from all fractions were pooled together and resuspended in 3% FCS with 2mM EDTA. A detailed protocol is described elsewhere (Dobeš *et al.*, 2018). To isolate DCs, pooled thymic cells were stained for 30 minutes with anti-CD11c antibody conjugated with biotin (Invitrogen) and $CD11c^+$ cells were then enriched by AutoMACS using anti-biotin microbeads. In the case of TECs isolation, $CD45^-$ fraction was enriched using anti-CD45 microbeads and MidiMACS. To eliminate erythrocytes from the $CD45^-$ fraction, ACK-lysis buffer was used. In some experiments, isolated cells were stained with TCRβ, CD3 and Ter119 antibodies conjugated with biotin (Biolegend) and T cells and erythrocytes were depleted by AutoMACS

using anti-biotin microbeads. If not specified otherwise, chemicals and materials used for MACS enrichment were from Miltenyi Biotec. The cell suspension of interest was resuspended in 3% FCS with 2mM EDTA and subjected to further analysis.

3. T cell isolation protocol

To isolate T cells, whole thymic tissue was mechanistically mashed through 40μm cell strainer (Biologix), the cell suspension was passed through 100μm filter (Sysmex), centrifuged at 4°C, 400g for 10 minutes and resuspended in 3% FSC with 2mM EDTA. Erythrocytes were depleted by ACK-lysis buffer. Approximately three million cells were used for further analysis.

4. Flow cytometry analysis and cell sorting

Fluorescent Activated Cell Sorting (FACS) analyses and cell sorting were performed by using LSRII and BD Influx cytometers (BD Biosciences), respectively. For surface FACS staining, cells were incubated (20-40 minutes) at 4°C with indicated fluorochrome-conjugated monoclonal antibodies (see **Table 1** below). For Foxp3 intracellular staining, after surface molecules staining, the cells were fixed and permeabilized (30 minutes) at room temperature using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and then stained (30 minutes) with anti-Foxp3 monoclonal antibody. Dead cells were excluded using Hoechst 33258 (Sigma) or fixable viability dye eFluor 450 or 506 (eBioscience). For the *in vitro* co-cultivation assays, cells were prepared as described previously and sorted with the use of BD Influx sorter. Fluorescence minus one (FMO) controls were used to set the positivity of antibody staining.

5. Antibodies

TARGET MOLECULE	CLONE	CONJUGATE	DILUTION	MANUFACTURER
B220	RA3-6B2	APC	1:200	Biolegend
CCR3	J073E5	APC	1:200	Biolegend
CD103	2E7	PE/Cy7	1:200	Biolegend
CD11b	M1/70	FITC	1:200	eBioscience
CD11b	M1/70	PE	1:200	eBioscience
CD11c	N418	APC/Cy7	1:200	Biolegend
CD11c	-	APC	1:200	Miltenyi
CD14	Sa2-8	APC	1:100	eBioscience
CD16/32	93	PE/Cy7	1:200	Biolegend
CD19	6D5	PE	1:200	Miltenyi
CD19	6D5	Percp/Cy5.5	1:200	Biolegend
CD25	3C7	PE/Cy7	1:150	Biolegend
CD3	145-2C11	APC	1:100	Biolegend
CD4	Gk1.5	FITC	1:200	EXBIO
CD40	1C10	APC	1:100	eBioscience
CD45	-	Pacific Blue	1:50	in house
CD80	16-10A1	APC	1:100	Biolegend
CD86	GL-1	APC/Cy7	1:150	Biolegend
CD8α	53-6.7	PE	1:400	Biolegend
CX3CR1	SA011F11	PE/Cy7	1:150	Biolegend
CX3CR1	SA011F11	Brilliant violet 421	1:150	Biolegend
EpCAM	G8.8	PE/Cy7	1:3000	Biolegend
F4/80	BM8	APC	1:200	Biolegend
Foxp3	FJk16s	APC	1:200	eBioscience
GR1	RB6-8C5	FITC	1:400	Biolegend
Ly51	6C3	Alexa Fluor 647	1:200	Biolegend
Mgl2	URA1	PE/Cy7	1:200	Biolegend
MHCII	M5/114.15.2	Percp/Cy5.5	1:500	Biolegend
MHCII	2G9	FITC	1:500	BD Pharmigen
PDCA1	927	Percp/Cy5.5	1:200	Biolegend
PD-L1	10F.9G2	PE/Cy7	1:200	Biolegend
Sirpα	P84	PE/Cy7	1:150	Biolegend
Sirpα	P84	APC	1:150	Biolegend
XCR1	ZET	Brilliant violet 421	1:200	Biolegend
XCR1	ZET	APC	1:200	Biolegend

Table 1. Antibodies used for flow cytometry

6. Antigen presentation assay

Antigen presentation assay was performed as described elsewhere (Aschenbrenner et al., 2007). DCs and mTECs were FACS sorted according to the protocol described above. Specifically, general DC population

and CX3CR1$^+$ mDCs were gated as described in Fig. 2A and Fig. 3F, respectively. mTECs were gated as described in Fig. 1C. Cells were sorted directly into DMEM high-glucose medium (Sigma Aldrich) supplemented with 10% FCS (Sigma Aldrich) and 1% Penicillin-Streptomycin (Sigma Aldrich) and cultivated in 96 well plate together with A5 hybridoma reporter cell line in a ratio 1:5 (~30 000 of APCs: ~150 000 of hybridoma cells). As a positive control, CX3CR1$^+$ mDCs were pulsed with HA peptide (107-119; customized by Thermofisher) in a concentration 10 μg/ml. After 17 hours, the level of eGFP expression in A5 hybridomas was analyzed by flow cytometry.

7. Antigen transfer assay analyzed by fluorescence microscopy

DCs and mTECs from MHCII-eGFP and Foxn1-Cre Rosa26-tdTomato, respectively, were FACS sorted according to the protocol described above. tdTomato$^+$ mTECs and eGFP$^+$ DCs were co-cultured in μ-slide 8 well (ibidi), comprising RPMI medium supplemented with 10% FCS and 1% Penicillin-Streptomycin, in a ratio 1:2 (30 000 TECs: 60 000 DCs). The detailed protocol was described elsewhere (Kroger et.al. 2017). After 30 minutes of incubation, potential antigen transfer was visualized by Deltavision widefield fluorescent microscope (Applied Precision) which enables long-term live imaging at physiological conditions (37^{o}C; 5% CO_2 atmosphere). Cells were observed for 96 minutes via 60x/1,42 Plan APO N oil objective (Applied Precision), with snapshots taken every 2 minutes.

8. Data analysis

All flow cytometry data were analyzed using FlowJo version 10 (Tree Star). Microscopic images were processed by Image J (Wayne Rasband; NIH). The graphs and statistical analysis shown here were carried out by GraphPad Prism (GraphPad Software).

D. RESULTS

The contribution of thymic DCs to CAT and their indirect presentation of mTEC-derived antigens is currently a well-established paradigm. However, the participation of other thymic cells and especially BM APCs, such as thymic B cells or thymic macrophages, in these processes has not been until recently explored (Lancaster *et al.*, 2019). The original aim of my thesis was to determine whether BM APCs, distinct from thymic DCs, participate in CAT. However, we failed to observe any contribution of thymic B cells to this process. Furthermore, we were unable to detect any CD11b$^+$ but CD11c$^-$ (an elementary marker of DCs) thymic macrophages participating in CAT. However, unpublished data from our laboratory from scRNA-seq of thymic DCs identified CD11c$^+$ CD11b$^+$ double positive cells, commonly considered as mDCs, which express combination of several monocyte/macrophage markers. Such phenotype points to unknown lineage origin of these cells. Moreover, their capacity to participate in CAT as well as their indirect presentation of antigens have not been studied so far. Thus, we have focused on CD11c$^+$ CD11b$^+$ double positive cells and set forth the following **aims**:

- To confirm that thymic CD11c$^+$ CD11b$^+$ cells (mDCs) express monocyte/macrophage markers on a protein level
- To find out whether and to what capacity the CD11c$^+$ CD11b$^+$ mDCs participate in CAT
- To determine whether CD11c$^+$ CD11b$^+$ mDCs exploit CAT to promote the indirect presentation
- To elucidate whether CD11c$^+$ CD11b$^+$ mDCs are relevant for the establishment of central tolerance

1. Experimental mouse model of cooperative antigen transfer

In order to investigate which thymus-homing cells participate in CAT, we utilized Foxn1-Cre-Rosa26-tdTomato mouse model, where cytoplasmic tdTomato protein is in the thymus exclusively produced by Foxn1-expressing TECs. This model is a perfect tool for studying CAT since it enables a direct detection of cells expressing or retaining tdTomato by FACS or fluorescent microscopy. In the thymus of these mice, all BM-derived cells, which display the positivity for tdTomato can acquire this antigen only by CAT from TECs. (**Fig. 1A**). As shown in **Fig. 1B**, FACS analysis of thymus from Foxn1-Cre-Rosa26-tdTomato model showed that CD45$^-$ cells were marked by significantly higher expression of tdTomato than their CD45$^+$ counterparts, suggesting the production rather than the transfer of tdTomato. Indeed, nearly all these cells also highly expressed EpCAM, a molecular marker of TECs. Further analysis of tdTomato^{LO} CD45$^+$ cell fraction (those cells which acquire tdTomato by CAT) revealed that neither CD3$^+$ cells (T cells), nor GR1$^+$ CD11c$^-$ cells and, importantly, nor thymic B cells, participate in CAT of tdTomato. As expected, tdTomato was transferred mainly to thymic CD11c$^+$ DCs and to unspecified CD45$^+$ cells which were negative for all markers tested (CD19, CD3, CD11c, GR1) (**Fig. 1B**). It is also important to emphasize, that CAT of tdTomato was confirmed by using BM chimera experiment, where lethally irradiated Foxn1-Cre-Rosa26-tdTomato mice were reconstituted by WT BM cells (data done in our laboratory and not shown in this thesis). To test whether tdTomato is expressed by distinct subtypes of TECs (**Fig. 1C**), we carried out flow cytometry experiment where cTECs, mTECs^{LO} and mTECs^{HI} can be distinguished and analyzed for their expression of tdTomato. Nearly 100% of cells from each indicated subset expressed high levels of tdTomato.

Taken together, we found that Foxn1-Cre-Rosa26-tdTomato mouse is a suitable model to study CAT, as tdTomato is expressed by all TECs subtypes and transferred to CD45$^+$ BM-derived cells. Specifically, we detected the tdTomato transfer to DCs (CD11c$^+$ cells) and to other unspecified CD45$^+$ cell population.

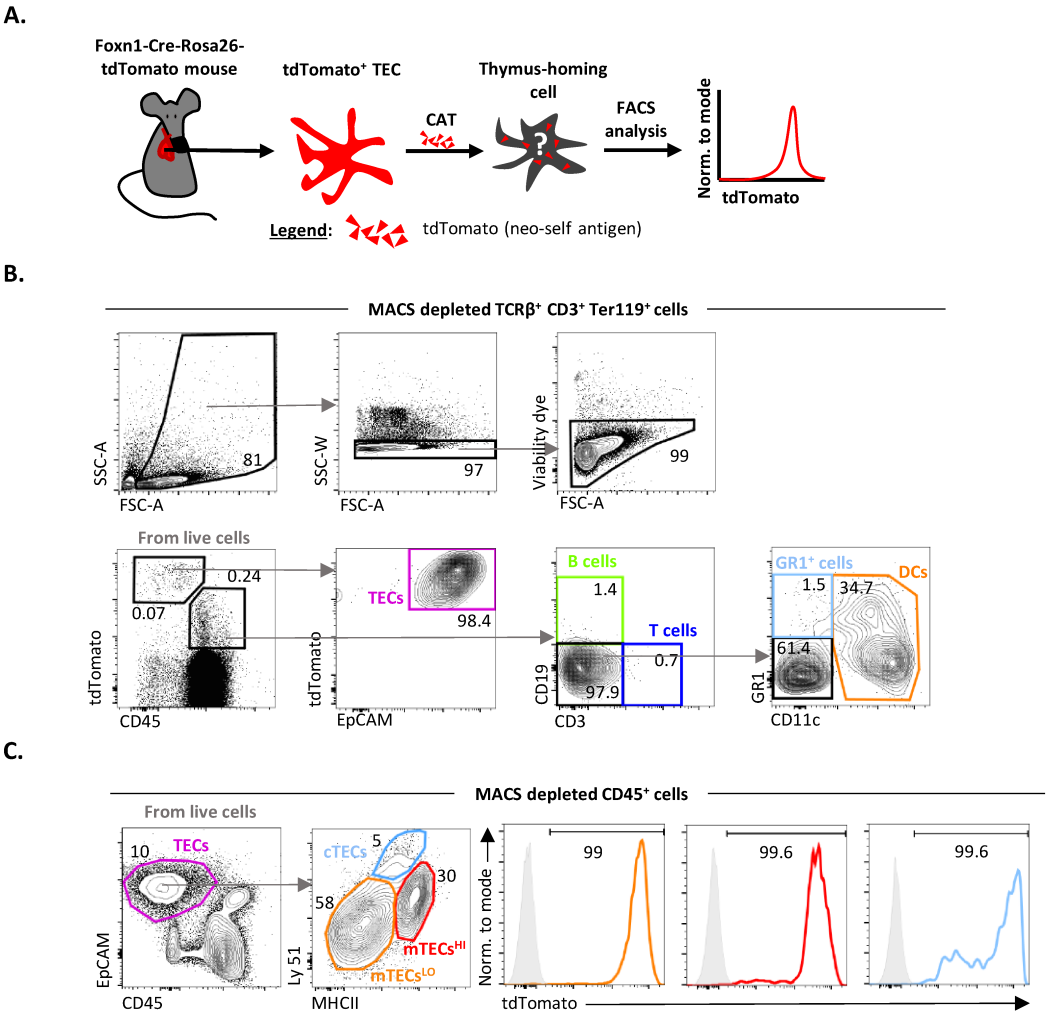


Figure 1. Experimental mouse model of CAT: A. *Design of the experiment using Foxn1-Cre-Rosa26-tdTomato mice* **B.** *Flow cytometry analysis of thymic tdTomato$^+$ cells. Thymus was enzymatically digested and erythrocytes and part of the thymocytes were MACS-depleted according to the Ter119$^+$, TCRβ$^+$ and CD3$^+$ staining. To deplete debris, doublets and dead cells, analyzed cells were gated according to FSC-A, SSC-A, SSC-W and viability dye. Rest of the cells were distinguished according to the tdTomato and CD45 expression. tdTomato^{HI} CD45$^-$ cells were gated according to EpCAM expression as TECs and tdTomato^{LO} CD45$^+$ cells according to CD19 and CD3 positivity as B cells and T cells, respectively. CD19$^-$CD3$^-$ double negative population was further divided by the expression of GR1 and CD11c as GR1$^+$CD11c$^-$ (GR1$^+$ cells) and GR1$^{-/+}$CD11c$^+$ (DCs). Foxn1-Cre mice were used as a negative control for tdTomato positivity. Representative experiment is shown (n=3 mice from 2 independent experiments)* **C.** *Flow cytometry analysis of tdTomato expression level in cTECs, mTECs^{LO} and mTECs^{HI}. Thymus was enzymatically digested and CD45$^+$ cells were MACS-depleted. TECs were gated as live, CD45$^-$EpCAM$^+$ which were further divided according to Ly51 and MHCII expression into cTECs (Ly51^{HI}MHCII$^+$), mTECs^{LO} (Ly51$^-$ MHCII^{LO}) and mTECs^{HI} (Ly51$^+$MHCII^{HI}). Foxn1-Cre mice were used as tdTomato$^-$ control (gray histogram). tdTomato positivity in each subset is visualized by colored histogram.*

2. Contribution of thymic dendritic cells to transfer of tdTomato

Based on the previous experiment (**Fig. 1B**) and several publications (Perry *et al.*, 2014; Leventhal *et al.*, 2016; Lancaster *et al.*, 2019), thymic DCs are considered as major cell population participating in CAT. Since thymic DCs are formed by three major subsets (See the chapter "Thymic dendritic cell subsets"), we decided to further evaluate whether there are any differences in CAT among these subtypes. We utilized commonly used flow cytometry gating strategy to distinguish mDCs, tDCs and pDCs (**Fig. 2A**) and evaluated the frequency of tdTomato$^+$ cells within these cell subsets (**Fig. 2B and C**). Importantly, we found that they all participate in CAT of tdTomato. However, mDCs and tDCs were much more efficient in CAT (close to 50% were tdTomato$^+$) compare to pDCs (on average 20%). This is consistent with a recent study which demonstrates analogous results in the case of MHCII CAT *in vitro* (Kroger *et al.*, 2017).

To confirm that CAT is occurring specifically from mTECs to DCs, we decided to visualize CAT by using fluorescence microscopy and *in vitro* antigen transfer assay. For this purpose, we sorted mTECs from Foxn1-Cre-Rosa26-tdTomato mice according to the protocol described in Fig. 1C and general thymic DC population gated as shown in Fig. 2A. To specifically visualize thymic DCs, the MHCII-eGFP mice were used. tdTomato$^+$ mTECs and eGFP$^+$ DCs were then co-cultivated and images acquired through the sequential scanning by fluorescent microscopy. After 12 minutes of cocultivation, the tdTomato$^+$ particle was released by mTEC and engulfed by eGFP$^+$ DC (**Fig. 2D**). This also confirms that CAT occurs only in one direction from mTECs to DCs, as we weren´t able to detect any eGFP transfer from DCs to mTECs. It is also interesting that CAT in this system is enabled through the engulfment of apoptotic bodies since only mTECs with fragmentized cytoplasm enabled the transfer of tdTomato$^+$ particle. The same phenomenon was also described with the transfer of Eα MHCII molecules (Perry et al., 2018). The MHCII-eGFP mouse model also enables the visualization of tdTomato processing inside the specific DC. Since MHCII molecules localize also inside of the cells, e.g. in the Golgi apparatus and endocytic vesicles (Cresswell, 1994), the co-localization of tdTomato with MHCII eGFP strongly suggests that tdTomato$^+$ particle was endocytosed. Indeed, after its acquisition, tdTomato signal overlapped with the brightest eGFP signal inside thymic DC, most likely with the Golgi apparatus (**Fig. 2D**).

Taken together, we showed that both mDCs and tDCs efficiently acquire TEC-derived tdTomato and that pDCs participate less efficiently in this process. Data from *in vitro* co-cultivation assay suggest that CAT is mediated mostly by the endocytosis of TEC-derived apoptotic bodies.

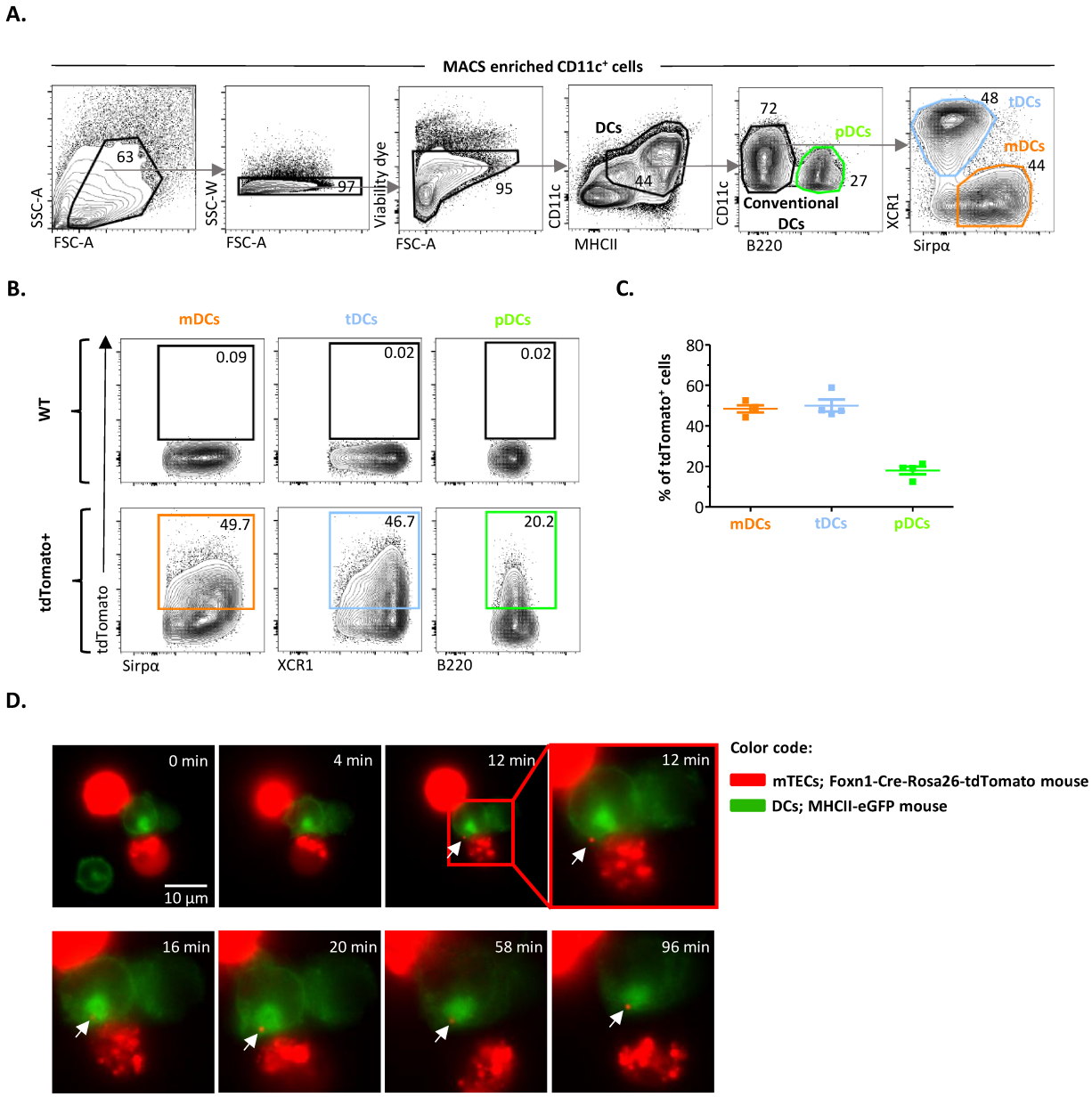


Figure 2. Contribution of thymic DCs to tdTomato transfer: A. *Flow cytometry gating strategy of all three major thymic DC subsets. Thymus was enzymatically digested and cells were MACS-enriched for CD11c$^+$ cells. Enriched cells were gated according to FSC-A, SSC-A, SSC-W and viability dye to separate debris, doublets and dead cells. Total thymic DCs were gated according to CD11c and MHCII expression. According to the expression of B220, DCs were discriminated into conventional DCs (B220$^-$) and pDCs (B220$^+$). Conventional DCs were further distinguished into Sirpα$^+$XCR1$^-$ mDCs and Sirpα$^-$XCR1$^+$ tDCs.* **B.** *Representative flow cytometry plots showing the frequency of tdTomato$^+$ cells within mDCs, tDCs and pDCs, gated according to Fig. 2A, from control Foxn1-Cre mouse (WT) and Foxn1-Cre-Rosa26-tdTomato mouse (tdTomato$^+$).* **C.** *Summarizing graph, related to Fig. 2B, which depicts the frequency of tdTomato$^+$ cells out of mDCs, tDCs and pDCs. n=4 mice from 2 independent experiments, error bars represent ±SEM.* **D.** *Antigen transfer assay. tdTomato$^+$ TECs (Foxn1-Cre-Rosa26-tdTomato mice) and MHCII-eGFP$^+$ thymic DCs (MHCII-eGFP mice) were co-cultivated for 96 minutes and observed by Deltavision widefield fluorescence microscope. White arrows mark the ongoing transfer of tdTomato$^+$ particle. Snapshot of the culture was taken every 2 minutes (48x), hence only representative images are shown.*

3. CX3CR1$^+$ migratory dendritic cells are potent in tdTomato transfer

As shown in **Fig. 1B**, CD11c$^+$ population of CD45$^+$ BM-derived cells isn´t the exclusive acceptor of tdTomato from TECs. To establish whether BM APCs, other than DCs, participate in CAT, we performed unsupervised flow cytometry analysis of all thymic tdTomato$^+$ cells (**Fig. 3A**). We utilized t-SNE algorithm which distinguished these cells according to the expression of MHCII (marks APCs), EpCAM (marks TECs), CD11c (marks DCs), CD11b (marks myeloid cells such as macrophages, monocytes, granulocytes, etc.) and GR1 (marks myeloid cells; not shown). The analysis of obtained clusters showed that the unspecified tdTomato$^+$ CD45$^+$ population of cells observed in **Fig. 1B** does not express MHCII and therefore does not complement BM APCs, which could have a direct role in thymic T cell selection (Perry *et al.*, 2014). Because we were specifically curious about thymic macrophages, which were previously shown to induce negative selection (Guerri *et al.*, 2013), we searched for the overlap between MHCII and CD11b expression within analyzed cell clusters. We found that those tdTomato$^+$ CD11b$^+$ cells which also expressed MHCII, concurrently co-expressed CD11c. By the definition, these cells are cDC2 which are in the thymus referred to as mDCs (See the chapter "Heterogeneity of dendritic cells"). Important conclusion from this part of our study, as demonstrated in **Fig. 3A,** is that the only population of BM APCs, which retains the capacity to acquire tdTomato antigen from TECs, are CD11c$^+$ cells.

As already discussed, our scRNA-seq experiment (data done in our laboratory and not shown in this thesis) show that some mDCs, which produced the highest mRNA levels of CD11c and CD11b, also express monocyte/macrophage markers. Based on this, we focused specifically on CD11c$^+$ CD11b$^+$ APCs and found that those cells are MHCII^{HI} (**Fig. 3B, left plot**). To verify that such APCs are mDCs, we performed flow cytometry experiment in which we analyzed their Sirpα (marker of mDCs), XCR1 (marker of tDCs) and B220 (marker of pDCs) expression (**Fig. 3B, central histograms**). Out of these markers, CD11c$^+$ CD11b$^+$ APCs expressed only Sirpα, which confirms that these cells belong to the mDC population. To verify the monocyte/macrophage phenotype of those CD11c$^+$ CD11b$^+$ cells, we used the CX3CR1 marker that according to the literature marks the mucosal MCs and other cells of monocyte origin (Varol *et al.*, 2009; Yona *et al.*, 2013). We found that more than half of these cells expressed CX3CR1 (**Fig. 3C**). Moreover, in comparison with CX3CR1$^-$ cells, CX3CR1$^+$ mDCs revealed substantially higher expression of other monocyte/macrophage markers, namely CD16/32, CD14, Mgl2 and F4/80. On the contrary, CX3CR1$^+$ as well as CX3CR1$^-$ mDCs showed negativity for GR1. Both mDC subsets were tested also for the expression of CD103, previously found to mark cDCs potent in CAT (Koble and Kyewski, 2009). The expression of CD103 was detected only in a very limited portion of both CX3CR1$^-$ and CX3CR1$^+$ mDC subsets (**Fig. 3D**).

Since macrophages are generally considered to be the most potent cells in the endocytosis of apoptotic bodies, we proposed that also CAT, which seems to occur by this process (**Fig. 2D**), will be enhanced in CX3CR1$^+$ mDCs. To test this, we specifically gated on thymic mDCs and compared the frequency of

tdTomato$^+$ cells between CX3CR1$^+$ and CX3CR1$^-$ mDCs. As expected, the frequency of tdTomato$^+$ cells was enriched in CX3CR1$^+$ mDCs compared to their CX3CR1$^-$ counterparts (**Fig. 3E and F**).

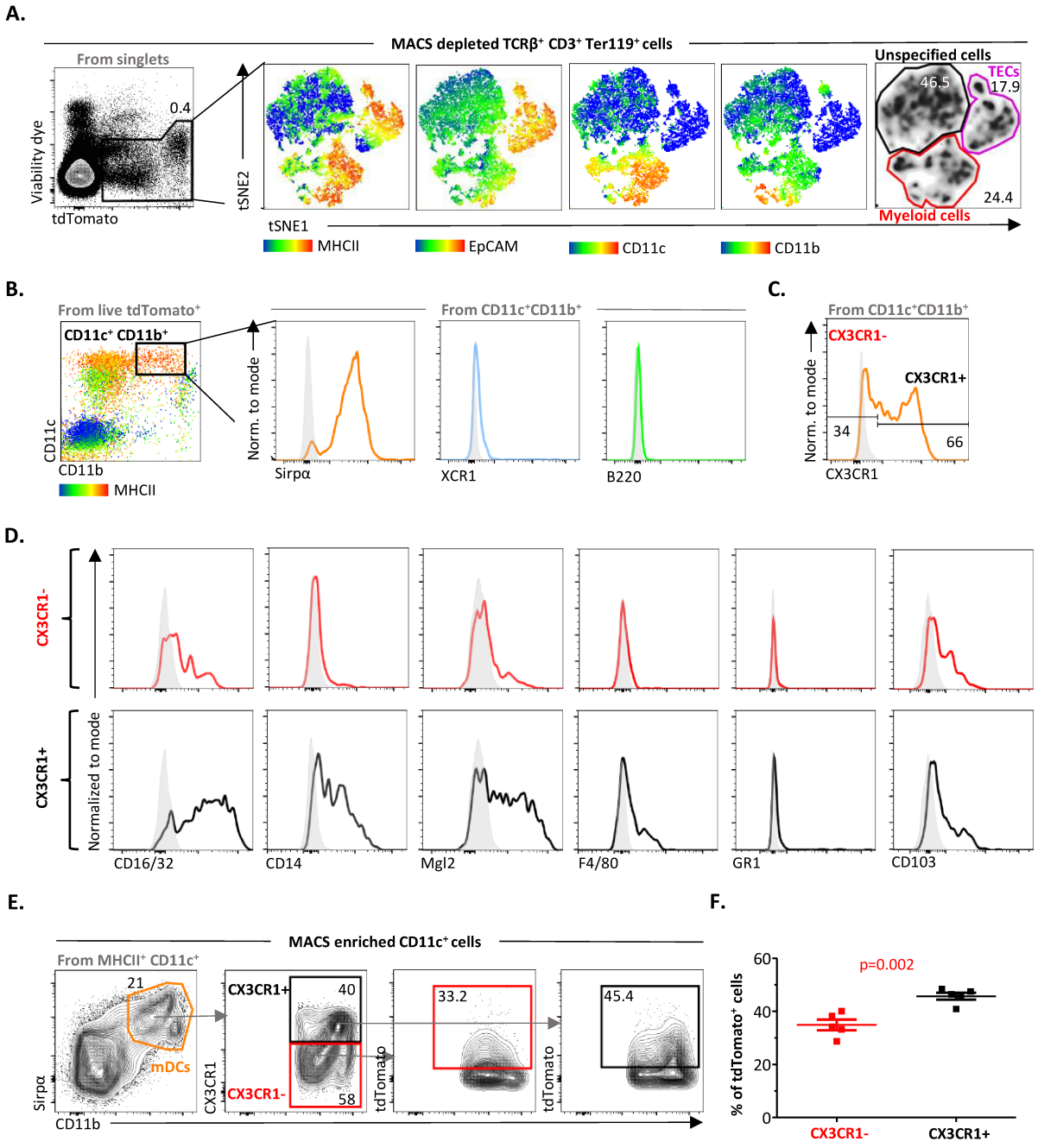


Figure 3. CX3CR1$^+$ mDCs are potent in tdTomato transfer: A. *Unsupervised flow cytometry analysis of tdTomato$^+$ cells. Thymic cells were MACS-depleted of TCRβ$^+$ CD3$^+$ Ter119$^+$ cells and gated as live and tdTomato$^+$. Distribution of tdTomato$^+$ cells into clusters was performed by t-SNE algorithm, according to the expression of EpCAM, MHCII, CD11c, CD11b and GR1 (not depicted). The expression of these molecules within cell clusters is visualized by heatmaps. Density plot demonstrates the distribution of three major populations in the t-SNE analysis. MHCII$^-$EpCAM$^-$CD11c$^-$CD11b$^-$ cells represent "unspecified cells" (black), MHCII$^+$EpCAM^{HI}CD11c$^-$ CD11b$^-$ represent TECs (purple) and MHCII$^+$EpCAM$^{-/+}$CD11c$^+$ CD11b$^{-/+}$ are myeloid cells (red).* **B.** *Flow cytometry plots showing expression of DC markers (MHCII, Sirpα, XCR1 and B220) by CD11c$^+$CD11b$^+$ population. The level of MHCII expression is visualized by heatmap. FMO controls for each marker are depicted in gray.* **C.** *Representative flow cytometry histogram showing the level of CX3CR1 expression within CD11c$^+$CD11b$^+$ double positive population. CX3CR1*

positivity was set according to the FMO control (gray histogram). CX3CR1$^-$ cells are depicted in red and CX3CR1$^+$ are shown in black. n=5 mice from 5 independent experiments. **D.** *The expression of monocyte/macrophage markers (CD16/32, CD14, Mgl2, F4/80 and GR1) and cDC marker CD103 in CX3CR1$^-$ and CX3CR1$^+$ mDCs visualized by histograms. The positivity was set in each case according to FMO controls (gray histograms).* **E.** *Representative flow cytometry analysis of the frequency of tdTomato$^+$ cells within CX3CR1$^-$ and CX3CR1$^+$ mDCs. Analyzed cells were gated as mDCs (Sirpα$^+$CD11b$^+$) and further distinguished according to the CX3CR1. tdTomato positivity was set according to the negative control (Foxn1-Cre mice).* **F.** *Summarizing graph, related to Fig. 3E, which demonstrates the frequency of tdTomato$^+$ cells within CX3CR1$^-$ and CX3CR1$^+$ mDCs. n=5 mice from 3 independent experiments, error bars represent ±SEM. Statistical analysis was performed by unpaired t-test, p ≤ 0.01=**, p ≤ 0.001=***.*

Taken together, these results demonstrate that among the all BM APC subtypes, only CD11c$^+$ DCs participate in CAT of tdTomato. On the other hand, we also revealed that subtype of mDCs which is marked by CX3CR1 expresses also other markers associated with monocyte/macrophage lineages, pointing to its monocyte-derived origin. Importantly and functionally relevant to CAT, these CX3CR1$^+$ mDCs were shown to be very potent in the acquisition of tdTomato from TECs.

4. CX3CR1$^+$ migratory dendritic cells are capable of indirect presentation

To find out whether CX3CR1$^+$ mDCs are capable to indirectly present the antigens acquired by CAT, we first tested the costimulatory properties of these cells. By using flow cytometry analysis, we found that they, along with the expression of MHCII (**Fig. 3B and Fig. 4A**), express high levels of costimulatory molecules CD80, CD86, CD40 and PD-L1, which predestine them to be very potent APCs (**Fig. 4A**). To test the indirect presentation capacity, we sorted out CX3CR1$^+$ mDCs (gating strategy shown in Fig. 3E) from Aire-HA mouse, where HA, produced under the promotor of Aire, is in the thymus exclusively expressed by mTECs^{HI} and co-cultivated them with A5 T cell hybridomas (further referred to as A5 hybridomas) (Aschenbrenner et al., 2007). This NFAT-GFP cell line bears the TCR specific for HA and its activation induces the expression of GFP (**Fig. 4B**). By using this approach, we found that about 40% of A5 hybridomas were activated after indirect presentation of HA by CX3CR1$^+$ mDCs (**Fig. 4C right histograms and Fig. 4D**). Importantly, however, the activation of A5 hybridomas by CX3CR1$^+$ mDCs was lower than in the case of direct presentation by mTECs^{HI} (~98%) or indirect presentation by the whole thymic DC population (~60%) (**Fig. 4C left histograms and Fig. 4D**). This suggests that other DC-populations are more potent in indirect presentation of HA. To verify that CX3CR1$^+$ mDCs are capable to present also exogenous antigens to developing thymocytes, we sorted out those cells from HA$^-$ (WT) mice, pulsed them with HA peptide and co-cultivated them with A5 hybridomas. This experimental approach demonstrated that the presentation of exogenous peptide by CX3CR1$^+$ mDCs activated about 80% of A5 hybridomas (**Fig. 4C right histograms and Fig. 4D**).

Taken together, we found that CX3CR1$^+$ mDCs are potent antigen presenting cells, since they reveal high expression of MHCII and costimulatory molecules. We also show that those cells are capable of indirect presentation of mTEC-derived HA to TCR-HA expressing T cell line.

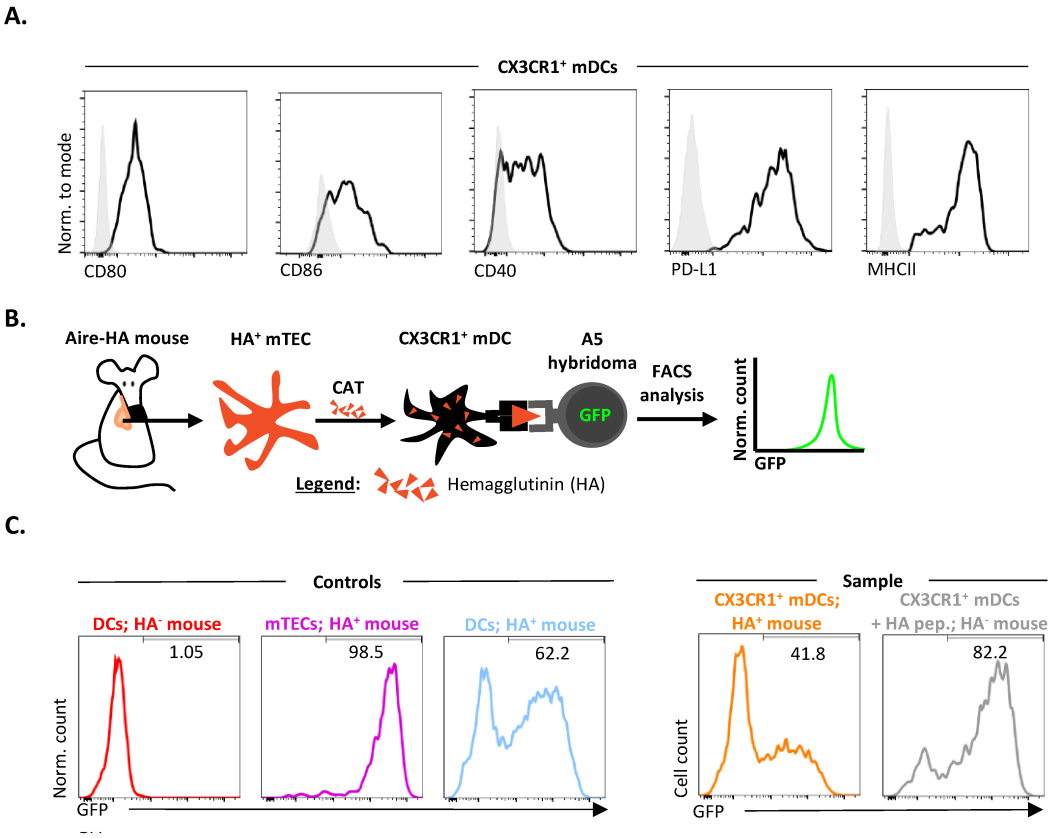


Figure 4. CX3CR1$^+$ mDCs are capable of indirect presentation: **A.** *Flow cytometry analysis of costimulatory molecules and MHCII expression by CX3CR1$^+$ mDCs. Cells were isolated as shown in Fig. 3E and analyzed for the expression of CD80, CD86, CD40, PD-L1 and MHCII. The positivity on each molecule analyzed was set according to FMO controls depicted in gray.* **B.** *Design of the experiment using Aire-HA mice and A5 T cell hybridomas.* **C.** *Indirect presentation of HA by CX3CR1$^+$ mDCs tested via antigen presentation assay. Representative flow cytometry histograms of GFP expression in A5 hybridomas after their co-cultivation with particular cell type: general thymic DCs (sorted as in Fig. 2A) from Aire-HA$^-$ mice (red, negative control), mTECs (sorted as in Fig. 1C) from Aire-HA$^+$ mice (purple, positive control), general thymic DCs (sorted as in Fig. 2A) from Aire-HA$^+$ mice (blue, positive control), CX3CR1$^+$mDCs (sorted as in Fig. 3E) from Aire-HA$^+$ mice (orange) and CX3CR1$^+$ mDCs (sorted as in Fig. 3E) from Aire-HA$^-$ mice pulsed with 107-119 HA peptide (gray, positive control).* **D.** *Summarizing graph, related to Fig. 4C, which demonstrates the frequency of GFP expressing A5 hybridomas. Color code used here corresponds to Fig. 4C. n=3 samples from single experiment.*

5. CX3CR1$^+$ migratory dendritic cells are depleted in CX3CR1-Cre-Rosa26-DTA mouse model

The results shown in the previous figures (**Fig. 4A, C and D**), demonstrate that CX3CR1$^+$ mDCs are very potent for induction of central tolerance mechanisms. To evaluate their dispensability for this process, we depleted CX3CR1$^+$ mDCs by crossing the CX3CR1-Cre with Rosa26-DTA mice (**Fig. 5A**). In offsprings of these mice, CX3CR1$^+$ cells should be selectively depleted with an active Cre-recombinase under the CX3CR1 promotor. To verify this model, we compared the frequency of all major thymic BM-derived populations between CX3CR1-Cre-Rosa26-DTA and CX3CR1-Cre$^-$ (control) littermates (**Fig. 5B and C**).

Clearly, we saw a dramatic decrease mainly in the frequency of CX3CR1$^+$ mDCs accompanied by the increased frequency of both XCR1$^+$ CX3CR1$^-$ DCs and XCR1$^-$ CX3CR1$^-$ DCs in CX3CR1-Cre-Rosa26-DTA mice, indicating that both tDCs and CX3CR1$^-$ mDCs were unaffected by DTA introduction. We also observed an increase in pDC compartment in majority of the CX3CR1-Cre-Rosa26-DTA mice (**Fig. 5B**). To analyze alterations in thymic cell composition more precisely, we compared the relative numbers of cells from CX3CR1-Cre-Rosa26-DTA and CX3CR1-Cre$^-$ controls (gated as in **Fig. 5B)**. As expected, we observed the significant decrease only in cell populations which expressed CX3CR1. Specifically, we found almost complete depletion of CX3CR1$^+$ mDCs and reduced numbers of GR1$^+$CX3CR1$^+$ double positive cells (**Fig. 5C**). Remarkably, the numbers of CD3$^+$ T cells showed increased tendency in CX3CR1-Cre-Rosa26-DTA mice (**Fig. 5C, first graph**).

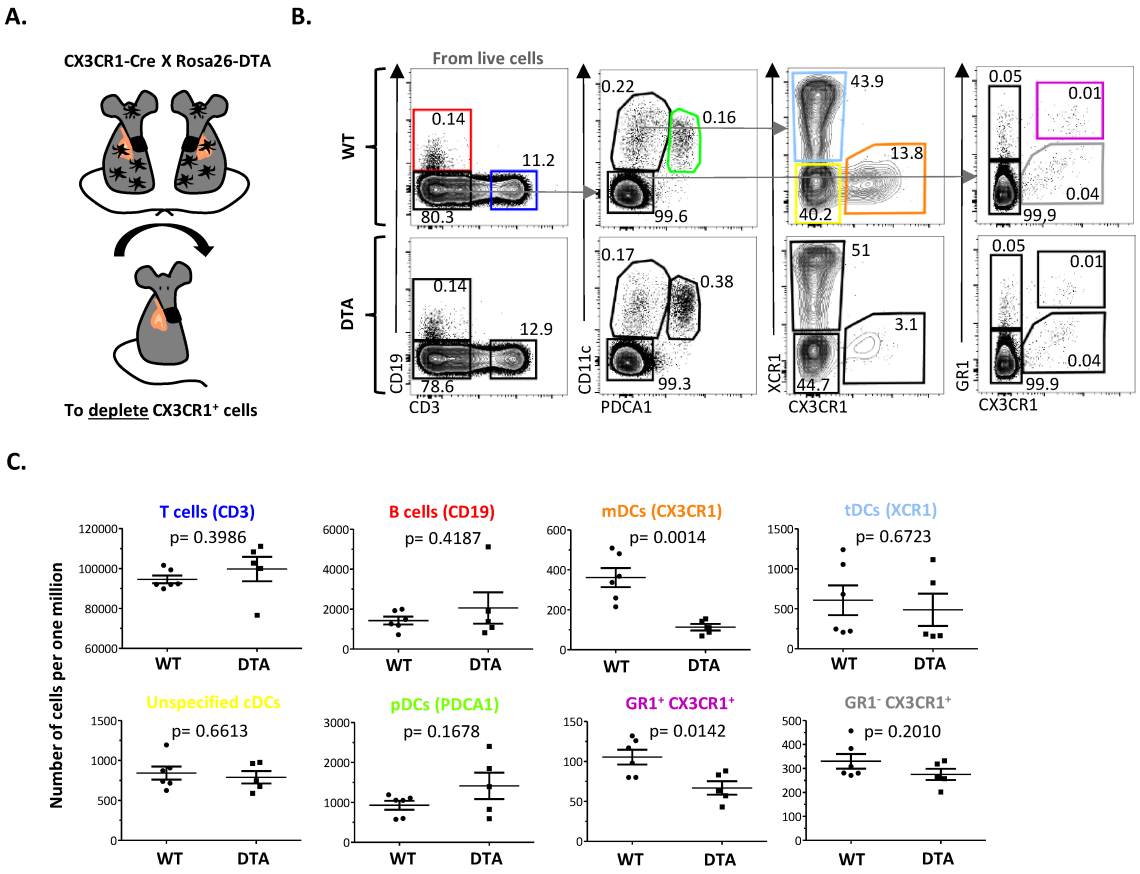


Figure 5. CX3CR1$^+$ mDCs are depleted in CX3CR1-Cre-Rosa26-tdTomato mouse model: A. *Crossbreeding scheme of CX3CR1-Cre and Rosa26-DTA mice.* **B.** *Representative flow cytometry analysis of cell composition in DTA (CX3CR1-Cre$^+$-Rosa26-DTA) and WT (CX3CR1-Cre$^-$-Rosa26-DTA, littermate control) mice. Cells were gated as live (see Fig. 1B) and subsequently divided into T cells (CD3$^+$CD19$^-$, blue), B cells (CD3$^-$CD19$^+$, red), conventional DCs (CD11c$^+$PDCA1$^-$), pDCs (CD11c$^+$PDCA1$^+$, green) and CD11c$^-$PDCA1$^-$ population. Conventional DCs were discriminated as tDCs (XCR1$^+$CX3CR1$^-$, light blue), CX3CR1$^+$mDCs (XCR1$^-$CX3CR1$^+$, orange) and unspecified cDCs (XCR1$^-$CX3CR1$^-$, yellow). CD11c$^-$PDCA1$^-$ population was further gated according to the expression of GR1 and CX3CR1 as GR1$^+$ CX3CR1$^+$ (purple), GR1$^-$CX3CR1$^+$ (gray) and GR1$^-$CX3CR1$^-$.* **C.** *Summarizing graphs demonstrating relative numbers of cells (per 1 million), from WT and DTA mice gated as in Fig. 5B. Color code used here corresponds to figure 5B. WT: n=6 mice, DTA: n=5 mice from 3 independent experiments, error bars represent ±SEM. Statistical analysis was performed by unpaired t-test, p ≤ 0.05= *, p ≤ 0.01= **, p ≤ 0.001= ***.*

Taken together, we determined that CX3CR1-Cre-Rosa26-DTA mice represent a suitable experimental model for specific depletion of CX3CR1$^+$ mDCs. Reduced numbers of these cells led to the increased numbers of total CD3$^+$ T cells, suggesting that depletion of CX3CR1$^+$ mDCs may affect the negative selection of developing thymocytes.

6. CX3CR1$^+$ migratory dendritic cells play a role in the negative selection of T cells

Previous studies have shown that indirect presentation of mTEC-derived antigen, specifically by mDCs, is crucial for negative selection of self-reactive thymocytes (Lancaster *et al.*, 2019) and for the generation of tTregs (Leventhal *et al.*, 2016). Also, as shown in the previous figure, the depletion of CX3CR1$^+$ subtype of mDCs resulted in increased numbers of CD3$^+$ T cells in the thymus (**Fig. 5C**). This prompted us to enumerate the relative number of all thymic T cell subsets in CX3CR1-Cre-Rosa26-DTA mice in comparison with CX3CR1-Cre$^-$ control mice. As a result, the flow cytometry analysis of DN, DP, CD8$^+$ and CD4$^+$ T cell subsets (**Fig. 6A**) revealed increasing trend in numbers of CD8$^+$ and CD4$^+$ SP thymocytes (**Fig. 6B, lower graphs**). This, together with increased numbers of CD3$^+$ T cells in CX3CR1-Cre-Rosa26-DTA (**Fig. 5C**), suggests that CX3CR1$^+$ mDCs are involved in the negative selection of SP thymocytes.

To reveal whether depletion of CX3CR1$^+$ cells affected also the development of Tregs, we enumerated the relative numbers of either mature Foxp3$^+$CD25$^+$ Tregs or its both immature populations, Foxp3$^+$CD25$^-$ and Foxp3$^-$CD25$^+$ (**Fig. 6C**), which have the potential to give rise to mature tTregs (Owen *et al.*, 2019). Even though the general population of mDCs was shown to contribute to tTregs selection (Leventhal *et al.*, 2016; Hu *et al.*, 2017), we failed to observe any significant differences in numbers of tTregs population and its precursor subsets in CX3CR1-Cre-Rosa26-DTA mice compared to CX3CR1-Cre$^-$ control mice (**Fig. 6D**). This suggests that CX3CR1$^+$ mDCs are not involved in the selection of tTregs.

Taken together, these results indicate that CX3CR1$^+$ mDCs contribute rather to negative selection of SP CD8$^+$ and CD4$^+$ T cells than to the generation of tTregs.

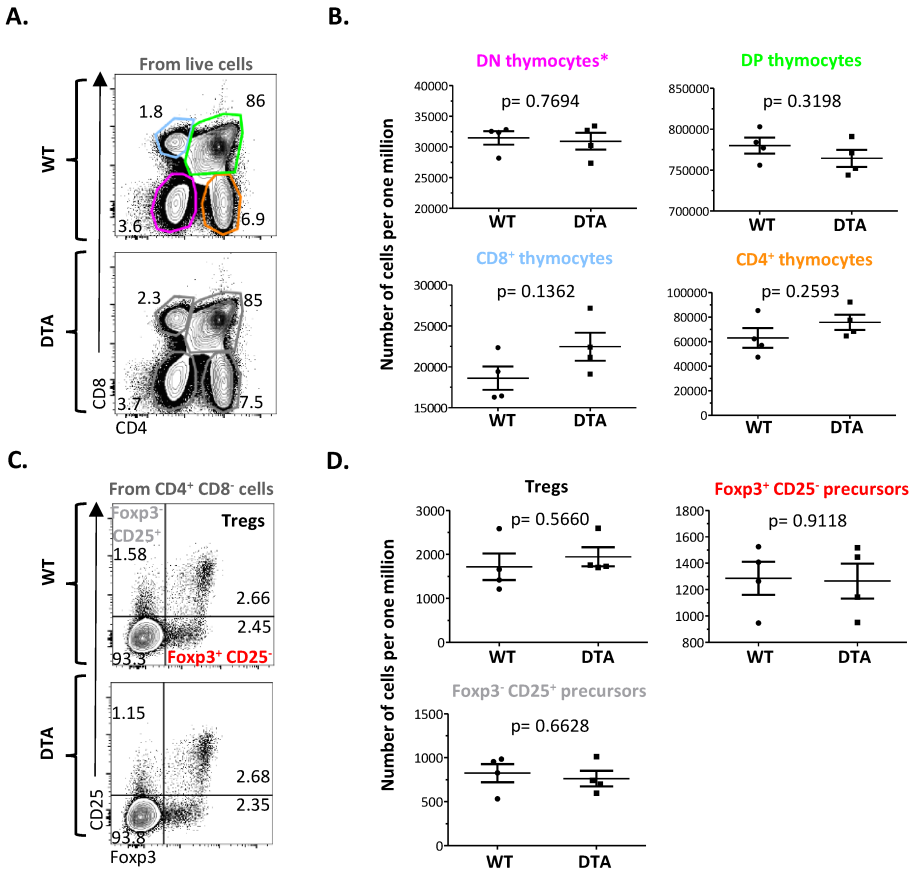


Figure 6. CX3CR1$^+$ mDCs play a role in the negative selection of T cells: A. *Representative flow cytometry analysis of T cells in DTA (CX3CR1-Cre-Rosa26-DTA) and WT (CX3CR1-Cre$^-$ littermate control) mice. Thymus was mashed and live cells (gated as in Fig. 1B) were divided according to the expression of CD4 and CD8 to DN thymocytes (CD4$^-$CD8$^-$, purple), DP thymocytes (CD4$^+$CD8$^+$, green), CD8$^+$ thymocytes (CD4$^-$CD8$^+$, blue) and CD4$^+$ thymocytes (CD4$^+$CD8$^-$, orange).* **B.** *Summarizing graphs demonstrating relative number (per 1 million) of cells, from WT and DTA mice gated as in Fig. 6A. Color code corresponds to Fig. 6A. n=4 mice from 2 independent experiments, error bars represent ±SEM.* **C.** *Representative flow cytometry analysis of Tregs and their precursors in DTA (CX3CR1-Cre-Rosa26-DTA) and WT (CX3CR1-Cre$^-$ littermate control) mice. Cells were prepared and gated as CD4$^+$ CD8$^-$ according to Fig. 6A and further separated by the expression of Foxp3 and CD25 into Tregs (Foxp3$^+$CD25$^+$, black), Foxp3$^-$CD25$^+$ precursors (gray) and Foxp3$^+$CD25$^-$ precursors (red).* **D.** *Summarizing graphs demonstrating relative number (per 1 million) of Tregs, Foxp3$^-$CD25$^+$ precursors and Foxp3$^+$CD25$^-$ precursors, from WT and DTA mice gated as in Fig. 6C. n=4 mice from 2 independent experiments, error bars represent ±SEM. Statistical analysis was performed by unpaired t-test, $p \leq 0.05$= *.*

E. DISCUSSION

Although the role of different subtypes of DCs in the processes of indirect antigen presentation and CAT has been extensively investigated in several recent studies (Perry *et al.*, 2014; Leventhal *et al.*, 2016; Kroger *et al.*, 2017; Perry *et al.*, 2018), the contribution of other BM APCs to these processes is still incompletely understood (Lancaster *et al.*, 2019). In this study, using Foxn1-Cre-Rosa26-tdTomato mouse model, we made several observations which provided new insight into the process of indirect antigen presentation and CAT in the thymus. First, we confirmed the results of a recently published study showing that only CD11c-

expressing cells are able to perform CAT from TECs (Lancaster *et al.*, 2019). Second, introducing a novel approach by combining an *in vitro* antigen transfer assay with advanced fluorescent microscopy, we were able, for the first time, to visualize the transfer of antigen (tdTomato) from mTECs to DCs. Third, we identified the subtype of thymic Sirpα^{+} cells which expresses CX3CR1 and several other signature markers associated with monocyte/macrophage lineage. Using CX3CR1-Cre-Rosa26-DTA mice, we also showed, that the depletion of CX3CR1-expressing cells in the thymus results in increased numbers of SP T cells in the thymus, indicating an impact on the mechanism of negative selection.

The fact, that we were not able to detect any other "CAT-participating" thymic BM APC population than CD11c^{+} DCs (**Fig. 1B and 3A**), was corroborated by recently published study (Lancaster *et al*., 2019). The authors demonstrated that thymic B cells as well as thymic CD11b^{+} F4/80^{+} macrophages were not capable to present the mTEC-derived OVA to OT-I or OT-II T cells. Although, it has been shown that thymic CD11b^{+} F4/80^{+} macrophages are important for negative selection of OT-II thymocytes *in vitro* (Guerri *et al*., 2013), these cells in steady-state conditions do not express MHCII and thus are not able to indirectly present mTEC-derived antigens to developing thymocytes (Lancaster *et al*., 2019).

On the other hand, in Foxn1-Cre-Rosa26-tdTomato model, in addition to thymic DCs, we detected a population of further unspecified CD45^{+} cells which acquire tdTomato (**Fig. 1B and 3A**). As thymus contains DN and DP population of thymocytes, which could not be stained by neither anti-CD3 nor anti-TCRβ antibodies, we predict that the population of tdTomato^{+} CD45^{+} MHCII$^{–}$ CD11c$^{–}$ cells represents mostly these immature T cells. This prediction is also supported by the fact, that both surface and cytosolic molecules were shown to be transferred from T cells to TECs, likely during the formation of the immunological synapse (Wang, Qiu and Zhong, 2016). Since these cells don´t express MHCII molecules (**Fig. 3A**) and costimulatory molecules (data not shown), we excluded them from further experimental setup, as without these critical surface molecules they can't be considered as potent APCs suitable for indirect presentation.

Our data, using Foxn1-Cre-Rosa26-tdTomato mouse model, clearly demonstrate that all subtypes of thymic DCs are capable to acquire the cytosolic tdTomato from TECs (**Fig. 2B**). These data contradict several previously reported observations where the transfer of surface MHCII molecules or intracellular GFP was limited only to tDCs or mDCs (Perry *et al.*, 2014). Moreover, in some cases, for example when Aire-GFP mouse model is used, the GFP was transferred predominantly to tDCs population (Perry et al. 2018). However, this is inconsistent with our observation from Foxn1-Cre-Rosa26-tdTomato model where tDCs and mDCs showed comparable participation in CAT (**Fig. 2B and C**). We suggest that such discrepancy is a direct consequence of the way how and by which TEC subsets are such neo-self-antigens produced. While the expression of Aire-GFP is restricted specifically to mTECs^{HI}, tdTomato in Foxn1-Cre-Rosa26-tdTomato mice is produced by all Foxn1-expressing TECs. Given that due to XCR1/XCL1 chemokine axis are tDCs specifically localized to the proximity of mTECs^{HI} (Lei *et al.*, 2011), it seems that this "position

effect" determines their predominant uptake of GFP in Aire-GFP mice (Perry *et al.*, 2018). On the other hand, tdTomato which is expressed by all TECs subtypes, including $mTECs^{LO}$ or cTECs, is readily accessible to all thymic DCs populations. This explanation can adequately reason that, even though $CX3CR1^{+}$ mDCs are very potent APCs (**Fig. 4A, C and D**), their indirect presentation of HA is less efficient than that observed by general thymic DCs (**Fig. 4C and D**). Since the expression of HA is driven by Aire promotor (same as in the case of Aire-GFP mouse model), the production of HA is restricted mostly to $mTECs^{HI}$ population and HA is mostly accessible to tDCs. It is also important to emphasize, that indirect presentation of HA by general $CD11c^{+}$ $MHCII^{+}$ thymic DCs was previously reported in another study (Aschenbrenner *et al.*, 2007). Compared to our results where almost 60% of thymic DCs present the HA to A5 hybridomas (**Fig. 4C and D**), they showed only limited indirect presentation capacity of such DCs (about 5%). As they also demonstrated much lower activation of A5 hybridomas by mTECs (20% to our 95%) (**Fig. 4C and D**), we assume that this discrepancy is likely caused by some unaccounted technical differences in experimental setups.

One of the caveats of the Foxn1-Cre-Rosa26-tdTomato mouse model is that tdTomato is expressed not only by mTECs but also cTECs (**Fig. 1C)**. Even though all populations of thymic DCs are considered to predominantly reside in thymic medulla (Sanos *et al.*, 2011; Hu *et al.*, 2015), it was also reported that mDCs, which migrate to the thymus through the bloodstream, could be found in its cortical regions (Baba, Nakamoto and Mukaida, 2009). Therefore, we could not formally exclude the possibility that the transfer of tdTomato to mDCs is mediated by cTECs. However, this is a highly unlikely scenario since cTECs do not drive PGE of TRAs (Danan-Gotthold *et al.*, 2016) and CAT from cTECs to donor cells has not been reported so far. Thus, the potential role of cTECs in CAT and consequent indirect antigen presentation originating in these cells is at present time being largely ignored.

It was previously described that the transfer of surface antigens from mTECs specifically to tDCs is promoted by endocytosis of apoptotic bodies through the scavenger receptor CD36 (Perry *et al*., 2018). Our visualization of *in vitro* antigen transfer assay also suggested that tdTomato is acquired by a similar mechanism (**Fig. 2D**). However, CD36 is important only for the transfer of cell-surface antigens (Perry *et al*., 2018). Therefore, the transfer of cytosolic tdTomato, from Foxn1-Cre-Rosa26-tdTomato mice, as observed in our experiment, is more likely CD36-independent. While the elucidation of an underlying mechanism of CAT await its resolution, based on our own observations and published data, we speculate that only apoptotic mTECs were able to transfer the tdTomato to DCs. Notably, we observed a series of direct contacts between thymic DCs and mTECs (**Fig. 2D, unpublished data**), but only those contacts, where mTECs with fragmentized cytoplasm were present, resulted in tdTomato transfer to DCs (**Fig. 2D**). In addition, mTECs, especially those which express Aire, undergo rapid apoptosis. The reason behind this is that Aire and its binding partners initiate TRA PGE by multiple double strand brakes in DNA (See the chapter "Aire-dependent mechanisms of promiscuous gene expression"), inducing apoptosis in mTECs within several days (Gray *et al*., 2007). Finally, it is worth to emphasize that our *in vitro* antigen transfer

assay possesses also several technical caveats. Specifically, as the cells were observed by widefield and not by confocal microscopy we cannot be sure that tdTomato$^+$ particle is localized inside of the thymic DC. The only indirect evidence in support of this assumption is that the particle moves concurrently with thymic DC and it localizes in the proximity of the brightest MHCII-eGFP signal, which likely represents the vesicular apparatus of thymic DC (**Fig. 2D**).

Originally, DCs accommodated in the thymus are subdivided based on their phenotype, origin and function to the three major subpopulations: B220$^+$ pDCs, XCR1$^+$ Sirpα$^-$ tDCs and XCR1$^-$ Sirpα$^+$ mDCs (Li *et al.*, 2009) (**Fig. 2A**). It is also generally accepted, that both tDCs and mDCs could be further distinguished by the exclusive expression of CCR7 and CD11b, respectively (Satpathy *et al.*, 2012; Ardouin *et al.*, 2016). Using unsupervised flow cytometry analysis, we have realized that CD11b$^+$ mDCs in the thymus are much more heterogeneous population than previously thought (**Fig. 3A**). Specifically, we found that CD11b$^+$ mDCs could be subdivided according to the expression of CX3CR1 to CX3CR1$^-$ and CXCR1$^+$ mDCs (**Fig. 3C**). The chemokine receptor CX3CR1 was shown to be mainly expressed by several myeloid populations including mucosal MCs (Varol *et al.*, 2009; Yona *et al.*, 2013). As already discussed, MCs phenotypically and functionally overlap with mDCs (cDC2), since they are potent in antigen presentation and express high levels of MHCII, CD11c and CD11b. However, these cells also share some features with "typical" macrophages (Satpathy *et al.*, 2012; Guilliams *et al.*, 2014; Gross, Salame and Jung, 2015). We also found that these CXCR1$^+$ mDCs are enriched for several signature markers associated with monocyte/macrophage lineage, such as the expression of CD16/32, CD14 or F4/80 (**Fig. 3D**). These data suggest, that thymic Sirpα$^+$ mDCs that express CX3CR1 are of different origin than "classical" cDC2 and most likely represent a novel population of thymic MCs. This notion is further corroborated by the fact that mDCs with monocyte/macrophage signature markers didn´t express gene encoding FLT3L receptor (expressed by "DC-specific" common DC precursor (CDP), Scheme 4) in our scRNA-seq analysis (data not shown). However, since the phenotype of those subclasses of myeloid cells is highly tissue specific (Varol *et al.*, 2009; Gross, Salame and Jung, 2015), the further experiments, using for example lineage-tracing systems, would be needed to delineate the exact origin of these cells.

To investigate the relevance of CX3CR1$^+$ mDCs for the establishment of central tolerance, we utilized CX3CR1-Cre-Rosa26-DTA mice. We found this experimental model specific, since the vast majority of CX3CR1$^+$ mDCs was depleted from the thymus of these mice (**Fig. 5B and C**). However, since CX3CR1 was shown to be expressed early during the ontogeny by MDP (the precursor of DCs, monocytes and some macrophages), the Cre-recombination in CX3CR1-Cre-Rosa26-DTA mice should occur in all MDP descendants (Yona *et al.*, 2013). To test this prediction, we crossed CX3CR1-Cre mice with Rosa26-tdTomato to generate the reporter system (data not shown). As expected, all CX3CR1$^+$ mDCs, and a substantial fraction of CX3CR1$^-$ mDCs, tDCs and pDCs also was found to express tdTomato, suggesting their MDP origin. However, this was largely inconsistent with the fact, that although the DTA and tdTomato are produced from the same Rosa26 locus, the DTA-mediated depletion was specific only to CX3CR1-

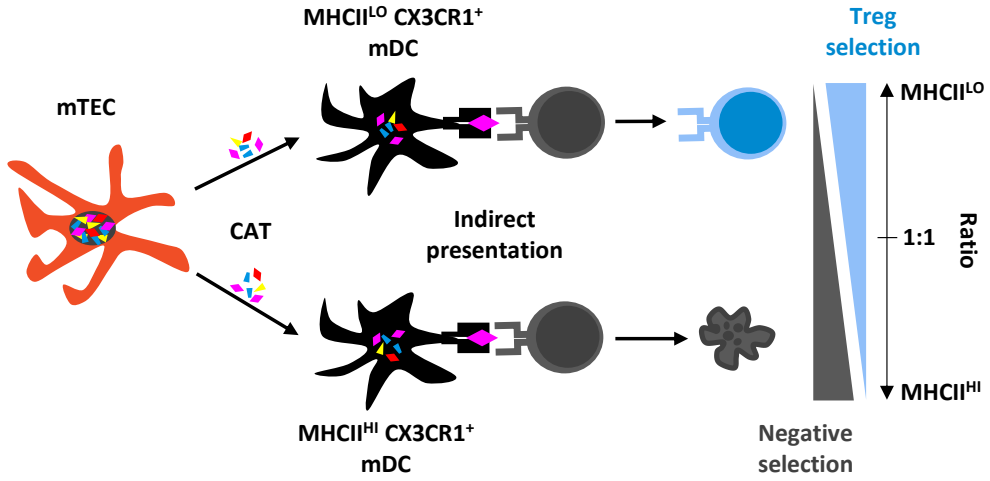
expressing cells (**Fig. 5B and C**)**.** We postulate, that due to a limited CX3CR1-Cre-driven recombination in tDCs and pDCs, the thymus still harbors undeleted cells or their precursors, which replenished the lost portion of these populations. This could happen due to the several reasons. Either tDCs, same as pDCs (Rodrigues *et al.*, 2018), are derived from different progenitors that never expressed CX3CR1, or the recombination capacity driven by CX3CR1-Cre transgene is insufficient to deplete all tDCs or pDCs and/or their progenitors. Since the incomplete recombination in CX3CR1-Cre mouse model was already reported (Yona *et al.*, 2013), we propose the latter possibility is operational in tDC and pDC subsets.

It has become increasingly clear that thymic DCs possess important role in the mechanisms of central tolerance since their depletion (CD11c-Cre-Rosa26-DTA mice) results in the increase of $CD4^+$ T cells in the periphery, which subsequently leads to the development of autoimmunity (Ohnmacht *et al.*, 2009). In association with altered deletion of $CD4^+$ T cells in DC-depleted mice (Ohnmacht *et al.*, 2009), our data, using CX3CR1-Cre-Rosa26-DTA mice, also suggest the impaired mechanisms of negative selection, since the cellularity of both $CD4^+$ and $CD8^+$ T cells was increased in the thymus (**Fig. 6B**). However, because so far only a limited number of performed measurements showed that neither the increase in whole thymic $CD3^+$ compartment (**Fig. 5C**) nor the enrichment in SP thymocytes (**Fig. 6B**) is statistically significant, the additional measurements are required to complete their statistical evaluation.

As described previously, the selection of tTregs was shown to be dependent not only on the direct antigen presentation by mTECs but also on their indirect presentation by DCs (Aschenbrenner *et al.*, 2007; Hinterberger *et al.*, 2010). Specifically, both $Sirp\alpha^+$ mDCs (Proietto *et al.*, 2008; Leventhal *et al.*, 2016; Hu *et al.*, 2017) and $CD8\alpha^+$ tDCs (Perry *et al.*, 2014) were shown to be important for this process. On the other hand, the deletion of $CX3CR1^+$ subset of mDCs did not result in changes in thymic Treg cell compartment (**Fig. 6C and D**). Nevertheless, the depletion of all DCs (CD11c-Cre-Rosa26-DTA mice) (Ohnmacht *et al.*, 2009) or specifically tDCs (Batf3 KO mice) (Perry *et al.*, 2014) also did not show changes in frequencies or total numbers of tTregs. Only the diminishment of MHCII molecules on DCs (CD11c-Cre-I-AB-flox mice) or deep sequencing of TCRs in Batf3 KO mice, revealed the alterations in Tregs selection, confirming the critical role of DCs in this process (Perry *et al.*, 2014; Leventhal *et al.*, 2016). So, to determine the exact role of $CX3CR1^+$ mDCs in tTreg generation, additional experiments are required.

Also, these data are in marked contrast with our so far unpublished study, showing that TLR9-induced enhanced migration of $CX3CR1^+$ mDCs into the thymus results in increased numbers of Tregs. However, it is important to note that such "immigrating" mDCs expressed low levels of MHCII which was recently found to mark mDCs with high potential to mediate Treg selection (Hu *et al.*, 2017). On the other hand, the $CX3CR1^+$ mDCs are at normal circumstances predominantly $MHCII^{HI}$ (**Fig. 4A**). Given that it was hypothesized that levels of MHCII expression by thymic APCs determine whether self-reactive T cell would be negatively selected or converted to the Treg lineage (Klein, Robey and Hsieh, 2019), we predict that $MHCII^{HI}$ $CX3CR1^+$ mDCs might mostly play a role in the negative selection of thymocytes and not in

their deviation to Tregs (**Fig. 6B and D**). This notion is also supported by the fact that MHCII^{HI} CX3CR1^{+} mDCs highly express PD-L1 (**Fig. 4A**), the engagement of which results in negative selection of T cells (Sharpe and Pauken, 2018). Hypothetical model of the role of CX3CR1^{+} mDCs in the central tolerance mechanisms is depicted in **Scheme 6**.



Scheme 6. Hypothetical model of the role of CX3CR1^{+} mDCs in the central tolerance mechanisms: *Both MHCII^{LO} and MHCII^{HI} CX3CR1^{+} mDCs acquire TRAs from mTECs, since they are highly potent in CAT, and they are capable to indirectly present antigens on MHCII molecules to mediate the mechanisms of central tolerance. While MHCII^{LO} CX3CR1^{+} mDCs are highly efficient in Treg selection, MHCII^{HI} CX3CR1^{+} mDCs are more efficient than their MHCII^{LO} counterparts in negative selection. Therefore, their ratio in the thymus maintains the balance between these selection processes which is visualized by gray (negative selection) and blue (Treg selection) triangle charts. Colored geometrical shapes represent diverse TRAs.*

F. CONCLUSIONS AND FUTURE PERSPECTIVES

The main aim of the thesis was to characterize the heterogeneity among the thymic BM APC subsets which participate in the mechanisms of CAT and indirect presentation of mTEC-derived antigens. Using Foxn1-Cre-Rosa26-tdTomato mouse model, we discovered the previously unrecognized population of thymic mDCs, which expressed CX3CR1 and other monocyte/macrophage markers and thus can be considered as thymic MCs. We also provide the experimental evidence that CX3CR1^{+} mDCs are very potent in CAT and presentation of mTEC-derived antigens to developing T cells. The physiological importance of these cells was demonstrated by reduced negative selection of CD4^{+} and CD8^{+} T cells in the thymus with selectively depleted CX3CR1^{+} mDCs. Since our data suggest that population of monocyte-derived APCs represents an important subset of BM-derived cells which participate in the selection of self-reactive T cells, it would be of utmost importance to determine their exact origin, localization, function and mode of action. Towards these goals, we are currently focusing on the generation of suitable murine transgenic models which will enable to target specifically thymic monocyte-derived cells and allow to manipulate their antigen presenting properties. Also, advanced techniques of fluorescence microscopy used for the description of antigen- and

cell type-dependent mechanism of CAT by *in vitro* antigen transfer assay, will provide us with needed tools to manipulate and study this process in order to elucidate its importance for the working central tolerance.

Together, inhere described work brought a new perspective on the complex process of thymic central tolerance, which represents a set of mechanisms preventing the onset and development of autoimmune reactions and diseases. Detailed knowledge about the specific subpopulations which take part in this process is essential to advance our understanding of how central immune tolerance works and how to manipulate it for potential therapeutic interventions in the future.

G. REFERENCES

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

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

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

Akiyama, T. *et al.* (2008) 'The Tumor Necrosis Factor Family Receptors RANK and CD40 Cooperatively Establish the Thymic Medullary Microenvironment and Self-Tolerance', *Immunity*, 29(3), pp. 423–437. doi: 10.1016/j.immuni.2008.06.015.

Anderson, G. *et al.* (1997) 'Fibroblast dependency during early thymocyte development maps to the CD25 ' CD44 ' stage and involves interactions with fibroblast matrix molecules', *European Journal of Immunology*, 27, pp. 1200–1206.

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

Anderson, M. S. *et al.* (2005) 'The cellular mechanism of Aire control of T cell tolerance', *Immunity*, 23(2), pp. 227–239. doi: 10.1016/j.immuni.2005.07.005.

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

Aschenbrenner, K. *et al.* (2007) 'Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells.', *Nature immunology*, 8(4), pp. 351–

358. doi: 10.1038/ni1444.

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

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

Bachem, A. *et al.* (2012) 'Expression of XCR1 characterizes the Batf3-dependent lineage of dendritic cells capable of antigen cross-presentation', *Frontiers in Immunology*, 3(July), pp. 1–12. doi: 10.3389/fimmu.2012.00214.

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

Blackburn, C. C. *et al.* (1996) 'The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors', *Proceedings of the National Academy of Sciences*, 93(June), pp. 5742–5746.

von Boehmer, H., Teh, H. S. and Kisielow, P. (1989) 'The thymus selects the useful, neglects the useless and destroys the harmful', *Immunology today*, 10(2), pp. 6–10. **Review**

Boes, M. *et al.* (2002) 'T-cell engagement of dendritic cells rapidly rearranges mhc class ii transport', *Nature*, 418(6901), pp. 983–988. doi: 10.1038/nature01004.

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

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

Bowlus, C. L. *et al.* (1999) 'Cloning of a novel MHC-encoded serine peptidase highly expressed by cortical epithelial cells of the thymus.', *Cellular immunology*, 196, pp. 80–86. doi: 10.1006/cimm.1999.1543.

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

Calabro, S. *et al.* (2016) 'Differential Intrasplenic Migration of Dendritic Cell Subsets Tailors Adaptive Immunity', *CellReports*. ElsevierCompany., 16(9), pp. 2472–2485. doi: 10.1016/j.celrep.2016.07.076.

Calderón, L. and Boehm, T. (2012) 'Synergistic, Context-Dependent, and Hierarchical Functions of Epithelial Components in Thymic Microenvironments', *Cell*, 149(1), pp. 159–172. doi: 10.1016/j.cell.2012.01.049.

Ceredig, R. and Rolink, T. (2002) 'A positive look at double-negative thymocytes.', *Nature reviews. Immunology*, 2(11), pp. 888–97. doi: 10.1038/nri937. **Review**

Chen, L., Xiao, S. and Manley, N. R. (2009) 'Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner', *Blood*, 113(3), pp. 567–574. doi: 10.1182/blood-2008-05-156265.

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

Cisse, B. *et al.* (2008) 'Transcription Factor E2-2 Is an Essential and Specific Regulator of Plasmacytoid Dendritic Cell Development', *Cell*, 135, pp. 37–48. doi: 10.1016/j.cell.2008.09.016.

Colonna, M., Trinchieri, G. and Liu, Y. (2004) 'Plasmacytoid dendritic cells in immunity', *Nature Reviews Immunology*, 5(12), pp. 1219–1226. doi: 10.1038/ni1141. **Review**

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

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

Cowan, J. E. *et al.* (2013) 'The thymic medulla is required for Foxp3 + regulatory but not conventional CD4 + thymocyte development', *Journal of Experimental Medicine*, 210(4), pp. 675–681. doi: 10.1084/jem.20122070.

Cowan, J. E. *et al.* (2018) 'Aire controls the recirculation of murine Foxp3 + regulatory T-cells back to the thymus', *European Journal of Immunology*, 48, pp. 844–854. doi: 10.1002/eji.201747375.

Cresswell, P. (1994) 'Assembly, Transport, and Function of MHC Class II Molecules', *Annual Review of Immunology*, 12(1), pp. 259–293. doi: 10.1146/annurev.iy.12.040194.001355. **Review**

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

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

Derbinski, J. *et al.* (2005) 'Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels.', *The Journal of experimental medicine*, 202(1), pp. 33–45. doi: 10.1084/jem.20050471.

Derbinski, J. *et al.* (2008) 'Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism.', *Proceedings of the National Academy of Sciences of the United States of America*, 105(2), pp. 657–62. doi: 10.1073/pnas.0707486105.

Diehl, G. E. *et al.* (2013) 'Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells.', *Nature*. Nature Publishing Group, 494(7435), pp. 116–20. doi: 10.1038/nature11809.

Dobeš, J. *et al.* (2018) 'A novel conditional Aire allele enables cell-specific ablation of the immune tolerance regulator Aire', *European Journal of Immunology*, 48(3), pp. 546–548. doi: 10.1002/eji.201747267.

Dress, R. J. *et al.* (2019) 'Plasmacytoid dendritic cells develop from Ly6D+ lymphoid progenitors distinct from the myeloid lineage', *Nature Immunology*. Springer US, 20(7), pp. 852–864. doi: 10.1038/s41590-019-0420-3.

Dubrot, J. *et al.* (2014) ' Lymph node stromal cells acquire peptide–MHCII complexes from dendritic cells and induce antigen-specific CD4 + T cell tolerance ', *The Journal of Experimental Medicine*, 211(6), pp. 1153–1166. doi: 10.1084/jem.20132000.

Dudziak, D. *et al.* (2007) 'Differential Antigen Processing by Dendritic Cell Subsets in Vivo', *Science*, 315(January), pp. 107–112.

Edelson, B. T. *et al.* (2010) ' Peripheral CD103 + dendritic cells form a unified subset developmentally related to CD8α + conventional dendritic cells ', *The Journal of Experimental Medicine*, 207(4), pp. 823–836. doi: 10.1084/jem.20091627.

Eisenbarth, S. C. (2019) 'Dendritic cell subsets in T cell programming: location dictates function', *Nature Reviews Immunology*. Springer US, 19(2), pp. 89–103. doi: 10.1038/s41577-018-0088-1. **Review**

Fergusson, J. R. *et al.* (2019) 'Maturing human CD127+ CCR7+ PDL1+ dendritic cells express AIRE in the absence of tissue restricted antigens', *Frontiers in Immunology*, 10(JAN), pp. 1–17. doi: 10.3389/fimmu.2018.02902.

Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003) 'Foxp3 programs the development and function of CD4+CD25+ regulatory T cells', *Nat Immunol*, 4(4), pp. 330–336. doi: 10.1038/ni904.

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

Gallo, V. *et al.* (2017) 'FOXN1 Deficiency: from the Discovery to Novel Therapeutic Approaches', *Journal of Clinical Investigation*. Journal of Clinical Immunology, 37, pp. 751–758. doi: 10.1007/s10875-017-0445-z. **Review**

Gao, Y. *et al.* (2013) 'Control of T Helper 2 Responses by Transcription Factor IRF4-dependent Dendritic Cells', *Immunity*, 39, pp. 722–732. doi: 10.1016/j.immuni.2013.08.028.

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

Gardner, J. M. *et al.* (2013) 'Extrathymic aire-expressing cells are a distinct bone marrow-derived population that induce functional inactivation of CD4 + T cells', *Immunity*. Elsevier Inc., 39(3), pp. 560–572. doi: 10.1016/j.immuni.2013.08.005.

Geissmann, F., Jung, S. and Littman, D. R. (2003) 'Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties', *Immunity*, 19, pp. 71–82.

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

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

Gordon, J. *et al.* (2004) 'Functional evidence for a single endodermal origin for the thymic epithelium.', *Nature immunology*, 5(5), pp. 546–553. doi: 10.1038/ni1064.

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

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

Gray, D. H. D. *et al.* (2006) 'Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells', *Blood*, 108(12), pp. 3777–3785. doi: 10.1182/blood-2006-02-004531.

Gross, M., Salame, T. M. and Jung, S. (2015) 'Guardians of the gut - murine intestinal macrophages and dendritic cells', *Frontiers in Immunology*, 6(JUN), pp. 1–10. doi: 10.3389/fimmu.2015.00254. **Review**

Guerri, L. *et al.* (2013) 'Analysis of APC Types Involved in CD4 Tolerance and Regulatory T Cell Generation Using Reaggregated Thymic Organ Cultures', *The Journal of Immunology*, 190(5), pp. 2102–2110. doi: 10.4049/jimmunol.1202883.

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

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

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

Hadeiba, H. and Butcher, E. C. (2013) 'Thymus-homing dendritic cells in central tolerance', *European Journal of Immunology*, 43(6), pp. 1425–1429. doi: 10.1002/eji.201243192. **Review**

Hanabuchi, S. *et al.* (2010) 'Thymic Stromal Lymphopoietin-Activated Plasmacytoid Dendritic Cells Induce the Generation of FOXP3 + Regulatory T Cells in Human Thymus', *Journal of Immunology*, 184, pp. 2999–3007. doi: 10.4049/jimmunol.0804106.

Heino, M. *et al.* (2000) 'RNA and protein expression of the murine autoimmune regulator gene (Aire) in normal, RelB-deficient and in NOD mouse', *European Journal of Immunology*, 30(7), pp. 1884–1893. doi: 10.1002/1521-4141(200007)30:7<1884::AID-IMMU1884>3.0.CO;2-P.

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

Hettinger, J. *et al.* (2013) 'Origin of monocytes and macrophages in a committed progenitor', *Nature immunology*, 14(8), pp. 821–830. doi: 10.1038/ni.2638.

Hildner, K. *et al.* (2008) 'Batf3 Deficiency Reveals a Critical Role for CD8a+ Dendritic Cells in Cytotoxic T Cell Immunity', *Science*, 322(November), pp. 1097–1101.

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

Hozumi, K. *et al.* (2008) 'Delta-like 4 is indispensable in thymic environment specific for T cell development.', *The Journal of experimental medicine*, 205(11), pp. 2507–13. doi: 10.1084/jem.20080134.

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

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

Hubert, F.-X. *et al.* (2009) 'Aire- Deficient C57BL/6 Mice Mimicking the Common Human 13-Base Pair Deletion Mutation Present with Only a Mild Autoimmune Phenotype', *The Journal of ImmunologyJ*. doi: 10.4049/jimmunol.0802124.

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

Jaitin, D. A. *et al.* (2014) 'Massively Parallel Single-Cell RNA-Seq for Marker-Free Decomposition of Tissues into Cell Types', *Science*, 343, pp. 776–779.

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

Josefowicz, S. Z., Lu, L.-F. and Rudensky, A. Y. (2012) 'Regulatory T Cells: Mechanisms of Differentiation and Function', *Annual Review of Immunology*, 30(1), pp. 531–564. doi: 10.1146/annurev.immunol.25.022106.141623. **Review**

Kernfeld, E. M. *et al.* (2018) 'A Single-Cell Transcriptomic Atlas of Thymus Organogenesis Resolves Cell Types and Developmental Maturation', *Immunity*, 48, pp. 1258–1270. doi: 10.1016/j.immuni.2018.04.015.

Ki, S. *et al.* (2017) 'EBI2 contributes to the induction of thymic central tolerance in mice by promoting rapid motility of medullary thymocytes', *European Journal of Immunology*, 47, pp. 1906–1917. doi: 10.1002/eji.201747020.

Kim, M. *et al.* (2018) 'Critical Role for the Microbiota in CX3CR1 + Intestinal Mononuclear Phagocyte Regulation of Intestinal T Cell Responses', *Immunity*. Elsevier Inc., 49(1), pp. 151-163.e5. doi: 10.1016/j.immuni.2018.05.009.

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

Kisand, Kai *et al.* (2010) 'Chronic mucocutaneous candidiasis in APECED or thymoma patients correlates with autoimmunity to Th17-associated cytokines', *The Journal of Experimental Medicine*, 207(2), pp. 299–308. doi: 10.1084/jem.20091669.

Klein, L. (2009) 'Dead man walking: How thymocytes scan the medulla', *Nature Immunology*. Nature Publishing Group, 10(8), pp. 809–811. doi: 10.1038/ni0809-809. **Review**

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

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

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

Koehli, S. *et al.* (2014) 'Optimal T-cell receptor affinity for inducing autoimmunity', *Proceedings of the National Academy of Sciences*, 111(48), pp. 17248–17253. doi: 10.1073/pnas.1402724111.

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

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

Konkel, J. E. *et al.* (2014) 'Thymocyte apoptosis drives the intrathymic generation of regulatory T cells', *Proceedings of the National Academy of Sciences*, 111(4), pp. E465–E473. doi: 10.1073/pnas.1320319111.

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

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

Kurobe, H. *et al.* (2006) 'CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance', *Immunity*, 24(2), pp. 165–177. doi: 10.1016/j.immuni.2005.12.011.

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

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

09727-4.

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

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

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

Leonardi, I. *et al.* (2018) 'CX3CR1+ mononuclear phagocytes control immunity to intestinal fungi', *Science*, 359(January), pp. 232–236.

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

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

Lind, E. F. *et al.* (2001) 'Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development.', *The Journal of experimental medicine*, 194(2), pp. 127–134. doi: 10.1084/jem.194.2.127.

Lio, C.-W. J. and Hsieh, C.-S. (2008) 'A Two-Step Process for Thymic Regulatory T Cell Development', *Immunity*, 28, pp. 100–111. doi: 10.1016/j.immuni.2007.11.021.

Liston, A. *et al.* (2003) 'Aire regulates negative selection of organ-specific T cells.', *Nature immunology*, 4(4), pp. 350–354. doi: 10.1038/ni906.

Liu, K. *et al.* (2009) 'In Vivo Analysis of Dendritic Cell Development and Homeostasis', *Science*, 324, pp. 392–398.

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

Lopes, N. *et al.* (2018) 'Lymphotoxin α fine-tunes T cell clonal deletion by regulating thymic entry of antigen-presenting cells', *Nature Communications*. Springer US, 9(1), pp. 1–16. doi:

10.1016/j.bbrc.2013.06.084.

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

Madisen, L. *et al.* (2010) 'A robust and high-throughput Cre reporting and characterization system for the whole mouse brain', *Nature Neuroscience*. Nature Publishing Group, 13(1), pp. 133–140. doi: 10.1038/nn.2467.

Malchow, S. *et al.* (2013) 'Aire-Dependent Thymic Development of Tumor Associated Regulatory T Cells', *Science*, 339, pp. 1219–1224.

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

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

Martínez-Martín, N. *et al.* (2011) 'T Cell Receptor Internalization from the Immunological Synapse Is Mediated by TC21 and RhoG GTPase-Dependent Phagocytosis', *Immunity*, 35(2), pp. 208–222. doi: 10.1016/j.immuni.2011.06.003.

Mashayekhi, M. *et al.* (2011) 'CD8α + Dendritic Cells Are the Critical Source of Interleukin-12 that Controls Acute Infection by Toxoplasma gondii Tachyzoites', *Immunity*. Elsevier Inc., 35(2), pp. 249–259. doi: 10.1016/j.immuni.2011.08.008.

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

Mccaughtry, T. M., Wilken, M. S. and Hogquist, K. A. (2007) 'Thymic emigration revisited', *Journal of Experimental Medicine*, 204(11), pp. 2513–2520. doi: 10.1084/jem.20070601.

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

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

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

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

Moore, N C *et al.* (1993) 'Analysis of cytokine gene expression in subpopulations of freshly isolated thymocytes and thymic stromal cells using semiquantitative polymerase chain reaction', *European Journal of Immunology*, 23, pp. 922–927.

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

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

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

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

Nakagawa, T. *et al.* (1998) 'Cathepsin L: Critical Role in li Degradation and CD4 T Cell Selection in the Thymus', *Science*, 280(5362), pp. 450–453. doi: 10.1126/science.280.5362.450.

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

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

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

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

Ouyang, W. *et al.* (2010) 'Transforming growth factor-β signaling curbs thymic negative selection promoting regulatory T cell development', *Immunity*. Elsevier Ltd, 32(5), pp. 642–653. doi: 10.1016/j.immuni.2010.04.012.

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

Owen, D. L. *et al.* (2019) 'Thymic regulatory T cells arise via two distinct developmental programs', *Nature Immunology*. Springer US, 20, pp. 195–205. doi: 10.1038/s41590-018-0289-6.

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

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

Perry, J. S. A. *et al.* (2018) 'CD36 Mediates Cell-Surface Antigens to Promote Thymic Development of the Regulatory T Cell Receptor Repertoire and Allo-tolerance', *Immunity*. Elsevier Inc., 48(5), pp. 923-936.e4. doi: 10.1016/j.immuni.2018.04.007.

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

Phee, H. *et al.* (2010) 'Regulation of thymocyte positive selection and motility by GIT2', *Nature Immunology*. Nature Publishing Group, 11(6), pp. 503–511. doi: 10.1038/ni.1868.

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

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

Plotkin, J. *et al.* (2003) 'Critical Role for CXCR4 Signaling in Progenitor Localization and T Cell Differentiation in the Postnatal Thymus', *Journal of Immunology*, 171, pp. 4521–4527. doi: 10.4049/jimmunol.171.9.4521.

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

Di Pucchio, T. *et al.* (2008) 'Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I', *Nature Immunology*, 9(5), pp. 551–557. doi: 10.1038/ni.1602.

Puel, A. *et al.* (2010) 'Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I', *The Journal of Experimental Medicine*, 207(2), pp. 291–297. doi: 10.1084/jem.20091983.

Rattay, K. *et al.* (2015) 'Homeodomain-Interacting Protein Kinase 2, a Novel Autoimmune Regulator Interaction Partner, Modulates Promiscuous Gene Expression in Medullary Thymic Epithelial Cells', *The Journal of Immunology*, 194(3), pp. 921–928. doi: 10.4049/jimmunol.1402694.

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

Rodewald, H. (2008) 'Thymus Organogenesis', *Annual Review of Immunology*, 26, pp. 355–388. doi: 10.1146/annurev.immunol.26.021607.090408. **Review**

Rodrigues, P. F. *et al.* (2018) 'Distinct progenitor lineages contribute to the heterogeneity of plasmacytoid dendritic cells', *Nature Immunology*. Springer US, 19(7), pp. 711–722. doi: 10.1038/s41590-018-0136-9.

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

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

Saare, M. *et al.* (2012) 'Autoimmune regulator is acetylated by transcription coactivator CBP/p300', *Experimental Cell Research*, 318(14), pp. 1767–1778. doi: 10.1016/j.yexcr.2012.04.013.

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

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

Sasaki, K. *et al.* (2015) 'Thymoproteasomes produce unique peptide motifs for positive selection of CD8(+) T cells.', *Nature communications*. Nature Publishing Group, 6(May), p. 7484. doi: 10.1038/ncomms8484.

Satpathy, A. T. *et al.* (2012) 'Re(de)fining the dendritic cell lineage', *Nature Reviews Immunology*, 13(12), pp. 1145–1154. doi: 10.1038/ni.2467. **Review**

Savina, A. *et al.* (2009) 'The Small GTPase Rac2 Controls Phagosomal Alkalinization and Antigen Crosspresentation Selectively in CD8+ Dendritic Cells', *Immunity*. Elsevier Ltd, 30(4), pp. 544–555. doi: 10.1016/j.immuni.2009.01.013.

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

Serbina, N. V *et al.* (2003) 'TNF / iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection', *Immunity*, 19, pp. 59–70.

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

Sharpe, A. H. and Pauken, K. E. (2018) 'The diverse functions of the PD1 inhibitory pathway', *Nature Reviews Immunology*, 18(3), pp. 153–167. doi: 10.1038/nri.2017.108. **Review**

Shi, Y. *et al.* (2016) 'LTBR controls thymic portal endothelial cells for haematopoietic progenitor cell homing and T cell regeneration', *Nature Communications*. Nature Publishing Group, 7, pp. 1–12. doi: 10.1038/ncomms12369.

Skogberg, G. *et al.* (2015) 'Human thymic epithelial primary cells produce exosomes carrying tissue-restricted antigens', *Immunology and Cell Biology*, 93(8), pp. 727–734. doi: 10.1038/icb.2015.33.

Stadinski, B. D. *et al.* (2019) 'A temporal thymic selection switch and ligand binding kinetics constrain neonatal Foxp3+ Treg cell development', *Nature Immunology*. Springer US. doi: 10.1038/s41590-019-0414-1.

Swiecki, M. *et al.* (2010) 'Plasmacytoid Dendritic Cell Ablation Impacts Early Interferon Responses and Antiviral NK and CD8+ T Cell Accrual', *Immunity*. Elsevier Inc., 33(6), pp. 955–966. doi: 10.1016/j.immuni.2010.11.020.

Tai, X. *et al.* (2005) 'CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2', *Nature Immunology*, 6(2), pp. 152–162. doi: 10.1038/ni1160.

Tai, X. *et al.* (2013) 'Foxp3 Transcription Factor Is Proapoptotic and Lethal to Developing Regulatory T Cells unless Counterbalanced by Cytokine Survival Signals', *Immunity*. Elsevier Inc., 38(6), pp. 1116–1128. doi: 10.1016/j.immuni.2013.02.022.

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

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

The Finnish-German APECED consortium (1997) 'An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains', *Nature genetics*, 17, pp. 399–403.

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

Thornton, A. M. *et al.* (2010) 'Expression of Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-Derived from Peripherally Induced Foxp3 + T Regulatory Cells', *Journal of Immunology*, 184, pp. 3433–3441. doi: 10.4049/jimmunol.0904028.

Trampont, P. C. *et al.* (2010) 'CXCR4 acts as a costimulator during thymic beta-selection', *Nature Immunology*. Nature Publishing Group, 11(2), pp. 162–170. doi: 10.1038/ni.1830.

Tussiwand, R. *et al.* (2012) 'Compensatory dendritic cell development mediated by BATF-IRF interactions', *Nature*, 490(7421), pp. 502–507. doi: 10.1038/nature11531.

Tykocinski, L. O. *et al.* (2010) 'Epigenetic regulation of promiscuous gene expression in thymic medullary epithelial cells', *Proceedings of the National Academy of Sciences of the United States of America*, 107(45), pp. 19426–19431. doi: 10.1073/pnas.1009265107.

Ueno, T. *et al.* (2004) 'CCR7 signals are essential for cortex-medulla migration of developing thymocytes', *The Journal of experimental medicine*, 200(4), pp. 493–505. doi: 10.1084/jem.20040643.

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

Vang, K. B. *et al.* (2008) 'IL-2, -7, and -15, but Not Thymic Stromal Lymphopoeitin, Redundantly Govern CD4+Foxp3+ Regulatory T Cell Development', *The Journal of Immunology*, 181(5), pp. 3285–3290. doi: 10.4049/jimmunol.181.5.3285.

Varol, C. *et al.* (2007) 'Monocytes give rise to mucosal, but not splenic, conventional dendritic cells', *The Journal of Experimental Medicine*, 204(1), pp. 171–180. doi: 10.1084/jem.20061011.

Varol, C. *et al.* (2009) 'Intestinal Lamina Propria Dendritic Cell Subsets Have Different Origin and Functions', *Immunity*, 31(3), pp. 502–512. doi: 10.1016/j.immuni.2009.06.025.

Venanzi, E. S. *et al.* (2008) 'The variable immunological self : Genetic variation and nongenetic noise in Aire-regulated transcription', *Proc Natl Acad Sci U S A*, 105(41), pp. 15860–15865.

Voehringer, D., Liang, H.-E. and Locksley, R. M. (2008) 'Homeostasis and effector function of lymphopenia-induced "memory-like" T cells in constitutively T cell-depleted mice.', *Journal of immunology*, 180(7), pp. 4742–53. doi: 10.4049/jimmunol.180.7.4742.

Walters, S. N. *et al.* (2014) 'A role for intrathymic B cells in the generation of natural regulatory T cells.', *Journal of immunology (Baltimore, Md. : 1950)*, 193(1), pp. 170–6. doi: 10.4049/jimmunol.1302519.

Wang, H. X., Qiu, Y. R. and Zhong, X. P. (2016) 'Intercellular protein transfer from thymocytes to thymic epithelial cells', *PLoS ONE*, 11(3), pp. 1–10. doi: 10.1371/journal.pone.0152641.

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

Waskow, C. *et al.* (2008) 'The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues', *Nature immunology*, 9(6), pp. 676–683. doi: 10.1038/ni.1615.

Watanabe, N. *et al.* (2005) 'Hassall ' s corpuscles instruct dendritic cells to induce CD4+ CD25+ regulatory T cells in human thymus', *Nature*, 436(August), pp. 1181–1185. doi: 10.1038/nature03886.

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

Weiss, J. M. *et al.* (2012) 'Neuropilin 1 is expressed on thymus-derived natural regulatory T cells , but not mucosa- generated induced Foxp3 + Treg cells', *Journal of Experimental Medicine*, 209(10), pp. 1723–1742. doi: 10.1084/jem.20120914.

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

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

Wong, K. *et al.* (2014) 'Multilineage potential and self-renewal define an epithelial progenitor cell population in the adult thymus', *Cell Reports*. The Authors, 8(4), pp. 1198–1209. doi: 10.1016/j.celrep.2014.07.029.

Wu, L. and Shortman, K. (2005) 'Heterogeneity of thymic dendritic cells', *Seminars in Immunology*, 17(4), pp. 304–312. doi: 10.1016/j.smim.2005.05.001. **Review**

Xing, Y., Jameson, S. C. and Hogquist, K. A. (2013) 'Thymoproteasome subunit-β5T generates peptide-MHC complexes specialized for positive selection.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(17), pp. 6979–84. doi: 10.1073/pnas.1222244110.

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

Yamano, T. *et al.* (2019) 'Aire-expressing ILC3-like cells in the lymph node display potent APC features', *Journal of Experimental Medicine*, pp. 1–11.

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

Yona, S. *et al.* (2013) 'Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages under Homeostasis', *Immunity*. Elsevier, 38(1), pp. 79–91. doi: 10.1016/j.immuni.2012.12.001.

Zheng, Y. *et al.* (2010) 'Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate', *Nature*. Nature Publishing Group, 463(7282), pp. 808–812. doi: 10.1038/nature08750.

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

Žuklys, S. *et al.* (2016) 'Foxn1 regulates key target genes essential for T cell development in postnatal thymic epithelial cells', *Nature immunology*, 17(10), pp. 1206–1217. doi: 10.1038/ni.3537.