Defining novel stages in medullary thymic epithelial cell differentiation: Implications for tolerance induction

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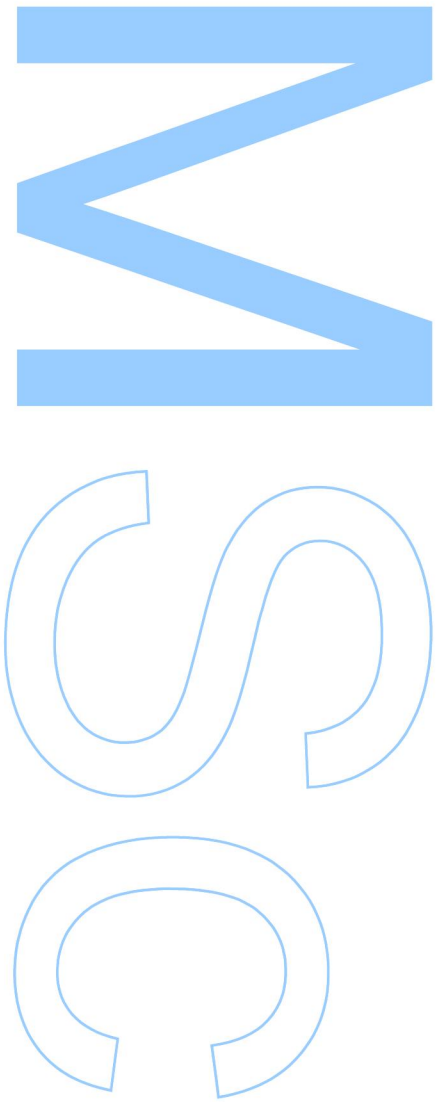

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Abstract

A competent immune system requires a functionally diverse repertoire of T cells that is able to respond to a variety of foreign antigens while being tolerant to self-constituents. These principles are in part imposed in the thymus through the action of highly specialized subtypes of thymic epithelial cells (TECs). In particular, medullary TECs (mTECs) have a critical role in tolerance induction, due to their unique ability to present tissue-restricted antigens (TRAs). The establishment of the medullary epithelial compartment is fostered by thymocyte-derived signals, through the engagement of signalling via the tumour necrosis factor receptor superfamily (TNFRSF) members RANK, CD40 and LTβR, expressed on mTECs and their precursors. The described mTEC developmental program includes immature $CD80^{low}MHCII^{low}$ precursors ($mTEC^{low}$) that give rise to mature $CD80^{high}$ $MHCII^{high}$ cells ($mTEC^{high}$), including cells that express Aire, an important regulator of TRA expression. Additionally, terminally differentiated $CD80^{low}MHCII^{low}$ post-Aire cells also reside within the $mTEC^{low}$. Further heterogeneity can also be defined by the existence of cells expressing Cld3/4, SSEA and podoplanin, which were shown to define mTEC-committed progenitors, or cells expressing CCL21, a key inducer of thymocyte migration into the medulla. As such, the mTEC niche encompasses highly diverse subsets, whose lineage and functional relationships remain difficult to characterize, mainly due to a lack of suitable markers to define existent subsets and further dissect new developmental stages.

In this thesis, we incorporate the study of CD24 and SCA1 expression in the standard flow cytometry analysis of TECs. With this strategy, we reveal three distinct subpopulations of mTECs that emerge from the perinatal period into early adulthood: $mTEC^{I}$ ($CD24^{+}SCA1^{-}$), $mTEC^{II}$ ($CD24^{+}SCA1^{+}$) and $mTEC^{III}$ ($CD24^{-}SCA1^{-}$). We find that $mTEC^{I}$ include a mixture of both $mTEC^{low}$ and $mTEC^{high}$, while the $mTEC^{II}$ and $mTEC^{III}$ subsets are enriched for $mTEC^{low}$ and $mTEC^{high}$, respectively. The production of CCL21 is specifically detected in a fraction of $mTEC^{II}$, while Aire expression is largely confined to the $mTEC^{III}$ subset. Using an *in vitro* lineage-tracing system, we show that $mTEC^{I}$ and $mTEC^{II}$ of $mTEC^{low}$ type have the potential to generate all subsets, including $Aire^{+}$ $mTEC^{high}$. Lastly, we show that while TNFRSF-induced mTEC maturation does not directly induce $mTEC^{I-III}$, signals provided by thymocytes at early stages of their development are sufficient to establish the $mTEC^{I-III}$ differentiation profile. Collectively, these results reveal a novel dimension of mTEC diversity defined by the differential expression of CD24 and SCA1. These newly-defined subpopulations provide a platform to further characterize their specific intrathymic functions. Ultimately, these findings extend our comprehension of the mTEC developmental program and introduce new markers that will allow a better insight of the mechanisms underlying mTEC development and tolerance induction.

Resumo

O sistema imunitário necessita de um vasto repertório de células T capazes de responder a inúmeros antigénios e que sejam simultaneamente tolerantes aos antigénios do próprio organismo. Estes princípios são impostos no timo pela ação das células epiteliais tímicas (TECs). Em particular, as TECs residentes na medula (mTECs) tem um papel crítico para o estabelecimento do estado de tolerância, devido à sua capacidade única de expressar antigénios restritos de tecidos (TRAs). O desenvolvimento das mTECs é dependente de sinais provenientes dos timócitos, que ativam os membros da superfamília de recetores de fator de necrose tumoral (TNFRSF) RANK, CD40 e LTβR, expressos pelas mTECs e as suas células progenitoras. O programa de desenvolvimento das mTECs atualmente descrito inclui células imaturas $CD80^{low}MHCII^{low}$ ($mTEC^{low}$), precursoras das células maturas $CD80^{high}MHCII^{high}$ ($mTEC^{high}$), que por sua vez incluem células que expressam Aire, um importante regulador da expressão de TRAs. Por último, mTECs num estado pós-Aire são $CD80^{low}MHCII^{low}$ e estão também inseridas na população $mTEC^{low}$. É ainda possível reconhecer uma maior heterogeneidade de mTECs através dos marcadores Cld3/4, SSEA e podoplanina, que identificam células progenitoras específicas da linhagem mTEC, ou CCL21, uma citocina que induz migração de timócitos para a medula. Deste modo, as mTECs possuem uma elevada diversidade, sendo que a relação entre as suas subpopulações e as respetivas funções permanecem pouco caracterizadas devido à escassez de marcadores para definir subpopulações existentes e elucidar novas fases de diferenciação.

No presente trabalho, foram introduzidos os marcadores CD24 e SCA1 na análise clássica de TECs por citometria de fluxo. Deste modo, mostramos que as mTECs se subdividem em três populações no período embrionário e pós-natal: $mTEC^{I}$ ($CD24^{+}SCA1^{-}$), $mTEC^{II}$ ($CD24^{+}SCA1^{+}$) e $mTEC^{III}$ ($CD24^{-}SCA1^{-}$). Revelamos que as $mTEC^{I}$ incluem $mTEC^{low}$ e $mTEC^{high}$. Enquanto as $mTEC^{II}$ e $mTEC^{III}$ abrangem maioritariamente $mTEC^{low}$ e $mTEC^{high}$, respetivamente. As $mTEC^{II}$ possuem células produtoras de CCL21, enquanto as $mTEC^{III}$ incluem a maioria das células $Aire^{+}$. A análise *in vitro* da descendência das subpopulações $mTEC^{I}$ ou $mTEC^{II}$ caracterizadas como $mTEC^{low}$ revelou que ambas são capazes de gerar as outras subpopulações, incluindo células $mTEC^{high}$ $Aire^{+}$. Por fim, apesar da maturação induzida *in vitro* através dos recetores TNFRSF não ter gerado diretamente as populações $mTEC^{I-III}$, os sinais provenientes de timócitos em fase inicial de desenvolvimento foram suficientes para induzir o perfil de diferenciação $mTEC^{I-III}$. Em conclusão, estes resultados revelam uma nova diversidade de mTECs definida pelo padrão de expressão de CD24 e SCA1. As novas populações descritas neste trabalho constituem uma importante ferramenta para a continuação da caracterização das suas funções no

timo. Por último, estas observações permitem expandir o atual modelo de diferenciação das mTECs e fornecem novos marcadores para o estudo dos mecanismos que controlam o seu desenvolvimento e a indução de tolerância.

Key words

Thymus medulla, Thymic epithelial cells, TEC progenitors, TEC differentiation, T cell development, Tolerance induction

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List of Abbreviations

Aire - Autoimmune regulator
APC - Antigen-presenting cell
BCR - B cell receptor
BM - Bone marrow
CCL - Chemokine (C-C motif) ligand
CCR - C-C Chemokine receptor
CD - Cluster of differentiation
cDC - Conventional dendritic cell
CD40L - Cluster of differentiation 40 ligand
Cld - Claudin
CMJ - Cortico-medullary junction
cTEC - Cortical thymic epithelial cell
CXCL - Chemokine (C-X-C motif) ligand
CXCR - C-X-C Chemokine receptor
DC - Dendritic cell
dGuo - 2-deoxyguanosine
DLL4 - Delta-like 4
DN - Double negative
DP - Double positive
DTR - Diphtheria toxin receptor
E - Embryonic day
EpCAM - Epithelial cell adhesion molecule
ETP - Early thymic progenitor
Fezf2 - FEZ family zing finger 2
FGF - Fibroblast growth factor
FoxP3 - Forkhead box P3
FoxN1 - Forkhead box N1
FTOC - Fetal thymus organ culture
Gcm2 - Glial cells missing homolog 2
GFP - Green fluorescent protein
HSC - Hematopoietic stem cell
ICAM - Intercellular adhesion molecule
IFN - Interferon
IGF - Insulin-like growth factor
IκB - Inhibitor of kappa B
IKK - IκB kinase
IL - Interleukin
ILC - Innate lymphoid cell
iNKT - Invariant natural killer T cell
Ivl - Involucrin
K - Cytokeratin
KI - Knockin
KLF2 - Kruppel-like factor 2
KO - Knockout
LTi - Lymphoid tissue inducer
LTβR - Lymphotoxin beta receptor
MHC - Major histocompatibility complex
mTEC - Medullary thymic epithelial cell
mTEP - Medullary thymic epithelial progenitor
MTS - Mouse thymic stroma
NC - Neural crest
NF-κB - Nuclear factor kappa B
NIK - NF-κB-inducing kinase
NK - Natural killer
OPG - Osteoprotegerin
pGE - Promiscuous gene expression
P - Postnatal day
PAMP - Pathogen-associated molecular pattern
pDC - Plasmacytoid dendritic cell
PDPN - Podoplanin
Plet-1 - Placenta-expressed transcript-1
PRR - Pattern recognition receptor
PSGL-1 - P-selectin glycoprotein ligand 1
RA - Retinoic acid

RAG - Recombination-activating gene

RANK - Receptor activator of nuclear factor kappa B

RANKL - Receptor activator of nuclear factor kappa B ligand

RelA - V-Rel reticuloendotheliosis viral oncogene homolog A

RelB - V-Rel reticuloendotheliosis viral oncogene homolog B

RFP - Red fluorescent protein

RTE - Recent thymic emigrant

RTOC - Reaggregate thymus organ culture

SCA1 - Stem cell antigen-1

S1P - Sphingosine-1-phosphate

S1P1 - Sphingosine-1-phosphate receptor 1

SM - Semimature

SCF - Stem cell factor

SP - Single positive

SSEA - Stage-specific embryonic antigen A

TCR - T cell receptor

TEC - Thymic epithelial cell

TEP - Thymic epithelial progenitor

TNFRSF - Tumour necrosis factor receptor superfamily

TRA - Tissue-restricted antigen

TRAF - Tumour necrosis factor receptor-associated factor

Treg - Regulatory T cell

TSSP - Thymus-specific serine protease

UEA - *Ulex europaeus* agglutinin

VCAM - Vascular cell adhesion molecule

WT - Wild Type

Introduction

An outline of the immune system

A competent immune system must confer an individual the ability to survive under the threat of constant and ever-evolving pathogens, including viral, bacterial, fungal and protozoa, as well as dysfunctional host cells. To do so, it must act rapidly and efficiently to recognize threats and activate different effector functions while still maintaining the flexibility to adapt to the changes in the internal and external environment. Importantly, recognition must include discrimination between "self" and "non-self" in order to maintain an immunological self-tolerant state. All the while, it must be efficient in its use of genes and cells, and not impose an excessive energetic burden to the host. Such requirements are met through features of both innate and adaptive immunity.

Innate immune defences constitute the first line of response to pathogens and are primarily mediated by germline-encoded receptors, termed pattern recognition receptors (PRRs), that have evolved to recognize highly conserved pathogen-associated molecular patterns (PAMPs). PRR engagement will rapidly induce a cell response characterized by the expression of different genes involved in cell activation, inflammation and production of microbicidal species [1].

In addition to the innate immune system, jawed vertebrates, beginning with cartilaginous fish, have developed an adaptive immune system mediated primarily by lymphocytes [2]. The lymphocyte receptor repertoire is highly diverse, being able to recognize antigens of any potential pathogen or toxin. This capability is the virtue of somatic recombination through the random rearrangement of variable (V), diversity (D) and joining (J) gene segments, which upon assembling produce antigen receptors expressed by T (for thymus-derived) and B (for bursa de Fabricius-derived) lymphocytes [3]. The random nature of this recombination process results in a vast clonally diverse repertoire of T cell receptors (TCRs) and B cell receptors (BCRs). However, this process inevitably generates receptor specificities against antigens of the host. Elimination or regulation of self-reactive lymphocytes and maintenance of immunological self-tolerance is safeguarded by central and peripheral tolerance mechanisms [4, 5].

Upon maturation in the bone marrow (BM) and thymus, T and B cells, respectively, migrate to secondary lymphoid tissues, which specialize in spatially organizing interactions between immune cells, such as antigen-presenting cells (APCs) and lymphocytes. With subsequent triggering by antigen recognition, these cells may undergo clonal expansion and differentiation into effector T lymphocytes or antibody-producing plasma cells or otherwise become memory cells that can act in case of reexposure to their specific antigens [3].

The thymus - Structural and functional elements

This thesis is centred on the thymus, a bilobed organ located in the central compartment of the thoracic cavity above the heart. It is the primary lymphoid site for the generation and selection of T cells bearing a TCR repertoire capable of responding to countless foreign antigens while being tolerant against self-antigens. The ability to accommodate such an essential and complex process is attributed to the unique thymic microenvironments, specialized in providing the necessary signals and cell-cell interactions to support thymocyte migration, survival, commitment, proliferation, and selection.

The thymus is an encapsulated and lobulated organ. The capsule consists of mesenchymal cells and connective tissue and penetrates into the thymus to form trabeculae. Below this layer is found the sub-capsular epithelium which overlies the outer cortex [6]. A peripheral cortex and central medulla are formed by three-dimensional networks of epithelial cells surrounded by developing T cells, or thymocytes, which make up for over 95% of the cellularity of the postnatal thymus **(Figure 1A)** [7].

The thymic stroma is mainly comprised of thymic epithelial cells (TECs), divided into two phenotypically and functionally distinct subtypes named according to their spatial location: cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs). Other stromal elements include endothelial cells, mesenchymal cells, and BM derived cells, such as dendritic cells (DCs) , macrophages, and B cells **(Figure 1B)** [6]. The principles underlying TEC development are the study subject of this thesis and will be reviewed in the following chapters.

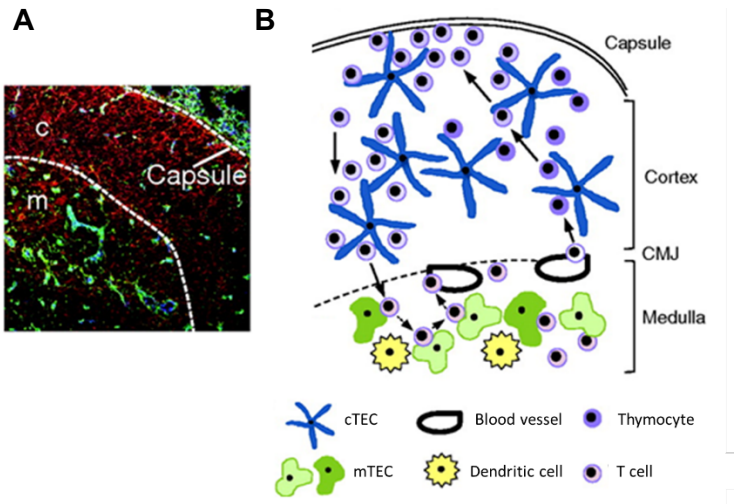

Figure 1 - Organization and cellular composition of the thymus. (A) Immunostaining showing cellular organization in the postnatal thymus. (c – cortex, m - medulla). pan-keratin (red) highlights cTECs and mTECs; CD31 (blue) labels endothelial cells; collagen IV (green) labels perivascular matrix; the dotted lines show the cortico-medullary boundary and the capsular boundary of the cortex. **(B)** Scheme image depicting the general cellular components of the adult thymus (CMJ – cortico-medullary junction). Images adapted from [8].

In the cortex, cTECs have an important role during the early stages of T cell development by promoting lineage commitment and expansion of early T cell progenitors and by providing the environment required for positive selection of thymocytes bearing self-

major histocompatibility complex (MHC)-restricted TCRs. In the medulla, mTECs and DCs mediate negative selection of autoreactive T cells and the generation of regulatory T cells [9]. The function of cTECs and mTECs in thymopoiesis will be described in more detail later on.

Ultimately, TECs are of vital importance in the establishment and maintenance of a functional T cell repertoire and population. This is evidenced by a clear link between TEC dysfunction and the emergence of multiple disorders, ranging from immunodeficiency to autoimmunity.

Thymus organogenesis

The establishment of the fully organized and competent thymic microenvironments is the result of a complex and tightly orchestrated process of thymus development. Thymus organogenesis occurs in the pharyngeal region of the embryo, initiating between embryonic day (E) 9 and 10. It is generated from the endoderm of the third pharyngeal pouch, in a shared primordium with the parathyroid gland [10]. Expression of the transcription factors Forkhead box N1 (Foxn1) and Glial cells missing homolog 2 (Gcm2) delineates the two non-overlapping organ-specific domains, orchestrating the differentiation of the thymus and parathyroid, respectively [11]. At E12.5, the two discrete organs begin to resolve and migrate to their final anatomical locations: the parathyroid locates adjacent to the thyroid gland, and the thymus migrates further into the chest cavity, where the two lobes meet above the heart [8]. Interestingly, an ectopic cervical thymus can also be identified in both mice and humans. The ectopic thymus emerges later, at E15.5, as a small patch of Foxn1-expressing cells [12] and possesses the same general organization as the thoracic thymus, as well as the ability to support T cell differentiation [13].

Foxn1 is the earliest thymus-specific marker and is an indispensable regulator of thymus organogenesis [14]. $Foxn1^{-/-}$ mice display the classical nude phenotype of congenital athymia and hairlessness. In nude mice, formation of the primordial organ is arrested between E11.5 and E12.0 and the primordium is not colonized by lymphocyte precursors, causing a severe primary T cell immunodeficiency [15]. Several other transcription factors and signalling pathways have been implicated in early thymus development [8, 15]. Still, thymic fate specification results from a complex molecular network that remains, for the most part, unknown.

The fetal thymus is seeded by hematopoietic stem cell (HSC)-derived lymphocyte precursors prior to vascularization, between E11 and E12. Lymphocytes provide key initial instructive signals for TEC development and proliferation [16]. Furthermore, the third pharyngeal pouch cells are surrounded by neural crest (NC)-derived mesenchymal cells

that will later form the capsule [17]. Proliferation and differentiation of the thymic epithelium is also highly dependent on signals supplied by the surrounding NC mesenchymal cells, such as fibroblast growth factor (FGF)-7 and -10 and insulin-like growth factor (IGF)-1 and -2 [18, 19]. In fact, NC cells provide several essential signals for thymus organogenesis. They are important regulators of the patterning of the third-pouch endoderm into the two organ-specific domains and subsequent migration and positioning of the thymus [20, 21]. Mesenchymal cells have also been shown to be the major source of retinoic acid (RA), an important regulator of TEC homeostasis, both in the embryonic and adult thymus [22]. Lastly, NC-derived cells stabilize blood-vessel structures by differentiating into perivascular cells [17].

In conclusion, thymic organogenesis is a dynamic process that involves cells from all three embryonic germ layers: endoderm-derived epithelium, ectoderm-derived NC cells and mesoderm-derived HSCs. Each undergoing tightly co-dependent and synchronised developmental programs.

TEC progenitors

During thymus organogenesis, the first immature TECs appear at E12.5 and develop from thymic epithelial progenitors (TEPs), which have the ability to differentiate into both cortical and medullary lineages. Bipotent TEPs are essential building blocks for thymus development and maintenance and have been detected in both the embryonic and postnatal thymus [23, 24]. The identification of TEPs is an area under intense investigation, however, a full consensus has not yet been reached. Some studies have suggested Placenta-expressed transcript-1 (Plet-1) as a TEP marker, which can be recognized by the monoclonal antibodies MTS20 and MTS24 [25]. Plet1^{+} TECs can be detected at E12 and progressively become rarer during thymus development. These cells were shown to possess the capacity to generate functional cortical and medullary thymic microenvironments [26, 27]. In contrast, a following report argued that both MTS24^{+} and MTS24^{-} cells have bipotent progenitor ability, challenging the view of Plet-1 as a TEP-specific marker [28]. Hence, the defining phenotype of bipotent TEPs remains unclear, as well as their location, abundance, and physiological contribution to the maintenance of the thymic compartments throughout life.

Follow-up reports showed that embryonic TEPs expressing several cTEC-associated markers, including β5t, CD205 and IL7, are able to generate both cTECs and mTECs [29-31]. Such findings argue against a model of TEC development in which bipotent TEPs synchronously diverge into cTEC and mTEC progenitors. Instead, they have led to the delineation of a serial progression model of TEC development, in which progenitors

pass through a transitional phase where they express cTEC traits prior to commitment to the cTEC or mTEC lineage [32]. Yet, the molecular principles controlling this cell-fate decision are still to be discovered (**Figure 2**).

Other studies have focused on the identification of mTEC lineage-committed progenitors (mTEPs) [33]. These cells emerge as early as E13 and can be defined by the expression of tight junction components claudin-3 and claudin-4 (Cld3/4) [34]. In addition, stage-specific embryonic antigen A (SSEA) is expressed in a small fraction of total Cld3/4^{+} cells and marks a more defined mTEP population whose self-renewing and progenitor potential, although decreased, can still be detected in adulthood [35]. Moreover, receptor activator of NF-κB (RANK)$^{+}$ unipotent mTEC progenitors arise from the Cld3/4^{+}SSEA^{-} fraction between E14 and E15 [36]. Lastly, a fraction of adult mTECs can also descend from a lineage-committed podoplanin (PDPN)$^{+}$ progenitor located at the cortico-medullary junction (CMJ) [37]. It remains however unclear whether these mTEP subsets are distinct stages of the same linear differentiation or if they represent divergent mTEC lineages (**Figure 2**).

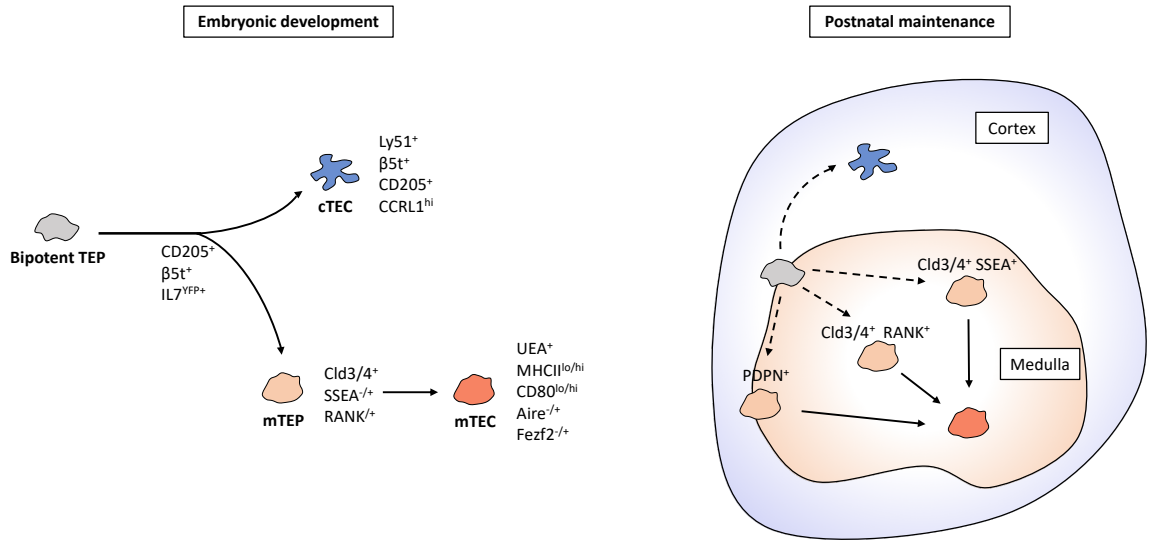

Figure 2 - TEC progenitors and their contribution to the development and maintenance of cTECs and mTECs. Bipotent TEPs express cTEC traits prior to commitment to the cTEC or mTEC lineage and have been suggested to have a reduced contribution to the maintenance of the postnatal thymus. Cld3/4^{+}SSEA^{+} and Cld3/4^{+}RANK^{+} cells constitute two mTEC-restricted progenitors that rise in the embryonic thymus, while PDPN^{+} mTEPs can also contribute to the maintenance of the mTEC compartment in the adult thymus. The lineage relationship between the represented mTEPs is not yet defined.

The cellular and molecular mechanisms controlling the maintenance and regeneration of the adult mTEC compartment are still under investigation [38]. Possibly, throughout life, the medullary epithelium could be maintained solely by embryonic derived-mTEPs, eventually leading to an exhaustion of this limited cell pool. Alternatively, new supply of mTEPs from bipotent progenitors could still occur in the adult thymus. In this

regard, bipotent β5t$^+$ TEPs can generate both Cld3/4$^+$SSEA1$^+$ and PDPN$^+$ mTEPs, however, this contribution to the mTEP pool fades during postnatal life, suggesting that the maintenance of the adult medullary epithelium is likely assured by mTEPs rather than bipotent progenitors [39, 40] (**Figure 2**).

Phenotypic markers of TECs

The development of novel antibodies and reporter mice has significantly aided the ongoing efforts to unveil all the developmental and functional complexity of TECs. Some markers are shared between cTECs and mTECs, for example, both are defined by the expression of epithelial cell adhesion molecule (EpCAM)/CD326 and MHCII, within the nonhematopoietic (CD45$^-$) fraction of the thymus [41]. Several phenotypic traits are routinely used to discriminate cTECs and mTECs and to identify subpopulations within each. Across studies, there is some variability in the method employed to discriminate TEC subsets, which is also dependent on the analytical tools used, such as flow cytometry and immunohistochemical analyses. cTECs are commonly defined by the expression of cytokeratin (K) 8 and 18, Ly51 (CD249), CD205 and ER-TR4, as well as more recently identified functional molecules, such as CCRL1, β5t, DLL4 and high levels of IL-7 [42]. On the other hand, mTECs are distinguished by the expression of K5 and 14, MTS10, ER-TR5 and the binding to lectin *Ulex europaeus* agglutinin (UEA) 1. Further discrimination of mTEC heterogeneity can be achieved based on the combined levels of expression of MHCII, CD40, CD80, Aire and CCL21 [32, 41]. This thesis will further explore the diversity within the mTEC compartment, as such, a more in-depth discussion of the described mTEC markers and subpopulations will be provided in the chapter “mTEC diversity”.

T cell development and its interdependence on thymic stroma

Early stages of differentiation

All blood cell types, including T cells, originate from HSCs. These cells are characterized by continuous progenitor ability and self-renewal, and are phenotypically negative for all lineage markers [43]. HSCs undergo a series of intermediate stages where they undergo progressive lineage restriction, and ultimately differentiate into cells of the lymphoid and myeloid lineage. Lymphoid cells include T, B, natural killer (NK) and innate lymphoid cells (ILCs), while the myeloid lineage gives rise to erythrocytes, megakaryocytes, granulocytes and macrophages [44]. Plasmacytoid DCs (pDCs) and conventional DCs (cDCs) differentiate from lymphoid- and myeloid-committed progenitors, respectively [44].

During fetal development, hematopoiesis occurs primarily in the liver, whereas in postnatal life, it resides within the bone marrow [43]. In order for HSCs to differentiate into competent self-tolerant T cells, they require the specialized thymic microenvironments. Therefore, these cells must egress from the adult bone marrow, traffic through the blood, and enter the thymus. Thymus homing of bone marrow-derived progenitors is guided by the expression of the chemokine receptors CCR7, CCR9 and CXCR4, while the corresponding chemokine ligands CCL19/CCL21, CCL25 and CXCL12, are provided by multiple stromal cell types, including mTECs, cTECs and non-TEC stroma [45, 46]. Other molecules involved in this trafficking are P-selectin glycoprotein ligand 1 (PSGL-1), α4β1 integrin, and αLβ2 integrin, present on bone marrow-derived progenitors, while the respective receptors, P-selectin, VCAM-1 and ICAM-1, are expressed on the endothelial cells of the thymus vasculature [47, 48]. Early migration to the unvascularized fetal thymus also relies on chemotaxis mediated by the three chemokine receptors CCR7, CCR9 and CXCR4 [45].

Upon entry through the blood vessels localized around the cortico-medullary junction [49], T cell precursors are commonly termed early thymic progenitors (ETP). ETPs lack the expression of CD4 and CD8, hence being referred to as double negative (DN) thymocytes. Within the DN stage, the developmental program can be subdivided into 4 stages, characterized by the coordinated expression of cell surface proteins CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$). DN1 cells are uncommitted, as they retain the potential to differentiate into T cells but also DCs, NKs, and possess some vestigial B cell and myeloid lineage potential [50, 51]. Engagement of the Notch signalling pathway is crucial at this stage to inhibit multiple cell fate potentials and gradually induce the genetic program for T cell fate determination. cTECs provide the essential and non-redundant ligand for Notch signalling, DLL4 [52]. The DN2 stage is characterized by a migratory movement to the subcapsular cortex. [49]. In this region, cTECs further support T lineage specification, differentiation and expansion by providing the necessary growth factors and cytokines, such as stem cell factor (SCF or kit ligand) and IL-7 [53, 54]. During this stage, alongside active proliferation, cells undergo recombination-activating gene (RAG) 1 and RAG2-mediated V(D)J rearrangements of the *Tcrb*, *Tcrg* and *Tcrd* loci, which are required for the assembly of the TCR [52]. Transition into the DN3 stage marks the complete and irreversible commitment to the T cell lineage [50]. Although during the DN3 stage, thymocytes can produce TCRγ and TCRδ chains and differentiate along the γδ T cell lineage, most cells undergo αβ TCR development pathways [53]. A successful *Tcrb* rearrangement will give rise to a functional TCRβ chain, which associates with an invariant pre-TCR α-chain and CD3 signalling molecules to form the pre-TCR complex [55]. The autonomous pre-TCR signalling, in non-redundant cooperation with Notch signalling, mediates a key developmental checkpoint, the β-selection event, which

rescues DN3 cells from apoptosis, promotes proliferation and ceases the recombination of the *Tcrb* locus [56]. Following β-selection, cells downregulate CD25, becoming DN4 or pre-double-positive cells, while initiating *Tcra* gene rearrangements. DN cells differentiate into $CD4^+CD8^+$ double positive (DP) thymocytes, which are the first cells to express a functional αβ TCR [57].

Positive selection

In the cortex, cTECs are self-antigen-presenting cells responsible for inducing positive selection. This process decides the fate of thymocytes (survival/differentiation versus apoptosis) according to the specificity and binding strength of the αβ TCR relatively to the self-peptide-MHC complex presented by cTECs. DP thymocytes carrying TCRs that interact with intermediate avidity are induced to survive and allowed to further differentiate [58]. Most commonly, cells express TCRs unable to interact with self-MHC molecules and undergo death by neglect [59]. On the other hand, high affinity interactions with the self-peptide-MHC complexes can lead to negative selection carried out by cTECs [59]. Ultimately, this checkpoint ensures a repertoire of cells carrying appropriate self-MHC restricted TCRs.

During positive selection, thymocytes pass through a brief intermediate $CD4^+CD8^{low}$ stage before differentiating into either $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) thymocytes [57]. The divergence into the CD4 helper or CD8 cytotoxic lineage depends on whether the TCR is restricted to MHC class II or MHC class I, respectively [60]. The production of the positively selecting peptides presented on the MHC molecules is also a determinant of positive selection, and is highly dependent on a set of proteolytic enzymes that are specifically expressed in cTECs. For instance, cTEC-restricted cathepsin L and thymus-specific serine protease (TSSP) mediate the production of peptides that are loaded onto MHC class II molecules in late endosomes and are crucial for $CD4^+$ T cell selection [61, 62]. Macroautophagy, a bulk protein degradation process, is also a source of unique peptides, important for the generation of certain MHC class II-restricted specificities [63]. On the other hand, MHC class I molecules present cytoplasm-derived peptides, which are produced by proteolytic complexes termed proteasomes. Proteasomes are formed by a set of three β catalytic subunits. While the β1, β2 and β5 subunits generate the constitutively expressed proteasome, interferon (IFN)-γ-stimulated cells and antigen presenting cells (such as dendritic cells and mTECs) express the β1i, β2i and β5i subunits, which form the immunoproteasome [54]. In contrast, cTECs exclusively express the proteasomal subunit

β5t, which is incorporated with β1i and β2i to form the thymoproteasome, resulting in the ability to process a unique repertoire of peptides important for $CD8^+$ T cell selection [64].

In conclusion, cTECs play a critical role in multiple stages of T cell development, including homing of T cell progenitors, lineage commitment and T cell expansion and development. Furthermore, they possess exclusive proteolytic machinery that allows them to present a unique self-peptide array for positive selection of αβ T cells.

Negative selection and regulatory T cell development

The random generation of the αβ TCR repertoire can produce receptors capable of recognizing self-antigens. The generation and escape of these potentially destructive autoreactive T cells is prevented through specialized functions of the medullary compartment. Positively selected thymocytes upregulate CCR7 on the cell surface, while CCR7 ligands CCL19 and CCL21 are expressed by mTECs. Consequently, CCR7-mediated chemotaxis attracts thymocytes to the medulla, where they will undergo further selection and development [65]. Once in the medulla, SP thymocytes interact with antigen-presenting cells, including mTECs, DCs and thymic B cells, that are able to screen them for self-reactive specificities [59, 66]. Cells bearing TCRs that bind to self-antigen-MHC complexes above a certain threshold are deleted through negative selection. Alternatively, $CD4^+$ SP cells bearing high binding capacity to self-antigens can be redirected to the $FoxP3^+$ regulatory T cell (Treg) lineage [67]. Tregs are essential for immune homeostasis and prevention of spontaneous autoimmunity [68]. Thus, clonal deletion of self-reactive T cells and Treg development are two complementary modes to impose self-tolerance.

Pivotal to both these processes, is the unique ability of mTECs to ectopically express and present tissue-restricted antigens (TRAs), a phenomenon termed promiscuous gene expression (pGE) [69]. TRA loci, whose expression is otherwise limited to specific tissues, become accessible in mTECs to allow their expression and subsequent exposure to developing T cells. Remarkably, mature mTECs express approximately 85% of the coding genome, in contrast to other tissues that typically express 60-65% [70]. The ectopic transcription of TRAs in mTECs is controlled in part by the Autoimmune regulator (Aire) protein [71], together with the more recently described FEZ family zinc finger 2 (Fezf2) [72]. Aire regulates the expression of nearly 4,000 genes by recruiting multi-protein complexes to induce transcription [73]. Still, a significant fraction of TRA transcripts is expressed in mTECs independently of Aire, which implicates the contribution of other factors to pGE [70, 74]. Interestingly, single-cell RNA sequencing revealed that, at a given time point, each TRA is expressed by 1-3% of mTECs, thus each individual mTEC expresses only a fraction of

the TRA repertoire and they ultimately add up at the population level to present a complete and stable TRA set [75]. This is thought to be a mechanism to avoid undesired physiological stress within the cell and also to increase the density of each TRA presented on the cell surface [76]. Although pGE reflects a stochastic mosaic pattern of TRA expression, it is subject to regulation. TRAs expressed at a single-cell level appear to cluster into co-expression groups, and a model has been suggested in which single mTECs can sequentially shift through distinct co-expression groups throughout their lifespan [77]. Central tolerance is highly dictated by the pool of TRAs presented in the medulla at a given time. Several mutations of the Aire gene have been shown to compromise TRA expression and negative selection of autoreactive T cells and are linked to the clinical manifestation of severe autoimmune symptoms [74, 78].

Tolerance induction is achieved through the collaboration of mTECs and DCs [79]. Thymic DCs localize predominantly in the medulla and are subdivided into three major subsets: two subtypes of cDCs, either of intrathymic or extrathymic origin, and pDCs [80]. Intrathymically-derived (or resident) cDCs are able to cross-present TRAs originally expressed by mTECs and play non-redundant roles in shaping the T cell repertoire through clonal deletion and Treg induction [81, 82]. Extrathymically-derived (or migratory) cDCs and pDCs broaden the range of self-antigens displayed in the thymus due to their ability to present peripherally-acquired self-antigens not covered by mTECs, and that otherwise would not be presented to developing thymocytes [83, 84].

During their residency in the medulla, thymocytes go through the selection process while also undergoing their final maturation steps. These last stages of thymocyte development before thymus exit are characterized by several cell surface protein changes. Initially, the recent positively selected or semimature (SM) SP thymocytes are phenotypically characterized as $CD69^{+}CD24^{+}CD62L^{-}Qa2^{-}$ [85] and are susceptible to negative selection [86]. As they upregulate MHC class I and downregulate CD69, they become competent to proliferate in response to TCR stimulation and express molecules associated with egress [85]. The transcription factor Kruppel-like factor 2 (KLF2) promotes CD62L and sphingosine-1-phosphate receptor 1 (S1P1) expression [87]. As they mature, SP thymocytes become $CD69^{-}CD24^{-}CD62L^{+}Qa2^{+}$ and thus acquire the functional competences to enter the peripheral T cell pool. S1P1 regulates the exit from the thymus into the circulation, where the chemotactic gradient of the corresponding S1P ligand is higher [88]. Alternatively, in the fetal thymus, thymocytes egress via a S1P-independent mechanism. Two known contributors to fetal thymus emigration are CCR7 and CXCR4, and the corresponding ligands CCL19 and CXCL12 [89, 90].

In sum, the thymic medulla is a key site in αβ T cell development, in which mTECs and DCs act in concert to provide different layers of tolerance induction, including deletion

of autoreactive T cell clones and Treg development. All the while, it provides the specific microenvironment to nurture the final maturation of the functionally competent and self-tolerant naive T cells.

Thymic involution

At the time of birth, mice possess a virtually empty peripheral T cell pool [91]. During early postnatal life, the thymus increases in size and operates at an extremely high rate of thymopoiesis, critical to establish a functional and diverse T cell compartment. Following this highly productive period, at around 6 weeks in mice and 1 year in humans, thymic size and output begin to reduce [92, 93]. This process, termed thymic involution, is evolutionarily conserved among all thymus-bearing species and is characterized by severe architectural alterations, with loss of true thymic tissue, and an increase in fat, connective tissue, and perivascular space [92]. As a result, fewer T cells are produced and exported as aging progresses. Consequently, the peripheral naive T cell compartment has a reduced frequency of recent thymic emigrants (RTEs), and a concomitant increase occurs in the memory T cell pool [94].

Thymic involution is a contributing factor to the age-related decline in immune system competence, which is associated with several detrimental effects, such as reduced effectiveness and response to vaccination, increased infection susceptibility, autoimmunity, and increased incidence of cancer [95]. Naturally, identifying the genetic or physiological programs and the cell types responsible for involution is of great interest. Several studies have indicated the ageing thymic stroma as the primary driving force of thymic atrophy, rather than changes in the T cell progenitors supplying the thymus [96]. With age, intrinsic and extrinsic factors contribute to the downregulation of several genes that are key in TEC function and homeostasis. Particularly, it has been shown that the downregulation of Foxn1, a key regulator of TEC development, is associated with the onset of thymic involution [97]. In fact, whereas attenuation of Foxn1 in the postnatal thymus leads to premature involution, up-regulation of Foxn1 in the fully involuted thymus improves architecture, gene expression and thymopoietic ability of TECs, which in turn regenerates thymic activity [98, 99].

Cellular and molecular aspects of mTEC development

mTEC diversity

The studies described earlier highlight the role of mTECs in safeguarding self-tolerance. As such, substantial efforts have been directed into the identification and characterization of mTEC diversity. A significant degree of phenotypic and functional heterogeneity has been described, and currently, different mTEC subpopulations are routinely identified by the expression of several molecular markers. The first level of diversity can be defined according to the expression of cell surface MHCII and CD80 molecules, which subdivides mTECs into $MHC^{low}CD80^{low}$ ($mTEC^{low}$) and $MHC^{high}CD80^{high}$ ($mTEC^{high}$) [100]. The $mTEC^{low}$ subset includes cells at distinct developmental stages. They were first shown to contain cells with the ability to give rise to mature $mTEC^{high}$, however, it was later revealed that a fraction of $mTEC^{low}$ expresses markers associated with terminal differentiation such as involucrin (Ivl) and keratin 10 (K10) and constitute a population of previously Aire-expressing cells, also termed post-Aire cells [101, 102]. Additionally, a fraction of $mTEC^{low}$ was shown to be responsible for the production of the chemokine CCL21, essential in the recruitment of thymocytes from the cortex to the medulla [103]. Thus, $mTEC^{low}$ are a heterogenous population, containing immature precursors of $mTEC^{high}$, but also terminally differentiated mTECs and a CCL21-producing subset with a specific role in T cell development **(Figure 3)**.

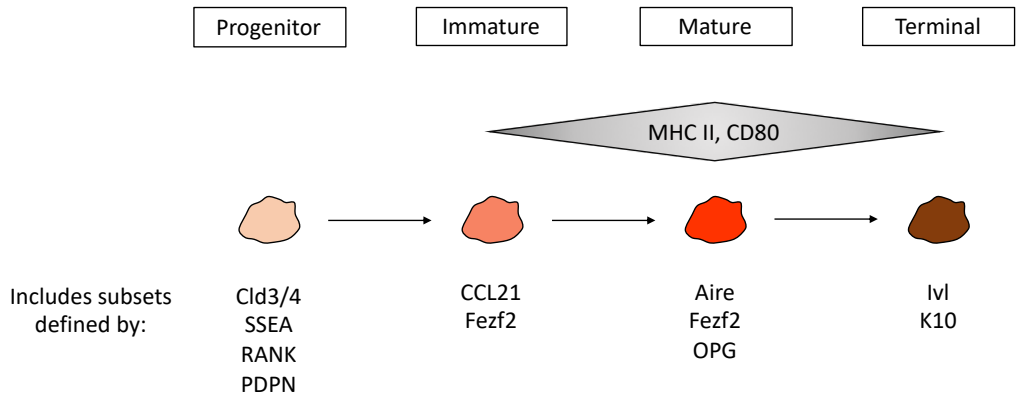

Figure 3 - mTEC differentiation stages possess cells with varying levels of MHC class II and CD80 expression and include subpopulations with other specific molecular markers. Several markers are associated with mTEC precursors (as discussed in "TEC progenitors"), including Cld3/4, SSEA, RANK and PDPN. mTECs characterized as $MHCII^{low}CD80^{low}$ ($mTEC^{low}$) include precursors of mature $MHCII^{high}CD80^{high}$ ($mTEC^{high}$), CCL21-producing cells, cells expressing Fezf2, and also terminally differentiated post-Aire cells expressing Ivl and K10. The $mTEC^{high}$ subset is also heterogeneous, with subpopulations defined by expression of Aire, Fezf2 and OPG.

Regarding the mTEC^{high} subset, these cells are characterized by a high turnover rate (2 to 3 weeks), while mTEC^{low} include only a small fraction of cycling cells [104]. At around E16, a fraction of mTEC^{high} begins expressing Aire, a crucial regulator of TRA gene expression for self-tolerance induction in thymocytes [100, 104]. Within Aire^{+} mTEC^{high}, further subdivision is possible based on the differential expression of osteoprotegerin (OPG), a regulator of mTEC cellularity and proliferation [105]. mTEC^{high} are also characterized by expression of the transcription factor Fezf2, which directly regulates TRA expression in an Aire-independent manner. Although at a lower frequency, Fezf2 is also detected in mTEC^{low} [72] **(Figure 3)**.

Due to the rapid turnover of Aire^{+} mTECs, it has been speculated that Aire expression might result in increased apoptosis [100]. This mechanism would allow the mTEC-derived TRAs to be passed onto DCs and improve the efficiency of cross-presentation. This notion was supported by the finding that in the absence of Aire, mTEC^{high} increase in proportion [100]. Additionally, it has been argued that Aire might not impact mTEC lifespan, but instead be required for the completion of the mTEC differentiation program and final transition into the mTEC^{low} post-Aire stage [106]. Thus, two different models are currently contemplated for the role of Aire in mTEC maturation. On one hand, Aire expression could interrupt mTEC maturation and only in its absence the cells would reach terminal differentiation. Alternatively, Aire could be a promoter of mTEC maturation, and thus, Aire deficiency would inhibit cells from completing their differentiation program [107].

Thymic crosstalk

During early thymic ontogenesis, the cortical and medullary areas are not yet organized in the typical three-dimensional arrangement observed in the adult thymus. Growth and organization of both compartments begins with colonization of the thymus by hematopoietic progenitors and is induced by instructive signals from the developing thymocytes. As such, a symbiotic relationship exists between thymocytes and TECs. These thymocyte-TEC interactions, commonly termed “thymic crosstalk”, are important to regulate TEC development, maintenance and function [16]. In the cortex, for instance, thymocyte-derived signals control the expression of Notch ligand DLL4 and IL-7 by cTECs, which are key factors during early T cell development [108, 109].

Crosstalk between thymocytes and mTECs is far more characterized and is an essential requirement for the development and maintenance of the thymic medulla. Thymocytes express ligands for several members of the tumour necrosis factor receptor

superfamily (TNFRSF), including receptor activator of NF-κB (RANK), CD40 and lymphotoxin β receptor (LTβR). These receptors are expressed in mTECs (and their precursors) and their engagement induces activation of the canonical and noncanonical nuclear factor kappa B (NF-κB) signalling pathways, which activate the transcriptional programs required for mTEC lineage specification and differentiation **(Figure 4)** [110]. As such, interference with elements of the NF-κB signal transduction pathways compromises mTEC development and induces autoimmunity [111]. For example, deficiency in NF-κB inducing kinase (NIK) or tumour necrosis factor receptor-associated factor 6 (TRAF6) results in a disorganized thymic medulla and absence of mature mTECs [112, 113]. Moreover, disruption at a more downstream point in the pathway further aggravates the phenotype, with deficiency in the RelB (V-Rel reticuloendotheliosis viral oncogene homolog B) subunit of the NF-κB complex resulting in a highly hypomorphic medulla **(Figure 4)** [114].

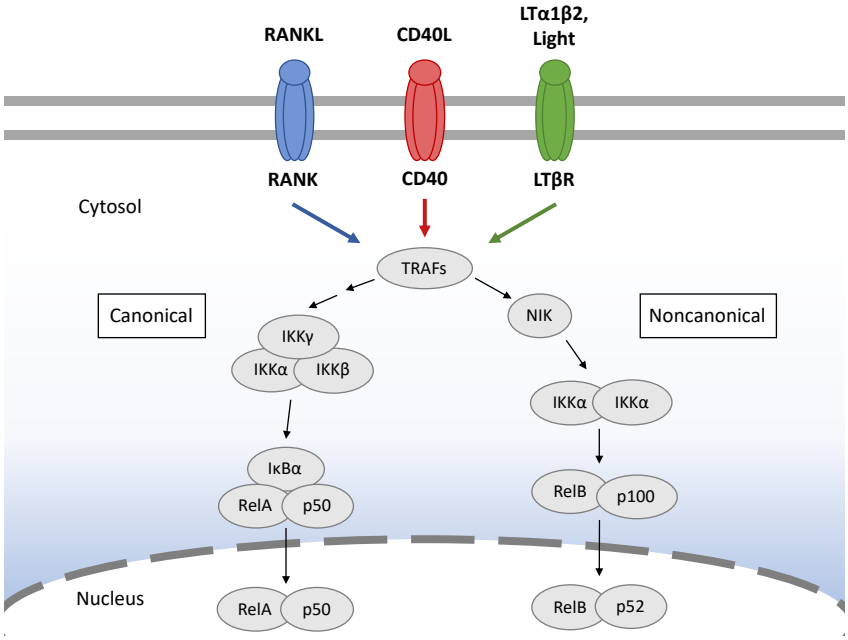

Figure 4 - RANK, CD40 and LTβR signalling lead to NF-κB activation pathways. Interaction of TNFRSF members with their respective ligands induces activation of NF-κB pathways. TNF receptor-associated factor (TRAF) family proteins bind to the cytoplasmic domains of TNF receptors and activate a downstream kinase cascade, which includes the NF-κB-inducing kinase (NIK) and IκB kinase (IKK) complexes. These kinases trigger the degradation of inhibitor of κb (IκB) proteins or the processing of p100, which sequester NF-κB in the cytosol, thereby leading to NF-κB nuclear translocation and transcriptional activation. In the canonical pathway, the NF-κB complex consists predominantly of RelA and p50, while the NF-κB subunits in the noncanonical pathway are typically RelB and p52 [115].

The TNFRSF ligands are differentially expressed by hemopoietic cells and their cellular source varies between developmental periods. In the embryonic thymus, prior to αβ T cell positive selection, innate $CD4^{+}3^{-}$ lymphoid tissue inducer (LTi) cells induce the development of the first $Aire^{+}$ mTECs through the expression of RANK ligand (RANKL) [116]. Invariant $Vγ5^{+}$ thymocytes accumulate in the embryonic medulla and are also able to trigger mTEC maturation through RANKL expression [117]. These findings support an initial role for innate LTi cells and γδ T cell progenitors in inducing $Aire^{+}$ mTECs to ensure

tolerance induction in the emergent αβ T cells. In adult mice, medulla formation is mainly maintained by positively selected $CD4^+$ and $CD8^+$ thymocytes and iNKT cells through RANKL/RANK signals [118, 119]. Furthermore, intrathymic CD40L is provided by $CD4^+$ SP T cells and CD40L-mediated antigen-specific interactions with self-reactive $CD4^+$ thymocytes are important to control mature mTEC cellularity [120].

While in adult RANKL-deficient thymus, $Aire^+$ mTECs are present at a drastically reduced frequency, CD40 deficiency results in a more modest reduction. Still, mice with combined deficiency in RANKL and CD40 show a more profound reduction in $Aire^+$ mTECs compared to mice lacking only RANK-mediated signalling [121]. Thus, CD40-mediated signalling appears to partially compensate for the absence of RANKL. Concluding, whereas RANK alone can induce $Aire^+$ $mTEC^{high}$ during embryogenesis, in adult mice, the establishment of the medullary microenvironment results from the cooperation between CD40 and RANK signals.

LTβR signalling is required for the organization of the medulla and export of mature T cells from the thymus [122]. Although it is not required for a normal $Aire^+$ mTEC frequency [123], it has been shown to control functional properties in $mTEC^{low}$. Particularly, it regulates the expression of medullary chemokines CCL19 and CCL21 by $mTEC^{low}$, which are required for thymocyte migration to the medulla and consequent negative selection [124]. Additionally, it is important for the expression of Aire-independent TRAs in $mTEC^{low}$ [125]. LTβR was also shown to be important in later mTEC development, particularly in regulating the transition into the terminally differentiated post-Aire stage [102].

It is possible that TNFRSF-mediated signalling may act stepwise during mTEC development. Embryonic $Cld3/4^+SSEA1^+$ mTEC progenitors are $LT\beta R^+RANK^-$ and were shown to emerge in the absence of RelB. On the other hand, production of temporally downstream $Cld3/4^+SSEA^-RANK^+$ mTEC progenitors is RelB-dependent, implicating the noncanonical NF-κB pathway [36]. Coincidently, LTβR signalling can elicit RANK expression and condition mTECs to receive the early RANK signal for differentiation [126]. Still, more insight is required to assume a direct lineage relationship between these two subsets. Lastly, an initial RANK signalling is in turn necessary to upregulate CD40 expression on developing mTECs [127].

Aims

mTECs represent a dynamic niche with precursor-product relationships between subsets with distinct functional abilities, regulated by several cooperative but non-redundant thymocyte-derived signals. Valuable past studies have established the foundations of a mTEC developmental program that is believed to be far more intricate. In this thesis, we aimed to further dissect mTEC diversity and provide new insights into the lineage and functional relationships between their subpopulations.

Taking advantage of multicolour flow cytometry analysis, we began by investigating the expression pattern of CD24 and stem cell antigen-1 (SCA1) in murine mTECs, in conjunction with well-established mTEC markers such as MHCII, CD80 and Aire, throughout thymic ontogeny. We next sought to investigate the lineage potential and molecular requirements of the novel subpopulations defined by the expression of CD24 and SCA1 using thymic organotypic cultures. Furthermore, we studied the requirement for Aire expression in the emergence of the mTEC subpopulations by analysing $Aire^{-/-}$ and Aire reporter mouse models. Lastly, using a CCL21 reporter mouse, we mapped CCL21 expression in the newly-defined mTEC diversity.

Characterizing the cellular and molecular determinants of mTEC development, as well as their functional contributions within this compartment, can ultimately elucidate and highlight the key steps involved in thymic medulla development and tolerance induction.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were kept and bred at the animal facility of Instituto de Investigação e Inovação em Saúde (i3S) under specific pathogen-free conditions. For fetal studies, the day of vaginal plug detection was designated as embryonic day (E) 0.5. Actin^{RFP} mice were purchased from The Jackson Laboratory and bred at the animal facility of i3S under specific pathogen-free conditions. Aire$^{-/-}$ and Aire/DTR KI [128] mouse models were analysed in collaboration with Mitsuru Matsumoto's laboratory and CCL21 reporter mice [129] were analysed in collaboration with Graham Anderson's laboratory. All studies were conducted in accordance with institutional guidelines.

Isolation and flow cytometry analysis of TECs

Thymi were dissected at the indicated time points and digested for 30 minutes at 37°C in trypsin (Sigma). A syringe was used to mechanically disrupt the thymic fragments every 5 minutes. Single-cell suspensions were enriched through the depletion of thymocytes using a MACS-based CD45^{+} cell depletion kit (Miltenyi Biotec) according to the manufacturer's instructions. Cell concentration was determined in a haemocytometer.
Cell surface staining was carried out for 30 minutes at 4°C with the following antibodies: PerCP-Cy5-conjugated anti-CD45.2 (clone 104), PE-conjugated anti-Ly51 (clone 6C3), APC-eFlour 780-conjugated anti-I-A/I-E (clone M5/114-15-2), Alexa eFluor 647-conjugated anti-EpCAM (clone G8.8), PE-conjugated anti-CD80 (clone 16-10A1) (all from eBioscience); BV421-conjugated anti-EpCAM (clone G8.8), BV650-conjugated anti-CD80 (clone 16-10A1), BV510-conjugated anti-CD24 (clone M1/69), BV786-conjugated anti-SCA1 (clone D7), APC-Fire-conjugated anti-CD24 (clone M1/69), Alexa 488-conjugated anti-SCA1 (clone D7) (all from Biolegend). Biotinylated UEA (vectorshield) was revealed with BV711-conjugated (Biolegend) or PE-Cy7-conjugated streptavidin (eBioscience). For intracellular staining, and upon cell surface labelling, cells were washed, fixed and permeabilized using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Intracellular staining was then carried out with eFlour 660-conjugated anti-AIRE and FITC-conjugated anti-Ki67 (both from eBioscience) for 30 minutes at 4°C. Analysis was performed using the LSRFortessa flow cytometer using the BD FACSDIVA software (BD Bioscience). The collected sample files were analysed using FlowJo v10.2 software (FlowJo, lcc).

Fetal thymus organ culture (FTOC)

E14 thymic lobes were dissected and used to establish fetal thymus organ cultures (FTOCs). Three to four lobes were used per condition to normalize intrathymic variations. Lobes were cultured in 6-well plates on a 0.8 μm Isopore membrane filter (Milipore) resting on a foam sponge placed in 1 mL DMEM medium supplemented with 10% FCS, 1% L-glutamine 200mM (GIBCO), and with or without 360mg/L of 2-deoxyguanosine (dGuo) (Sigma). After 4 days, the dGuo-treated FTOCs were cultured in either medium alone or with 5μg/ml of recombinant CD40L (CD40L) (R&D Systems), 2μg/ml of agonist anti-RANK (αRANK) (R&D Systems) or 10μg/ml of agonist anti-LTβR (αLTβR) mAB AC.H6 (provided by Jeff Browning, Biogenidec, US). After 2 or 4 days in culture, the lobes were digested and analysed by flow cytometry.

Reaggregate thymus organ culture (RTOC)

Thymic single-cell suspensions from embryonic (E14 to E17) $Actin^{RFP}$ mice or newborn (P1 to P3) C57BL/6 mice were stained as previously described and acquired in a FACSAria II (BD Biosciences) for the sorting of the following mTEC ($CD45^{-}EpCAM1^{+}UEA^{+}$) populations: $CD24^{+}SCA1^{-}CD80^{-}$, $CD24^{+}SCA1^{+}CD80^{-}$ and $CD24^{-}SCA1^{-}CD80^{+}$. Isolated mTEC populations were mixed with single-cell suspensions from E14 C57BL/6 or BALB/c thymic lobes and reaggregates were performed as previously described [130] under the organ culture conditions described above. Chimeric reaggregate thymus organ cultures (RTOCs) were established by mixing 25.000 $Actin^{RFP}$ mTECs with $5x10^{7}$ E14 C57BL/6 thymic cells or 20.000-30.000 of the different sorted populations with $5x10^{7}$ E14 BALB/c thymic cells. After 7 days in culture, $Actin^{RFP}$ RTOCs were assessed for fluorescence in a IN Cell Analyzer 2000 (GE Healthcare). All RTOCs were subsequently dissociated and analysed by flow cytometry.

Results

The differential expression of CD24 and SCA1 defines new mTEC subsets

An in depth characterization of mTEC differentiation has been prevented due to the limited number of traits to define distinct developmental and functional states [131]. Our initial approach was to search for additional mTEC markers to further resolve the maturation program of mTECs. Using multicolour flow cytometry, we integrated the analysis of the expression of CD24 and SCA1 in the prototypical study of cTECs and mTECs. These two markers have been associated with stem/progenitor cell potential in other tissues [132, 133]. For this purpose, thymi were isolated from C57BL/6 mice ranging from embryonic day 14 (E14) to 6 weeks-old (6W). TECs were defined as $CD45^-$ $EpCAM^+$ (supplemental Fig. 1) and subdivided into UEA^+Ly51^- mTECs, UEA^-Ly51^+ cTECs and UEA^-Ly51^- TECs [41]. At E14, all TECs were uniformly $CD24^+SCA1^-$. In both cTECs and UEA^-Ly51^- TECs, the expression of CD24 was reduced throughout embryonic life and these cells were mostly $CD24^-SCA1^-$ at the neonatal period. However, at a later point of development, $CD24^-SCA1^+$ cells, as well as rare $CD24^+SCA1^-$ and $CD24^+SCA1^+$ cells, appeared in cTECs and UEA^-Ly51^- TECs (Fig. 5).

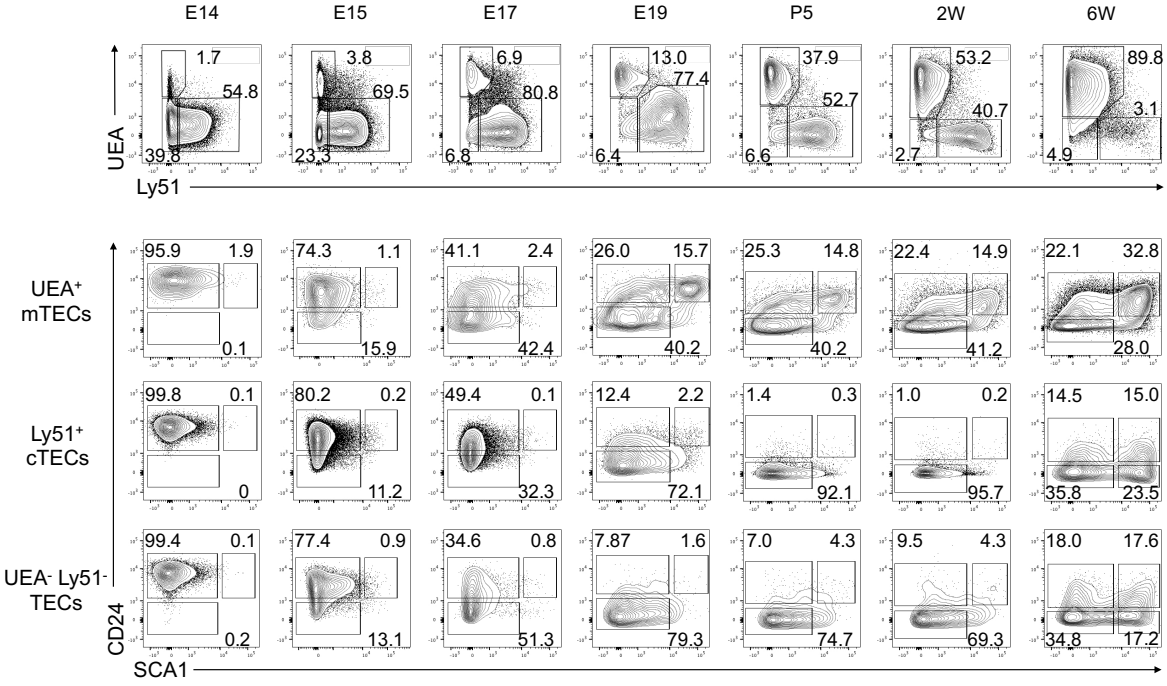

Figure 5 - **Differential expression CD24 and SCA1 defines novel subsets of mTECs**. Thymi from C57BL/6 mice were isolated at the indicated time points and total TECs (defined hereafter as $CD45^-EpCAM^+$) were analysed for expression of Ly51 and UEA binding by flow cytometry (FC). UEA^+Ly51^- mTECs, UEA^-Ly51^- TECs and UEA^-Ly51^+ cTECs were analysed for CD24 and SCA1 expression. Numbers in plots indicate the frequency of cells found within each gate. E – Embryonic day; P – Postnatal day; W – Weeks. Plots presented are representative of at least three analysis per time point.

Interestingly, only a fraction of mTECs became $CD24^-$ during embryonic development, and a distinct $CD24^+SCA1^+$ subset appeared in the perinatal period (E19) (Fig. 5). Consequently, three mTEC subpopulations could be defined as $CD24^+SCA1^-$, $CD24^+SCA1^+$ and $CD24^-SCA1^-$, hereafter referred to as $mTEC^I$, $mTEC^{II}$ and $mTEC^{III}$. The diversity within the mTEC compartment defined by the expression pattern of CD24 and SCA1 was maintained in young adult mice (6W) (Fig. 5). Overall, these results reveal that the differential expression of CD24 and SCA1 allows the identification of novel mTEC subpopulations, whose developmental and functional characterization became the main focus of this work.

$mTEC^{II}$ and $mTEC^{III}$ subsets are respectively enriched for $mTEC^{low}$ and $mTEC^{high}$

Within mTECs, two major subpopulations can be defined according to the co-expression of MHCII and CD80. On one hand, $MHCII^{low}CD80^{low}$ mTECs, also known as $mTEC^{low}$, include a mixture of immature precursors and terminally differentiated mTECs [102]. On the other hand, $MHCII^{high}CD80^{high}$ mTECs, known as $mTEC^{high}$, include mostly mature cells, including $Aire^+$ cells [100]. Thus, we sought to investigate how $mTEC^I$, $mTEC^{II}$ and $mTEC^{III}$ were phenotypically related with $mTEC^{low}$ and $mTEC^{high}$. Throughout time, $mTEC^I$ included both $mTEC^{low}$ and $mTEC^{high}$ (Fig. 6, *red*). Notably, the $mTEC^{II}$ encompassed mostly $mTEC^{low}$, while the $mTEC^{III}$ were consistently enriched for $mTEC^{high}$ (Fig. 6, *green and blue*).

$mTEC^{high}$ present a further degree of heterogeneity, as a large fraction of these cells expresses the Aire protein and represents one of the later stages of mTEC maturation [100]. We next analysed how $Aire^+$ cells were related with $mTEC^I$, $mTEC^{II}$ and $mTEC^{III}$ populations. Throughout development, while $Aire^-$ $mTEC^{high}$ distributed equally throughout the three subsets, $Aire^+$ $mTEC^{high}$ were located predominantly in $mTEC^{III}$ (Fig. 7). As such, the lack of SCA1 and CD24 expression is correlated with a mature $Aire^+$ mTEC stage.

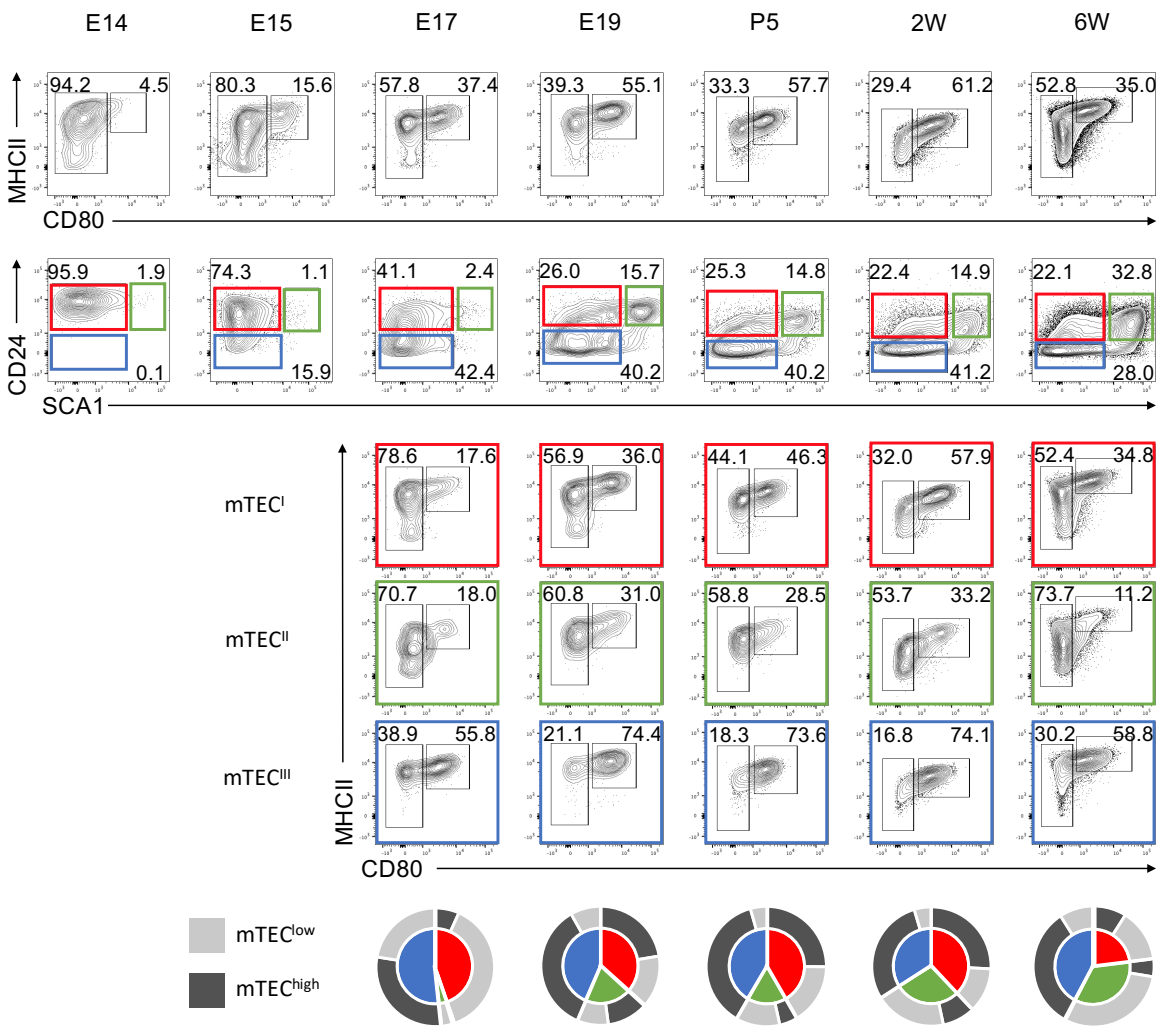

Figure 6 - Correlation between the mTEC^{I-III} subsets and mTEC^{low} and mTEC^{high}. Thymi from C57BL/6 mice were isolated at the indicated time points and UEA^{+} mTECs were analysed for CD80, MHCII, CD24 and SCA1 expression by FC. The subsets defined by the coloured gates, mTEC^{I} (red), mTEC^{II} (green) and mTEC^{III} (blue), were analysed for the expression of CD80 and MHCII. Plots are representative of at least three analysis per time point. Pie charts represent the average proportion of mTEC^{low} (light grey) and mTEC^{high} (dark grey) within each of the colour-coded mTEC subsets. E – Embryonic day; P – Postnatal day; W – Weeks.

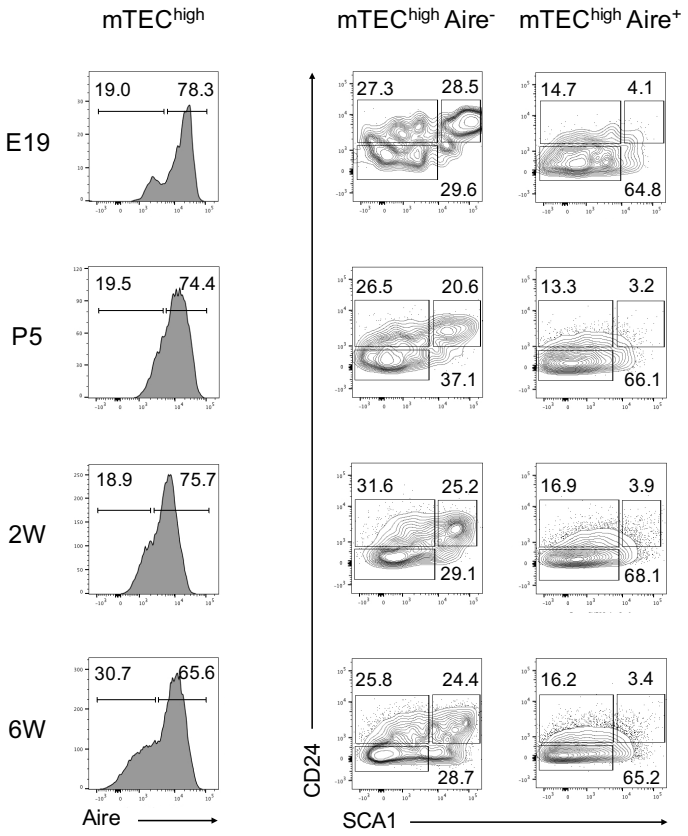

Figure 7 - **Aire expression is largely confined to the mTEC^{III} subset.** Thymi from C57BL/6 mice were isolated at the indicated time points and UEA^{+}CD80^{+} mTEC^{high} were analysed for expression of Aire by FC. Aire^{-} mTEC^{high} and Aire^{+} mTEC^{high} were analysed for CD24 and SCA1 expression. Plots are representative of at least three analysis per time point. E – Embryonic day; P – Postnatal day; W – Weeks.

In addition, mTEC heterogeneity has also been described based on the distinct proliferation/turnover rates of mTEC^{low} and mTEC^{high} [104]. To further characterize the new mTEC subsets, we also investigated their proliferative status by analysing the expression of the proliferative marker Ki67 [134]. These results revealed that the pool of cycling mTECs in the postnatal thymus comprised mostly mTEC^{III} (Supplemental Fig. 2). This observation is in agreement with the fact that mTEC maturation correlates with an increased turnover rate [104].

Collectively, these results indicate that mTEC^{III} are a mostly mature subset, enriched for cycling mTEC^{high} and Aire-expressing cells. On the other hand, mTEC^{II} define a distinct mTEC^{low} subset and mTEC^{I} encompass both mTEC^{low} and mTEC^{high}.

Lineage relationship between mTEC^{I}, mTEC^{II} and mTEC^{III}

We next investigated the precursor-product relationship between the newly-defined mTEC subsets. Given that the mTEC compartment was comprised of mTEC^{I} CD80^{low} in the E14 thymus (Fig. 6, *E14 column*), we first sought to determine whether this subset was able to generate the diversity observed in postnatal life. To do so, we isolated mTEC^{I} CD80^{low} from Actin reporter E14 to E17 thymi and mixed them with unlabelled fetal thymus cells to establish *in vitro* reaggregate thymus organ cultures (RTOCs) (Fig. 8A-B) [130]. In Actin reporter mice, the gene encoding red fluorescent protein (RFP) is inserted under the control of the *Actin* promoter, inducing ubiquitous and continuous expression of RFP [135]. Thus, we used RFP labelling to track the progeny of the initial population of interest. After 7 days in culture, the specificity of the detection of RFP^{+} cells was confirmed by analysis of the control RTOC composed only of carrier fetal thymus cells (Fig. 8C, *left*). Analysis of the chimeric RTOC revealed that the existing RFP^{+} cells contained mTEC^{I}, mTEC^{II} and mTEC^{III}, as well as CD80^{low} and CD80^{high} cells (Fig. 8C, *right*). These findings demonstrate that embryonic mTEC^{I} CD80^{low} contain precursors capable of differentiating into cells of the remaining subsets.

To further expand our analysis, we proceeded to assess the lineage potential of the three mTEC subsets (mTEC^{I} CD80^{low}, mTEC^{II} CD80^{low} and mTEC^{III} CD80^{high}) in the postnatal thymus. The three different cell types were isolated by cell sorting from newborn C57BL/6 mice and used to establish individual RTOCs with fetal thymus cells of BALB/c mice (Fig. 9A-B). In this system, the different MHCI haplotypes allowed us to distinguish the distinct spiked mTEC subsets of interest (H-2K^{b+}) from the carrier cells (H-2K^{d+}). In addition, this complementary experimental setup allowed the evaluation of the expression of intracellular markers (*e.g.* Aire). After 7 days in culture, we confirmed the specificity of detection of H-2K^{b+}-derived cells by analysis of the control RTOC formed only by BALB/c cells (Fig. 9C, *left*). Regarding mTEC^{I} CD80^{low}, we found that these cells generated all the subsets, including CD80^{high} and Aire^{+} cells (Fig. 9C, *red*). These results are similar to the ones obtained in the experiments conducted with Actin^{RFP}-derived cells, although we find some variation in the frequency of the generated mTEC subsets.

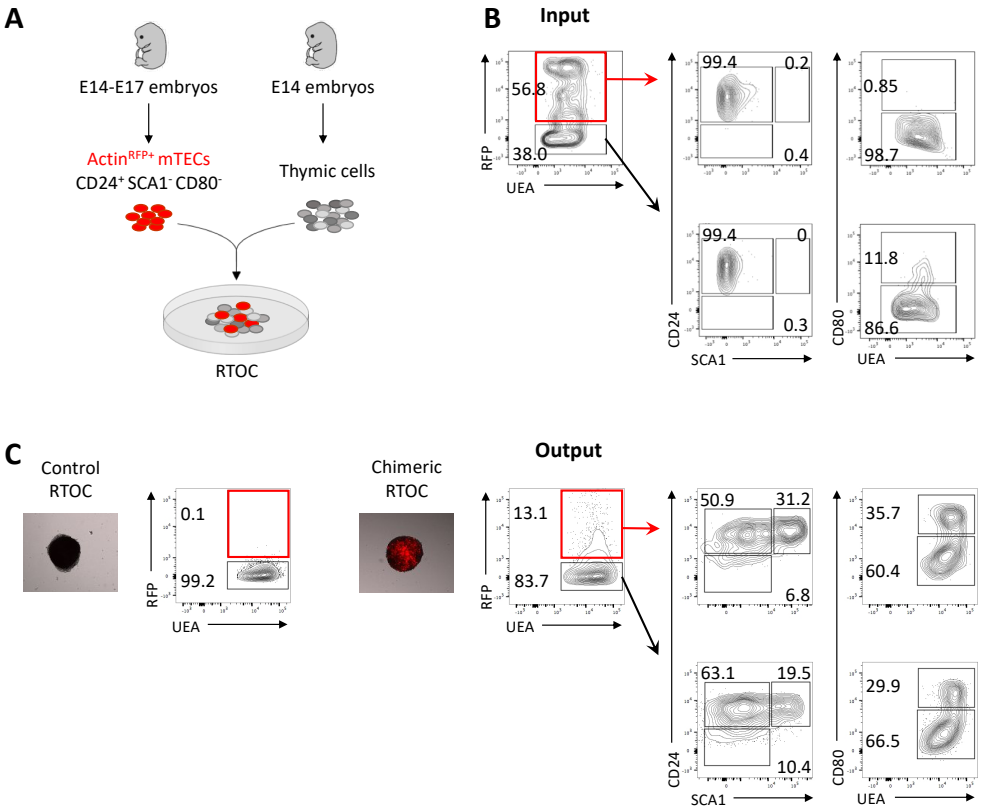

Figure 8 – mTEC^{I}CD80^{low} give rise to the remaining mTEC subsets. (A) Chimeric RTOCs were established with disaggregated fetal thymus cells placed in co-culture with mTEC^{I} CD80^{low} isolated from Actin^{RFP} E14-E17 embryos. (B) Analysis by FC of UEA^{+} mTECs in the cell suspension used to perform the chimeric RTOC at day 0 (input). (C) After 7 days in culture, control and chimeric RTOCs were dissociated and UEA^{+} mTECs were analysed by FC (output). Data presented correspond to a representative analysis out of 3 experiments

The mTEC^{II} CD80^{low} were also capable of differentiating into mTEC^{I} and mTEC^{III}, including CD80^{high} and Aire^{+} cells. Nevertheless, a large fraction of mTEC^{II} CD80^{low} maintained their original phenotype (Fig. 9C, *green*). Lastly, we found that mTEC^{III} CD80^{high} remained mostly unaltered, although mTEC^{I} and mTEC^{II} were also detected. As expected, the progeny resulting from these cells had the highest frequency of CD80^{high} and Aire^{+} cells (Fig. 9C, *blue*), in accordance with the more advanced maturation state of the initial population. Overall, these results show that mTEC^{I} CD80^{low} and mTEC^{II} CD80^{low} include precursors capable of differentiating into CD80^{high} Aire^{+} cells and generating the other mTEC subsets.

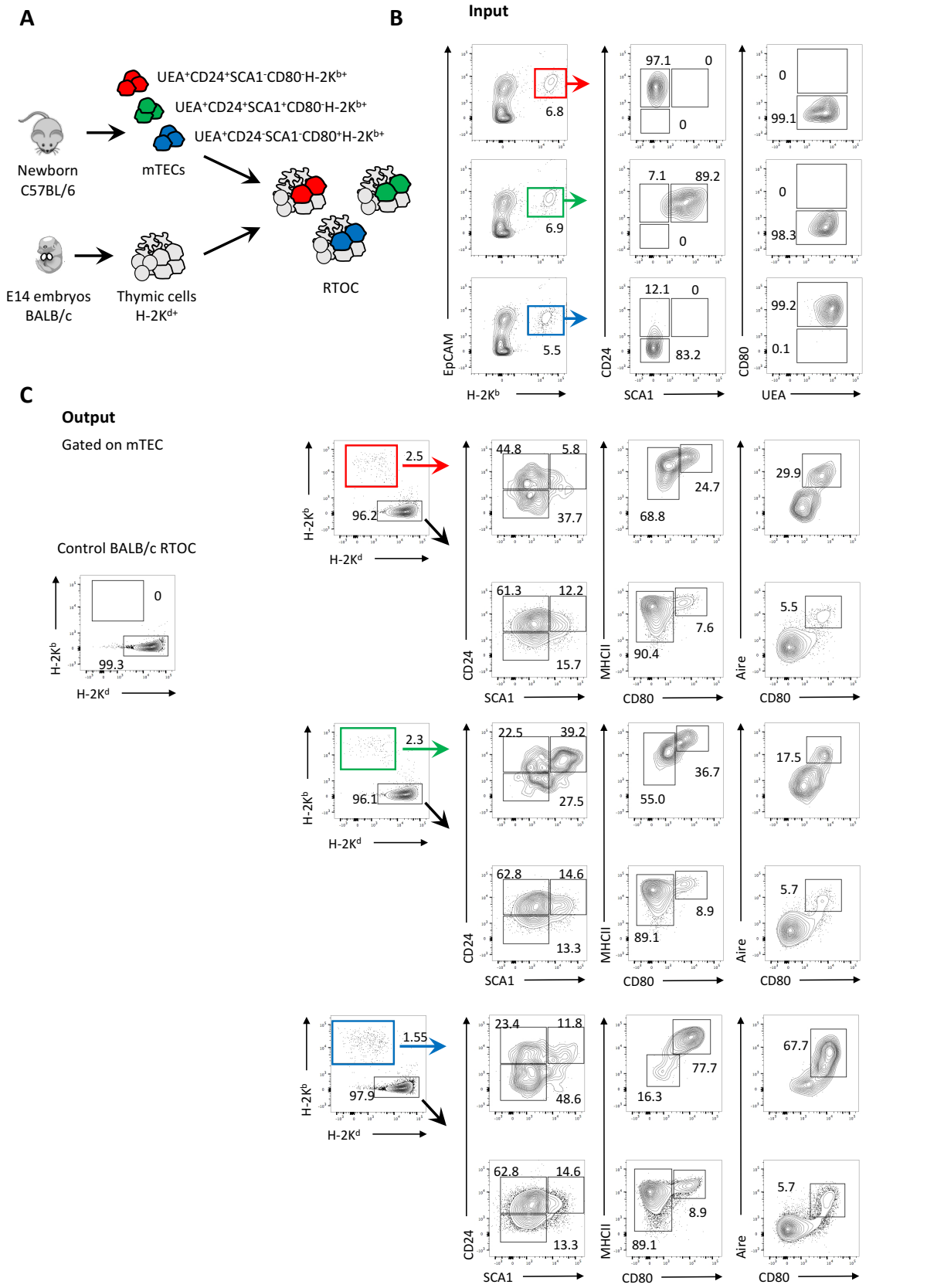

Figure 9 – ***In vitro*** **lineage potential of mTEC^{I-III} subsets.** Chimeric RTOCs were established with disaggregated fetal thymus cells from BALB/c mice and placed in co-culture with mTEC^{I} CD80^{low}, mTEC^{II} CD80^{low} or mTEC^{III} CD80^{high} isolated from newborn C57BL/6 mice. (B) Analysis by FC of the cell suspension used to perform the chimeric RTOCs at day 0 (input). (C) After 7 days in culture, control and chimeric RTOCs were dissociated and UEA^{+} mTECs were analysed by FC (output). C57BL/6 and BALB/c cells were separated based on H-2K^{b+} and H-2K^{d+} expression, respectively. Data presented correspond to a representative analysis out of 2 experiments for the mTEC^{I} CD80^{low} and mTEC^{II} CD80^{low} and of 1 experiment for the mTEC^{III} CD80^{high}.

Differentiation of mTEC^{I-III} is uncoupled from *in vitro* TNFRSF-induced maturation and independent of post-β-selected thymocytes

The development and differentiation of the mTEC compartment is primarily driven by signals transduced by several members of the TNFRSF, including LTβR, CD40 and RANK, whose respective ligands are presented mainly by developing thymocytes (and other hematopoietic populations) [110]. Considering that these signals promote mTEC maturation, we next explored if the emergence of mTEC^{I}, mTEC^{II} and mTEC^{III} would also be under the control of TNFRSF signalling. To identify the molecular requirements for the generation of the described mTEC diversity, we mimicked mTEC development using fetal thymus organ culture (FTOC) [136]. In this *in vitro* system, E14 thymi were depleted of thymocytes with a 2-deoxyguanosine (dGUO) treatment and cultured with LTβR, RANK or CD40 agonists for 2 or 4 days. As expected, in the non-depleted FTOCs, thymocyte-TEC crosstalk induced significant mTEC development and maturation, defined by UEA^{+} mTECs and mTEC^{high} (Fig. 10A-B). In contrast, the maturation of mTECs was completely abrogated in the dGUO-treated control FTOCs (Fig. 10A-B). LTβR activation did not induce mTEC^{high} and its effect was limited to the mTEC^{low} population, where it induced MHCII upregulation (Fig. 10A-B). In contrast, CD40 and RANK stimulation induced UEA^{+} mTECs and mTEC^{high}, with an increased effect in the 4-day cultures (Fig. 10A-B). These results are in line with the dominant contribution of RANK and CD40 signalling for mTEC development [121]. However, under these conditions, mTEC^{high} remained largely as mTEC^{I}, while mTEC^{II} and mTEC^{III} failed to develop (Fig. 10A-B). Therefore, the generation of CD80^{+} mTEC^{high} occurred uncoupled from the described mTEC diversity. This observation is in agreement with the *ex vivo* data (Fig. 6, *E15 and E17 columns*), in which the first mTEC^{high} population was detected before the complete establishment the mTEC^{I-III} subsets. While the RANK and CD40-activated conditions failed to induce the diversity observed in the *ex vivo* analysis, the mTEC^{I-III} profile was partially replicated in the FTOC condition (Fig. 10A-B). Our data indicate that differentiation induced *in vitro* through TNFRSF signalling does not directly establish the mTEC^{I-III} subsets, and imply a requirement for other crosstalk-derived signals. To further address which thymocyte subpopulations provide the differentiating signals that induce the development of the novel mTEC subsets, we analysed Rag2$^{-/-}$ mice. In these mice, thymocyte development is blocked at the β-selection event, therefore thymocytes only reach the DN3 stage [137]. The impaired thymic crosstalk caused a severe reduction of the mTEC compartment (Fig. 10C). Nevertheless, mTEC differentiation was similar to that observed in WT mice (Fig. 10C; Fig. 6, *2W column*), including the establishment of mTEC^{I},

mTEC^{II} and mTEC^{III}. These results indicate that the signals provided by DN1 to DN3 thymocytes are sufficient to induce the reported mTEC diversity.

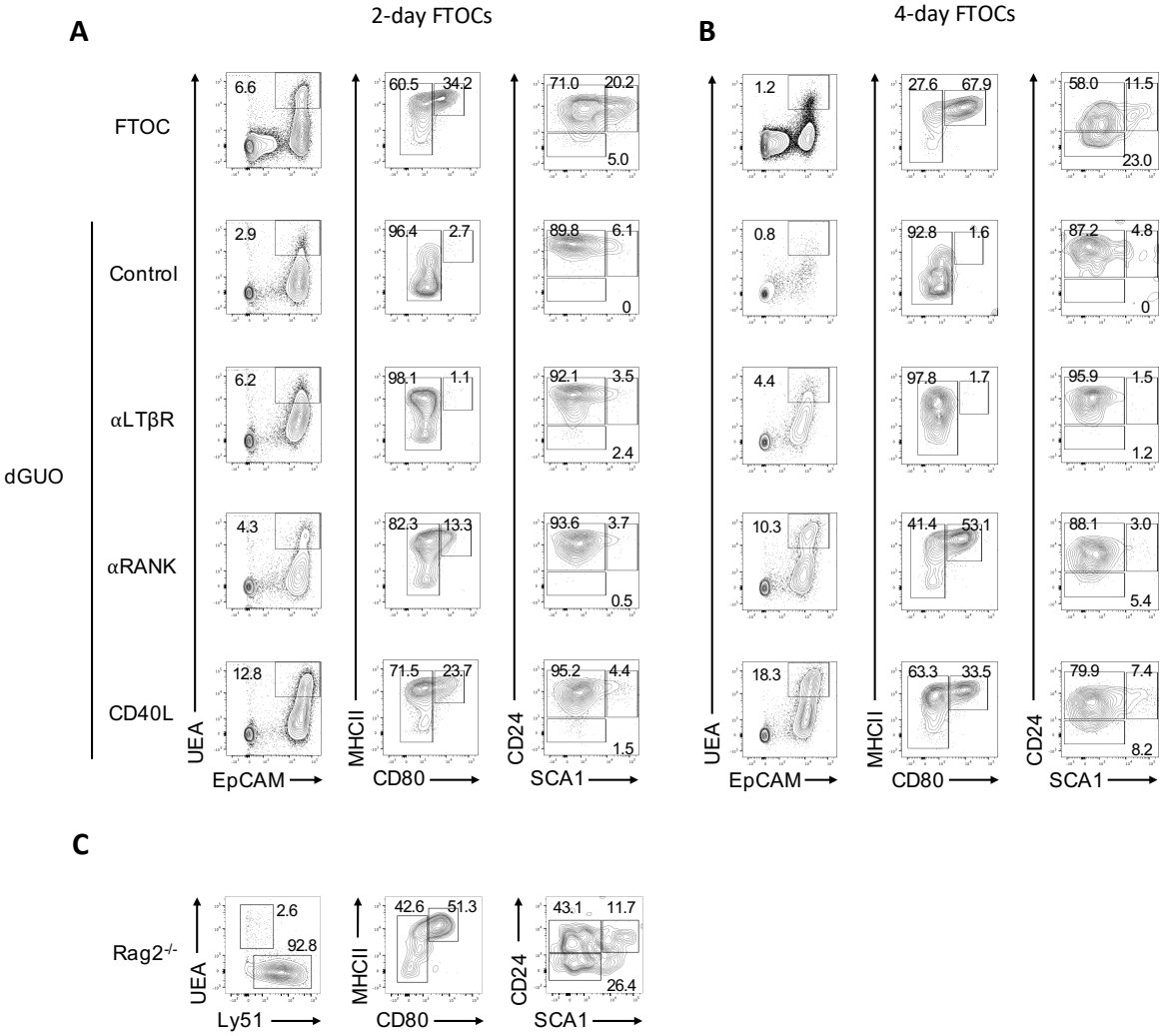

Figure 10 - Establishment of mTEC^{I-III} is uncoupled from *in vitro* TNFRSF-induced maturation and independent of crosstalk with post-β-selected thymocytes. dGUO treated thymi were cultured in medium (Control) or in the presence of anti-LTβR (αLTβR) or anti-RANK (αRANK) agonist antibodies, or recombinant CD40L (CD40L), for either 2 (A) or 4 days (B). Total cells were analysed by FC for UEA binding and expression of EpCAM. UEA$^+$ mTECs were analysed for CD80, MHCII, CD24 and SCA1 expression. Data representative of 3 (2-day FTOCs) and 2 (4-day FTOCs) independent experiments. (C) TECs from 2 weeks-old Rag2$^{-/-}$ thymi were analysed for expression of Ly51 and UEA binding by flow cytometry. UEA$^+$ mTECs were analysed for expression of CD80, MHCII, CD24 and SCA1. Data representative of two independent experiments.

The frequency of mTEC^{I-III} is regulated by Aire-dependent differentiation

Beyond the primary role of Aire in the transcriptional control of TRAs in mTECs, different studies have highlighted a possible role for Aire in mTEC differentiation [107, 128]. Particularly, Aire$^{-/-}$ mice show an increased proportion and number of mTEC^{high} [106]. We then proceeded to explore if alterations in mTEC maturation that result from Aire deficiency were accompanied by changes in mTEC^{I}, mTEC^{II} and mTEC^{III}. In collaboration with the laboratory of Mitsuru Matsumoto, we analysed the novel mTEC subsets in adult Aire$^{-/-}$ mice. Absence of Aire induced an expected increase in mTEC^{high} (Fig. 11A). Interestingly, this resulted in a reduced frequency of mTEC^{II} and a concomitant accumulation of mature mTEC^{III} (Fig. 11A). These results demonstrate that the absence of Aire alters the kinetic of mTEC^{I-III} differentiation.

To further characterize mTEC^{I-III} in the context of Aire-dependent differentiation, we also analysed Aire reporter mice (also provided by Mitsuru Matsumoto's laboratory), in which the coding sequence of diphtheria toxin receptor (DTR) fused with green fluorescent protein (GFP) is inserted into the *Aire* gene locus in a manner that homozygous insertion disrupts functional Aire protein expression [128]. In heterozygous Aire/DTR-knockin (KI) (Aire$^{+/DTR}$) mice, GFP-labelled cells mark Aire^{+} mTECs. These mice displayed a mTEC^{I-III} profile similar to that observed in WT mice (Fig. 11B). In addition, Aire^{+} mTECs were largely characterized as mTEC^{III} (Fig. 11B), confirming the analysis previously shown (Fig. 7). On the other hand, in homozygous (Aire$^{DTR/DTR}$) mice, GFP^{+} cells define mTECs that are transcriptionally committed for Aire expression but lack the Aire protein [128]. In these Aire-deficient mice, we found an expected increase of mTEC^{III} and a concomitant reduction in the mTEC^{II} population (Fig. 11B), similarly to the Aire$^{-/-}$ data (Fig. 11A). Interestingly, GFP^{+} were mTEC^{III} (Fig. 11B), indicating that the emergence of the mTEC^{III} subset precedes the acquisition of Aire expression.

Overall, the data acquired from both mouse models highlight a role for Aire in mTEC differentiation. Additionally, we found that Aire fine-tunes the rate of establishment of the mTEC^{I-III} populations.

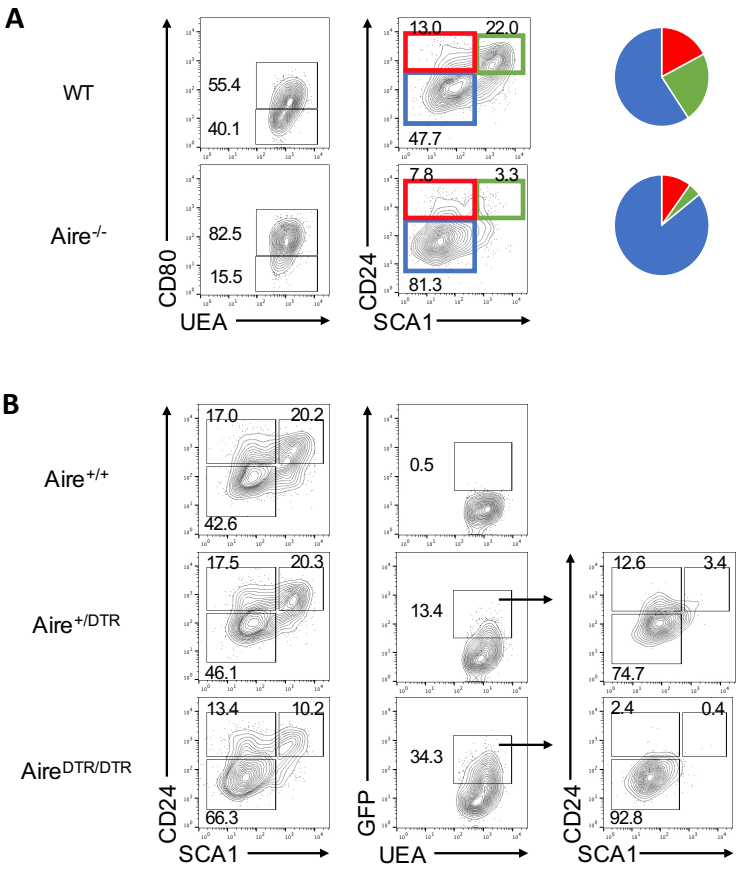

Figure 11 – **Aire deficiency affects mTEC^{I-III} differentiation.** (A) UEA^{+} mTECs from 5 weeks-old Aire$^{-/-}$ and wild-type (WT) thymi were analysed by FC for CD80, CD24 and SCA1 expression. Plots are representative of 3 independent experiments. Pie charts represent the average proportion of the colour-coded mTEC subsets. (B) UEA^{+} mTECs from 5 weeks-old WT (Aire$^{+/+}$), heterozygous (Aire$^{+/DTR}$) and homozygous (Aire$^{DTR/DTR}$) Aire/DTR-KI mice were analysed by FC for CD24, SCA1 and GFP expression. Plots are representative of one experiment.

CCL21-expressing cells are confined to the mTEC^{II} subset

Having established that mTEC^{III} are mostly mature mTEC^{high}, we next sought to determine if mTEC^{I} and mTEC^{II} could represent functionally distinct subsets. Both subsets were mapped to the mTEC^{low} subpopulation. In addition to immature precursors and terminally differentiated post-Aire cells, mTEC^{low} include functionally distinct mTECs that express CCL21, a critical cytokine that regulates the migration of positively selected thymocytes from the cortex to the medulla [102, 103]. Considering the described mTEC^{low} diversity, we sought to examine if the differential expression of SCA1 within CD24^{+} cells (*i.e.* mTEC^{I} or mTEC^{II}) could resolve discrete CCL21-expressing mTEC^{low}. In collaboration with the lab of Graham Anderson, we analysed CCL21 expression in TECs using thymi isolated from a CCL21^{GFP} reporter mouse [129]. These mice carry one allele in which the gene encoding GFP was inserted at the translation initiation site of the *Ccl21a* gene [129]. We confirmed that CCL21-producing cells were found within UEA^{+} mTECs (Fig. 12A) and

were mostly detected in the mTEC^{low} population (Fig. 12B). Strikingly, we found that CCL21^{high} cells were specifically enriched within mTEC^{II} (Fig. 12C). These results suggest mTEC^{II} as a surrogate population for CCL21-producing mTEC^{low} in WT mice, providing additional phenotypic characterization of this specialized mTEC subpopulation.

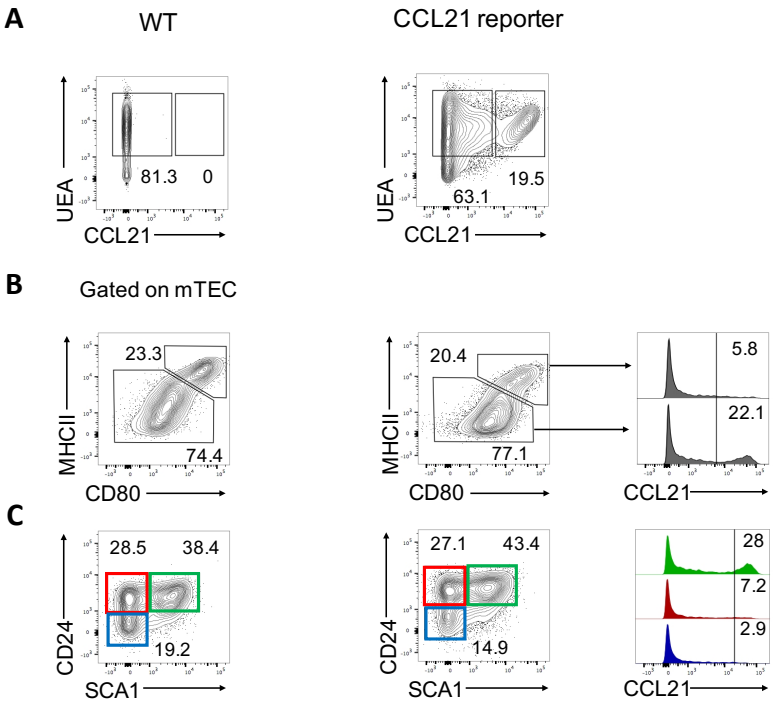

Figure 12 – CCL21 production is mapped to the mTEC^{II}. (A) Total TEC from 7 weeks-old WT and CCL2 reporter thymi were analysed by flow cytometry for UEA binding and CCL21 expression. (B) UEA^{+} mTECs were analysed for MHCII and CD80 expression. Histograms depict the frequency of CCL21^{+} cells within the mTEC^{low} and mTEC^{high} gates. (C) UEA^{+} mTECs were analysed for CD24 and SCA1 expression. Histograms depict the frequency of CCL21^{+} cells within the colour-coded subsets. Plots presented are representative of 3 independent analysis.

Discussion and Final Remarks

The thymus is a lymphoid organ that is essential for the development of a functional adaptive immune system. As such, it has the ability to attract T lymphoid progenitors, induce their differentiation and selection, and ultimately, export functionally competent and self-tolerant T cells. In the medulla, mTECs in association with DCs and other rare leukocyte populations (*e.g.* B cells), screen potentially autoreactive thymocytes for their ability to recognise self-peptide-MHC complexes, inducing their negative selection or clonal deviation into the regulatory T cell lineage [9]. The role of mTECs in the establishment of central T cell tolerance relies greatly on their ability to drive the promiscuous gene expression and presentation of TRAs, a function that is in part controlled by the transcriptional regulators Aire and Fezf2 [69, 72]. Several advances have provided an improved understanding of the phenotypic and functional diversity within mTECs. These studies, however, revealed that this population also bears a large degree of heterogeneity. This knowledge led to the initial characterization of mTEC subsets on the basis of differential expression of CD80 and MHCII. These markers allowed the distinction of $mTEC^{low}$ ($CD80^{low}MHCII^{low}$) and $mTEC^{high}$ ($CD80^{high}$ $MHCII^{high}$), the latter containing mature Aire-expressing cells [100]. Further diversity within these subsets has since been identified. Particularly, $mTEC^{low}$ have been shown to encompass not only precursors of mature $Aire^{+}$ $mTEC^{high}$, but also CCL21-producing mTECs, and terminally differentiated post-Aire cells [102, 103]. Nevertheless, the cellular and molecular relation between the different subsets, and how these mechanisms integrate in the mTEC developmental program, remain poorly defined.

In this thesis, we describe the lineage relationship, molecular requirements, and functional traits of novel mTEC subsets, providing new insights into the program of mTEC differentiation. Our findings reveal that differential expression of CD24 and SCA1 defines three new mTEC subpopulations, which we have named as $mTEC^{I}$ ($CD24^{+}SCA1^{-}$), $mTEC^{II}$ ($CD24^{+}SCA1^{+}$) and $mTEC^{III}$ ($CD24^{-}SCA1^{-}$). These two markers (CD24 and SCA1) have been previously used to probe TEC diversity in conjunction with other markers [138, 139]. However, to the best of our knowledge, this is the first study wherein both markers are used in conjunction to identify the aforementioned populations and integrate them into defined stages of the mTEC developmental program. In this regard, $mTEC^{I}$ display a mix of $mTEC^{low}$ and $mTEC^{high}$. Strikingly, $mTEC^{II}$ are a predominantly $mTEC^{low}$ subset that encompasses the CCL21-expressing cells. The CCL21-producing mTECs are a key population for the recruitment of thymocytes to the medullary compartment [103]. However, due to the lack of specific cell surface markers, this subset has exclusively been studied exploiting reporter mice. Thus, our results provide an additional mean to facilitate the phenotypic and molecular characterization of this specialized mTEC subpopulation. Lastly, $mTEC^{III}$ are mostly comprised of $mTEC^{high}$ and map the majority of cycling and $Aire^{+}$ cells. Our initial findings

do not directly show a possible linear transition between the three mTEC populations. Using RTOCs as a lineage-tracing experimental model, we demonstrated that in the postnatal thymus, both $mTEC^{I}$ and $mTEC^{II}$ can generate $mTEC^{III}$, including $Aire^{+}$ $mTEC^{high}$. The two RTOC experimental settings (with $Actin^{RFP}$- or $H\text{-}2K^{b+}$-derived cells) used to characterize the progeny of $CD80^{low}mTEC^{I}$ yielded distinct frequencies of $mTEC^{II}$ and $mTEC^{III}$. These differences could be a result of intrathymic variations in thymocyte-TEC interactions and/or variations in the established *in vitro* culture conditions. Additionally, $Actin^{RFP}$ and $H\text{-}2K^{b+}$ $mTEC^{I}$ were respectively purified from embryonic and postnatal thymus. As such, the differences observed in their progeny might reflect changes in the lineage potential of $CD80^{low}mTEC^{I}$ that occur throughout development. Moreover, the $CD80^{high}$ cells isolated from $mTEC^{III}$, while remaining largely $mTEC^{high}$, also developed a minor $mTEC^{low}$ population. It could be speculated that the generated $mTEC^{low}$ include cells in the post-Aire stage. In order to define the presence of post-Aire cells in $mTEC^{I\text{-}III}$, we will use in the future, in collaboration with the laboratory of Mitsuru Matsumoto, a fate-mapping mouse model to visualize this population [106]. In these mice, tamoxifen-inducible Cre recombinase is under control of the *Aire* promoter, while GFP is inserted in a ubiquitously expressed gene (*e.g. Rosa26*) downstream of a floxed termination sequence. With this system, tamoxifen-induced Cre activity permanently labels $Aire^{+}$ cells with GFP allowing the analysis of the post-Aire cells [106].

Thymocyte-derived signals transmitted through TNFRSF members RANK, CD40 and LTβR, are central for the establishment of the medullary compartment and for the accomplishment of the mTEC differentiation program [110]. Surprisingly, we found that while RANK or CD40 signalling induced $mTEC^{high}$ in FTOCs, the emergence of $mTEC^{II}$ or $mTEC^{III}$ was not directly induced by TFNRSF signalling. One possibility is that the differentiation of $mTEC^{II}$ and $mTEC^{III}$ requires additional combinatory signals provided by developing thymocytes. Concordantly, in the FTOC condition with thymic lobes not depleted of thymocytes, $mTEC^{I\text{-}III}$ subsets achieved a profile that partially replicated the one established *in vivo*. Subsequent analysis of $Rag2^{-/-}$ mice provided evidence of the cellular requirements for $mTEC^{I\text{-}III}$ differentiation. As described, these mice exhibit severe defects in medulla formation [16]. However, the existing mTECs possess $mTEC^{high}$ and $mTEC^{I\text{-}III}$ subsets in comparable frequencies to the ones observed in WT mice. Thus, these results show that DN1 to DN3 thymocytes provide the necessary ligands to induce the establishment of $mTEC^{I\text{-}III}$.

Aire has been previously implicated in mTEC differentiation. Aire deficiency causes changes in medullary TEC organization, leading to a significant increase in the frequency of $mTEC^{high}$ [106, 140]. However, the precise mechanism through which Aire controls mTEC maturation remains elusive [107]. Our analysis of $Aire^{-/-}$ mice confirmed the influence of Aire

in mTEC development. Interestingly, and in accordance with the above discussed results, we found that the increased frequency of $mTEC^{high}$ was accompanied by a reduction of $mTEC^{II}$ and an accumulation of $mTEC^{III}$. This altered kinetic of $mTEC^{I-III}$ differentiation could be explained based on the two current models for the role of Aire during mTEC differentiation. On one hand, considering Aire a regulator of mTEC differentiation, Aire deficiency could increase the transition into the $mTEC^{III}$ state. Alternatively, in the case that Aire interrupts the mTEC developmental program, the $mTEC^{III}$ in Aire-deficient mice would be spared from Aire's proapoptotic activity [141], resulting in their accumulation. In this sense, the usage of the Aire/DTR-KI mouse model provided further information regarding the relationship between the newly-established mTEC subsets and Aire expression. $Aire^{DTR/DTR}$ mice represent an alternative Aire-deficient model, which allows the identification of cells that actively transcribe the *Aire* gene but lack the Aire protein [128]. Although we had shown that Aire expression was largely confined to $mTEC^{III}$, these mice revealed that *Aire* transcription was almost exclusively restricted to this population. These data suggest that the differentiation of $mTEC^{III}$ precedes the commitment into the $Aire^{+}$ lineage. Additionally, we could also hypothesise that, in this mouse model, cells that fail to express the Aire protein are accumulated in $mTEC^{III}$. If this were the case, it would imply a requirement for Aire in the completion of the mTEC developmental program, as it has been previously suggested [106]. In this regard, future studies could benefit from the establishment of the new $mTEC^{I-III}$ subsets to assess possible contributions to these two models.

We report that at E14 all TEC subpopulations express CD24, and that in the perinatal period, CD24 expression becomes only detected in a fraction of UEA^{+} mTECs. CD24 is a marker that has been associated with progenitor states in other tissues [132]. More recently, embryonic mTEC-committed precursors were characterized as $UEA^{+}MHCII^{low}CD24^{high}$ and were shown to generate an intermediate mTEC progenitor characterized as $UEA^{+}RANK^{+}MHCII^{inter}$ upon RANK and LtβR stimulation [138]. Our results further segregated $CD24^{+}mTEC^{low}$ into $mTEC^{I}$ and $mTEC^{II}$, thus it would be interesting to assess if these mTEC progenitors can be mapped to one of the new subsets. Later in development, from E19 until 2W, we detect SCA1 expression only in UEA^{+} mTECs. However, in young adult mice, UEA^{-} TECs contain a $SCA1^{+}$ population, as well as some $CD24^{+}$ cells. Expression of SCA1 has also been previously linked to a bipotent progenitor function in TECs [139]. Interestingly, it was shown that a TEC subset in the adult thymus characterized as $UEA^{-}MHCII^{low}$α6-$integrin^{high}SCA1^{high}$ is enriched for colony-forming progenitors and contains cells that can give rise to both cTEC and mTEC lineages in reaggregate thymic grafts. In addition, some enrichment for clonogenic capacity was verified in the $CD24^{+}$ fraction of adult $UEA^{-}MHCII^{low}$ TECs [139]. This study also detected

mTEC precursors in adult $UEA^{+}MHCII^{low}$ TECs, which were not further phenotypically dissected. Additionally, some clonogenic activity has been evidenced within postnatal mTECs [142]. In this regard, future studies can be carried out to assess if differential expression of CD24 and SCA1 ($mTEC^{I-III}$) allow to enrich for colony-forming precursors in distinct mTEC subsets.

mTEC-restricted progenitors are theoretically positioned downstream of bipotent TEPs and can be regarded as the starting point of the mTEC lineage(s). During ontogeny, the first $Cld3/4^{+}$ UEA^{+} cells possess self-renewal properties and are capable of specific and long-term generation of $Aire^{+}$ mTECs [34]. From E16.5 into adulthood, $UEA^{+}Cld3/4^{-}$ cells emerge and become the most abundant population, while the $UEA^{+}Cld3/4^{+}$ population is largely characterized as $MHCII^{high}CD80^{high}$ and $Aire^{+}$ [34]. The mTEC progenitor ability of embryonic $Cld3/4^{+}$ TECs is further enriched in a minor $SSEA^{+}$ fraction. Although total $Cld3/4^{+}$ cells are predominantly $mTEC^{high}$, the $Cld3/4^{+}SSEA^{+}$ subset shows an enriched frequency of $MHCII^{low}$ cells in the postnatal thymus [35]. Our future studies are planned to determine how $Cld3/4^{+}$ and $SSEA^{+}$ cells relate to $mTEC^{I-III}$. Ultimately, we aim to assess if in light of the new mTEC diversity, the phenotype of mTEC progenitors can be further elucidated. Furthermore, adult mTECs have been shown to develop from lineage-committed precursors localized at the cortico-medullary junction and characterized by expression of podoplanin [37]. However, a specific segregation of $podoplanin^{+}$ mTEPs in the $mTEC^{I-III}$ populations was not observed, regardless of $mTEC^{low}$ or $mTEC^{high}$ subdivision (data not shown). Although some mTEC progenitors have been shown to arise temporally downstream from others, no concrete evidence has revealed direct precursor-product relationships between them [36]. Therefore, it remains unknown whether the phenotypically distinct mTEPs so far described represent consecutive stages of mTEC differentiation. The possibility remains that these mTEPs could give rise to distinct mTEC lineages, perhaps with non-overlapping functions. The new mTEC diversity here described also provides an important insight for the study of mTEPs and their lineage potential.

In addition to the previously discussed mTEC precursor subsets, other functionally distinct subpopulations have also been described. Of notice, Fezf2 is a recently reported transcription factor that controls TRA expression in both $mTEC^{high}$ and $mTEC^{low}$ subsets [72]. In addition, a fraction of $mTEC^{high}$ is known to express OPG, a decoy receptor for RANKL that regulates the $mTEC^{high}$ pool [115]. In order to better understand the possible relationship between the aforementioned mTEC subpopulations and the new $mTEC^{I-III}$ subsets, we are currently determining the genome-wide transcription profile of $mTEC^{I-III}$ subsets through RNA-sequencing. With this analysis, we aim to elucidate the genetic and functional features associated with each phenotype and reveal their singular and overlapping traits. This genome-wide molecular characterisation might lead to a better

understanding of the lineage relationship between the different mTEC subsets as well as expose any specific role in mTEC development or function.

In conclusion, mTECs form a remarkably diverse niche that provides a crucial instructive microenvironment for central tolerance induction in T cells. As such, the developmental and functional characterization of mTECs has remained a challenging field. Our results reveal an unreported degree of mTEC heterogeneity defined by the dynamic pattern of CD24 and SCA1 expression. Hence, our findings extend the previously established three-stage mTEC differentiation program and elucidate specific functional roles for the novel mTEC subpopulations. Ultimately, these findings provide new tools for mTEC characterization, contributing to the ongoing efforts to define the intricate mechanisms that underlie tolerance induction by the thymic medulla.

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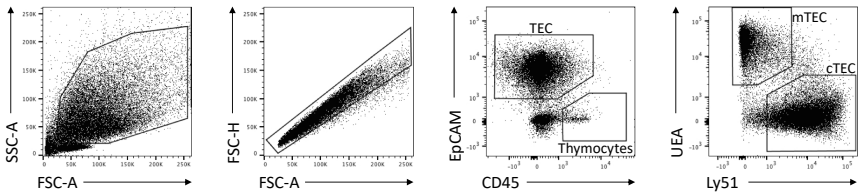
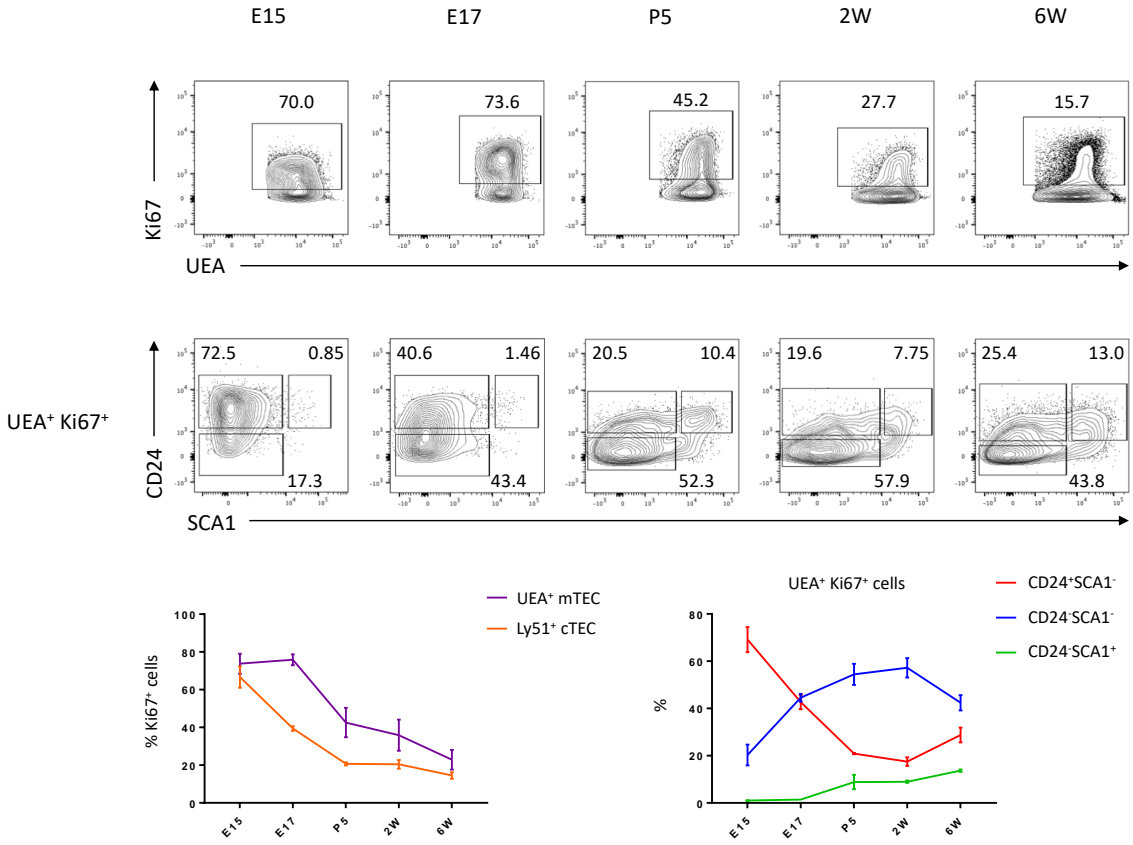
Supplemental Information



Supplemental Figure 1 - Flow cytometry gating strategy for analysis of TECs. Representative flow cytometry profile derived from postnatal day 5 wild type C57BL/6 mice.



Supplemental Figure 2 - **Proliferating mTECs in the postnatal thymus are enriched for mTEC^{III}.** Thymi from C57BL/6 mice were isolated at the indicated time points and UEA$^+$ mTECs were analysed for expression of Ki67 by FC. Ki67$^+$ mTECs were analysed for CD24 and SCA1 expression. Plots are representative of at least three analysis per time point. Graphs depict the means and standard deviations of the frequency of Ki67$^+$ cells within mTECs and cTECs (left) and the frequency of the CD24/SCA1 subsets within Ki67$^+$ mTECs (right). E – Embryonic day; P – Postnatal day; W – Weeks.