U.PORTO

DOUTORAMENTO
BIOLOGIA MOLECULAR E CELULAR

Delineating the molecular control of medullary thymic epithelial cell homeostasis and function - Implications for self-tolerance induction

Maria Camila Trindade Ribeiro

D

2024

MARIA CAMILA TRINDADE RIBEIRO

DELINEATING THE MOLECULAR CONTROL OF MEDULLARY THYMIC EPITHELIAL CELL HOMEOSTASIS AND FUNCTION - IMPLICATIONS FOR SELF-TOLERANCE INDUCTION

Tese de Candidatura ao grau de Doutor em
Biologia Molecular e Celular

Programa Doutoral da Universidade do Porto
Instituto de Ciências Biomédicas Abel Salazar

Orientador – Doutor Nuno Miguel de Oliveira Lages Alves
Categoria – Investigador Principal
Afiliação – Instituto de Investigação e Inovação em Saúde (i3S)

Co-orientador – Doutor Pedro Miguel do Mar Ferreirinha
Categoria – Investigador Júnior
Afiliação – Instituto de Investigação e Inovação em Saúde (i3S)

PUBLICATIONS

Ribeiro C, Alves NL, Ferreirinha P. Medullary thymic epithelial cells: *Deciphering the functional diversity beyond promiscuous gene expression.* Immunol Lett. 2019 Nov;215:24-27. doi: 10.1016/j.imlet.2019.01.012. Epub 2019 Mar 7. PMID: 30853502.

Ferreirinha P, **Ribeiro C**, Morimoto J, Landry JJM, Matsumoto M, Meireles C, White AJ, Ohigashi I, Araújo L, Benes V, Takahama Y, Anderson G, Matsumoto M, Alves NL. *A novel method to identify Post-Aire stages of medullary thymic epithelial cell differentiation.* Eur J Immunol. 2021 Feb;51(2):311-318. doi: 10.1002/eji.202048764. Epub 2020 Sep 15. PMID: 32845012; PMCID: PMC7891440.

Ribeiro C, Ferreirinha P, Landry JJM, Macedo F, Sousa L, Pinto R, Benes V, Alves NL. *Foxo3 regulates cortical and medullary thymic epithelial cell homeostasis with implications in T cell development.* Cell Death & Disease. 2024 May 21;15(5):352. doi: 10.1038/s41419-024-06728-0. PMID: 38773063; PMCID: PMC11109193.

TABLE OF CONTENTS

ACKNOWLEDGMENTS VIII
SUMMARY IX
RESUMO X
LIST OF ABBREVIATIONS XII
LIST OF FIGURES XIV

CHAPTER I - INTRODUCTION 1

THE THYMUS: INSTRUCTING T-CELL DEVELOPMENT AND SHAPING IMMUNOLOGICAL SELF-TOLERANCE 3
T-cell development 5
T-cell fate determination 5
β-selection 7
Positive selection 7
Antigen presentation in the thymic cortex 8
Negative selection 9
Regulatory T cell development 9
Antigen presentation in the thymic medulla 11
Maturation of SP thymocytes and thymic egress 13
iNKT cell development 13
THE ORIGIN AND FATES OF THYMIC EPITHELIAL CELLS 16
Thymus organogenesis 16
Thymic epithelial cell progenitors and lineage diversion 17
Functional heterogeneity within the cTEC and mTEC lineages 19
Cortical TEC diversity 19
Medullary TEC diversity 20
Signalling requirements for TEC differentiation 23
INTRINSIC AND EXTRINSIC SIGNALS CONTROLLING TEC HOMEOSTASIS 27
Age-related thymic involution 27
Impact of aging in the thymic epithelium 28
The TEC-autonomous role of FOXN1 30
Hormonal and metabolic factors 30
Genomic integrity 32
Acute DNA damage 34
FOXO TRANSCRIPTION FACTORS – SENTINELS FOR CELLULAR INTEGRITY 36
FOXO3 in longevity and stress resistance 37

AIMS 40
REFERENCES 41

CHAPTER II - MEDULLARY THYMIC EPITHELIAL CELLS: DECIPHERING THE FUNCTIONAL DIVERSITY BEYOND PROMISCUOUS GENE EXPRESSION 61

ABSTRACT 63
INTRODUCTION 64
MTEC PROGENITORS AND THEIR MATURATION REQUIREMENTS 64
PHENOTYPIC AND FUNCTIONAL COMPLEXITY OF MTECS 66
CONCLUDING REMARKS 68
REFERENCES 69

CHAPTER III - A NOVEL METHOD TO IDENTIFY POST-AIRE STAGES OF MEDULLARY THYMIC EPITHELIAL CELL DIFFERENTIATION 73

ABSTRACT 75
INTRODUCTION 76
RESULTS AND DISCUSSION 77
CD24 and Sca1 expression identifies new subsets of mTEC^{hi} with distinct levels of Aire 77
The genetic program mTEC$^{A\text{-}C/hi}$ maps successive stages of Aire^{+} mTEC differentiation. 78
mTEC$^{B/hi}$ and mTEC$^{C/hi}$ are absent in *Aire*-deficient mice. 81
CONCLUDING REMARKS 83
MATERIAL AND METHODS 84
REFERENCES 87
SUPPLEMENTARY INFORMATION 89

CHAPTER IV - FOXO3 REGULATES CORTICAL AND MEDULLARY THYMIC EPITHELIAL CELL HOMEOSTASIS WITH IMPLICATIONS IN T-CELL DEVELOPMENT 93

ABSTRACT 95
INTRODUCTION 96
RESULTS 98
The deletion of *Foxo3* in TECs leads to a reduction in the mTEC compartment. 98

Foxo3 deficiency induces changes in the transcriptome of cTECs and mTECs. 100
Foxo3 deficiency alters the DNA damage response in mTECs. 102
TEC-specific deletion of *Foxo3* affects the development of regulatory T cells and iNKT cells. 104
Effect of TEC-specific deletion of *Foxo3* in peripheral immune tolerance. 107
DISCUSSION 110
METHODS AND MATERIALS 114
REFERENCES 120
SUPPLEMENTARY INFORMATION 125

CHAPTER V - GENERAL DISCUSSION 131

UNVEILING THE DYNAMICS OF AIRE$^+$ MTEC DIFFERENTIATION 133
Prospective isolation of mTECs entering the post-Aire stage 133
Future insights into TEC development 135
EXPLORING THE PATHWAYS OF TEC HOMEOSTASIS: IMPLICATIONS FOR T-CELL DEVELOPMENT 137
The FOXO3 transcription factor safeguards mTEC homeostasis 137
TRA-expressing mTECs rely on FOXO3 139
FOXO3 supports thymic medullary functions for T-cell development 140
A FOXO3-p53 interplay guards the mTEC genome 143
Future study directions for FOXO3 in thymus biology 144
FINAL REMARKS 146
REFERENCES 147

ACKNOWLEDGMENTS

I am profoundly grateful to my supervisor Nuno Alves, whose support, expertise, and guidance have not only shaped my research but also fostered my academic growth.

I extend my sincere gratitude to Pedro Ferreirinha and Rute Pinto, whose invaluable insights, support, and friendship have enriched every aspect of my time at i3S.

I would like to recognize the contributions of my fellow PhD colleagues, Laura Sousa and Rúben Pinheiro, and of all current and past members of the Thymus Development and Function lab, including Rita Ribeiro, Ana Hage, Francisco Sobral, João Cardoso, Leonor Araújo, Nuno David, and Carolina Monteleone.

Acknowledgment is also due to the Animal Facility, Translational Cytometry, and HEMS scientific platforms at i3S, our collaborators at EMBL, and the FCT and ERC funding agencies.

Finally, I want to express my heartfelt gratitude to my mother and sister, for their love and encouragement.

SUMMARY

The specialized thymic stromal microenvironments defined by thymic epithelial cells (TECs) control the development of competent and self-tolerant T cells. Notably, thymic stromal integrity and T-cell production are sensitive to several physiological and environmental factors, such as ageing, stress, infection, and cytoablative regimens. As a result, these conditions are associated with deficient T-cell output and increased susceptibility to opportunistic infections, cancer, and autoimmunity. Understanding the mechanisms underlying TEC development and function directly aids the design of clinical strategies to improve immune reconstitution or correct inadequate T-cell responses.

Whereas cortical TECs (cTECs) promote T-cell lineage fate and positive selection, medullary TECs (mTECs) regulate negative selection of self-reactive thymocytes and regulatory T cell differentiation. mTECs play a central role in tolerance induction due to their unique ability to induce tissue-restricted antigen (TRA) expression, a function partially dependent on the autoimmune regulator (Aire). mTECs undergo a complex multi-lineage differentiation program, that includes mTEC-committed progenitors, thymocyte-attractant $CCL21^{+}$ mTECs, and specialized TRA-expressing cells, such as $Aire^{+}$ and post-Aire mimetic cells. Following a revision of the existing literature and open questions concerning mTECs, included in **Chapter II**, we proceed to develop a new method to dissect mTEC diversity. Our results, described in **Chapter III,** show that differential expression of CD24 and Sca1 defines consecutive $Aire^{+}$ and post-Aire mTEC developmental stages, thus providing a new strategy for their assessment.

Past findings suggest that mTECs depend on genome-protecting pathways. Specifically, Aire-mediated transcription promotes double-strand DNA breaks and recruits proteins related to the DNA damage response. In this regard, our laboratory further showed that p53, a classical regulator of the DNA damage response, plays a key role in mTEC homeostasis and tolerance induction. In **Chapter IV**, we investigate a new molecular regulator of TEC biology. Specifically, we target the transcription factor Forkhead box O3 (FOXO3) in TECs, given its diverse functions in homeostasis regulation and genome preservation, including cell cycle arrest, apoptosis, ROS resistance, and DNA repair. In this work, we expand the pervasive functions of FOXO3 and underscore a novel role in supporting mTEC homeostasis and function.

The results presented in this thesis contribute to elucidating the regulatory mechanisms underlying the development and function of the TEC microenvironments. Ultimately, this will improve therapeutic approaches targeting the intrathymic mechanisms guiding effective and self-tolerant T-cell responses.

RESUMO

O estroma do timo possui microambientes especializados definidos pelas células epiteliais do timo (TECs), as quais são responsáveis por controlar o desenvolvimento de células T imuno-competentes e tolerantes aos antigénios do próprio organismo. A integridade do estroma tímico e a produção de células T são sensíveis a vários fatores fisiológicos e ambientais, como envelhecimento, stress, infeção e regimes cito-ablativos. Como resultado, estes fatores estão associados a uma produção deficiente de células T e a um aumento da suscetibilidade a infeções oportunistas, cancro e autoimunidade. Deste modo, compreender os mecanismos subjacentes ao desenvolvimento e função das TECs é essencial para a criação de novas estratégias clínicas de reconstituição imunológica ou de correção de respostas de células T inadequadas.

Enquanto as TECs corticais (cTECs) promovem o início da diferenciação das células T e a sua seleção positiva, as TECs medulares (mTECs) regulam a seleção negativa de timócitos auto-reativos e a diferenciação de células T reguladoras. As mTECs desempenham um papel central na indução de auto-tolerância devido à sua capacidade única de induzir a expressão de antigénios restritos de tecidos (TRA), uma função parcialmente dependente do regulador autoimune (Aire). As mTECs passam por um programa complexo de diferenciação, que inclui progenitores restritos de mTECs, mTECs $CCL21^{+}$ que medeiam a quimioatração de timócitos, e mTECs especializadas em expressão de TRAs, como as células $Aire^{+}$ e as células miméticas pós-Aire. Após uma revisão da literatura e das questões abertas sobre mTECs, incluídas no **Capítulo II**, procedemos ao desenvolvimento de um novo método para dissecar a diversidade de mTECs. Os nossos resultados, descritos no **Capítulo III**, mostram que a expressão diferencial de CD24 e Sca1 define etapas de desenvolvimento consecutivas de mTECs $Aire^{+}$ e pós-Aire, fornecendo assim uma nova estratégia para sua análise.

Descobertas anteriores sugerem que as mTECs dependem de vias de proteção do genoma. Especificamente, a transcrição mediada por Aire pode promover quebras de DNA de cadeia dupla e recrutar proteínas relacionadas à resposta a danos no DNA. A este respeito, o nosso laboratório mostrou ainda que o p53, um regulador clássico da resposta ao dano no DNA, desempenha um papel fundamental na homeostasia das mTECs e na indução de tolerância. No **Capítulo IV**, investigamos um novo regulador molecular da biologia das TECs. Concretamente, procedemos à eliminação da expressão do fator de transcrição Forkhead box O3 (FOXO3) em TECs, dadas as suas diversas funções na regulação da homeostasia e preservação do genoma, incluindo saída do ciclo celular, apoptose, resistência a ROS e reparo de DNA. Neste trabalho, expandimos a lista de

funções do FOXO3 e reportamos um novo papel no apoio à homeostasia e função das mTECs.

Os resultados apresentados nesta tese contribuem para elucidar os mecanismos que regulam o desenvolvimento e função dos microambientes estabelecidos pelas TECs. Por último, o estudo destes mecanismos permitirá melhorar as abordagens terapêuticas que atuam sobre os mecanismos intra-tímicos que direcionam respostas mediadas células T imuno-competentes e tolerantes a antigénios próprios.

LIST OF ABBREVIATIONS

Aire – Autoimmune regulator;
AMPK – 5' adenosine monophosphate-activated protein kinase;
APC – Antigen-presenting cell;
BCR – B-cell receptor;
BM – Bone marrow;
BMP – Bone morphogenic protein;
BMT – Bone marrow transplant;
CCL – Chemokine (C-C motif) ligand;
CCR – C-C Chemokine receptor;
CD – Cluster of differentiation;
CD40 – Cluster of differentiation 40;
CD40L – Cluster of differentiation 40 ligand;
CDK – Cyclin-dependent kinase,
cKO – Conditional knockout;
Cld – Claudin;
CMJ – Cortico-medullary junction;
CR – Caloric restriction;
cTEC – Cortical thymic epithelial cell;
CTR – Control;
CXCL – Chemokine (C-X-C motif) ligand;
CXCR – C-X-C Chemokine receptor;
DC – Dendritic cell;
DDR – DNA damage response;
Dll4 – Delta-like 4;
DN – Double negative;
DP – Double positive;
DSB – Double-strand breaks;
E – Embryonic day;
EpCAM – Epithelial cell adhesion molecule;
ETP – Early thymic progenitor;
Eya1 – Eyes absent 1 homologue;
Fezf2 – FEZ family zinc finger 2;
FGF – Fibroblast growth factor;
FOXO3 – Forkhead box O3;
FOXP3 – Forkhead box P3;
FOXN1 – Forkhead box N1;
Gcm2 – Glial cells missing homolog 2;
GO – Gene ontology;
HSC – Hematopoietic stem cells;
Hoxa3 – Homeobox A3;
ICAM – Intracellular adhesion molecule;
IFN – Interferon;
IGF – Insulin-like growth factor;
IIS – Insulin and/or IGF-1 signalling;
IL – Interleukin;
IR – Ionizing radiation;
iNKT – Invariant natural killer T cell;
JNK – c-Jun N-terminal kinase
jTEC – Junctional thymic epithelial cell;
K – Cytokeratin;
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 – mTEC-committed thymic epithelial progenitor;
mTOR – Mechanistic target of rapamycin;
MST1 – Mammalian sterile 20-like kinase 1;
MTS – Mouse thymic stroma;
NC – Neural crest;
NF-κB – Nuclear factor kappa B;
NIK – NF-κB-inducing kinase;
NLS – Nuclear localization sequence;
OPG – Osteoprotegerin;

PAMP – Pathogen-associated molecular pattern;
Pax – Paired box;
PDPN – Podoplanin;
PGE – Promiscuous gene expression;
Plet1 – Placenta-expressed transcript-1;
PLZF – Promyelocytic leukaemia zinc finger;
PRR – Pattern recognition receptors;
PSGL-1 – P-selectin glycoprotein ligand 1;
PTM – Post-transcriptional modification;
RA – Retinoic acid;
RAG – Recombination activating gene;
RANK – Receptor activator of nuclear factor kappa B;
RANKL – Receptor activator of nuclear factor kappa B ligand;
RelB – V-Rel reticuloendotheliosis viral oncogene homolog B;
ROS – Reactive oxygen species;
ROR – RAR-related orphan receptor;
RTE – Recent thymic emigrant;
RTOC – Reaggregate thymic organ culture;
RUNX – Runt-related transcription factor;
S1P – Sphingosine-1-phosphate;
S1P1 – Sphingosine-1-phosphate receptor 1;
SCF – Stem cell factor;
SGK1 – Serum/glucocorticoid-inducible kinase 1;
Six1 – Sine oculis homologue 1;
SL-TBI – Sub-lethal total body irradiation;
SM – Semimature;
SP – Single positive;
SSEA – Stage-specific embryonic antigen;
TAC – Transit-amplifying cell;
TCR – T-cell receptor;
ThPOK – T-helper inducing POZ/Kruppel-like factor;
TEC – Thymic epithelial cell;
TEP – Thymic epithelial cell progenitor;
TNC – Thymic nurse cell;
TNFRSF – Tumour necrosis factor receptor super family;
TRA – Tissue-restricted antigen;
TRAF – Tumour necrosis factor receptor-associated factor;
Trp53 – Transforming related protein 53;
Treg – Regulatory T cell;
TSSP – Thymus-specific serine protease;
UEA – Ulex europaeus agglutinin;
VCAM – Vascular cell adhesion molecule;
Wnt – Wingless/Integrated;

LIST OF FIGURES

CHAPTER I - INTRODUCTION

Figure 1 – Overview of T-cell development orchestrated by thymic stromal cells. 5

Figure 2 – cTEC functions for positive selection and T-cell lineage commitment. 8

Figure 3 – mTEC functions for T-cell repertoire selection and Treg cell induction. 12

Figure 4 – Proposed model for cTEC and mTEC differentiation according to identified TEP populations and single-cell transcriptomic analysis. 19

Figure 5 – Diversity of specialized mTEC subsets for TRA expression and tolerance induction. 22

Figure 6 – RANK, CD40 and LTβR signalling is required for NF-κB pathway activation and mTEC differentiation. 24

Figure 7 – Age-dependent alterations in thymus morphology and peripheral T-cell composition. 28

Figure 8 – FOXO regulatory pathways and transcriptional programs involved in cellular homeostasis. 37

CHAPTER II - MEDULLARY THYMIC EPITHELIAL CELLS: DECIPHERING THE FUNCTIONAL DIVERSITY BEYOND PROMISCUOUS GENE EXPRESSION

Figure 1 – Schematic representation of mTEC development and of the characterized phenotypic and functional subsets. 68

CHAPTER III - A NOVEL METHOD TO IDENTIFY POST-AIRE STAGES OF MEDULLARY THYMIC EPITHELIAL CELL DIFFERENTIATION

Figure 1 – The combined analysis of CD24 and Sca1 defines new mature mTEC subsets with distinct patterns of Aire expression. 78

Figure 2 – Transcriptomic analysis of $mTEC^{A-C}$ within $mTEC^{lo}$ and $mTEC^{hi}$ and their relationship to the genetic profile of distinct stages of $Aire^{+}$ mTEC differentiation. 80

Figure 3 – Altered $mTEC^{A-C/hi}$ composition in *Aire*-deficient mice. 82

Supplementary figure 1 89

Supplementary figure 2 90

Supplementary figure 3 – Reaggregate thymus organ cultures (RTOCs) with discrete $mTEC^{A-C/hi}$ subsets. 91

CHAPTER IV - FOXO3 REGULATES CORTICAL AND MEDULLARY THYMIC EPITHELIAL CELL HOMEOSTASIS WITH IMPLICATIONS IN T-CELL DEVELOPMENT

Figure 1 – FOXO3 supports the size and functional diversity of the mTEC compartment. 99

Figure 2 – FOXO3^{cKO} TEC subsets display altered transcriptional signatures. 102

Figure 3 – FOXO3^{cKO} mTECs present an impaired DNA damage response. 104

Figure 4 – Thymopoiesis in FOXO3^{cKO} mice. 105

Figure 5 – FOXO3^{cKO} mice exhibit compromised regulatory T cell and iNKT cell development. 107

Figure 6 – TEC-specific *Foxo3* deletion affects peripheral tolerance induction. 108

Supplementary figure 1 – Targeting *Foxo3* expression in TECs. 125

Supplementary figure 2 – Transcriptome analysis of FOXO3^{cKO} cTECs and mTECs. . 126

Supplementary figure 3 – Analysis of genetic signature of mTEC subsets in FOXO3^{cKO} mTECs. 127

Supplementary figure 4 – Analysis of TEC cellularity recovery following SL-TBI. 128

Supplementary figure 5 – T-cell development in the FOXO3^{cKO} thymus. 129

Supplementary figure 6 – Effects of TEC-specific *Foxo3* deletion in Aire-independent mTEC lineages and in the autoimmune syndrome of Aire^{KO} mice. 130

CHAPTER V - GENERAL DISCUSSION

Figure 1 – Proposed model for the progression through mTEC^{high} A-C subsets during Aire lineage mTEC differentiation. 135

Figure 2 – Summary of thymic molecular, structural, and functional alterations driven by TEC-specific *Foxo3* deletion. 143

CHAPTER I - INTRODUCTION

THE THYMUS: INSTRUCTING T-CELL DEVELOPMENT AND SHAPING IMMUNOLOGICAL SELF-TOLERANCE

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, such as cancer cells. To do so, it must act rapidly and efficiently to recognize those threats and activate different effector functions. Importantly, this recognition must include the discrimination between “self” and “non-self”, to maintain an immunological self-tolerant state. At the same time, it must be efficient in its use of genes, molecules, and cells, and not impose an excessive energetic burden on the host. Such requirements are met through the unique and complementary features of innate and adaptive immunity.

Innate immunity represents the first line of defence against pathogen invasion by providing a rapid and broad response and relies on the detection of conserved pathogen-specific features or signs of infection in host cells. The innate immune mechanisms include physical barriers, molecular mediators (such as the complement system and defensins), the recognition of highly conserved pathogen-associated molecular patterns (PAMPs) by germline-encoded pattern recognition receptors (PRRs), and the actions of phagocytic and natural killer (NK) cells [1].

In addition to the innate immune system, jawed vertebrates, beginning with cartilaginous fish, have developed an adaptive immune system centred on antigen-specific recognition and mediated by lymphocytes [2]. The lymphocyte receptor repertoire can recognize antigens from any potential foreign element. This capability is the virtue of antigen receptors expressed by T (for thymus-derived) and B (for bursa de Fabricius-derived) lymphocytes, which arise from the somatic recombination of variable (V), diversity (D), and joining (J) gene segments of the T and B cell receptors [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 non-useful or even undesired specificities, such as those recognizing antigens of the host. Thus, to prevent the activation and propagation of self-specific immune responses (termed autoimmunity), central and peripheral tolerance mechanisms act synergistically to eliminate or regulate self-reactivity [4, 5].

The importance of the thymus lies, initially, in providing the specialized lymphoid site for T-cell precursor recruitment and the inductive microenvironment for T-cell lineage commitment. Then, the thymus orchestrates the essential processes of expansion and

selection of T-cell precursors, and ultimately, induces the maturation of T cells bearing a carefully curated TCR repertoire, capable of responding to numerous potential foreign antigens while being tolerant against self-antigens. The ability to accommodate this intricate process is attributed to distinct thymic niches, which have dedicated functions dictated by the resident thymic stromal components and work in concert to provide the necessary signals and cell-cell interactions to support thymocyte migration, commitment, proliferation, and selection.

The thymus is a lobulated organ encapsulated by connective tissue formed by mesenchymal cells. Below this layer, the sub-capsular epithelium overlies the outer cortex [6]. Then, a peripheral cortex and central medulla are formed by three-dimensional networks of thymic epithelial cells (TECs) surrounded by developing T cells, or thymocytes, which make up for over 95% of the cellularity of the postnatal thymus [7]. TECs are the main thymic stromal population in the post-natal thymus and are subdivided into two phenotypically and functionally distinct types 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 bone marrow (BM)-derived cells, such as dendritic cells (DCs), macrophages, and B cells [6] (**Figure 1**). In the cortex, cTECs play important roles during the early stages of T-cell development by promoting T-cell fate commitment and expansion of early T-cell progenitors and by providing the required environment for positive selection of thymocytes bearing self-major histocompatibility complex (MHC)-restricted TCRs. In the medulla, mTECs and DCs mediate the negative selection of autoreactive T cells and the generation of regulatory T (Treg) cells [8] (**Figure 1**). This thesis specifically focuses on the development and function of TECs. Still, before dwelling on the intricate details of TEC biology, this initial Section of the Introduction Chapter aims to provide a fundamental overview of the role of TECs in T-cell development.

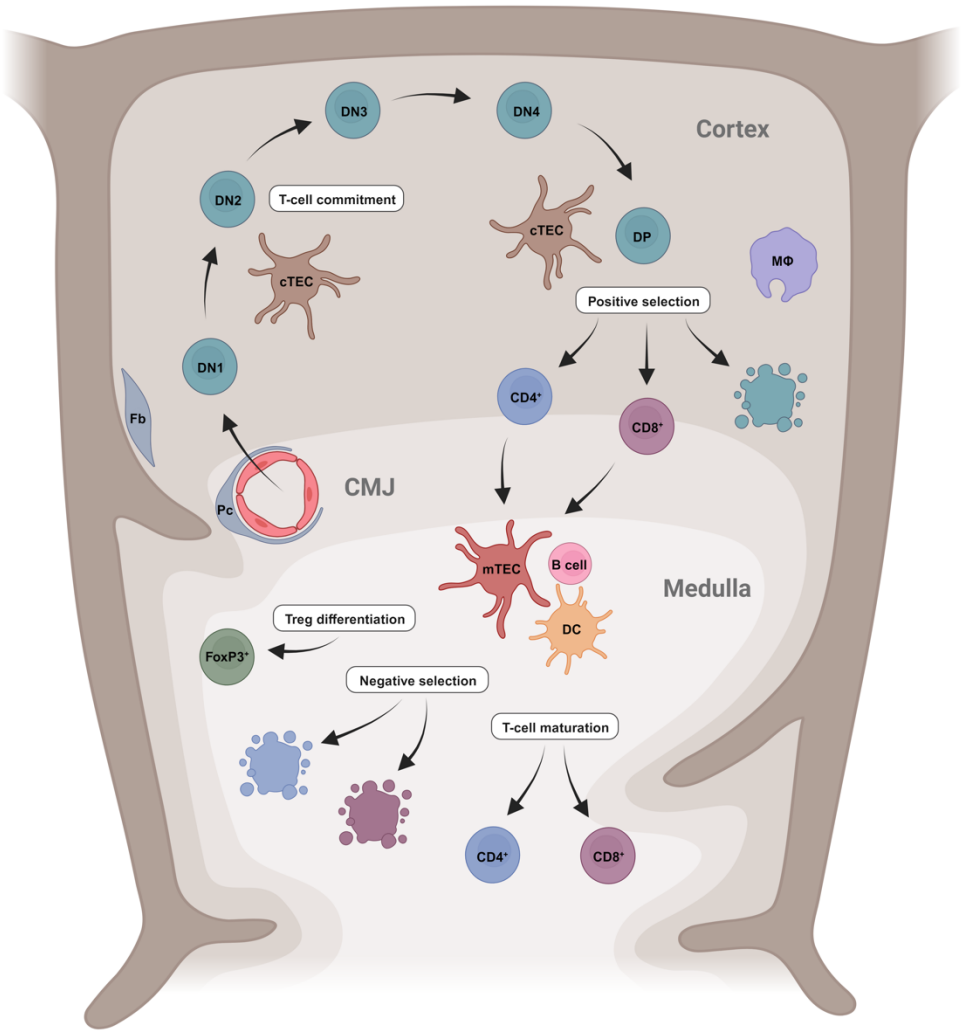


Figure 1 – Overview of T-cell development orchestrated by thymic stromal cells. The thymic stroma includes cortical and medullary thymic epithelial cells (cTECs and mTECs), endothelial cells, mesenchymal cells, such as fibroblasts (Fb) and pericytes (Pc), dendritic cells (DCs), macrophages (MΦ), and B cells. In the postnatal thymus, T-cell precursors enter the thymic cortex through blood vessels located at the cortico-medullary junction (CMJ) and, under the control of cTECs, will undergo sequential stages of T-cell fate commitment and positive selection. Following positive selection, SP thymocytes migrate to the medulla, wherein mTECs and other medullary antigen-presenting cells (APCs) will induce negative selection, differentiation of regulatory T (Treg) cells and T-cell maturation. A detailed description of the aforementioned T-cell development stages will be provided in the present Section of the Introduction Chapter.

T-cell development

T-cell fate determination

All blood cell types originate from multipotent hematopoietic stem cells (HSCs), which are phenotypically devoid of mature lineage markers and possess continuous self-renewal and progenitor ability [9]. HSCs undergo a series of intermediate stages where they progressively become lineage-restricted, and ultimately, differentiate into lymphoid or myeloid cells. Lymphoid cells include T, B, NK, and innate lymphoid cells (ILCs), while the

myeloid lineage gives rise to erythrocytes, megakaryocytes, granulocytes, and macrophages [10]. Plasmacytoid DCs (pDCs) and conventional DCs (cDCs) differentiate from lymphoid- and myeloid-committed progenitors, respectively [10]. Hematopoiesis occurs primarily in the fetal liver during embryonic development, whereas in postnatal life, it occurs within the bone marrow [9]. For HSCs to differentiate into competent self-tolerant T cells, they require specialized thymic microenvironments. In postnatal life, these cells must egress from the bone marrow and traffic through the blood to 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 and cTECs [11, 12]. Other molecules involved in this trafficking include 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 by the endothelial cells in the thymic vasculature [13, 14].

T-cell precursors enter the thymus through the blood vessels localized at the cortico-medullary junction [15], a stage at which they are called early thymic progenitors (ETPs). ETPs lack the expression of CD4 and CD8, hence being referred to as double-negative (DN) thymocytes. During the DN stage, the developmental program can be further subdivided into 4 stages, characterized in mice by the dynamic expression of cell surface proteins CD44 and CD25: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) (**Figure 1**). 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 [16, 17]. At this stage, engagement of the Notch signalling pathway is crucial to inhibit multiple cell fate potentials and gradually induce the genetic program for T-cell fate determination. The essential ligand for Notch signalling, DLL4, is provided by cTECs [18, 19]. The following DN2 stage is characterized by a migratory movement to the subcapsular cortex [15]. In this region, cTECs further support T-cell lineage specification, differentiation, and expansion by providing the necessary growth factors and cytokines, such as IL-7 and stem cell factor (SCF or c-kit ligand) [20-22]. 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, required to start the assembly of the TCR [23]. The complete and irreversible commitment to the T-cell lineage is marked by the transition into the DN3 stage [16]. DN3 stage thymocytes can either differentiate into αβ T cells or produce TCRγ and TCRδ chains and differentiate along the γδ T-cell lineage [24]. The focus of this thesis, and this Section in particular, will be on the development of αβ T cells, which represent the major cellular product of the thymus.

β-selection

A successful *Tcrb* gene rearrangement will give rise to a functional TCRβ chain at the DN3 stage, which associates with an invariant pre-TCR α-chain and CD3 signalling molecules to form the pre-TCR complex [25]. Autonomous pre-TCR signalling, in association 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 [26]. Following β-selection, cells downregulate CD25, becoming DN4 or pre-double-positive cells, while initiating *Tcra* gene rearrangements. Thus, the subsequent $CD4^+CD8^+$ double-positive (DP) thymocyte population includes the first cells expressing a functional αβ TCR [27].

Positive selection

Within the cortex, cTECs are the self-antigen-presenting cells responsible for inducing positive selection, a process that decides the fate of thymocytes (survival and CD4/CD8 lineage commitment versus apoptosis) according to the αβ TCR specificity and binding strength to the self-peptide-MHC complex presented by cTECs. DP thymocytes carrying TCRs that interact with intermediate affinity are induced to survive and allowed to further differentiate [28]. Most commonly, cells express TCRs unable to interact with self-peptide-MHC molecules and undergo death by neglect [29]. Alternatively, high-affinity interactions with the self-peptide-MHC complexes can also lead to clonal deletion carried out by cTECs [29]. Ultimately, this checkpoint ensures a repertoire of thymocytes carrying appropriate self-MHC-restricted TCRs.

During positive selection, DP thymocytes differentiate into either the $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive (SP) lineage, a decision based on whether the TCR is restricted to MHCII or MHCI, respectively [27]. When a DP thymocyte expresses a TCR that interacts with self-peptide:MHCII, the CD4 co-receptor assists this interaction, leading to the loss of expression of the uninvolved co-receptor (CD8) and upregulation of the transcription factor ThPOK [30]. Subsequently, these $CD4^+$ thymocytes will undergo chromatin remodelling so that upon their activation in the periphery, the $CD4^+$ T cells will have helper cell effector functions (**Figure 2**). On the other hand, when the TCR is self-peptide:MHCI-restricted, the CD8 co-receptor assists the interaction, leading to loss of CD4 expression and upregulation of the transcription factor RUNX3 [31], resulting in $CD8^+$ T cells becoming poised to perform cytotoxic or killer cell effector functions once activated (**Figure 2**). This process of differentiation into $CD4^+$ helper or $CD8^+$ cytotoxic T cells is designated as lineage commitment [32].

Antigen presentation in the thymic cortex

Positive selection requires the production of the positively selecting self-peptides presented on the MHC molecules. In this regard, cTECs possess an exclusive set of proteolytic enzymes and degradation processes that are suggested to allow them to display self-peptides different from those found on other APCs. For instance, cTEC-restricted cathepsin L and thymus-specific serine protease (TSSP) appear to mediate the production of self-peptides that are loaded onto MHCII molecules in late endosomes and are crucial for $CD4^+$ T-cell selection [33, 34] (**Figure 2**). Additionally, macroautophagy, a bulk protein degradation process, is also a source of unique peptides for the selection of certain MHCII-restricted specificities [35] (**Figure 2**). On the other hand, MHCI 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 [36]. Notably, cTECs exclusively express the proteasomal subunit β5t, which is incorporated with β1i and β2i to form an additional form of the proteasome called thymoproteasome [37]. This configuration is considered to confer upon cTECs the ability to process a unique repertoire of peptides important for $CD8^+$ T-cell selection [38] (**Figure 2**).

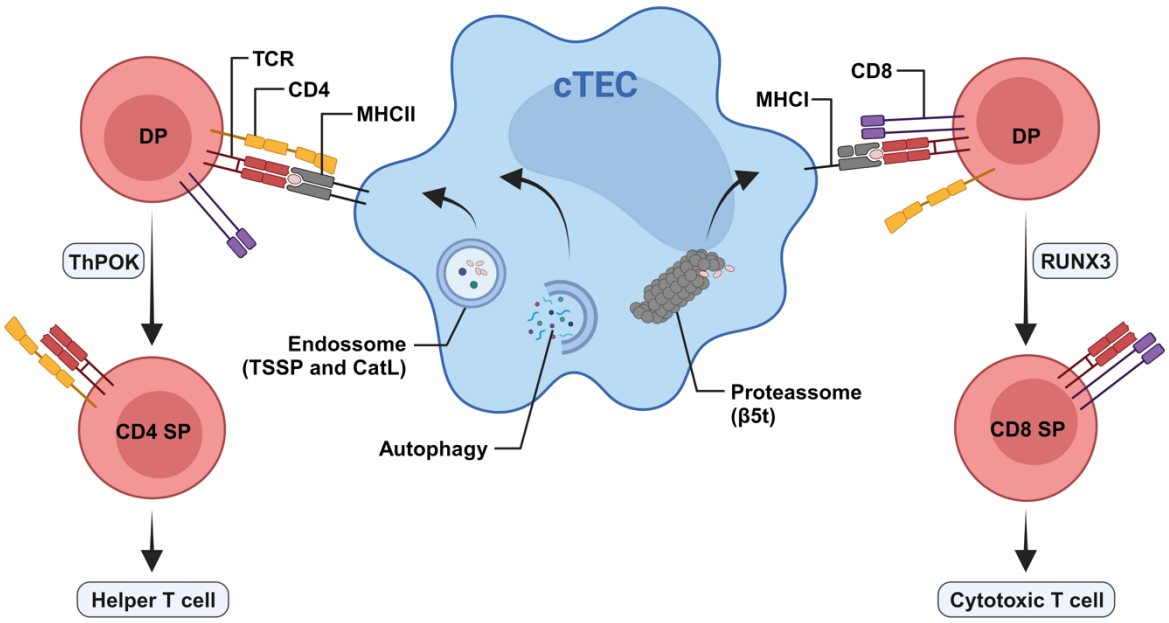


Figure 2 – cTEC functions for positive selection and T-cell lineage commitment. In cTECs, specific antigen-processing mechanisms produce the self-peptides for presentation by MHCI and MHCII. Positive selection of thymocytes by self-peptides presented on either MHCII or MHCI, respectively, triggers the mutually exclusive retention of the CD4 or CD8 coreceptors, upregulation of transcription factors ThPOK or RUNX3 and, consequently, dictates $CD4^+$ helper versus $CD8^+$ cytotoxic T-cell fate decision.

Altogether, the cTEC compartment plays a critical role in multiple stages of T-cell development, including homing of T-cell precursors, T-cell fate determination, expansion, and differentiation. Furthermore, the cTEC-specific proteolytic machinery allows them to present an exclusive peptidome during positive selection, a process that will dictate T-cell self-MHC restriction, as well as coordinate $CD4^+$ helper versus $CD8^+$ cytotoxic lineage specification. Positively selected thymocytes upregulate CCR7 and CCR4 on the cell surface, while the respective ligands (CCL19 or CCL21, and CCL17 or CCL22) are expressed by mTECs [39, 40]. Consequently, thymocytes are attracted to the medulla, where they will undergo further selection and development.

Negative selection

The random genetic recombination mechanisms underlying the generation of the αβ TCR repertoire can produce receptors capable of recognizing self-antigens. In addition to the clonal deletion events that take place in the cortex, the medullary compartment provides further central tolerance induction to prevent the escape of potentially destructive autoreactive T cells [41]. Once in the medulla, SP thymocytes interact with antigen-presenting cells, including mTECs, DCs, and thymic B cells, that will screen them for self-reactive specificities [41]. Thymocytes with high-affinity interactions between TCRs and self-antigen-MHC complexes will suffer clonal deletion through apoptosis induction, a process referred to as negative selection, or will deviate into regulatory T cells (discussed next) [29]. Negative selection involves the upregulation of a pro-apoptotic protein BIM [42] and requires co-stimulation through CD28 [43] (**Figure 3**). The process of eliminating high-affinity self-reactive clones from the TCR repertoire, although efficient, can be imperfect, allowing the development of some self-reactive T cells [44]. Still, under most circumstances, traces of conventional T-cell self-reactivity have no significant detrimental effects due to the actions of other tolerance-inducing processes, such as thymic Treg development and peripheral tolerance mechanisms [45].

Regulatory T cell development

In concert with clonal deletion, $CD4^+$ SP cells exhibiting overt reactivity to self-antigens on medullary APCs can be redirected to the Treg lineage [46]. Thus, thymic Treg cell selection will simultaneously limit self-reactivity within the conventional $CD4^+$ T-cell repertoire and supply the peripheral Treg cell pool with self-reactive specificities that were rescued from negative selection. The Treg lineage is essential for the maintenance of immune homeostasis and the prevention of spontaneous autoimmunity by inhibiting immune activation against self-antigens [47, 48]. The factors that influence the decision

between Treg cell development or clonal deletion, although not completely defined, have been addressed in several studies [49]. The availability of IL-2 was shown to be a determining factor in rescuing self-reactive thymocytes receiving TCR and costimulatory signals from apoptosis and inducing Treg cell differentiation [50-52]. In the absence of IL-2, Treg cell induction can also occur through the partial compensatory roles of IL-15 and IL-7 signalling [53]. Moreover, other pathways have been shown to favour Treg cell induction by antagonizing apoptotic pathways, such as TGF-β receptor signalling [54] and CD27 co-stimulation [55]. Treg cell selection can also be dictated by the frequency at which the selecting antigen is present in the medulla. In this regard, rare or less abundant antigens favour Treg cell development, while highly abundant antigens have an increased number of encounters with the respective thymocytes and a higher likelihood of negative selection [56]. Other suggested factors that influence Treg fate determination, which are not mutually exclusive and are possibly interrelated, include the early or late timing of TCR signalling, the persistent or disrupted TCR/peptide-MHC binding [57], as well as the APC type or age-dependent effects [49].

The transcription factor forkhead box protein 3 (FOXP3) is the master regulator of Treg cell development and function [58]. Mature thymic Treg cells are characterized by the stable expression of FOXP3 and the high-affinity IL-2 receptor (CD25) [58] (**Figure 3**). While the main requirements for Treg cell maturation are clearly defined, namely TCR ligation, CD28 and CD27 co-stimulation, and IL-2 signalling, it remains unclear whether the sensing and integration of the signals for Treg cell induction by Treg precursors are stochastic or follow specific spatial and/or temporal rules [49]. In this regard, two developmental pathways have been proposed. The first is through $CD25^{+}$ $FOXP3^{-}$ precursors, which are poised to express FOXP3 upon cytokine exposure without receiving further antigenic signals [52]. This pathway supports a two-step model of Treg cell differentiation, with an initial phase of TCR signalling, followed by a second cytokine-dependent phase to consolidate the Treg cell fate [52]. Other studies have suggested an alternative developmental pathway where Treg cells progress from $CD25^{-}FOXP3^{lo}$ precursors, which can also mature into $CD25^{+}FOXP3^{+}$ Treg cells upon IL-2 exposure [51, 59]. Notably, while mature Treg cells can arise from the two different Treg precursors independently, a recent study has described a possible linear relationship between $CD25^{+}$ $FOXP3^{-}$ and $CD25^{-}$ $FOXP3^{lo}$ precursors [57]. Hence, given the key roles of Treg cells in both immune homeostasis and disease, ongoing studies are exploring the mechanisms coordinating Treg fate commitment and the determinants and implications of their alternative developmental pathways.

Antigen presentation in the thymic medulla

Pivotal to self-antigen presentation and T-cell repertoire selection in the medulla is the unique ability of mTECs to ectopically express tissue-restricted antigens (TRAs), a phenomenon termed promiscuous gene expression (PGE) [60]. 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 have been estimated to express approximately 85-89% of the coding genome [61, 62]. Moreover, mTECs display a relatively high level of alternative splicing and RNA editing, which further ensures a comprehensive representation of peripheral self-antigens in the thymus [62]. Notably, single-cell RNA-sequencing estimated that, at any given time, each TRA is expressed by 1-3% of mTECs. This suggests that TRA expression follows a mosaic pattern, wherein individual mTECs express only a fraction of the TRAs, ultimately combining at the population level to present a complete and stable TRA repertoire [63, 64]. This is thought to be a mechanism to avoid undesired physiological stress within the cell and to favour a tolerogenic outcome by increasing the amount of each TRA presented on the cell surface of mTECs [64].

The ectopic transcription of TRAs in mTECs is controlled in part by the Autoimmune regulator (Aire) [65] (**Figure 3**). Aire does not function as a conventional transcription factor as it lacks DNA sequence specificity [66]. Instead, Aire targets chromatic regions in a repressive state and induces transcription by repurposing general mechanisms, such as release of stalled RNA polymerase [67], polycomb silencing [61], and chromatin looping [68]. Consequently, Aire-regulated transcription affects (directly and indirectly) a wide range of genes (nearly 4000 genes) and occurs in a random and probabilistic manner [61]. Several mutations of the Aire gene have been shown to compromise TRA expression, negative and Treg selection, and are associated with manifestations of autoimmunity in mice and humans [69, 70]. Central tolerance is, therefore, dictated by the pool of TRAs presented in the thymic medulla. Nevertheless, a significant portion of TRA transcripts is expressed by mTECs independently of Aire, implicating the contribution of other regulators [62, 69]. Indeed, the FEZ family zinc finger 2 (Fezf2) transcription factor is another described contributor to PGE [71] (**Figure 3**).

The initial studies focusing on PGE described a stochastic pattern of TRA expression [72, 73], an observation that was concordant with the non-specific nature of the mechanisms through which Aire induces transcription in silenced chromatin. Some degree of coordination was later discovered, as Aire-mediated PGE was shown to exhibit small co-expression groups of TRAs with no biological association. Thus, a model of ordered stochasticity was suggested, in which single mTECs shift through distinct co-expression

groups throughout their lifespan [64, 74-76]. More recently, advances in PGE characterization have provided further insights regarding coordinated TRA expression in mTECs. In particular, Michelson *et al*. have defined a variety of highly distinct mTEC subtypes, named “mimetic cells” [77, 78], which will be discussed in more detail in the next Section of the Introduction Chapter. These subsets express lineage-defining transcription factors and transcriptional signatures akin to extrathymic cell types (such as muscle, skin, and neuronal cells) and were suggested to promote tolerance to the antigens expressed in the corresponding peripheral tissues [77, 78]. Therefore, the discovery of the mimetic mTECs implies that TRAs expression can also occur in a biologically coordinated manner.

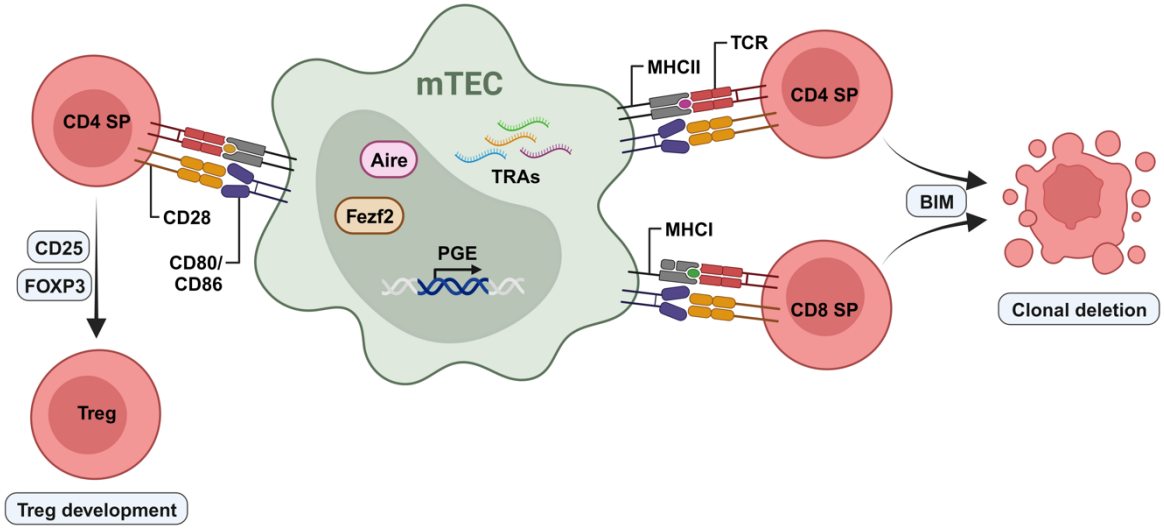


Figure 3 – mTEC functions for T-cell repertoire selection and Treg cell induction. Promiscuous gene expression in mTECs is partially controlled by Aire and Fezf2. Thymocytes with high affinity interactions with self-peptide-MHC complexes can undergo clonal deletion through the upregulation of the pro-apoptotic protein BIM. Alternatively, $CD4^+$ thymocytes with high self-antigen reactivity can upregulate the IL-2 receptor CD25 and the FOXP3 transcription factor and undergo Treg cell differentiation.

Tolerance induction is also dependent on a unique haematopoietic APC compartment within the medulla [41]. Notably, an important collaboration is established between DCs and mTECs [79]. Thymic DCs localize predominantly in the medulla and are subdivided into three major subsets: two subtypes of conventional DCs (cDC1 and cDC2), either of intrathymic or extrathymic origin, respectively, and plasmacytoid DCs (pDCs) [80]. Intrathymically-derived (or resident) cDC1 have been shown to acquire and cross-present TRAs originally expressed by mTECs and play non-redundant roles in shaping the T-cell repertoire through clonal deletion and Treg cell induction [81, 82]. Extrathymically-derived (or migratory) cDC2 and pDCs broaden the range of self-antigens displayed in the thymus due to their ability to present peripherally-acquired self-antigens that otherwise would not be presented to developing thymocytes [83, 84]. In sum, by mimicking antigen presentation

from peripheral tissues and harbouring specialized APCs, the thymic medulla is uniquely designed for providing different regulatory layers for tolerance induction.

Maturation of SP thymocytes and thymic egress

During their residency in the medulla, thymocytes that survive both selection processes undergo the final maturation steps. These last stages of thymocyte development, before thymus exit, are mapped by changes in cell surface proteins. 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 following strong TCR signalling [86]. As they upregulate MHCI and downregulate CD69, they become competent to proliferate in response to TCR stimulation and to participate in peripheral immune responses upon antigen recognition [85]. Late-stage maturation is driven by the transcription factor Kruppel-like factor 2 (KLF2) [87]. KLF2 promotes sphingosine-1-phosphate receptor 1 (S1P1) expression, which in turn, mediates the exit from the thymus into the blood, where the chemotactic gradient of the corresponding S1P ligand is higher [88] KLF2 also induces upregulation of L-selectin, or CD62L, which interacts with ligands on high endothelial venules in lymph nodes [87]. As a result, the $CD69^{-}CD24^{-}CD62L^{+}Qa2^{+}$ mature SP thymocytes acquire the functional competences to enter the circulation as recent thymic emigrants (RTEs) and to home to secondary lymphoid organs. Thus, in addition to central tolerance induction, the thymic medulla provides the specific microenvironment to nurture the maturation of functionally competent and self-tolerant naive T cells.

iNKT cell development

While conventional T cells are largely responsible for the control of adaptive immunity, unconventional T cells present properties that bridge the innate and adaptive arms of the immune system [89, 90]. Unconventional T cells are functionally poised and capable of responding more rapidly and have a propensity for a more localized tissue distribution within the body. Still, a particular defining feature that distances unconventional T cells from their conventional counterparts is the method of antigen recognition. As previously discussed, conventional T cells integrate a complex system centred on their ability to collectively possess highly diverse TCRs to recognize processed antigenic peptides presented on MHC molecules. In contrast, unconventional T cells have a more limited TCR repertoire, expressing, for instance, an alternative γδ TCR, or a relatively nondiverse αβ TCR, like the one found in invariant natural killer T (iNKT) cells [89, 90]. Indeed, most iNKT cells express an identical TCRα chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) coupled with a set of TCRβ chains formed from a limited number of TCRβ

variable segments [91]. This TCR configuration confers iNKT cells conventional MHC-independent antigen recognition properties, particularly, the ability to recognize glycolipid antigens presented by CD1d, a non-polymorphic MHCI-like molecule [92]. CD1d binds lipid antigens from various commensal and pathogenic microorganisms, as well as self-lipid antigens [93].

The emergence of the iNKT cell lineage occurs within the thymus [94]. Notably, the nature of the selecting self-lipids presented within the thymus remains unclear [94], and it has been documented that microbiota-derived lipids do not mediate the positive selection of iNKT cells [95]. Unlike conventional T cells, iNKT cells are already functionally primed to respond before emigrating from the thymus and populating peripheral tissues or secondary lymphoid organs [96]. Thymic iNKT cells can produce cytokines in steady-state, including IL-4, and exhibit an antigen-experienced phenotype that allows them to respond with rapid and robust cytokine production immediately upon TCR and/or cytokine signals [96].

Before acquiring their distinctive functional features relative to peptide-reactive αβ T cells, iNKT cells begin their divergence from the conventional route in early T-cell development. In the thymic cortex, iNKT cell precursors emerge within the DP thymocyte population during positive selection [91]. Importantly, unlike the cTEC-mediated positive selection of conventional T cells, iNKT cell positive selection requires homotypic interactions between DP thymocytes and is independent of cTEC-mediated antigen presentation [91]. The resulting signalling pathway induces an iNKT cell precursor, also called developmental stage 0, characterized by the expression of CD24, CD69, and the early growth response transcription factor Egr-2 [97]. For the subsequent stages of the iNKT cell developmental pathway, a “lineage diversification model” was suggested and has gained generalized acceptance [98]. This model evolves from its ancestor, the “linear model”, which described 3 consecutive stages of iNKT cell development: stage 1 ($CD24^-$ $CD44^-$ $NK1.1^-$), stage 2 ($CD24^-$ $CD44^+$ $NK1.1^-$), and stage 3 ($CD24^-$ $CD44^+$ $NK1.1^+$) [99]. The current model incorporates the categorization of iNKT cells into functionally distinct subsets, NKT1, NKT2, and NKT17, according to their respective expression of transcription factors and cytokines upon activation, analogous to the $CD4^+$ T helper sub-lineages [98]. The $NK1.1^+$ stage 3 cells of the linear model correspond to a $Tbet^+$ and IFNγ-producing subset, termed iNKT1 cells. Moreover, $NK1.1^-$ stage 1 and 2 cells, previously deemed as immature, are shown to be enriched for iNKT2 and iNKT17 sub-lineages. While $PLZF^{hi}$ iNKT2 cells produce IL-4 in steady-state and after stimulation, iNKT17 cells are $PLZF^{int}$ $ROR\gamma t^+$ and produce IL-17 after stimulation [94, 98]. Of note, the precursor-product relationship initially described between stage 2 cells (iNKT2/iNKT17) and stage 3 cells (iNKT1) has been conciliated with the current model by fate-mapping studies demonstrating that nearly all iNKTs pass through a $PLFZ^+$ IL-4-producing iNKT2 stage before final differentiation [94].

While the thymic cortex harbours iNKT cells in their most primordial stages, the migration of CCR7$^+$ iNKT precursors to the medulla is an essential requirement for the continued differentiation of iNKT cells [100]. Simultaneously, the medullary microenvironment is greatly impacted by the presence of iNKT cells, particularly iNKT2 cells, due to their steady-state production of IL-4 [98]. iNKT2-derived IL-4 promotes the development and function of Tregs [59, 101], drives chemokine production and clonal deletion by thymic DCs [98, 102], induces thymic emigration of mature T cells [103], and supports thymic regeneration [104]. Hence, IL-4 influences the thymic stroma, T-cell development, and tolerance induction. Reciprocally, on the axis connecting iNKT-derived IL-4 and thymus medulla function, iNKT cells require the medullary compartment for their maturation, as evidenced by the reduction in CD44$^+$ iNKT cells in mTEC-deficient *Relb* KO mice [105]. Additionally, the lineage fate of developing iNKT cells is shaped by medullary APCs. For instance, mTECs are a major source of thymic IL-15, which contributes to the generation of iNKT1 cells, while a mimetic mTEC subset, named thymic tuft cells, was shown to influence the differentiation of iNKT2 cells, possibly through their production of IL-25 [105-107]. Additionally, medullary macrophages are the predominant APCs required for the licensing of NKT2 for steady-state production of IL-4 [108]. Therefore, the functional role of the medulla extends beyond the negative selection of self-reactive αβ T cells and Treg cell generation, including also the development of functionally distinct iNKT cell lineages.

THE ORIGIN AND FATES OF THYMIC EPITHELIAL CELLS

Thymus organogenesis

The early establishment of organized and functionally competent thymic microenvironments is the result of a complex and tightly orchestrated process. Thymus organogenesis begins in the mouse embryo between embryonic day (E) 9 and 10, as a shared primordium of the thymus and the parathyroid gland develops from the endoderm of the third pharyngeal pouch [109]. Subsequently, the expression of the transcription factors Forkhead box N1 (FOXN1) and Glial cells missing homolog 2 (Gcm2) will delineate the two organ-specific domains and orchestrate the differentiation of the thymus and parathyroid, respectively [110]. At E12.5, the two organ rudiments separate and begin to migrate to their final anatomical locations: the parathyroid becomes adjacent to the thyroid gland, and the thymus migrates further into the chest cavity, where its two lobes meet above the heart [111]. 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 and possesses the same general organization and functional features as the thoracic thymus [112] [113].

FOXN1 is the most well-characterized thymus-specific marker and is an indispensable regulator of thymus organogenesis [114]. FOXN1$^{-/-}$ mice display the classical “nude” phenotype of hairlessness and congenital athymia, as the formation of the primordial organ is arrested between E11.5 and E12.0, and the thymic rudiment is not colonized by lymphocyte precursors, causing a severe primary T-cell immunodeficiency [115, 116]. Several other transcription factors and signalling pathways have been implicated in early thymus development, including eyes absent homologue 1 (Eya1), homeobox a3 (Hoxa3), paired box 1 (Pax1), Pax9, sine oculis homologue 1 (Six1), Six4 and T-box 1 (Tbx1) [111, 117]. Still, despite the significant progress in this field, our understanding of the complex molecular network that controls thymic fate specification is still incomplete.

The fetal thymus is seeded by fetal liver-derived HSC prior to vascularization, between E11 and E12, which are key for providing the first wave of T-cell precursors and initial instructive signals for TEC development and proliferation [118]. The nature of HSC-derived signals required for the formation of the thymic epithelium will be detailed later in this Section. Additionally, neural crest (NC)-derived mesenchymal cells surrounding the third pharyngeal pouch supply other important signals for the proliferation and differentiation of the thymic epithelium, such as fibroblast growth factor (FGF)-7 and -10 and insulin-like growth factor (IGF)-1 and -2 [119, 120]. Furthermore, during thymus organogenesis, NC cells 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 [121, 122]. 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 [123]. Ultimately, NC-derived cells form the surrounding capsule of the thymus and also stabilize blood-vessel structures by differentiating into perivascular cells [124]. Altogether, the dynamic process of thymic organogenesis involves the participation of cells from all three embryonic germ layers - endoderm-derived epithelium, ectoderm-derived NC cells, and mesoderm-derived HSCs, each undergoing co-dependent and synchronised developmental programs.

Thymic epithelial cell progenitors and lineage diversion

During thymus organogenesis, the first immature TECs appear at E12.5 and develop from thymic epithelial cell progenitors (TEPs), which have the ability to differentiate into both cortical and medullary lineages. These bipotent TEPs are essential building blocks for thymus development and maintenance and have been detected in both the embryonic and postnatal thymus [125, 126]. The identification of TEPs is a subject still under investigation. Some studies have suggested Placenta-expressed transcript-1 (Plet1) as a TEP marker, which can be recognized with antibodies against MTS20 and MTS24 [127]. 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 [128, 129]. In contrast, a subsequent report showed that both MTS24^{+} and MTS24^{-} cells possess bipotent progenitor ability, supporting the existence of TEPs that lack Plet1 expression [130]. Hence, the phenotypic definition 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 IL-7, have the potential to generate both cTECs and mTECs [131-133] (**Figure 4**). Such findings argued 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 first express cTEC-associated traits before committing to the mature cTEC or mTEC lineages, a cell-fate decision whose underlying molecular principles remain undefined [134].

Other studies have focused on the characterization of lineage-restricted progenitors downstream of bipotent TEPs, such as mTEC-committed progenitors (mTEPs) [135]. 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) [136]. In addition, stage-specific embryonic antigen A (SSEA) is expressed in a small fraction of total Cld3/4$^+$ cells and marks a more defined population of mTEPs, termed mTEC stem cells due to their self-renewing and progenitor potential that, although decreased, can still be detected in adulthood [137] (**Figure 4**). Moreover, a receptor activator of NF-κB (RANK)$^+$ unipotent mTEC progenitor also arises from the Cld3/4$^+$SSEA$^-$ fraction between E14 and E15 [138] (**Figure 4**). Lastly, other studies have revealed anatomically restricted mTEPs by showing that a fraction of adult mTECs can descend from a lineage-committed podoplanin (PDPN)$^+$ progenitor located at the cortico-medullary junction (CMJ), also called junctional TECs [139] (**Figure 4**). Further work is needed to elucidate whether these mTEP subsets establish linear relationships or represent divergent mTEC differentiation programs in the embryonic and postnatal thymus.

The cellular processes that allow the continued generation of mTECs in adult life constitute an enigmatic subject for which two possible scenarios have been considered [140]. One possibility is that, throughout life, the medullary epithelium could be maintained solely by embryonically derived mTEPs, eventually leading to an exhaustion of this limited cell pool. Alternatively, a new supply of mTEPs from bipotent TEPs could still occur in the adult thymus. In this regard, while bipotent β5t$^+$ TEPs can generate both Cld3/4$^+$SSEA1$^+$ and PDPN$^+$ mTEPs, this contribution to the mTEP pool fades during postnatal life [141, 142]. These results implicate that the maintenance of the adult medullary epithelium could be assured by either β5t-independent bipotent TEPs or through the contribution of mTEPs instead of bipotent progenitors.

Some new insights on TEC developmental pathways were gained from recent single-cell RNA-sequencing studies. Work by Baran-gale *et al*. identified a cluster of single-cells with a heterogeneous transcriptional signature overlapping with mature cTECs and mTECs, which were termed intertypical TECs [143]. This cluster expressed markers associated with bipotent TEPs, like Plet1, and encompassed junctional TECs. A combination of fate-mapping and pseudotime analyses showed that intertypical TECs are mostly downstream of β5t$^+$ TEPs. Still, the existence of intertypical TECs without a β5t-expressing ancestor implied also the existence of β5t$^-$ TEPs. In turn, intertypical TECs followed mTEC differentiation trajectories that passed through a cluster of cells in a highly proliferative state, called proliferating TECs [143] (**Figure 4**). Notably, intertypical TECs quiesce with age, resulting in cells becoming increasingly blocked in an intertypical TEC state and reducing their contribution for the maintenance of the mTEC compartment [143]. In agreement with these findings, a separate study by Wells *et al*. showed that a transit-amplifying cell (TAC) population of cycling TECs, a possibly equivalent cluster to the proliferating TEC cluster defined by Baran-gale *et al*., may represent a precursor of mTECs

[144] (**Figure 4**). Despite these contributions, further investigation will be needed to define a complete profile of the developmental relationships established between bipotent TEPs, lineage-committed TEPs, and mature TECs across different stages of life.

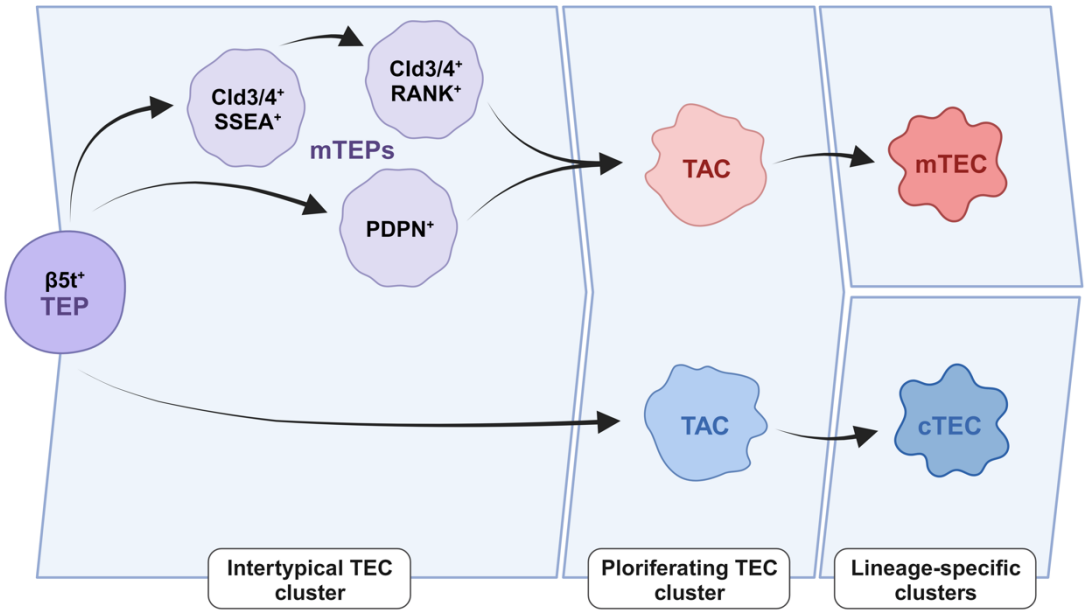


Figure 4 – Proposed model for cTEC and mTEC differentiation according to identified TEP populations and single-cell transcriptomic analysis. Bipotent TEPs with cTEC-associated markers, such as β5t, can give rise to mTEC lineage-committed mTEPs defined by the expression of Cld3/4, SSEA, RANK and PDPN. Transcriptional single-cell analysis defined a heterogeneous intertypical TEC cluster downstream of β5t$^+$ TEPs and containing marker genes of mTEPs. On the trajectory of mTEC differentiation, intertytpical TECs pass through a proliferating TEC cluster, also called transit-amplifying cells (TAC) TECs. The intertypical and proliferating TEC clusters possess both mTEC and cTEC signatures, suggesting that they represent common states that occur during the differentiation of both lineages.

Functional heterogeneity within the cTEC and mTEC lineages

Cortical TEC diversity

Despite the contribution of cTECs for T-cell development becoming increasingly more defined, the functional heterogeneity within the cTEC population is still relatively unknown. Some of the difficulties in the dissection of the mature cTEC compartment can be attributed to the relatively low yield of cells when isolated from the adult thymus, in comparison to mTECs. On the other hand, particularly in the embryonic thymus, shared markers between TEPs and mature cTECs might perturb the discrimination of these two populations.

Although phenotypic heterogeneity has been shown to exist within mature cTECs, namely differential expression of markers such as CD83, CD40, HVEM (CD270), Ly51, and MHCII [145], whether such markers discriminate functionally distinct cTEC subsets is still

mostly unknown. Notably, Baran-Gale *et al*. have described a transcriptionally distinct population of cTECs, which were termed perinatal cTECs due to their presence being restricted to the first weeks of life [143]. The perinatal cTEC population was further phenotypically characterized as $CD83^{+}CD40^{+}Sca1^{-}$ and shown to possess an increased capacity for positive selection when compared to non-perinatal ($CD83^{-}CD40^{-}$) cTECs, thus suggesting possible specific or enhanced functional abilities [145].

A unique feature of cTECs that constitutes another example of possible functional heterogeneity are thymic nurse cells (TNCs). TNCs are large epithelial cell complexes formed *in vivo* by persistent cTEC-thymocyte interactions, in which single cTECs encase between four and eight viable DP thymocytes [146]. These complexes are not present in the embryonic thymus and are only detected after postnatal day 5 with a frequency of approximately 10% [147]. TNC complexes possess gene expression profiles distinct from total cTECs, with increased mRNA expression levels of genes coding for CD205, CXCL12, TGF-β, TSSP, and VCAM-1 [147]. Although TNCs are not essential for T-cell differentiation and positive selection, thymocytes within TNCs are enriched for long-lived DPs that underwent secondary TCR-Vα rearrangement, suggesting a role for TNCs in optimizing T-cell selection through secondary TCR rearrangement [147].

Medullary TEC diversity

Given the role of mTECs in safeguarding self-tolerance, over the years, substantial efforts have been directed to the characterization of the mTEC compartment, revealing a high degree of phenotypic, molecular, and functional heterogeneity. Initially, the sole method to dissect mTEC diversity was based on the differential expression levels of cell surface MHCII and CD80, which subdivides mTECs into $MHC^{low}CD80^{low}$ ($mTEC^{low}$) and $MHC^{high}CD80^{high}$ ($mTEC^{high}$) [148]. Cells in the $mTEC^{high}$ subset are characterized by a high turnover rate (2 to 3 weeks), while $mTEC^{low}$ include only a small fraction of cycling cells [149, 150]. At around E16, a fraction of $mTEC^{high}$ begins expressing Aire [149, 150]. Within $Aire^{+}$ $mTEC^{high}$, further subdivision is possible based on the differential expression of osteoprotegerin (OPG), a regulator of mTEC cellularity and proliferation [151]. $mTEC^{high}$ are also characterized by the expression of the transcription factor Fezf2, which directly regulates the expression of some Aire-independent TRAs [71]. Although at a lower frequency, Fezf2 is also detected in $mTEC^{low}$ [71], supporting the notion that TRA expression is not restricted to $mTEC^{high}$.

The $mTEC^{low}$ subset was originally characterized as a population containing precursors of mature $mTEC^{high}$ [150]. However, it was later revealed that a portion of $mTEC^{low}$ expresses markers associated with terminal differentiation, such as involucrin (Ivl) and keratin 10 (K10), and constitutes a population of previously Aire-expressing cells,

termed post-Aire cells [152, 153]. Additionally, a subset of mTEC^{low} was shown to produce the chemokine CCL21, essential in the recruitment of thymocytes from the cortex to the medulla [154]. These studies brought to light the notion that the mTEC^{low} subset harbours a high degree of developmental and functional heterogeneity, as this population contains precursors of mTEC^{high}, functionally mature cells, such as CCL21^{+} cells, and terminally differentiated post-Aire mTECs.

With the development of single-cell genomic technologies, subsequent studies have achieved increasingly high degrees of resolution regarding mTEC diversity. In a study by Bornstein *et al*., single-cell profiling of the thymic stroma revealed four major mTEC populations, named mTEC I-IV [155]. The mTEC I population was characterized by high expression of *Itga6* and *Sca1*, the mTEC II expressed mature mTEC markers, such as *Aire*, *Fezf2*, *Cd40*, and *H2-Aa*, and mTEC III represented a heterogeneous population containing cells expressing the post-Aire associated genes *Ivl* and *Krt10*. Notably, the mTEC IV subset did not express canonical cTEC or mTEC markers and instead constituted a novel divergent TEC lineage with characteristics of the gut chemosensory epithelial tuft cells, such as expression of the canonical taste transduction pathway and IL-25 [155]. In a concomitant study, using an Aire fate-mapping mouse model, Miller *et al*. reported that these cells, referred to as thymic tuft cells, can be found in the post-Aire mTEC population, while some differentiated in an Aire-independent manner [156].

While historically, light microscopy observations had suggested that the thymic medulla includes histologically distinct epithelial cell types, it was only recently that studies have successfully identified and characterized the ample variety of highly distinct mTEC subtypes in the mouse and human thymus, particularly within the mTEC^{low} [77, 143, 155-158]. In this regard, by assaying single-cell chromatin accessibility, Michelson *et al*. uncovered signatures of lineage-defining transcription factors of extra-thymic tissues, such as skin, lung, liver, and intestines, in distinct single-cell clusters enriched within post-Aire mTECs [77]. These subsets mirrored the chromatic landscapes, transcriptional programs, and TRA expression profiles of the corresponding peripheral cell types, thus being labelled mimetic cells [77]. Currently, the catalogue of mimetic mTEC subsets includes tuft cells, keratinocyte, ciliated, muscle, secretory/neuroendocrine, microfold, ionocyte, and enterocyte/hepatocyte subsets [78] (**Figure 5**).

The effects of Aire expression in mTEC differentiation have been discussed in several studies. Initially, due to the notoriously high turnover rate of Aire-expressing mTECs, Aire was once believed to have an apoptotic effect [149]. This notion coincided with the observation that in the absence of Aire, the mTEC^{high} compartment significantly expands [149]. It was later revealed that rather than impacting mTEC lifespan, Aire expression was required for the completion of the mTEC differentiation program and the transition into the

mTEC^{low} post-Aire stage [159]. This proposed role for Aire in mTEC differentiation is in agreement with the current identification of the aforementioned diversity of mTEC subsets downstream of Aire expression. Notably, similarly to the initially described tuft cell subset, the additional mimetic cells described by Michelson *et al.*, although existing within post-Aire mTECs, were partially and variably dependent on Aire [77]. These observations indicate that mimetic cell differentiation can occur through both Aire-dependent and Aire-independent pathways (**Figure 5**). In contrast, the accumulation of mimetic subsets depends strictly on the respective lineage-defining transcription factors. *Pou2f3* was identified as the master regulator of thymic tuft cell development [155], while *SpiB* and *Sox8* are required for microfold mTEC differentiation [77], and *Insm1* for the development of endocrine mTECs [160].

The collective work on PGE characterization has revealed that Aire and mimetic cell lineage-defining transcription factors operate in developmentally distinct mTEC populations and mediate TRA expression through different mechanism [78]. Therefore, it is possible to reconcile the research reporting Aire-mediated stochastic co-expression groups lacking biological significance [64, 74-76] with the recent demonstration of biologically coherent TRA expression in mimetic cells enriched within the post-Aire mTEC^{low} [77] (**Figure 5**). Nevertheless, the distinct stochastic and coordinated manners of TRA expression are cooperative and not independent, as Aire is still an indirect promoter of mimetic cell TRA expression by generally favouring the accumulation of mimetic cell subtypes [77].

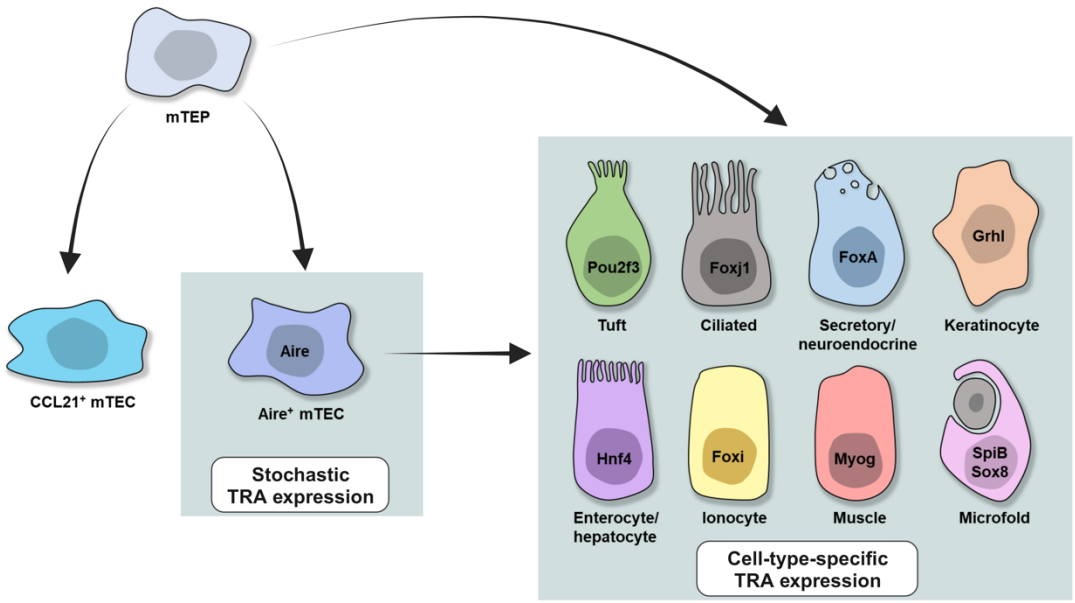


Figure 5 – Diversity of specialized mTEC subsets for TRA expression and tolerance induction. mTEPs give rise to different mTEC subsets, including CCL21^{+} mTECs and Aire^{+} mTECs. Aire^{+} mTECs can further differentiate into several mimetic cell subsets through the expression of lineage-defining transcription factors of peripheral cell types. Some mimetic cells may differentiate independently of Aire. Aire drives quasi-random expression of TRAs, while lineage-defining transcription factors drive the expression of biologically coordinated TRAs.

To support a physiological contribution of mimetic mTECs to tolerance induction, different experimental strategies have been used. First, athymic nude mice transplanted with tuft cell-deficient thymi, in which IL-25 expression is abrogated, were shown to generate an IL-25-specific antibody response following immunization [156]. In a subsequent report, induced expression of a model antigen in ciliated or muscle mimetic mTECs was sufficient to promote negative selection of the cognate antigen-specific T cells [77], and most recently, the specific deletion of thymic endocrine mimetic cells resulted in increased autoreactivity to some endocrine-rich tissues [160].

Moreover, several studies have also supported that mimetic cell subtypes assume additional functions beyond the provision of TRAs. The most characterized is the role of thymic tuft cells in supporting iNKT cell development and polarization and, consequently, promoting an IL-4-enriched medullary microenvironment [106, 156]. Thymic tuft cells have also been associated with the control of thymic type-2 innate lymphoid cells (ILC2s) [155], and microfold mTECs were suggested to control thymic B cells [77] and their differentiation into IgA^+ plasma cells [160]. Lastly, endocrine and microfold mimetic cells, through the production of ghrelin and OPG, respectively, have been proposed to regulate TEC compartment size and thymic function [160]. Follow-up studies will be key to characterize the emergence and functionality of these still ill-defined subsets and understand the roles of lineage-defining transcription factors and mimetic mTECs in both self-tolerance and thymus biology.

Signalling requirements for TEC differentiation

During early thymic ontogenesis, the cortical and medullary areas are not yet organized in the typical three-dimensional structure observed in the adult thymus. Growth and organization of both compartments begin with colonization of the thymus by hematopoietic progenitors and is induced by instructive signals from the developing thymocytes [118]. 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 [118]. 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 [161, 162].

The molecular cues that established the crosstalk between thymocytes and mTECs are far more characterized and are an essential requirement for the development and maintenance of the thymic medulla. Specific thymocyte subsets express ligands for several members of the tumour necrosis factor receptor superfamily (TNFRSF), including RANK, CD40 and lymphotoxin β receptor (LTβR) [163] (**Figure 6**). These receptors are expressed

by mTECs and mTEC precursors, and their engagement induces activation of the canonical and non-canonical nuclear factor kappa B (NF-κB) signalling pathways, which activate the transcriptional programs required for mTEC lineage specification and differentiation [163] (**Figure 6**). As such, the disruption of elements of the NF-κB signal transduction pathways compromises mTEC development and induces autoimmunity [164]. For instance, deficiency in TNF receptor-associated factor 6 (TRAF6) or NF-κB inducing kinase (NIK), signal transducers of the canonical and non-canonical pathways respectively, results in a disorganized thymic medulla and absence of mature mTECs [165, 166]. Moreover, disruption of a more downstream factor in the non-canonical 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 [167] (**Figure 6**).

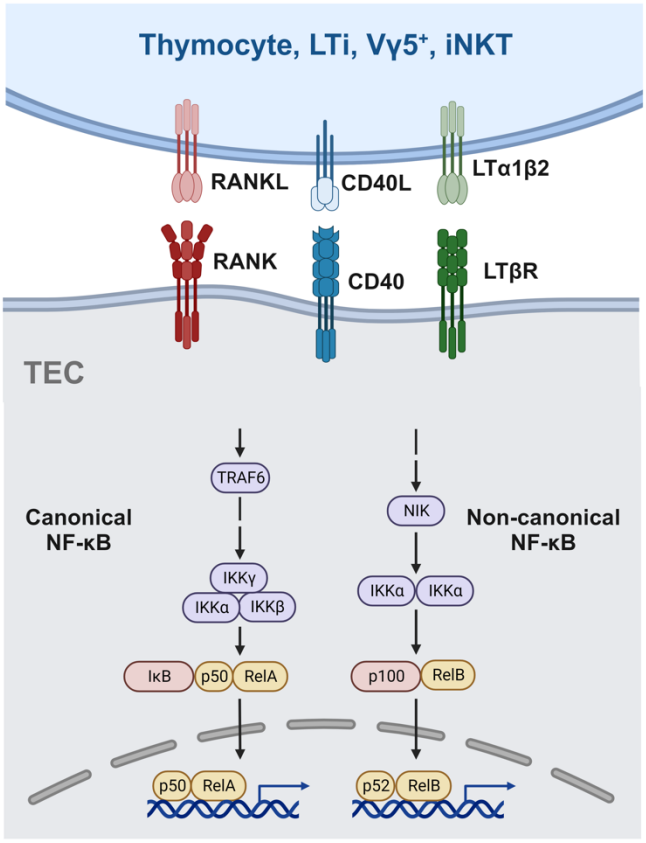


Figure 6 – RANK, CD40 and LTβR signalling is required for NF-κB pathway activation and mTEC differentiation. TEC-expressed TNFRSF members interact with their respective ligands present on distinct hematopoietic cells to induce activation of NF-κB pathways. TNF receptor-associated factor (TRAF) family proteins bind to the cytoplasmic domains of TNF receptors and activate downstream kinase cascades, which include 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 p50 and RelA, while the NF-κB subunits in the non-canonical pathway are typically p52 and RelB [172].

TNFRSF ligands are differentially expressed by hemopoietic cells, and their cellular source varies between developmental periods. In the embryonic thymus, before αβ T-cell positive selection, innate $CD4^+3^-$ lymphoid tissue inducer (LTi) cells promote the development of the first $Aire^+$ mTECs through the expression of RANK ligand (RANKL) [168]. Invariant $Vγ5^+$ thymocytes accumulate in the embryonic medulla and are also able to trigger mTEC maturation through RANKL expression [169]. These findings support an initial role for innate LTi cells and γδ T-cell progenitors in securing the development of $Aire^+$ mTECs prior to the emergence of conventional αβ T cells bearing potentially autoreactive TCRs. In adult mice, medulla formation is mainly maintained by positively selected $CD4^+$ and $CD8^+$ thymocytes and iNKT cells through RANKL/RANK signals [105, 170]. Furthermore, CD40/CD40L-mediated interactions between mTECs and self-reactive $CD4^+$ thymocytes are important in controlling mature mTEC cellularity [171] (**Figure 6**).

While the frequency of $Aire^+$ mTECs is markedly reduced in adult RANKL-deficient thymi, CD40 deficiency results in a more modest reduction. Still, mice with a combined deficiency of RANKL and CD40 show a more profound reduction in $Aire^+$ mTECs compared to mice lacking only RANK-mediated signalling [173]. Thus, CD40-mediated signalling appears to partially compensate for the absence of RANKL. Collectively, these observations suggest that 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 signalling.

Regarding LTβR signalling, a seminal study revealed its requirement for the organization of the medulla and export of mature T cells from the thymus [174]. Furthermore, although LTβR is not required for a normal $Aire^+$ mTEC frequency [175], it has been shown to control functional diversity within the $mTEC^{low}$. Particularly, it regulates the expression of medullary chemokines CCL19 and CCL21 by $mTEC^{low}$, which are required for thymocyte migration to the medulla and, consequently, negative selection [176]. A dependency on LTβR signalling was also found for Aire-independent TRA expression in $mTEC^{low}$ [177], as well as for the transition of mTECs into the post-Aire stage [153]. Moreover, LTβR was also shown to influence thymic medulla function through a role that is unconnected from its impact on mTECs, namely by controlling the intrathymic DC pool required for negative selection [178]. More recently, a study by Lucas *et al*. further elucidated that LTβR regulates thymic tuft cell development [106]. In this study, the authors highlight that LTβR expression in TECs, by controlling the differentiation of thymic tuft cell and $CD104^+CCl21^+$ $mTEC^{low}$ subsets, respectively promotes IL-25 production and IL-15 trans-presentation in the thymic medulla, and consequently, supports the intrathymic and peripheral availability of iNKT cells [106].

In the context of mTEP populations, LTβR was also shown to contribute to the maintenance of the Cld3/4^{+}SSEA1^{+} mTEC progenitors, particularly in neonatal mice [179]. Interestingly, collective evidence suggests a possible sequential requirement for TNFRSF-mediated signalling during mTEP differentiation into mTECs. On one hand, embryonic Cld3/4^{+}SSEA1^{+} mTEC progenitors, which are LTβR^{+}RANK^{-}, were shown to emerge in the absence of RelB [138]. In contrast, the production of temporally downstream Cld3/4^{+}SSEA^{-} RANK^{+} mTEC progenitors is RelB-dependent, implicating the noncanonical NF-κB pathway [138]. Coincidently, LTβR signalling can elicit RANK expression and prepare mTECs to respond to early RANK-mediated differentiation [180], while initial RANK signalling is, in turn, necessary to upregulate CD40 expression on developing mTECs [181]. Still, more insight is required to establish the direct lineage relationships between mTEC precursors according to the sequential requirement and expression of TNFRSF members.

The collection of studies discussed in this Section demonstrates that cooperative and non-redundant thymocyte-derived signals influence the development of a remarkable variety of specialized TEC niches. Characterizing the cellular and molecular determinants for the development of different TEC lineage fates, as well as their functional contributions within the thymic microenvironments, can ultimately improve our understanding of the mechanisms that govern T-cell generation and tolerance induction.

INTRINSIC AND EXTRINSIC SIGNALS CONTROLLING TEC HOMEOSTASIS

Age-related thymic involution

The thymus undergoes an evolutionarily conserved process of involution with an earlier onset than most other organs [182]. Human thymic involution is thought to begin at 1 year of age, while the murine thymus begins its decline at 4-6 weeks of age [183, 184]. During early life, the thymus operates at its highest rate of thymopoiesis to establish a robust T-cell compartment characterized by a broad TCR repertoire [185]. This highly productive time window is critical for neonates, as the exposure to new pathogens is maximal during this period. Simultaneously, key thymic tolerogenic mechanisms are being implemented to preserve tissue homeostasis [186, 187]. Later in life, immune memory responses against previously encountered pathogens also contribute to immune surveillance. One hypothesis to explain the early decline of thymic function is that the high energy expense of thymopoiesis can be redirected to support other organ functions [188, 189]. Sustaining the initial high rate of thymopoiesis may not only become redundant in terms of TCR diversity generation but could also result in increased chances of T-cell leukaemia and autoimmune disorders [190, 191]. Therefore, it has been argued that the physiological early-onset thymic involution may represent a biologically beneficial trade-off [188, 189]. Nevertheless, late-phase involution shares more resemblance to the natural aging process of other organs and is a contributing cause for the reduced immune surveillance in the elderly, a hallmark of systematic immune senescence [188]. The age-related thymic decline reduces the production and output of new naive T cells and, subsequently, leads to the homeostatic expansion of existing T cells and the narrowing of the peripheral TCR repertoire [192, 193] (**Figure 7**). The increased frequency of memory T cells, however, does not account for the responses to newly encountered antigens, including new pathogens, tumour-associated antigens, and vaccines [194]. While some residual thymic function persists into old age [195], naive T cells in aged organisms exhibit altered functional properties, including compromised proliferation in response to antigen stimulation and mobilization [196, 197]. Additionally, the fidelity of tolerance induction mechanisms decreases with age [198]. Eventually, the reduction in thymic function contributes to the age-related decline in immune system competence and is associated with several detrimental effects, such as reduced effectiveness of vaccination, increased infection susceptibility, autoimmunity, and increased incidence of cancer [199]. Ultimately, thymic involution plays an intriguing physiological role due to the duality of its implications. In early life, it can be considered a balancing, efficient and protective process, whereas in aged organisms, the downsides of this evolutionary trade-off begin to unravel, creating weak spots in immune surveillance.

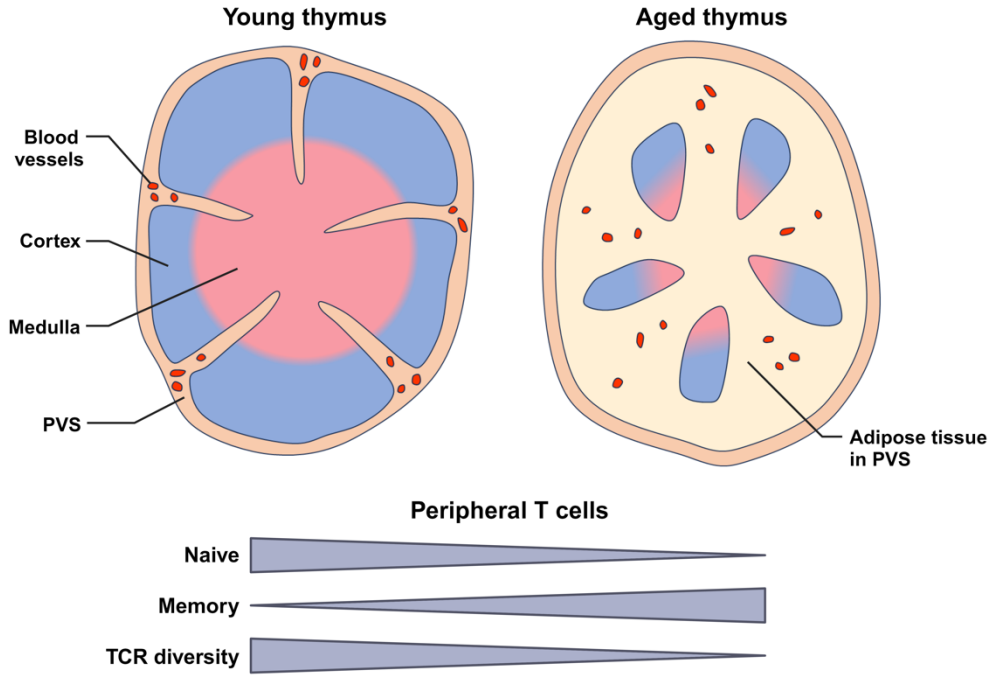


Figure 7 – Age-dependent alterations in thymus morphology and peripheral T-cell composition. Thymus involution is characterized by a progressive reduction of cortical and medullary areas, increase in perivascular space (PVS), and adipocyte accumulation. The age-dependent reduction in thymic function and T-cell output causes qualitative changes in the peripheral T-cell pool, which include a decline in naive T cells, expansion of memory T cells and narrowing of the TCR repertoire.

Impact of aging in the thymic epithelium

Considering that thymopoiesis relies on the continuous seeding by lymphoid progenitors, this process has been evaluated as a potential limiting factor in the function of the aged thymus. Some studies have highlighted that, with age, defects arise in the BM T-cell progenitor potential and in the ability of intrathymic ETPs to reconstitute T-cell development [200, 201]. Despite some evidence suggesting that aging in the hematopoietic system might be a contributing factor, other studies have discarded ETP-intrinsic defects as the main cause of thymic involution. In this regard, the transfer of young HSC or ETPs into old mice was shown not to reverse thymus involution [202, 203]. Furthermore, old and young mouse-derived lymphoid progenitors have shown similar abilities to seed a transplanted fetal thymic microenvironment [203, 204]. Indeed, growing evidence supports that a possible primary driving force of thymic atrophy is the age-related degeneration of the thymic stroma, particularly of the TEC compartment [205]. Such studies also provide an important characterization of the structural and functional changes that affect TECs during aging.

Obvious morphological changes in the thymus can be observed due to age-dependent thymic stroma disruption [184] (**Figure 7**). The classical reticular and globular

pattern of cortical and medullary TEC, respectively, and the definition of cortico-medullary junctions gradually disappear with age [206]. Concomitantly, fibroblasts and adipocytes expand and TEC-free areas and perivascular spaces increase [183, 206]. On the other hand, TEC areas contract, and TEC cellularity is estimated to reduce by 50% between 4 and 16 weeks of age [143, 148]. The most notable reduction is in the mTEC compartment, which results in a progressive decrease in the mTEC-to-cTEC ratio [148]. Whether cTECs undergo a similar but less pronounced decline in cellularity is still arguable. Venables *et al*. have suggested that the reduction in the cortical area might be due to a contraction in cTEC cell projections and not a reduction in cell numbers, which could be an artifact of inefficient TEC isolation methods [207, 208].

With the refinement of singe-cell RNA-Seq technology, more studies have provided informative comparisons of TEC subset composition and transcriptional profiles in young versus aged organisms [143, 158, 209, 210]. In particular, Baran-Gale *et al*. have provided the most recent characterization of mouse age-related thymic epithelium remodeling. This study demonstrated that the perinatal cTEC subset has the earliest and most abrupt fluctuation in relative abundance, going from an initial 30% frequency to 1% at postnatal week 4. Moreover, most mTEC subtypes follow a gradual reduction with age, although a more drastic reduction is detected in the mature mTEC subset or mTEC^{high}, which begins as the most represented subset at 4 weeks. Conversely, mature cTECs and intertypical TECs increase in proportion over time. A clear reduction is also noted for the proliferating TEC subset, which is consistent with the age-related reduction in TEC proliferation and self-renewing capacity reported over the years, initially described using the proliferation maker Ki67 [148] and further dissected in recent transcriptional analyses [211-213]. These studies report that cTECs and mTECs, as early as 6 weeks, show a decline in the expression of cell cycle-associated genes, including targets of transcription factor E2F3 [211, 212]. In another study detecting the downregulation of cell cycle and ribosomal biogenesis genes, the authors attributed these features of aged TECs to a decline in the activity of the transcription factor Myc, which regulates the high rate of proliferation during early TEC development [213]. Importantly, several studies demonstrated the upregulation of a pro-inflammatory signature in the aged thymus, a characteristic of senescent tissues. This includes an increased expression of inflammatory cytokines by the aged non-TEC stroma, including *Il1a*, *Il1b*, *Il6*, *Il12b*, *Il18*, and *Tnf* [211], some of which had been shown to accelerate thymic involution [214, 215]. Consequently, an increased inflammatory signature is detected in aged mature cTEC and mTEC subsets [143]. Altogether, despite the inherent obstacles of stromal cell analysis, several studies have provided important insights into TEC compositional and transcriptional changes during thymic aging.

The TEC-autonomous role of FOXN1

A critical aspect of thymic atrophy is the downregulation of key genes that partake in TEC maintenance, function, and homeostasis. Specifically, the predominant mechanism causally associated with thymic involution is the decrease in expression of the TEC-defining transcription factor FOXN1 [114, 216-220]. FOXN1 is required for thymic organogenesis and TEC maintenance, and its progressive decline in expression is one of the first markers of the onset of thymic involution [221]. The cause-effect relationship between FOXN1 decline and thymic atrophy has been addressed using different FOXN1-targeting mouse models [216-218]. For instance, a study by Sun *et al*. showed a direct correlation between changes in FOXN1 and thymic involution using a mouse model carrying loxP-floxed-*Foxn1* and a ubiquitous CreERT transgene with a low level of spontaneous activation. In this study, the progressive reduction in FOXN1 levels was positively correlated with thymic involution [218]. In contrast, an exogenous supply of FOXN1 into the aged thymus, either via plasmid or transgene, greatly corrected most of the changes caused by thymic involution, including thymic size, ETP frequency, TEC numbers, and naive T-cell production [218-220]. Furthermore, FOXN1 reporter mice have been particularly useful in elucidating the timeline and dynamics of FOXN1 expression in thymic organogenesis and aging. In particular, it has been suggested that the downregulation of FOXN1 in cTECs, and consequently impaired cTEC function, might be the event initiating thymic involution [221].

While several studies have shown that the onset and progression of TEC degeneration results from a decline in FOXN1 expression, the upstream molecular mechanisms controlling FOXN1 maintenance or downregulation in aging TECs remain elusive. Two main pathways have been suggested to promote the induction of FOXN1 expression in early TEC development, the Wingless/Integrated (Wnt) and Bone morphogenetic protein (Bmp) pathways [222, 223]. Coincidently, a drastic downregulation of the Wnt signalling pathway occurs in the aged thymus [224], concomitant with an age-related increase in levels of Wnt signalling inhibitors [225, 226]. Together, these findings imply that age-related attenuation of the FOXN1-inducing Wnt signalling pathway contributes to TEC degeneration. While comprehensive information about the pathways regulating FOXN1 expression in the adult thymus is still lacking, given its position at the forefront of thymic involution, FOXN1 constitutes an attractive target for the development of strategies aimed at inducing TEC differentiation and function.

Hormonal and metabolic factors

In addition to TEC-intrinsic molecular mechanisms, thymic function and involution are affected by several hormonal factors. In particular, the association between sex

hormones and thymic atrophy was the earliest to be reported, with the observation that surgical castration causes enlargement of the thymus [227]. As steroid hormone levels change dramatically with age, their role in thymic involution is supported by the fact that age-related degeneration is most evident after puberty, when steroid hormone production is at its peak [205]. Additionally, thymic involution is also more rapid in males than in females, implying a more significant impact for androgens [205]. Indeed, androgens were shown to drive involution by acting directly on TEC-expressed androgen receptors [228]. Moreover, androgen signalling was shown to have a significant impact on the TEC transcriptome, controlling the expression of cell survival and viability genes, such as *Foxn1*, and repressing key cTEC genes for T-cell development and function, including *Dll4*, *Psmb11*, and *Ctsl* [229, 230]. Similarly, progesterone receptor signalling in thymic stromal cells has also been linked to thymic involution, particularly during pregnancy, when progesterone levels rise significantly [231]. Despite the key role of sex hormone signalling in TEC atrophy, castration-induced regeneration of the aged thymus is only transient [224], suggesting that thymic involution results from the culmination of several underlying mechanisms.

Metabolic activity is another important modulator of thymic function, dictating both degenerative and regenerative pathways in TEC homeostasis. Particularly, caloric restriction (CR), which has been associated with extended longevity, has also been shown to significantly delay thymic involution, inhibit thymic adipogenesis, and improve thymopoiesis [232, 233]. On the contrary, diet-induced obesity has deleterious effects on the thymus, accelerating adipocyte accumulation and loss of function [234].

Metabolic hormones play key roles in physiological homeostasis by mediating important cross-talk among the neuroendocrine and immune systems. Several neuroendocrine hormones possess demonstrated effects on thymic function and age-related involution. Among the first reports, growth hormone (GH) and its proximal mediator IGF-1 were shown to have significant protective properties in thymic involution. While circulating levels of GH decrease with age, partial restoration of thymic function can be achieved through GH administration [235, 236]. Similarly, exogenous administration of IGF-1 induces TEC expansion in mice and enhances thymopoiesis [237]. Subsequent reports have also demonstrated thymopoietic effects for leptin and ghrelin during aging. Leptin is an adipokine with numerous roles, being most extensively characterized as a regulator of satiety in response to energy expenditure. Leptin deficiency leads to severe obesity and thymic involution that can be partially rescued through leptin administration [238]. A later study further showed that the leptin receptor is mainly expressed in mTECs and can protect overall TEC cellularity in induced acute atrophy [239]. Moreover, Ghrelin is a stomach hormone that induces strong GH-releasing activity. Its expression within the thymus

declines with age, and when administered, it restores TEC cellularity and thymopoiesis [240]. Another example of a metabolic hormone mediating beneficial effects on thymic function through ectopic lipid mobilization is Fibroblast growth factor 21 (FGF-21), an activator of fatty acid metabolism and ketogenesis. FGF-21 expression in TECs reduces with age, contributing to thymic epithelium degeneration [241]. Of note, in prior studies, another fibroblast growth factor, FGF-7 (or keratinocyte growth factor), has been shown to significantly regenerate the thymic stroma [242]. Accordingly, during CR, the expression of leptin, ghrelin, and IGF-1 [243], as well as FGF-21 [241] is augmented in thymic tissues, suggesting that thymic stromal components undergo transcriptional reprogramming.

A separate stromal cell-intrinsic aspect demonstrating the role of metabolism in thymic atrophy was revealed by Griffith *et al*. The authors have reported that thymic stromal cells, in contrast to lymphocytes, are inherently deficient in the hydrogen peroxide-reducing enzyme catalase [244]. Due to lymphoid cell expansion, the thymus constitutes a highly anabolic environment, causing prolonged exposure of the unprotected stromal cells to reactive oxygen species (ROS) and, consequently, causing the accumulation of oxidative damage. The authors further showed that genetic or biochemical restoration of antioxidant activity improved thymic atrophy [244]. Altogether, the hindering of thymic involution through the elevation of antioxidant activity or downmodulation of metabolic activity (*e.g.*, CR) demonstrates the strong link between thymic atrophy and TEC susceptibility to metabolic damage. This intrinsic homeostatic challenge, together with the physiological age-associated fluctuations in the signalling of an array of hormones and growth factors, constitute some of the elements taking part in the erosion of thymic stromal cell integrity and function.

Genomic integrity

Besides the biological diversity and selective advantages that can arise from a small degree of genomic instability, such as random mutations, the preservation of the genomic material is key for proper cellular function and to ensure its faithful transmission to progeny. Genomic instability is a constant side effect of DNA metabolizing processes, such as replication and recombination [245]. Still, processes that directly damage DNA impose the biggest burden on the genome. These include environmental agents such as ultraviolet (UV) light, ionizing radiation (IR), and genotoxic chemicals, as well as endogenous sources, namely ROS and alkylating agents [246]. Indeed, maintaining the stability and function of the genome is a major cellular task, as is it estimated that on average a single cell suffers 10^4–10^5 DNA lesions per day [247]. Uncleared lesions in the DNA will impair the proper functioning of the genome and contribute to the accelerated development of age-related

diseases and carcinogenesis [246]. To safeguard genomic integrity, DNA damage response (DDR) mechanisms have evolved to allow cells to mount different orchestrated responses to an array of genotoxic insults. The DDR consists of a complex signalling network involving spatially and temporally controlled functional components. These include factors that monitor DNA and detect structural abnormalities, elements that transduce and amplify the damage signal, and lastly, effectors that mediate the required cellular processes. Such processes include cell cycle checkpoints and arrest, DNA repair, regulation of specific transcriptional programs, apoptosis, and senescence [248].

The DDR triggers replication arrest and senescence when cells reach their replicative limit or accumulate irreparable DNA damage [248]. In a study by Aw *et al*., increased cellular senescence, revealed by β-galactosidase activity, was observed within aged thymi [206]. Furthermore, the search for markers of activated DDR, namely double-strand breaks (DSBs) identified by phosphorylated histone H2AX (γH2AX) and the p53 binding protein (53BP1), revealed increased levels in aged thymus sections, for which TECs may be partially accountable [206]. These observations supported the notion that the accumulation of DNA damage could be a contributing factor to the age-related decline in TECs. Concordantly, the subsequent study by Griffith *et al*., also added that metabolic stress in stromal cells manifested as oxidative DNA damage [244]. When compared to lymphoid cells derived from the same tissue, stromal cell DNA showed increases in the two types of lesions measured, 2-deoxyguanosine (2dG) oxidation and chromatin unwinding [244].

The central tumour suppressor p53 guards the genome by orchestrating a variety of DDR mechanisms. Different cellular stresses stabilize p53, resulting in the activation of various p53-induced transcriptional programs that mediate key functions, such as induction of apoptosis, cell cycle arrest, and facilitation of various DNA repair systems [249]. A study by Rodrigues *et al*. in our laboratory investigated the role of p53 in the thymic epithelium by using a mouse model with TEC-specific *Trp53* deletion [250]. This study demonstrated that p53 is required for the integrity of the postnatal and adult medullary compartment, including its responsiveness to RANK signalling, transcriptomic signature, and functions in tolerance induction. Although a mild defect emerged in adult *Trp53*-deficient cTECs, it was intriguing to observe that mTECs were specifically dependent on p53-mediated pathways [250]. This raised the hypothesis that DNA damage and DDR activation might be increased in mTECs.

Coincidently, other studies have demonstrated a functional link between mTEC-specific transcriptional mechanisms and DDR activation [251, 252]. Initially, a study by Abramson *et al*. aiming to dissect Aire-mediated induction of ectopic TRA expression, performed a comprehensive screening of proteins that interact with Aire. The identified Aire partners integrated several functional classes and included a protein complex that is

involved in both transcriptional elongation and DNA repair, namely DNA-PK, TOP2a, PARP1, H2AX, and Ku80 [252]. Furthermore, Aire was found to promote TOP2a-initiated DSBs *in vitro*. Accordingly, *in vivo* Aire-sufficient mTEC^{high}, but not Aire^{KO} mTEC^{high}, contain a subpopulation highly expressing γH2AX [252]. DSBs ease superhelical tensions in the DNA generated by RNA polymerase II and promote transcriptional elongation. Additionally, these lesions recruit and activate the members of the aforementioned complex, which can promote histone eviction, chromatin relaxation, and facilitate transcription [252]. A posterior study further revealed that, while TOP2 was involved at later stages of transcription, TOP1 is a primary Aire partner with an earlier role seeding the assembly of Aire-containing multi-protein complexes at super-enhancer regions through recruitment of elements of the detected DDR complex [251]. Both TOP1 and TOP2 contributed to Aire-dependent TRA expression, negative selection, and peripheral self-tolerance, further highlighting the importance of the transient DNA brakes created by these enzymes and the mobilization of the DDR [251]. Altogether, these findings raise the notion that the mechanisms involved in Aire-mediated TRA expression might be a source of genomic instability in mTECs, contributing to an increased baseline level of DNA damage and, consequently, rendering mTECs highly dependent on the activation of protective DDR.

Acute DNA damage

In addition to chronic age-dependent thymus involution, acute thymic involution can occur in response to DNA-damaging agents, such as chemotherapy or radiotherapy, which are necessary before bone marrow transplantation (BMT) [253, 254]. Acute involution constitutes a qualitatively distinct process from age-related involution, being characterized as a transient deterioration that can be reversed with the removal of the causative factors [255]. Importantly, besides the direct genomic injury inflicted on TECs, DNA-damaging regimens also exert indirect effects on TEC homeostasis by targeting hematopoietic cells. The depletion of developing T cells eradicates the key thymocyte-derived signals (*e.g.*, CD40 and RANK) necessary for lymphoepithelial interactions, which in turn, impacts TEC development and regeneration [256].

Although TECs were historically considered radio-resistant cells, several studies have shown that TECs are affected by irradiation, particularly mTECs, whose post-irradiation defects persist even after the recovery of mature thymic T cells [250, 257-260]. Moreover, a study by Calvo-Asensio *et al*. using TEC lines demonstrated the direct effects of IR-induced DNA damage on TECs, which had detrimental effects on TEC survival and a broad impact on the expression of TEC functional genes [261]. Interestingly, they further showed that cTECs and mTECs respond differently to DNA damage. Particularly, mTEC radio-resistance was superior at high IR doses, mTECs remained in cell cycle arrest for a

longer period, and notably, showed higher expression levels of DDR genes involved in DNA repair and cell cycle regulation, compared to cTECs [261]. Overall, these observations suggested that mTECs have more robust DDR activation upon acute genotoxic stress.

In conclusion, besides the primary deleterious effects of BMT preparative regimens on the hematopoietic compartment, these also affect, directly and indirectly, the TEC niche. Following these treatments, the thymic microenvironment is essential to support immune reconstitution, as severe repercussions can arise from prolonged periods of T-cell deficiency or defective *de novo* T-cell development [262]. As such, understanding the TEC-intrinsic factors and pathways that aggravate or amend the DNA-damaging effect of these regimens provides an additional angle for the search for potential targets to enhance thymic function and the outcomes of BMT.

FOXO TRANSCRIPTION FACTORS – SENTINELS FOR CELLULAR INTEGRITY

The Forkhead family of transcription factors is characterized by an evolutionarily conserved winged-helix DNA-binding domain at the N-terminal region, the “Forkhead box”, or FOX [263]. The FOXO subfamily is conserved from *C. elegans* to mammals. While only one FOXO gene exists in invertebrates, mammals have four genes, FOXO1, FOXO3, FOXO4, and FOXO6 [264]. Besides the DNA-binding domain (DBD), FOXOs contain a nuclear localization sequence (NLS), a nuclear export sequence (NES), and a C-terminal transactivation domain. Several of these regions are highly conserved between the FOXO isoforms, including the DBD [264]. As a result, all FOXOs activate gene transcription by recognizing promoters containing the consensus motif TTGTTTAC and thus show redundancy in target genes. However, functional specificity between the individual members is achieved through their different tissue expression patterns, as well as through cell type-specific upstream regulators, binding partners, or pathway crosstalk [265, 266]. FOXO proteins are key factors in homeostatic maintenance and protection against environmental stressors, such as oxidative stress, metabolic stress, or growth factor deprivation. FOXOs integrate multiple upstream signalling pathways and orchestrate essential cellular processes, including cell cycle arrest, oxidative stress resistance, apoptosis, DNA damage repair, and energy metabolism [267].

For cells and tissues to survive changes in nutrient accessibility, a series of essential adaptation mechanisms need to be activated for the regulation of energy balance and glucose homeostasis. In conditions of normal nutrient availability and absence of environmental challenges, FOXOs are inactive due to negative regulation by the insulin and/or IGF-1 signalling pathway (IIS) [268]. The IIS pathway, mediated by PI3K-AKT, allows the phosphorylation of FOXO factors by the serine/threonine kinase AKT. AKT-mediated phosphorylation of FOXOs generates binding sites for chaperone proteins, resulting in the retention of FOXO transcription factors in the cytoplasm, and consequent downregulation of FOXO target genes [269] (**Figure 8**). Conversely, stress signals or nutrient/growth factor scarcity activate FOXOs and their essential transcriptional programs. The FOXO-mediated responses to environmental changes are downstream of a complex and dynamic regulatory network that modulates FOXO activity via post-transcriptional modifications (PTMs). Numerous kinases induced by different stimuli will recognize specific sites on FOXOs and may exert opposing effects on their activity [266]. For instance, IIS-mediated inhibition is antagonized by oxidative stress, which activates FOXOs through phosphorylation by c-Jun N-terminal kinase (JNK) or mammalian sterile 20-like kinase 1 (MST1) [270, 271]. AMP-activated protein kinase (AMPK) activates FOXOs under energy stress conditions [272], while DNA damage influences FOXOs through another set of kinases, including cyclin-

dependent kinases (CDKs) and ataxia telangiectasia mutated (ATM) [273, 274] (**Figure 8**). Besides phosphorylation, other FOXO PTMs include acetylation/deacetylation, methylation, and ubiquitination. Hence, various stress-induced regulators control FOXO activity through distinct modifications. The induced PTMs will influence FOXO subcellular localization, protein levels, DNA binding, transcriptional activity, and interaction with cofactors [267]. Consequently, several environment-sensing pathways can be integrated to generate a specific FOXO-mediated transcription program and functional outcome.

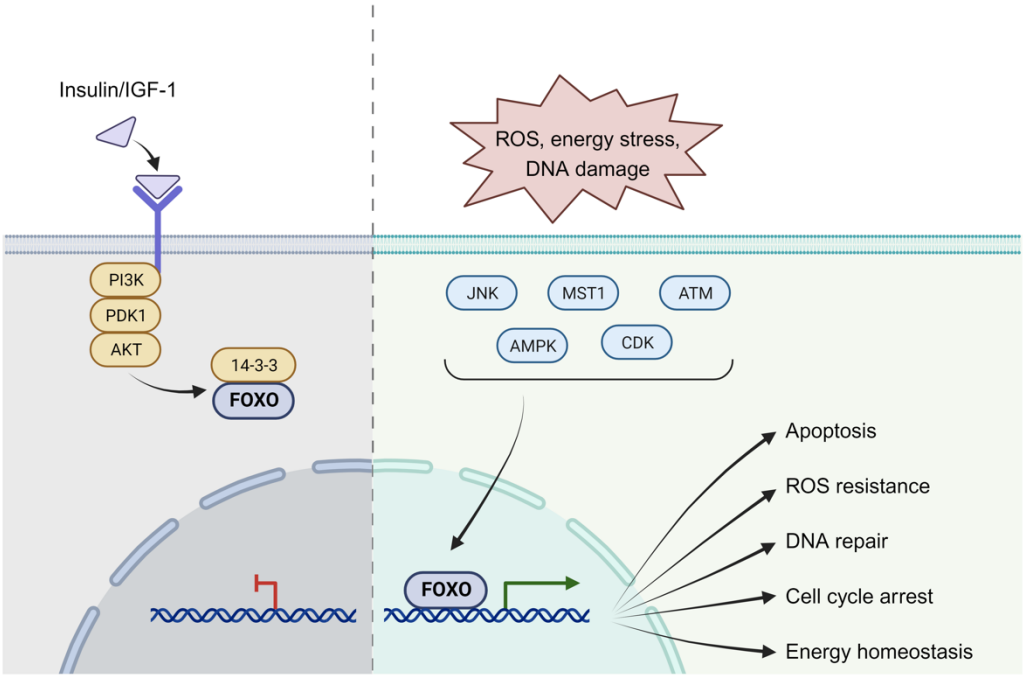


Figure 8 – FOXO regulatory pathways and transcriptional programs involved in cellular homeostasis. While insulin or IGF-1 signalling negatively regulate FOXO transcriptional activity, stress stimuli promote FOXO nuclear translocation and transcription of target genes that orchestrate different cellular processes for stress resistance.

FOXO3 in longevity and stress resistance

Longevity constitutes a complex and enigmatic phenotype influenced by countless genetic and environmental factors. Notably, FOXO transcription factors have shown a strong association with prolonged lifespan in different model organisms [275]. DAF-16, the single FOXO homolog in *C. elegans*, promotes longevity by increasing resistance to oxidative stress, pathogens, and protein damage. Longevity can be induced through mutations perturbing IIS signalling, an effect that is DAF-16-dependent [276]. Likewise, in *D. melanogaster*, overexpression of dFOXO, the equivalent of mammalian FOXO3, also promotes longevity [277]. Similarities in FOXO target genes across invertebrates and mammals suggest that FOXO functions are evolutionarily conserved. Additionally, accumulating evidence supports that FOXO transcription factors also play an important role

in human aging. Data gathered from global population studies has consistently linked FOXO3 genetic variants with extreme longevity and identified FOXO3 as a protective gene in cancer, as well as in cardiovascular and neurodegenerative diseases [269, 275]. One of the hallmarks of aging across different organisms is the deregulation of nutrient sensing via the IIS pathway [278]. Correspondingly, the longevity response induced by CR regulates several nutrient-sensing pathways, including AMPK, mTOR, SIRTs, and the IIS pathways. FOXO transcription factors mediate cellular responses to CR by serving as a downstream effector for the IIS, AMPK, and SIRTs pathways [275]. A study by Shimokawa *et al*. has suggested a non-redundant role for FOXO3 in response to CR, showing that it is specifically required for the longevity effect induced in mice [279].

Under normal physiological conditions, ROS fluctuations have important functions in cell signalling, homeostasis, and infection clearance. However, different environmental stressors can elevate ROS levels dramatically, resulting in cellular damage to proteins, lipids, and DNA. Beyond the IIS–FOXO axis, redox signalling is a second major regulatory signal for FOXO activation, and FOXO-mediated antioxidant properties are a key mechanism in their contribution to longevity. In addition to FOXO factor induction by ROS-stimulated kinases, deacetylation and monoubiquitylation can also be provoked by oxidative stress and stimulate FOXO transcriptional activity. In parallel with PTM-induced activity, FOXOs can also directly sense the cellular redox status through reversible oxidation and reduction of cysteine residues [280]. Multiple studies have highlighted the essential role of FOXO3 in conferring oxidative stress resistance, particularly through the induction of stress-related gene products and antioxidant enzymes, such as manganese superoxide dismutase (MnSOD) and catalase [281-284]. While the control of ROS levels is a primary FOXO3 function to prevent DNA damage, when genomic lesions occur, FOXO3 mediates different responses. For instance, under steady-state conditions, FOXO3 promotes the expression of DNA repair machinery and prevents the accumulation of oxidative stress-induced DNA damage [285]. Alternatively, when stress stimuli rise beyond a certain threshold, FOXO3 can promote a pro-apoptotic program in response to DNA damage [286].

Compromised genomic integrity causes cycling cells to arrest at one of two critical cell cycle checkpoints, before and after DNA replication (G1-S or G2-M), thus allowing the action of further DDR mechanisms. Several studies have demonstrated that FOXO3 has multiple roles in cell cycle control in response to stress stimuli. FOXO3 can induce cell cycle arrest at the G1-S boundary, as well as delay the G2-M checkpoint, during which FOXO3 stimulates DNA repair through the induction of GADD45 [287-289]. Furthermore, FOXO3 was shown to be the predominant FOXO member activated during replication stress [290]. Altogether, the multifactorial role of FOXO3 in maintaining genomic stability offers another intuitive explanation for its effect on longevity.

Given the role of FOXO3 in conferring protection from DNA damage and facilitating the removal of genomically compromised cells, this transcription factor has gathered attention in a manner reminiscent of the tumour suppressor protein p53. Besides their functional similarities, FOXOs and p53 also share upstream regulatory enzymes, namely SIRT1, MDM2, USP7, and JNK. Interestingly, SIRT1 and USP7 have opposite effects on FOXOs and p53, whereas both transcription factors are stimulated by JNK under stress conditions [266]. FOXO and p53 functions are balanced through their shared regulators but also through direct reciprocal control, which has been verified specifically with FOXO3. However, the effects of the p53-FOXO3 co-regulatory relationship appear to be context-dependent. FOXO3 is negatively regulated by p53 through the activity of p53-induced serum/glucocorticoid-inducible kinase 1 (SGK1) [291]. Additionally, under oxidative stress, p53 can inhibit FOXO3 transcriptional activity [292]. Conversely, p53 can directly upregulate FOXO3 mRNA expression [293]. On the other hand, activated FOXO3 can promote p53 stabilization and p53-driven apoptosis [294]. Thus, p53 and FOXO3 can act individually or simultaneously and may establish a synergic or antagonistic relationship. Ultimately, the complex nature of the p53-FOXO3 crosstalk may be influenced by several factors, including nutrient availability, the degree and duration of the stress signals, and the type of stress.

AIMS

The mTEC compartment is unique in its functional specialization for TRA expression and central self-tolerance induction. Past studies have provided valuable insights into the mTEC developmental program and the ongoing work in this field is progressively unravelling an unprecedented degree of mTEC diversity. Before conducting the original experimental work described in this thesis, we sought to assess the current state of knowledge regarding mTECs. An overview of the predominant studies and questions dominating the field at that time was published in the format of a review, included in **Chapter II**. Subsequently, in **Chapter III** of this thesis, we searched for new means to dissect mTEC heterogeneity and obtain new insights into the lineage relationships between subpopulations. With these findings, we aim to provide an additional tool to study the mTEC compartment and its role in tolerance induction. We employed flow cytometry, transcriptional and functional analyses to dissect different stages of Aire^{+} mTEC differentiation.

In TECs, intrinsic and extrinsic signals are integrated to collectively dictate the balance between TEC maintenance, degeneration, or regeneration. In particular, DNA damage has been highlighted as a key stress signal in mTECs, which rely on DNA damage response mechanisms for homeostasis maintenance as well as Aire-mediated TRA expression. Furthermore, acute TEC DNA damage occurs in patients undergoing cytoablative therapies, for which a rapid and accurate recovery of T-cell development and selection is paramount. Thus, understanding which factors partake in TEC DNA damage response is key for uncovering potential therapeutic targets to correct or restore thymic function. In **Chapter IV**, we investigated the TEC-intrinsic role of the transcription factor FOXO3, a regulator of stress resistance and homeostasis. To do this, we generated mouse models with TEC-specific deletion of *Foxo3* under intact or deficient *Aire* expression conditions. We performed an extensive phenotypic and transcriptional characterization of *Foxo3*-deficient TEC subsets and evaluated their functional competence to support T-cell development and induce self-tolerance.

Collectively, these findings aim to contribute to the elucidation of the mechanisms controlling the development and function of the thymic epithelial microenvironments. Ultimately, supporting a deeper understanding and improved therapeutic targeting of the central mechanisms that dictate the principles of T-cell immunity.

REFERENCES

1. Riera Romo, M.*, et al.*, 2016, *Innate immunity in vertebrates: an overview,* Immunology, **148**(2), 125-39.
2. Boehm, T. and Swann, J.B., 2014, *Origin and evolution of adaptive immunity,* Annu Rev Anim Biosci, **2**, 259-83.
3. Parkin, J. and Cohen, B., 2001, *An overview of the immune system,* Lancet, **357**(9270), 1777-89.
4. Bluestone, J.A., 2011, *Mechanisms of tolerance,* Immunol Rev, **241**(1), 5-19.
5. Basten, A. and Silveira, P.A., 2010, *B-cell tolerance: mechanisms and implications,* Curr Opin Immunol, **22**(5), 566-74.
6. Boyd, R.L.*, et al.*, 1993, *The thymic microenvironment,* Immunol Today, **14**(9), 445-59.
7. O'Neill, K.*, et al.*, *Chapter 43 - Thymus and Parathyroid Organogenesis*, in *Principles of Tissue Engineering (Fourth Edition)*, R. Lanza, R. Langer, and J. Vacanti, Editors. 2014, Academic Press: Boston. p. 869-897.
8. Takahama, Y., 2006, *Journey through the thymus: stromal guides for T-cell development and selection,* Nat Rev Immunol, **6**(2), 127-35.
9. Mikkola, H.K. and Orkin, S.H., 2006, *The journey of developing hematopoietic stem cells,* Development, **133**(19), 3733-44.
10. Kondo, M., 2010, *Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors,* Immunol Rev, **238**(1), 37-46.
11. Zhang, S.L. and Bhandoola, A., 2014, *Trafficking to the thymus,* Curr Top Microbiol Immunol, **373**, 87-111.
12. Abramson, J. and Anderson, G., 2017, *Thymic Epithelial Cells,* Annu Rev Immunol, **35**, 85-118.
13. Rossi, F.M.*, et al.*, 2005, *Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1,* Nat Immunol, **6**(6), 626-34.
14. Scimone, M.L.*, et al.*, 2006, *A multistep adhesion cascade for lymphoid progenitor cell homing to the thymus,* Proc Natl Acad Sci U S A, **103**(18), 7006-11.
15. Lind, E.F.*, et al.*, 2001, *Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development,* J Exp Med, **194**(2), 127-34.
16. Bhandoola, A.*, et al.*, 2007, *Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from,* Immunity, **26**(6), 678-89.

17. Luc, S.*, et al.*, 2012, *The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential,* Nat Immunol, **13**(4), 412-9.
18. Koch, U.*, et al.*, 2008, *Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment,* J Exp Med, **205**(11), 2515-23.
19. Hozumi, K.*, et al.*, 2008, *Delta-like 4 is indispensable in thymic environment specific for T cell development,* J Exp Med, **205**(11), 2507-13.
20. Alves, N.L.*, et al.*, 2009, *Characterization of the thymic IL-7 niche in vivo,* Proc Natl Acad Sci U S A, **106**(5), 1512-7.
21. Shitara, S.*, et al.*, 2013, *IL-7 produced by thymic epithelial cells plays a major role in the development of thymocytes and TCRgammadelta+ intraepithelial lymphocytes,* J Immunol, **190**(12), 6173-9.
22. Rodewald, H.R.*, et al.*, 1995, *Intrathymically expressed c-kit ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes in vivo,* Immunity, **3**(3), 313-9.
23. Koch, U. and Radtke, F., 2011, *Mechanisms of T cell development and transformation,* Annu Rev Cell Dev Biol, **27**, 539-62.
24. Ciofani, M. and Zuniga-Pflucker, J.C., 2007, *The thymus as an inductive site for T lymphopoiesis,* Annu Rev Cell Dev Biol, **23**, 463-93.
25. von Boehmer, H., 2005, *Unique features of the pre-T-cell receptor alpha-chain: not just a surrogate,* Nat Rev Immunol, **5**(7), 571-7.
26. Ciofani, M.*, et al.*, 2004, *Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation,* J Immunol, **172**(9), 5230-9.
27. Singer, A.*, et al.*, 2008, *Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice,* Nat Rev Immunol, **8**(10), 788-801.
28. Klein, L.*, et al.*, 2009, *Antigen presentation in the thymus for positive selection and central tolerance induction,* Nat Rev Immunol, **9**(12), 833-44.
29. Klein, L.*, et al.*, 2014, *Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see),* Nat Rev Immunol, **14**(6), 377-91.
30. He, X.*, et al.*, 2005, *The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment,* Nature, **433**(7028), 826-33.
31. Taniuchi, I.*, et al.*, 2002, *Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development,* Cell, **111**(5), 621-33.
32. Teh, H.S.*, et al.*, 1988, *Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells,* Nature, **335**(6187), 229-33.

33. Honey, K.*, et al.*, 2002, *Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands,* J Exp Med, **195**(10), 1349-58.
34. Gommeaux, J.*, et al.*, 2009, *Thymus-specific serine protease regulates positive selection of a subset of CD4+ thymocytes,* Eur J Immunol, **39**(4), 956-64.
35. Nedjic, J.*, et al.*, 2008, *Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance,* Nature, **455**(7211), 396-400.
36. Takada, K.*, et al.*, *Development and Function of Cortical Thymic Epithelial Cells*, in *Thymic Development and Selection of T Lymphocytes*, T. Boehm and Y. Takahama, Editors. 2014, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 1-17.
37. Murata, S.*, et al.*, 2007, *Regulation of CD8+ T cell development by thymus-specific proteasomes,* Science, **316**(5829), 1349-53.
38. Nitta, T.*, et al.*, 2010, *Thymoproteasome shapes immunocompetent repertoire of CD8+ T cells,* Immunity, **32**(1), 29-40.
39. Ueno, T.*, et al.*, 2004, *CCR7 signals are essential for cortex-medulla migration of developing thymocytes,* J Exp Med, **200**(4), 493-505.
40. Hu, Z.*, et al.*, 2015, *CCR4 promotes medullary entry and thymocyte-dendritic cell interactions required for central tolerance,* J Exp Med, **212**(11), 1947-65.
41. Breed, E.R.*, et al.*, 2018, *Directing T cell fate: How thymic antigen presenting cells coordinate thymocyte selection,* Semin Cell Dev Biol, **84**, 2-10.
42. Bouillet, P.*, et al.*, 2002, *BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes,* Nature, **415**(6874), 922-6.
43. Breed, E.R.*, et al.*, 2019, *Measuring Thymic Clonal Deletion at the Population Level,* J Immunol, **202**(11), 3226-3233.
44. Yu, W.*, et al.*, 2015, *Clonal Deletion Prunes but Does Not Eliminate Self-Specific alphabeta CD8(+) T Lymphocytes,* Immunity, **42**(5), 929-41.
45. Xing, Y. and Hogquist, K.A., 2012, *T-cell tolerance: central and peripheral,* Cold Spring Harb Perspect Biol, **4**(6).
46. Klein, L. and Jovanovic, K., 2011, *Regulatory T cell lineage commitment in the thymus,* Semin Immunol, **23**(6), 401-9.
47. Josefowicz, S.Z.*, et al.*, 2012, *Regulatory T cells: mechanisms of differentiation and function,* Annu Rev Immunol, **30**, 531-64.
48. Bennett, C.L.*, et al.*, 2001, *The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3,* Nat Genet, **27**(1), 20-1.
49. Savage, P.A.*, et al.*, 2020, *Regulatory T Cell Development,* Annu Rev Immunol, **38**, 421-453.

50. Burchill, M.A.*, et al.*, 2008, *Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire,* Immunity, **28**(1), 112-21.
51. Tai, X.*, et al.*, 2013, *Foxp3 transcription factor is proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine survival signals,* Immunity, **38**(6), 1116-28.
52. Lio, C.W. and Hsieh, C.S., 2008, *A two-step process for thymic regulatory T cell development,* Immunity, **28**(1), 100-11.
53. Vang, K.B.*, et al.*, 2008, *IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development,* J Immunol, **181**(5), 3285-90.
54. Ouyang, W.*, et al.*, 2010, *Transforming growth factor-beta signaling curbs thymic negative selection promoting regulatory T cell development,* Immunity, **32**(5), 642-53.
55. Coquet, J.M.*, et al.*, 2013, *Epithelial and dendritic cells in the thymic medulla promote CD4+Foxp3+ regulatory T cell development via the CD27-CD70 pathway,* J Exp Med, **210**(4), 715-28.
56. Malhotra, D.*, et al.*, 2016, *Tolerance is established in polyclonal CD4(+) T cells by distinct mechanisms, according to self-peptide expression patterns,* Nat Immunol, **17**(2), 187-95.
57. Tai, X.*, et al.*, 2023, *How autoreactive thymocytes differentiate into regulatory versus effector CD4(+) T cells after avoiding clonal deletion,* Nat Immunol, **24**(4), 637-651.
58. Fontenot, J.D.*, et al.*, 2003, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells,* Nat Immunol, **4**(4), 330-6.
59. Owen, D.L.*, et al.*, 2019, *Thymic regulatory T cells arise via two distinct developmental programs,* Nat Immunol, **20**(2), 195-205.
60. Kyewski, B. and Klein, L., 2006, *A central role for central tolerance,* Annu Rev Immunol, **24**, 571-606.
61. Sansom, S.N.*, et al.*, 2014, *Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia,* Genome Res, **24**(12), 1918-31.
62. Danan-Gotthold, M.*, et al.*, 2016, *Extensive RNA editing and splicing increase immune self-representation diversity in medullary thymic epithelial cells,* Genome Biol, **17**(1), 219.
63. Rattay, K.*, et al.*, 2016, *Evolutionary conserved gene co-expression drives generation of self-antigen diversity in medullary thymic epithelial cells,* J Autoimmun, **67**, 65-75.

64. Brennecke, P.*, et al.*, 2015, *Single-cell transcriptome analysis reveals coordinated ectopic gene-expression patterns in medullary thymic epithelial cells,* Nat Immunol, **16**(9), 933-41.
65. Mathis, D. and Benoist, C., 2009, *Aire,* Annu Rev Immunol, **27**, 287-312.
66. Koh, A.S.*, et al.*, 2008, *Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity,* Proc Natl Acad Sci U S A, **105**(41), 15878-83.
67. Giraud, M.*, et al.*, 2012, *Aire unleashes stalled RNA polymerase to induce ectopic gene expression in thymic epithelial cells,* Proc Natl Acad Sci U S A, **109**(2), 535-40.
68. Bansal, K.*, et al.*, 2021, *Aire regulates chromatin looping by evicting CTCF from domain boundaries and favoring accumulation of cohesin on superenhancers,* Proc Natl Acad Sci U S A, **118**(38).
69. Anderson, M.S.*, et al.*, 2002, *Projection of an immunological self shadow within the thymus by the aire protein,* Science, **298**(5597), 1395-401.
70. Akirav, E.M.*, et al.*, 2011, *The role of AIRE in human autoimmune disease,* Nat Rev Endocrinol, **7**(1), 25-33.
71. Takaba, H.*, et al.*, 2015, *Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance,* Cell, **163**(4), 975-87.
72. Derbinski, J.*, et al.*, 2008, *Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism,* Proc Natl Acad Sci U S A, **105**(2), 657-62.
73. Villasenor, J.*, et al.*, 2008, *Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated,* Proc Natl Acad Sci U S A, **105**(41), 15854-9.
74. Pinto, S.*, et al.*, 2013, *Overlapping gene coexpression patterns in human medullary thymic epithelial cells generate self-antigen diversity,* Proc Natl Acad Sci U S A, **110**(37), E3497-505.
75. Meredith, M.*, et al.*, 2015, *Aire controls gene expression in the thymic epithelium with ordered stochasticity,* Nat Immunol, **16**(9), 942-9.
76. Dhalla, F.*, et al.*, 2020, *Biologically indeterminate yet ordered promiscuous gene expression in single medullary thymic epithelial cells,* EMBO J, **39**(1), e101828.
77. Michelson, D.A.*, et al.*, 2022, *Thymic epithelial cells co-opt lineage-defining transcription factors to eliminate autoreactive T cells,* Cell, **185**(14), 2542-2558 e18.
78. Michelson, D.A. and Mathis, D., 2022, *Thymic mimetic cells: tolerogenic masqueraders,* Trends Immunol, **43**(10), 782-791.

79. Klein, L.*, et al.*, 2011, *Autonomous versus dendritic cell-dependent contributions of medullary thymic epithelial cells to central tolerance,* Trends Immunol, **32**(5), 188-93.
80. Wu, L. and Shortman, K., 2005, *Heterogeneity of thymic dendritic cells,* Semin Immunol, **17**(4), 304-12.
81. Kyewski, B. and Feuerer, M., 2014, *Love is in the Aire: mTECs share their assets,* Immunity, **41**(3), 343-345.
82. Perry, J.S.A.*, et al.*, 2014, *Distinct contributions of Aire and antigen-presenting-cell subsets to the generation of self-tolerance in the thymus,* Immunity, **41**(3), 414-426.
83. Bonasio, R.*, et al.*, 2006, *Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus,* Nat Immunol, **7**(10), 1092-100.
84. Hadeiba, H.*, et al.*, 2012, *Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance,* Immunity, **36**(3), 438-50.
85. Hogquist, K.A.*, et al.*, 2015, *T Cell Adolescence: Maturation Events Beyond Positive Selection,* J Immunol, **195**(4), 1351-7.
86. Kishimoto, H. and Sprent, J., 1997, *Negative selection in the thymus includes semimature T cells,* J Exp Med, **185**(2), 263-71.
87. Carlson, C.M.*, et al.*, 2006, *Kruppel-like factor 2 regulates thymocyte and T-cell migration,* Nature, **442**(7100), 299-302.
88. Matloubian, M.*, et al.*, 2004, *Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1,* Nature, **427**(6972), 355-60.
89. Godfrey, D.I.*, et al.*, 2015, *The burgeoning family of unconventional T cells,* Nat Immunol, **16**(11), 1114-23.
90. Mayassi, T.*, et al.*, 2021, *A multilayered immune system through the lens of unconventional T cells,* Nature, **595**(7868), 501-510.
91. Gapin, L., 2016, *Development of invariant natural killer T cells,* Curr Opin Immunol, **39**, 68-74.
92. Kawano, T.*, et al.*, 1997, *CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides,* Science, **278**(5343), 1626-9.
93. Brigl, M. and Brenner, M.B., 2010, *How invariant natural killer T cells respond to infection by recognizing microbial or endogenous lipid antigens,* Semin Immunol, **22**(2), 79-86.
94. Krovi, S.H.*, et al.*, 2022, *Current insights in mouse iNKT and MAIT cell development using single cell transcriptomics data,* Semin Immunol, **60**, 101658.
95. Park, S.H.*, et al.*, 2000, *Unaltered phenotype, tissue distribution and function of Valpha14(+) NKT cells in germ-free mice,* Eur J Immunol, **30**(2), 620-5.

96. Crosby, C.M. and Kronenberg, M., 2018, *Tissue-specific functions of invariant natural killer T cells,* Nat Rev Immunol, **18**(9), 559-574.
97. Seiler, M.P.*, et al.*, 2012, *Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling,* Nat Immunol, **13**(3), 264-71.
98. Lee, Y.J.*, et al.*, 2013, *Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells,* Nat Immunol, **14**(11), 1146-54.
99. Godfrey, D.I.*, et al.*, 2010, *Raising the NKT cell family,* Nat Immunol, **11**(3), 197-206.
100. Wang, H. and Hogquist, K.A., 2018, *CCR7 defines a precursor for murine iNKT cells in thymus and periphery,* Elife, **7**.
101. Kang, B.H.*, et al.*, 2016, *PLZF(+) Innate T Cells Support the TGF-beta-Dependent Generation of Activated/Memory-Like Regulatory T Cells,* Mol Cells, **39**(6), 468-76.
102. Breed, E.R.*, et al.*, 2022, *Type 2 cytokines in the thymus activate Sirpalpha(+) dendritic cells to promote clonal deletion,* Nat Immunol, **23**(7), 1042-1051.
103. White, A.J.*, et al.*, 2017, *A type 2 cytokine axis for thymus emigration,* J Exp Med, **214**(8), 2205-2216.
104. Cosway, E.J.*, et al.*, 2022, *Eosinophils are an essential element of a type 2 immune axis that controls thymus regeneration,* Sci Immunol, **7**(69), eabn3286.
105. White, A.J.*, et al.*, 2014, *An essential role for medullary thymic epithelial cells during the intrathymic development of invariant NKT cells,* J Immunol, **192**(6), 2659-66.
106. Lucas, B.*, et al.*, 2020, *Diversity in medullary thymic epithelial cells controls the activity and availability of iNKT cells,* Nat Commun, **11**(1), 2198.
107. Tao, H.*, et al.*, 2021, *Thymic Epithelial Cell-Derived IL-15 and IL-15 Receptor alpha Chain Foster Local Environment for Type 1 Innate Like T Cell Development,* Front Immunol, **12**, 623280.
108. Wang, H.*, et al.*, 2019, *Myeloid cells activate iNKT cells to produce IL-4 in the thymic medulla,* Proc Natl Acad Sci U S A, **116**(44), 22262-22268.
109. Nowell, C.S.*, et al.*, 2007, *Thymus organogenesis and development of the thymic stroma,* Methods Mol Biol, **380**, 125-62.
110. Gordon, J.*, et al.*, 2001, *Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch,* Mech Dev, **103**(1-2), 141-3.
111. Gordon, J. and Manley, N.R., 2011, *Mechanisms of thymus organogenesis and morphogenesis,* Development, **138**(18), 3865-78.

112. Terszowski, G.*, et al.*, 2006, *Evidence for a functional second thymus in mice,* Science, **312**(5771), 284-7.
113. Corbeaux, T.*, et al.*, 2010, *Thymopoiesis in mice depends on a Foxn1-positive thymic epithelial cell lineage,* Proc Natl Acad Sci U S A, **107**(38), 16613-8.
114. Romano, R.*, et al.*, 2013, *FOXN1: A Master Regulator Gene of Thymic Epithelial Development Program,* Front Immunol, **4**, 187.
115. Nehls, M.*, et al.*, 1994, *New member of the winged-helix protein family disrupted in mouse and rat nude mutations,* Nature, **372**(6501), 103-7.
116. Nehls, M.*, et al.*, 1996, *Two genetically separable steps in the differentiation of thymic epithelium,* Science, **272**(5263), 886-9.
117. Boehm, T.*, et al.*, 2003, *Genetic dissection of thymus development in mouse and zebrafish,* Immunol Rev, **195**, 15-27.
118. van Ewijk, W.*, et al.*, 1994, *Crosstalk in the mouse thymus,* Immunol Today, **15**(5), 214-7.
119. Revest, J.M.*, et al.*, 2001, *Development of the thymus requires signaling through the fibroblast growth factor receptor R2-IIIb,* J Immunol, **167**(4), 1954-61.
120. Jenkinson, W.E.*, et al.*, 2007, *PDGFRalpha-expressing mesenchyme regulates thymus growth and the availability of intrathymic niches,* Blood, **109**(3), 954-60.
121. Griffith, A.V.*, et al.*, 2009, *Increased thymus- and decreased parathyroid-fated organ domains in Splotch mutant embryos,* Dev Biol, **327**(1), 216-27.
122. Foster, K.E.*, et al.*, 2010, *EphB-ephrin-B2 interactions are required for thymus migration during organogenesis,* Proc Natl Acad Sci U S A, **107**(30), 13414-9.
123. Sitnik, K.M.*, et al.*, 2012, *Mesenchymal cells regulate retinoic acid receptor-dependent cortical thymic epithelial cell homeostasis,* J Immunol, **188**(10), 4801-9.
124. Foster, K.*, et al.*, 2008, *Contribution of neural crest-derived cells in the embryonic and adult thymus,* J Immunol, **180**(5), 3183-9.
125. Rossi, S.W.*, et al.*, 2006, *Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium,* Nature, **441**(7096), 988-91.
126. Bleul, C.C.*, et al.*, 2006, *Formation of a functional thymus initiated by a postnatal epithelial progenitor cell,* Nature, **441**(7096), 992-6.
127. Depreter, M.G.*, et al.*, 2008, *Identification of Plet-1 as a specific marker of early thymic epithelial progenitor cells,* Proc Natl Acad Sci U S A, **105**(3), 961-6.
128. Bennett, A.R.*, et al.*, 2002, *Identification and characterization of thymic epithelial progenitor cells,* Immunity, **16**(6), 803-14.
129. Gill, J.*, et al.*, 2002, *Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells,* Nat Immunol, **3**(7), 635-42.

130. Rossi, S.W.*, et al.*, 2007, *Redefining epithelial progenitor potential in the developing thymus,* Eur J Immunol, **37**(9), 2411-8.
131. Ohigashi, I.*, et al.*, 2013, *Aire-expressing thymic medullary epithelial cells originate from beta5t-expressing progenitor cells,* Proc Natl Acad Sci U S A, **110**(24), 9885-90.
132. Baik, S.*, et al.*, 2013, *Generation of both cortical and Aire(+) medullary thymic epithelial compartments from CD205(+) progenitors,* Eur J Immunol, **43**(3), 589-94.
133. Ribeiro, A.R.*, et al.*, 2013, *Thymocyte selection regulates the homeostasis of IL-7-expressing thymic cortical epithelial cells in vivo,* J Immunol, **191**(3), 1200-9.
134. Alves, N.L.*, et al.*, 2014, *Serial progression of cortical and medullary thymic epithelial microenvironments,* Eur J Immunol, **44**(1), 16-22.
135. Rodewald, H.R.*, et al.*, 2001, *Thymus medulla consisting of epithelial islets each derived from a single progenitor,* Nature, **414**(6865), 763-8.
136. Hamazaki, Y.*, et al.*, 2007, *Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin,* Nat Immunol, **8**(3), 304-11.
137. Sekai, M.*, et al.*, 2014, *Medullary thymic epithelial stem cells maintain a functional thymus to ensure lifelong central T cell tolerance,* Immunity, **41**(5), 753-61.
138. Baik, S.*, et al.*, 2016, *Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK(+) medullary epithelial progenitors,* Eur J Immunol, **46**(4), 857-62.
139. Onder, L.*, et al.*, 2015, *Alternative NF-kappaB signaling regulates mTEC differentiation from podoplanin-expressing precursors in the cortico-medullary junction,* Eur J Immunol, **45**(8), 2218-31.
140. Alves, N.L. and Ribeiro, A.R., 2016, *Thymus medulla under construction: Time and space oddities,* Eur J Immunol, **46**(4), 829-33.
141. Ohigashi, I.*, et al.*, 2015, *Adult Thymic Medullary Epithelium Is Maintained and Regenerated by Lineage-Restricted Cells Rather Than Bipotent Progenitors,* Cell Rep, **13**(7), 1432-1443.
142. Mayer, C.E.*, et al.*, 2016, *Dynamic spatio-temporal contribution of single beta5t+ cortical epithelial precursors to the thymus medulla,* Eur J Immunol, **46**(4), 846-56.
143. Baran-Gale, J.*, et al.*, 2020, *Ageing compromises mouse thymus function and remodels epithelial cell differentiation,* Elife, **9**.
144. Wells, K.L.*, et al.*, 2020, *Combined transient ablation and single-cell RNA-sequencing reveals the development of medullary thymic epithelial cells,* Elife, **9**.

145. Klein, F.*, et al.*, 2023, *Combined multidimensional single-cell protein and RNA profiling dissects the cellular and functional heterogeneity of thymic epithelial cells,* Nat Commun, **14**(1), 4071.
146. Ohigashi, I.*, et al.*, 2019, *Trans-omics Impact of Thymoproteasome in Cortical Thymic Epithelial Cells,* Cell Rep, **29**(9), 2901-2916 e6.
147. Nakagawa, Y.*, et al.*, 2012, *Thymic nurse cells provide microenvironment for secondary T cell receptor alpha rearrangement in cortical thymocytes,* Proc Natl Acad Sci U S A, **109**(50), 20572-7.
148. Gray, D.H.*, et al.*, 2006, *Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells,* Blood, **108**(12), 3777-85.
149. Gray, D.*, et al.*, 2007, *Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire,* J Exp Med, **204**(11), 2521-8.
150. Gabler, J.*, et al.*, 2007, *Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells,* Eur J Immunol, **37**(12), 3363-72.
151. McCarthy, N.I.*, et al.*, 2015, *Osteoprotegerin-Mediated Homeostasis of Rank+ Thymic Epithelial Cells Does Not Limit Foxp3+ Regulatory T Cell Development,* J Immunol, **195**(6), 2675-82.
152. Metzger, T.C.*, et al.*, 2013, *Lineage tracing and cell ablation identify a post-Aire-expressing thymic epithelial cell population,* Cell Rep, **5**(1), 166-79.
153. White, A.J.*, et al.*, 2010, *Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells,* J Immunol, **185**(8), 4769-76.
154. Lkhagvasuren, E.*, et al.*, 2013, *Lymphotoxin beta receptor regulates the development of CCL21-expressing subset of postnatal medullary thymic epithelial cells,* J Immunol, **190**(10), 5110-7.
155. Bornstein, C.*, et al.*, 2018, *Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells,* Nature, **559**(7715), 622-626.
156. Miller, C.N.*, et al.*, 2018, *Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development,* Nature, **559**(7715), 627-631.
157. Park, J.E.*, et al.*, 2020, *A cell atlas of human thymic development defines T cell repertoire formation,* Science, **367**(6480).
158. Bautista, J.L.*, et al.*, 2021, *Single-cell transcriptional profiling of human thymic stroma uncovers novel cellular heterogeneity in the thymic medulla,* Nat Commun, **12**(1), 1096.
159. Nishikawa, Y.*, et al.*, 2014, *Temporal lineage tracing of Aire-expressing cells reveals a requirement for Aire in their maturation program,* J Immunol, **192**(6), 2585-92.

160. Givony, T.*, et al.*, 2023, *Thymic mimetic cells function beyond self-tolerance,* Nature, **622**(7981), 164-172.
161. Fiorini, E.*, et al.*, 2008, *Cutting edge: thymic crosstalk regulates delta-like 4 expression on cortical epithelial cells,* J Immunol, **181**(12), 8199-203.
162. Alves, N.L.*, et al.*, 2010, *Cutting Edge: a thymocyte-thymic epithelial cell cross-talk dynamically regulates intrathymic IL-7 expression in vivo,* J Immunol, **184**(11), 5949-53.
163. Anderson, G. and Takahama, Y., 2012, *Thymic epithelial cells: working class heroes for T cell development and repertoire selection,* Trends Immunol, **33**(6), 256-63.
164. Derbinski, J. and Kyewski, B., 2005, *Linking signalling pathways, thymic stroma integrity and autoimmunity,* Trends Immunol, **26**(10), 503-6.
165. Akiyama, T.*, et al.*, 2005, *Dependence of self-tolerance on TRAF6-directed development of thymic stroma,* Science, **308**(5719), 248-51.
166. Kajiura, F.*, et al.*, 2004, *NF-kappa B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner,* J Immunol, **172**(4), 2067-75.
167. Burkly, L.*, et al.*, 1995, *Expression of relB is required for the development of thymic medulla and dendritic cells,* Nature, **373**(6514), 531-6.
168. Rossi, S.W.*, et al.*, 2007, *RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla,* J Exp Med, **204**(6), 1267-72.
169. Roberts, N.A.*, et al.*, 2012, *Rank signaling links the development of invariant gammadelta T cell progenitors and Aire(+) medullary epithelium,* Immunity, **36**(3), 427-37.
170. Hikosaka, Y.*, et al.*, 2008, *The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator,* Immunity, **29**(3), 438-50.
171. Irla, M.*, et al.*, 2008, *Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity,* Immunity, **29**(3), 451-63.
172. Akiyama, T.*, et al.*, 2012, *TNF receptor family signaling in the development and functions of medullary thymic epithelial cells,* Front Immunol, **3**, 278.
173. Akiyama, T.*, et al.*, 2008, *The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance,* Immunity, **29**(3), 423-37.
174. Boehm, T.*, et al.*, 2003, *Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR,* J Exp Med, **198**(5), 757-69.

175. Venanzi, E.S.*, et al.*, 2007, *Lymphotoxin pathway and Aire influences on thymic medullary epithelial cells are unconnected,* J Immunol, **179**(9), 5693-700.
176. Zhu, M.*, et al.*, 2007, *Lymphotoxin beta receptor is required for the migration and selection of autoreactive T cells in thymic medulla,* J Immunol, **179**(12), 8069-75.
177. Seach, N.*, et al.*, 2008, *The lymphotoxin pathway regulates Aire-independent expression of ectopic genes and chemokines in thymic stromal cells,* J Immunol, **180**(8), 5384-92.
178. Cosway, E.J.*, et al.*, 2017, *Redefining thymus medulla specialization for central tolerance,* J Exp Med, **214**(11), 3183-3195.
179. Wu, W.*, et al.*, 2017, *Epithelial LTbetaR signaling controls the population size of the progenitors of medullary thymic epithelial cells in neonatal mice,* Sci Rep, **7**, 44481.
180. Mouri, Y.*, et al.*, 2011, *Lymphotoxin signal promotes thymic organogenesis by eliciting RANK expression in the embryonic thymic stroma,* J Immunol, **186**(9), 5047-57.
181. Desanti, G.E.*, et al.*, 2012, *Developmentally regulated availability of RANKL and CD40 ligand reveals distinct mechanisms of fetal and adult cross-talk in the thymus medulla,* J Immunol, **189**(12), 5519-26.
182. Chaudhry, M.S.*, et al.*, 2016, *Thymus: the next (re)generation,* Immunol Rev, **271**(1), 56-71.
183. Steinmann, G.G.*, et al.*, 1985, *The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study,* Scand J Immunol, **22**(5), 563-75.
184. Manley, N.R.*, et al.*, 2011, *Structure and function of the thymic microenvironment,* Front Biosci (Landmark Ed), **16**(7), 2461-77.
185. Linton, P.J. and Dorshkind, K., 2004, *Age-related changes in lymphocyte development and function,* Nat Immunol, **5**(2), 133-9.
186. Guerau-de-Arellano, M.*, et al.*, 2009, *Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity,* J Exp Med, **206**(6), 1245-52.
187. Yang, S.*, et al.*, 2015, *Immune tolerance. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance,* Science, **348**(6234), 589-94.
188. Aw, D. and Palmer, D.B., 2012, *It's not all equal: a multiphasic theory of thymic involution,* Biogerontology, **13**(1), 77-81.
189. Boehm, T. and Swann, J.B., 2013, *Thymus involution and regeneration: two sides of the same coin?,* Nat Rev Immunol, **13**(11), 831-8.
190. Martins, V.C.*, et al.*, 2014, *Cell competition is a tumour suppressor mechanism in the thymus,* Nature, **509**(7501), 465-70.

191. Coder, B.D.*, et al.*, 2015, *Thymic involution perturbs negative selection leading to autoreactive T cells that induce chronic inflammation,* J Immunol, **194**(12), 5825-37.
192. Surh, C.D. and Sprent, J., 2000, *Homeostatic T cell proliferation: how far can T cells be activated to self-ligands?,* J Exp Med, **192**(4), F9-F14.
193. Murray, J.M.*, et al.*, 2003, *Naive T cells are maintained by thymic output in early ages but by proliferation without phenotypic change after age twenty,* Immunol Cell Biol, **81**(6), 487-95.
194. Haynes, L. and Swain, S.L., 2006, *Why aging T cells fail: implications for vaccination,* Immunity, **24**(6), 663-666.
195. Douek, D.C. and Koup, R.A., 2000, *Evidence for thymic function in the elderly,* Vaccine, **18**(16), 1638-41.
196. Akbar, A.N. and Henson, S.M., 2011, *Are senescence and exhaustion intertwined or unrelated processes that compromise immunity?,* Nat Rev Immunol, **11**(4), 289-95.
197. Mo, R.*, et al.*, 2003, *T cell chemokine receptor expression in aging,* J Immunol, **170**(2), 895-904.
198. Lancaster, J.N.*, et al.*, 2022, *Central tolerance is impaired in the middle-aged thymic environment,* Aging Cell, **21**(6), e13624.
199. Lynch, H.E.*, et al.*, 2009, *Thymic involution and immune reconstitution,* Trends Immunol, **30**(7), 366-73.
200. Min, H.*, et al.*, 2004, *Reduction in the developmental potential of intrathymic T cell progenitors with age,* J Immunol, **173**(1), 245-50.
201. Zediak, V.P.*, et al.*, 2007, *Multiple prethymic defects underlie age-related loss of T progenitor competence,* Blood, **110**(4), 1161-7.
202. Mackall, C.L.*, et al.*, 1998, *Thymic function in young/old chimeras: substantial thymic T cell regenerative capacity despite irreversible age-associated thymic involution,* Eur J Immunol, **28**(6), 1886-93.
203. Zhu, X.*, et al.*, 2007, *Lymphohematopoietic progenitors do not have a synchronized defect with age-related thymic involution,* Aging Cell, **6**(5), 663-72.
204. Gui, J.*, et al.*, 2007, *The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells,* Int Immunol, **19**(10), 1201-11.
205. Gui, J.*, et al.*, 2012, *Thymus Size and Age-related Thymic Involution: Early Programming, Sexual Dimorphism, Progenitors and Stroma,* Aging Dis, **3**(3), 280-90.

206. Aw, D.*, et al.*, 2008, *Architectural changes in the thymus of aging mice,* Aging Cell, **7**(2), 158-67.
207. Venables, T.*, et al.*, 2019, *Dynamic changes in epithelial cell morphology control thymic organ size during atrophy and regeneration,* Nat Commun, **10**(1), 4402.
208. Sakata, M.*, et al.*, 2018, *Cellularity of Thymic Epithelial Cells in the Postnatal Mouse,* J Immunol, **200**(4), 1382-1388.
209. Yue, S.*, et al.*, 2019, *Cell-type-specific role of lamin-B1 in thymus development and its inflammation-driven reduction in thymus aging,* Aging Cell, **18**(4), e12952.
210. Lee, S.*, et al.*, 2023, *Comprehensive Transcriptomic Analysis for Thymic Epithelial Cells of Aged Mice and Humans,* Immune Netw, **23**(5), e36.
211. Ki, S.*, et al.*, 2014, *Global transcriptional profiling reveals distinct functions of thymic stromal subsets and age-related changes during thymic involution,* Cell Rep, **9**(1), 402-415.
212. Wu, H.*, et al.*, 2018, *Time-course transcriptome analysis of medullary thymic epithelial cells in the early phase of thymic involution,* Mol Immunol, **99**, 87-94.
213. Cowan, J.E.*, et al.*, 2019, *Myc controls a distinct transcriptional program in fetal thymic epithelial cells that determines thymus growth,* Nat Commun, **10**(1), 5498.
214. Morrissey, P.J.*, et al.*, 1988, *In vivo administration of IL-1 induces thymic hypoplasia and increased levels of serum corticosterone,* J Immunol, **141**(5), 1456-63.
215. Sempowski, G.D.*, et al.*, 2000, *Leukemia inhibitory factor, oncostatin M, IL-6, and stem cell factor mRNA expression in human thymus increases with age and is associated with thymic atrophy,* J Immunol, **164**(4), 2180-7.
216. Chen, L.*, et al.*, 2009, *Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner,* Blood, **113**(3), 567-74.
217. Cheng, L.*, et al.*, 2010, *Postnatal tissue-specific disruption of transcription factor FoxN1 triggers acute thymic atrophy,* J Biol Chem, **285**(8), 5836-47.
218. Sun, L.*, et al.*, 2010, *Declining expression of a single epithelial cell-autonomous gene accelerates age-related thymic involution,* Aging Cell, **9**(3), 347-57.
219. Zook, E.C.*, et al.*, 2011, *Overexpression of Foxn1 attenuates age-associated thymic involution and prevents the expansion of peripheral CD4 memory T cells,* Blood, **118**(22), 5723-31.
220. Bredenkamp, N.*, et al.*, 2014, *Regeneration of the aged thymus by a single transcription factor,* Development, **141**(8), 1627-37.
221. O'Neill, K.E.*, et al.*, 2016, *Foxn1 Is Dynamically Regulated in Thymic Epithelial Cells during Embryogenesis and at the Onset of Thymic Involution,* PLoS One, **11**(3), e0151666.

222. Bleul, C.C. and Boehm, T., 2005, *BMP signaling is required for normal thymus development,* J Immunol, **175**(8), 5213-21.
223. Balciunaite, G.*, et al.*, 2002, *Wnt glycoproteins regulate the expression of FoxN1, the gene defective in nude mice,* Nat Immunol, **3**(11), 1102-8.
224. Griffith, A.V.*, et al.*, 2012, *Persistent degenerative changes in thymic organ function revealed by an inducible model of organ regrowth,* Aging Cell, **11**(1), 169-77.
225. Yang, H.*, et al.*, 2009, *Axin expression in thymic stromal cells contributes to an age-related increase in thymic adiposity and is associated with reduced thymopoiesis independently of ghrelin signaling,* J Leukoc Biol, **85**(6), 928-38.
226. Ferrando-Martinez, S.*, et al.*, 2015, *WNT signaling suppression in the senescent human thymus,* J Gerontol A Biol Sci Med Sci, **70**(3), 273-81.
227. Henderson, J., 1904, *On the relationship of the thymus to the sexual organs: I. The influence of castration on the thymus,* J Physiol, **31**(3-4), 222-9.
228. Olsen, N.J.*, et al.*, 2001, *Androgen receptors in thymic epithelium modulate thymus size and thymocyte development,* Endocrinology, **142**(3), 1278-83.
229. Dumont-Lagace, M.*, et al.*, 2015, *Sex hormones have pervasive effects on thymic epithelial cells,* Sci Rep, **5**, 12895.
230. Velardi, E.*, et al.*, 2014, *Sex steroid blockade enhances thymopoiesis by modulating Notch signaling,* J Exp Med, **211**(12), 2341-9.
231. Tibbetts, T.A.*, et al.*, 1999, *Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility,* Proc Natl Acad Sci U S A, **96**(21), 12021-6.
232. Messaoudi, I.*, et al.*, 2006, *Delay of T cell senescence by caloric restriction in aged long-lived nonhuman primates,* Proc Natl Acad Sci U S A, **103**(51), 19448-53.
233. Yang, H.*, et al.*, 2009, *Inhibition of thymic adipogenesis by caloric restriction is coupled with reduction in age-related thymic involution,* J Immunol, **183**(5), 3040-52.
234. Yang, H.*, et al.*, 2009, *Obesity accelerates thymic aging,* Blood, **114**(18), 3803-12.
235. Kelley, K.W.*, et al.*, 1986, *GH3 pituitary adenoma cells can reverse thymic aging in rats,* Proc Natl Acad Sci U S A, **83**(15), 5663-7.
236. French, R.A.*, et al.*, 2002, *Age-associated loss of bone marrow hematopoietic cells is reversed by GH and accompanies thymic reconstitution,* Endocrinology, **143**(2), 690-9.
237. Chu, Y.W.*, et al.*, 2008, *Exogenous insulin-like growth factor 1 enhances thymopoiesis predominantly through thymic epithelial cell expansion,* Blood, **112**(7), 2836-46.

238. Howard, J.K.*, et al.*, 1999, *Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice,* J Clin Invest, **104**(8), 1051-9.
239. Gruver, A.L.*, et al.*, 2009, *Leptin receptor is expressed in thymus medulla and leptin protects against thymic remodeling during endotoxemia-induced thymus involution,* J Endocrinol, **203**(1), 75-85.
240. Dixit, V.D.*, et al.*, 2007, *Ghrelin promotes thymopoiesis during aging,* J Clin Invest, **117**(10), 2778-90.
241. Youm, Y.H.*, et al.*, 2016, *Prolongevity hormone FGF21 protects against immune senescence by delaying age-related thymic involution,* Proc Natl Acad Sci U S A, **113**(4), 1026-31.
242. Min, D.*, et al.*, 2007, *Sustained thymopoiesis and improvement in functional immunity induced by exogenous KGF administration in murine models of aging,* Blood, **109**(6), 2529-37.
243. Omeroglu Ulu, Z.*, et al.*, 2018, *Transcriptome Analysis of the Thymus in Short-Term Calorie-Restricted Mice Using RNA-seq,* Int J Genomics, **2018**, 7647980.
244. Griffith, A.V.*, et al.*, 2015, *Metabolic Damage and Premature Thymus Aging Caused by Stromal Catalase Deficiency,* Cell Rep, **12**(7), 1071-9.
245. Mognato, M.*, et al.*, 2021, *Interplay between DNA replication stress, chromatin dynamics and DNA-damage response for the maintenance of genome stability,* Mutat Res Rev Mutat Res, **787**, 108346.
246. Wolters, S. and Schumacher, B., 2013, *Genome maintenance and transcription integrity in aging and disease,* Front Genet, **4**, 19.
247. Yousefzadeh, M.*, et al.*, 2021, *DNA damage-how and why we age?,* Elife, **10**.
248. Giglia-Mari, G.*, et al.*, 2011, *DNA damage response,* Cold Spring Harb Perspect Biol, **3**(1), a000745.
249. Hernandez Borrero, L.J. and El-Deiry, W.S., 2021, *Tumor suppressor p53: Biology, signaling pathways, and therapeutic targeting,* Biochim Biophys Acta Rev Cancer, **1876**(1), 188556.
250. Rodrigues, P.M.*, et al.*, 2017, *Thymic epithelial cells require p53 to support their long-term function in thymopoiesis in mice,* Blood, **130**(4), 478-488.
251. Bansal, K.*, et al.*, 2017, *The transcriptional regulator Aire binds to and activates super-enhancers,* Nat Immunol, **18**(3), 263-273.
252. Abramson, J.*, et al.*, 2010, *Aire's partners in the molecular control of immunological tolerance,* Cell, **140**(1), 123-35.

253. Fletcher, A.L.*, et al.*, 2009, *Ablation and regeneration of tolerance-inducing medullary thymic epithelial cells after cyclosporine, cyclophosphamide, and dexamethasone treatment,* J Immunol, **183**(2), 823-31.
254. Williams, K.M.*, et al.*, 2009, *Single cell analysis of complex thymus stromal cell populations: rapid thymic epithelia preparation characterizes radiation injury,* Clin Transl Sci, **2**(4), 279-85.
255. Ansari, A.R. and Liu, H., 2017, *Acute Thymic Involution and Mechanisms for Recovery,* Arch Immunol Ther Exp (Warsz), **65**(5), 401-420.
256. Duah, M.*, et al.*, 2021, *Thymus Degeneration and Regeneration,* Front Immunol, **12**, 706244.
257. Hauri-Hohl, M.M.*, et al.*, 2008, *TGF-beta signaling in thymic epithelial cells regulates thymic involution and postirradiation reconstitution,* Blood, **112**(3), 626-34.
258. Dudakov, J.A.*, et al.*, 2012, *Interleukin-22 drives endogenous thymic regeneration in mice,* Science, **336**(6077), 91-5.
259. Alawam, A.S.*, et al.*, 2022, *Failures in thymus medulla regeneration during immune recovery cause tolerance loss and prime recipients for auto-GVHD,* J Exp Med, **219**(2).
260. Horie, K.*, et al.*, 2023, *Acute irradiation causes a long-term disturbance in the heterogeneity and gene expression profile of medullary thymic epithelial cells,* Front Immunol, **14**, 1186154.
261. Calvo-Asensio, I.*, et al.*, 2017, *Differential Response of Mouse Thymic Epithelial Cell Types to Ionizing Radiation-Induced DNA Damage,* Front Immunol, **8**, 418.
262. Krenger, W. and Hollander, G.A., 2010, *The role of the thymus in allogeneic hematopoietic stem cell transplantation,* Swiss Med Wkly, **140**, w13051.
263. Carlsson, P. and Mahlapuu, M., 2002, *Forkhead transcription factors: key players in development and metabolism,* Dev Biol, **250**(1), 1-23.
264. van der Vos, K.E.*, et al.*, 2012, *Regulation of autophagy by Forkhead box (FOX) O transcription factors,* Adv Biol Regul, **52**(1), 122-36.
265. Furuyama, T.*, et al.*, 2000, *Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues,* Biochem J, **349**(Pt 2), 629-34.
266. van der Horst, A. and Burgering, B.M., 2007, *Stressing the role of FoxO proteins in lifespan and disease,* Nat Rev Mol Cell Biol, **8**(6), 440-50.
267. Eijkelenboom, A. and Burgering, B.M., 2013, *FOXOs: signalling integrators for homeostasis maintenance,* Nat Rev Mol Cell Biol, **14**(2), 83-97.

268. Brunet, A.*, et al.*, 1999, *Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor,* Cell, **96**(6), 857-68.
269. Zhao, Y. and Liu, Y.S., 2021, *Longevity Factor FOXO3: A Key Regulator in Aging-Related Vascular Diseases,* Front Cardiovasc Med, **8**, 778674.
270. Lehtinen, M.K.*, et al.*, 2006, *A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span,* Cell, **125**(5), 987-1001.
271. Essers, M.A.*, et al.*, 2004, *FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK,* EMBO J, **23**(24), 4802-12.
272. Greer, E.L.*, et al.*, 2007, *The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor,* J Biol Chem, **282**(41), 30107-19.
273. Yata, K. and Esashi, F., 2009, *Dual role of CDKs in DNA repair: to be, or not to be,* DNA Repair (Amst), **8**(1), 6-18.
274. Tsai, W.B.*, et al.*, 2008, *Functional interaction between FOXO3a and ATM regulates DNA damage response,* Nat Cell Biol, **10**(4), 460-7.
275. Martins, R.*, et al.*, 2016, *Long live FOXO: unraveling the role of FOXO proteins in aging and longevity,* Aging Cell, **15**(2), 196-207.
276. Kenyon, C.*, et al.*, 1993, *A C. elegans mutant that lives twice as long as wild type,* Nature, **366**(6454), 461-4.
277. Hwangbo, D.S.*, et al.*, 2004, *Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body,* Nature, **429**(6991), 562-6.
278. Kenyon, C.J., 2010, *The genetics of ageing,* Nature, **464**(7288), 504-12.
279. Shimokawa, I.*, et al.*, 2015, *The life-extending effect of dietary restriction requires Foxo3 in mice,* Aging Cell, **14**(4), 707-9.
280. Dansen, T.B.*, et al.*, 2009, *Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity,* Nat Chem Biol, **5**(9), 664-72.
281. Li, M.*, et al.*, 2006, *Down-regulation of manganese-superoxide dismutase through phosphorylation of FOXO3a by Akt in explanted vascular smooth muscle cells from old rats,* J Biol Chem, **281**(52), 40429-39.
282. Olmos, Y.*, et al.*, 2009, *Mutual dependence of Foxo3a and PGC-1alpha in the induction of oxidative stress genes,* J Biol Chem, **284**(21), 14476-84.
283. Nemoto, S. and Finkel, T., 2002, *Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway,* Science, **295**(5564), 2450-2.
284. Kops, G.J.*, et al.*, 2002, *Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress,* Nature, **419**(6904), 316-21.

285. Bigarella, C.L.*, et al.*, 2017, *FOXO3 Transcription Factor Is Essential for Protecting Hematopoietic Stem and Progenitor Cells from Oxidative DNA Damage,* J Biol Chem, **292**(7), 3005-3015.

286. Chung, Y.M.*, et al.*, 2012, *FOXO3 signalling links ATM to the p53 apoptotic pathway following DNA damage,* Nat Commun, **3**, 1000.

287. Medema, R.H.*, et al.*, 2000, *AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1,* Nature, **404**(6779), 782-7.

288. Tran, H.*, et al.*, 2002, *DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein,* Science, **296**(5567), 530-4.

289. Furukawa-Hibi, Y.*, et al.*, 2002, *FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress,* J Biol Chem, **277**(30), 26729-32.

290. Hornsveld, M.*, et al.*, 2021, *A FOXO-dependent replication checkpoint restricts proliferation of damaged cells,* Cell Rep, **34**(4), 108675.

291. You, H.*, et al.*, 2004, *p53-dependent inhibition of FKHRL1 in response to DNA damage through protein kinase SGK1,* Proc Natl Acad Sci U S A, **101**(39), 14057-62.

292. Miyaguchi, Y.*, et al.*, 2009, *P53 negatively regulates the transcriptional activity of FOXO3a under oxidative stress,* Cell Biol Int, **33**(8), 853-60.

293. Renault, V.M.*, et al.*, 2011, *The pro-longevity gene FoxO3 is a direct target of the p53 tumor suppressor,* Oncogene, **30**(29), 3207-21.

294. You, H.*, et al.*, 2006, *Regulation of transactivation-independent proapoptotic activity of p53 by FOXO3a,* Proc Natl Acad Sci U S A, **103**(24), 9051-6.

CHAPTER II - MEDULLARY THYMIC EPITHELIAL CELLS: DECIPHERING THE FUNCTIONAL DIVERSITY BEYOND PROMISCUOUS GENE EXPRESSION

Camila Ribeiro[1,2], Nuno L. Alves[1,2] and Pedro Ferreirinha[1,2]

[1]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto.
[2]IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto.

Immunology Letters
2019
doi: 10.1016/j.imlet.2019.01.012.

ABSTRACT

Within the thymus, cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) provide unique microenvironments for the development of T cells that are responsive to diverse foreign antigens while self-tolerant. Essential for tolerance induction, mTECs play a critical role in negative selection and T regulatory cell differentiation. In this article, we review the current knowledge on the functional diversity within mTECs and discuss how these novel subsets contribute to tolerance induction and are integrated in the complex blueprint of mTEC differentiation.

INTRODUCTION

T cells express a variety of T-cell receptors (TCRs) that respond to a myriad of pathogen and cancer cell-derived antigens while being simultaneously tolerant to our own tissues. This diverse pattern of recognition depends on the random rearrangement of V(D)J gene segments of TCR during T-cell development, which apart from of generating T cells with favourable specificities, can also produce autoreactive T cells [1]. Cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) are essential to impose the basic principles that control T-cell immunity and self-tolerance [2]. While cTECs promote T-cell lineage commitment and positive selection, mTECs regulate negative selection, T regulatory cell differentiation and the final steps of single positive thymocyte maturation [3]. Despite defining functionally distinct compartments [4], cTECs and mTECs arise from common bipotent TEC progenitors (TEPs) that exist in the embryonic and postnatal thymus [5, 6]. TEC differentiation is strictly dependent on the forkhead box protein N1 (FOXN1) transcription factor (reviewed in detail in [7]).

The chief role of mTECs in tolerance induction depends on their capacity to express virtually all tissue restricted antigens (TRAs) [8]. This process anticipates the presentation of peripheral self-antigens to developing T cells, so that potentially autoreactive thymocytes are eliminated or deviated to the T regulatory cell lineage. Autoimmune regulator (Aire) and recently described forebrain embryonic zinc finger-like protein 2 (Fezf2) are two known regulators of promiscuous gene expression (PGE) in mTECs [9]. The advent of single-cell RNA analysis has revealed that individual mTECs express unique sets of TRA genes, so that the comprehensive representation of all genes in the genome is only achieved by multiple mTECs [10-12]. Apart from this heterogeneous expression pattern, recent findings highlight that mTECs are composed of functionally distinct subsets that might not be immediately linked to their promiscuous gene expression capacity. Here, we discuss how unravelling mTEC complexity could help decode the mechanisms that regulate their development and function in tolerance induction.

MTEC PROGENITORS AND THEIR MATURATION REQUIREMENTS

Although it was initially considered that cTECs and mTECs synchronously differentiate from common bipotent progenitors, recent studies have provided evidence for the existence of TEPs that express cTEC traits (*e.g.*, β5t, CD205, IL-7 and CCRL1) and have the potential to generate both cTECs and mTECs [13-16]. These observations led to a refined differentiation model whereby TEC progenitors transverse through the

cTEC lineage prior to the commitment into mTECs [17] (**Figure 1**). Still, the identity of bipotent TEPs or unipotent cTEC precursors at the single-cell level as well as the molecular blueprint that drives the differentiation of cTECs remains unknown. In contrast, the nature of mTEC-restricted precursors (mTEPs) and the molecular principles that drive the maturation and maintenance of mTECs are better understood [18]. In particular, initial reports identified Claudin3$^+$ and Claudin4$^+$ (Cld3/4$^+$) mTEPs in the early embryonic thymus that exclusively generate mTECs [19]. A subsequent study revealed that the expression of stage-specific embryonic antigen A (SSEA) further enriched for mTEPs within Cld3/4$^+$ cells [20], which derive from β5t-expressing cells [21]. More recently, Cld3/4$^+$SSEA$^-$RANK$^+$ mTEPs have also been defined to exist in the embryonic thymus [22]. In the post-natal thymus, Podoplanin (PDPN)$^+$ cells identify another mTEP population that resides at the cortico-medullary junction and substantially contributes to the maintenance of the adult mTEC compartment [23]. Despite these advances, the precursor-product relationship between these novel mTEC subsets is unclear (**Figure 1**). In particular, future studies should address whether these subsets constitute sequential stages of the same differentiation program or separate lineages.

The development of mTECs is highly dependent on instructive signals provided by the hematopoietic cells within the thymus [24, 25]. The thymocyte-TEC interactions, commonly termed “thymic crosstalk”, engage the canonical and noncanonical nuclear factor kappa B (NF-κB) signalling pathways in mTECs and their progenitors, which in turn activate the transcriptional programs required for their maturation [26]. As such, the disruption of genes in these pathways results in impaired medulla organization and autoimmunity [27-29]. Later studies coupled the induction of NF-κB signalling to the upstream engagement of specific members of the tumour necrosis factor receptor superfamily (TNFRSF), including receptor activator of NF-κB (RANK), CD40 and lymphotoxin β receptor (LTβR) [4]. In this regard, LTβR signalling has been implicated in the maintenance of the Cld3/4$^+$SSEA$^+$ mTEP population, particularly in the neonatal thymus [30], and in the induction of RANK expression [31]. Accordingly, Cld3/4$^+$SSEA$^+$ cells express LTβR [20] and emerge prior to the RelB-dependent Cld3/4$^+$SSEA$^-$RANK$^+$ mTEPs [22]. Additionally, the engagement of RANK signalling upregulates the expression of CD40 during mTEC development [32]. Collectively, these findings suggest a sequential requirement for activation of the TNFRSF members to implement the mTEC differentiation program.

PHENOTYPIC AND FUNCTIONAL COMPLEXITY OF MTECS

Over the last years, several studies revealed a high degree of functional heterogeneity within mTECs, which correlates with the distinct thymopoietic stages supported by these cells. Earlier reports uncovered a first level of mTEC diversity via the characterization of cells expressing low and high levels of MHCII and CD80, ($mTEC^{low}$ and $mTEC^{high}$, respectively), which share a precursor-product relationship [33]. Subsequently, it was discovered that a fraction of $mTEC^{high}$ contains Aire-expressing cells [34]. RANK signalling plays a dominant role in the development of $Aire^+$ mTECs, whereas CD40 also contributes to this process [35]. Although LTβR signalling was initially linked to the development of the $Aire^+$ lineage [36], subsequent studies uncoupled these two processes [37, 38]. Instead, LTβR signalling was shown to be involved in the organization of the medullary compartment [39]. The characterization of RANK reporter mice revealed that RANK expression can be found in both the $mTEC^{low}$ and $mTEC^{high}$, including $Aire^+$ cells, and therefore, it seems not to demark a discrete mTEC population [40]. Importantly, the intensity of RANK signalling is tightly controlled *in vivo.* In particular, Osteoprotegerin (OPG) is a decoy receptor for RANKL that inhibits RANK signalling in mTECs [41]. Interestingly, OPG is produced within the $mTEC^{high}$ population, predominantly by $Aire^+$ cells [40], revealing a further degree of functional heterogeneity with the medullary microenvironment (**Figure 1**).

Beyond its role in TRA expression, several reports suggested an alternative function for the Aire protein in the regulation of mTEC homeostasis [42]. Supporting this hypothesis, $mTEC^{high}$ accumulate in *Aire*-deficient mice [33, 43] and present an altered mTEC morphology [44, 45]. One of the models to explain this phenotype argues for a pro-apoptotic effect of Aire, so that $mTEC^{high}$ display an increased survival and higher numbers in its absence [33]. An alternative scenario has been constructed in which Aire expression is required for the completion of mTEC differentiation [46]. In this regard, $Aire^+$ cells differentiate into a post-Aire stage, which is typified by the downregulation of MHCII and CD80, reduced TRA expression and the expression of markers associated with terminal keratinocyte differentiation [47-49]. Although both models are not necessarily mutually exclusive, the identification of the post-Aire stage within the $mTEC^{low}$ subset revealed a further degree of diversity within this compartment. More recently, it has been shown that $mTEC^{low}$ also include CCL21-producing cells, which develop in a LTβR-dependent manner and possess an important role in the migration of positively selected thymocytes to the medulla [50]. As such, disruption of CCL21 expression leads to a defective localization of thymocytes to the medulla and perturbs tolerance induction [51].

The existence of Aire-independent TRA expression [52] implies that PGE is controlled in mTECs by other regulators. The transcription factor Fezf2 has been recently defined as a regulator of the expression of Aire-independent TRAs [53]. Fezf2^{+} cells are found both in mTEC^{low} and mTEC^{high} [53] and represent another example that highlights the functional heterogeneity of mTECs. Although the development of Fezf2^{+} cells was initially described as LTβR-dependent [53], a subsequent study has linked its expression to RANK signalling [39]. Further studies are warranted to settle the role of this seemingly important population for tolerance induction. Overall, these findings highlight that mTEC^{low} represent a multifunctional population, containing not only mTEC^{high} precursors, but also terminally differentiated post-Aire cells and other functionally competent mTECs with specific roles in T-cell development (**Figure 1**).

The latest addition to the expanding catalogue of mTEC subsets has been provided by two complementary studies. Taking advantage of unsupervised single-cell RNA-sequencing and fate-mapping analyses, both reports have identified a novel mTEC subpopulation that intriguingly resembles mucosal-associated epithelial tuft cells [54, 55]. Tuft-like mTECs have a unique transcriptional program that differs from the remaining mTEC subsets and are characterized, among others, by the production of IL-25, a characteristic cytokine of mucosal tuft cells [54, 55]. This population exist within both pre- and post-Aire mTEC^{low} (**Figure 1**), and their development is critically dependent on the transcription factor Pou2f3 [54]. Albeit with a normal T cell differentiation, mice deficient in tuft cells, via genetic ablation of Pou2f3, present specific alterations in type-2 cells. While Miller *et al.* show that Pou2f3$^{-/-}$ mice present a defective tolerance to tuft cell-associated antigens, including IL-25, and a reduction in NKT2 cells [54], Bornstein *et al*. demonstrate that these mutants have an increased frequency of thymic ILC2s [55]. The presence of this unique population in the thymus raises multiple questions on the basis of their development and function. It is unlikely that these cells result from a coincidental assembly of randomly expressed tuft-restricted TRAs. A more favourable possibility is that they arise from a programmed mTEC differentiation pathway. These findings raise the possibility that other specialized populations of epithelial cells from other tissues could be represented within the thymus, perhaps with additional effects in tolerance induction.

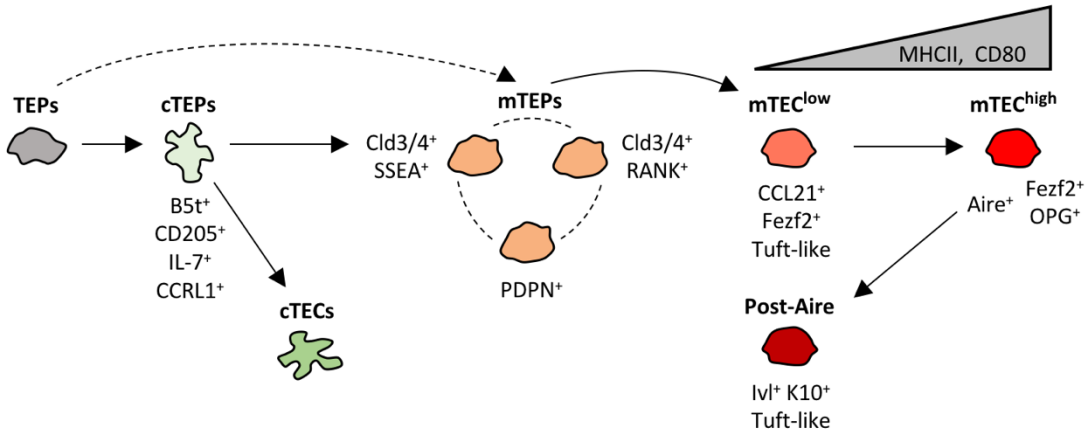


Figure 1 – Schematic representation of mTEC development and of the characterized phenotypic and functional subsets. Medullary thymic epithelial cells (mTECs) derive from bipotent thymic epithelial progenitors (TEPs). TEPs expressing cTEC-associated traits (cTEPs) participate in the differentiation of mTEC-committed progenitors (mTEPs). The highly heterogeneous mTEC compartment contains several subsets with specific roles in T-cell development. mTECs can be broadly divided into mTEC^{low} and mTEC^{high}, based on the expression levels of MHCII and CD80, the latter also containing Aire-expressing cells. In turn, Aire-expressing cells can transition into a post-Aire stage, which is characterized by the downregulation of MHCII and CD80. Beyond the populations defined by MHCII and CD80 expression levels, mTECs possess a higher degree of phenotypic and functional diversity, defined by the expression of the listed cell surface or secreted phenotypic markers, chemokines, transcription factors or specific transcriptional programs.

CONCLUDING REMARKS

The distinction of self and non-self is an essential aspect of T-cell immunobiology that is highly dependent on the selection processes governed by mTECs. Overall, the findings described in this review portray the mTEC compartment as an expanding universe of functionally distinct but perhaps interconnected subsets (**Figure 1**), which warrant further exploration. Given the inherent complexity of this population, a comprehensive blueprint of the mTEC developmental program has not yet been defined. Regarding the origin of mTECs, we still lack a solid understanding of the mechanisms that regulate the maintenance of the different mTEP pools and their possible lineage relationship. Also, the molecular roadmap that leads to the establishment of the aforementioned different functional subsets remains elusive. In this regard, it has been proposed that all mTECs follow a differentiation program predestined to Aire expression [56]. Alternatively, Aire$^+$ cells might constitute only one of several distinct possible lineages. Future research will benefit from the establishment of novel stage-specific markers, which in combination with RNA-Seq and fate-mapping mouse models, can further dissect new developmental stages in mTECs. These efforts can ultimately uncover the inner workings of this unique cell population and provide a steppingstone for the understanding of human mTEC biology, thus opening novel avenues for the development of immunotherapies.

REFERENCES

1. Boehm, T. and Swann, J.B., 2014, *Origin and evolution of adaptive immunity,* Annu Rev Anim Biosci, **2**, 259-83.
2. Takaba, H. and Takayanagi, H., 2017, *The Mechanisms of T Cell Selection in the Thymus,* Trends Immunol, **38**(11), 805-816.
3. Klein, L.*, et al.*, 2014, *Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see),* Nat Rev Immunol, **14**(6), 377-91.
4. Abramson, J. and Anderson, G., 2017, *Thymic Epithelial Cells,* Annu Rev Immunol, **35**, 85-118.
5. Rossi, S.W.*, et al.*, 2006, *Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium,* Nature, **441**(7096), 988-91.
6. Bleul, C.C.*, et al.*, 2006, *Formation of a functional thymus initiated by a postnatal epithelial progenitor cell,* Nature, **441**(7096), 992-6.
7. Romano, R.*, et al.*, 2013, *FOXN1: A Master Regulator Gene of Thymic Epithelial Development Program,* Front Immunol, **4**, 187.
8. Kyewski, B. and Klein, L., 2006, *A central role for central tolerance,* Annu Rev Immunol, **24**, 571-606.
9. Passos, G.A.*, et al.*, 2018, *Update on Aire and thymic negative selection,* Immunology, **153**(1), 10-20.
10. Pinto, S.*, et al.*, 2013, *Overlapping gene coexpression patterns in human medullary thymic epithelial cells generate self-antigen diversity,* Proc Natl Acad Sci U S A, **110**(37), E3497-505.
11. Brennecke, P.*, et al.*, 2015, *Single-cell transcriptome analysis reveals coordinated ectopic gene-expression patterns in medullary thymic epithelial cells,* Nat Immunol, **16**(9), 933-41.
12. Rattay, K.*, et al.*, 2016, *Evolutionary conserved gene co-expression drives generation of self-antigen diversity in medullary thymic epithelial cells,* J Autoimmun, **67**, 65-75.
13. Ribeiro, A.R.*, et al.*, 2013, *Thymocyte selection regulates the homeostasis of IL-7-expressing thymic cortical epithelial cells in vivo,* J Immunol, **191**(3), 1200-9.
14. Ohigashi, I.*, et al.*, 2013, *Aire-expressing thymic medullary epithelial cells originate from beta5t-expressing progenitor cells,* Proc Natl Acad Sci U S A, **110**(24), 9885-90.
15. Baik, S.*, et al.*, 2013, *Generation of both cortical and Aire(+) medullary thymic epithelial compartments from CD205(+) progenitors,* Eur J Immunol, **43**(3), 589-94.

16. Ribeiro, A.R.*, et al.*, 2014, *Intermediate expression of CCRL1 reveals novel subpopulations of medullary thymic epithelial cells that emerge in the postnatal thymus,* Eur J Immunol, **44**(10), 2918-24.
17. Alves, N.L.*, et al.*, 2014, *Serial progression of cortical and medullary thymic epithelial microenvironments,* Eur J Immunol, **44**(1), 16-22.
18. Alves, N.L. and Ribeiro, A.R., 2016, *Thymus medulla under construction: Time and space oddities,* Eur J Immunol, **46**(4), 829-33.
19. Hamazaki, Y.*, et al.*, 2007, *Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin,* Nat Immunol, **8**(3), 304-11.
20. Sekai, M.*, et al.*, 2014, *Medullary thymic epithelial stem cells maintain a functional thymus to ensure lifelong central T cell tolerance,* Immunity, **41**(5), 753-61.
21. Ohigashi, I.*, et al.*, 2015, *Adult Thymic Medullary Epithelium Is Maintained and Regenerated by Lineage-Restricted Cells Rather Than Bipotent Progenitors,* Cell Rep, **13**(7), 1432-1443.
22. Baik, S.*, et al.*, 2016, *Relb acts downstream of medullary thymic epithelial stem cells and is essential for the emergence of RANK(+) medullary epithelial progenitors,* Eur J Immunol, **46**(4), 857-62.
23. Onder, L.*, et al.*, 2015, *Alternative NF-kappaB signaling regulates mTEC differentiation from podoplanin-expressing precursors in the cortico-medullary junction,* Eur J Immunol, **45**(8), 2218-31.
24. Ritter, M.A. and Boyd, R.L., 1993, *Development in the thymus: it takes two to tango,* Immunol Today, **14**(9), 462-9.
25. van Ewijk, W.*, et al.*, 1994, *Crosstalk in the mouse thymus,* Immunol Today, **15**(5), 214-7.
26. Anderson, G. and Takahama, Y., 2012, *Thymic epithelial cells: working class heroes for T cell development and repertoire selection,* Trends Immunol, **33**(6), 256-63.
27. Akiyama, T.*, et al.*, 2005, *Dependence of self-tolerance on TRAF6-directed development of thymic stroma,* Science, **308**(5719), 248-51.
28. Kajiura, F.*, et al.*, 2004, *NF-kappa B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner,* J Immunol, **172**(4), 2067-75.
29. Burkly, L.*, et al.*, 1995, *Expression of relB is required for the development of thymic medulla and dendritic cells,* Nature, **373**(6514), 531-6.

30. Wu, W.*, et al.*, 2017, *Epithelial LTbetaR signaling controls the population size of the progenitors of medullary thymic epithelial cells in neonatal mice,* Sci Rep, **7**, 44481.
31. Mouri, Y.*, et al.*, 2011, *Lymphotoxin signal promotes thymic organogenesis by eliciting RANK expression in the embryonic thymic stroma,* J Immunol, **186**(9), 5047-57.
32. Desanti, G.E.*, et al.*, 2012, *Developmentally regulated availability of RANKL and CD40 ligand reveals distinct mechanisms of fetal and adult cross-talk in the thymus medulla,* J Immunol, **189**(12), 5519-26.
33. Gray, D.*, et al.*, 2007, *Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire,* J Exp Med, **204**(11), 2521-8.
34. Gabler, J.*, et al.*, 2007, *Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells,* Eur J Immunol, **37**(12), 3363-72.
35. Akiyama, T.*, et al.*, 2008, *The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance,* Immunity, **29**(3), 423-37.
36. Chin, R.K.*, et al.*, 2003, *Lymphotoxin pathway directs thymic Aire expression,* Nat Immunol, **4**(11), 1121-7.
37. Venanzi, E.S.*, et al.*, 2007, *Lymphotoxin pathway and Aire influences on thymic medullary epithelial cells are unconnected,* J Immunol, **179**(9), 5693-700.
38. Seach, N.*, et al.*, 2008, *The lymphotoxin pathway regulates Aire-independent expression of ectopic genes and chemokines in thymic stromal cells,* Journal of Immunology, **180**(8), 5384-5392.
39. Cosway, E.J.*, et al.*, 2017, *Redefining thymus medulla specialization for central tolerance,* J Exp Med, **214**(11), 3183-3195.
40. McCarthy, N.I.*, et al.*, 2015, *Osteoprotegerin-Mediated Homeostasis of Rank+ Thymic Epithelial Cells Does Not Limit Foxp3+ Regulatory T Cell Development,* J Immunol, **195**(6), 2675-82.
41. Akiyama, N.*, et al.*, 2014, *Limitation of immune tolerance-inducing thymic epithelial cell development by Spi-B-mediated negative feedback regulation,* J Exp Med, **211**(12), 2425-38.
42. Matsumoto, M., 2011, *Contrasting models for the roles of Aire in the differentiation program of epithelial cells in the thymic medulla,* Eur J Immunol, **41**(1), 12-7.
43. Hubert, F.X.*, et al.*, 2009, *Aire-deficient C57BL/6 mice mimicking the common human 13-base pair deletion mutation present with only a mild autoimmune phenotype,* J Immunol, **182**(6), 3902-18.

44. Yano, M.*, et al.*, 2008, *Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance,* J Exp Med, **205**(12), 2827-38.
45. Gillard, G.O.*, et al.*, 2007, *Aire-dependent alterations in medullary thymic epithelium indicate a role for Aire in thymic epithelial differentiation,* J Immunol, **178**(5), 3007-15.
46. Nishikawa, Y.*, et al.*, 2014, *Temporal lineage tracing of Aire-expressing cells reveals a requirement for Aire in their maturation program,* J Immunol, **192**(6), 2585-92.
47. Metzger, T.C.*, et al.*, 2013, *Lineage tracing and cell ablation identify a post-Aire-expressing thymic epithelial cell population,* Cell Rep, **5**(1), 166-79.
48. White, A.J.*, et al.*, 2010, *Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells,* J Immunol, **185**(8), 4769-76.
49. Wang, X.*, et al.*, 2012, *Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens,* Front Immunol, **3**(March), 19.
50. Lkhagvasuren, E.*, et al.*, 2013, *Lymphotoxin beta receptor regulates the development of CCL21-expressing subset of postnatal medullary thymic epithelial cells,* J Immunol, **190**(10), 5110-7.
51. Kozai, M.*, et al.*, 2017, *Essential role of CCL21 in establishment of central self-tolerance in T cells,* J Exp Med, **214**(7), 1925-1935.
52. Derbinski, J.*, et al.*, 2005, *Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels,* J Exp Med, **202**(1), 33-45.
53. Takaba, H.*, et al.*, 2015, *Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance,* Cell, **163**(4), 975-87.
54. Miller, C.N.*, et al.*, 2018, *Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development,* Nature, **559**(7715), 627-631.
55. Bornstein, C.*, et al.*, 2018, *Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells,* Nature, **559**(7715), 622-626.
56. Kawano, H.*, et al.*, 2015, *Aire Expression Is Inherent to Most Medullary Thymic Epithelial Cells during Their Differentiation Program,* J Immunol, **195**(11), 5149-58.

CHAPTER III - A NOVEL METHOD TO IDENTIFY POST-AIRE STAGES OF MEDULLARY THYMIC EPITHELIAL CELL DIFFERENTIATION

Pedro Ferreirinha[1,2], Camila Ribeiro[1,2], Junko Morimoto[3], Jonathan J. M. Landry[4], Minoru Matsumoto[3], Catarina Meireles[1], Andrea J. White[5], Izumi Ohigashi[6], Leonor Araújo[1,2], Vladimir Benes[4], Yousuke Takahama[7], Graham Anderson[5], Mitsuru Matsumoto[3], Nuno Alves[1,2]

[1] Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto.

[2] Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto.

[3] Division of Molecular Immunology, Institute for Enzyme Research, Tokushima University, Tokushima, Japan

[4] Genomics Core Facility, European Molecular Biology Laboratory, Heidelberg, Germany

[5] Institute of Immunology and Immunotherapy, College of Medical and Dental Sciences, Medical School, University of Birmingham, Birmingham, UK.

[6] Division of Experimental Immunology, Institute of Advanced Medical Sciences, University of Tokushima, Tokushima, Japan.

[7] National Cancer Institute, NIH, Bethesda, Maryland, US.

European Journal of Immunology

2020

doi: 10.1002/eji.202048764.

ABSTRACT

Autoimmune regulator^{+} (Aire) medullary thymic epithelial cells (mTECs) play a critical role in tolerance induction. Several studies demonstrated that Aire^{+} mTECs differentiate further into Post-Aire cells. Yet, the identification of terminal stages of mTEC maturation depends on unique fate-mapping mouse models. Herein, we resolve this limitation by segmenting the mTEC^{hi} (MHCII^{hi}CD80^{hi}) compartment into mTEC$^{A/hi}$ (CD24^{-} Sca1^{-}), mTEC$^{B/hi}$ (CD24^{+}Sca1^{-}) and mTEC$^{C/hi}$ (CD24^{+}Sca1^{+}). While mTEC$^{A/hi}$ included mostly Aire-expressing cells, mTEC$^{B/hi}$ contained Aire^{+} and Aire^{-} cells and mTEC$^{C/hi}$ were mainly composed of cells lacking Aire. The differential expression pattern of Aire led us to investigate the precursor-product relationship between these subsets. Strikingly, transcriptomic analysis of mTEC$^{A/hi}$, mTEC$^{B/hi}$ and mTEC$^{C/hi}$ sequentially mirrored the specific genetic program of Early-, Late- and Post-Aire mTECs. Corroborating their Post-Aire nature, mTEC$^{C/hi}$ downregulated the expression of tissue-restricted antigens, acquired traits of differentiated keratinocytes and were absent in *Aire*-deficient mice. Collectively, our findings reveal a new and simple blueprint to survey late stages of mTEC differentiation.

INTRODUCTION

Within the thymus, cortical (c) and medullary (m) thymic epithelial cells (TECs) provide dedicated microenvironments for the development and selection of T cells that are simultaneously reactive to foreign antigens and tolerant to the body's own constituents[1, 2]. While cTECs direct T-cell commitment and positive selection, mTECs regulate negative selection of autoreactive thymocytes or their deviation into the T regulatory cell lineage [2]. The critical role of mTECs in tolerance induction depends on their capacity to express virtually all tissue-restricted antigens (TRAs), which are otherwise confined to peripheral tissues [3]. This process is regulated, in part, by the Autoimmune regulator (Aire) protein, which is encoded by the gene mutated in human autoimmune disorder APECED. Deficiency in Aire alters TRA expression in mTECs, causes failures in negative selection and T regulatory cell differentiation, leading to an increased susceptibility to autoimmunity [4].

Since the discovery of Aire, there have been considerable advances in the understanding of the development of $Aire^+$ mTECs, which represent a fraction of mature $mTEC^{hi}$ ($MHCII^{hi}CD80^{hi}$) and derive from precursors within $mTEC^{lo}$ ($MHCII^{lo}CD80^{lo}$) [2]. Although the expression of Aire was initially considered to identify a terminal step of mTEC maturation [5], subsequent studies demonstrated that $Aire^+$ mTECs can further differentiate into a Post-Aire stage [6-10]. Post-Aire mTECs downregulate the expression of Aire, certain TRAs, CD80 and MHCII, while acquiring traits of terminally differentiated keratinocytes, including keratin 10 (K10) and involucrin (Ivl) [6-8, 10]. Still, the identification of later stages of $Aire^+$ mTEC maturation remains dependent on the use of unique fate-mapping mouse models and advanced high-content single-cell RNA-sequencing, the latter being incompatible with functional studies. In this study, we provide an efficient method to prospectively identify $Aire^+$ mTECs and their Post-Aire descendants, which will prove useful to examine the murine medullary microenvironment, and ultimately, study its role in tolerance induction.

RESULTS AND DISCUSSION

CD24 and Sca1 expression identifies new subsets of mTEC^{hi} with distinct levels of Aire.

Compelling evidence indicates that the cartography of mature mTEC differentiation is more complex than initially recognized [4]. To resolve the heterogeneity of mTECs (defined as indicated in **Supp. Fig. 1A**), we integrated in their conventional phenotypic profiling (CD45$^-$EpCAM$^+$UEA$^+$MHCII$^{lo/hi}$CD80$^{lo/hi}$) the analysis of two markers, CD24 and Sca1, which are routinely used to mark progenitor cells in a variety of hematopoietic, mammary and intestinal epithelial lineages [11, 12]. Examination at defined stages of postnatal life revealed the existence of three prime novel mTEC subsets: CD24$^-$Sca1$^-$ (referred hereafter as mTEC^A), CD24$^+$Sca1$^-$ (mTEC^B) and CD24$^+$Sca1$^+$ (mTEC^C) (**Fig. 1A**). The representation of these subsets was largely maintained over time, and their numbers increase with the expansion of the mTEC compartment during early adulthood (**Fig. 1A**). Next, we assessed whether the mTEC^{A-C} subtypes specifically segregated into conventional mTEC^{lo} or mTEC^{hi} (defined as in **Supp. Fig. 1B**). We found that mTEC^{lo} contained comparable proportions of mTEC^A (mTEC$^{A/lo}$), mTEC^B (mTEC$^{B/lo}$) and mTEC^C (mTEC$^{C/lo}$) during postnatal life, with a reduction in mTEC$^{A/lo}$ and an accumulation of mTEC$^{C/lo}$ unfolding at 6 weeks (**Fig. 1B and Supp. Fig. 1C**). In contrast, mTEC^{hi} were continuously enriched in mTEC^A (mTEC$^{A/hi}$) (**Fig. 1C**). We also found rare cells with a CD24$^-$Sca1$^+$ phenotype, which appeared as an extension of mTEC$^{C/lo}$ (**Fig. 1B**) and mTEC$^{A/hi}$ (**Fig. 1C**) instead of defined populations. Thus, we focused our study on mTEC^{A-C}. Since Aire$^+$ mTECs represent a specialized functional subset within mature mTEC^{hi} [4], we determined their relationship to mTEC$^{A-C/hi}$ subtypes. Notably, mTEC$^{A/hi}$ were mainly Aire$^+$, mTEC$^{B/hi}$ included a combination of Aire$^{+/-}$ cells, and mTEC$^{C/hi}$ largely lacked Aire expression (**Fig. 1C**). Complementary analysis confirmed that the majority of Aire$^+$ cells were confined to the mTEC$^{A/hi}$ subtype (**Fig. 1D**)**.** The sequential changes in the pattern of Aire expression within mTEC$^{A-C/hi}$ was intriguing and led us to further study whether these subsets identified discrete stages of Aire$^+$ mTEC differentiation. Since mTEC$^{C/hi}$ and mTEC$^{B/hi}$ largely lack or express intermediate levels of Aire, we hypothesized that they could either represent immediate precursors of Aire$^+$ mTECs (Pre-Aire) or cells completing their final stages of maturation (Post-Aire). Alternatively, mTEC$^{C/hi}$ could represent a subset with an independent lineage relationship to Aire$^+$ mTECs. The subsequent experiments were designed to address these possibilities.

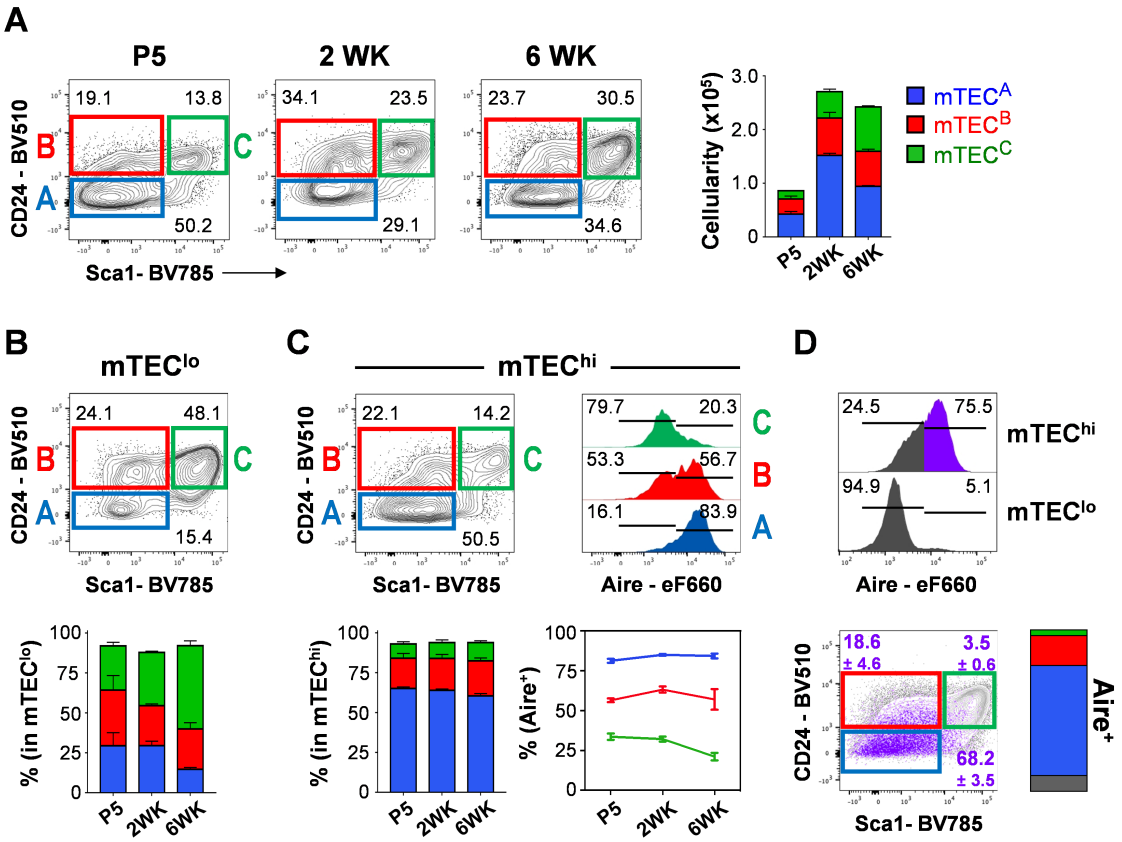


Figure 1 – The combined analysis of CD24 and Sca1 defines new mature mTEC subsets with distinct patterns of Aire expression. (A) Thymi from C57BL/6 mice were isolated at the indicated time points and cells were stained with PerCP-Cy5-conjugated anti-CD45.2, PE-conjugated anti-Ly51, APC-eFlour 780-conjugated anti-MHCII (I-A/I-E), eFlour 660-cojugated anti-AIRE, BV421-conjugated anti-EpCAM, BV650-conjugated anti-CD80, BV510-conjugated anti-CD24, BV786-conjugated anti-Sca1; Biotinylated UEA: BV711-conjugated streptavidin. Total mTECs (CD45$^-$EpCAM$^+$UEA$^+$) were subdivided into three subpopulations based on the expression pattern of CD24 and Sca1: mTEC^A (CD24$^-$Sca1$^-$, blue), mTEC^B (CD24$^+$Sca1$^-$, red) and mTEC^C (CD24$^+$Sca1$^+$, green). Bar graph displays the number of mTEC subsets at different time points. Representation of mTEC^A, mTEC^B and mTEC^C within mTEC^{lo} **(B)** and mTEC^{hi} **(C)** at different time points. (A-C) Data are representative of at least three experiments per time point with 2-4 mice per experiment. Numbers in plots indicate the frequency of cells found within each gate. Bar graph displays the frequency of mTEC^A, mTEC^B and mTEC^C within mTEC^{lo} and mTEC^{hi}, as mean plus SD. P – Postnatal day; WK – Postnatal weeks. Histograms in **(C)** show Aire expression within mTEC$^{A\text{-}C/hi}$ subsets. Numbers in plots indicate the frequency of cells found within each gate. Bar graph below displays the frequency of Aire$^+$ cells within mTEC$^{A\text{-}C/hi}$ subsets across time. **(D)** Aire expression in mTEC^{hi} (top) and mTEC^{lo} (bottom). The expression of CD24 and Sca1 was analysed on Aire$^+$ mTEC^{hi} (pseudo-coloured purple). Total mTECs were plotted in gray. Column chart illustrates the relative composition of mTEC$^{A/hi}$ (blue), mTEC$^{B/hi}$ (red) and mTEC$^{C/hi}$ (green) subsets within the total Aire$^+$ mTEC^{hi}. Data are representative of at least three experiments per time point with 2-4 mice per experiment.

The genetic program mTEC$^{A\text{-}C/hi}$ maps successive stages of Aire$^+$ mTEC differentiation.

To determine whether mTEC$^{B\text{-}C/hi}$ defined early or later stages of Aire$^+$ mTEC differentiation, we performed RNA-sequencing analysis of mTEC$^{A\text{-}C/hi}$, including mTEC$^{A\text{-}C/lo}$ as additional reference populations. The overall snap-shot of their transcriptome revealed that the biological replicates of each subset clustered together, emphasizing

that these populations had stable genetic programs and low intrapopulation variability (**Fig. 2A**). Secondly, mTEC$^{A\text{-}C/hi}$ primarily segregated from their mTEC^{lo} counterparts and presented the highest number of detected genes, indicating a presumable increased promiscuous gene expression capacity. Interestingly, mTEC$^{A/hi}$ and mTEC$^{B/hi}$ were more closely related to each other compared to mTEC$^{C/hi}$ (**Fig. 2A**). To further study the functional significance of mTEC$^{A\text{-}C/hi}$, we extracted a publicly available list of Aire-dependent and Aire-independent TRAs [13] and determined their expression pattern in the analysed subsets. We found that Aire-dependent and Aire-independent TRA genes were predominantly upregulated in mTEC$^{A/hi}$ and mTEC$^{B/hi}$, with Aire-dependent targets displaying the most marked augment in these subsets (**Fig. 2B and Supp. Table 1**). These observations are in line with the expression pattern of Aire in mTEC$^{A\text{-}B/hi}$ subsets (**Fig.1D**) and the TRA expression capacity of mature Aire^{+} mTECs [13].

Recent single-cell RNA-sequencing studies have reclassified mTECs into four subtypes, termed mTEC I-IV [14]. Using their available transcriptome and *in silico* analysis, we defined the top 200 genes specifically upregulated in mTEC I-IV (details under material and methods) and cross-examined how their expression oscillated in mTEC$^{A\text{-}C/lo\text{-}hi}$ subtypes. We found that the genetic signatures of mTEC I, mTEC II, mTEC III and mTEC IV were respectively augmented in mTEC$^{C/lo}$, mTEC$^{A/hi}$, mTEC$^{C/hi}$ and mTEC$^{B/hi}$. In contrast, mTEC$^{A/lo}$ and mTEC$^{B/lo}$ did not reveal a particular link to any of the mTEC I-IV subtypes (**Fig. 2C, Supp. Table 2**). While the molecular similarities between mTEC I:mTEC$^{C/lo}$ and mTEC II:mTEC$^{A/hi}$ pairs were in line with their mTEC$^{lo/hi}$ nature and the expression of Aire, the association between mTEC III:mTEC$^{C/hi}$ and mTEC IV:mTEC$^{B/hi}$ was intriguing. Since mTEC III and mTEC IV contained cells that expressed molecular features linked to Post-Aire cells [14], we examined the possibility that mTEC$^{B\text{-}C/hi}$ defined later stages of Aire^{+} mTEC differentiation. To do so, we curated recently published transcriptional profiles of Pre-, Early-, Late- and Post-Aire mTECs, which were originally obtained from the analysis of an Aire-Cre fate-mapping mouse model [15]. Following a similar strategy to the aforementioned, we deconvoluted the top 500 genes specifically enriched in Pre-, Early-, Late- and Post-Aire mTECs and surveyed their expression pattern in mTEC$^{A\text{-}C/lo\text{-}hi}$ subtypes (**Fig. 2D, Supp. Table 3**). As above, mTEC$^{A\text{-}B/lo}$ did not reveal a particular association to the analysed genetic profiles. We found that gene sets specifically associated to Pre- and Early-Aire mTECs were respectively upregulated in mTEC$^{C/lo}$ and mTEC$^{A/hi}$ subtypes. The genetic program of Late-Aire cells was detected across mTEC$^{A\text{-}C/hi}$, being mostly marked in mTEC$^{B/hi}$. Strikingly, specific genetic features of Post-Aire cells were upregulated in mTEC$^{B/hi}$ and mTEC$^{C/hi}$. Supportive of their Post-Aire nature, the expression of genes associated to Post-Aire cells, and shared by terminally differentiated keratinocytes: *Krt10* (keratin-10), *Ivl*

(Involucrin), *Dsg3* (Desmoglein-3), *Dsc1* (Desmocollin-1), *Asprv1* (Aspartic Peptidase Retroviral Like 1) and *Krt6a and Krt6b* [6-8, 10, 15], was progressively augmented in mTEC$^{B\text{-}C/hi}$. Contrarily, the expression of *Cd80*, *Cd86* and *Aire* was gradually downregulated from mTEC$^{A/hi}$ to mTEC$^{B\text{-}C/hi}$, replicating the dynamics observed in the transition from Early- to Late and Post-Aire stages (**Supp. Fig. 2**) [7, 10, 14-16]. Together, our genome-wide transcriptional analysis suggested the following developmental trajectory: mTEC$^{A/hi}$ (Aire^{+}; *i.e.,* Early-Aire) turn on CD24 and Sca1 expression during their transition to mTEC$^{B\text{-}C/hi}$ (Aire$^{+/-}$ to Aire^{-}; *i.e.,* Late to Post-Aire stages).

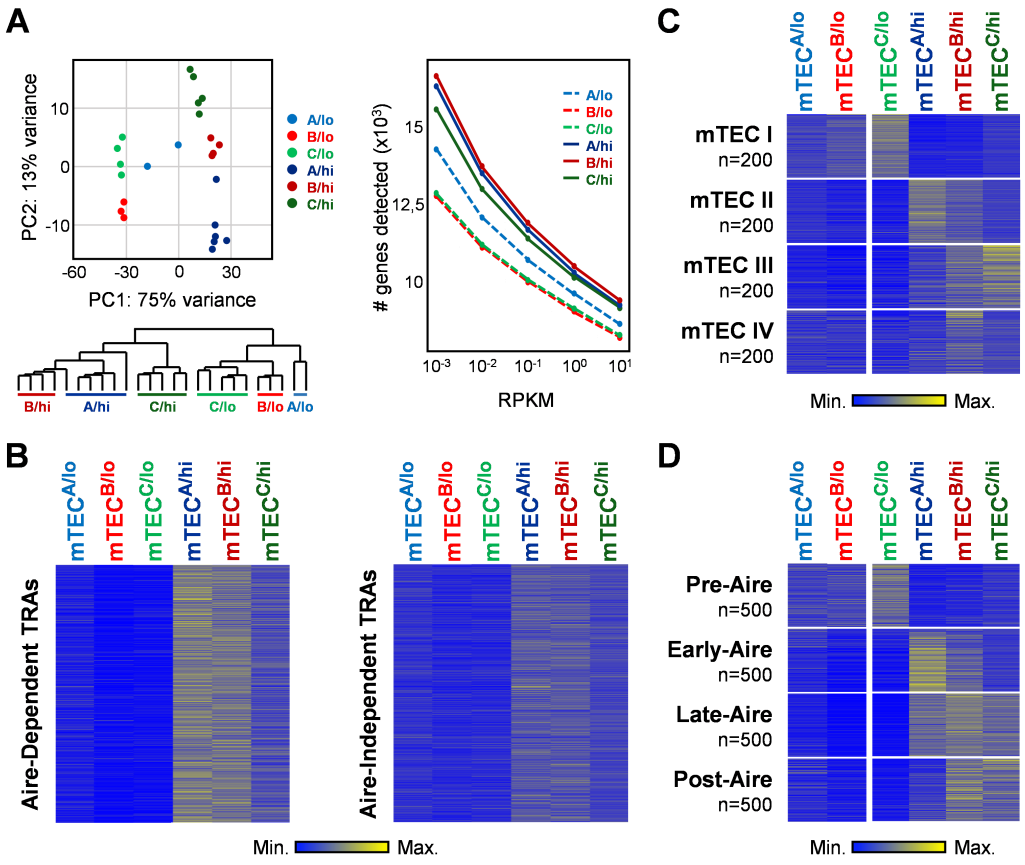


Figure 2 – Transcriptomic analysis of mTEC$^{A\text{-}C}$ within mTEC^{lo} and mTEC^{hi} and their relationship to the genetic profile of distinct stages of Aire^{+} mTEC differentiation. RNA-Seq analysis was performed on independently sorted biological replicates of mTEC$^{A/lo}$ (n=2), mTEC$^{B/lo}$ (n=3), mTEC$^{C/lo}$ (n=5), mTEC$^{A/hi}$ (n=6), mTEC$^{B/hi}$ (n=4) and mTEC$^{C/hi}$ (n=5). **(A)** Principal component analysis (PCA) and dendrogram shows the hierarchical clustering between the distinct biological samples; graph shows the total amount of genes detected in each subset. **(B)** Heat maps representing the deviation to the average expression of Aire-dependent (left) and Aire-independent (right) TRAs in mTEC$^{A\text{-}C/lo\text{-}hi}$. **(C)** Heat maps represent the deviation to the average expression of the top 200 specifically upregulated genes of the mTEC I-IV subsets in mTEC$^{A\text{-}C/lo\text{-}hi}$. **(D)** Heat maps represent the deviation to the average expression of the top 500 specifically upregulated genes of Pre-, Early-, Late- and Post-Aire subsets in mTEC$^{A\text{-}C/lo\text{-}hi}$.

mTEC$^{B/hi}$ and mTEC$^{C/hi}$ are absent in *Aire*-deficient mice.

The findings described above implicated that mTEC$^{B-C/hi}$ derived from Aire-expressing mTEC$^{A/hi}$. To study the precursor-product relationship, we purified (cell sorting) mTEC$^{A-C/hi}$ and determined their developmental potential using reaggregate thymus organ cultures (RTOCs). Sorted C57BL/6-derived mTEC subsets were mixed with BALB/c-derived embryonic thymic cells (carriers), and differences in MHCI expression (H2-K^b for C57BL/6 and H2-K^d for BALB/c) were used to trace their progeny within hybrid RTOCs (**Supp. Fig. 3A-B**). Since mTEC^{lo} contained precursors of Aire$^+$ mTEC^{hi} [2] and mTEC$^{C/lo}$ presented transcriptomic traits coupled to the pre-Aire stage (**Fig. 2D**)**,** we first evaluated the capacity of mTEC$^{A-C/lo}$, which lacked Aire, to contribute to the Aire lineage. Interestingly, both mTEC$^{B/lo}$ and mTEC$^{C/lo}$ subsets contained cells that were able to differentiate into mature mTEC^{hi}, including Aire$^+$ mTEC^{hi}. While some cells maintained their original mTEC$^{B-C/lo}$ phenotype, Aire$^+$ cells that developed under these conditions resided within the mTEC$^{A/hi}$ subset as their *in vivo* counterparts (**Supp. Fig. 3C**). We failed to detect the progeny of mTEC$^{A/lo}$ (data not shown). Further studies are required to deconstruct whether mTEC$^{B-C/lo}$ represent developmentally unrelated precursors or transitional stages of the same differentiation pathway, as well as their relationship to recently defined embryonic (*e.g.,* Cld3/4$^+$SSEA$^-$RANK) and post-natal (Podoplanin (PDPN)$^+$) mTEC-restricted precursors [17]. Still, our results are in line with the observations that Aire$^+$ mTEC precursors (*e.g.,* mTEC$^{B-C/lo}$) downregulate CD24 expression during their differentiation into Aire$^+$ cells [18].

Relatively to the developmental potential of mTEC$^{A-C/hi}$ subsets, we found that a large fraction of mTEC$^{A/hi}$ remained mTEC^{hi} and either lacked or maintained Aire expression. While Aire$^+$ generally maintained their mTEC$^{A/hi}$ phenotype, emerging Aire$^-$ contained cells that acquired mTEC$^{B-C/hi}$ traits (**Supp. Fig. 3D**). Yet, we failed to detect the progeny of mTEC$^{B-C/hi}$ (data not shown), suggesting that these *ex vivo* isolated subtypes had a poor survival in RTOCs. We infer that the reduced ability to engraft and thrive in RTOCs may be related to their terminal maturation stage. Albeit our data suggest a linear relationship between mTEC$^{A/hi}$ and mTEC$^{B-C/hi}$, hybrid RTOCs experiments have limitations related with the integration and sensitivity of detection of "spiked" mature mTECs and their competition with embryonic-derived carrier cells. Thus, we undertook a complementary *in vivo* genetic approach to provide further mechanistic evidence that mTEC$^{B-C/hi}$ represent end-stages of Aire$^+$ mTEC maturation.

Apart from its role in regulating the expression of TRAs, Aire expression regulates mTEC differentiation [9, 19-21]. This notion is supported by the skewed $mTEC^{lo}/mTEC^{hi}$ ratio, in favour of $mTEC^{hi}$, and the absence of Post-Aire cells in $Aire^{-/-}$ mice [9, 14, 15, 20, 22]. To study whether the homeostasis of $mTEC^{B\text{-}C/hi}$ was Aire-dependent, we compared their frequency in control and $Aire^{-/-}$ mice. As expected, $Aire^{-/-}$ mice presented an accumulation of $mTEC^{hi}$. Interestingly, the $mTEC^{hi}$ compartment of *Aire*-deficient mice was mostly composed of $mTEC^{A/hi}$ and virtually lacked $mTEC^{B/hi}$ and $mTEC^{C/hi}$ subsets (**Fig. 3A**). We consider that the absence of $mTEC^{B\text{-}C/hi}$ does not result from a defective expression of CD24 and Sca1 in *Aire*-deficient mice. CD24 and Sca1 have been reported as Aire-independent genes [13] and CD24 and Sca1 were expressed in $mTEC^{B\text{-}C/lo}$. Instead, we favour that the accumulation of $mTEC^{A/hi}$ in *Aire*-deficient mice define mature cells, which immediately preceded Aire expression but ceased their differentiation at this stage as a result of the lack of Aire protein. Lastly, the absence of $mTEC^{B\text{-}C/hi}$ (Post-Aire) under *Aire*-deficient conditions further supports the notion that Aire controls mTEC differentiation.

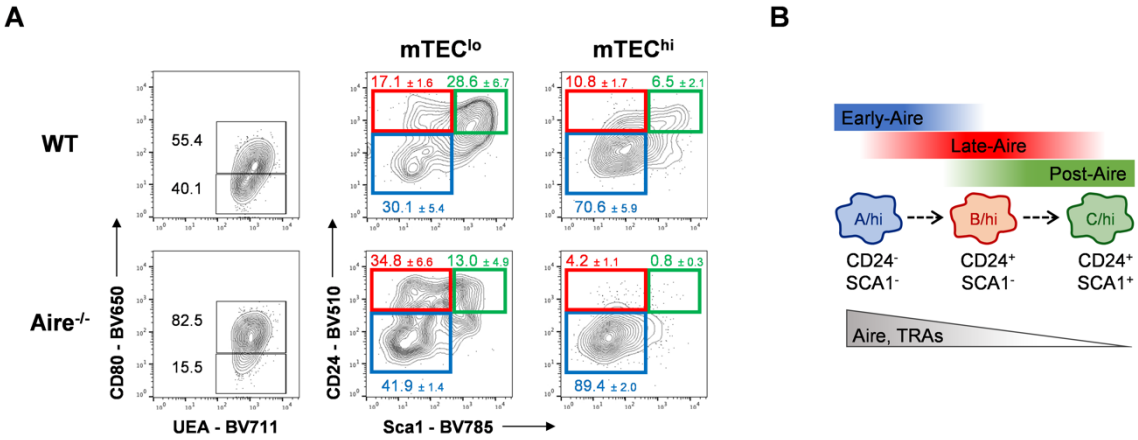


Figure 3 – Altered $mTEC^{A\text{-}C/hi}$ composition in *Aire*-deficient mice. (A) Thymic cells were stained with PerCP-Cy5-conjugated anti-CD45.2, PE-conjugated anti-Ly51, APC-eFlour 780-conjugated anti-MHCII (I-A/I-E), BV421-conjugated anti-EpCAM, BV650-conjugated anti-CD80, BV510-conjugated anti-CD24, BV786-conjugated anti-Sca1; Biotinylated UEA: BV711-conjugated streptavidin. Total mTECs ($CD45^{-}EpCAM^{+}UEA^{+}$) from 6-week-old $Aire^{-/-}$ and control (WT) C57BL/6 mouse thymi were analysed for the expression of CD80, and $mTEC^{A\text{-}C}$ subsets were respectively identified with $mTEC^{lo}$ and $mTEC^{hi}$. Plots shown are of a representative analysis out of three independent experiments with 1-2 mice of each genotype per experiment. Frequencies for $mTEC^{A\text{-}C}$ populations are shown as a mean plus SD. **(B)** Proposed model for the phenotypic discrimination of Early-, Late, and Post-Aire stages of mTEC differentiation.

CONCLUDING REMARKS

Our understanding of the role of Aire^{+} mTECs has considerably improved in the last years. Albeit the expression of Aire was initially considered to mark a terminal stage of mTEC maturation, several studies have shown that mTECs extend their differentiation beyond Aire (Post-Aire) [19]. The most recent evidence of the complexity of mTEC cartography is the identification of tuft-like mTECs, some of which progress through an Aire-expressing stage before terminating Aire expression [14, 15]. The isolation of Post-Aire cells has been hampered by the paucity of a reliable set of cell surface markers to define them. To date, the identification of these dedicated mTEC populations depends on lineage-tracing systems and complex single-cell RNA-sequencing approaches, which are neither always easily accessible nor compatible with functional analysis. Our study solves this conundrum, offering an efficient and novel phenotypic chart for the direct analysis of Early-, Late- and Post-Aire stages of mTEC maturation. To our knowledge, the simultaneous analysis of CD24 and Sca1 expression within mTECs provides the first mean to prospectively segregate later stages of Aire^{+} mTEC differentiation. Firstly, our observation that mTEC$^{C/hi}$ mirror cells in a Post-Aire stage are consistent with the genome-wide molecular signature of Post-Aire mTECs obtained from the analysis of fate-mapping mouse models [6, 8, 9]. Secondly, the expression of Aire-dependent TRAs was downregulated in mTEC$^{C/hi}$ compared to mTEC$^{A\text{-}B/hi}$, an observation that matches the pattern of Aire expression in mTEC$^{C/hi}$ (Aire^{-}). Lastly, mTEC$^{C/hi}$ express markers linked to terminally differentiated keratinocytes and depend on Aire. Although Post-Aire cells terminate their differentiation process as mTEC^{lo}, our populational RNAseq analysis was not able to dissect if those cells are diluted into a specific mTEC$^{A\text{-}C/lo}$ subset. Still, as the majority of cells that lost Aire expression downregulate MHCII and CD80 but remain as mTEC^{hi} [8] or reside at the mTEC$^{hi/lo}$ boundary [6, 9], we reason that our phenotypic definition (CD24^{+}Sca1^{+}MHCII^{+}CD80^{+}) enables the prospective isolation of a large fraction of Post-Aire cells. Hence, we propose that mTEC$^{A/hi}$-mTEC$^{B/hi}$-mTEC$^{C/hi}$ sequentially mark transitional steps of Aire^{+} mTEC differentiation into a Post-Aire stage (**Fig. 3B**). Our study provides an integrative road map that will prove of general interest to survey the dynamic of the medullary microenvironment in several experimental and autoimmune conditions.

MATERIAL AND METHODS

Mice

C57BL/6 mice were housed under specific pathogen-free conditions at i3S' animal facility. Aire$^{-/-}$ mice were described previously [22] and housed at the animal facility of Tokushima University. Experiments were performed under the European guidelines for animal experimentation.

Isolation and flow cytometry analysis of TECs

Thymi were dissected at the indicated time points and TEC isolation and staining was performed as previously described [23]. The following antibodies were used for flow cytometry analysis of isolated cells: 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) and eFlour 660-cojugated anti-AIRE (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). Flow cytometry analyses were performed on a LSRFortessa and cells sorted on a FACS ARIA II (both from BD Bioscience) with purities above 95%. Data were analysed on FlowJo software (Tree Star Inc). Our study was conducted according to the guidelines for the use of flow cytometry and cell sorting in immunological studies [24].

RNA-sequencing

Total RNA library preparation and high-throughput sequencing of sorted postnatal (P5) mTEC^{A-C} subsets were performed at Gene Core facility (EMBL, Germany), as previously described [23]. Thirteen sequencing libraries (three for mTEC$^{A/lo}$, five for mTEC$^{B/lo}$, two for mTEC$^{C/lo}$, four for mTEC$^{A/hi}$, five for mTEC$^{B/hi}$ and six for mTEC$^{C/hi}$) were prepared using NEB Next RNA ultra protocol (#E7530 NEB). Obtained libraries were quantified fluorimetrically, pooled in equimolar amounts and sequenced on the Illumina NextSeq sequencer (NextSeqHi-75) in single-end mode (75 bases), following manufacturer's instructions (Illumina). Sequencing reads were submitted to ENA (http://www.ebi.ac.uk/ena) and are accessible under the accession number PRJEB32793. The reads were mapped to the mouse genome (mm10) using STAR (version 2.4.2a) with mm10 GTF annotation. The number of reads per gene was

generated during alignment step (quantMode GeneCounts) and gene counts were then analysed with DESeq2 package [25]. Reads per kb of genes per million mapped reads (RPKM) values were computed from normalized read counts. Gene ontology (GO) enrichment analysis was done using model-based gene set analysis (MGSA) [26].

In silico comparative transcriptomic analyses

To obtain the deviation to average expression in mTEC I, mTEC II, mTEC III and mTEC IV, we calculated the mean of the deviation to the average expression in clusters X3-X18, X19-X28, X29-33 and X34-36 [14]. To determine the gene sets specifically upregulated in mTEC I-IV, we further identified the top 200 most upregulated genes from each population considering a 2-fold increase relatively to the remaining three. To acquire the gene sets specifically upregulated in Early-, Pre-, Late- and Post-Aire mTECs [15], we calculated the deviation of expression of each gene in each subset (mean of the four biological replicates) relatively to the average expression across all samples. To identify the top 500 most upregulated genes in each subpopulation, we considered genes with an expression >10 rpkm and genes in which the upregulation in the selected population is specifically (at least) 2-fold higher relatively to any of the other three.

Reaggregate thymus organ culture (RTOC)

Reaggregate thymus organ cultures (RTOCs) were established as previously described [27] by combining $7x10^5$ total thymic cells from E14.5 BALB/c thymus and 2-$3x10^4$ sorted mTECs ($EpCAM^+CD45^-UEA^+$) of $mTEC^{A\text{-}C/lo\text{-}hi}$ subsets obtained from newborn C57BL/6 thymic lobes. After 7 days in culture, RTOCs were dissociated and analysed by flow cytometry.

Author Contributions

N.L.A. conceived and performed experiments, wrote the manuscript, and secured funding, P.F and C.R, conceived and performed experiments and wrote the manuscript, J.M. J.J.M.L., C.M., L.A. performed experiments, and M.M., V.B., M.M. provided scientific expertise and feedback.

Acknowledgements

This work was supported by a starting grant from the European Research Council (ERC) under the project 637843 and by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior in the framework of the project POCI-01-0145-FEDER-029129 (PTDC/MED-IMU/29129/2017). N.L.A is supported by the FCT program “Scientific Employment Stimulus”. M.M. is supported by the Japanese Agency for Medical Research and Development-Core Research for Evolutional Science and Technology (JSPS KAKENHI Grant Numbers JP16K21731, JP16H06496 and JP16H05342). We thank Drs. Sofia Lamas and the caretakers from the animal facility for technical assistance. We thank Dr. Pedro Rodrigues for discussions and critical reading.

Conflict of interest

The authors have no commercial or financial conflict of interests.

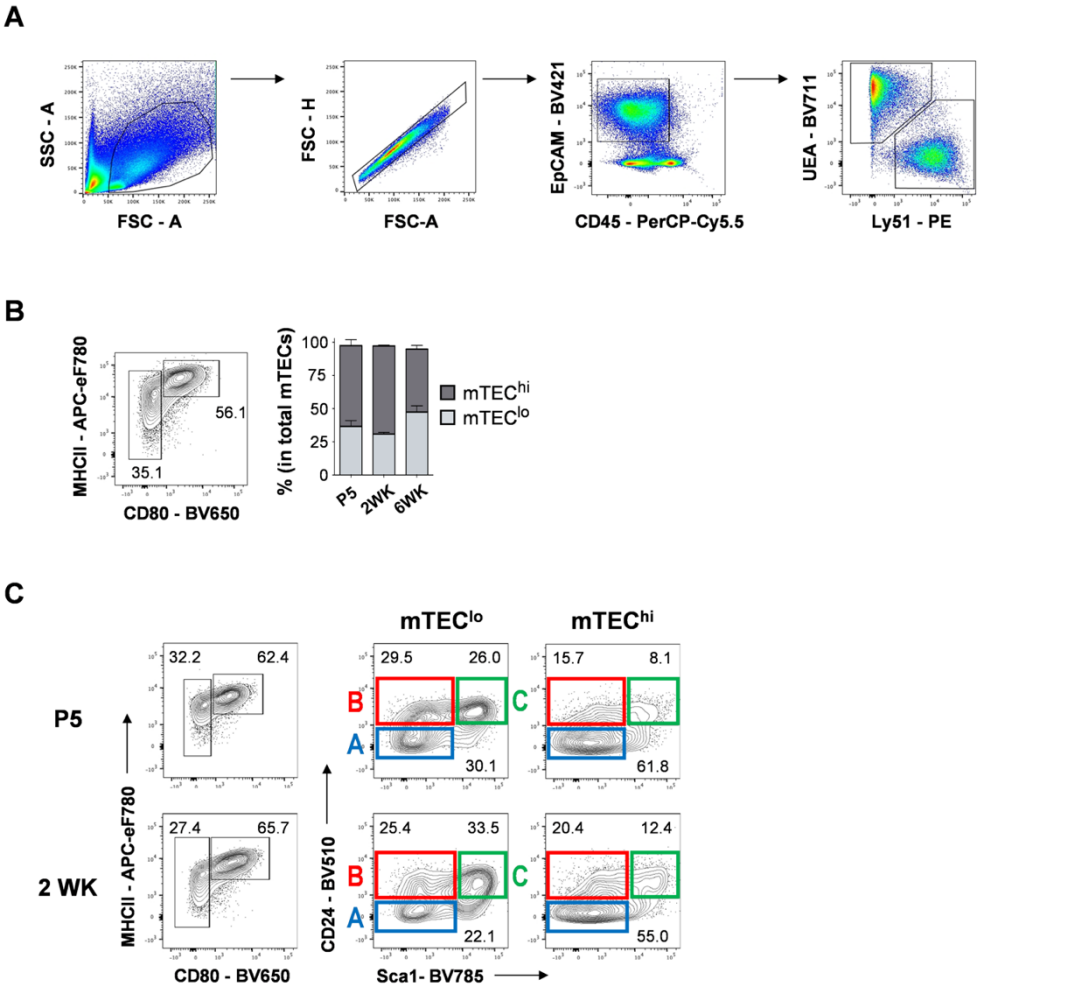
REFERENCES

1. Alves, N.L.*, et al.*, 2014, *Serial progression of cortical and medullary thymic epithelial microenvironments,* Eur J Immunol, **44**(1), 16-22.
2. Anderson, G. and Takahama, Y., 2012, *Thymic epithelial cells: working class heroes for T cell development and repertoire selection,* Trends Immunol, **33**(6), 256-63.
3. Klein, L.*, et al.*, 2014, *Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see),* Nat Rev Immunol, **14**(6), 377-91.
4. Abramson, J. and Anderson, G., 2017, *Thymic Epithelial Cells,* Annu Rev Immunol, **35**, 85-118.
5. Gray, D.*, et al.*, 2007, *Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire,* J Exp Med, **204**(11), 2521-8.
6. Nishikawa, Y.*, et al.*, 2010, *Biphasic Aire expression in early embryos and in medullary thymic epithelial cells before end-stage terminal differentiation,* J Exp Med, **207**(5), 963-71.
7. White, A.J.*, et al.*, 2010, *Lymphotoxin signals from positively selected thymocytes regulate the terminal differentiation of medullary thymic epithelial cells,* J Immunol, **185**(8), 4769-76.
8. Metzger, T.C.*, et al.*, 2013, *Lineage tracing and cell ablation identify a post-aire-expressing thymic epithelial cell population,* Cell Rep, **5**(1), 166-79.
9. Nishikawa, Y.*, et al.*, 2014, *Temporal lineage tracing of Aire-expressing cells reveals a requirement for Aire in their maturation program,* J Immunol, **192**(6), 2585-92.
10. Wang, X.*, et al.*, 2012, *Post-Aire maturation of thymic medullary epithelial cells involves selective expression of keratinocyte-specific autoantigens,* Front Immunol, **3**(March), 19.
11. Shackleton, M.*, et al.*, 2006, *Generation of a functional mammary gland from a single stem cell,* Nature, **439**(7072), 84-8.
12. Holmes, C. and Stanford, W.L., 2007, *Concise review: stem cell antigen-1: expression, function, and enigma,* Stem Cells, **25**(6), 1339-47.
13. Sansom, S.N.*, et al.*, 2014, *Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia,* Genome Res, **24**(12), 1918-31.
14. Bornstein, C.*, et al.*, 2018, *Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells,* Nature, **559**(7715), 622-626.

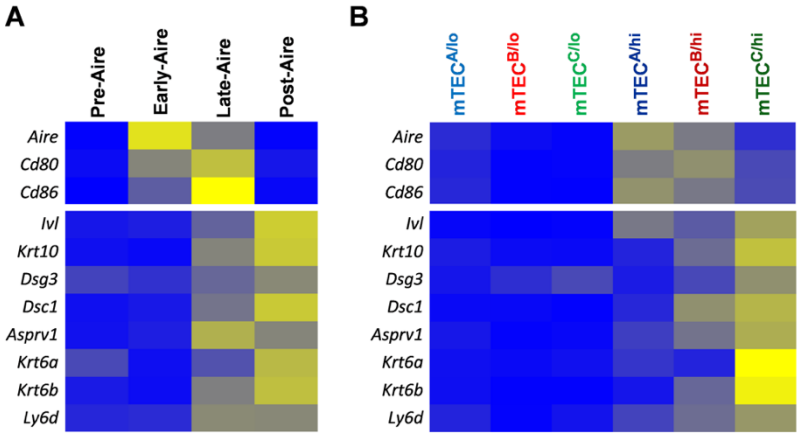
15. Miller, C.N.*, et al.*, 2018, *Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development,* Nature, **559**(7715), 627-631.
16. Wang, J.*, et al.*, 2019, *Hassall's corpuscles with cellular-senescence features maintain IFNalpha production through neutrophils and pDC activation in the thymus,* Int Immunol, **31**(3), 127-139.
17. Alves, N.L. and Ribeiro, A.R., 2016, *Thymus medulla under construction: Time and space oddities,* Eur J Immunol, **46**(4), 829-33.
18. Akiyama, N.*, et al.*, 2016, *Identification of embryonic precursor cells that differentiate into thymic epithelial cells expressing autoimmune regulator,* J Exp Med, **213**(8), 1441-58.
19. Matsumoto, M., 2011, *Contrasting models for the roles of Aire in the differentiation program of epithelial cells in the thymic medulla,* Eur J Immunol, **41**(1), 12-7.
20. Yano, M.*, et al.*, 2008, *Aire controls the differentiation program of thymic epithelial cells in the medulla for the establishment of self-tolerance,* J Exp Med, **205**(12), 2827-38.
21. Gillard, G.O.*, et al.*, 2007, *Aire-dependent alterations in medullary thymic epithelium indicate a role for Aire in thymic epithelial differentiation,* J Immunol, **178**(5), 3007-15.
22. Kawano, H.*, et al.*, 2015, *Aire Expression Is Inherent to Most Medullary Thymic Epithelial Cells during Their Differentiation Program,* J Immunol, **195**(11), 5149-58.
23. Rodrigues, P.M.*, et al.*, 2017, *Thymic epithelial cells require p53 to support their long-term function in thymopoiesis in mice,* Blood, **130**(4), 478-488.
24. Cossarizza, A.*, et al.*, 2019, *Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition),* Eur J Immunol, **49**(10), 1457-1973.
25. Love, M.I.*, et al.*, 2014, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2,* Genome Biol, **15**(12), 550.
26. Bauer, S.*, et al.*, 2010, *GOing Bayesian: model-based gene set analysis of genome-scale data,* Nucleic Acids Res, **38**(11), 3523-32.
27. Ribeiro, A.R.*, et al.*, 2013, *Thymocyte Selection Regulates the Homeostasis of IL-7-Expressing Thymic Cortical Epithelial Cells In Vivo,* J Immunol, **191**(3), 1200-9.

SUPPLEMENTARY INFORMATION

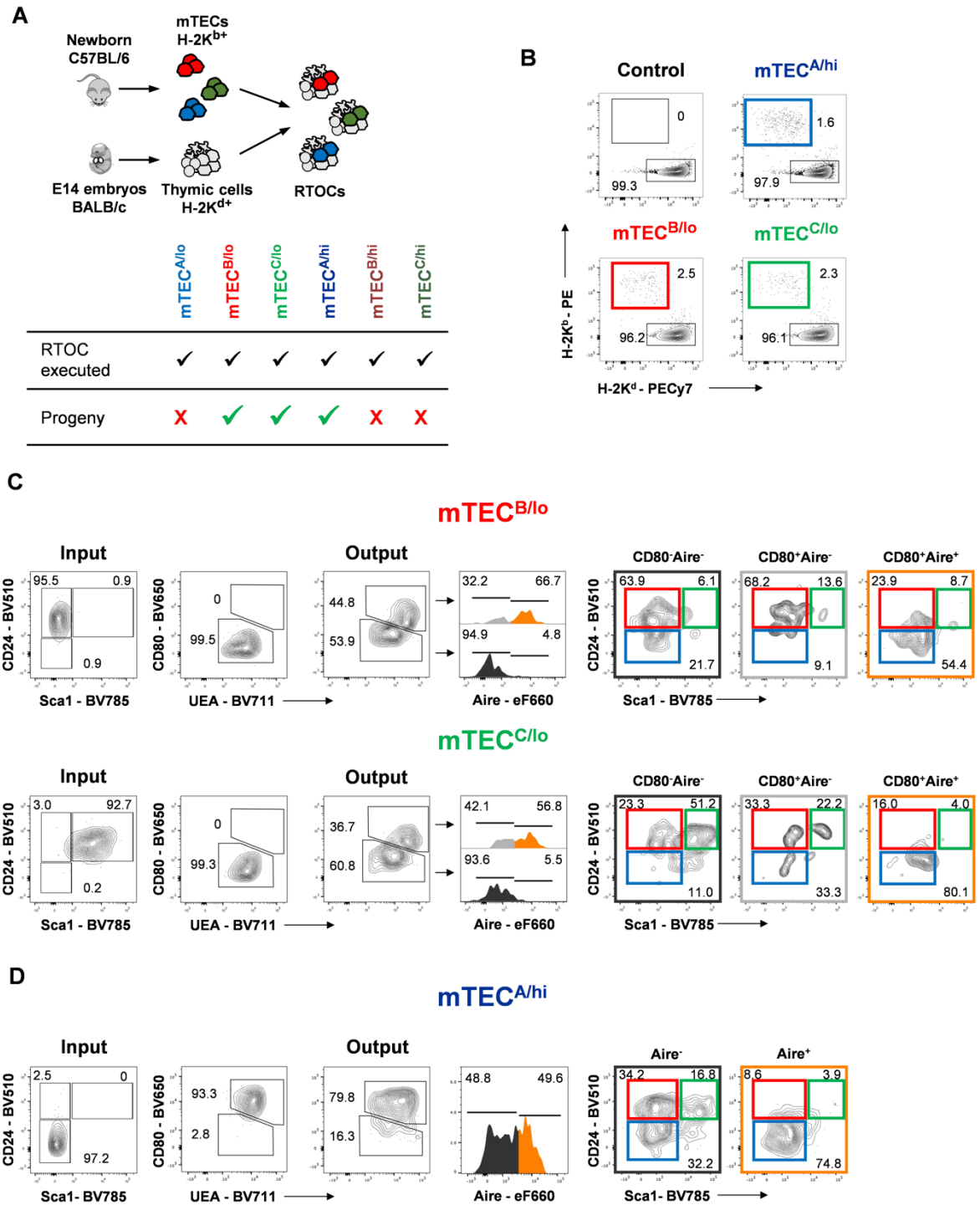
Supplementary tables are available on the online version of this article.



Supplementary figure 1 – (A) Flow cytometry analysis and gating strategy used to identify TECs ($CD45^-EpCAM^+MHCII^+$) and mTECs (UEA^+Ly51^-). Cells were stained with PerCP-Cy5-conjugated anti-CD45.2, PE-conjugated anti-Ly51, APC-eFlour 780-conjugated anti-MHCII, BV421-conjugated anti-EpCAM, BV650-conjugated anti-CD80, BV510-conjugated anti-CD24, BV786-conjugated anti-Sca1, Biotinylated UEA: BV711-conjugated streptavidin. **(B)** Thymi from C57BL/6 mice were isolated at the indicated time points. Total mTECs ($CD45^-EpCAM^+UEA^+$) were analysed for CD80 and MHCII expression and subdivided in $mTEC^{lo}$($MHCII^{lo}CD80^{lo}$) and $mTEC^{hi}$ ($MHCII^{hi}CD80^{hi}$). Bar graphs correspond to the frequency of $mTEC^{lo}$ and $mTEC^{hi}$ at different time points and are displayed as mean plus SD. **(C)** Representation of $mTEC^{A-C}$ subsets within $mTEC^{lo}$ and $mTEC^{hi}$. Representative data from three independent experiments per time point. Numbers in plots indicate the frequency of cells found within each gate. P – Postnatal day; WK – Postnatal weeks.



Supplementary figure 2 – Heatmap showing the deviation to the average expression level of selected genes associated with post-Aire mTECs in Pre-, Early-, Late- and Post-Aire cells (as defined in [15] and in mTEC$^{A\text{-}C/lo\text{-}hi}$.



Supplementary figure 3 – Reaggregate thymus organ cultures (RTOCs) with discrete mTEC$^{A\text{-}C/hi}$ subsets. (A) Schematic outlines of the established chimeric RTOCs. Table depicts the success of recovering spiked populations and their progeny. RTOCs were established by spiking the 6 mTEC$^{A\text{-}C/hi\text{-}lo}$ subsets isolated from P3 C57BL/6 mice into E14 total thymic cells from BALB/c mice. Cells were stained with PerCP-Cy5-conjugated anti-CD45.2, PE-conjugated anti-H-2Kb, PE-Cy7-conjugated anti-H-2Kd, BV421-conjugated anti-EpCAM, eFlour 660-cojugated anti-AIRE, BV650-conjugated anti-CD80, BV510-conjugated anti-CD24, BV786- conjugated anti-Sca1, Biotinylated UEA: BV711-conjugated streptavidin. **(B)** Detection of mTEC$^{A/hi}$ (blue), mTEC$^{B/lo}$ (red) and mTEC$^{C/lo}$ (green) cells isolated from P3 C57BL/6 mice (H-2Kb) in 7-day-old RTOCs established with fetal thymus cells from BALB/c mice (H-2Kd). **(C)** Lineage potential of mTEC$^{B/lo}$ and mTEC$^{C/lo}$. Input: purity assessment on day 0; Output: After 7 days in culture, RTOCs were dissociated and the progeny of C57BL/6-derived mTECs was analysed for the expression of

CD24, Sca1, CD80 and UEA binding in total mTEC population ($CD45^-EpCAM^+UEA^+$). Histograms depict the expression of Aire in $mTEC^{lo/hi}$ cells that derived from $mTEC^{B\text{-}C/lo}$ subsets after culture (output). $Aire^-$ and $Aire^+$ cells were further analysed for the expression of CD24 and Sca1. Data corresponds to a representative analysis of at least three experiments. **(D)** Lineage potential of $mTEC^{A/hi}$. Input: purity assessment on day 0; Output: After 7 days in culture, RTOCs were dissociated and the progeny of C57BL/6-derived mTECs was analysed for the expression of CD80, UEA binding and Aire in total mTEC population ($CD45^-EpCAM^+UEA^+$). $Aire^-$ and $Aire^+$ cells that derived from $mTEC^{A/hi}$ after culture were further analysed for the expression of CD24 and Sca1. Data corresponds to a representative analysis of at least three experiments.

CHAPTER IV - FOXO3 REGULATES CORTICAL AND MEDULLARY THYMIC EPITHELIAL CELL HOMEOSTASIS WITH IMPLICATIONS IN T-CELL DEVELOPMENT

Camila Ribeiro[1,2], Pedro Ferreirinha[1,2], Jonathan J. M. Landry[3], Fátima Macedo[1,2,4], Laura Sousa[1,2], Rute Pinto[1,2], Vladimir Benes[3], Nuno Alves[1,2]

[1] i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal;
[2] IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal;
[3] Genomics Core Facility, European Molecular Biology Laboratory, Heidelberg, Germany;
[4] Departamento de Ciências Médicas, Universidade de Aveiro, Portugal;

Cell Death & Disease
2024
doi: 10.1038/s41419-024-06728-0.

ABSTRACT

Within the thymus, thymic epithelial cells (TECs) create dedicated microenvironments for T-cell development and selection. Considering that TECs are sensitive to distinct pathophysiological conditions, uncovering the molecular elements that coordinate their thymopoietic role has important fundamental and clinical implications. Particularly, medullary thymic epithelial cells (mTECs) play a crucial role in central tolerance. Our previous studies, along with others, suggest that mTECs depend on molecular factors linked to genome-protecting pathways, but the precise mechanisms underlying their function remain unknown. These observations led us to examine the role of FOXO3, as it is expressed in TECs and involved in DNA damage response. Our findings show that mice with TEC-specific deletion of *Foxo3* (FOXO3^{cKO}) displayed a disrupted mTEC compartment, with a more profound impact on the numbers of CCL21^{+} and thymic tuft mTEC^{lo} subsets. At the molecular level, FOXO3 controls distinct functional modules in the transcriptome of cTECs and mTECs under normal conditions, which includes the regulation of ribosomal biogenesis and DNA damage response, respectively. These changes in the TEC compartment resulted in a reduced total thymocyte cellularity and specific changes in regulatory T cell and iNKT cell development in the FOXO3^{cKO} thymus. Lastly, the thymic defects observed in adulthood correlated with mild signs of altered peripheral immunotolerance in aged FOXO3^{cKO} mice. Moreover, the deficiency in *Foxo3* moderately aggravated the autoimmune predisposition observed in *Aire*-deficient mice. Our findings highlight the importance of FOXO3 in preserving the homeostasis of TECs and in supporting their role in T-cell development and tolerance.

INTRODUCTION

Within the thymus, the development of immunologically competent and self-tolerant T cells relies on inductive signals provided by specialized microenvironments constituted by thymic epithelial cells (TECs) [1]. Notably, deficits in TEC function arise with age, infection and cancer cytoablative chemotherapies, resulting in reduced T-cell production and skewed T-cell receptor (TCR) repertoire. Such failures in TEC function contribute to poor T-cell responses and immune surveillance or may also promote autoimmunity, thereby increasing morbidity and mortality in the elderly and in patients undergoing bone marrow transplantation or receiving anticancer treatments [2]. Thus, understanding the molecular mechanisms controlling TEC homeostasis is essential for developing novel therapeutic strategies that can enhance immune reconstitution or correct insufficient T-cell responses.

The thymic epithelium is broadly subdivided into cortical (cTEC) and medullary (mTEC) subsets, which arise from common bipotent TEC progenitors (TEPs) [3]. While cTECs promote T-cell lineage commitment and positive selection, mTECs regulate the elimination of self-reactive thymocytes via negative selection or their deviation into regulatory T cells. The crucial role of mTECs in tolerance induction is, in part, attributed to their ability to promiscuously express tissue-restricted antigens (TRAs) [3]. The complex molecular mechanisms governing mTEC function are under intense scrutiny and depend, to some extent, on the autoimmune regulator (Aire). Studies have suggested that Aire interacts with proteins involved in DNA damage response, leading to the induction of double-strand DNA breaks and facilitating the transcription of TRA-encoding genes [4]. Interestingly, we previously showed that p53, a well-known regulator of the DNA damage response, serves as a guardian of mTECs and their function in T-cell development and self-tolerance [5]. These findings suggest the possibility that promiscuous gene expression of TRAs in mTECs leads to genomic instability, which requires protecting mechanisms to preserve the integrity of the medullary microenvironment. Yet, the pathways controlling the balance of the mTEC niche under physiological conditions are still not fully understood.

In this study, we sought to identify novel transcription factors involved in the DNA damage pathway, which might protect the genomic integrity of mTECs while maintaining their role in thymopoiesis. Particularly, we focused on investigating the role of forkhead box O 3 (FOXO3) in TECs. FOXO3 is a member of the FOXO family of transcriptions factors, which also includes FOXO1, FOXO4 and FOXO6. FOXO proteins have a pleiotropic role in several cellular processes, including survival, proliferation and metabolism [6]. In particular, the *Foxo3* gene has been implicated in increased human

longevity and a reduced risk of age-related diseases. FOXO3 is believed to contribute to longevity by regulating responses to various stress signals, including cell cycle arrest, DNA damage and oxidative stress [7]. In this study we examine the link between FOXO3 and the homeostasis of the TEC microenvironment, and its role in T-cell development.

RESULTS

The deletion of *Foxo3* in TECs leads to a reduction in the mTEC compartment.

The observation that p53 controls mTEC homeostasis prompted us to search for novel transcription factors involved in p53-mediated DNA damage response. To do so, we examined transcriptomic data sets from cTECs and mTECs of the 2-week-old thymus [5, 8] (**Figure S1A**). Given the high expression of *Foxo3* in TECs, we sought to determine its role in TEC differentiation and function. To specifically delete *Foxo3* in TECs, we developed a FOXO3 conditional KO model by crossing mice carrying loxP-flanked *Foxo3* alleles ($FOXO3^{fl/fl}$) [9] with mice expressing the Cre recombinase under the control of the *Foxn1* promoter ($FOXN1^{Cre}$) [10]. $FOXN1^{Cre}FOXO3^{fl/fl}$ mice ($FOXO3^{cKO}$) were born without any apparent abnormalities and showed a similar development to their control $FOXO3^{fl/fl}$ littermates ($FOXO3^{Ctr}$). PCR analysis of genomic DNA showed that the *Foxo3* floxed allele was deleted in TECs from $FOXO3^{cKO}$ thymus, but not in thymocytes. Moreover, the *Foxo3* floxed allele was not deleted in TECs from $FOXO3^{Ctr}$ thymus (**Figure S1B-C**). These results suggest that *Foxo3* expression is successfully inactivated in TECs from $FOXO3^{cKO}$ mice.

We began by comparing the thymic epithelium composition of $FOXO3^{Ctr}$ and $FOXO3^{cKO}$ mice during key time points of postnatal life. Although unaltered in the postnatal day 4 thymus, the total number of TECs was significantly reduced in the 2- and 10-week-old $FOXO3^{cKO}$ thymus (**Figure S1D**). Despite moderate variations in the frequency of cTECs/mTECs, all major TEC subsets, including cTECs, $mTEC^{lo}$, $mTEC^{hi}Aire^{-}$ and $mTEC^{hi}Aire^{+}$, were detected in the thymus of $FOXO3^{cKO}$ at postnatal day 4 (**Figure 1A, Figure S1E and data not shown**). These results indicated that the first stages of TEC differentiation appeared to proceed normally in the absence of FOXO3. Notably, the number of mTECs, but not cTECs, became significantly reduced in the $FOXO3^{cKO}$ thymus from 2-week-old onwards (**Figure 1A**). Further analysis of $MHCII^{low}CD40^{low}$ ($cTEC^{lo}$) and $MHCII^{hi}CD40^{hi}$ ($cTEC^{hi}$) subsets showed no differences between $FOXO3^{Ctr}$ and $FOXO3^{cKO}$ in the adult thymus (**Figure S1F**). In contrast, the adult mTEC compartment of the $FOXO3^{cKO}$ thymus showed marked alterations in the frequency of $MHCII^{low}CD80^{low}$ ($mTEC^{lo}$) and $MHCII^{hi}CD80^{hi}$ ($mTEC^{hi}$) subsets, leading to a more pronounced reduction in the numbers of the $mTEC^{lo}$ population (**Figure 1B**). We next examined the composition of functionally distinct subsets included within $mTEC^{hi}$ ($Aire^{+}$) and $mTEC^{lo}$ ($CCL21^{+}$ and thymic tuft cells) populations [3]. Although there were differences in the frequency of $mTEC^{hi}$, the fraction of the $Aire^{+}$ subset within $mTEC^{hi}$ was comparable between $FOXO3^{Ctr}$ and $FOXO3^{cKO}$ groups (**Figure 1C**). In contrast, concomitant to a reduction in the frequency of $mTEC^{lo}$, the percentages of $CCL21^{+}$ and

tuft-like DCLK1^{+} cells within mTEC^{lo} were further diminished in the mutant thymus. Thus, despite the reduced cellularity of Aire^{+} cells, the deficiency in *Foxo3* led to a more significant decrease in the number of mTEC^{lo} subsets (**Figure 1D-E**). The histological analysis of adult FOXO3^{cKO} thymi showed reduced overall thymic areas, while the cortex and medulla areas appeared to segregate normally. Despite the relative proportion of the medullary area to the total thymic area found in the FOXO3^{cKO} thymus being comparable to that in controls, its structural organization showed a reduced number of medullary islets (**Figure S1G**). These results suggest that a FOXO3-dependent pathway is essential for maintaining normal homeostasis of the mTEC compartment.

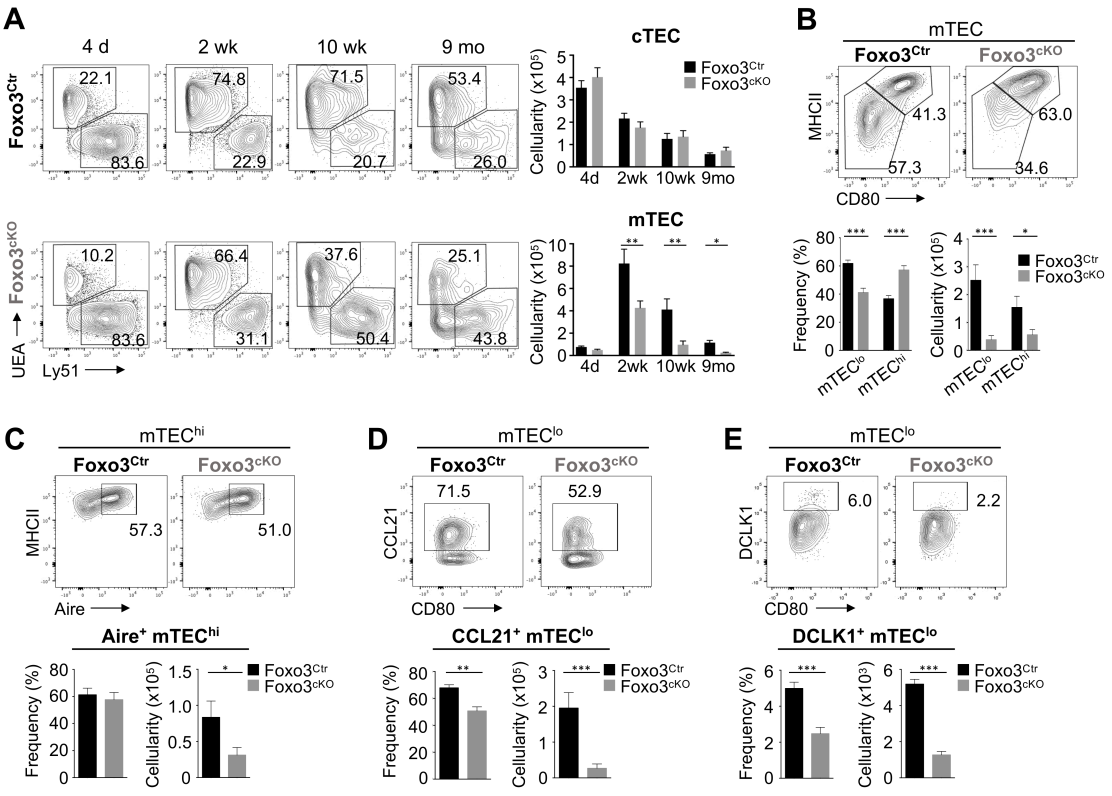


Figure 1 – FOXO3 supports the size and functional diversity of the mTEC compartment. (A) Thymi were isolated from FOXO3^{Ctr} (FOXO3$^{fl/fl}$) and FOXO3^{cKO} (FOXN1^{Cre}:FOXO3$^{fl/fl}$) mice at the indicated time points. TECs (CD45^{-}EpCAM^{+}) were analysed for cTEC (Ly51^{+}) and mTEC (UEA^{+}) composition. Bar graphs show absolute cell numbers. **(B)** mTEC^{lo} (MHCII^{low}CD80^{low}) and mTEC^{hi} (MHCII^{high}CD80^{high}) composition within total mTECs of the 10-week-old adult thymus. **(C)** Aire expression within mTEC^{hi}. **(D)** CCL21 expression within mTEC^{lo}. **(E)** Expression of tuft cell marker DCLK1 within mTEC^{lo}. Bar graphs show absolute cell numbers and percentages. Data are representative of 2 or 3 independent experiments per time-point (n= 6-9 independent samples). All data are represented as mean ± SEM. d - days; wk - weeks; mo - months. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

***Foxo3* deficiency induces changes in the transcriptome of cTECs and mTECs.**

To elucidate the mechanism underlying the role of FOXO3 in TEC maintenance, we performed bulk RNA-sequencing (RNA-Seq) analysis and compared the transcriptomes of cTECs and mTECs isolated from 6-week-old FOXO3^{Ctr} and FOXO3^{cKO} mice. We selected this age to ensure the mTEC phenotype unfolds in FOXO3^{cKO} mice and, consequently, to capture potential transcriptional changes, all while obtaining a sufficient number of cTECs and mTECs for analysis. Hierarchical clustering of all biological samples showed that cTECs and mTECs separated primarily according to cell types (**data not shown**). The quantification of reads covering exon 2 of *Foxo3* was markedly reduced in FOXO3^{cKO}-derived TEC subsets, confirming the specific inactivation of the gene in mutant mice (**Figure S2A**). Next, we compared the impact of *Foxo3* deficiency in the transcriptome of cTECs and mTECs. Approximately 600 genes for cTECs and 900 genes for mTECs showed statistically significant differences, which segregated control and mutant samples in the Principal Component 1 (PC1) (**Figure 2A-B, Figure S2B, Supp. Tables 1-4**). There was a small overlap in the identity of differentially expressed genes (DEGs) in cTECs and mTECs, suggesting that *Foxo3* deficiency has specific effects on the transcriptome of each cell population (**Figure S2C**). Given the functional interplay between p53 and FOXO3 in the DNA damage response [6, 7], and our previous observations that p53 controls a broad molecular network of the mTEC transcriptome, including *Foxo3* [5], we compared the overlap between statistically significant DEGs affected by *Trp53* and *Foxo3* deficiency in cTEC and mTEC subsets. The deficiency in *Trp53* had a limited impact on the cTEC transcriptome compared to the effects of *Foxo3* deficiency (**Figure S2D**) [5]. In contrast, the influence of *Trp53* deletion was broader than that of *Foxo3* in mTECs. Yet, only approximately 46% (435/935) of DEGs in FOXO3^{cKO} mTECs were commonly affected by *Trp53* deficiency, indicating that FOXO3 and p53 control the expression of common and specific targets in mTECs (**Figure S2D**). Despite the changes imposed by *Foxo3* deficiency in cTECs, the expression of particular genes associated with cTEC-dependent functions (migration, commitment, selection) showed no difference between FOXO3^{Ctr} and FOXO3^{cKO} cTECs (**Figure S2E**). On the contrary, the expression of particular genes linked to mature mTECs, such as *Aire* and *CD80*, was increased in mutant mTECs (**Figure S2F**). These changes may be attributed to the RNA-Seq analysis being done in total mTECs, which show an increased frequency of mTEC^{hi} in the FOXO3^{cKO} thymus (as shown in **Figure 1**).

We began by examining whether *Foxo3* deficiency caused alterations in transcriptional signatures of mTEC subsets defined by their history of Aire expression.

We isolated the genetic signatures of pre-, early-, late-, and post-Aire mTECs [11], and examined the variation in the expression of the top 200 enriched genes within each cluster between control and mutant mTECs. While gene sets associated with pre-, early- and late-Aire cells lacked a specific pattern, the genetic signature of the post-Aire subset was downregulated in FOXO3^{cKO} mTECs (**Figure 2C**). Similar complementary analysis of mTEC I-IV [12] populations revealed that the genetic signature of mTEC IV, which includes Post-Aire cells, was similarly downregulated in mutant mTECs (**Figure S3A**). Recent findings indicated that Post-Aire cells harbour additional heterogeneity, consisting of cells that have genetic programs resembling those of extra-thymic cells, such as tuft cells, keratinocytes, ionocytes, enterocytes and more [13, 14]. We examined how *Foxo3* deficiency affected the genetic signatures of these mTEC mimetics [13]. Although our analysis was conducted on bulk mTEC populations and these specialized subsets are rare within mTECs, we found that gene sets, associated with Tuft- and Neuroendocrine-like cells were downregulated, whereas the ones linked to Microfold-like cells appeared slightly enriched in FOXO3^{cKO} mTECs (**Figure S3B**). Moreover, we obtained a list of Aire-dependent and Aire-independent TRAs genes, identified through statistical comparison of their expression on wild type (WT) and Aire knockout (Aire^{KO}) mTECs [8], and determined their presence among the DEGs of FOXO3^{cKO} mTECs. Downregulated DEGs included 41% of TRAs (14% Aire-dependent and 27% Aire-independent). Conversely, upregulated DEGs contained 29% TRAs (2% Aire-dependent and 27% Aire-independent) (**Figure 2D**). These results suggested that *Foxo3* deficiency mostly affected the representation of Aire-independent TRAs.

To further dissect the molecular role of FOXO3 in TECs, we performed gene ontology (GO) analysis on cTEC and mTEC DEGs. GO analysis in downregulated DEGs in mutant cTECs or mTECs yielded no significant term enrichment. In contrast, upregulated DEGs in mutant cTECs showed enrichment in processes linked with ribosomes and their biogenesis (**Figure 2E, Supp. Tables 5-6**). Notably, GO analysis of upregulated DEGs in FOXO3^{cKO} mTECs revealed enrichment in the intrinsic apoptotic signalling pathway, protein degradation, MHCI antigen presentation and processes that promote T-cell activation (**Figure 2E, Supp. Tables 7-8**). These results indicate that FOXO3 has a pervasive but diverse impact in the transcriptional programs of cTECs and mTECs.

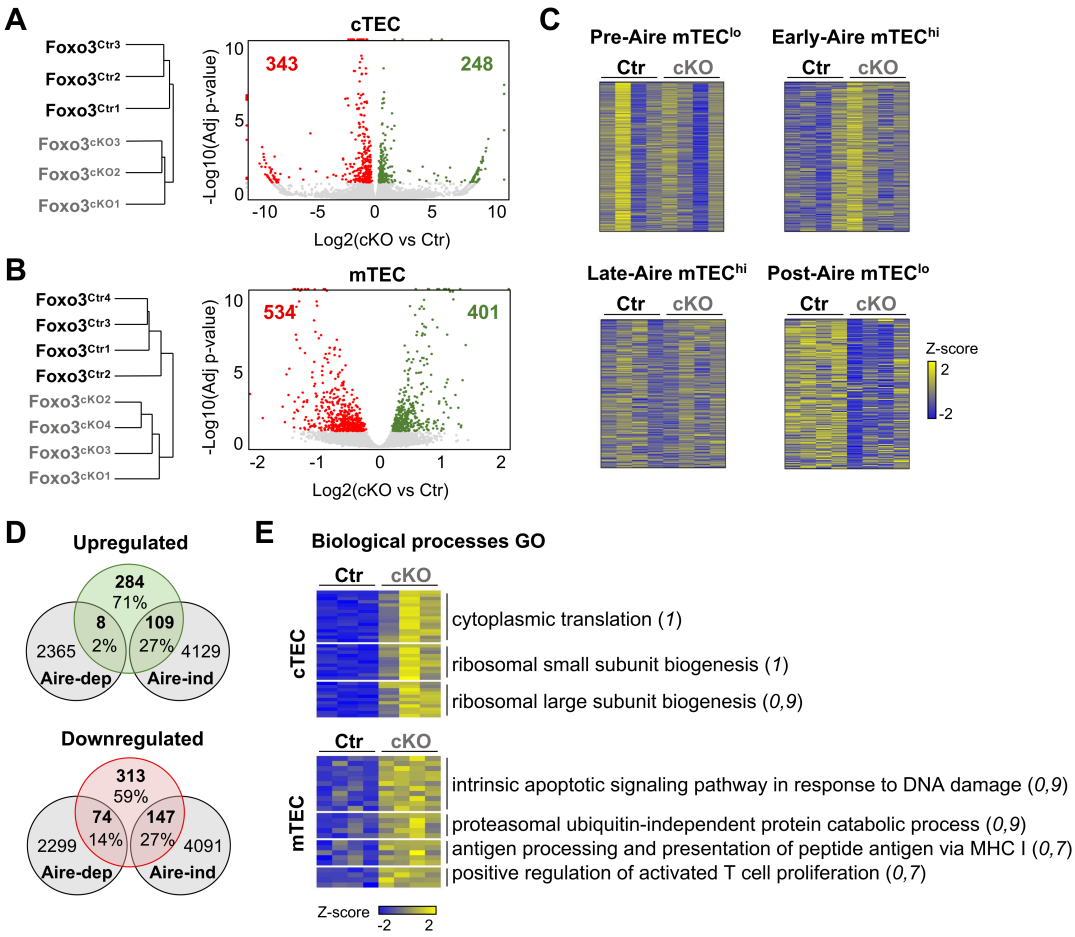


Figure 2 – FOXO3^{cKO} TEC subsets display altered transcriptional signatures. (A-B) RNA-Seq analysis of FACS-sorted cTECs **(A)** and mTECs **(B)** purified from 6-week-old FOXO3^{Ctr} and FOXO3^{cKO} mice. Hierarchical clustering of all biologically independent samples (left). Volcano plot depicting downregulated and upregulated DE genes in FOXO3^{cKO} versus FOXO3^{Ctr} samples (right). In volcano plots, off-scale points were collapsed to the nearest axis. **(C)** Relative expression level of genes specifically upregulated in pre-, early-, late- and post-Aire mTECs (data obtained from [11]) in FOXO3^{Ctr} and FOXO3^{cKO} mTECs. **(D)** Venn diagrams depict the frequency and number of FOXO3^{cKO} mTEC upregulated (top) and downregulated (bottom) genes classified as Aire-dependent (Aire-dep) or Aire-independent (Aire-ind) tissue-restricted antigens, as defined in [8]. **(E)** Representation of the most enriched biological processes obtained by Gene Ontology (GO) analysis of upregulated genes in cTECs and mTECs. Terms represented show statistical significance with a marginal posterior probability estimate that is > 0.7 (enclosed within parentheses). Heatmap rows represent the relative expression level of the differentially expressed genes for each biological process. Their identity is specified in Supp. Table 6 (for cTECs) and Supp. Table 8 (for mTECs).

Foxo3 deficiency alters the DNA damage response in mTECs.

Given the recognized role of FOXO3 in DNA damage response, we were particularly intrigued by the fact that the most significant augmented pathway in FOXO3^{cKO} mTECs was apoptosis in response to DNA damage. Concordantly, the analysis of the expression of Bcl-2 family members showed a statistically significant upregulation of some BH3-only and BAX/BAK-like genes in FOXO3^{cKO} mTECs. A similar trend was observed in the remaining pro-apoptotic genes, whereas the levels of their pro-survival counterparts were unaltered in mutant mTECs (**Figure S3C**). Moreover,

assessment of double-strand DNA breaks and apoptosis showed an increase in the frequency of γH2AX in mTEC^{hi}, and a higher frequency of annexin V^{+} cells in mTEC^{lo} of the FOXO3^{cKO} thymus (**Figure 3A-B**). Due to the role of FOXO3 in promoting cell cycle arrest after DNA damage [15], we also analysed the proliferative rate of TECs and observed a higher proportion of Ki67^{+} FOXO3^{cKO} mTEC^{lo} (**Figure 3C**). This observation was consistent with an increase in the genetic signature of recently identified proliferating mTECs, also termed transit-amplifying cells [16, 17], in mutant mTECs (**Figure S3D**)**.** To further determine whether FOXO3^{cKO} mTECs possess an impaired DNA damage response, we examined the regenerative capacity of mutant TECs after acute damage induced by ionizing radiation. Analysis of cTEC recovery on days 3 and 21 post-radiation showed a comparable dynamic between the control and mutant thymus. Despite showing numerical differences at steady state (untreated), the numbers of mTEC^{lo} and mTEC^{hi} significantly decreased by day 3 post-radiation in the control and mutant thymus (**Figure 3D; Figure S4A**). The high sensitivity of mTECs to radiation-induced damage was consistent with previous observations [5, 18, 19]. Yet, the radiation-induced decline was less pronounced in the FOXO3^{cKO} thymus, suggesting that deficiency in FOXO3 may confer some short-term resistance to apoptosis provoked by acute radiation (**Figure 3D; Figure S4A**). In both groups, mTEC^{lo} showed no significant regeneration by day 21 after treatment. Although the number of mTEC^{hi} was comparable by day 3 in both groups, their recovery was affected in FOXO3^{cKO} thymus by day 21 (**Figure 3D; Figure S4A**). Furthermore, despite an equivalent level of depletion in thymocyte cellularity by day 3 in both groups, the recovery was also affected in the FOXO3^{cKO} thymus by day 21 post-treatment (**Figure 3E; Figure S4B**). These results suggest that the regenerative response upon radiation-induced damage is inhibited in the FOXO3^{cKO} thymus.

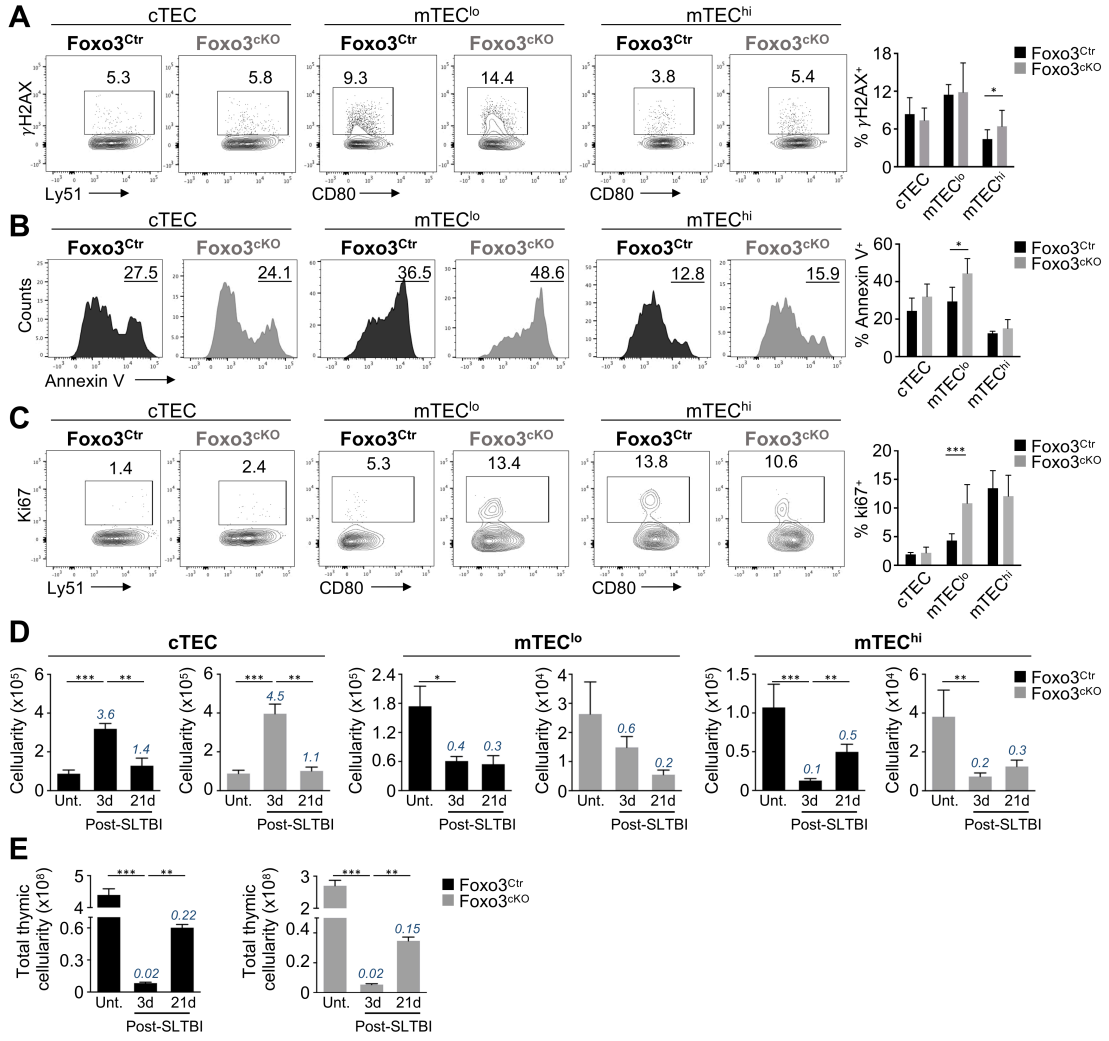


Figure 3 – FOXO3^{cKO} mTECs present an impaired DNA damage response. (A-C) Frequency of γH2AX$^+$ cells **(A)**, Annexin V$^+$ cells in 7-AAD$^-$ **(B)** and Ki67$^+$ cells **(C)** in cTECs, mTEC^{lo} and mTEC^{hi} in FOXO3^{Ctr} and FOXO3^{cKO} adult thymus. Data are representative of 3 independent experiments (n=7 to 9 independent samples) and represented as mean ± SD. **(D-E)** 10-week-old FOXO3^{Ctr} and FOXO3^{cKO} mice were subjected to sublethal total-body irradiation (SLTBI) and analysed at day 3 and day 21 post-irradiation. 10-week-old FOXO3^{Ctr} and FOXO3^{cKO} untreated mice (Unt.) were also analysed. The cellularity of cTECs, mTEC^{lo} and mTEC^{hi} **(D)** and total thymic cellularity **(E)** were determined at the indicated time-points. Numbers inserted above day 3 and day 21 columns depict the normalized recovery index of each population (average fold change relative to the corresponding untreated cellularity, which was set as 1). Data are representative of 2 or 3 independent experiments (n=6-9 independent samples) and represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

TEC-specific deletion of *Foxo3* affects the development of regulatory T cells and iNKT cells.

We next proceeded to examine how the described cellular and molecular alterations within the TEC microenvironments influence T-cell development in the FOXO3^{cKO} thymus. While slightly similar at postnatal day 4, the number of thymocytes was notably reduced in the 10-week-old mutant thymus. These differences became however attenuated in the 9-month-old thymus (**Figure 4A**). In the adult thymus, TEC-

specific deletion of *Foxo3* did not lead to abnormalities in the frequency of the main thymocyte subsets, such as double-negative (DN), double-positive (DP), and CD4 and CD8 single-positive (SP) cells (**Figure 4B**). The composition of immature DN subsets (DN1, $CD25^{-}CD44^{+}$; DN2, $CD25^{+}CD44^{+}$; DN3, $CD25^{+}CD44^{-}$; DN4, $CD25^{-}CD44^{-}$), gated on lineage negative (Lin^{-}) markers (CD4, CD8, NK1.1; CD11b, CD11c, CD19, TCRβ, Ter-119, GR1), was also similar in the mutant thymus, except for a significant reduction in the frequency and numbers of DN1 cells (**Figure 4C**). Additionally, the frequency of DP thymocytes initiating selection ($TCR\beta^{int}CD69^{int}$), post-positive selection SP thymocytes ($TCR\beta^{hi}CD69^{hi}$) and mature SP thymocytes ($TCR\beta^{hi}CD69^{-}$) [20] was similar in $FOXO3^{Ctr}$ and $FOXO3^{cKO}$ thymus. Still, their numbers were diminished as a result of the decreased total thymocyte cellularity in the $FOXO3^{cKO}$ thymus (**Figure 4D**).

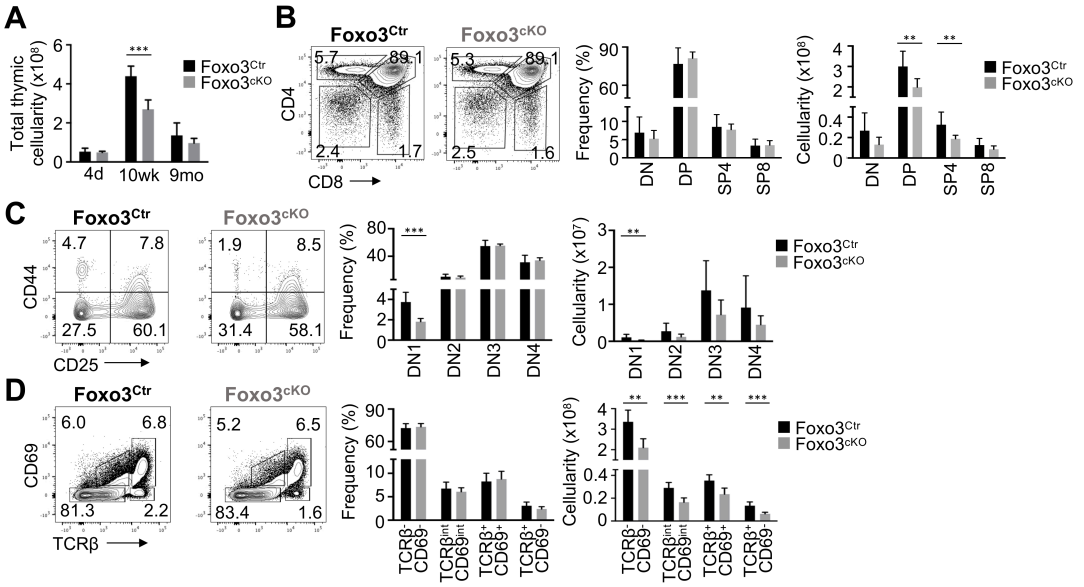


Figure 4 – Thymopoiesis in $FOXO3^{cKO}$ mice. (A) Total thymic cellularity at the indicated time points. **(B)** CD4 and CD8 expression on total thymocytes in the 10-week-old thymus of $FOXO3^{Ctr}$ and $FOXO3^{cKO}$ mice. **(C)** CD44 and CD25 expression on Lin^{-} $CD4^{-}$ $CD8^{-}$ DN thymocytes. **(D)** CD69 and TCRβ expression on total thymocytes. Bar graphs show absolute cell numbers and percentages. Data are representative of 2 to 4 independent experiments (n=6-12 independent samples). All data are represented as mean ± SD. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Subsequently, we determined whether the more significant changes in mTEC numbers of the $FOXO3^{cKO}$ thymus could directly impact mTEC-dependent T-cell developmental stages, such as negative selection, SP4 maturation, regulatory T cell and iNKT cell differentiation [1]. The co-expression of Helios and PD1 [21] on both DP thymocytes and $CD4^{+}$ $FOXP3^{-}$ SP thymocytes showed that cortical and medullary negative selection, respectively, remained unaltered in the 10-week-old $FOXO3^{cKO}$ thymus (**Figure S5A**). However, the mutant thymus showed a moderate increase in the

proportion of immature $CD24^{hi}CD62L^{lo}$ CD4 SP thymocytes. Despite the partial block in SP4 maturation, the numbers of both immature and mature SP4 were diminished in adult $FOXO3^{cKO}$ thymus, likely due to the aforementioned decline in total thymocyte cellularity (**Figure S5B**). Strikingly, the percentage and number of mature ($CD25^{+}FOXP3^{+}$) regulatory T cells were significantly decreased in the adult $FOXO3^{cKO}$ thymus (**Figure 5A**). Although *Foxo3* deficiency did not impact the percentages of immature regulatory T cells ($CD25^{+}FOXP3^{-}$ and $CD25^{-}FOXP3^{+}$ SP4s) [22], their numbers were also reduced in the mutant thymus (**Figure 5A**). A complementary analysis excluding $CD44^{hi}$ recirculating cells [22] confirmed that *Foxo3* deficiency significantly affected the frequency of newly generated regulatory T cells, resulting in a tendency towards a reduction in their numbers (**Figure S5C**). We next set *in vitro* suppression assays using equal numbers of regulatory T cells from the control or mutant thymus, and assessed their cell-intrinsic capacity to suppress TCR-mediated polyclonal proliferation of WT conventional T cells ($TCR\beta^{+}CD4^{+}CD25^{-}$). The precursor frequency and average division of conventional T cells were higher when co-cultured with regulatory T cells isolated from the $FOXO3^{cKO}$ thymus compared to the control (**Figure S5D**), indicating that regulatory T cells derived from the $FOXO3^{cKO}$ thymus showed lower suppressive function.

Besides their well-defined role in conventional $\alpha\beta$ T-cell development, mTECs also control the development of CD1d-restricted iNKT cells [23]. Using CD1d:PBS57 loaded tetramer, we found a significant reduction in the frequency and absolute number of iNKT cells in the adult $FOXO3^{cKO}$ thymus (**Figure 5B**). Following recognition of CD1d/glycolipid complexes, iNKT cells undergo a stepwise maturation program characterized by the loss of CD24 and acquisition of CD44 and NK1.1 [24]. We found a specific and significant decrease in the numbers of stage 1 ($CD24^{-}CD44^{-}NK1.1^{-}$) and stage 2 ($CD24^{-}CD44^{+}NK1.1^{-}$) iNKT subsets in the mutant thymus (**Figure S5E**). Lastly, we analysed the expression of T-bet, PLZF and RORγt to determine whether *Foxo3* deficiency affected iNKT1, iNKT2 and iNKT17 cells [25]. Although the numbers of all three iNKT subsets decreased, the decline in iNKT2 cells in the $FOXO3^{cKO}$ thymus was statistically more significant (**Figure 5C**).

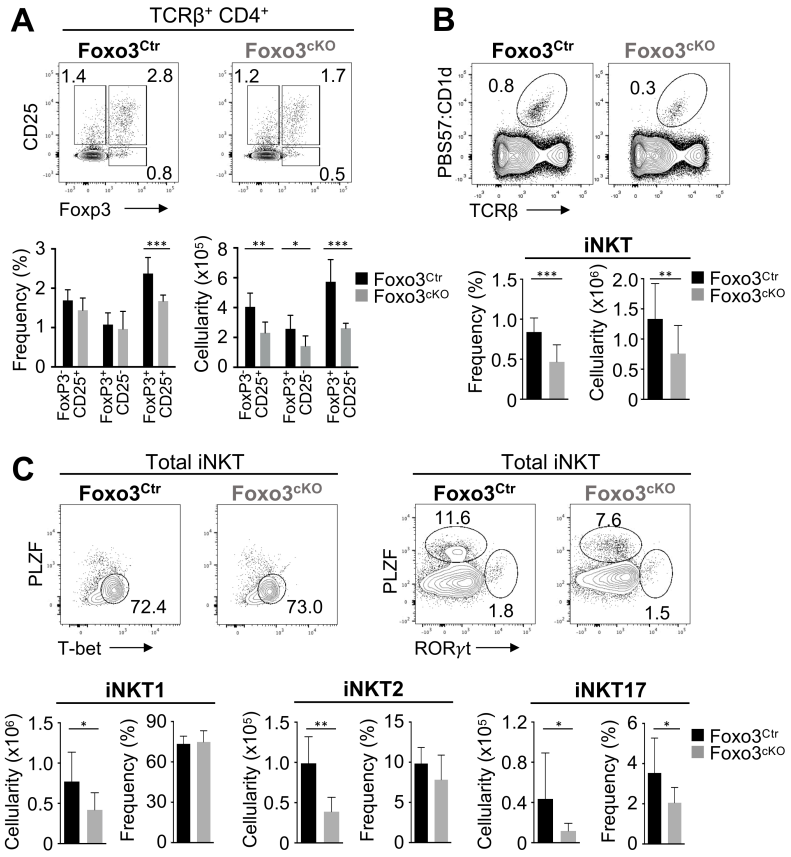


Figure 5 – FOXO3^{cKO} mice exhibit compromised regulatory T cell and iNKT cell development. (A) CD25 and FOXP3 expression on TCRβ$^+$ CD4$^+$ thymocytes of 10-week-old thymus of FOXO3^{Ctr} and FOXO3^{cKO} mice. **(B)** Expression of TCRβ and reactivity with PBS57-loaded CD1d tetramer in total thymocytes. **(C)** Detection of T-bet$^+$ iNKT1, PLZF$^+$ iNKT2 and RORγt$^+$ iNKT17 in total thymic iNKTs. Bar graphs show absolute cell numbers and percentages. Data are representative of 2 to 4 independent experiments (n=6-12 independent samples). All data are represented as mean ± SD. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Effect of TEC-specific deletion of *Foxo3* in peripheral immune tolerance.

Lastly, we investigated whether the aforementioned thymic phenotypes predisposed to changes in peripheral immune tolerance in aged (10-month-old) FOXO3^{cKO} mice. It is worth noting that FOXO3^{cKO} mice were generated in a C57Bl/6 background, which is known for its resistance to autoimmunity resulting from defects in central tolerance, such as impaired mTEC and/or Aire function [14, 26]. Although not statistically significant, the frequency of mice with severe lymphocytic infiltrations in the salivary glands was increased in aged FOXO3^{cKO} mice (**Figure 6A-B**). Despite these mild alterations, we did not find changes in antinuclear antibodies in the serum of aged mutant mice (data not shown).

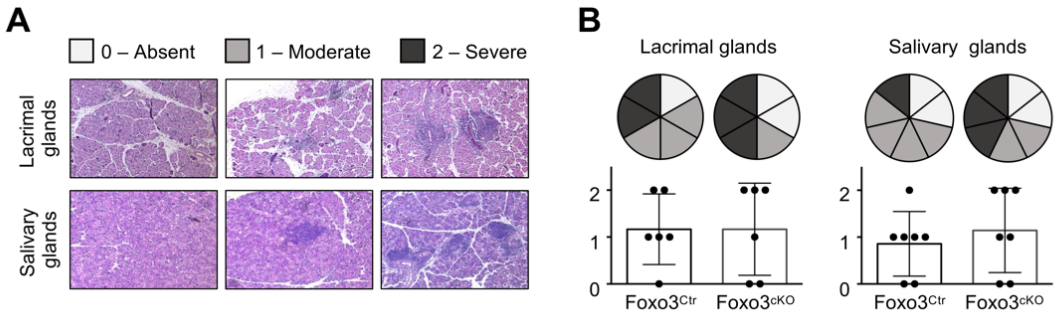


Figure 6 – TEC-specific *Foxo3* deletion affects peripheral tolerance induction. (A) Representative H&E staining of the different degrees of infiltration. **(B)** Severity scores for inflammatory lymphocytic infiltration in salivary and lacrimal glands of 10 months-old FOXO3^{Ctr} and FOXO3^{cKO} mice. Pie charts represent absent, moderate and severe lesions as light grey, dark grey and black, respectively. Bar graphs represent absent, moderate and severe lesions scored as 0-2, respectively. Data are represented as mean ± SD.

The mild signs of disturbed tolerance in aged FOXO3^{cKO} mice led us to determine the role of FOXO3 in the context of a genetically susceptible model for autoimmunity. To do so, we crossed FOXO3^{cKO} mice onto an Aire^{KO} background (Aire^{KO}FOXO3^{cKO}) and determined whether *Foxo3* deficiency aggravated the autoimmune predisposition previously reported in *Aire*-deficient mice (Aire^{KO}) [3]. Considering the description of the impact of *Foxo3* deficiency on the homeostasis and genetic program of TECs, T-cell development, and tolerance (**Figures 1-5**), as well as the challenges associated with establishing dual-deficient Aire^{KO}FOXO3^{cKO} mice, we did not reanalyse single FOXO3^{cKO} mice within this experimental complementary setup, which include Aire^{WT}, Aire^{KO} and Aire^{KO}FOXO3^{cKO} groups. Thus, we did not directly compare the independent or interdependent role of FOXO3 and Aire.

Aire^{+} mTECs represent one of the most dominant mature mTEC lineages in the postnatal thymus [11, 12, 17, 27] and *Aire* deficiency is associated with defects in mTECs, regulatory T cell and iNKT cell differentiation [11, 16, 28-31]. We confirmed that Aire^{KO} (Aire^{KO}FOXO3^{Ctr}) adult thymus had a reduced number of cTECs, mTEC^{lo} and an increase in mTEC^{hi} when compared to Aire^{WT} controls (Aire^{WT}FOXO3^{Ctr}). Yet, the cellularity of cTEC, mTEC^{lo} and mTEC^{hi} subsets was further decreased in the Aire^{KO}FOXO3^{cKO} age-matched thymus (**Figure S6A**). Furthermore, we confirmed that the percentage and number of regulatory T cells and iNKT cells were reduced in the adult Aire^{KO} thymus, and these defects were also further reduced in their Aire^{KO}FOXO3^{cKO} counterparts (**Figure S6B-C**). These results suggested that *Foxo3* deficiency further accelerated the defects in TECs, regulatory T cell and iNKT cell development in Aire^{KO} mice. Considering that Aire^{KO} mice represent an early-onset autoimmunity model, we chose to anticipate the assessment of altered peripheral tolerance to 6 months of age. In line with previous studies in the C57BL/6 background

[14, 26], aged $Aire^{KO}$ mice showed moderate to severe lymphocytic infiltrates, mostly affecting the salivary and lacrimal glands, when compared to age-matched controls. Although lacking statistical significance, $Aire^{KO}FOXO3^{cKO}$mice showed a moderate aggravation compared to $Aire^{KO}$ mice, with a higher number of animals displaying severe lymphocytic infiltrates in salivary glands (**Figure S6D**). Analysis of other organs, including lacrimal glands, kidney and colon, showed no apparent differences between $Aire^{KO}$ and $Aire^{KO}FOXO3^{cKO}$ mice (**Figure S6D**), suggesting that this exacerbation effect could be organ-specific. Collectively, our findings suggest that *Foxo3* deficiency in TECs may predispose to failures in central tolerance, ultimately leading to impaired peripheral tolerance.

DISCUSSION

The differentiation of the TEC compartment is initiated during embryonic development and persists through adult life. This dynamic process is supported by the coordinated functioning of specialized bipotent precursors and restricted c/mTEC precursors [3]. Our findings imply that FOXO3 primarily controls the cellularity of mTECs. There are several possible explanations for our observations that are not mutually exclusive. Mature mTECs, particularly the Aire^{+} subset, are estimated to have a turnover rate of 7–10 days [32, 33], implicating the need for replacement by upstream progenitors. Thus, the reduction in FOXO3^{cKO} mTECs may stem from a failure in mTEC precursors and/or their capacity to replace mature lineages. As mTEC precursors exist within mTEC^{lo} subset [3], the more pronounced effect in the mTEC^{lo} subset would align with this possibility. Yet, mTEC^{lo} also include differentiated populations such as CCL21^{+} and Post-Aire cells. An alternative, and not mutually exclusive, possibility is that *Foxo3* deficiency may render mTECs more susceptible to death. The increase in annexin V^{+} in FOXO3^{cKO} mTEC^{lo} and the enrichment in genes involved in DNA-damage apoptosis found in FOXO3^{cKO} mTECs would support this hypothesis, suggesting that the mTEC^{lo} subsets are more sensitive to the absence of FOXO3. Further studies that aid in deciphering the signals responsible for activating FOXO3 in TECs and determining whether the function of FOXO3 is differentially regulated in distinct c/mTEC subsets will offer insights concerning the regulation of FOXO3-dependent pathways in TECs.

Although *Foxo3* deficiency predominantly affected mTEC cellularity, *Foxo3* was more expressed in cTECs than mTECs. Our transcriptomic data suggest that FOXO3 controls distinct genetic programs in each TEC subset. At present, the mechanisms governing cTEC homeostasis and biology remain unclear. Our results showed that *Foxo3* deficiency also influences the cTEC genetic program. While cTEC numbers were seemingly unaltered under steady-state conditions, the transcriptomic alterations in cTECs correlated with a decrease in the frequency of DN1 thymocytes and an overall reduction in cellularity of all thymocyte subsets in the adult FOXO3^{cKO} thymus. Yet, despite the reduction in thymic subsets, the percentages of cells in distinct subsequent stages of cortical T-cell development (DN2-DN4, DP) were comparable between mutant and control mice. We can only speculate on how *Foxo3* deficiency affects the function of cTECs resulting in a reduced number of cortical DN1 and DP thymocytes in the adult thymus. cTECs provide key chemokines (Cxcl12, Ccl19, Ccl21), ligands (Dll4) and cytokines (Il7, Scf1) to regulate the attraction, commitment and proliferation of early T-cell precursors, as well as self-peptides:MHC ligands for positive selection [3]. The absence of transcriptional alterations of these essential cTEC-derived factors may

suggest that they are not the underlying cause for the reduction in the number of DP thymocytes in the adult FOXO3^{cKO} thymus. Concordantly, *Foxo3* deficiency appeared not to affect the rate at which DN intrathymic T-cell precursors progress through T-cell commitment and positive selection, as indicated by the similar frequency of cells within DN2-DN4, and pre and post-positive selection DP stages compared to controls. One possibility is that *Foxo3* deficiency affects the entry of early thymic precursor and/or the DN1 niche may be affected in the mutant thymus, which would lead to a reduction in the number of DN1 cells and their downstream progeny, DN2-DN4 and DPs. Our data suggest that FOXO3 restrains ribosomal biogenesis in cTECs. Previous studies in *D. melanogaster* and *C. elegans* have demonstrated that FOXO3 suppresses protein translation in response to reduced insulin signalling or stress signals, such as nutrient deprivation or reactive oxygen species [34, 35]. In this scenario, *Foxo3* deficiency in cTECs may lead to enhanced translation, resulting in an enrichment of functional processes that control protein synthesis, including ribosome biogenesis. It remains to be determined whether these molecular changes in cTECs, or the ones reported in mTECs, contribute to anatomic alterations in the cortical niche and/or perturb the cortico-medullary junction, thereby affecting the entry, migration or nurture of DN1 cells. This scenario could also involve a possible complex functional and structural interplay between cTECs, mTECs and other non-epithelial thymic stromal cells (*e.g.*, endothelia and mesenchyme). Seminal research has shown that distinct clonally derived medullary islets formed during the embryonic period expand during postnatal life and merge into larger medullary areas in the adult thymus [36]. The reduced mTEC compartment in the adult FOXO3^{cKO} thymus was associated with fewer medullary islets, which may affect the spatial organization of mTECs and their relationship with other stromal components. Future studies 3D reconstruction studies of the FOXO3^{cKO} thymus should investigate the effects of FOXO3 on the organization and position of diverse cTEC and mTEC subsets, and determine their potential impact on T-cell development.

The role of FOXO3 in regulating the integrity of the mTEC compartment is in line with its role in preserving genomic stability and control of cell cycle [7, 15]. Moreover, we infer that the higher frequency of cycling cells in *Foxo3*-deficient mTEC^{lo} could be due to a compensatory response triggered by apoptosis following DNA damage and/or related to the absence of FOXO3-dependent regulation of cell cycle arrest [7]. The most significant difference in the frequency of cells with DNA damage (γH2AX^{+}) was found in the FOXO3^{cKO} mTEC^{hi} subset. Intriguingly, previous studies have connected double-strand DNA breaks to Aire-mediated TRA expression [37]. While most of the studies have primarily focused on mTEC^{hi}, it is worth noting that TRA expression occurs in both Aire^{-} mTEC^{hi} and mTEC^{lo} subsets, partly regulated by Fezf2 and other unidentified

transcriptional regulators [8, 38]. It is conceivable that the unique epigenetic and transcriptional states of mTECs, particularly related to TRA expression, initiate DNA breaks that require a reparative DNA damage response. Our transcriptional analysis revealed that FOXO3^{cKO} mTECs predominantly altered the expression of Aire-independent TRAs. Given that mTECs express clusters of TRAs at a single-cell level [38-40], the susceptibility of *Foxo3*-deficient cells to apoptosis may lead to alterations in the representation of particular mTEC clusters, and TRAs, in mutant mTECs. In this regard, the decline in the mTEC compartment and altered transcriptome of mTECs caused by *Foxo3* deficiency mirrors some of the changes found in the context of *Trp53* deficiency [5]. p53 directly targets and activates the expression of *Foxo3* [41], and FOXO3 can promote p53 stabilization and p53-driven apoptosis [42]. Conversely, FOXO3 can also be negatively regulated by p53 through the activity of serum and glucocorticoid-inducible kinase 1 (SGK1) [43]. Additionally, under oxidative stress, p53 can inhibit FOXO3 transcriptional activity [44]. Thus, the functional relationship between p53 and FOXO3 appears to be context-dependent. Albeit *Foxo3* is downregulated in p53^{cKO} mTECs, *Trp53* remains unaltered in FOXO3^{cKO} mTECs, suggesting that FOXO3 may operate downstream of p53 in controlling mTEC homeostasis. In this regard, approximately 46% of DEGs found in FOXO3^{cKO} mTECs were also affected by *Trp53* deficiency, indicating that FOXO3 and p53 control the expression of common targets in mTECs. Still, the significant number of DEGs affected only by *Foxo3* deficiency infers the existence of other p53-independent mechanisms to control FOXO3 in mTECs. We hypothesize that FOXO3 and p53 collaboratively control common and specific regulatory pathways within mTECs. Future studies should investigate how FOXO3 controls DNA repair and cell cycle in mTECs, and how these processes connect to promiscuous gene expression and the maintenance of their function.

The perturbation in the mTEC compartment of FOXO3^{cKO} mice predominantly impacts specific stages of T-cell development, namely iNKT cells and regulatory T cells. These observations are in line with previous studies implicating mTECs in the development of these specific T-cell subsets [23, 45]. CCL21^{+} and thymic tuft mTECs have been shown to control the development of functional iNKT sublineages [46]. Specifically, the decline in iNKT2 cells in the FOXO3^{cKO} thymus aligns with the reduction in tuft cell-like mTECs, known to regulate iNKT2 cell development [12, 46]. Moreover, our findings indicate that the FOXO3^{cKO} mTEC microenvironment compromises the development and function of regulatory T cells, potentially predisposing to failures in peripheral immune tolerance. There are several possibilities for the reduced development and suppressive function of regulatory T cells in a polyclonal non-antigen-specific setting, which may not be mutually exclusive. These could involve a reduction in

the size of the mTEC compartment, alterations in processing or presentation of self-antigen:MHCII complexes by FOXO3^{cKO} mTECs and/or failure in other mTEC-derived signals critical for T regulatory cell differentiation [3]. Future studies should clarify if these defects in regulatory T cells are general or antigen-specific. The observations that *Foxo3* deficiency further potentiated the previously reported defects in mTEC, iNKT and regulatory T cell development in Aire^{KO} mice were intriguing. Further studies are required to define the interdependent or independent roles of FOXO3 and Aire in mTECs and how these affect iNKT and regulatory T cell development and function. Still, the mild autoimmune manifestations detected in FOXO3^{cKO} mice, either in Aire^{WT} or Aire^{KO} settings, may be attributed to the resistant C57BL/6 background and/or the presence of extrathymic T regulatory cell-dependent compensatory mechanisms [26, 47], which cooperatively might be sufficient to prevent the development of overt autoimmunity. Previous studies have shown that Aire-dependent regulatory T cells, which are established during the perinatal stages of life, play a crucial role in protecting against autoimmune responses later in life [47, 48]. Notably, the differentiation of mTECs and regulatory T cells in FOXO3^{cKO} thymus seemed to be normal during the perinatal period, with the more severe thymic phenotype unfolding in adulthood. We reason that the FOXO3^{cKO} thymus may establish a functional pool of regulatory T cells during early life, which serves to prevent an overt autoimmune response in aged mice. Additionally, in C57BL/6 mice, PD1-expressing conventional T cells and regulatory T cells accumulate in nonlymphoid organs of Aire^{KO} mice [26, 47], presumably contributing to an immunosuppressive state for otherwise more severe autoimmunity observed in different genetic backgrounds.

Our findings on FOXO3 align with previous studies that establish a link between mTEC homeostasis, TRA expression, the initiation of a DNA damage response and induction of immunological tolerance [4, 5]. Worth noting, FOXO3 has additional roles in response to other physiologic stress stimuli, including growth factor deprivation, metabolic stress and oxidative stress [6, 49-52]. In this regard, other FOXO family proteins, including FOXO1, FOXO4 and FOXO6, control basic cellular processes in response to nutrient deprivation and stress [6, 7]. Future studies must clarify the individual and cooperative functions of other FOXO proteins in the control of TEC homeostasis, akin to the collaborative roles attributed to the FOXO family in other tissues [53-55]. In conclusion, our study underscores the important influence of FOXO3 in controlling TEC biology and function.

METHODS AND MATERIALS

Mice

All mice were in a C57BL/6 background and housed under specific pathogen-free conditions. FOXO3$^{fl/fl}$ and Aire$^{-/-}$ were purchased from The Jackson Laboratory [9, 56]. FOXN1^{Cre} mice were obtained from Dr. Thomas Boehm [57]. The required number of animals in each experimental setting was estimated based on results obtained with similar procedures to those in previous studies [5], considering both biological and technical variability. Apart from categorization based on genotype, mice were randomly assigned to particular treatment and timepoint experimental groups, irrespectively of sex, and no exclusions were made. Whenever feasible, sample preparation and data acquisition were conducted in a blinded manner with respect to the genotype. In radiation recovery studies, mice received sublethal total-body irradiation (450 rads) with a Cs^{137} radiation source (Gammacell 1000, Nordion). Experiments were performed in accordance with European guidelines for animals used for scientific purposes (Directive 2010/63/EU).

PCR genotyping

Foxo3 inactivation was confirmed by PCR analysis (using primers *a*, *b* and *d*) to detect the excision of exon 2, the first coding exon, which encodes the start codon and the N-terminal half of the protein and forkhead DNA binding domain [9]. Primers spanning the *loxP* site in intron 1, *a* (F 5′-AACAACCTCACACATGTGCC-3′) and *b* (R 5′-AGTGTCTGATACCGAAGAGC-3′) were used to detect the *Foxo*3^{fl} allele. The excision was detected using primers *a* and *d* (R 5′-CATGCAGTCCGAGAGATTTG-3).

Flow Cytometry and cell sorting

For hematopoietic cell analysis, thymocytes were obtained from mechanically disrupted thymus samples and subsequently stained with antibodies listed below for T-cell development characterization [5, 58, 59]. This procedure liberates a significant fraction of hematopoietic cells while preserving most of thymic stroma cells. For stromal cell analysis, the remaining thymic fragments underwent enzymatic digestion, resulting in a cell suspension that was subjected to CD45$^+$ cell depletion using anti-CD45 microbeads and LS columns (Miltenyi Biotec), as previously described [5, 58-60]. The total thymocyte cellularity was estimated by adding the number of hematopoietic cells (CD45$^+$) obtained from the mechanically liberated fraction and the number of hematopoietic cells (CD45$^+$) obtained from the digested fraction before anti-CD45 depletion. The number of distinct T-cell subsets was estimated from the total thymocyte

cellularity. The total TEC cellularity was estimated by determining the number of TECs ($CD45^{-}EpCAM^{+}$) obtained from the digested fraction after CD45 depletion, as previously described [5, 58, 59]. Enriched TEC samples were analysed using the following antibodies: anti-CD45.2 (104, eBioscience, cat. no.: 45-0454-82), anti-EpCAM (G8.8, Biolegend, cat. no.: 118225) or anti-EpCAM (G8.8, Biolegend, cat. no.: 118212), anti-Ly51 (6C3, eBioscience, cat. no.: 12-5891-82), anti-I-A/I-E (M5/114-15-2, eBioscience, cat. no.: 47-5321-82), anti-CD80 (16-10A1, Biolegend, cat. no.: 104731). Biotinylated UEA-1 (Vector Laboratories) was detected with streptavidin Brilliant Violet 711 conjugate (Biolegend, cat. no.: 405241) or streptavidin PE-Cy7 conjugate (eBioscience, cat. no.: 25-4317-82). For intracellular stainings, we used a FOXP3 staining buffer set (eBioscience). Intracellular markers were analysed using the following antibodies: anti-CCL21 (59106, R&D Systems, IC457G-100UG), anti-Aire (5H12, eBioscience, cat. no.: 50-5934-82), anti-γH2AX (2F3, Biolegend, cat. no.: 613405) and anti-Ki67 (11F6, Biolegend, cat. no.: 151204), as described [5, 58, 59]. Intracellular detection of DCLK1 was achieved using anti-DCLK1 (DCAMKL1, Abcam, cat. no.: Ab31704), followed by donkey anti-rabbit Alexa Fluor 488 (Invitrogen, cat. no.: A21206). Apoptosis was assessed using FITC Annexin V (Biolegend, cat. no.: 640905), 7-AAD (Biolegend, cat. no.: 420403) and Annexin V Binding Buffer (Biolegend, cat. no.: 422201).

For hematopoietic cell analysis, thymocyte samples obtained from mechanically disrupted thymus were stained for flow cytometry analysis. The complete list of antibodies can be found in **Table 1**. CD1d tetramers loaded with PBS57 or unloaded CD1d tetramers were obtained from the National Institutes of Health Tetramer Core Facility. All intracellular staining was performed using a FOXP3 staining buffer set (eBioscience). Flow cytometry analyses were performed on an LSR Fortessa (Becton Dickinson) and cell sorting was performed on a FACS Aria II (Becton Dickinson). Data were analysed on FlowJo software.

RNA-sequencing

The total RNA library preparation and high-throughput sequencing of sorted cTEC/mTEC samples from 6 weeks-old $FOXO3^{cKO}$ and $FOXO3^{Ctr}$ littermates were performed at Gene Core facility (EMBL, Germany) as previously described [5, 58]. Fourteen sequencing libraries (three for cTEC control, three for cTEC $FOXO3^{cKO}$, four for mTEC control, four for mTEC $FOXO3^{cKO}$) were prepared using NEB Next RNA ultraprotocol (#E7530 NEB). Obtained libraries were quantified fluorimetrically, pooled in equimolar amounts and sequenced on the Illumina NextSeq sequencer (NextSeq 2000 P3 flowcell) in single-end mode (75 bases), following manufacturer's instructions (Illumina). The reads were aligned to the mouse genome (mm10) using STAR (version

2.4.2a) with mm10 GTF annotation. The number of reads per gene was generated during alignment step (quantMode GeneCounts) and gene counts were then analysed with DESeq2 package [61], as previously done [5, 58, 62]. An average of 58 million reads were mapped per $FOXO3^{Ctr}$ cTEC and $FOXO3^{cKO}$ cTEC samples, and 49 and 51 million reads were mapped per $FOXO3^{Ctr}$ mTEC and $FOXO3^{cKO}$ mTEC samples, respectively. Reads per kb of genes per million mapped reads (RPKM) values were calculated based on the normalized read counts. We selected differentially expressed genes based on the adjusted p-value lower than 0.1. Reads per kb of exon model per million mapped reads (RPKM) values were computed from normalized read counts. Gene ontology (GO) enrichment analysis was performed using model-based gene set analysis (MGSA) [63]. The analysis was performed with 20 independent runs of the Markov chain of 1.109 steps each. For each parameter, we used a regularly spaced grid with 11 points. The search intervals for the parameters p, alpha, and beta were set to [0.001, 0.1], [0, 0.2], and [0.5, 0.9] respe36ctively for the search on biological process terms, and [0.001, 0.1], [0, 0.15], and [0.5, 0.9] for the search on molecular function terms. Functional categories with a marginal posterior probability estimate higher than 0.65 were retained for further analysis. The hierarchical clustering, represented as a dendrogram, of TEC populations were performed using the hclust function in R on euclidean distances between the variance of the rlog-transformed read counts for each genes across samples. The sequencing reads have been submitted to ENA (http://www.ebi.ac.uk/ena) and further information and resources, code and methods will be accessible under the following project number: PRJEB64863 (available upon publication). Requests for further information should be directed to and will be fulfilled by the corresponding author.

Comparative transcriptomic analysis

Analysis of publicly available RNA-Seq data and identification of the specific genes of different mTEC subpopulations was performed as previously described [58]. Briefly, the specific gene signatures of mTEC I, mTEC II, mTEC III, and mTEC IV [64] were obtained by extracting the top 200 most expressed genes for which the relative expression in the selected population was ≥ 2-fold the expression in all other populations. The specific gene signatures of early-, pre-, late-, and post-Aire mTEC [11] were obtained by extracting the top 200 most expressed genes with z-score ≥ 1.5. Mimetic and transit-amplifying subset signatures were derived from differentially expressed genes by filtering for genes unique to each cluster with fold-change > 2 and adjusted p < 0.01 [13]. The proliferating TEC signature was used as provided [16, 17].

Regulatory T cell suppression assay

For *in vitro* suppression assays, $5x10^4$ control conventional T cells ($CD4^+CD25^-$) were labelled with1 μM CFSE after cell sorting and cultured in duplicates for 3 days in round bottom 96-well plates in the presence of 1 μg/ml anti-CD3 (145-2C11; BD Pharmingen), γ-irradiated splenocytes (200 rads; $1x10^5$ /well), and different ratios of either control or $FOXO3^{cKO}$-derived regulatory T cells ($CD4^+CD25^+$). Suppressive ability of regulatory T cells was assessed based on conventional T-cell division, measured by the number of cells found within discrete levels of CFSE dilution. The average number of divisions was calculated for cells that performed at least one division. The precursor frequency of dividing cells (percentage of cells in the initial population that had undergone division) was calculated as follows: $[\sum_{n\geq1}(P_n/2^n)]/[\sum_{n\geq0}(P_n/2^n)]$, where *n* is the division number that cells have gone through and P_n is the number of cells in division *n* [65].

Histology

Paraffin-embedded tissue sections of thymus, lacrimal gland, salivary glands, stomach, colon, pancreas, kidney and liver were stained with haematoxylin and eosin (H&E). Analysis was done on a light microscope (Olympus CX31) and images were captured using a brightfield microscope (Leica DM2000 LED). Thymic section images were processed using Fiji software. Histopathology was scored in a blind and randomized fashion by four independent observers. For organs in which no lymphocytic infiltration is detected in any animal of the genotypes under comparison, data are not shown.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. The 2-tailed Mann-Whitney test was used for all statistical analyses. Sample sizes (n) and number of experiment replications are reported in the respective figure legends. Samples correspond to biological replicates. Significant *P* values are as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Nonsignificant differences are not specified.

Specificity	Clone	Supplier	Cat. No.
CD177	2B8	eBioscience	47-1171-82
CD11b	M1/70	eBioscience	50-0112-82
CD11c	N418	eBioscience	50-0114-82
CD19	eBio1D3	eBioscience	50-0193-82
CD24	M1/69	eBioscience	48-0242-82
CD24	M1/69	Biolegend	101831
CD25	PC.61.5	eBioscience	25-0251-82
CD4	GK1.5	Biolegend	100453
CD44	IM7	Biolegend	103022
CD44	IM7	Biolegend	103049
CD5	53-7.3	Biolegend	100605
CD62L	MEL-14	Biolegend	104437
CD69	H1.2F3	Biolegend	104511
CD8	53-6.7	Biolegend	100741
FoxP3	FJK-16s	eBioscience	17-5773-80
GR1	RB6-8CS	eBioscience	50-5931-82
Helios	22F6	Biolegend	137204
NK 1.1	PK136	Biolegend	108713
NK1.1	PK136	eBioscience	17-5941-82
PD1	29F-1A12	Biolegend	135229
PLZF	Mags.21F7	eBioscience	53-9320-80
RORgt	Q31-378	BD	564723
T-bet	4B10	Biolegend	644832
TCRβ	H57-597	eBioscience	12-5961-82
TCRβ	H57-597	eBioscience	45-5961-80
TER-119	TER-119	eBioscience	17-5921-82
γδ TCR	eBioGL3	eBioscience	17-5711-82

Table 1 – Antibodies used in flow cytometry analysis of hematopoietic populations in thymus samples.

Acknowledgements

We thank Dr. Lamas, the caretakers of the animal facility and Translational Cytometry platform for technical assistance, and the Genomics Core Facility (EMBL, Germany) for assistance with sequencing. The authors also acknowledge the NIH Tetramer Core Facility, Emory University, Atlanta, GA, USA for the CD1d-PBS57 tetramer.

Conflict of Interest

The authors have no conflict nor competing financial interests.

Author Contributions

C.R. conceived and performed experiments, analysed data, wrote the manuscript; P.F. performed experiments and feedback on the manuscript; J.J.M.L., F.M, L.S., R.P., performed experiments, V.B. provided scientific expertise; N.L.A. conceived experiments, wrote the manuscript, and secured funding,

Ethics Statement

This work involved animal experimentation. Experiments with mice were performed in accordance with European guidelines for animals used for scientific purposes (Directive 2010/63/EU).

Funding

This study was supported by a starting grant from the European Research Council (ERC) under the project 637843 and by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through FCT - Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior in the framework of the project PTDC/MED-IMU/1416/2020. Moreover, the study was supported by funds from the 10th Edition of the “Crioestaminal” Prize awarded to N.A. with the support of the Association "Viver a Ciência" (VAC) and “Crioestaminal”. Crioestaminal award attributed to N.L.A.

Availability of Data and Materials

Data was deposited in the GEO database (PRJEB64863). Requests for further information should be directed to and will be fulfilled by the corresponding author.

REFERENCES

1. Takahama, Y.*, et al.*, 2017, *Generation of diversity in thymic epithelial cells,* Nat Rev Immunol, **17**(5), 295-305.
2. Hollander, G.A.*, et al.*, 2010, *Emerging strategies to boost thymic function,* Curr Opin Pharmacol, **10**(4), 443-53.
3. Abramson, J. and Anderson, G., 2017, *Thymic Epithelial Cells,* Annu Rev Immunol, **35**, 85-118.
4. Abramson, J. and Goldfarb, Y., 2016, *AIRE: From promiscuous molecular partnerships to promiscuous gene expression,* Eur J Immunol, **46**(1), 22-33.
5. Rodrigues, P.M.*, et al.*, 2017, *Thymic epithelial cells require p53 to support their long-term function in thymopoiesis in mice,* Blood, **130**(4), 478-488.
6. Eijkelenboom, A. and Burgering, B.M., 2013, *FOXOs: signalling integrators for homeostasis maintenance,* Nat Rev Mol Cell Biol, **14**(2), 83-97.
7. Calissi, G.*, et al.*, 2021, *Therapeutic strategies targeting FOXO transcription factors,* Nat Rev Drug Discov, **20**(1), 21-38.
8. Sansom, S.N.*, et al.*, 2014, *Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia,* Genome Res, **24**(12), 1918-31.
9. Castrillon, D.H.*, et al.*, 2003, *Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a,* Science, **301**(5630), 215-8.
10. Bleul, C.C. and Boehm, T., 2005, *BMP signaling is required for normal thymus development,* J Immunol, **175**(8), 5213-21.
11. Miller, C.N.*, et al.*, 2018, *Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development,* Nature, **559**(7715), 627-631.
12. Bornstein, C.*, et al.*, 2018, *Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells,* Nature, **559**(7715), 622-+.
13. Michelson, D.A.*, et al.*, 2022, *Thymic epithelial cells co-opt lineage-defining transcription factors to eliminate autoreactive T cells,* Cell.
14. Givony, T.*, et al.*, 2023, *Thymic mimetic cells function beyond self-tolerance,* Nature, **622**(7981), 164-172.
15. Tsai, W.B.*, et al.*, 2008, *Functional interaction between FOXO3a and ATM regulates DNA damage response,* Nat Cell Biol, **10**(4), 460-7.
16. Lindh, E.*, et al.*, 2010, *AIRE deficiency leads to impaired iNKT cell development,* J Autoimmun, **34**(1), 66-72.

17. Wells, K.L.*, et al.*, 2020, *Combined transient ablation and single-cell RNA-sequencing reveals the development of medullary thymic epithelial cells,* Elife, **9**.
18. Zhang, S.L.*, et al.*, 2014, *Chemokine treatment rescues profound T-lineage progenitor homing defect after bone marrow transplant conditioning in mice,* Blood, **124**(2), 296-304.
19. Dudakov, J.A.*, et al.*, 2012, *Interleukin-22 drives endogenous thymic regeneration in mice,* Science, **336**(6077), 91-5.
20. Yamashita, I.*, et al.*, 1993, *CD69 cell surface expression identifies developing thymocytes which audition for T cell antigen receptor-mediated positive selection,* Int Immunol, **5**(9), 1139-50.
21. Daley, S.R.*, et al.*, 2013, *Helios marks strongly autoreactive CD4+ T cells in two major waves of thymic deletion distinguished by induction of PD-1 or NF-kappaB,* J Exp Med, **210**(2), 269-85.
22. Owen, D.L.*, et al.*, 2019, *Thymic regulatory T cells arise via two distinct developmental programs,* Nat Immunol, **20**(2), 195-205.
23. White, A.J.*, et al.*, 2014, *An essential role for medullary thymic epithelial cells during the intrathymic development of invariant NKT cells,* J Immunol, **192**(6), 2659-66.
24. Das, R.*, et al.*, 2010, *Transcriptional control of invariant NKT cell development,* Immunol Rev, **238**(1), 195-215.
25. Crosby, C.M. and Kronenberg, M., 2018, *Tissue-specific functions of invariant natural killer T cells,* Nat Rev Immunol, **18**(9), 559-574.
26. Jiang, W.*, et al.*, 2005, *Modifier loci condition autoimmunity provoked by Aire deficiency,* J Exp Med, **202**(6), 805-15.
27. Baran-Gale, J.*, et al.*, 2020, *Ageing compromises mouse thymus function and remodels epithelial cell differentiation,* Elife, **9**.
28. Lkhagvasuren, E.*, et al.*, 2013, *Lymphotoxin beta receptor regulates the development of CCL21-expressing subset of postnatal medullary thymic epithelial cells,* J Immunol, **190**(10), 5110-7.
29. Lei, Y.*, et al.*, 2011, *Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development,* J Exp Med, **208**(2), 383-94.
30. Morimoto, J.*, et al.*, 2022, *Aire suppresses CTLA-4 expression from the thymic stroma to control autoimmunity,* Cell Rep, **38**(7), 110384.
31. Nishijima, H.*, et al.*, 2022, *Aire Controls Heterogeneity of Medullary Thymic Epithelial Cells for the Expression of Self-Antigens,* J Immunol, **208**(2), 303-320.

32. Gray, D.*, et al.*, 2007, *Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire,* J Exp Med, **204**(11), 2521-8.
33. Nishikawa, Y.*, et al.*, 2014, *Temporal lineage tracing of Aire-expressing cells reveals a requirement for Aire in their maturation program,* J Immunol, **192**(6), 2585-92.
34. Puig, O.*, et al.*, 2003, *Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway,* Genes Dev, **17**(16), 2006-20.
35. Junger, M.A.*, et al.*, 2003, *The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling,* J Biol, **2**(3), 20.
36. Rodewald, H.R.*, et al.*, 2001, *Thymus medulla consisting of epithelial islets each derived from a single progenitor,* Nature, **414**(6865), 763-8.
37. Abramson, J.*, et al.*, 2010, *Aire's partners in the molecular control of immunological tolerance,* Cell, **140**(1), 123-35.
38. Dhalla, F.*, et al.*, 2020, *Biologically indeterminate yet ordered promiscuous gene expression in single medullary thymic epithelial cells,* Embo Journal, **39**(1).
39. Meredith, M.*, et al.*, 2015, *Aire controls gene expression in the thymic epithelium with ordered stochasticity,* Nat Immunol, **16**(9), 942-9.
40. Brennecke, P.*, et al.*, 2015, *Single-cell transcriptome analysis reveals coordinated ectopic gene-expression patterns in medullary thymic epithelial cells,* Nat Immunol, **16**(9), 933-41.
41. Renault, V.M.*, et al.*, 2011, *The pro-longevity gene FoxO3 is a direct target of the p53 tumor suppressor,* Oncogene, **30**(29), 3207-21.
42. You, H.*, et al.*, 2006, *Regulation of transactivation-independent proapoptotic activity of p53 by FOXO3a,* Proc Natl Acad Sci U S A, **103**(24), 9051-6.
43. You, H.*, et al.*, 2004, *p53-dependent inhibition of FKHRL1 in response to DNA damage through protein kinase SGK1,* Proc Natl Acad Sci U S A, **101**(39), 14057-62.
44. Miyaguchi, Y.*, et al.*, 2009, *P53 negatively regulates the transcriptional activity of FOXO3a under oxidative stress,* Cell Biol Int, **33**(8), 853-60.
45. Cowan, J.E.*, et al.*, 2013, *The thymic medulla is required for Foxp3+ regulatory but not conventional CD4+ thymocyte development,* J Exp Med, **210**(4), 675-81.
46. Lucas, B.*, et al.*, 2020, *Diversity in medullary thymic epithelial cells controls the activity and availability of iNKT cells,* Nat Commun, **11**(1), 2198.

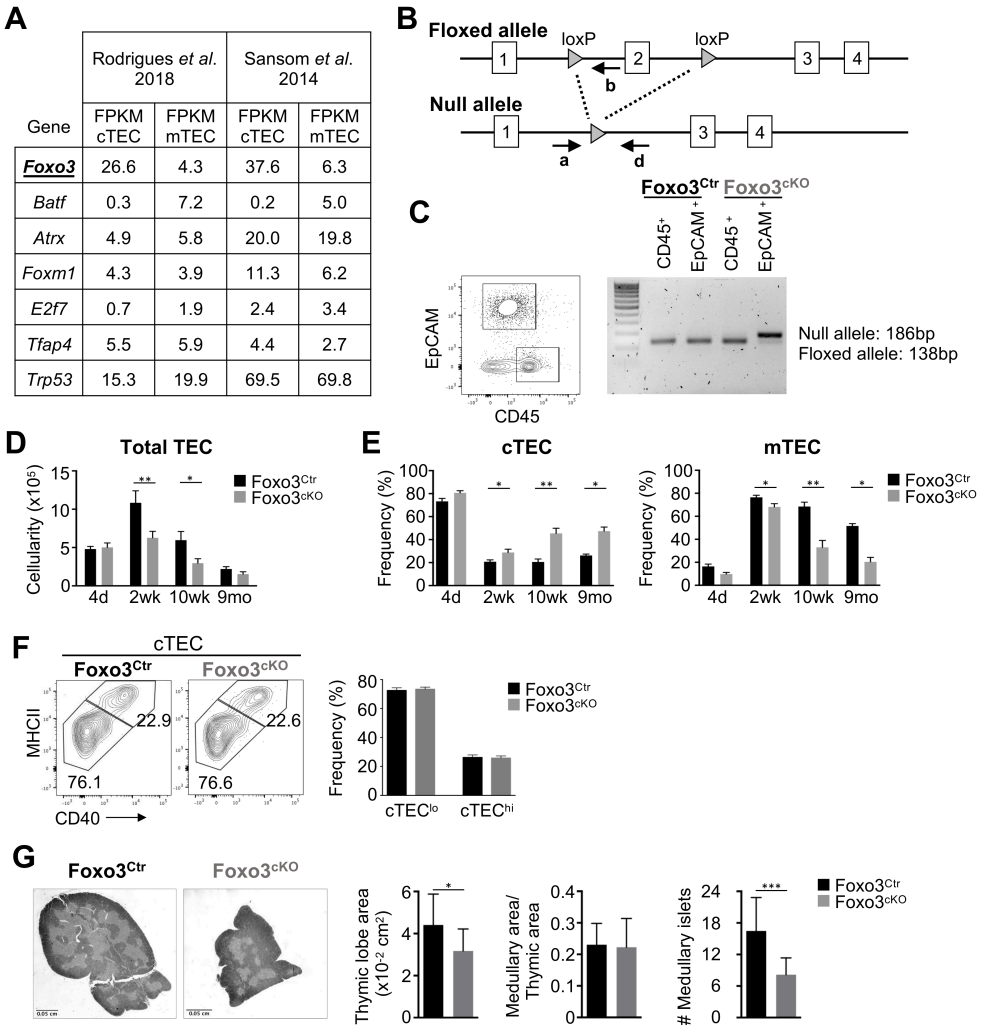
47. Guerau-de-Arellano, M.*, et al.*, 2009, *Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity,* J Exp Med, **206**(6), 1245-52.
48. Yang, S.*, et al.*, 2015, *Immune tolerance. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance,* Science, **348**(6234), 589-94.
49. Tothova, Z.*, et al.*, 2007, *FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress,* Cell, **128**(2), 325-39.
50. Stahl, M.*, et al.*, 2002, *The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2,* J Immunol, **168**(10), 5024-31.
51. Ekoff, M.*, et al.*, 2007, *The BH3-only protein Puma plays an essential role in cytokine deprivation induced apoptosis of mast cells,* Blood, **110**(9), 3209-17.
52. You, H.*, et al.*, 2006, *FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal,* J Exp Med, **203**(7), 1657-63.
53. Ouyang, W.*, et al.*, 2010, *Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells,* Nat Immunol, **11**(7), 618-27.
54. Paik, J.H.*, et al.*, 2009, *FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis,* Cell Stem Cell, **5**(5), 540-53.
55. Paik, J.H.*, et al.*, 2007, *FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis,* Cell, **128**(2), 309-23.
56. Anderson, M.S.*, et al.*, 2002, *Projection of an immunological self shadow within the thymus by the aire protein,* Science, **298**(5597), 1395-401.
57. Soza-Ried, C.*, et al.*, 2008, *Maintenance of thymic epithelial phenotype requires extrinsic signals in mouse and zebrafish,* J Immunol, **181**(8), 5272-7.
58. Ferreirinha, P.*, et al.*, 2021, *A novel method to identify Post-Aire stages of medullary thymic epithelial cell differentiation,* Eur J Immunol, **51**(2), 311-318.
59. Rodrigues, P.M.*, et al.*, 2018, *Intrathymic Deletion of IL-7 Reveals a Contribution of the Bone Marrow to Thymic Rebound Induced by Androgen Blockade,* J Immunol, **200**(4), 1389-1398.
60. Gray, D.H.*, et al.*, 2002, *Analysis of thymic stromal cell populations using flow cytometry,* J Immunol Methods, **260**(1-2), 15-28.
61. Love, M.I.*, et al.*, 2014, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2,* Genome Biol, **15**(12), 550.
62. Ferreirinha, P.*, et al.*, 2022, *Identification of fibroblast progenitors in the developing mouse thymus,* Development, **149**(10).
63. Bauer, S.*, et al.*, 2010, *GOing Bayesian: model-based gene set analysis of genome-scale data,* Nucleic Acids Res, **38**(11), 3523-32.

64. Bornstein, C.*, et al.*, 2018, *Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells,* Nature, **559**(7715), 622-626.

65. Alves, N.L.*, et al.*, 2003, *IL-15 induces antigen-independent expansion and differentiation of human naive CD8+ T cells in vitro,* Blood, **102**(7), 2541-6.

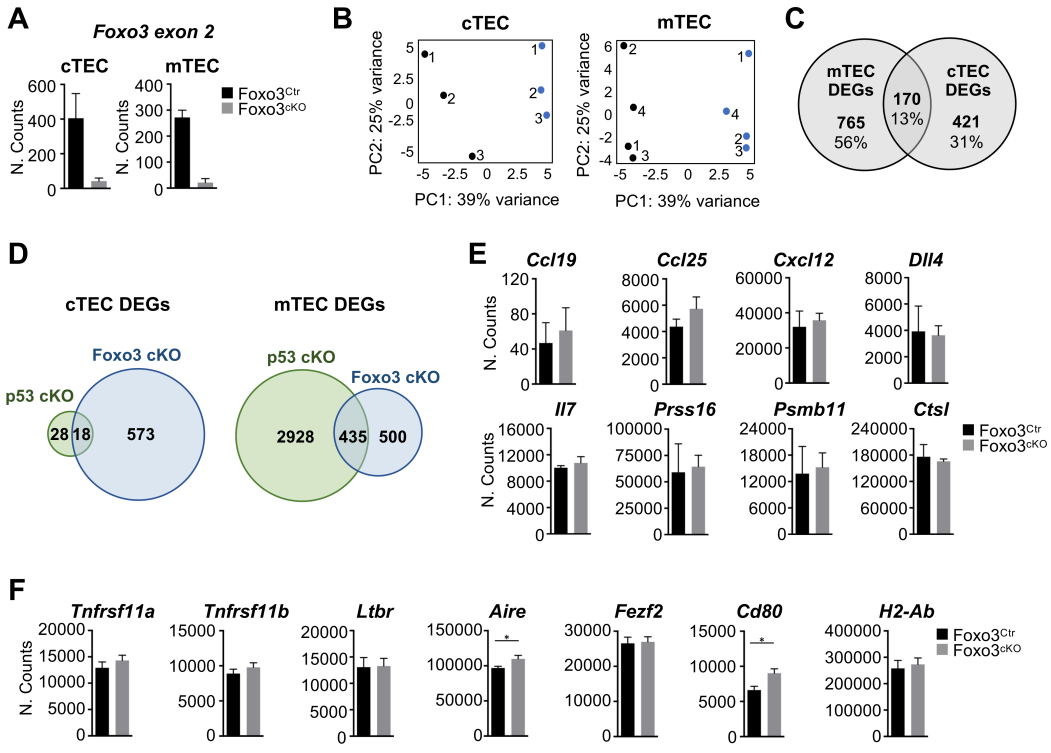
SUPPLEMENTARY INFORMATION

Supplementary tables are available on the online version of this article.

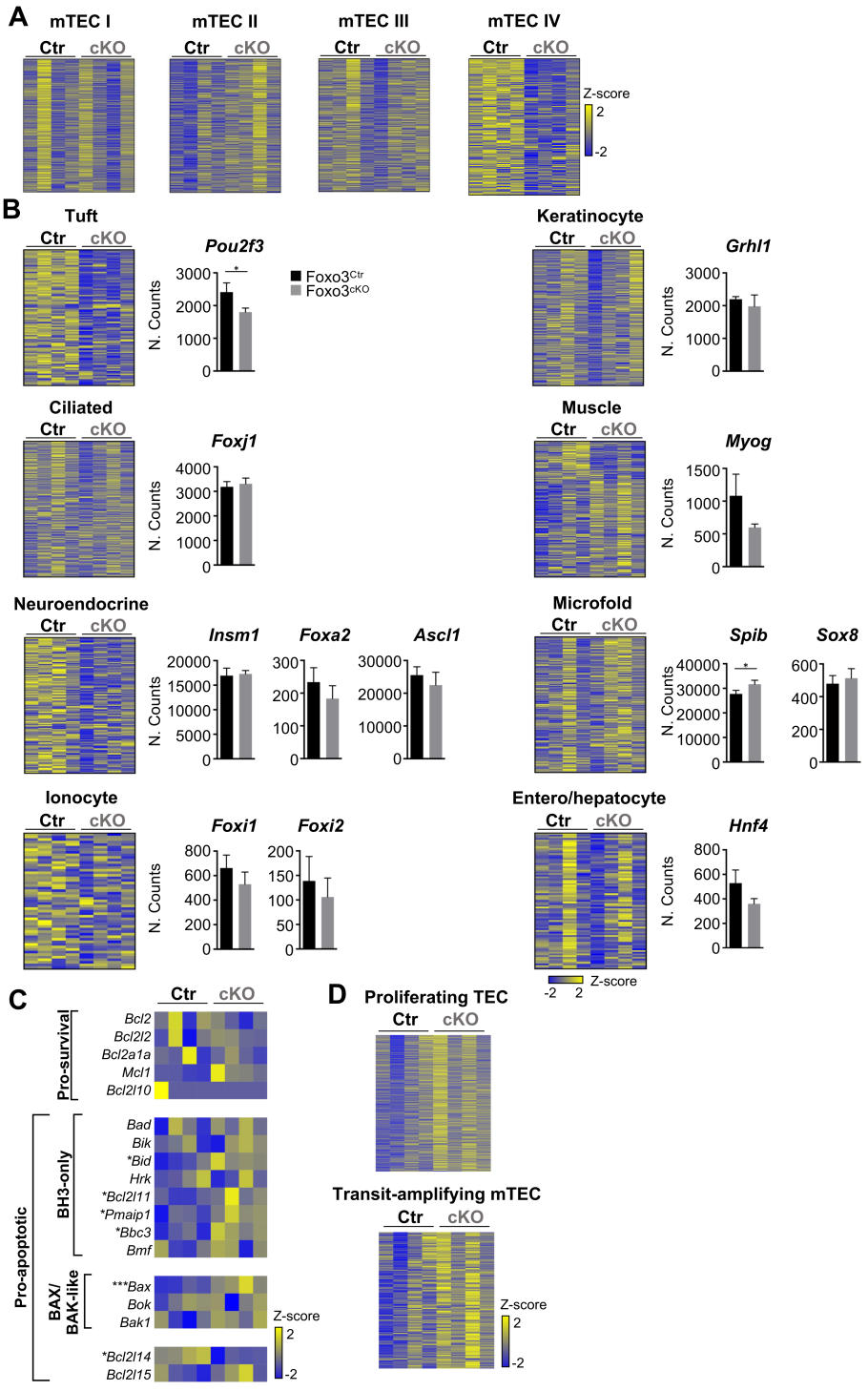


Gene	Rodrigues *et al.* 2018		Sansom *et al.* 2014	
	FPKM cTEC	FPKM mTEC	FPKM cTEC	FPKM mTEC
Foxo3	26.6	4.3	37.6	6.3
Batf	0.3	7.2	0.2	5.0
Atrx	4.9	5.8	20.0	19.8
Foxm1	4.3	3.9	11.3	6.2
E2f7	0.7	1.9	2.4	3.4
Tfap4	5.5	5.9	4.4	2.7
Trp53	15.3	19.9	69.5	69.8

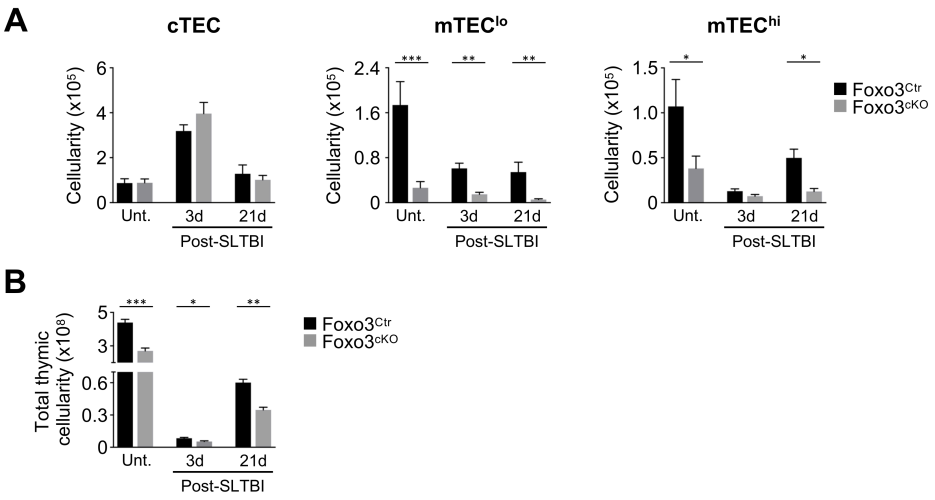
Supplementary figure 1 – Targeting *Foxo3* expression in TECs. (A) The gene list shows the transcription factor-coding genes found in *Mus musculus* annotated under the gene ontology term GO:0030330 "DNA damage response, signal transduction by p53 class mediator". FPKM was obtained from RNA-sequencing analysis of cTECs and mTECs from postnatal thymus. **(B)** Genomic structure of *Foxo3*^{floxed} and *Foxo3*^{cKO} null alleles. Primers used for PCR (a + b + d) are indicated by small arrows. Grey triangles represent the loxP sequences. Represented exon and intron sizes are not to scale. **(C)** Confirmation of FOXN1^{Cre}-driven *Foxo3* exon 2 deletion by genomic PCR analysis of FACS-sorted thymocytes (CD45^{+}) and TECs (EpCAM^{+}) from FOXO3^{Ctr} (FOXO3$^{fl/fl}$) and FOXO3^{cKO} (FOXN1^{Cre}:FOXO3$^{fl/fl}$) mice. **(D)** Total TEC numbers in thymi isolated at the indicated time points. **(E)** Frequency of cTECs and mTECs at the indicated time points. **(F)** cTEC^{lo} (MHCII^{low}CD40^{low}) and cTEC^{hi} (MHCII^{high}CD40^{high}) composition within total cTECs of the 10-week-old adult thymus. Data are representative of 2 or 3 independent experiments per time-point (n=6-9 independent samples). All data are represented as mean ± SEM. **(G)** Thymic sections from 10-week-old FOXO3^{Ctr} and FOXO3^{cKO} mice stained with hematoxylin and eosin (H&E). Bar graphs depict mean and SD of thymic lobe area, medulla/total area ratio and medullary islet number measured on 3 sections per thymus from 5 FOXO3^{Ctr} and 5 FOXO3^{cKO} mice. wk - weeks; mo - months. *P < 0.05; **P < 0.01; ***P < 0.001.



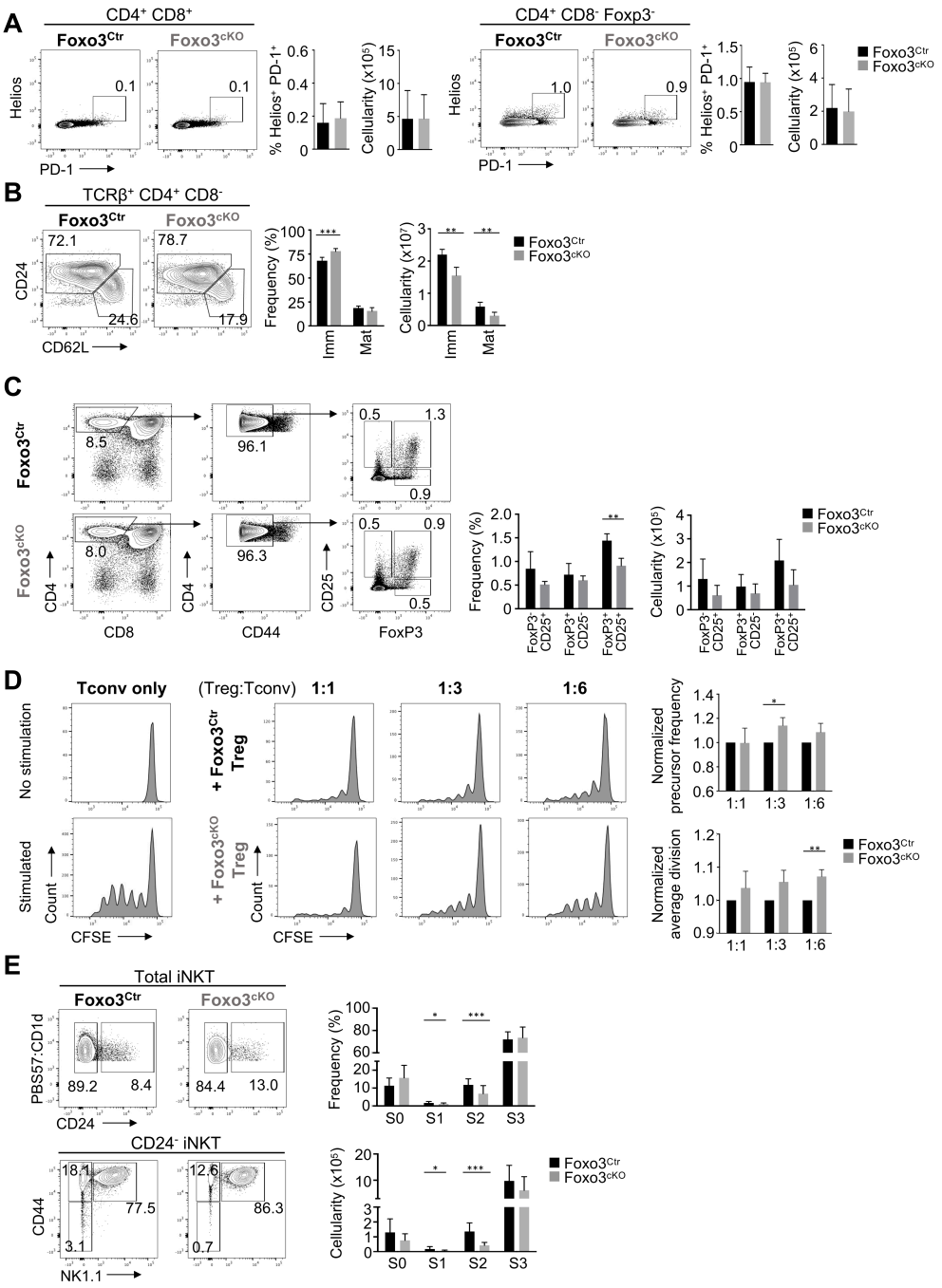
Supplementary figure 2 – Transcriptome analysis of FOXO3^{cKO} cTECs and mTECs. (A) Expression of exon 2 of *Foxo3* gene obtained from RNA-Seq analysis of FACS-sorted cTECs and mTECs purified from 6-week-old FOXO3^{Ctr} and FOXO3^{cKO} mice. **(B)** Principal component analysis (PCA) obtained from RNA-Seq analysis of cTECs and mTECs isolated from FOXO3^{Ctr} and FOXO3^{cKO} thymus. **(C)** Venn diagram depicts the number of differentially expressed genes detected in cTECs, mTECs and commonly detected in both cTECs and mTECs. **(D)** Venn diagrams depict the specific and common DEGs resulting from *Trp53* or *Foxo3* deficiency in cTECs (left) and mTECs (right). **(E)** Expression of cTEC-specific genes in FOXO3^{Ctr} and FOXO3^{cKO} cTECs. **(F)** Expression of mTEC-specific genes in FOXO3^{Ctr} and FOXO3^{cKO} mTECs. All data are represented as mean ± SD. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.



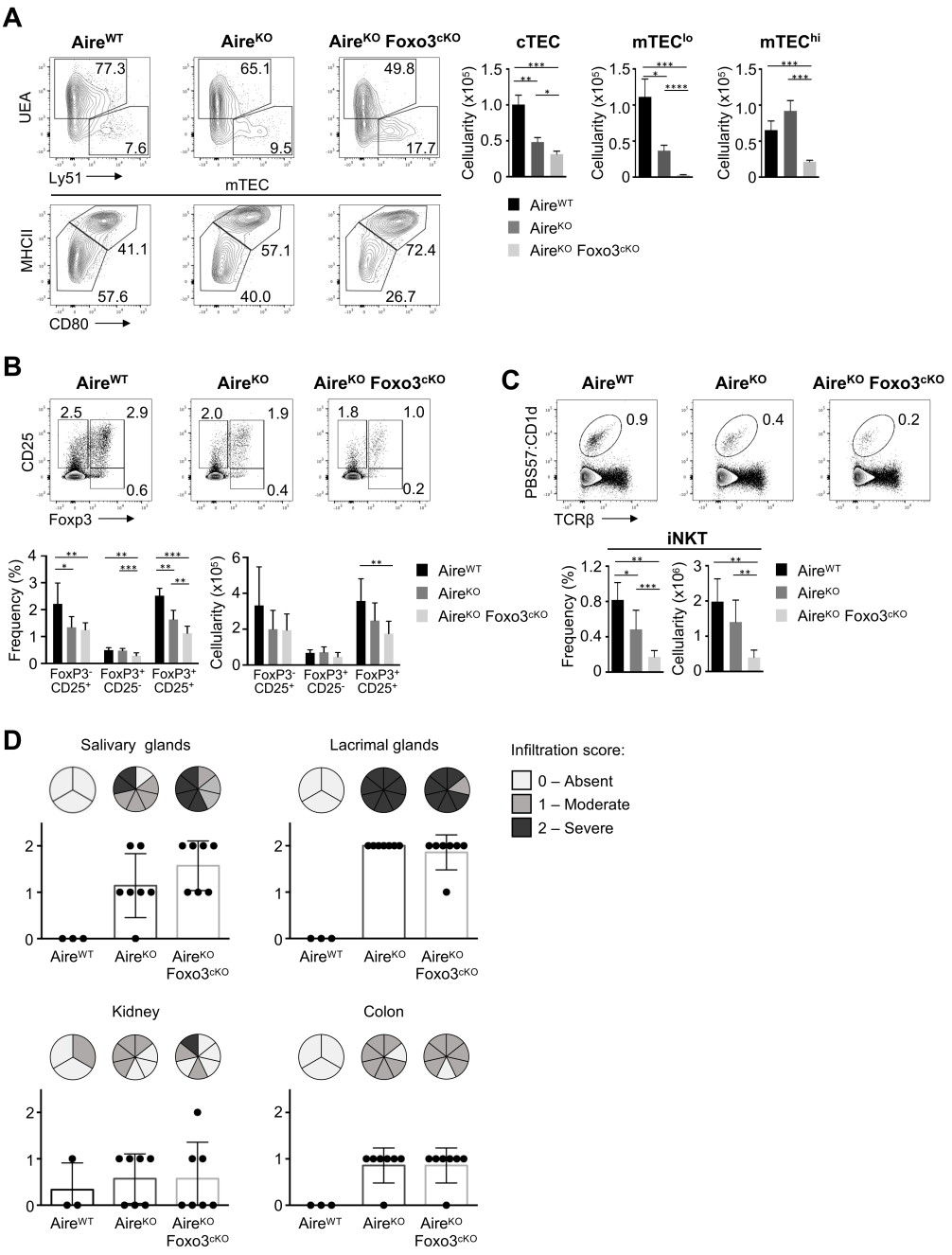
Supplementary figure 3 – Analysis of genetic signature of mTEC subsets in FOXO3^{cKO} mTECs. (A) Relative expression level of genes specifically upregulated in mTEC I, mTEC II, mTEC III and mTEC IV (data from [12]) in FOXO3^{Ctr} and FOXO3^{cKO} mTECs. **(B)** Relative expression level of genes specifically upregulated in mimetic mTEC subsets (as defined by Michelson et al., 2022) in FOXO3^{Ctr} and FOXO3^{cKO} mTECs. The expression of subset-specific transcription factor genes was measured in FOXO3^{Ctr} and FOXO3^{cKO} mTECs and is depicted on the right of the corresponding heatmaps. Bar graphs show mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001. **(C)** FOXO3^{Ctr} and FOXO3^{cKO} mTEC samples were analysed for the relative expression of genes coding for pro-survival and pro-apoptotic Bcl2 family members. Statistically significant differentially expressed genes are marked as * (P< 0.05), ** (P< 0.01) or *** (P< 0.001). **(D)** Relative expression level of genes specifically upregulated in proliferating and transit-amplifying mTECs (as defined by Baran-Gale et al., 2020, and Michelson et al., 2022) in FOXO3^{Ctr} and FOXO3^{cKO} mTECs.



Supplementary figure 4 – Analysis of TEC cellularity recovery following SL-TBI. 10-week-old FOXO3^{Ctr} and FOXO3^{cKO} mice were subjected to sublethal total-body irradiation (SLTBI) and analysed at day 3 and day 21 post-irradiation. 10-week-old FOXO3^{Ctr} and FOXO3^{cKO} untreated mice (Unt.) were also analysed. The cellularity of cTECs, mTEC^{lo} and mTEC^{hi} **(A)** and total thymic cellularity **(B)** were determined at the indicated time-points. Data are representative of 2 or 3 independent experiments (n=6-9 independent samples) and represented as mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.



Supplementary figure 5 – T-cell development in the FOXO3^{cKO} thymus. (A) Expression of Helios and PD-1 on CD4$^+$ CD8$^+$ DP thymocytes (left) and on CD4$^+$ CD8$^-$ FoxP3$^-$ thymocytes (right). **(B)** Expression of CD24 and CD62L on TCRβ$^+$ CD4$^+$ CD8$^-$ thymocytes. Bar graphs show absolute cell numbers and percentages. Data are representative of 3 independent experiments (n=9 independent samples). **(C)** CD25 and FOXP3 expression on non-recirculatory CD44$^{-/lo}$ SP4 thymocytes in the 10-week-old thymus of FOXO3^{Ctr} and FOXO3^{cKO} mice. All data are represented as mean ± SD. **(D)** *In vitro* suppression assay measuring CFSE labelling in conventional T cells (Tconv) from FOXO3^{Ctr} thymus (WT) on day 3 of stimulation and co-culture with FOXO3^{Ctr} or FOXO3^{cKO}-derived thymic regulatory T cells (Treg) at the indicated Treg:Tconv ratios. Graphs represent the average number of divisions and precursor frequency normalized relatively to the corresponding conditions with FOXO3^{Ctr} Tregs, which were set to 1. Results are presented as mean ± SEM of 4 independent experiments. **(E)** Expression of CD24, CD44 and NK1.1 on iNKTs for the quantification of stage 0 (CD24$^+$), stage 1 (CD24$^-$CD44$^-$NK1.1$^-$), stage 2 (CD24$^-$CD44$^+$NK1.1$^-$) and stage 3 (CD24$^-$CD44$^+$NK1.1$^+$). Bar graphs show absolute cell numbers and percentages. Data are representative of 2 to 4 independent experiments (n=6 to 12 independent samples). Data are represented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.



Supplementary figure 6 – Effects of TEC-specific *Foxo3* deletion in Aire-independent mTEC lineages and in the autoimmune syndrome of Aire^{KO} mice. (A) TECs from 10-week-old Aire^{WT}, Aire^{KO} and Aire^{KO}FOXO3^{cKO} thymus were analysed for cTEC and mTEC composition (top). Total mTECs were analysed for mTEC^{lo} and mTEC^{hi} composition (bottom). Bar graphs show absolute cell numbers. Data are representative of 4 independent experiments (n=5-10 independent samples). Data are represented as mean ± SEM. **(B)** TCRβ^{+} CD4^{+} thymocytes from Aire^{WT}, Aire^{KO} and Aire^{KO}FOXO3^{cKO} thymus were analysed for CD25 and FOXP3 expression **(C)** Total thymocytes from Aire^{WT}, Aire^{KO} and Aire^{KO}FOXO3^{cKO} thymus were analysed for expression of TCRβ and reactivity with PBS57-loaded CD1d tetramer. Bar graphs show absolute cell numbers and percentages. Data are representative of 4 independent experiments (n=5-10 biologically independent samples). Data are represented as mean ± SD. **(D)** Severity scores for inflammatory lymphocytic infiltration in salivary glands, lacrimal glands, kidney and colon of 6 months-old Aire^{WT}, Aire^{KO} and Aire^{KO}FOXO3^{cKO} mice. Pie charts represent absent, moderate and severe lesions as light grey, dark grey and black, respectively. Bar graphs represent absent, moderate and severe lesions scored as 0-2, respectively. Data are represented as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

CHAPTER V - GENERAL DISCUSSION

The generation of a functionally diverse and self-tolerant T-cell pool relies on specialized thymic niches established by cTECs and mTECs. Both TEC lineages play well-defined roles in coordinating distinct phases of T-cell differentiation and repertoire selection [1]. With age, cellular and functional alterations within the TEC compartment contribute to physiological aged-related thymic involution [2]. Furthermore, TECs also undergo acute damage in response to infection, chemotherapy, or irradiation [3]. Both chronic and acute perturbations on the thymic microenvironment reduce T-cell production and can compromise the tolerogenic mechanisms of the thymus. This thesis focused on elucidating the cellular and molecular mechanisms that dictate TEC differentiation and homeostasis. Our understanding of these processes is key to the development of strategies for thymic function rejuvenation or correction in the context of acquired or congenital immunodeficiencies or autoimmunity. While Chapter III describes a novel methodology to monitor Aire^{+} mTEC differentiation, Chapter IV reports the role of the transcription factor FOXO3 in regulating mTEC homeostasis. The present Chapter reflects upon the reported findings and unanswered questions, integrating how these insights shed light on new trajectories for the investigation of the mechanisms that underlie thymic function.

UNVEILING THE DYNAMICS OF AIRE^{+} MTEC DIFFERENTIATION

Prospective isolation of mTECs entering the post-Aire stage

The transcription regulator Aire defines a subpopulation of mTECs and is the most characterized regulator of their ability to activate the transcription of peripheral tissue-restricted genes in otherwise silenced chromatin regions [4]. As a result, Aire is responsible for a significant portion of TRAs presented by mTECs to developing thymocytes and has a critical contribution to the central tolerance induction mechanisms enforced by the thymic medulla. As the roadmap of mTEC differentiation continues to be unravelled, Aire expression has been shown to demark a key entry point into a transcriptional state that favours the emergence of phenotypically divergent post-Aire subsets [5, 6]. Chapter III of this thesis focuses on increasing the resolution of this specific window of mTEC differentiation. Our results reveal that the differential cell surface expression of CD24 and Sca1 represents a novel strategy to map the entry of Aire^{+} mTEC^{high} into the post-Aire stage. Particularly, we show that CD24^{-} Sca1^{-} mTEC$^{A/hi}$ are mostly defined as Aire^{+} cells (approximately 80%). Concordantly, this subset shows the highest upregulation of previously described mTEC II and early-Aire gene signatures

and of Aire-dependent TRA genes. Notably, the mTEC^{high} includes two other subsets, the CD24^{+}Sca1^{-} mTEC$^{B/hi}$ and the CD24^{+}Sca1^{+} mTEC$^{C/hi}$. The mTEC$^{B/hi}$ includes similar fractions of Aire^{+} and Aire^{-} cells and its transcriptional profile shows intermediate levels of mTEC II and mTEC III signature genes and Aire-dependent TRAs. Additionally, we detected the highest levels of late-Aire-specific genes in this subset. Lastly, mTEC$^{C/hi}$ is the least abundant mTEC^{high} subset and includes the smallest fraction of Aire-expressing cells (approximately 20%). The mTEC$^{C/hi}$ subset is characterized by a drastic downregulation of all TRA genes, a mixed profile of late- and post-Aire genes, and a high upregulation of mTEC III and terminally differentiated keratinocyte genes. Altogether, these observations suggested a developmental path in which mTEC$^{A/hi}$, mTEC$^{B/hi}$, and mTEC$^{C/hi}$ constitute sequential stages of development from Aire-expressing cells into the post-Aire stage (**Figure 1**).

We then proceed with both *in vitro* and *in vivo* strategies to demonstrate the precursor-product relationships between these new subsets. While we could not detect the progeny of isolated mTEC$^{B/hi}$ and mTEC$^{C/hi}$ using hybrid RTOCs, mTEC$^{A/hi}$ showed successful engraftment and their progeny included Aire^{-} cells characterized as mTEC$^{B/hi}$ and mTEC$^{C/hi}$. These results implied that mTEC$^{A/hi}$ contained precursors of the mTEC$^{B/hi}$ and mTEC$^{C/hi}$ subsets. In turn, these two subsets might represent more terminal differentiation states, which could explain their inability to engraft and differentiate *in vitro.* Furthermore, analysis of Aire^{KO} mice revealed that their mTEC^{high} population was mostly composed of mTEC$^{A/hi}$ and did not possess the mTEC$^{B/hi}$ and mTEC$^{C/hi}$ subsets. This observation led us to hypothesize that, in the Aire^{KO} mouse model, mature mTECs that become poised to begin Aire expression but are devoid of the Aire protein become retained in the mTEC$^{A/hi}$ differentiation stage. These cells, due to the absence of Aire, will not proceed to late-Aire or post-Aire stages, thus explaining the absence of mTEC$^{B/hi}$ and mTEC$^{C/hi}$ in the Aire^{KO} thymus (**Figure 1**). Altogether, our results show that mTEC$^{A/hi}$, mTEC$^{B/hi}$, and mTEC$^{C/hi}$ mark three consecutive states of differentiation starting at Aire acquisition and ending at Aire downregulation and upregulation of post-Aire signature genes. Still, while post-Aire cells can be found in a transitory state between mTEC^{high} and mTEC^{low} cells, they complete their terminal differentiation within mTEC^{low}. Worth noting, the three CD24 and Sca1-defined subsets within the mTEC^{low} (CD24^{-}Sca1^{-} mTEC$^{A/lo}$, CD24^{+}Sca1^{-} mTEC$^{B/lo}$, and CD24^{+}Sca1^{+} mTEC$^{C/lo}$) had no particular enrichment for post-Aire associated genes, suggesting a dilution of this signature among the different subsets. Hence, we report that neither of the A-B subsets within the mTEC^{low} maps terminally differentiated post-Aire cells. Instead, A-B subsets within the mTEC^{high} solely identify the end of the Aire-expressing stage and the passage into the post-Aire

state. Thus, the detection of CD24 and Sca1 constitutes a simple surface marker-based strategy to assess a key transition in the mTEC developmental program.

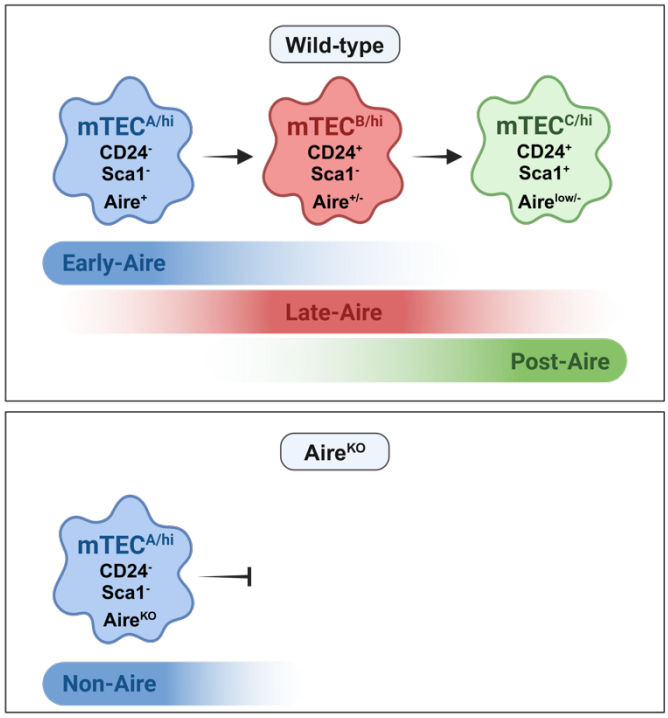


Figure 1 – Proposed model for the progression through mTEC^{high} A-C subsets during Aire lineage mTEC differentiation. Differential expression of CD24 and Sca1 resolves three mTEC^{high} subsets - CD24^{-} Sca1^{-} mTEC$^{A/hi}$, CD24^{+}Sca1^{-} mTEC$^{B/hi}$, and CD24^{+}Sca1^{+} mTEC$^{C/hi}$ - which map sequential stages of early-, late-, and post-Aire mTEC differentiation. In the context of *Aire* deficiency, mTEC^{high} become poised to begin Aire expression during the CD24^{-}Sca1^{-} mTEC$^{A/hi}$ stage but are precluded from further progression due to the absence of Aire.

Future insights into TEC development

The analysis of Sca1 expression is routinely used to identify stem cells of different lineages [7] and has also been associated with a bipotent progenitor function in TECs [8]. Similarly, CD24 is a marker of stem cells in the mammary tissue [9]. Our RTOC lineage-tracing experiments showed that both mTEC$^{B/lo}$ and mTEC$^{C/lo}$ contained cells that had the potential to differentiate into mTEC^{high} and Aire-expressing cells *in vitro*. This observation is in line with the previous report that the maturation of Aire^{+} mTEC precursors is associated with CD24 downregulation [10]. Furthermore, the mTEC^{low} progeny of mTEC$^{B/lo}$ and mTEC$^{C/lo}$, in varying frequencies, were found to convert into the other mTEC$^{A\text{-}B/lo}$ subsets. Although the progeny of mTEC$^{A/lo}$ could not be detected, these results are not sufficient to formulate conclusions of the precursor potential within this subset, as such culture conditions possess limitations in viability and detection of the rare “spiked” cells. Further studies are required to elucidate the developmental relationship between mTEC$^{A\text{-}C/lo}$ subsets and their potential for differentiation into distinct mTEC lineages. As this study did not target TEP characterization, future strategies

should also investigate the association of the reported pattern of CD24 and Sca1 expression within $mTEC^{low}$ with the reported subtypes of mTEPs [11].

Regarding the applicability of using the CD24 and Sca1 expression pattern for the identification of post-Aire mTECs, a limitation of the reported strategy pertains to the time point of analysis. These analyses were performed during the post-natal period until 6 weeks of age, as such, the pertinence of these findings in adult or aged thymi remains to be determined. It would be important to assess if, as the mTEC subpopulation landscape changes during ageing, CD24 and Sca1 detection still recapitulates these findings and/or if other subset associations arise. Furthermore, since the time of publication of this study, mTEC diversity has been increasingly defined, particularly, with the reports of the new post-Aire mimetic subtypes [12]. Although, as mentioned previously, we found no association between any particular $mTEC^{A\text{-}C/lo}$ subset and the general post-Aire gene signature, the restricted mimetic cell type signatures could also be investigated in the context of CD24 and Sca1-defined $mTEC^{low}$ subsets to reveal any possible associations. Despite the considered shortcomings, this study provides a useful tool for future research by providing a post-Aire mTEC detection strategy that does not require RNA-Seq or fate-mapping approaches, and therefore, is easily accessible and compatible with functional studies. Thus, the identification of these novel mTEC subsets not only elucidates key steps in the roadmap of $Aire^{+}$ mTEC lineage differentiation but can also be incorporated into research efforts aimed at elucidating the dynamics of TEC development and function.

EXPLORING THE PATHWAYS OF TEC HOMEOSTASIS: IMPLICATIONS FOR T-CELL DEVELOPMENT

The FOXO3 transcription factor safeguards mTEC homeostasis

The first reports designed to understand the role of FOXO3 in immune homeostasis described its vital role in limiting lymphoid cell proliferation and survival upon growth factor and cytokine withdrawal [13-16]. Subsequent studies further revealed that FOXO3 can functionally regulate T cells, B cells, neutrophils, and dendritic cells [17]. More recently, complementary studies have emphasized the significance of FOXO3 in the pathogenesis of autoimmunity [18]. Nevertheless, the potential contribution of FOXO3 within the thymic microenvironment, including its role in TEC differentiation and function, and possible implications for T-cell development, were not yet addressed. In Chapter IV of this thesis, through the generation and characterization of TEC-specific FOXO3^{cKO} mice, we report that *Foxo3* deficiency primarily causes a severe defect in the cellularity of the mTEC compartment. The loss of mTECs becomes more pronounced in early adulthood and is associated with alterations in subset composition, namely, a reduction in the proportion of mTEC^{low} and the relative abundance of the CCL21^{+} and tuft cell mTEC^{low} subsets. These results suggested a role for FOXO3 in mTEC homeostasis and functional diversification and prompted the investigation of FOXO3-mediated pathways in TECs.

FOXO3 target genes have pleiotropic roles in cellular homeostasis [19]. Interestingly, our analysis of the *Foxo3*-deficient mTEC transcriptional signature revealed that *Foxo3* deletion increased the expression of pro-apoptotic genes in response to DNA damage. The upregulation of this pathway is in line with the established role of FOXO3 in the DNA damage response, which includes the regulation of DNA repair mechanisms, cell cycle checkpoints and arrest, and apoptosis induction [20-24]. Further supporting an accumulation of genomic damage in mTECs, we detected and increased frequency of γH2AX^{+} cells in FOXO3^{cKO} mTECs. Interestingly, the increase in DSBs was specific to the mTEC^{high} subset, an observation that aligns with previous studies associating Aire-mediated TRA expression with DSB generation and the formation of multi-protein complexes with Aire and DNA damage response proteins [25-27]. Still, whether FOXO3 participates in the upregulation of Aire partners and/or facilitates the DNA repair mechanisms adjacent to Aire-dependent TRA expression warrants investigation.

The increased FOXO3^{cKO} mTEC predisposition to apoptosis suggested in our transcriptional analysis was supported by the detection of an increased frequency of

Annexin V^+ early apoptotic mTECs, particularly within the $mTEC^{low}$ subset. As $FOXO3^{cKO}$ $mTEC^{low}$ were the mTEC subpopulation suffering the most pronounced cellularity loss, this suggests that increased cell death is a contributing factor to the depletion of this subset.

Cell cycle arrest is an important mechanism to modulate the rate of cell turnover and/or expansion in a manner compatible with the available resources, environmental cues, and genomic integrity, ultimately supporting the sustainable division of genomically intact cells. FOXO3 plays several roles in cell cycle checkpoints and is an important inducer of cell cycle exit [22, 24, 28]. Coincidently, we found that $FOXO3^{cKO}$ $mTEC^{low}$ have a higher frequency of cycling cells. One possibility is that this higher proliferative status can result from the division of cells lacking FOXO3-mediated regulation, and that in FOXO3-expressing conditions would not meet the requirements to undergo division. Furthermore, considering the severe reduction of $mTEC^{low}$ cellularity, a second scenario is that the higher percentage of proliferating cells can be a consequence of a favoured depletion of non-cycling $mTEC^{low}$ relative to cycling cells. A third hypothesis is that the reduction of the $FOXO3^{cKO}$ mTEC niche could induce a compensatory expansion of some $mTEC^{low}$. In addition to the detected increase in the proportion of $ki67^+$ mTECs in the absence of FOXO3, we detected an upregulation of the gene signature of proliferating and transit-amplifying TECs in the $FOXO3^{cKO}$ mTEC transcriptome. Notably, the studies dissecting the signature of proliferating and transit-amplifying mTECs have proposed a differentiation model in which these cells constitute a cellular state that precedes mTEC differentiation and lineage diversification [29, 30]. Regardless of which of the aforementioned hypotheses explains the increased proportion of proliferating cells, assessing the impact of FOXO3 in mTEC differentiation constitutes an intriguing future trajectory for investigation.

Altogether, these findings implied that $FOXO3^{cKO}$ mTECs possess compromised DNA damage response mechanisms, contributing to an accumulation of DNA lesions in steady-state conditions. This prompted us to investigate whether *Foxo3* deletion could have further consequences in the context of TEC regeneration following acute IR-induced DNA damage. Sub-lethal total body irradiation (SL-TBI) of adult mice revealed that *Foxo3* deficiency precludes the regeneration of the $mTEC^{high}$ subpopulation during the first 21 days after treatment. In the same period, we also detected a significant defect in the *de novo* thymopoiesis achieved in $FOXO3^{cKO}$ mice relative to the controls. Thus, our findings suggest that FOXO3 supports mTEC homeostasis in response to physiological levels of genomic stress signals and additionally, in induced conditions of acute mTEC damage, FOXO3 supports their regenerative ability.

In contrast to the effects of *Foxo3* deletion in mTECs, *Foxo3*-deficient cTEC cellularity and the expression levels of key cTEC functional genes were unaltered. Additionally, we found no evidence of altered DNA damage response in either the transcriptional, *ex vivo,* or *in vivo* analyses. Although intriguing, this observation aligns with previous reports describing more robust DNA damage response activation and increased radiation sensitivity in mTECs, relative to cTECs [31-36]. Still, it is important to note that, like in any other study utilizing thymus enzymatic digestion, our findings are limited to the fraction of TECs that are successfully liberated from the thymic tissue and that survive the digestion process. The isolated TECs may not only underrepresent the actual *in situ* cell numbers but may also not faithfully depict TEC subpopulation diversity [37]. Thus, the interpretations and conclusions derived from these findings must recognize a possible bias towards enzymatic digestion-resistant TECs, which can generate blind spots in both flow cytometry analyses and transcriptome profiling. Despite these limitations, it was intriguing to observe that cTECs isolated from FOXO3^{cKO} mice revealed an enrichment for pathways related to ribosomal biogenesis. This observation is in agreement with a reported role for FOXO3 in suppressing protein translation in other model organisms [38-40]. Nevertheless, the analyses performed in this study did not detect the possible homeostatic and/or functional impact of increased ribosome biogenesis or translation in *Foxo3*-deficient cTECs. Further cTEC-targeted studies are necessary to fully address the possible role of FOXO3-mediated pathways in the cTEC compartment.

TRA-expressing mTECs rely on FOXO3

The thymic medulla provides a comprehensive self-antigen repertoire for thymocyte selection through different TRA expression mechanisms [12]. As revealed by pioneering studies, Aire-regulated TRA expression by mTEC^{high} exhibits a stochastic mosaic pattern, with small co-expression groups lacking biological association [41-44]. Our analysis of differentially expressed genes in FOXO3^{cKO} mTECs detected previously labelled Aire-dependent TRAs. These genes, although rare among FOXO3^{cKO} upregulated genes (2%), occupied a larger fraction of downregulated genes (14%), suggesting possible quantitative differences in the Aire-dependent TRA repertoire presented by FOXO3^{cKO} mTECs.

Importantly, Aire also performs an important role in mTEC differentiation by inducing the emergence and/or favouring the accumulation of post-Aire subsets [5, 6]. The recent discovery of post-Aire mimetic cell types brought to light the unique ability of the mTEC^{low} compartment to perform biologically coherent PGE under the control of

several lineage-defining transcription factors [6]. These findings also highlighted that the category of Aire-dependent TRA genes comprises not only direct targets of Aire-mediated TRA expression but also genes that are indirectly promoted by Aire, or Aire-influenced, as is the case of mimetic cell antigens. In the present work, upon de detection of a general downregulation of the post-Aire gene signature in FOXO3^{cKO} mTECs, we further investigated possible fluctuations in the representation of mimetic subset-specific genes. This analysis revealed a robust downregulation of tuft cell genes, corroborating our prior detection of reduced DCLK1^{+} mTECs, and hinted at possible reductions of the neuroendocrine and ionocyte subset signatures. Still, the employed bulk RNA-Seq strategy is inadequate for the study of these rare populations. Therefore, the precise characterization of possible alterations in mimetic subset diversity and cell-type-specific TRA expression in *Foxo3*-deficient mTECs requires more detailed genomic strategies, such as single-cell RNA-sequencing.

Lastly, a vast number of TRAs are Aire-independent [45, 46], such as the TRAs whose expression is regulated by Fezf2 [47]. We found that *Foxo3* ablation significantly alters the expression levels of Aire-independent TRAs, which constituted 27% of both upregulated and downregulated genes in FOXO3^{cKO} mTECs. In line with this observation, we have found, through the analysis of FOXO3^{cKO}Aire^{KO} mouse TECs, that the *in vivo* differentiation of Aire-independent mTECs may share a similar requirement for FOXO3 as the differentiation of Aire-sufficient mTECs. In the future, it would be important to investigate if, in similarity to Aire-mediated TRA expression [25-27], the mechanisms underlying Aire-independent PGE also lead to the generation of DNA damage. Such studies, for instance, could employ genome-wide DSB profiling [48] to map these lesions in Aire-independent TRA genes and asses their dependency on FOXO3 and the DNA damage response. Ultimately, this could clarify the indirect or direct contribution of FOXO3-mediated pathways to Aire-independent TRA expression.

FOXO3 supports thymic medullary functions for T-cell development

We investigated the ability of *Foxo3*-deficient TECs to support T-cell development and observed that the early-adult FOXO3^{cKO} thymus exhibited a general cellularity reduction, affecting all major thymocyte subsets. Nevertheless, DN, DP, and SP subpopulations and sequential stages of positive selection did not reveal any major alterations in frequency, suggesting an unaffected progression of thymocytes through these developmental stages. In contrast, the perturbations in the mTEC compartment of FOXO3^{cKO} mice were associated with defects in specific aspects of T-cell development that are dependent on interactions with mTECs. Of note, the analyses performed in this

study are insufficient to properly exclude alterations in cTEC function driven by *Foxo3* deficiency. Indeed, the significantly lower scale of thymopoiesis detected in adult FOXO3^{cKO} mice suggests the existence of other underlying defects. Future studies should investigate whether changes in *Foxo3*-deficient cTECs and/or mTECs may compromise thymus seeding by BM-derived progenitors and, consequently, limit the extent of thymopoiesis.

While FOXO3 has reported T-cell-intrinsic roles in the induction of FOXP3 expression and Treg development [49], in this study, we further reveal that thymic Treg induction requires FOXO3 expression on the thymic epithelium, particularly, on mTECs. Our analyses suggest that neither CD25^{+}FOXP3^{-} nor CD25^{-}FOXP3^{low} Treg precursors were affected in frequency in the FOXO3^{cKO} thymus. Instead, our results imply a defect in the differentiation of mature CD25^{+}FOXP3^{+} Tregs. Furthermore, polyclonal T-cell activation *in vitro* was less suppressed in the presence of FOXO3^{cKO}-derived thymic Tregs compared to FOXO3^{Ctr}-derived Tregs. Thus, in addition to a lower rate of Treg generation, the FOXO3^{cKO} mTEC microenvironment may produce functionally compromised Tregs.

Furthermore, we report a significant defect in PBS57/CD1d tetramer-labeled iNKT cells in the FOXO3^{cKO} thymus. Concordant with a defect downstream of iNKT cell positive selection, stage 0 iNKT cells were not affected, whereas the mTEC-dependent stage 2 iNKT cells showed a drastic reduction in frequency and numbers. Moreover, while iNKT1, iNKT2, and iNKT17 effector populations all showed cellularity reductions, the most significant reduction was detected in iNKT2. Notably, CCL21^{+} mTECs and thymic tuft cells are involved in controlling the development of functionally diverse iNKT lineages [50]. In particular, the decrease in iNKT2 cells in the FOXO3^{cKO} thymus is consistent with the decline in thymic tuft cells, which regulate iNKT2 cell development via IL-25 production [50, 51].

In the present study, the medullary functions that become deficient as a result of the decline in FOXO3^{cKO} mTECs, namely Treg and iNKT cell development, are quantitatively similar to reported defects in Aire^{KO} mice [52-54]. The defects in these two processes in the FOXO3^{cKO} thymus could be driven by the reduction in Aire lineage mTECs. However, another non-mutually exclusive possibility is that the functional contribution of Aire-independent mTECs was also compromised by *Foxo3* deficiency. Notably, we observed that Treg and iNKT cell development were less affected when sustained by mTECs with only *Aire* deletion relative to mTECs with combined deletion of *Aire* and *Foxo3*, which showed an enhanced defect. These findings suggest that the thymic Treg and iNKT cell generation that occur independently of Aire may be also supported by FOXO3. Whether the two regulators influence mTEC-dependent Treg

development and iNKT cell maturation through cooperative or separate mechanisms remains to be further studied.

The observed defects in FOXO3^{cKO} thymic medulla homeostasis and compromised thymic Treg development and function led us to search for peripheral immune tolerance defects. Our quantification of autoimmune manifestations in FOXO3^{cKO} mice revealed only a mild increase in lymphocytic infiltrates in selective peripheral tissues, both in *Aire*-sufficient and *Aire*-deficient groups. Thus, in spite of the significantly smaller mTEC compartment and reported Treg defects, tolerance induction in FOXO3^{cKO} mice is not gravely impacted. Concordantly, our assessment of overall negative selection-induced apoptosis through PD-1 and Helios detection did not reveal alterations in the FOXO3^{cKO} thymus. Of note, both Treg and negative selection analyses were done at a polyclonal scale and may overlook antigen-specific defects. Still, in the event of any defect in T-cell repertoire selection in the FOXO3^{cKO} thymus, the development of a more severe autoimmune phenotype could be hindered by several different factors. For instance, our studies were conducted on the C57BL/6 mouse genetic background, which has an inherent resistance to autoimmune disease [55]. Furthermore, the specific pathogen-free environment in which the mice were housed can also impact the immune system, and possibly the development of autoimmunity [56]. Indeed, the development of an autoimmune syndrome is a multifactorial process, as important peripheral mechanisms can efficiently promote self-tolerance, such as peripheral Tregs and several tolerogenic APCs [57]. Therefore, possible shortcomings in central tolerance induction by the FOXO3^{cKO} thymus may not necessarily drive an autoimmune syndrome. Further studies using additional autoimmunity-prone experimental models can help uncover the possible mechanisms through which FOXO3 may influence central tolerance induction.

Collectively, our results demonstrate that FOXO3 in mTECs contributes to preserving genomic stability and prevents the decline of the mTEC compartment. Otherwise, *Foxo3* deficiency in mTECs results in an altered TRA repertoire, defective Treg and iNKT cell development, and drives a predisposition to mild organ-specific autoimmunity (**Figure 2**).

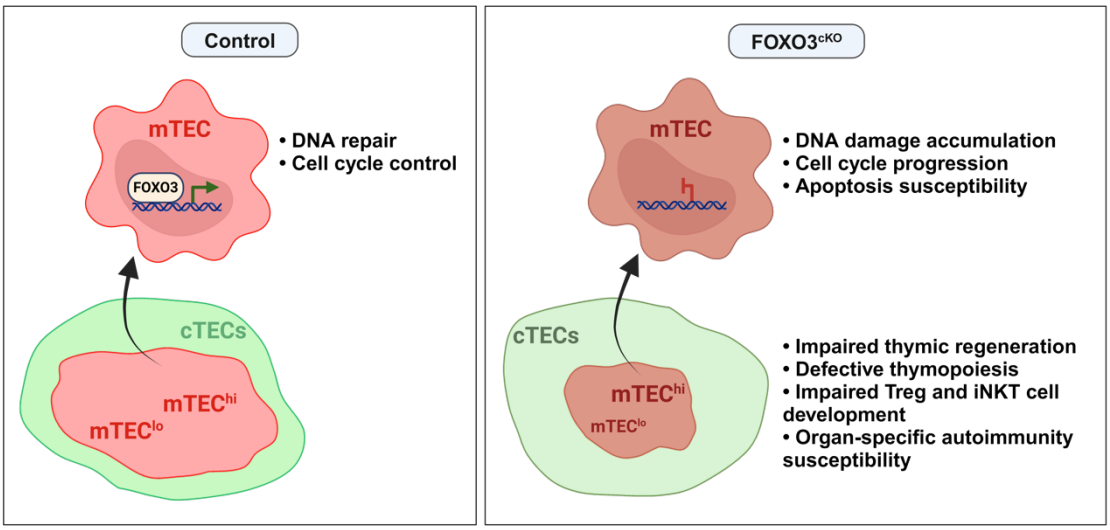


Figure 2 – Summary of thymic molecular, structural, and functional alterations driven by TEC-specific *Foxo3* deletion. In the control thymus, FOXO3 preserves mTEC genomic integrity by orchestrating DNA repair pathways and cell cycle checkpoints. In contrast, FOXO3^{cKO} mTECs have compromised DNA damage response pathways, resulting in the accumulation and propagation of DNA damage. The genomic defects in FOXO3^{cKO} mTECs render them susceptible to apoptosis and drive a decline in mTEC cellularity, more severely in the mTEC^{low} subset. Both cTECs and mTECs show broad transcriptome alterations as a result of *Foxo3* deletion. Altogether these changes drive several defects in the FOXO3^{cKO} thymus, including impaired regeneration following IR-induced damage, defective thymopoiesis, Treg and iNKT cell development, and increased susceptibility to organ-specific autoimmunity.

A FOXO3-p53 interplay guards the mTEC genome

The results reported in this thesis provide new evidence to support an mTEC-inherent dependency on the DNA damage response. Of note, this has also been implied in a past study from our laboratory revealing the specific requirement for p53 in mTEC homeostasis [31]. FOXO3 and p53 are tumour suppressors with shared functions and some common upstream regulatory pathways [58]. It has been demonstrated that p53 and FOXO3 can have synergic effects in DNA damage response pathways [59], in particular, FOXO3 can regulate p53-driven apoptosis [60]. Still, as they integrate several different stress stimuli, depending on the type or intensity of the signals, p53 and FOXO3 can favour different cellular outcomes (*i.e.,* cell death versus resistance). Thus, the two transcription factors can also be activated separately or perform antagonizing functions [61, 62]. In the context of mTEC homeostasis, the comparison of p53-controlled and FOXO3-controlled genes reveals the existence of both shared and unique targets, highlighting the existence of both redundant and individual functions. Furthermore, our past *Trp53*-deficient mTEC RNA-Seq data suggested that p53 expression is upstream of *Foxo3* induction. Notably, the upregulation of initiator and effector pro-apoptotic Bcl2 family members, including the p53 targets Puma (*Bbc3*), Noxa (*Pmaip1*), and Bax (*Bax*), in *Foxo3*-deficient mTECs suggests that p53-driven apoptosis in mTECs can occur in a

FOXO3-independent manner. Yet, our observation of DNA damage accumulation in FOXO3^{cKO} mTECs also demonstrates that intact p53-mediated DNA damage response mechanisms are not sufficient to preserve mTEC genomic stability without the contribution of FOXO3. Therefore, p53 and FOXO3 may act as two key complementary players in mTEC genomic and homeostatic preservation that perform non-redundant roles.

We also contemplated the possibility of functional collaboration between FOXO family transcription factors, which identify a single consensus sequence and, theoretically, possess redundant target genes. Nevertheless, functional specificity has been described between the different FOXO members due to their individual spatial-temporal patterns of expression [58, 63]. By searching previously acquired TEC RNA-Seq data, we observed that *Foxo3* is the most expressed FOXO member in embryonic and early postnatal TECs [31, 64]. The data acquired in the current study further showed that only *Foxo3* possesses a significant expression level in adult TECs, while the expression of the other members (*Foxo1*, *Foxo4* and *Foxo6*) remains vestigial, thus supporting the disregard of redundant FOXO target gene regulation in TECs. Furthermore, FOXO3^{cKO} TECs showed no alterations in the expression levels of the remaining FOXO transcription factors, implying that no compensatory mechanisms induce their upregulation in response to FOXO3 insufficiency.

The DNA damage response is the culmination of several molecular processes that monitor, repair, and deter the propagation of DNA lesions to preserve genomic integrity and cellular functionality. Our findings provide new evidence to strengthen the link between this response and mTEC compartment maintenance and highlight FOXO3 as another sentinel, in addition to p53 [31], of mTEC genomic integrity. Still, further research is needed to mechanistically characterize FOXO3-mediated DNA repair, cell cycle arrest, and apoptosis in mTECs. Such studies can also elucidate possible functional partnerships or pathway crosstalk with other tumour suppressors.

Future study directions for FOXO3 in thymus biology

Notably, FOXO3 target genes that restrain genomic lesions represent only a fraction of the FOXO3-induced transcriptional programs. Several cellular stress signals, such as metabolic or oxidative stress, and insulin or growth factor reduction, induce signalling cascades that activate FOXO3 transcriptional activity [19]. Interestingly, many reported FOXO3-activating signalling pathways have been shown to partake in thymic stromal homeostasis and are implicated in both thymic involution and recovery. For instance, CR produces a robust and well-defined delay in age-related thymic involution

[65, 66]. CR acts through several nutrient-sensing pathways that act on FOXO3, such as the IIS, AMPK, and SIRTs pathways [67]. Therefore, future work could investigate whether a CR-induced delay in thymic involution is still observed in the context of *Foxo3* deficiency in TECs. Such studies could dissect the possible role of FOXO3 in CR-induced TEC signalling pathways and in the effect of CR on thymic function. Moreover, FOXO3 is an evolutionarily conserved longevity gene, consistently associated with healthy ageing and prolonged lifespan in humans [67, 68]. Interestingly, a study in mice has implied a requirement for FOXO3 for the life-extending effect of CR [69]. Thus, another potential hypothesis to explore is whether the role of FOXO3 in CR-driven longevity includes a protective effect on thymic function. Lastly, it has been suggested that the propensity of the thymus to metabolic damage is associated with an inherent susceptibility of the thymic stroma to oxidative damage [70]. Since elevation of ROS levels is another major stress signal driving FOXO3 activation, future studies can elucidate whether FOXO3-mediated antioxidant activity can better equip TEC or non-TEC stromal cells to combat oxidative damage. Altogether, several hypothetical associations can be conjectured considering the work on thymus biology and the research on FOXO3. The findings presented in this thesis, by establishing an unprecedented link between these two fields, expose enticing new research avenues to examine a possible multifactorial role for FOXO3 in thymic involution and regeneration.

FINAL REMARKS

Since its importance was first documented in 1961 [71], the thymus has continuously captivated researchers. The collective efforts in the field have yielded valuable insights into the development, maintenance, and function of this organ. These studies have also highlighted the complexity of the molecular pathways that govern the differentiation of TECs and dictate their diverse functional natures. Hence, to this day, the map of the intricate mechanisms that underlie the function of the thymus remains incomplete. This knowledge is crucial for enabling interventions that can enhance deficient thymic output or correct inadequacies in T-cell repertoire-selecting mechanisms, which are vital in multiple clinical settings. This thesis provides two main contributions that expand our understanding of distinct aspects of TEC physiology. First, the findings reported in Chapter III provide a new angle for the assessment of the mTEC compartment. This study shows that the differential expression of CD24 and Sca1 defines three novel subsets that represent sequential states in the differentiation of $Aire^{+}$ mTECs into post-Aire mTECs. Moreover, Chapter IV describes a new role for the transcription factor FOXO3 in the molecular control of TEC stress resistance and homeostasis. Particularly, by demonstrating that FOXO3 expression is essential for maintaining the mTEC compartment, preventing genomic stress accumulation, and sustaining mTEC-mediated functions. The combined discoveries here presented improve our comprehension of the thymic epithelial microenvironments while paving the way for new research strategies. Ultimately, by establishing a comprehensive groundwork, we can unravel the intricacies of thymic function and design effective therapeutic interventions to support immune reconstitution and self-tolerance induction.

REFERENCES

1. Abramson, J. and Anderson, G., 2017, *Thymic Epithelial Cells,* Annu Rev Immunol, **35**, 85-118.
2. Gray, D.H.*, et al.*, 2006, *Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells,* Blood, **108**(12), 3777-85.
3. Chaudhry, M.S.*, et al.*, 2016, *Thymus: the next (re)generation,* Immunol Rev, **271**(1), 56-71.
4. Mathis, D. and Benoist, C., 2009, *Aire,* Annu Rev Immunol, **27**, 287-312.
5. Nishikawa, Y.*, et al.*, 2014, *Temporal lineage tracing of Aire-expressing cells reveals a requirement for Aire in their maturation program,* J Immunol, **192**(6), 2585-92.
6. Michelson, D.A.*, et al.*, 2022, *Thymic epithelial cells co-opt lineage-defining transcription factors to eliminate autoreactive T cells,* Cell, **185**(14), 2542-2558 e18.
7. Holmes, C. and Stanford, W.L., 2007, *Concise review: stem cell antigen-1: expression, function, and enigma,* Stem Cells, **25**(6), 1339-47.
8. Wong, K.*, et al.*, 2014, *Multilineage potential and self-renewal define an epithelial progenitor cell population in the adult thymus,* Cell Rep, **8**(4), 1198-209.
9. Shackleton, M.*, et al.*, 2006, *Generation of a functional mammary gland from a single stem cell,* Nature, **439**(7072), 84-8.
10. Akiyama, N.*, et al.*, 2016, *Identification of embryonic precursor cells that differentiate into thymic epithelial cells expressing autoimmune regulator,* J Exp Med, **213**(8), 1441-58.
11. Alves, N.L. and Ribeiro, A.R., 2016, *Thymus medulla under construction: Time and space oddities,* Eur J Immunol, **46**(4), 829-33.
12. Michelson, D.A. and Mathis, D., 2022, *Thymic mimetic cells: tolerogenic masqueraders,* Trends Immunol, **43**(10), 782-791.
13. Brunet, A.*, et al.*, 1999, *Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor,* Cell, **96**(6), 857-68.
14. Dijkers, P.F.*, et al.*, 2002, *FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity,* J Cell Biol, **156**(3), 531-42.
15. Stahl, M.*, et al.*, 2002, *The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2,* J Immunol, **168**(10), 5024-31.

16. Lin, L.*, et al.*, 2004, *Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a,* Immunity, **21**(2), 203-13.
17. Dejean, A.S.*, et al.*, 2011, *Highly specialized role of Forkhead box O transcription factors in the immune system,* Antioxid Redox Signal, **14**(4), 663-74.
18. Xu, S.*, et al.*, 2021, *Role of Forkhead box O3a transcription factor in autoimmune diseases,* Int Immunopharmacol, **92**, 107338.
19. Eijkelenboom, A. and Burgering, B.M., 2013, *FOXOs: signalling integrators for homeostasis maintenance,* Nat Rev Mol Cell Biol, **14**(2), 83-97.
20. Bigarella, C.L.*, et al.*, 2017, *FOXO3 Transcription Factor Is Essential for Protecting Hematopoietic Stem and Progenitor Cells from Oxidative DNA Damage,* J Biol Chem, **292**(7), 3005-3015.
21. Chung, Y.M.*, et al.*, 2012, *FOXO3 signalling links ATM to the p53 apoptotic pathway following DNA damage,* Nat Commun, **3**, 1000.
22. Medema, R.H.*, et al.*, 2000, *AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1,* Nature, **404**(6779), 782-7.
23. Tran, H.*, et al.*, 2002, *DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein,* Science, **296**(5567), 530-4.
24. Furukawa-Hibi, Y.*, et al.*, 2002, *FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress,* J Biol Chem, **277**(30), 26729-32.
25. Abramson, J.*, et al.*, 2010, *Aire's partners in the molecular control of immunological tolerance,* Cell, **140**(1), 123-35.
26. Bansal, K.*, et al.*, 2017, *The transcriptional regulator Aire binds to and activates super-enhancers,* Nat Immunol, **18**(3), 263-273.
27. Fang, Y.*, et al.*, 2024, *AIRE relies on Z-DNA to flag gene targets for thymic T cell tolerization,* Nature, **628**(8007), 400-407.
28. Hornsveld, M.*, et al.*, 2021, *A FOXO-dependent replication checkpoint restricts proliferation of damaged cells,* Cell Rep, **34**(4), 108675.
29. Baran-Gale, J.*, et al.*, 2020, *Ageing compromises mouse thymus function and remodels epithelial cell differentiation,* Elife, **9**.
30. Wells, K.L.*, et al.*, 2020, *Combined transient ablation and single-cell RNA-sequencing reveals the development of medullary thymic epithelial cells,* Elife, **9**.

31. Rodrigues, P.M.*, et al.*, 2017, *Thymic epithelial cells require p53 to support their long-term function in thymopoiesis in mice,* Blood, **130**(4), 478-488.
32. Hauri-Hohl, M.M.*, et al.*, 2008, *TGF-beta signaling in thymic epithelial cells regulates thymic involution and postirradiation reconstitution,* Blood, **112**(3), 626-34.
33. Dudakov, J.A.*, et al.*, 2012, *Interleukin-22 drives endogenous thymic regeneration in mice,* Science, **336**(6077), 91-5.
34. Alawam, A.S.*, et al.*, 2022, *Failures in thymus medulla regeneration during immune recovery cause tolerance loss and prime recipients for auto-GVHD,* J Exp Med, **219**(2).
35. Horie, K.*, et al.*, 2023, *Acute irradiation causes a long-term disturbance in the heterogeneity and gene expression profile of medullary thymic epithelial cells,* Front Immunol, **14**, 1186154.
36. Calvo-Asensio, I.*, et al.*, 2017, *Differential Response of Mouse Thymic Epithelial Cell Types to Ionizing Radiation-Induced DNA Damage,* Front Immunol, **8**, 418.
37. Sakata, M.*, et al.*, 2018, *Cellularity of Thymic Epithelial Cells in the Postnatal Mouse,* J Immunol, **200**(4), 1382-1388.
38. Puig, O.*, et al.*, 2003, *Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway,* Genes Dev, **17**(16), 2006-20.
39. Junger, M.A.*, et al.*, 2003, *The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling,* J Biol, **2**(3), 20.
40. Tullet, J.M., 2015, *DAF-16 target identification in C. elegans: past, present and future,* Biogerontology, **16**(2), 221-34.
41. Brennecke, P.*, et al.*, 2015, *Single-cell transcriptome analysis reveals coordinated ectopic gene-expression patterns in medullary thymic epithelial cells,* Nat Immunol, **16**(9), 933-41.
42. Pinto, S.*, et al.*, 2013, *Overlapping gene coexpression patterns in human medullary thymic epithelial cells generate self-antigen diversity,* Proc Natl Acad Sci U S A, **110**(37), E3497-505.
43. Meredith, M.*, et al.*, 2015, *Aire controls gene expression in the thymic epithelium with ordered stochasticity,* Nat Immunol, **16**(9), 942-9.
44. Dhalla, F.*, et al.*, 2020, *Biologically indeterminate yet ordered promiscuous gene expression in single medullary thymic epithelial cells,* EMBO J, **39**(1), e101828.

45. Danan-Gotthold, M.*, et al.*, 2016, *Extensive RNA editing and splicing increase immune self-representation diversity in medullary thymic epithelial cells,* Genome Biol, **17**(1), 219.
46. Anderson, M.S.*, et al.*, 2002, *Projection of an immunological self shadow within the thymus by the aire protein,* Science, **298**(5597), 1395-401.
47. Takaba, H.*, et al.*, 2015, *Fezf2 Orchestrates a Thymic Program of Self-Antigen Expression for Immune Tolerance,* Cell, **163**(4), 975-87.
48. Yan, W.X.*, et al.*, 2017, *BLISS is a versatile and quantitative method for genome-wide profiling of DNA double-strand breaks,* Nat Commun, **8**, 15058.
49. Ouyang, W.*, et al.*, 2010, *Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells,* Nat Immunol, **11**(7), 618-27.
50. Lucas, B.*, et al.*, 2020, *Diversity in medullary thymic epithelial cells controls the activity and availability of iNKT cells,* Nat Commun, **11**(1), 2198.
51. Bornstein, C.*, et al.*, 2018, *Single-cell mapping of the thymic stroma identifies IL-25-producing tuft epithelial cells,* Nature, **559**(7715), 622-626.
52. Lindh, E.*, et al.*, 2010, *AIRE deficiency leads to impaired iNKT cell development,* J Autoimmun, **34**(1), 66-72.
53. Lei, Y.*, et al.*, 2011, *Aire-dependent production of XCL1 mediates medullary accumulation of thymic dendritic cells and contributes to regulatory T cell development,* J Exp Med, **208**(2), 383-94.
54. Morimoto, J.*, et al.*, 2022, *Aire suppresses CTLA-4 expression from the thymic stroma to control autoimmunity,* Cell Rep, **38**(7), 110384.
55. Jiang, W.*, et al.*, 2005, *Modifier loci condition autoimmunity provoked by Aire deficiency,* J Exp Med, **202**(6), 805-15.
56. Beura, L.K.*, et al.*, 2016, *Normalizing the environment recapitulates adult human immune traits in laboratory mice,* Nature, **532**(7600), 512-6.
57. Xing, Y. and Hogquist, K.A., 2012, *T-cell tolerance: central and peripheral,* Cold Spring Harb Perspect Biol, **4**(6).
58. van der Horst, A. and Burgering, B.M., 2007, *Stressing the role of FoxO proteins in lifespan and disease,* Nat Rev Mol Cell Biol, **8**(6), 440-50.
59. Renault, V.M.*, et al.*, 2011, *The pro-longevity gene FoxO3 is a direct target of the p53 tumor suppressor,* Oncogene, **30**(29), 3207-21.
60. You, H.*, et al.*, 2006, *Regulation of transactivation-independent proapoptotic activity of p53 by FOXO3a,* Proc Natl Acad Sci U S A, **103**(24), 9051-6.
61. You, H.*, et al.*, 2004, *p53-dependent inhibition of FKHRL1 in response to DNA damage through protein kinase SGK1,* Proc Natl Acad Sci U S A, **101**(39), 14057-62.

62. Miyaguchi, Y.*, et al.*, 2009, *P53 negatively regulates the transcriptional activity of FOXO3a under oxidative stress,* Cell Biol Int, **33**(8), 853-60.
63. Furuyama, T.*, et al.*, 2000, *Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues,* Biochem J, **349**(Pt 2), 629-34.
64. Sansom, S.N.*, et al.*, 2014, *Population and single-cell genomics reveal the Aire dependency, relief from Polycomb silencing, and distribution of self-antigen expression in thymic epithelia,* Genome Res, **24**(12), 1918-31.
65. Messaoudi, I.*, et al.*, 2006, *Delay of T cell senescence by caloric restriction in aged long-lived nonhuman primates,* Proc Natl Acad Sci U S A, **103**(51), 19448-53.
66. Yang, H.*, et al.*, 2009, *Inhibition of thymic adipogenesis by caloric restriction is coupled with reduction in age-related thymic involution,* J Immunol, **183**(5), 3040-52.
67. Martins, R.*, et al.*, 2016, *Long live FOXO: unraveling the role of FOXO proteins in aging and longevity,* Aging Cell, **15**(2), 196-207.
68. Zhao, Y. and Liu, Y.S., 2021, *Longevity Factor FOXO3: A Key Regulator in Aging-Related Vascular Diseases,* Front Cardiovasc Med, **8**, 778674.
69. Shimokawa, I.*, et al.*, 2015, *The life-extending effect of dietary restriction requires Foxo3 in mice,* Aging Cell, **14**(4), 707-9.
70. Griffith, A.V.*, et al.*, 2015, *Metabolic Damage and Premature Thymus Aging Caused by Stromal Catalase Deficiency,* Cell Rep, **12**(7), 1071-9.
71. Miller, J.F., 1961, *Immunological function of the thymus,* Lancet, **2**(7205), 748-9.

INSTITUTO DE **CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**