

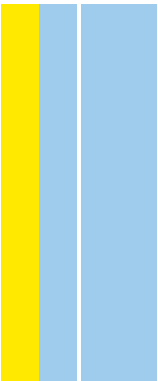
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BIOLOGIA MOLECULAR E CELULAR

Learning from TECs: Exploring the mechanisms that govern the maturation of the thymic epithelial cell microenvironments and their role in the education of T cell development

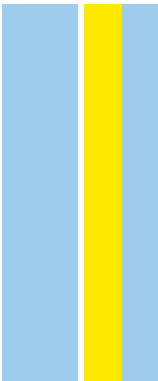
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Learning from TECs: Exploring the mechanisms that govern the maturation of the thymic epithelial cell microenvironments and their role in the education of T cell development

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LAURA ISABEL GUEDES DE SOUSA

**LEARNING FROM TECS: EXPLORING THE MECHANISMS THAT
GOVERN THE MATURATION OF THE THYMIC EPITHELIAL CELL
MICROENVIRONMENTS AND THEIR ROLE IN THE EDUCATION
OF T CELL DEVELOPMENT**

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Summary

The thymus is the organ where specialized cells within the thymic microenvironment provide instructive cues to promote the development and selection of T cells that bear a functional, self-tolerant and diverse T cell receptor (TCR) repertoire. Thymopoietic signals are mainly delivered by thymic epithelial cells (TECs), which comprise unique stromal cells with a non-redundant role in T cell development. TECs are classically subdivided into two main lineages: cortical (c) and medullary (m) TECs, based on their distinct anatomical localization and functional properties in dedicated stages of T cell differentiation. Specifically, cTECs support the earlier stages of thymopoiesis such as T cell lineage commitment and positive selection. On the other hand, mTECs safeguard the later stages of differentiation, which comprise negative selection and T regulatory cell differentiation, and are critical for tolerance induction. To promote the selection of developing T cells, cTECs and mTEC present self-peptide-MHC complexes generated via unique antigen processing and presentation properties.

Despite being functionally distinct, cTECs and mTECs arise from common bipotent TEC progenitor cells that reside within the thymic microenvironment. The development and functional maturation of TECs are dependent on the transcription factor Forkhead box N1 (Foxn1), which remains to date a master regulator of TEC differentiation. Autosomal-recessive mutation in Foxn1 results in a TEC deficiency in mice and humans and is the basis of the T⁻B⁺NK⁺ nude/SCID athymic phenotype. Yet, the details that control the induction and maintenance of Foxn1 expression remain unknown. Given the essential role of TECs in thymopoiesis, understanding the elements that coordinate their function will advance our basic knowledge in thymus biology and provide insights that can be integrated into therapeutics to harness thymic function.

This thesis focuses on investigating the molecular mechanisms that regulate the development and function of TECs. In **Chapter II**, we investigate the regulatory mechanisms that control Foxn1 expression in the thymus. Exploring novel reporter mouse models that enable to monitor *Foxn1* promoter activation, we describe that TEC precursors in a Foxn1-deficient setting can activate the *Foxn1* gene at early stages of embryonic and postnatal life, but not in adulthood. Notably, fate-mapping

analysis shows that cells that expressed *Foxn1* persist in the adult *Foxn1*-deficient thymic rudiment, suggesting that they lose the capacity to activate the *Foxn1* promoter over time. Furthermore, single-cell RNA sequencing analysis shows that *Foxn1*-deficient TEC precursors display an unappreciated transcriptional complexity. Our study offers the possibility to further understand the modulation of *Foxn1* expression in TECs, providing future translational insights to medically intervene in disorders associated with dysfunctional thymic function.

In **Chapter III**, we document a novel role for the lysosomal-associated membrane protein 2 (LAMP2) in the macroautophagy/autophagy-lysosomal protein degradation pathway of cTECs and its impact on CD4 T cell selection. We report that genetic inactivation of *Lamp2* in thymic stromal cells specifically affects the development of CD4 T cells that completed positive selection, without misdirecting MHC II-restricted cells into the CD8 lineage. Our data further suggests that LAMP2 controls the processing and the presentation of self-peptide:MHC II complexes on cTECs, thereby influencing the selection of CD4 T cells and the generation of a broad CD4 TCR repertoire. These findings led us to summarize in a review article the most recent studies on the role of TECs in T cell selection (integrated in the general discussion).

Collectively, this thesis describes new perspectives on the regulatory mechanisms controlling the development and role of TECs. The elucidation of the dynamics of *Foxn1* expression in TECs, along with the identification of a novel pathway controlling their role in the selection of polyclonal CD4 T cells, contributes to deepen our knowledge of thymic development and function.

Resumo

O timo é o órgão onde células especializadas do microambiente tímico providenciam sinais que promovem o desenvolvimento e a seleção de células T que possuem um recetor (TCR) que é funcional, tolerante ao próprio e diverso. Os sinais que instruem o desenvolvimento destas células são produzidos maioritariamente pelas células epiteliais do timo (TECs), que são células estromais tímicas com um papel indispensável para a diferenciação das células T. As TEC são normalmente divididas em duas linhagens distintas: as células corticais (c) e as células medulares (m), de acordo com a sua localização anatômica e a função que desempenham no desenvolvimento das células T. As cTECs suportam os estádios iniciais deste desenvolvimento, tais como a especialização na linhagem T e a seleção positiva, enquanto as mTECs controlam as fases tardias de diferenciação, nomeadamente a seleção negativa e a diferenciação das células T reguladoras. De modo a promover a seleção dos linfócitos T em desenvolvimento, as cTECs e as mTECs apresentam péptidos do próprio através de moléculas de MHC, péptidos estes que são gerados graças às suas capacidades únicas de processamento e de apresentação dos mesmos.

Apesar de serem funcionalmente distintas, as cTECs e as mTECs desenvolvem-se a partir de células progenitoras bipotentes que se encontram no ambiente tímico. O desenvolvimento e a maturação das TEC depende da expressão do fator de transcrição Foxn1, que até à data se mantém como o único fator que regula a diferenciação das TEC. Mutações autossomais recessivas no gene que codifica para a proteína Foxn1 levam a uma deficiência nas TECs, quer em humanos ou em murgos, e é refletida pelos fenótipos SCID/nude T⁻B⁺NK⁺. Contudo, o mecanismo que controla a expressão deste fator de transcrição continua indefinido. Atendendo ao seu papel fundamental para a função das TEC e para o desenvolvimento das células T, a elucidação dos mecanismos que coordenam a sua função irá aumentar os conhecimentos básicos da biologia do timo e contribuir para o desenvolvimento de terapias que pretendem controlar a função tímica.

Esta tese foca-se em investigar os mecanismos moleculares que regulam o desenvolvimento e a função das TEC. No **capítulo II**, investigámos os mecanismos que controlam a expressão de Foxn1 no timo. Ao explorar novos animais que

reportam a expressão de proteínas que nos permitem avaliar a ativação do promotor do *Foxn1*, nós descrevemos que as células precursoras das TEC são capazes de ativar este promotor na ausência de Foxn1 durante os estádios embrionários e pós-natais, mas não na sua vida adulta. A análise de “fate-mapping” demonstrou que as células que expressavam Foxn1 persistem na vida adulta na ausência deste fator de transcrição, o que sugere que estas perdem a capacidade de ativar o promotor do *Foxn1* ao longo do tempo. Para além disso, a análise de sequenciação de RNA em células individuais demonstrou que existe uma complexidade transcripcional nos precursores que são deficientes em Foxn1, o que nunca foi observado anteriormente. O nosso estudo oferece a possibilidade de explorar a modulação da expressão de Foxn1 nas TEC, o que equaciona a hipótese de intervir em alterações que estão associadas a uma função tímica disfuncional.

No **capítulo III**, descrevemos o papel da proteína lisossomal LAMP2 na via autofágica/lisossomal na degradação dos péptidos pelas cTECs e o seu impacto na seleção das células T CD4. Verificámos que a deleção do gene que codifica para a proteína LAMP2 nas células que compõem o estroma do timo afetava especificamente o desenvolvimento das linfócitos T CD4 que completavam a seleção positiva, sem as redirecionar para a linhagem CD8. Os nossos dados também sugerem que a proteína LAMP2 controla o processamento e a apresentação dos péptidos do próprio via moléculas de MHC classe II pelas cTECs, o que por sua vez, tem um impacto na seleção de células T CD4 e no estabelecimento do seu repertório diversificado de TCR. Estas observações levaram-nos a sumarizar este e outros estudos recentes referentes ao papel das TECs na seleção de células T num artigo de revisão (integrado na discussão geral).

Em suma, esta tese descreve novas perspetivas referentes aos mecanismos que controlam o desenvolvimento e a função das TEC. A perceção da dinâmica da expressão de Foxn1 nas TECs, acoplada com a identificação de uma nova via que controla o papel destas células na seleção de células T CD4 policlonais, permitem o alargamento do nosso conhecimento no que diz respeito ao desenvolvimento e à função do timo.

List of Abbreviations

Aire - Autoimmune regulator	DP - Double positive
ACKR - Atypical chemokine receptor	DN - Double negative
AL – Autolysosome	E - Embryonic day
AP - Autophagosome	ECM - Extracellular matrix
APC - Antigen-presenting cell	EGFP - Enhanced green fluorescent protein
Atg5 - Autophagy related 5	ER - Endoplasmic reticulum
BCR - B cell receptor	ETP - Early thymic progenitor
BMP - Bone morphogenetic protein	Eya1 - Eyes absent 1 homologue
B2m - Beta-2 microglobulin	EYFP - Enhanced yellow fluorescence protein
CCL - Chemokine (C-C motif) ligand	FBS - Fetal bovine serum
CCR7 - C-C chemokine receptor type 7	Fezf2 - FEZ family zinc finger 2
CD - Cluster of differentiation	FGF - Fibroblast growth factor
CFU - Colony-forming units	Foxn1 - Forkhead box N1
cKO - Conditional knockout	Foxp3 - Forkhead box p3
Clec16a - C-Type Lectin Domain Containing 16A	FRC - Fibroblastic reticular cell
CLIP - Class II-associated invariant chain peptide	GATA3 - GATA-binding protein 3
CLR - C-type lectin receptor	Gcm2 - Glial cells missing homolog 2
CMJ - Cortico-medullary junction	GO - Gene ontology
CRISPR - Clustered regularly interspaced short palindromic repeats	GPCR - G-protein coupled receptor
cTECs - Cortical thymic epithelial cells	HDAC3 - Histone deacetylase 3
CtsL - Cathepsin L	Hoxa3 - Homeobox A3
CXCL - C-X-C motif chemokine	ICAM-1 - Intercellular adhesion molecule 1
D - Diversity	IFN - Interferon
DC - Dendritic cell	IGF - Insulin-like growth factor
DETC - Dendritic epidermal T cell	li - Invariant chain
DII4 - Delta-like ligand 4	IL – Interleukin
	ILC - Innate lymphoid cell
	ISP8 - Immature CD8 single positive

Itga6 - Integrin Subunit Alpha 6

J – Joining

K - Cytokeratin

KLF2 - Krüppel-like factor 2

KO - Knockout

LAMP2 - Lysosomal-associated
membrane protein 2

Lm - *Listeria monocytogenes*

LTβR - Lymphotoxin β receptor

MAIT - Mucosal-associated invariant
T cell

mESC - Mouse embryonic stem cell

MFI - Mean fluorescence intensity

MHC - Major histocompatibility
complex

MIIC - MHC II processing
compartment

MN - Mature naïve

mTECs - Medullary thymic epithelial
cells

mTEP - mTEC-restricted progenitor

NC - Neural crest-derived

NF-κB - Nuclear factor kappa B

NK - Natural killer

NLRs - Nucleotide-binding
oligomerization domain-like receptors

P - Postnatal day

PAMP - Pathogen-associated
molecular pattern

PAX - Paired box gene

pGE - Promiscuous gene expression

PLZF - Promyelocytic leukemia zinc-
finger protein

PP - Pharyngeal pouch

PRR - Pattern recognition receptor

RA - Retinoic acid

RAG - Recombination activating gene

RANK - Receptor activator of NF-κB

Rb – Retinoblastoma

RE - Regulatory element

RLR - Retinoic acid-inducible gene-I
(RIG-I)-like receptor

RTE - Recent thymic emigrant

RUNX3 - Runt-related transcription
factor 3

Sca1 - Stem cell antigen 1

SCF - Stem cell factor

SCID - Severe combined
immunodeficiency

scRNAseq - Single cell RNA
sequencing

Six1 - Sine oculis-related homeobox
1 homologue

SP - Single positive

Sox9 - SRY-box transcription factor 9

SSEA-1 - Stage-specific embryonic
antigen1

S1PR₁ - Sphingosine-1-phosphate
receptor 1

Tbx1 - T-box protein 1

TCR - T cell receptor

TEC - Thymic epithelial cell

TEP – Thymic epithelial progenitor

Tc - T cytotoxic

Th - T-helper

Th-POK - T-helper inducing
POZ/Kruppel-like factor

TLR - Toll-like receptor

TNC - Thymic nurse cell

TOX - Thymus high mobility group
box protein

TR

A - Tissue-restricted antigen

TRAF3 - TNF receptor-associated
factor 3

TSP - Thymic seeding progenitor

TSSP - Thymus-specific serine
protease

tTEP - Transitional progenitor

UEA-1 - Ulex europaeus agglutinin 1

V - Variable

VCAM-1 - Vascular cell adhesion
molecule 1

VEGF - Vascular endothelial growth
factor

Wnt - Wingless-related MMTV
integration

WT - Wild-type

YFP - Yellow fluorescent protein

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Chapter I

Introduction

An overview of the immune system

Every living organism relies on a complex and sophisticated network of cellular and molecular mediators that together, protect them against diverse threats while preventing excessive damage to their own tissues. This intricate network, described collectively as the immune system, has evolved throughout the years. Specifically, the complexity that we find nowadays in the vertebrate immune system is believed to have arisen from the evolutionary pressure of their rapid development and consequent exposure to a wider spectrum of pathogens (Boehm, 2012). While it was initially proposed that this system would only be activated in the presence of a pathogen, there is growing evidence that is constantly being challenged (Eberl, 2016). This is, therefore, a dynamic system, exemplified by the symbiotic and tolerogenic relationship between the host and its gut microbiota. Furthermore, the immune system is involved in other several processes that span from sustaining tissue homeostasis to establishing a crosstalk with other organs to promote normal physiology such as in the case of the nervous system (Eva et al., 2023). The permanent balance between the challenges that the immune system faces and the induction of tolerance mechanisms assures the homeostasis of the tissues.

The immune system has been classically subdivided into the innate and adaptive branches, although there is an interplay between them in their responses. As it represents the primary line of immune protection, the innate immunity is usually typified by the absence of classical memory response. By contrast, adaptive immunity comprises a range of highly specific responses that rely on the recognition of antigens by highly specialized receptors (Medzhitov & Janeway, 1997; Vivier & Malissen, 2005). The innate arm of the immune system provides an immediate and less specific response which ranges from non-specific anatomical barriers and molecular mediators, such as complement proteins and defensins to the expression of germ-line encoded pattern recognition receptors (PRRs). These receptors, which include toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), recognize a strict set of molecules that are conserved and shared by different microorganisms, usually referred to as pathogen-associated molecular patterns (PAMPs). While this initial line of response is moderately efficient in dealing with potential threats to the host, organisms developed a more specialized type of response: adaptive immunity. Importantly, this type of immune response promotes the formation of immunological memory, enabling a more specific and efficient response following a subsequent challenge with the same stimuli. Its main cellular effectors are B and T lymphocytes, which express B- and T-cell receptors (BCRs and TCRs), respectively. Together, they provide a catalogue of diverse BCR and TCR repertoires, capable of virtually

respond to all types of threats. This diversity is generated through a process of random somatic rearrangement of variable (V), diversity (D) and joining (J) (V(D)J) gene segments of antigen receptor loci, increasing the chances of both BCR and TCR to recognize, and respond, to any possible antigen encountered throughout life (Schatz & Ji, 2011). The randomness associated with this process will potentially allow the generation of receptors bearing self-reactivity and, as such, the tight balance of the self-tolerant state is assured by the coordinated action of central and peripheral tolerance mechanisms. Collectively, while fundamentally distinct in several aspects, innate and adaptive immune responses are intimately interconnected, relying on each other for the optimal maintenance of a fully functional immune system. In this thesis, I will focus my attention on one of the effector cells of the adaptive immunity, T lymphocytes, and on the site where these cells are generated: The thymus.

Thymus: The founder of T lymphocytes

What was once perceived as a vestigial organ with no apparent role in the immune system, became recognized in the 1960s as a vital primary lymphoid organ. The thymus represents the unique generative site of functionally diverse and self-tolerant T lymphocytes (Miller, 2011). The thymus is a bilobed organ that is located behind the sternum right above the heart. Each lobe is encapsulated by connective tissue and anatomically organized in two distinct areas: the outer cortex and the inner medulla. The immunological function of this organ was first documented in 1961 by Jacques Miller's elegant studies on virus induced-leukemia in neonatal thymectomized mice (Miller, 1961). His work demonstrated that surgical removal of the thymus during the neonatal period led to severe immunodeficiency, as mice exhibited impaired immune responses, which were later found to be T-cell dependent. These observations undoubtedly highlighted the essential role of the thymus in T cell development and, consequently, in mounting an efficient adaptive immune response.

Within the stromal compartment of the thymus, a specialized set of epithelial cells known as thymic epithelial cells (TECs) represent the most critical element for T cell development and selection. TECs are classically segregated into cortical TECs (cTECs) and medullary TECs (mTECs), with each presenting a specific anatomical location and associated function. These cells are organized in a three-dimensional network and provide the essential instructive cues for the differentiation of T cell precursors, also known as thymocytes. In addition to TECs, the thymic stroma is also composed of other specialized cell types of non-hematopoietic origin, such as endothelial and mesenchymal cells, as well

as cells of hematopoietic origin, including dendritic cells (DCs), macrophages and B cells (Takahama, 2006). Together, these cells function cooperatively, playing a crucial role in supporting key events of T cell development.

Among its peculiar features, the thymus begins to involute early in life (Shanley et al., 2009). A series of functional and structural age-related alterations in the organ (which will be covered later in more detail) contribute to the gradual loss of its capacity to support T cell development. This physiological process is referred to as thymic involution (Shanley et al., 2009). The disruption of the cortical and medullary architecture of the thymus, along with phenotypic and functional alterations on TEC microenvironments, contributes to an increased susceptibility to infectious diseases, and neoplastic or autoimmune disorders in the elderly (Glaser et al.). Therefore, the thymus represents a unique and indispensable organ, as it provides a specialized microenvironment that supports the maturation, selection, and functional specialization of T cells. The recognition of the thymus as a central organ in the immune system has paved the way for a deeper understanding of the complex processes underlying T cell-mediated immunity, immune tolerance, and the prevention of autoimmune diseases.

The fundamentals of T cell development

Within the immune system, T lymphocytes comprise a group of cells that are highly specialized in cell-mediated immunity, due to their distinctive ability to recognize a diverse array of foreign antigens and to mount highly antigen-specific immune responses. The thymus generates two main lineages of T lymphocytes, which segregate based on the expression of either $\alpha\beta$ or $\gamma\delta$ TCRs (Takahama, 2006). In my thesis, I will mainly focus on the cellular and molecular aspects underlying the development of $\alpha\beta$ T cells. T cell differentiation is an intricate and tightly regulated process that generates a diverse repertoire of T cells with distinct effector functions. It is a very stringent differentiation route, as several developmental checkpoints assure the selection of $\alpha\beta$ T cells expressing TCRs that are major histocompatibility complex (MHC)-restricted, self-tolerant and reactive to foreign antigens. As such, only a minor fraction (around 3%) of developing thymocytes survive and are exported to secondary lymphoid organs (Klein et al., 2014).

In mice, fetal liver-derived hematopoietic progenitors start colonizing the thymus around the embryonic day (E) 12 (Soares-da-Silva et al., 2023). Sustained T cell production in the postnatal and adult thymus requires then a continual influx of bone marrow-derived hematopoietic progenitor cells, which are considered to populate the thymus in a cyclic fashion rather than in a continuous way (Foss et al., 2001). The differentiation of T

lymphocytes is a very dynamic process, with T cell progenitors (thymocytes) migrating through different areas of the organ as they progress further in their differentiation. Thymic colonization by hematopoietic progenitor cells is mediated by two distinct pathways: a vasculature-independent seeding that takes place only during early embryonic development (around E12), and a later vasculature-dependent entry, once thymic vasculature is established (Glaser et al.). It is believed that the vascular-independent colonization of the thymus is regulated by the chemotactic recruitment of the chemokine (C-C motif) ligands 21 (CCL21), 25 (CCL25) and the C-X-C motif chemokine 12 (CXCL12) (Liu et al., 2006; Liu et al., 2005). As soon as thymic vasculature is established, thymic seeding progenitors (TSPs) enter the thymus through the blood vessels located near the cortico-medullary junction (CMJ) (**Figure 1**, in yellow). Within the postnatal thymus, thymic colonization appears to be additionally facilitated by the expression of several adhesion molecules expressed by the thymic endothelium, namely P-selectin (Gossens et al., 2009; Rossi et al., 2005), the chemotactic recruitment by the chemokines CCL25 and CXCL12 and the binding of the $\alpha 4\beta 1$ and $\alpha L\beta 2$ integrins to vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), respectively (Scimone et al., 2006).

a) Early thymopoietic events in the cortex: T cell lineage commitment and positive selection

Following their entry into the thymus, cells dwell within the cortex, migrating from the perimedullary to the outer cortical areas according to their defined stage of differentiation (Porritt et al., 2003). These immature thymocytes are typified by the lack of mature T cell markers, namely the cluster of differentiation 4 (CD4) and CD8 co-receptors (CD4⁻CD8⁻) (**Figure 1**, in orange) and are referred to as double negative (DN). The differentiation of DN thymocytes is further subdivided into DN1 to DN4 stages, according to the expression profile of CD25 and CD44 molecules. DN1 thymocytes (CD44⁺ CD25⁻) spend approximately 10 days in a restricted area near the site of thymic entry. Within this DN1 population, CD117⁺ early thymic progenitors (ETPs) bear the potential to generate either $\alpha\beta$ or $\gamma\delta$ T cells, while retaining a limited capacity to generate other lineages such as B cells, DCs or natural killer (NK) cells (Ciofani & Zuniga-Pflucker, 2007). DN2 thymocytes upregulate the expression of CD25 (CD44⁺ CD25⁺) and undergo active proliferation. Their expansion and survival are supported by stem cell factor (SCF or kit ligand) (Rodewald et al., 1997) and interleukin 7 (IL-7) (Alves et al., 2009). The (irreversible) commitment towards the T cell lineage requires the interaction between delta-like ligand 4 (Dll4) expressed by cTECs and its transmembrane receptor Notch 1 expressed in developing thymocytes (Sambandam et al., 2005). As DN thymocytes enter the subsequent DN3 stage (CD44⁻ CD25⁺), recombination

activating gene (RAG) enzymes promote the rearrangement of the V(D)J gene segments of the β , δ and γ TCR alleles (Schlissel, 2003). A minor fraction of these cells successfully progress towards the $\gamma\delta$ T cell lineage (Capone et al., 1998). At this stage, a crucial developmental checkpoint termed β selection ensures the differentiation of DN3 cells expressing a productive TCR β chain. The recently generated TCR β chain pairs with an invariant pre-TCR α chain and forms a pre-TCR, along with CD3 signaling molecules (von Boehmer, 2005). Pre-TCR signaling, along with signaling via Notch-1 and CXCR4, ceases TCR β locus recombination, stimulates proliferation and promotes further differentiation towards the DN4 (CD44⁺ CD25⁻) stage (Maillard et al., 2006; Trampont et al., 2010). The rearrangement of the TCR α chain allows the assembly of a full $\alpha\beta$ TCR and cells progress towards an intermediate immature CD8 single positive (ISP8) state, wherein they acquire the expression of CD8 before the upregulation of CD4. With the acquisition of both CD4 and CD8 co-receptors, thymocytes progress to the double positive (DP) stage. DP cells are composed of mostly unselected TCR $\alpha\beta$ -expressing thymocytes (around 85% of thymocytes), and undergo at this stage an indispensable checkpoint in T cell development: positive selection (**Figure 1, in grey**).

During positive selection, cTECs screen DP thymocytes expressing TCRs for their ability to recognize with the proper affinity self-peptides presented via MHC molecules (Klein et al., 2009). While low to intermediate TCR-peptide/MHC interactions permit DP thymocytes to receive survival signals and allow cells to progress towards the single positive (SP) stage, cells with high-affinity interactions or that express TCR unable to recognize self-peptide/MHC complexes are eliminated. Thymocytes expressing TCRs that recognize self-peptide:MHC class I molecules are selected into the CD8 SP lineage (**Figure 1, in blue**) and those expressing TCRs that recognize self-peptide:MHC class II molecules become committed towards the CD4 lineage (**Figure 1, in green**). The molecular basis for CD4 or CD8 lineage fate is believed to depend on the strength and duration of signals derived from TCR and the upregulation of downstream programs initiated by lineage-specific transcription factors. Two models were proposed to explain CD4 vs CD8 lineage specification according to the length and strength of TCR signaling (Brugnera et al., 2000; Hernandez-Hoyos et al., 2000; Suzuki et al., 1995). While the “strength of signal” model suggests that CD4 and CD8 lineage relies respectively on strong and weaker TCR-peptide:MHC molecules interactions, the “kinetic signaling model” integrates that the decision-making between the CD4 or CD8 lineage depends also on the duration of the TCR-peptide:MHC interaction. Continuous TCR signaling would promote the differentiation towards the CD4 lineage, whereas disruption of TCR signaling would favor CD8 lineage differentiation. A recent groundbreaking study where co-receptor proteins encoded by *Cd4* and *Cd8* genes were swapped has demonstrated that *CD4* locus promoted weak and

persisting TCR signaling, while co-receptors encoded by *CD8* gene had strong and short-lived TCR signaling (Shinzawa et al., 2022). These observations argue against the “strength of signal” model and instead elucidate that, during positive selection, co-receptor genes regulate the extent of TCR signaling duration. Alongside with these interactions between the TCR and self-peptide:MHC complexes, the upregulation of specific transcription factors has been described to imprint the differentiation program towards a specific lineage. T-helper inducing POZ/Kruppel-like factor (Th-POK) (Egawa & Littman, 2008), GATA-binding protein 3 (GATA3) (Pai et al., 2003) and thymus high mobility group box protein (TOX) (Aliahmad & Kaye, 2008) play a key role in CD4 lineage commitment. Specifically, Th-POK is upregulated during MHC II-induced selection and appears to prevent Runx-dependent differentiation, with GATA3 proposed to be important to induce Th-POK expression (Wang et al., 2008). Conversely, runt-related transcription factor 3 (RUNX3) is described to be essential for the silencing of the *CD4* gene and to promote the expression of CD8 lineage-associated genes, such as the ones encoding for perforin or granzyme B (Sato et al., 2005) (Wang & Bosselut, 2009). Its expression is controlled by IL-7-derived signals after TCR signal cessation (Park et al., 2010). The expression of these transcription factors is, therefore, vital to reinforce CD4 and CD8 lineage decisions.

b) Late stages of T cell development and tolerance induction in the medullary area: Negative selection and T regulatory cell differentiation

Following positive selection, SP thymocytes upregulate the expression of the C-C chemokine receptor type 7 (CCR7). The ligands for CCR7, CCL19 and CCL21, are produced by mTECs and attract developing SPs towards the medulla (Ueno et al., 2004). Here, negative selection and T regulatory cell differentiation act as key developmental checkpoints to impose immunological central tolerance. They respectively promote the clonal deletion or deviation of potential autoreactive SP thymocytes, which bear TCRs with high affinity for self-antigens:MHC molecules (Glaser et al.). It is considered that a fraction of autoreactive SP4 thymocytes can escape clonal deletion and instead be diverted into the Treg lineage. The differentiation into this lineage requires, among other factors, the upregulation of the transcription factor forkhead box p3 (Foxp3) (Fontenot et al., 2003). Thymic-derived Treg cells comprise a population of T cells with the unique competence to safeguard peripheral immunological tolerance through the suppression of autoimmune reactions. Collectively, negative selection and T reg differentiation are critically dependent on mTECs and on their unique ability to virtually mirror the expression of all tissue-restricted antigens (TRAs) of our body (Derbinski et al., 2001). This process is known as promiscuous gene expression (pGE) and it is partially under the control of the autoimmune regulator, also known as Aire (Anderson et al., 2002), and FEZ family zinc finger 2 (Fezf2) (Takaba

et al., 2015). Therefore, cTECs and mTECs represent two distinct but complementary gatekeepers of selection and tolerance induction. The details of the specific machineries that each cell type possesses to promote thymocyte selection along with their specific role in each stage of T cell development will be covered later in more detail.

While in the medulla, SP thymocytes expressing TCR reactive to foreign antigens complete their maturation process before exiting the thymus. Several sequential stages of maturation have been defined and can be segregated into semi-mature $CD69^{hi} CD24^{hi} CD62L^{low} Qa-2^{low}$, mature (M1) $CD69^{int} CD24^{+} CD62L^{low} Qa-2^{low}$ and further mature (M2) $CD69^{low} CD24^{low} CD62L^{hi} Qa-2^{hi}$ expressing cells (Hogquist et al., 2015). Along with this progressive maturation, SP thymocytes acquire the functional capacity to egress the thymus. This is an active process controlled by the sphingosine-1-phosphate receptor 1 (S1PR₁), a G-protein coupled receptor (GPCR) (Matloubian et al., 2004). The expression of this receptor is under the transcriptional regulation of krüppel-like factor 2 (KLF2), which is induced after termination of the TCR signaling and downregulation of CD69 (Carlson et al., 2006). The concentration of S1P is higher in serum than in most tissues and its chemotactic effects drive the migration of mature SPs towards the CMJ to leave the thymus. The population of cells that have just exited the thymus is known as recent thymic emigrants (RTEs) (Fink, 2013). Before they become fully mature naïve (MN) T cells, RTEs require additional maturation steps in the peripheral lymphoid organs. RTEs enter secondary lymphoid organs and interact with other cell types, including fibroblastic reticular cells (FRCs) and peripheral DCs, which have been shown to be essential for T cell maturation and survival (Houston et al., 2012). In peripheral organs, T cells are continually recirculating and scanning the existence of possible threats from the tissues.

The recognition of foreign antigens by T cells, presented by antigen-presenting cells (APCs) via MHC molecules, is a crucial step in initiating an immune response. This recognition, along with co-stimulation and specific cytokine signals, leads to the activation and differentiation of different subsets of effector T cells. Within the CD4 helper lineage, various T-helper (Th) effector subsets have been characterized, each playing specific roles in immune responses. These subsets produce distinct sets of cytokines (Th1 producing interferon (IFN)- γ and IL-2, Th2 producing IL-4 and IL-13 and Th17 cells producing IL-17A and IL-22), contributing to the coordination and regulation of the immune response. Similarly, within the T cytotoxic (Tc) CD8 lineage, several subsets have been proposed, each characterized by distinct cytokine profiles and functions: IFN- γ -producing Tc1s, IL-4 producing Tc2s, IL-9 producing Tc9s, IL-17 producing Tc17s and IL-22 producing Tc22s (St Paul & Ohashi, 2020). The differentiation and establishment of a functional $\alpha\beta$ T cell pool is an extremely complex and tightly regulated process that promotes the export of self-tolerant

T lymphocytes that guarantee under normal conditions an adequate immune response against foreign threats.

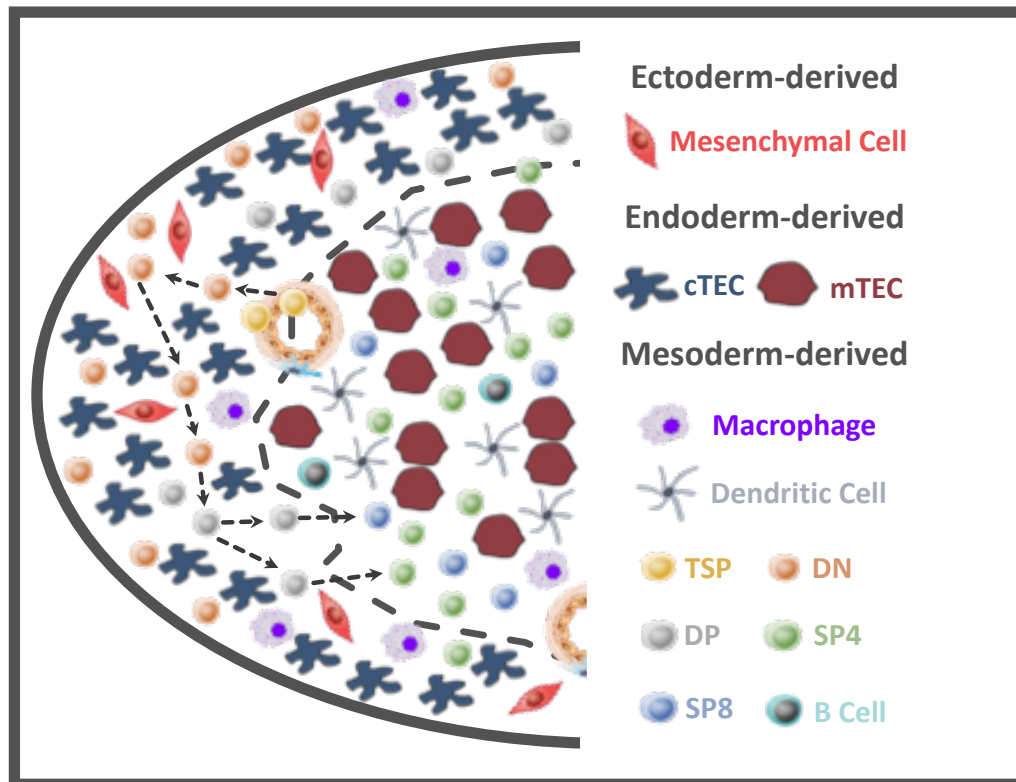


Figure 1- Stages of $\alpha\beta$ T cell development in the thymus. Graphical representation of the cellular composition and organization of the adult thymus. Once inside the thymic microenvironment, thymic seeding progenitors (TSPs) undergo a complex differentiation pathway towards the outer regions of the thymic cortex as $CD4^-CD8^-$ double negative (DN) thymocytes. After T cell receptor (TCR) rearrangement and β -selection, DN cells mature into $CD4^+CD8^+$ double positive (DP) thymocytes and undergo positive selection. This checkpoint promotes their commitment into a single positive (SP) SP4 or SP8 lineage. Positively selected SP thymocytes then migrate towards the medulla, wherein cells expressing TCRs that are both restricted to self-MHC and tolerant to self-antigens complete their maturation and egress into the peripheral lymphoid organs. The arrows represented in solid depict the transition of cells through the distinct stages of thymocyte development. Adapted from (Minoru Matsumoto & Tsuneyama, 2019).

c) Differentiation of unconventional innate-like T cell lineages

Although my thesis is mostly focused on the development of classical $\alpha\beta$ T cells, the thymus also generates other specialized subsets of T cells, namely $\gamma\delta$ T cells, natural killer T cells and Mucosal-associated invariant T cells.

$\gamma\delta$ T cell differentiation

In addition to $\alpha\beta$ T cells, $\gamma\delta$ T lymphocytes undergo somatic gene rearrangement for the generation of antigen receptors within the thymus, although representing a distinct subset of cells with specific requirements (Ribot et al., 2021). They participate and regulate a wide array of immune functions, interconnecting features of innate and adaptive immune

responses, such as being prevalent at a barrier organ like the skin and recognizing certain ligands via their TCR (Parker & Ciofani, 2020). As previously mentioned, a fraction of DN2 thymocytes start rearranging the δ and γ gene segments from TCR alleles and commit into the $\gamma\delta$ T cell lineage, while remaining phenotypically as DN for CD4 and CD8 markers. In contrast to $\alpha\beta$ thymocytes, $\gamma\delta$ T cells express TCRs with limited diversity. They can be functionally subdivided according to the cytokines they produce: IFN- γ producing $\gamma\delta 1$ and IL-17A-producing $\gamma\delta 17$ (Glaser et al.).

A peculiar feature of $\gamma\delta$ T cell differentiation is the fact that it takes place in coordinated waves that arise at specific times during embryogenesis, with these waves correlating with specific tissue-resident cells and their function (Prinz et al., 2013). The first wave (around E13) gives rise to $V\gamma 5^+$ dendritic epidermal T cells (DETCs), that will reside in the skin and are known to produce IFN- γ . At around E16, a second wave gives rise to IL-17A-producing T cells, which include $V\gamma 6^+$, $V\gamma 4^+$ and $V\gamma 1^+$. A third functional wave promotes the generation of $\gamma\delta$ NK T cells (Prinz et al., 2013). These express the promyelocytic leukemia zinc-finger protein (PLZF) similarly to NK T cells and its development is controlled by TCR signal strength (Alonzo et al., 2010). Moreover, they are described to be continuously produced after the postnatal period and reside in the spleen and liver. $\gamma\delta$ T cells represent, therefore, a peculiar cell type with preferential homing to certain tissues, which might be important to expand immune responses to places where B and T cells are not so dominant while acting as a first line of defense against ligands that do not require antigen presentation and T cell activation. We will, however, mainly focus our attention on the development of the most prevalent $\alpha\beta$ T cells and how TECs control their differentiation and selection.

Natural killer T cell differentiation

NK T cells equally differentiate within the thymus and are described to recognize lipid-based antigens presented via MHC class-I-like molecule CD1d (Pellicci et al., 2020). They segregate into three distinct classes, NKT type 1, NKT type 2 and NKT type 17, resembling in part the system used to identify the distinct subsets of CD4 helper cells. In mice, NKT 1 cells express an $\alpha\beta$ TCR with limited combinations of $V\alpha 14$ and $V\beta 8$, $V\beta 7$ and $V\beta 2$ chains, with the remaining subsets displaying equally restricted TCR usage (Bendelac et al., 2007). These follow a similar differentiation pathway as conventional $\alpha\beta$ T cells until, at the DP stage, TCR engagement with CD1d commits developing thymocytes towards the NKT cell lineage. NKT type 1 cells express low levels of PLZF and high levels of T-bet ($PLZF^{\text{low}}$ T-bet^{hi}) while NKT2 are $PLZF^{\text{hi}}$ T-bet^{low} and NKT17 are $PLZF^{\text{int}}$ ROR γ t⁺ (Lee et al., 2013). In parallel with the helper cell subsets, type 1 NKT cells produce IFN- γ , type 2 produce IL-4 and type 17 produce IL-17. NK T cells have been described to be involved in several immune functions that span from protection against microbes to tumor rejection (Seino &

Taniguchi, 2005). Interestingly, recent studies have elucidated the critical role of thymic-resident NKT 2 cells as the predominant source of IL-4 inside the thymus (White et al., 2017). The development of NKT 2 depends on recently identified thymic tuft cells (Glaser et al.). It has been demonstrated that the production of IL-4 by this type of NK T cells plays a role in the export of mature thymocytes from the thymus (Glaser et al.) (Miller et al., 2018; White et al., 2017).

Mucosal-associated invariant T cells

Similarly to NK T cells, the mucosal-associated invariant T cell (MAIT) compartment displays a limited $\alpha\beta$ TCR diversity (Pellicci et al., 2020). The TCR consists of a V α 19 and a V β 8 or V β 6 chain and it recognizes vitamin B metabolites. These cells are selected in the thymus by MR1-expressing DP thymocytes (Seach et al., 2013). In mice, there are two main subsets of MAIT cells: MAIT1 and MAIT17 cells. The latter is the most prevalent, with cells being PLZF^{int} ROR γ t⁺ and producing IL-17 (Pellicci et al., 2020). MAIT1 cells are PLZF^{low} T-bet⁺ and produce IFN- γ , which resembles the NK T cell subsets. As these cells recognize microbial-derived vitamin B metabolites, they are believed to be important for anti-microbial immunity (Legoux et al., 2017). Furthermore, differences in the frequency of these cells have been associated with the appearance of certain diseases such as autoimmune-associated conditions (Rouxel & Lehuen, 2018).

The ontogeny of the murine thymus

The establishment of a fully functional thymic microenvironment is indispensable for the proper differentiation and selection of T lymphocytes. The generation of the thymus involves a series of highly coordinated events common to general organ development. Early stages include the positioning and both initiation and outgrowth of the thymic anlage, while this structure starts to migrate to its final position. Conversely, later phases of organogenesis involve the expansion and differentiation of the thymic epithelium in a thymocyte-independent fashion (Glaser et al.).

The development of the thymus is intimately linked with the formation of the parathyroid gland, with both organs emerging from a common primordium derived from the third pharyngeal pouches (3PP) of the foregut endodermal tube around murine E11 (Blackburn & Manley, 2004) (**Figure 2**). At E12.5, both the parathyroid and the thymic domain start to grow and by E13.5 they physically separate and begin moving towards their final positions: the thymic primordium within the chest cavity and the parathyroid on the back of the thyroid gland. The patterning of each specific domain is critically dependent on the respective expression of the transcription factors glial cells missing homolog 2 (Gcm2) for the

parathyroid and forkhead box N1 (Foxn1) for the thymus. While Gcm2 is found to be expressed in the anterior-dorsal region of the 3PP epithelium as early as E9.5 (Liu et al., 2007), Foxn1 is first detected in the posterior-ventral side of the pharyngeal pouch at E11.25 (Blackburn et al., 1996). After induction of Foxn1 expression, TEC differentiation is believed to start from a common bipotent TEC progenitor cell (TEP) that will give rise to both cTEC and mTEC lineages. Further maturation of these cell types requires signals provided by developing thymocytes, in a process known as thymic crosstalk (covered later in more detail). In addition to the thoracic thymus, mice and humans can possess a small, yet functional, cervical thymus (Dooley et al., 2006; Terszowski et al., 2006). It shares the same structural organization as the thoracic thymus and retains the ability to, to some extent, attract hematopoietic precursors and promote T cell differentiation (Glaser et al.). Cells that reside within the cervical thymus express MHC II and Foxn1, but at that stage, the structure of the thymus is still primitive and it only accommodates immature thymocytes (Glaser et al.). Curiously, the appearance of the thoracic and cervical thymus is not synchronized, with the latter arising shortly after birth (Rodewald, 2008).

Thymus organogenesis involves, among other processes, the production of morphogenic factors that control each stage of development (Rodewald, 2008). During this period of organogenesis, neural crest-derived (NC) mesenchymal cells form an envelope around the developing primordium and provide supporting and differentiation signals such as fibroblast growth factors (FGF) 7 and FGF10, along with retinoic acid (RA) and insulin-like growth factors (IGF) 1 and IGF2, which induce the proliferation and homeostasis of the thymic epithelium (Jenkinson et al., 2007; Revest et al., 2001; Sitnik et al., 2012). These cells surround the thymic epithelium and will give rise to the perivascular mesenchyme (Glaser et al.). Apart from their role in thymus development, some studies have also proposed that NC mesenchymal cells participate in stage-specific maturation of developing thymocytes, particularly in the earlier stages of their development (Anderson et al., 1993). These may include the production of extracellular matrix (ECM) components that may capture essential maturation signals, such as IL-7 (Banwell et al., 2000).

The understanding of the precise molecular determinants that control thymic organogenesis remains incomplete. Some of the transcription factors involved in these early events have been identified. These include homeobox A3 (Hoxa3) (Chisaka & Capecchi, 1991), paired box genes 1 (Pax1) (Wallin et al., 1996) and Pax9 (Hetzer-Egger et al., 2002), eyes absent 1 homologue (Eya1) (Xu et al., 2002), sine oculis-related homeobox 1 homologue (Six1) (Xu et al., 2002) and T-box protein 1 (Tbx1) (Jerome & Papaioannou, 2001). Mutations on either of these genes can impair the migration of the thymus towards its final location or lead to thymus aplasia or hypoplasia. Yet, at which specific event each

one of them is particularly required is undetermined. It is, therefore, challenging to understand the primary function of these genes due to their pleiotropic defects on the organism and targeted studies are still required. Understanding the intricate cellular and molecular events that drive thymus organogenesis would provide valuable insights into the foundations that control the formation of a fully operational thymus.

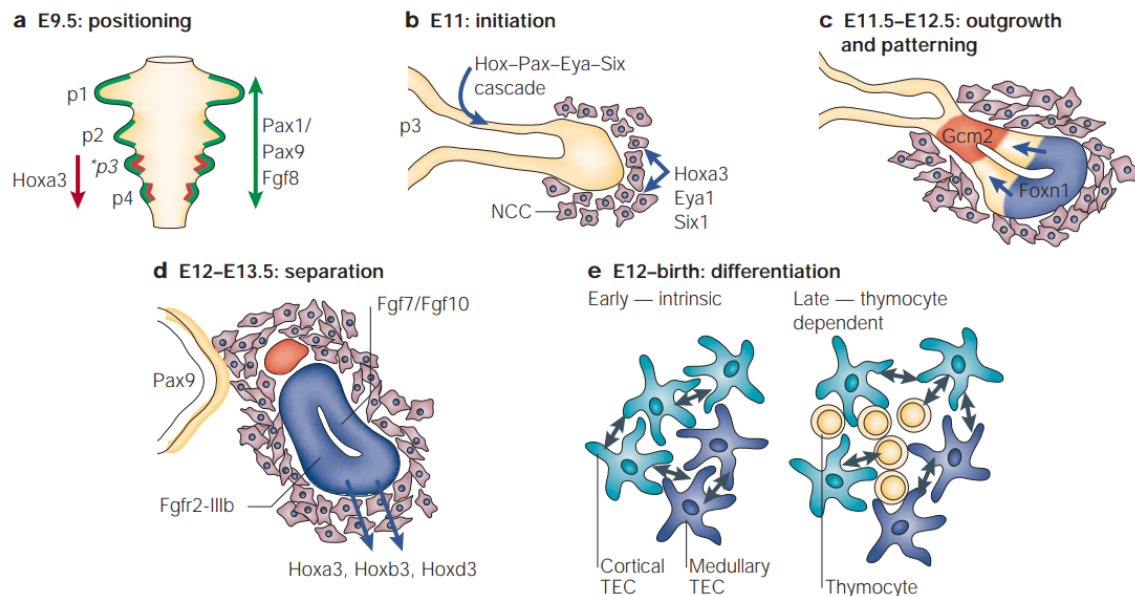


Figure 2- Overview of murine thymus organogenesis. The scheme represents an overview of the murine thymus development. Briefly, both the thymic and parathyroid domains arise from the third pharyngeal pouches (3PP) of the foregut endodermal tube. Early stages of organ development include the initiation of complex molecular pathways that will eventually culminate in the formation of a common primordium that will give rise to both the parathyroid and the thymic domains. Once the transcription factors glial cells missing homolog 2 (Gcm2) at the anterior-dorsal region and forkhead box N1 (Foxn1) at the posterior-ventral side are induced, the specific outgrowth and patterning of each domain starts and the organs physically separate and begin to migrate towards their final position. Once the thymic domain is established due to Foxn1 induction, lymphoid progenitors colonize the organ and the signals provided by developing thymocytes promote the maturation of thymic epithelial cells (TECs). Adapted from (Blackburn & Manley, 2004).

Foxn1: The master regulator of TEC differentiation and function

Among the hierarchy of elements described to be involved in thymus organogenesis, Foxn1 remains to date as one of the key transcription factors with a non-redundant role in TEC differentiation (Romano et al., 2013; Zuklys et al., 2016). Foxn1 belongs to the evolutionarily conserved forkhead box family of transcription factors, that commonly possess a distinctive winged-helix/forkhead DNA-binding domain and a transcriptional activation domain (Schuddekopf et al., 1996). These transcription factors are described to be involved in several biochemical and cellular processes, that span from development and homeostasis to aging or cancer (Golson & Kaestner, 2016). Foxn1 is believed to have arisen in jawed vertebrates from the genomic duplication of its paralog Foxn4 (Bajoghli et al., 2009). Strikingly, Foxn4 expression is also found within the pharyngeal endoderm (Glaser

et al.), possibility highlighting the endoderm as a conserved site for the expression of both *Foxn4* and *Foxn1* genes. The appearance of the *Foxn1* gene might have also paved the way for the appearance of the genetic circuits that control thymopoiesis (Swann et al., 2014). Specifically, *Foxn4* is able to drive thymopoiesis, although this process does not give rise exclusively to T cells, supporting both B and T cell development. These results indicate that thymus appears to have evolved from a lymphopoietic organ with bipotent capabilities, with the evolutionary emergence of *Foxn1* transforming it into a site exclusively dedicated to the development of T lymphocytes (Swann et al., 2014).

Foxn1 is expressed both in the thymic epithelium and keratinocytes, where it controls numerous differentiation pathways (Schlake et al., 2000). Specifically, *Foxn1* expression in TECs represents the master regulator of TEC lineage specification and function. Apart from being essential for the early events that promote TEC lineage specification and early embryonic TEC differentiation, it is also continuously required during postnatal life to regulate TEC homeostasis (Romano et al., 2013; Vaidya et al., 2016). Furthermore, *Foxn1* controls the expression of chief regulators of thymopoiesis in TECs, which include genes that contribute to T cell lineage commitment (such as *Dll4*), antigen processing and presentation ($\beta 5t$, thymus-specific serine protease, TSSP) and lymphocyte migration (*CCL25*) (Zuklys et al., 2016). The intricate downstream pathways triggered by this transcription factor undoubtedly highlight the pivotal role of *Foxn1* in TEC development and function. Of note, reduction in the expression of *Foxn1* has been described to contribute to thymic involution (O'Neill et al., 2016), and this notion will be further discussed later.

Disruption of the normal function of *Foxn1* provokes the athymic nude phenotype in mice and humans and gives rise to severe combined immunodeficiency (SCID) (Blackburn et al., 1996). In cases where *Foxn1* is not expressed or a non-functional protein is produced, there is an early developmental arrest in TEC differentiation (Blackburn et al., 1996). Under these conditions, a functional thymus is not formed, and hematopoietic precursors are not able to colonize the organ, leading to severe T cell immunodeficiency. Akin to mice, *FOXN1* mutations in humans are associated with athymia, complete hair loss (alopecia totalis), and nail dystrophy (Markert et al., 2011). Conducting T cell output screenings on newborns with early-onset and recurrent life-threatening infections is critical as they allow the identification of this immunodeficient state in these patients. Patients with mutations on the *Foxn1* gene might benefit from mature thymic tissue transplantation to promote the reconstitution of the T cell pool and ameliorate their effective immune responses (Markert et al., 2011).

During murine thymus ontogeny, although low levels of *Foxn1* can be already detected as early as E9.5 (O'Neill et al., 2016), higher expression levels are only found around

E11.25, where it is required for TEC lineage specification (Glaser et al.). The analysis of nude mice demonstrated that the early stages of thymic organogenesis could proceed normally in these mice until around E11 with the formation of the common thymus-parathyroid primordium (Nehls et al., 1996). Therefore, early thymus organogenesis does occur in these animals, and they can retain a small and alymphoid cystic thymic rudiment that can be functionalized upon ectopic expression of Foxn1 (Bleul et al., 2006). These observations suggest that Foxn1 might be dispensable for the initial thymic/parathyroid fate specification but is critical for the subsequent differentiation and maturation of the TEC compartment.

Despite its chief role in TEC lineage specification and function, the molecular mechanisms that regulate the induction of Foxn1 *in vivo* remain elusive (Vaidya et al., 2016). Evidence suggests that bone morphogenetic proteins (BMP), wingless-related MMTV integration (Wnt) and retinoblastoma (Rb) family of proteins might have a role in the regulation of Foxn1 expression (Glaser et al.). Specifically, using *in vitro* assays and lineage-tracing mice, BMP4 was shown to act upstream of FGF7 and FGF10 within the thymic stroma and to promote the upregulation of Foxn1 (Soza-Ried et al., 2008; Tsai et al., 2003). Similarly, Wnt proteins were able to induce Foxn1 expression *in vitro* using TEC cell lines, acting both in autocrine and paracrine mode (Balciunaite et al., 2002). The Rb family of proteins appears play instead an inhibitory role, with increased levels of Foxn1 being found in animals where the function of Rb proteins was ablated (Garfin et al., 2013). Interestingly, evidence suggests that Foxn1 itself might also regulate its own expression, through a positive feedback loop (Zook et al., 2011). These observations highlight that the mechanisms underlying the induction of Foxn1 are complex and several signaling pathways might cooperate to enable physiological expression of this key factor for thymopoiesis. The identification of the molecular triggers of Foxn1 induction *in vivo* is, therefore, an open question and solving this would expand our knowledge of the early events during ontogeny as well as provide means to functionalize TECs in therapies targeting thymic disorders.

Thymic epithelial cells: Origins and phenotypical characterization

Thymic epithelial cells represent highly specialized cells that are classically subdivided into cTECs and mTECs based on their spatial, functional and phenotypic proprieties (Abramson & Anderson, 2017). These cells can be identified through the expression of a myriad of both common- and lineage-specific markers (**Figure 3**). Despite this traditional segregation into two distinct sublineages, recent studies based on single cell RNA sequencing (scRNAseq) have highlighted how the cTEC and mTEC niches are formed by

heterogenous subpopulations, each with distinct molecular and functional properties (Kadouri et al., 2020). cTECs represent the least characterized subset of TECs. Nonetheless, they can be identified by the specific expression of cytokeratins 8 (K8) and K18, Ly-51 (also known as BP1/CD249), CD205 (also commonly known as DEC-205) and the atypical chemokine receptor 4 (ACKR4, also known as CCRL1). Additional markers such as high levels of IL-7 and Dll4, and differential expression of MHC II, CD40 or the thymoproteosome subunit $\beta 5t$ can be used to identify discrete cTEC subsets (Minoru Matsumoto & Tsuneyama, 2019; Ohigashi et al., 2016). A population of perinatal cTECs was identified previously based on scRNA seq analysis (Baran-Gale et al., 2020), but their recent phenotypic characterization using massively parallel flow cytometry allowed for a more detailed dissection of these novel cTEC subsets (Klein et al., 2023). Conversely, mTECs represent a more thoroughly characterized population. They are typified by the expression of K5 and K14, as well as the specific binding to the lectin *Ulex europaeus* agglutinin 1 (UEA-1) (Farr & Anderson, 1985). Although mTECs were conventionally segregated into mTEC^{low} (expressing low levels of CD80 and MHC II) and mTEC^{hi} (expressing high levels of both CD80 and MHC II and with a fraction of them expressing the autoimmune regulator-Aire), recent studies have also further resolved mTEC heterogeneity. The mTEC^{low} compartment contains precursors of mTEC^{hi} cells, CCL21-expressing mTECs and terminally differentiated post-Aire mTECs, which comprises a fraction of cells that once expressed Aire (Kadouri et al., 2020). Initial scRNAseq analysis revealed four subsets of mTECs described as: mTEC I appear to represent more immature cells that express high levels of markers such as Integrin Subunit Alpha 6 (*Itga6*) and stem cell antigen 1 (*Sca1*); mTEC II are enriched for the expression of markers of mature mTECs, such as Aire and CD74; mTEC III express unique genes (*Irf1* and *Krt10*) that resemble post-Aire mTECs; and the newly identified mTEC IV thymic tuft cells, that express a unique panel of genes (such as *Dclk1* or *Sox9*) and mimic intestinal tuft cells (Bornstein et al., 2018). Recently, additional post-Aire subsets were identified and collectively termed mimetic cells. These express lineage-specific signatures of several peripheral cell types such as enterocyte and ciliated-like cells (Michelson et al., 2022). These cells are believed to promote the expression of dedicated gene programs within the thymus, providing specific patterns of TRAs that would contribute to the establishment of central tolerance (Michelson et al., 2022).

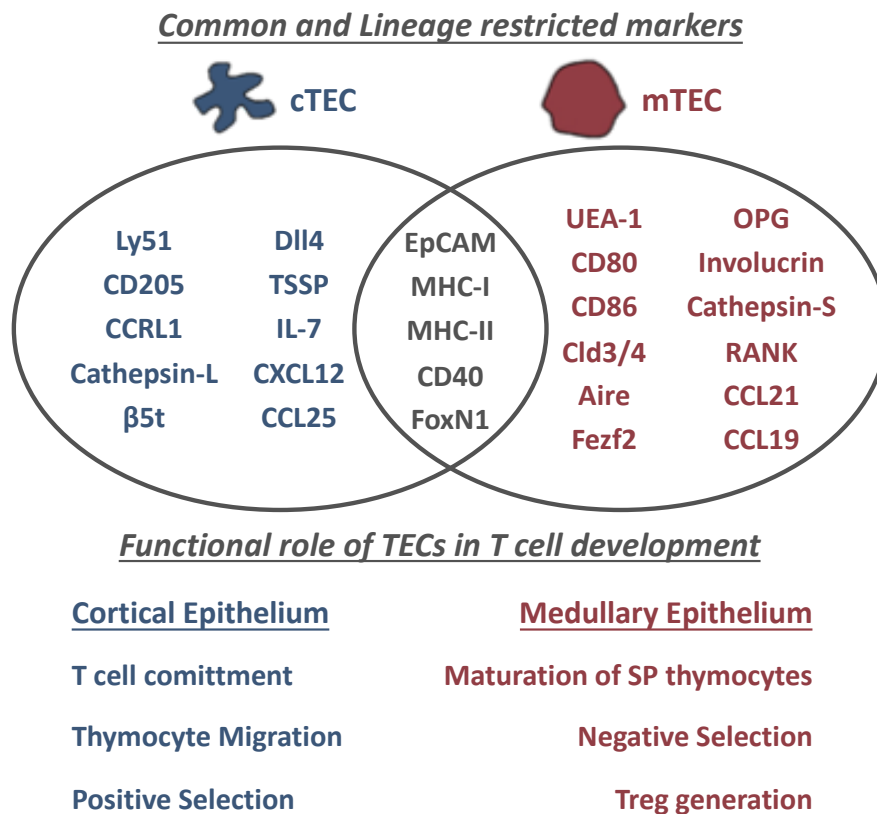


Figure 3- cTEC and mTEC comprise phenotypical and functional distinct subpopulations within the thymus. Inside the thymus, TECs represent the predominant stromal compartment. These cells can be divided into cortical TECs (or cTECs) and medullary TECs (or mTECs). Despite functional distinct, cTEC and mTEC can be identified through the usage of common or specific panel of markers. While cTECs are responsible for regulating the earlier stages of thymopoiesis such as T cell lineage commitment and positive selection, mTECs support the later stages of T cell differentiation and are critical for the establishment of central tolerance and for the differentiation of the regulatory T cell (Treg) lineage. Adapted from (Minoru Matsumoto & Tsuneyama, 2019).

While it was initially postulated that cTECs and mTECs developed independently, several studies unveiled that both TEC subpopulations arise from common bipotent TEC progenitors (TEPs) that are present both within the embryonic and the postnatal thymus (**Figure 4**) (Kadouri et al., 2020; Ropke et al., 1995; Rossi et al., 2006). The existence of bipotent TEPs was initially demonstrated by a series of complementary studies. Using a lineage tracing system wherein single yellow fluorescent protein (YFP)-tagged E12 TEC were injected into a native thymus, the authors demonstrated that these labelled cells were able to give rise to both cTECs and mTECs (Rossi et al., 2006). Furthermore, the analysis of an animal wherein a mutant allele of Foxn1 was reverted to its wild-type form in TEC indicated that bipotent progenitor cells persisted in the postnatal thymus of nude mice and could be reactivated by Foxn1 (Bleul et al., 2006). Despite the compelling evidence for the existence of these bipotent progenitor cells, their precise identification and molecular properties remain a matter of debate. Initial studies have suggested that E14 MTS24⁺ progenitor TECs would have the capacity to generate both cTECs and mTECs (Gill et al.,

2002). Yet, complementary analysis demonstrated that both MTS24⁻ and MTS24⁺ expressing cells presented bipotent properties, arguing against the use of this marker to identify progenitor-specific cells (Rossi, Chidgey, et al., 2007). Subsequent studies have revealed that TEPs share the expression of cortical-restricted markers such as CD205, $\beta 5t$ and high expression of IL-7 (Gill et al., 2002; Ohigashi et al., 2013; Ribeiro et al., 2013). These observations suggested that TEPs would transition through a stage where they would exhibit cellular and molecular traits associated with cTECs before commitment to the cTEC or mTEC lineage. These observations led to the proposal of a “serial progression model” (Alves et al., 2014), wherein transient cTEC-like TEPs transverse first through the cTEC lineage and require the subsequent activation of the mTEC-specific transcriptional program to commit towards the mTEC lineage (Goldfarb et al., 2016). Furthermore, recent scRNAseq analysis supported the observation that embryonic TECs represent a relatively homogeneous population and were enriched for cTEC-associated markers (Bornstein et al., 2018).

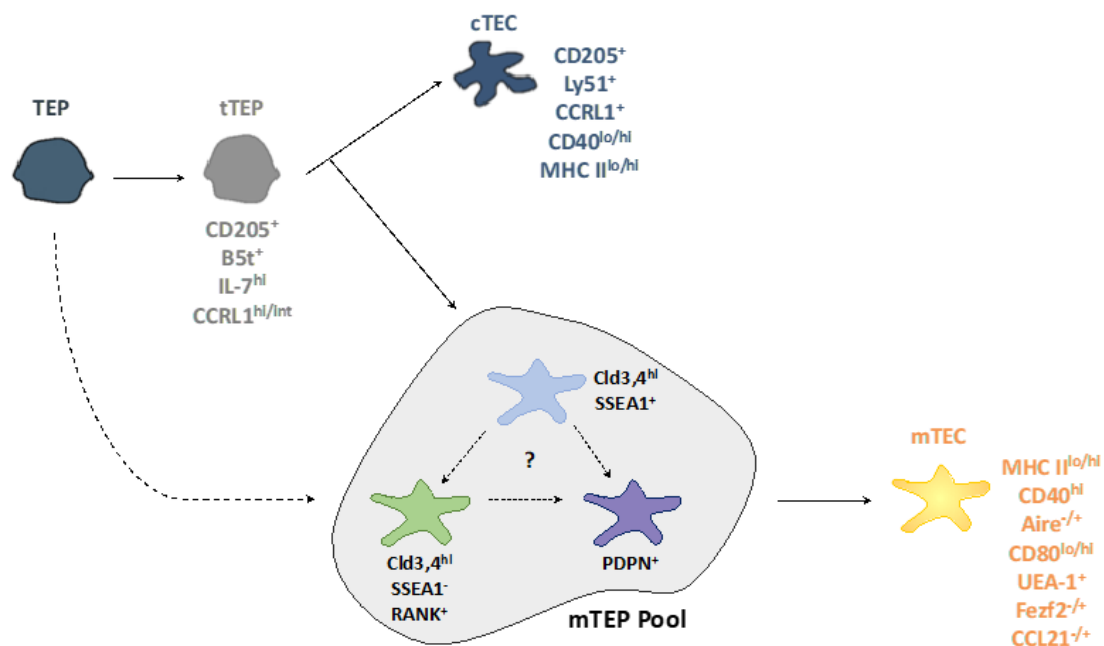


Figure 4- Model of thymic epithelial cell development. Although cTEC and mTEC comprise two distinct subpopulations, these cells arise from common bipotent TEC progenitors (TEPs). Before committing to either a cTEC or mTEC lineage, TEPs transverse through a “transitional progenitor (tTEP)” stage that exhibit cellular and molecular traits associated with cTEC. Recently, novel subsets of mTEC-restricted progenitors (mTEP) have been proposed. The dashed arrow represents the possible direct contribution of TEPs for the mTEC pool and the undefined towards the precursor-product relationship among the different mTEC-committed progenitors (also represented by “?”). Adapted from (Minoru Matsumoto & Tsuneyama, 2019).

Recent studies have also validated the presence of bipotent TEPs within the adult thymus (Ohigashi et al., 2013; Ulyanchenko et al., 2016; Wong et al., 2014). scRNAseq analysis, along with clustered regularly interspaced short palindromic repeats (CRISPR)–

Cas9 technology, allowed for the elucidation and the precise identification of a population containing early bipotent TEPs. These cells were biased towards the differentiation of cTECs (referred to as “early progenitors”). Additionally, the study identified a population of postnatal bipotent TEPs that were enriched for mTEC-biased differentiation, referred to as “postnatal progenitors” (Nusser et al., 2022). It is interesting to note that a linear correlation was found between the size of the initial functional TEC progenitor pool and the overall thymopoiesis (Jenkinson et al., 2008). This indicates that the bioavailability and function of TEP is a deterministic variable in thymic function. Although there is still not a complete picture of TEC developmental pathways, the advent of new high-content analytic technologies (scRNAseq seq, Mass Cytometry) and the ability to analyze rare populations at the single cell level will undoubtedly expand our knowledge and fill the gaps remaining along the narrative of TEC lineage specification and differentiation.

Apart from bipotent TEPs, mTEC-restricted progenitors have also been described, but their relative contribution to the maintenance of distinct mTEC lineages across time remains incomplete. A pool of cells expressing the tight junction components Claudins 3 and 4 were found to represent a restricted reservoir of mTEC-dedicated progenitor cells, specifically for Aire-expressing mTECs (Hamazaki et al., 2007). A subsequent study identified a subpopulation of stage-specific embryonic antigen (SSEA-1)-expressing cells within this restricted pool of mTEC progenitors that possess self-renewable clonogenic abilities (Sekai et al., 2014). Furthermore, a fraction of these SSEA-1⁺ precursor cells might give rise to a population of receptor activator of NF- κ B (RANK)-expressing progeny (Baik et al., 2016). Apart from these identified subpopulations that share some degree of lineage relationship, cells residing in the CMJ and expressing podoplanin (jTECs) were also proposed to contribute for the pool of mature adult mTECs (Onder et al., 2015). More recently, two distinct studies have proposed the presence of cells expressing specific markers that potentially uncover novel subsets of mTEC-restricted progenitors, although their precise relationship remains undetermined. First, a population of embryonic K19⁺ cells was demonstrated to sustain the differentiation of several mTEC subsets in the adult life (CCL21⁺ mTEC^{lo}, Aire⁺ mTEC^{hi} and thymic tuft cells) (Lucas et al., 2023). A second study, through the use of scRNAseq analysis, has revealed a population of embryonic mTEC-restricted progenitors that expresses the SRY-box transcription factor 9 (Sox9) (Farley et al., 2023). Collectively, these observations challenge the idea that bipotent TEPs represent the immediate source of mature mTECs, providing evidence for the existence of lineage-restricted mTEC precursors.

cTECs and their role during early thymopoiesis and positive selection

cTECs are responsible for providing instructive signals that regulate the early stages of T cell development, including cell migration, survival, lineage specification and positive selection. As mentioned before, cTECs promote the homing of TSPs through the production of the cytokines CCL25 and CXCL12 and guide their migration within the cortex as these cells start to differentiate (Liu et al., 2006; Liu et al., 2005). Commitment of progenitor cells towards the T cell lineage is achieved through interactions with the Notch ligand Dll4 expressed by cTECs (Koch et al., 2008). Furthermore, cTECs express IL-7, which mediates not only cell survival and proliferation (along with SCF) of DN thymocytes, but its expression was also demonstrated to have a role in the differentiation of both $\alpha\beta$ and $\gamma\delta$ T cells (Moore et al., 1996). Important to note, a particular uncharacterized subset of cTECs referred to as thymic nurse cells (TNCs) represent a population of cTECs that are tightly associated with thymocytes. This highlights the importance and distinct roles that cTECs play within the cortical area (Nakagawa et al., 2012). TNCs are considered to sustain prolonged interactions with several DP thymocytes to optimize the process of positive selection (Nakagawa et al., 2012).

Apart from their role in T cell lineage commitment, cTECs act as the exclusive mediators of positive selection at the DP stage. This process ensures the differentiation of developing thymocytes expressing $\alpha\beta$ TCRs that recognize with the proper affinity self-peptides presented via MHC molecules by cTECs (Klein et al., 2014). Apart from MHC, the other critical element to consider during positive selection is the precise nature of the presented self-peptides. cTECs display a specialized array of selecting self-peptides (referred to as “private” peptides) that seems different from those presented by mTECs and thymic DCs in the medulla (Takada et al., 2017) (**Figure 5**). This unique ability is due to their specialized protein-degradation and antigen-processing machineries, although the details of each process are still not fully understood. The selection of CD8 T cells is critically dependent on the presentation of selecting MHC I-associated self-peptides produced by a distinct form of the proteasome in cTECs. The thymoproteasome incorporates the $\beta 5t$ subunit (encoded by *Psm111*) and it is exclusively found in cTECs (Murata et al., 2007). Mice deficient in $\beta 5t$ exhibit a severe block in the development of CD8 T cells and the residual cells that arise display an altered $\alpha\beta$ TCR repertoire as well as being functionally compromised (Murata et al., 2007; Takada et al., 2015). The precise details of how the thymoproteasome controls CD8 positive selection are still obscure. Two distinct, yet possibly complementary, models have been proposed to explain how these selection events take place: the low-affinity model and the peptide-switch model. The first argues that the thymoproteasome produces low-affinity peptides and the subsequent low-affinity interactions with DP thymocytes promote

their differentiation towards the CD8 lineage (Xing et al., 2013). The peptide-switch model instead proposes that the unique and non-overlapping peptides produced by the thymoproteosome allow for the positive selection of more TCR clones that would otherwise be negatively selected in the medulla by mTECs or DCs (Kincaid et al., 2016). These models are not mutually exclusive and further studies may help elucidate the precise mechanisms that control the MHC I-driven positive selection of CD8 SP thymocytes. On the other hand, the molecular determinants that control the positive selection of SP4 thymocytes have also been described, but the entire process is not completely understood. The generation of MHC-II-associated self-peptides is partially dependent on the TSSP and the lysosomal protease cathepsin L (Nakagawa et al., 1998; Viret et al., 2011). The deficiency in either of these proteases, although with distinct effects, has an impact on the selection of a panel of CD4⁺ SP cells. TSSP appears to be important for the positive selection of particular MHC-II-restricted transgenic TCRs (Glaser et al.). Along the MHC II processing pathway, cTECs include the autophagic-mediated shuttling of cytoplasmatic proteins into the lysosomes as the major route for the generation of selecting peptides (Klein et al., 2014). Newly generated MHC II molecules are paired with an invariant chain (Ii) that promotes the stabilization of the MHC heterodimer (Crotzer & Blum, 2009). These are then incorporated into the endosomal-lysosomal compartments. Here, cathepsin L is a crucial player in the processing of the invariant chain which is degraded into a smaller fragment commonly known as the class II-associated invariant chain peptide or CLIP. The CLIP acts as a guardian, preventing the premature binding of a processed peptide. Whenever a mature MHC II molecule is loaded with a peptide, these are shuttled towards the cell surface and cTECs can screen and positively select a panel of CD4 T cells (Glaser et al.). Subsequent studies have suggested a role for the macroautophagy pathway in shaping the composition of the MHC-II ligandome, as TECs display a high constitutive level of this processing pathway (Nedjic et al., 2008). Autophagy related 5 (Atg5)-deficient mice present a defect in the selection of a restricted array of MHC II-selected TCR specificities (Schuster et al., 2015). Furthermore, the analysis of Clec16a deficient mice, a gene associated with autoimmune manifestations, provided a functional link between autophagy in TEC and T cell selection and reactivity (Schuster et al., 2015). These observations suggest that cTECs might possess distinct yet complementary mechanisms to effectively promote the selection of MHC II-restricted CD4 T cells.

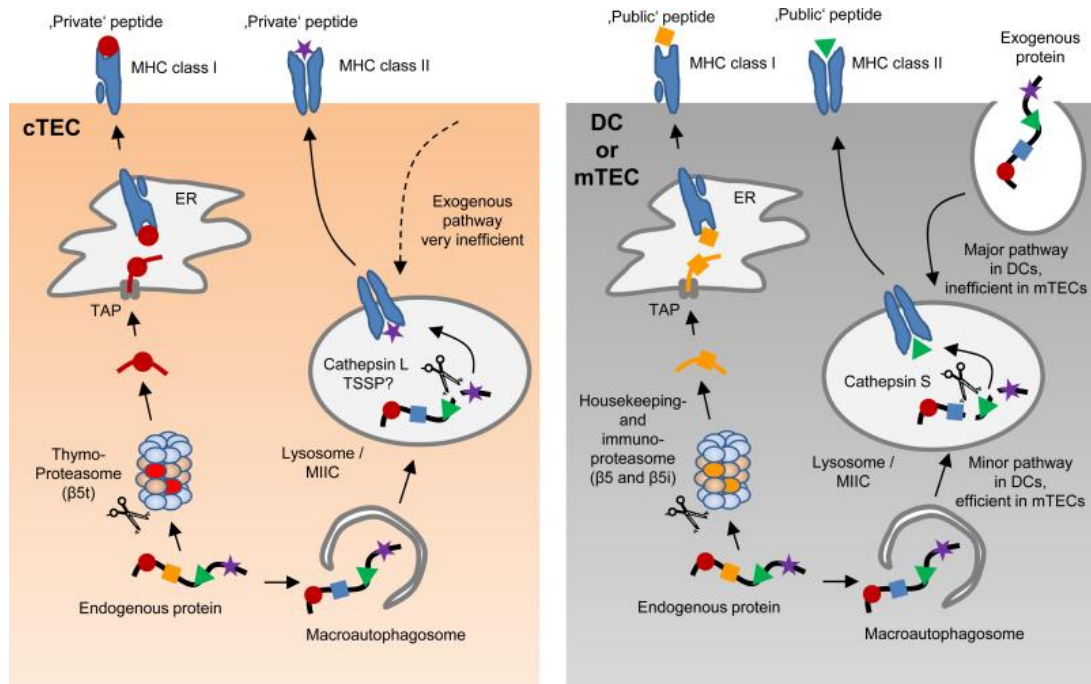


Figure 5- Generation of “private” MHC I- and MHC II-associated peptides by cTECs. While mTECs use conventional antigen processing machinery, cTECs possess specialized protein-degradation and antigen-processing machineries that generate the so-called “private” set of peptides that do not overlap with the ones presented in the medulla by mTECs and DCs. The generation of MHC I-associated peptides is dependent on the presence of the specific form of proteasome exclusively found in cTECs, the thymoproteasome. On the other hand, the generation of MHC II-associated peptides is, at least in part, dependent on the thymus specific serine protease (TSSP) and the lysosomal protease cathepsin L. Adapted from (Klein et al., 2014)

mTECs as the gatekeepers of central tolerance

After being positively selected in the cortical area, thymocytes will encounter the unique microenvironment of the thymic medulla whose main role is to impose central tolerance. mTECs act as a unique peripheral catalogue of antigens as they possess the capacity to display distinct TRAs and screen developing thymocytes in order to eliminate potential clones with auto-reactive $\alpha\beta$ TCRs, a process known as negative selection, or deviate them to T reg differentiation pathway (Glaser et al.). At this stage, these interactions between mTECs (as well as with thymic DCs) and thymocytes are crucial, as this is an opportunity to expose them to antigens that would be otherwise exclusively limited to peripheral tissues. As previously mentioned, this unique feature of mTECs is known as pGE and it is described to be, at least in part, regulated by Aire and Fezf2. Aire acts as the main regulator of pGE as it has been described to govern the expression of a large fraction of these genes (St-Pierre et al., 2015). Interestingly to note, Aire expression within a precise window of the perinatal period is required for the establishment of tolerance mechanisms that are sustained throughout life (Guerau-de-Arellano et al., 2009). Despite the fact that mTECs were thought to express TRAs in a rather homogenous fashion, each individual mTEC was

demonstrated to actually comprise specific clusters that appear to follow an individual co-expression pattern, much like a mTEC-individual mosaic (Brennecke et al., 2015; Meredith et al., 2015). Furthermore, mTECs contribute to the induction of immune tolerance by promoting the differentiation of T regulatory cells (Tregs). Tregs represent a specialized subset of CD4⁺ T cells that help maintain immune homeostasis and suppress excessive immune responses. These are generated through the diversion of a fraction of self-reactive CD4⁺ SP cells. Apart from its pivotal role in the induction of TRAs, Aire arises as a unique pleiotropic molecule that has been described to control several processes that contribute to mTEC maturation and function, which might include its involvement in the selection of Tregs (Nishikawa et al., 2014; Nomura & Sakaguchi, 2007).

In order to promote T cell selection, mTECs employ distinct yet complementary antigen processing and presentation features from cTECs (Klein et al., 2014). They express the conventional proteolytic pathways to present the proposed “public” MHC-bound peptides to developing thymocytes. To do so, they are believed to make use of the housekeeping- or the immune-proteasomes as well as the use of macroautophagy-mediated endogenous loading pathway (Klein et al., 2014). These non-overlapping antigen processing machineries between cTECs and mTECs might allow mTECs to present distinct peptides from those uniquely presented by cTECs during positive selection.

The basic role of “thymic crosstalk” in TEC differentiation

As the instructive cues provided by TECs are vital for the differentiation of immunocompetent T cells, signals from developing thymocytes are equally required for the establishment of a functional thymic microenvironment (Irla, 2022). The bidirectional relationship referred to as “thymic crosstalk”, although described several years ago (van Ewijk et al., 1994), still lacks the complete resolution in terms of the molecular details that underlie this process. cTECs, as previously mentioned, support the earlier stages of thymopoiesis and, although the initial stages of cTEC differentiation can occur independently of thymocytes, which includes the emergence of K8⁺K5⁺ cells within the thymus (Klug et al., 2002), their further maturation and the establishment of their characteristic reticular network require the presence of DN1-DN3 thymocytes (Klug et al., 1998). Without the signals from these immature thymocytes, there was a lack of a histologically organized thymic architecture as well as a lack of mature cTECs, depicted by the differentiation of a K8⁺K5⁺ precursor subset into a more differentiated K8⁺K5⁻ subset (Glaser et al.). Still, the precise nature of such signals and how these early DN thymocytes influence cTEC development remains largely unknown. In contrast, the signaling pathways

that control the maintenance of mTEC are better understood. Embryonic mTECs development requires signals provided by lymphoid tissue inducer, also known as Lti, and $\gamma\delta$ T cells through the activation of the nuclear factor kappa B (NF- κ B) signaling pathway (Roberts et al., 2012; Rossi, Kim, et al., 2007). Specifically, during the embryonic period, the lymphotoxin β receptor (LT β R) and RANK promote the emergence of mature mTECs (Lopes et al., 2018; Mouri et al., 2011; Rossi, Kim, et al., 2007). Postnatal mTECs continue to expand and instead require interactions with positively selected SP4 thymocytes, wherein they provide both RANK and CD40 ligands. Of note, the interactions between autoreactive SP4 thymocytes and mTECs were revealed to be also critical for the optimal maintenance and architectural organization of the medullary compartment (Irla et al., 2008; Lopes et al., 2022). Hence, the thymus represents a peculiar microenvironment in which both developing thymocytes and differentiating TECs require each other for the correct establishment of thymic function.

In addition to the abovementioned regulators, other molecules have been described to modulate the mTEC developmental program. Specifically, the deletion of the TNF receptor-associated factor 3 (TRAF3) in TECs, which serves as an inhibitor of the non-classical NF- κ B signaling pathway, was shown to be sufficient to allow mTEC development in a thymocyte-independent fashion (Jenkinson et al., 2013). These results indicate that the deletion of this molecule can potentially be used to overcome a defective differentiation of mTECs in a thymocyte-deprived microenvironment. Yet, the activation of this signaling pathway was able to overcome the need for both CD40- and LT β R-mediated signaling pathway, but not for RANK, which interestingly demonstrates distinct requirements within the circuits that control mTEC homeostasis. Furthermore, a complementary study has also provided a role for the histone deacetylase 3 (HDAC3) in the establishment of the mTEC microenvironment (Goldfarb et al., 2016). This deacetylase promotes the activation of the transcriptional program that will promote mTEC lineage specification and differentiation, while repressing cTEC-associated transcription.

Thymic involution and regeneration

Despite its essential role in the production of a self-restricted and tolerant T cell pool, the thymus represents one of the first organs that progressively deteriorates with age, an evolutionary conserved physiological process referred to as "thymic involution" (Shanley et al., 2009). Interestingly, the onset of thymic involution may differ between species. For instance, while the murine thymus starts to decline around the time of sexual maturity, in humans it is described to begin as early as around one year of life (Sempowski et al., 2002;

Steinmann et al., 1985). Thymic atrophy is accompanied by a reduced capacity to support T cell differentiation, which contributes to a higher susceptibility to opportunistic infections, neoplastic transformations or autoimmune disorders. The quantitative and qualitative alterations observed in the peripheral T cell compartment correlates with several defects in the thymic epithelium, including a reduction in TEC cellularity, the loss of cortical and medullary compartmentalization and the expansion of adipose tissue and fibroblasts around the perivascular space (Taub & Longo, 2005). Yet, the molecular mechanisms behind thymic involution remain largely uncharacterized. They appear to be multifactorial and involve both intrinsic and extrinsic age-dependent alterations of the thymic stroma. An increased inflammatory signature has been described within the aged thymic microenvironment (Sempowski et al., 2000). Specifically, thymic involution is aggravated after administration of the inflammatory cytokines IL-1 or IL-6 to mice, with alterations on the thymic epithelium being dose-dependent (Morrissey et al., 1988). Fluctuations in the levels of sex hormones have been also proposed to aggravate the atrophy of the thymus, as the rapid decline in thymic function occurs during puberty (Grossman, 1985). Surgical or pharmacological blockade of sex steroid production reverses thymic atrophy, with the rebuilding of the thymic microenvironment and a concomitant boost in T cell production (Heng et al., 2005; Sutherland et al., 2005). Furthermore, the downregulation of Foxn1 expression appears to be one of the first triggers of thymic involution (O'Neill et al., 2016). The progressive accumulation of Foxn1-negative cells within the cortex and the medulla suggested that the maintenance of Foxn1 expression is critical to sustaining the properties of the thymic microenvironment (Rode et al., 2015). Using mice in which Foxn1 expression was modulated to decrease and reach 30% of its physiological levels, the authors observed a premature thymic atrophy, phenocopying the molecular events that occur at the onset of thymic involution (Chen et al., 2009). Interestingly, these effects were dose-dependent, as cortex-specific downregulation of Foxn1 had a greater impact on the expression of genes involved in cTEC-dependent T cell differentiation, in accordance with their higher levels of Foxn1 expression over mTECs (O'Neill et al., 2016). Ubiquitous deletion of Foxn1 in the postnatal thymus promoted significant thymic atrophy, further highlighting the need for Foxn1 in the homeostasis of both the embryonic and the postnatal thymus (Cheng et al., 2010; Corbeaux et al., 2010). These observations propose that either the onset and/or the progression of thymic involution may be linked to a decline in Foxn1 expression. The use of transgenic mice overexpressing Foxn1 (Zook et al., 2011), along the timely induction of Foxn1 in aged animals has demonstrated the capacity of this transcription factor to attenuate the effects of thymic involution in the thymic microenvironment and its functionality, with increased thymus size and T cell output (Bredenkamp, Nowell, et al., 2014). Restoration of Foxn1 levels also upregulates the expression of Foxn1-dependent

genes, such as *Dll4* and *CCL25* (Bredenkamp, Nowell, et al., 2014), positioning this key transcription factor as an attractive therapeutic target for repairing and restoring thymic function in distinct pathophysiological conditions.

A gain-of-function study has shown that in the absence of Foxn1, TEPs persisted and, once Foxn1 is expressed, these cells maintained the capacity to differentiate to functional TECs (Bleul et al., 2006). This was achieved by rescuing the impaired thymic function in nude mice. Furthermore, the enforced expression of Foxn1 *in vitro* was sufficient to reprogram mouse embryonic fibroblasts into functional TECs (Bredenkamp, Ulyanchenko, et al., 2014). While these observations demonstrated that Foxn1 is required for the differentiation of TEPs into TECs, it proposes that these cells arise independently of this transcription factor and appear to retain the molecular machinery to differentiate toward functional TECs. Collectively, given the vital role of Foxn1 in thymus development and function, understanding the molecular machineries that govern its expression is an urgent priority. The possibility to control thymic function would open new avenues to repair thymic function and potentially reduce comorbidities associated with a defective thymic-derived response.

Aims

The overarching aim of this thesis is to explore the fundamental aspects underlying the molecular mechanisms that govern the induction and maintenance of thymic function. Specifically, in **Chapter II**, we developed a strategy to unravel the molecular determinants that control Foxn1 induction. To do so, we generated a novel mouse model and employed state-of-the-art scRNAseq and multicolor flow cytometry analysis to study early events during TEC development. In **Chapter III**, we investigated the role of the lysosomal-associated membrane protein 2 (LAMP2) in the thymic stroma and its effect on the generation of functionally diverse T cells expressing a broad TCR repertoire. To address this question, we analyzed distinct complementary knockout and reporter mouse models, characterizing them through multicolor flow cytometry analysis, confocal microscopy, next-generation sequencing technology and *in vitro* functional assays.

Understanding thymic function will pave the way to solve one of the lingering challenges in modern immunology: the possibility to modulate thymic function through the functional activation of TECs. The identification of the molecular pathways that control TEC differentiation, along with the comprehension of the mechanisms governing thymic selection, will ultimately deepen our understanding of thymic function. This will in turn bring researchers one step closer to potentially intervening medically in disorders associated with a dysfunctional thymic function.

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Chapter II

Resolving the *in vivo* dynamics of Foxn1 expression in thymic epithelial cells: insights from the analysis of a novel Foxn1^{GFP}:Foxn1^{nu/nu} mouse model

(Manuscript in preparation)

Abstract

The thymus serves as the generational site of T lymphocytes. Herein, thymic epithelial cells (TECs) comprise the predominant component of the thymic stroma in the non-involuting thymus and provide unique microenvironments to support T cell development and tolerance induction. Thus, understanding how TECs develop is of utmost importance not only to comprehend the mechanisms behind the generation of functional T cells but also to clinically intervene in thymic-related disorders. Forkhead box N1 (Foxn1) remains to date a critical master transcription factor with a non-redundant role in TEC differentiation. Mice with a disruptive mutation in the *Foxn1* gene, *Foxn1*^{nu/nu} (nude), display an athymic phenotype. Whereas previous studies suggested the involvement of particular pathways in the regulation of Foxn1 expression, the precise molecular mechanisms that control the induction of Foxn1 *in vivo* remain undetermined. To analyze the mechanisms underlying Foxn1 expression, we generated a novel mice model in which we crossed previously developed Foxn1^{GFP} reporter mice into the Foxn1^{nu/nu} background, referred as Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice. In Foxn1^{GFP} reporter animals, detection of enhanced green fluorescent protein (EGFP) expression phenocopies the dynamic expression of this transcription factor. As previously reported, the majority of TECs from Foxn1^{GFP} reporter mice expressed Foxn1^{GFP} in the embryonic and adult thymus. Similarly, analysis of Foxn1^{GFP} mice into a Rag^{-/-}γc^{-/-} background revealed that Foxn1^{GFP} expression remained stable in the young adult alymphoid thymus, indicating that thymocyte-derived signals are dispensable for the induction and maintenance of *Foxn1* expression. In Foxn1^{nu/nu}:Foxn1^{GFP} mice, the analysis of Foxn1^{GFP} expression allows us to explore the dynamics of *Foxn1* induction in the absence of a Foxn1-dependent program. Notably, we found that the embryonic thymic rudiment of Foxn1^{nu/nu}:Foxn1^{GFP} mice contained primitive EpCAM⁺ TECs that initiated Foxn1^{GFP} expression but subsequently disappeared in the postnatal life. These results suggest that *Foxn1*-expressing cells may either die or lose the capacity to sustain *Foxn1* expression in Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice. A fate-mapping mouse model, Foxn1^{Cre}:ROSA26^{loxP-stop-loxP-EYFP}, crossed into the Foxn1^{nu/nu} background showed that primitive EpCAM⁺ TECs retaining a history of *Foxn1* induction were not lost in Foxn1-deficient conditions, but instead, represent a subset that actively downregulated *Foxn1* expression. Furthermore, single cell transcriptomic analysis of embryonic primitive Foxn1-deficient TECs (EpCAM⁺) from the Foxn1^{nu/nu}:Foxn1^{GFP} thymic rudiment unexpectedly revealed distinct cell clusters with specific patterns of *Foxn1* expression. Further analysis of the genetic program of TEC clusters within the nude thymic rudiment offers a unique platform to identify the molecular mechanisms involved in the control of *Foxn1* expression.

Understanding how TECs initiate and maintain Foxn1 expression would provide the opportunity to intervene in pathologies associated with a dysfunctional thymic function.

Introduction

Within the thymus, thymic epithelial cells (TECs) represent a highly specialized epithelial population that promotes the generation of self-restricted and tolerant T cells. Two major subsets of TECs can be distinguished based on their anatomical localization and functional properties: cortical (c) and (m) medullary TECs (Abramson & Anderson, 2017). While cTECs are responsible for supporting the earlier stages of thymopoiesis, such as T cell lineage commitment and positive selection, mTECs safeguard the later stages of T cell differentiation, namely negative selection and T regulatory cell differentiation. Although cTECs and mTECs segregate into two functionally distinct subsets, they derive from common bipotent TEC progenitors (TEPs) that persist both in the embryonic period and during postnatal life (Kadouri et al., 2020).

The differentiation of TEPs critically depends on the expression of forkhead box N1 (Foxn1) (Romano et al., 2013). The induction of Foxn1 is initiated during the early stages of thymus organogenesis (around E11.5) and it is essential to promote TEC maturation, differentiation and maintenance over time. The critical role of this transcription factor is underscored by the athymia in Foxn1^{nu/nu} (nude) mice caused by a disruptive mutation on the *Foxn1* gene (Schuddekopf et al., 1996). Despite the pivotal acknowledged role of Foxn1 in thymic function, the underlying events that regulate its induction remain to date not entirely understood. Although this topic remains a long-standing question, recent efforts have tried to establish a connection between some regulators such as bone morphogenetic proteins (BMP), wntless-related MMTV integration (Wnt) or the retinoblastoma (Rb) family of proteins and Foxn1 induction (Balciunaite et al., 2002; Garfin et al., 2013; Soza-Ried et al., 2008; Tsai et al., 2003). These proteins were shown to potentially regulate the expression of Foxn1. Yet, their role was mostly assessed by *in vitro* assays and therefore the effective inducers of Foxn1 expression *in vivo* remain unidentified.

Here, we aimed to explore the microenvironment of the thymic rudiment of Foxn1^{nu/nu} mice and unravel the molecular determinants that control the induction of *Foxn1* expression in the absence of a complex Foxn1-mediated downstream transcriptional program. To achieve this goal, we generated a novel mouse model by crossing Foxn1^{nu/nu} mice with previously established Foxn1^{GFP} reporter animals (Foxn1^{nu/nu}:Foxn1^{GFP} mice) (Terszowski et al., 2006). This strategy allowed us to exclude any potential complex array of downstream targets of Foxn1 that could interfere with the identification of its upstream inductive signals.

Understanding the molecular circuits behind Foxn1 induction would resolve one of the most long-standing and appealing questions in the field of thymic biology and function.

Results

Temporal dynamics of Foxn1^{GFP} expression in TECs from embryonic to adult immunocompetent mice

The transcription factor Foxn1 is dynamically regulated during the embryonic and the postnatal life, being essential for both embryonic TEC differentiation and continuous TEC maintenance in the adult thymic microenvironment (O'Neill et al., 2016). The downregulation of Foxn1 is believed to be associated with the initiation of thymic involution and the consequent expansion of a population of Foxn1-negative TECs (O'Neill et al., 2016). In this study, we aimed at understanding the cellular and molecular determinants that control the induction of Foxn1 expression. Terszowski et al. previously generated a Foxn1^{GFP} reporter mice capable of identifying Foxn1-expressing cells through the detection of an enhanced green fluorescent protein (EGFP) (Terszowski et al., 2006). Transgenic Foxn1^{GFP} reporter mice contain a construct wherein the EGFP coding sequence was inserted downstream of a 27970 base pairs-long sequence that is described to contain the regulatory elements (RE) required for Foxn1 expression (Terszowski et al., 2006) (**Supplemental Figure 1A**). Exploring Foxn1^{GFP} reporter mice, we first performed a temporal characterization of the thymus throughout embryonic and postnatal life and confirmed that their total thymic cellularity, TEC numbers and overall cTEC/mTEC profile were comparable to those from controls counterparts (**Supplemental Figure 1B-C**), indicating that the presence of the transgene did not alter normal thymic development. The detection of EGFP was specific, as it did not appear in non-transgenic animals (**Supplemental Figure 1D**). Analysis of the dynamics of Foxn1^{GFP} expression over time showed that a large majority of TECs were labelled in the embryonic period and, as mice aged, there was an accumulation of Foxn1^{GFP} negative cells with a concomitant decrease in the level of the mean fluorescence intensity (MFI) of Foxn1^{GFP} expression with time (**Figure 1A-C**). These Foxn1^{GFP} negative cells may correspond to the described Foxn1-negative cells that arise with age (O'Neill et al., 2016). We further found that Foxn1^{GFP} expression levels vary within TEC subpopulations, with cTECs expressing distinct levels compared with mTECs (**Supplemental Figure 1E**). In accordance with previous study (O'Neill et al., 2016), within mTECs, mTEC^{hi} (MHC II^{hi}, CD80^{hi}) express higher levels of Foxn1^{GFP} than their mTEC^{lo} (MHC II^{lo}, CD80^{lo}) counterparts (**Supplemental Figure 1F**). Collectively, these results, along with previous observations (Nusser et al., 2022;

Terszowski et al., 2006), support the efficacy of this mouse model to phenocopy the expected *in vivo* dynamics of Foxn1 induction and expression in the thymus throughout life.

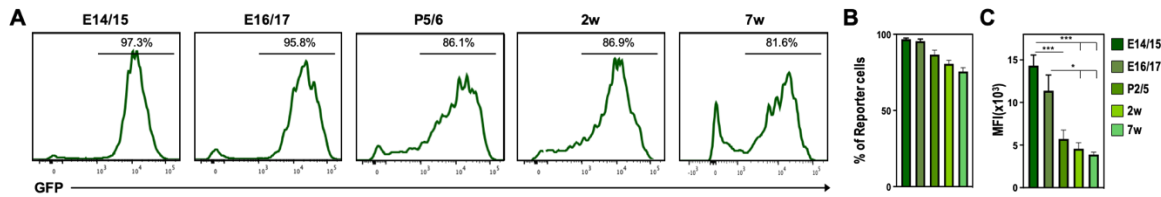


Figure 1. The detection of Foxn1^{GFP} expression in TECs in the embryonic and adult life. (A) Foxn1^{GFP} expression in total TECs (CD45⁺EpCAM⁺) from Foxn1^{GFP} reporter mice at indicated time points: Embryonic days (E)14/15, E16/17, P5/6, 2 weeks old (2w) and 7w old thymus. Data representative of 7 independent experiments (n = 5-11 animals/group). (B) Bars graphs represent the frequency of Foxn1^{GFP}-expressing cells within total TECs (CD45⁺EpCAM⁺) at indicated time points: from left to right bars represent E14/15 (in dark grey), E16/17 (in dark green), P5/6 (in white) and 2 weeks old (2w) (in dark red) thymus. Data representative of 7 independent experiments (n = 5-11 animals/group). (C) Bars graphs represent mean fluorescence intensity (MFI) of Foxn1^{GFP} expression within total TECs (CD45⁺EpCAM⁺) at indicated time points: from left to right bars represent E14/15, E16/17, P5/6, 2w and 7w old thymus. Data representative of 7 independent experiments (n = 5-12 animals/group). Results in B and C are shown as mean ± SEM.

Foxn1^{GFP} expression in TECs is not affected in alymphoid *Rag2^{-/-}Il2rg^{-/-}* thymus

The proper establishment of both cTEC and mTEC functional microenvironments requires signals that are provided by developing thymocytes at distinct stages of differentiation, a symbiotic relationship that is commonly known as thymic crosstalk (Abramson & Anderson, 2017; Rossi et al., 2007). Given the importance of these lympho-epithelial interactions, we next examined whether the absence of thymocyte-inductive signals would impact on Foxn1^{GFP} expression. To address this question, we crossed Foxn1^{GFP} reporter mice with immunodeficient *Rag2^{-/-}Il2rg^{-/-}* animals (referred hereafter as Foxn1^{GFP}:*Rag^{-/-}γc^{-/-}* mice). The deletion of the Rag2 enzyme along with the deficiency in the common IL-2 receptor γ chain renders these mice deficient in T, B, innate lymphoid cells (ILCs) and NK cells. Deficiency in the Rag2 enzyme causes a developmental block in DN2/DN3 stages of thymocyte development, while the absence of the common IL-2 receptor γ chain has an impact in several cytokine-derived signaling pathways, such as the ones encompassing IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Mazurier et al., 1999; Shinkai et al., 1992). Similarly, it affects T cell development in a very early stage, as DN2 thymocytes require IL-7 for their survival. Due to this early block in T cell differentiation, the establishment of the TEC compartment is severely compromised and the thymus becomes a small alymphoid structure (**Figure 2A**). Still, TECs (CD45⁺EpCAM⁺) can be isolated in these mice, comprising mostly cells with a cTEC-like phenotype (**Figure 2A and B**). The absence of thymocytes has a particularly detrimental effect on mTECs. In particular, the growth and maturation of mTECs are critically dependent on signals, such as RANK and

CD40 ligands, provided by distinct hematopoietic cells. These maturation signals promote the activation of the NF- κ B signaling pathway in mTECs and their precursors (Rossi et al., 2007; van Ewijk et al., 1994). In accordance with previous studies (Meireles et al., 2017), we observed that the differentiation of mTECs was markedly compromised in Foxn1^{GFP}:Rag^{-/-} γ c^{-/-} mice (**Figure 2B**). Strikingly, the frequency and expression level of Foxn1^{GFP}-expressing cells within total TECs (CD45⁻EpCAM⁺) from Foxn1^{GFP}:Rag^{-/-} γ c^{-/-} young adult (6-7 weeks of age) mice remained similar to those found in their immunocompetent (**Figure 2C, D and E**). Overall, these results suggest that lymphoepithelial interactions do not influence the induction and maintenance of Foxn1 expression by TECs in the young adult thymus.

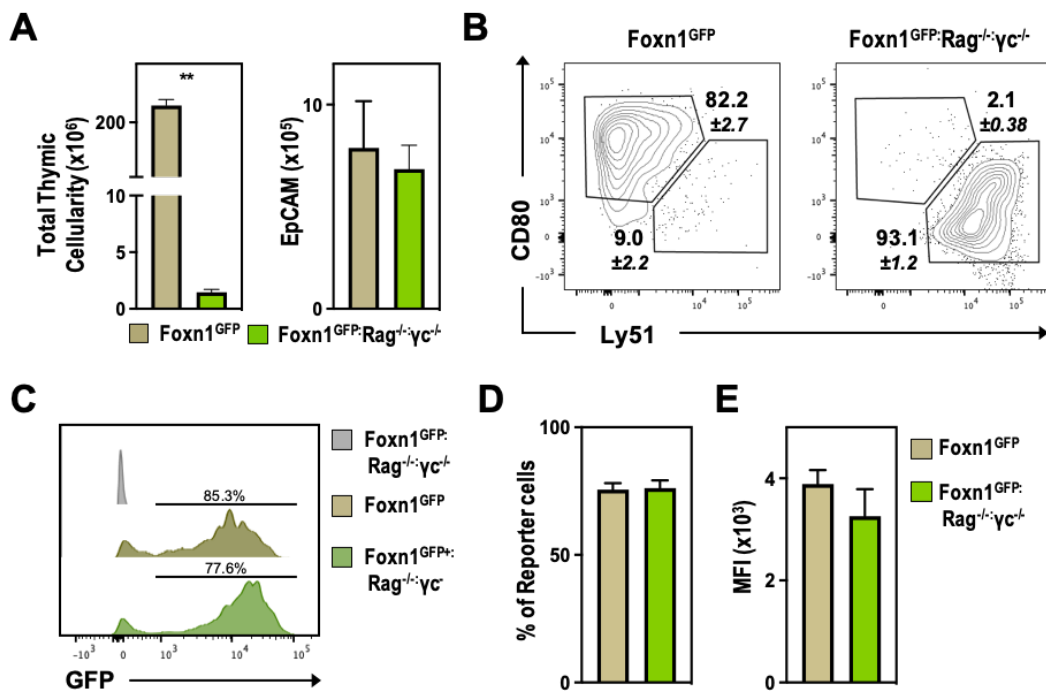


Figure 2. Thymic crosstalk is dispensable for the maintenance of Foxn1^{GFP} expression in TECs. (A) The graphs represent the average total thymic cellularity (left graph) and TEC (CD45⁻ EpCAM⁺) cellularity (right graph) of immunocompetent (Foxn1^{GFP}), in gold and immunodeficient (Foxn1^{GFP}:Rag^{-/-} γ c^{-/-}) Foxn1 reporter mice, in light green, at 6-7 weeks of age from 2 independent experiments (n = 3-8 animals/group). (B) Representative flow cytometric profile of cTEC (CD45⁻ EpCAM⁺ Ly51⁺ CD80⁺) and mTEC (CD45⁻ EpCAM⁺ Ly51⁺ CD80⁻) composition of wild type (Foxn1^{GFP}) and immunodeficient (Foxn1^{GFP}:Rag^{-/-} γ c^{-/-}) Foxn1 reporter mice at 6-7 weeks of age. Dot plots show a representative Ly51 and CD80 profile in the indicated timepoints from 2 independent experiments (n=3-8 animals/group). (C) GFP expression in total TECs (CD45⁻EpCAM⁺) from Foxn1^{GFP}:Rag^{-/-} γ c^{-/-} (in grey), Foxn1^{GFP} (in gold) and Foxn1^{GFP}:Rag^{-/-} γ c^{-/-} reporter mice (in light green) at 6-7 weeks old thymus. Data representative of 5 independent experiments (n= 3-8 animals/group). (D) Bars graphs represent the frequency of GFP-expressing cells within total TECs (CD45⁻EpCAM⁺) from Foxn1^{GFP} (in gold) and Foxn1^{GFP}:Rag^{-/-} γ c^{-/-} reporter mice (in light green) at 6-7 weeks old thymus (E) Bars graphs represent the mean fluorescence intensity (MFI) of GFP-expressing cells within total TECs (CD45⁻EpCAM⁺) from Foxn1^{GFP} (in gold) and Foxn1^{GFP}:Rag^{-/-} γ c^{-/-} reporter mice at 6-7 weeks old thymus. Data representative of 4 independent experiments (n = 3-5 animals/group). Results in A, D and E are shown as mean $\pm$ SEM. ** p < 0.01.

The induction of Foxn1^{GFP} expression is initiated but is not sustained under Foxn1-deficient conditions

Despite the pivotal role of Foxn1 in TEC differentiation and function, the identification of the precise molecular machinery that controls the induction and maintenance of its expression *in vivo* remains challenging to study. This hurdle is, in part, due to the complex cascade of signaling pathways that Foxn1 induces once it is expressed in TECs, including its direct targets and their downstream products (Zuklys et al., 2016). These comprise not only genes that are necessary for TEC differentiation but also gene sets that support their function in T cell development. The very early events of murine thymus organogenesis seem to occur independently of Foxn1 and as such, Foxn1^{nu/nu} (known as “nude”) mice retain a small, cystic thymic rudimental structure, which was curiously observed several decades ago (Cordier & Haumont, 1980). The thymic rudiment of nude mice contains epithelial cells that are considered to represent the earliest TEC progenitor cells (pro-TECs) that remained blocked in their differentiation program, due to the absence of Foxn1 (Blackburn et al., 1996; Bleul et al., 2006). We will refer to these cells as primitive or pro-TECs.

To study the upstream molecular mechanisms that control the induction and maintenance of Foxn1 expression during thymic organogenesis, in the absence of Foxn1 protein expression and without the confounding effects of the molecular pathways induced by Foxn1 itself, we developed a novel mouse model by crossing Foxn1^{GFP} mice into the Foxn1^{nu/nu} nude background (**Supplemental Figure 2A**). In Foxn1^{nu/nu}:Foxn1^{GFP} mice, the expression of Foxn1^{GFP} is marking cells that are actively capable of activating the Foxn1 promoter in the transgene but remain blocked in their differentiation. We isolated the thymic rudiments from Foxn1^{nu/nu}:Foxn1^{GFP} mice and analyzed them at distinct stages of embryonic and postnatal periods. In agreement with previous studies (Balciunaite et al., 2002; Blackburn et al., 1996), the introduction of homozygous Foxn1^{nu/nu} mutation severely blocked both thymus and TEC development (**Supplemental Figure 2B and C**). The cellularity of total thymic and pro-TECs (EpCAM⁺) was markedly reduced compared to Foxn1^{nu/wt} and Foxn1^{wt/wt} (**Supplemental Figure 2B and C**). Although there is a dose-effect of Foxn1 at some stages, the analysis of Foxn1^{nu/wt} mice revealed no relevant alterations on their cTEC/mTEC composition, as previously reported (**Supplemental Figure 2B-D**) (Chen et al., 2009; Scheiff et al., 1978). Strikingly, we found Foxn1^{nu/nu}:Foxn1^{GFP+} TECs within the thymic rudiments of nude both at embryonic and early postnatal periods, showing a frequency comparable that found in mature TECs from immunocompetent Foxn1^{GFP} counterparts. Notably, the proportion of Foxn1^{nu/nu}:Foxn1^{GFP+} TECs and the level of

$Foxn1^{GFP}$ expression decreased over time, in contrast with $Foxn1^{GFP}$ and $Foxn1^{nu/wt}:Foxn1^{GFP}$ reporter models (Figure 3A-C). These observations suggest that $Foxn1^{nu/nu}$ thymic rudiments contain cells that retain the transcriptional machinery necessary for the activation of the *Foxn1* promoter and the initiation of *Foxn1* transcription.

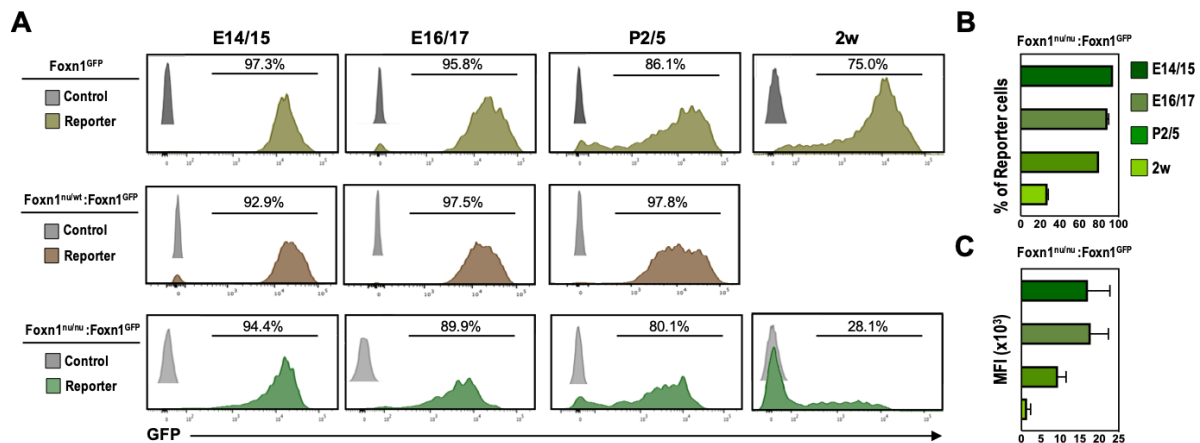


Figure 3. Generation of a novel mice model to identify the molecular determinants of Foxn1 induction. (A) Representative GFP expression (by flow cytometry) in EpCAM-expressing cells (CD45⁺EpCAM⁺) from $Foxn1^{GFP}$ reporter mice (control in dark grey and reporter in gold), $Foxn1^{nu/wt}:Foxn1^{GFP}$ reporter mice (control in grey and reporter in brown) and $Foxn1^{nu/nu}:Foxn1^{GFP}$ reporter mice (control in light grey and reporter in dark green) at embryonic days 14/15 (E14/15), E16/17, postnatal day 2/5 (P2/5) and 2 weeks (2w) of age. Data representative of 12 independent experiments (n = 1-7 animals/group). (B) Bars graphs represent the frequency of GFP-expressing cells within total TECs (CD45⁺EpCAM⁺) at indicated time points: from top to bottom bars represent E14/15, E16/17, P2/5 and 2 weeks old thymus. Data representative of 4 independent experiments (n = 1-2 animals/group). (C) Bars graphs represent mean fluorescence intensity (MFI) of GFP expression within $Foxn1^{GFP}$ -expressing total TECs (CD45⁺EpCAM⁺) at indicated time points: from top to bottom bars represent E14/15, E16/17, P2/5 and 2 weeks old thymus (in dark red). Data representative of 4 independent experiments (n = 1-2 animals/group). Results in B and C are shown as mean ± SEM.

A lineage tracing model reveals that a fraction of pro-TECs that previously expressed *Foxn1* is sustained within the $Foxn1^{nu/nu}$ thymic microenvironment

Our previous observations demonstrated that $Foxn1^{nu/nu}:Foxn1^{GFP}$ TECs started to gradually disappear around 2 weeks of age. The loss of these cells within the thymic rudiment of $Foxn1^{nu/nu}$ mice could be attributed to several non-mutually exclusive possibilities. $Foxn1^{nu/nu}:Foxn1^{GFP}$ TECs might disappear due to (1) inability to survive under *Foxn1*-deficient conditions, (2) becoming cell-autonomously unresponsive to extrinsic signals that drive *Foxn1* expression, (3) lacking the cell-extrinsic signals required for *Foxn1* expression in the postnatal life, or (4) lacking *Foxn1* itself, a factor that has been suggested to maintain its own expression in an auto-regulatory loop (Larsen et al., 2019).

To address these possibilities, we crossed Foxn1^{nu/nu} mice with Foxn1^{Cre}:Rosa26^{loxP-stop-loxP-EYFP} animals (referred hereafter as Foxn1^{nu/nu}:Foxn1^{Cre}:R26^{EYFP}) (**Figure 4A**). In this fate-mapping model, the expression of the enhanced yellow fluorescence protein (EYFP) is under the control of the ubiquitous ROSA26 promoter, but flanked by loxP-stop-loxP sites that, due to the transcriptional stop sequences, prevent the constitutive expression of EYFP (Srinivas et al., 2001). Once R26^{stop-EYFP} mice were crossed to Foxn1^{Cre} transgenic mice, loxP-flanked stop sequences (LoxP sites) will be excised in cells expressing Foxn1. As a result, these cells will permanently express EYFP. Hence, EYFP expression will label cells that are either actively able to activate the transgenic *Foxn1* promoter and express Cre or that had activated the promoter at a given period of time during their development. Previous lineage tracing studies showed that almost all TECs from the adult thymus derived from *Foxn1*-expressing progenitors (Corbeaux et al., 2010; Srinivas et al., 2001). These results suggest that Foxn1-negative TECs described in the literature to arise with age possibly derive from previous *Foxn1*-expressing cells. We analyzed Foxn1^{nu/wt}:Foxn1^{Cre}:R26^{EYFP} along with Foxn1^{nu/nu}:Foxn1^{Cre}:R26^{EYFP} and their respective Foxn1^{nu/wt}:R26^{EYFP} and Foxn1^{nu/nu}:R26^{EYFP} controls and observed that the introduction of the Foxn1^{nu/nu} mutation, as expected, disrupted TEC differentiation (**Figure 4B**). Moreover, the expression of EYFP labeled virtually all Foxn1^{nu/wt} TECs (**Figure 4C**). Interestingly, when we analyzed the dynamics of Foxn1^{Cre}-mediated R26-driven EYFP expression within the thymic rudiments from Foxn1^{nu/nu}:Foxn1^{Cre}:R26^{EYFP} mice, we found that a large fraction of the pro-TEC compartment was maintained in the early postnatal and adult life (**Figure 4C and D**). These results contrasted with what we observed in Foxn1^{nu/nu}:Foxn1^{GFP} mice (**Figure 3A and B**), and indicated that pro-TECs do not completely disappear in Foxn1^{nu/nu}, but instead, they are not able of maintaining the activation of transgenic *Foxn1* promoter in a Foxn1-deficient setting.

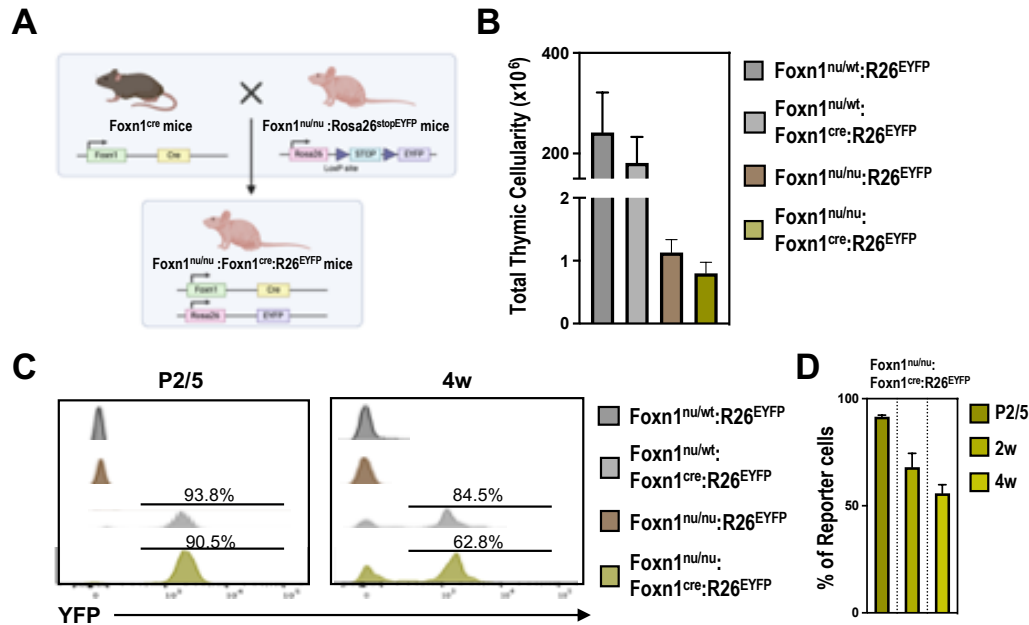


Figure 4. Fate-mapping *in vivo* analysis demonstrate that pro-TECs are sustained in a *Foxn1*-deficient setting. (A) *Foxn1^{Cre}* mice, wherein the Cre enzyme is expressed under the control of the *Foxn1* promoter, was crossed to *Rosa26^{loxP-stop-loxP-EYFP}* reporter in which an EYFP is flanked by a LoxP-STOP-LoxP sequence (LoxP sites) and inserted into the *Rosa26* locus. In animals expressing both constructs (*Foxn1^{Cre}; Rosa26^{loxP-stop-loxP-EYFP}*), Cre specifically activates the reporter in cells that express the promoter by excising the STOP sequence. These were then crossed onto the *Foxn1^{nu/nu}* background, to generate *Foxn1^{nu/nu}; Foxn1^{Cre}; Rosa26^{EYFP}* mice. (B) Graphs represent the average total thymic cellularity in *Foxn1^{nu/nu}; Rosa26^{EYFP}* (in dark grey), *Foxn1^{nu/nu}; Foxn1^{Cre}; Rosa26^{EYFP}* (in light grey), *Foxn1^{nu/nu}; Rosa26^{EYFP}* (in brown) and *Foxn1^{nu/nu}; Foxn1^{Cre}; Rosa26^{EYFP}* reporter mice (in yellow) at 2 weeks old thymus, from 5 independent experiments (n = 2–9 animals/group). (C) Plot represents YFP expression (by flow cytometry) in EpCAM-expressing cells (CD45⁺EpCAM⁺) from *Foxn1^{nu/nu}; Rosa26^{EYFP}* (in dark grey), *Foxn1^{nu/nu}; Foxn1^{Cre}; Rosa26^{EYFP}* (in light grey), *Foxn1^{nu/nu}; Rosa26^{EYFP}* (in brown) and *Foxn1^{nu/nu}; Foxn1^{Cre}; Rosa26^{EYFP}* reporter mice (in yellow) at indicated time points: Postnatal days (P)2/5 and 4 weeks (4w) of age. Data representative of 8 independent experiments (n= 2-9 animals/group). (D) Bars graphs represent frequency of EYFP-expressing cells within total TECs (CD45⁺EpCAM⁺) at indicated time points: from left to right bars represent P2/5, 2 weeks old and 4 weeks old thymus. Data representative of 14 independent experiments (n = 4-7 animals/group). Results in B and D are shown as mean ± SEM.

Single-cell RNA sequencing analysis reveals an unexpected complexity within the pro-TEC compartment of *Foxn1^{nu/nu}* mice

Our data indicated that a fraction of pro-TECs from *Foxn1^{nu/nu}; Foxn1^{GFP}* mice developed early during embryonic periods, were maintained during perinatal stage, and declined during early postnatal life. To study the heterogeneity of pro-TECs within *Foxn1^{nu/nu}; Foxn1^{GFP}* thymic rudiment, we performed an initial single-cell RNA sequencing (scRNAseq) analysis of E16-derived *Foxn1^{GFP}*⁻ and *Foxn1^{GFP}*⁺ pro-TECs (EPCAM⁺) from *Foxn1^{nu/nu}; Foxn1^{GFP}* mice, along with both cTECs and mTECs from aged-matched *Foxn1^{nu/nu}; Foxn1^{GFP}* animals, that were used as internal controls for source of *Foxn1*-expressing subsets (Figure 5A). This particular timepoint was chosen due to the comparable levels of *Foxn1^{GFP}* expression between *Foxn1^{nu/nu}* and *Foxn1^{GFP}* (as shown in

Figure 3A). We reason that the analysis at the embryonic stage will offer a fundamental temporal window to study the early transcriptomic events that arise in Foxn1^{nu/nu}:Foxn1^{GFP+} cells and may control the initiation and maintenance of Foxn1 expression.

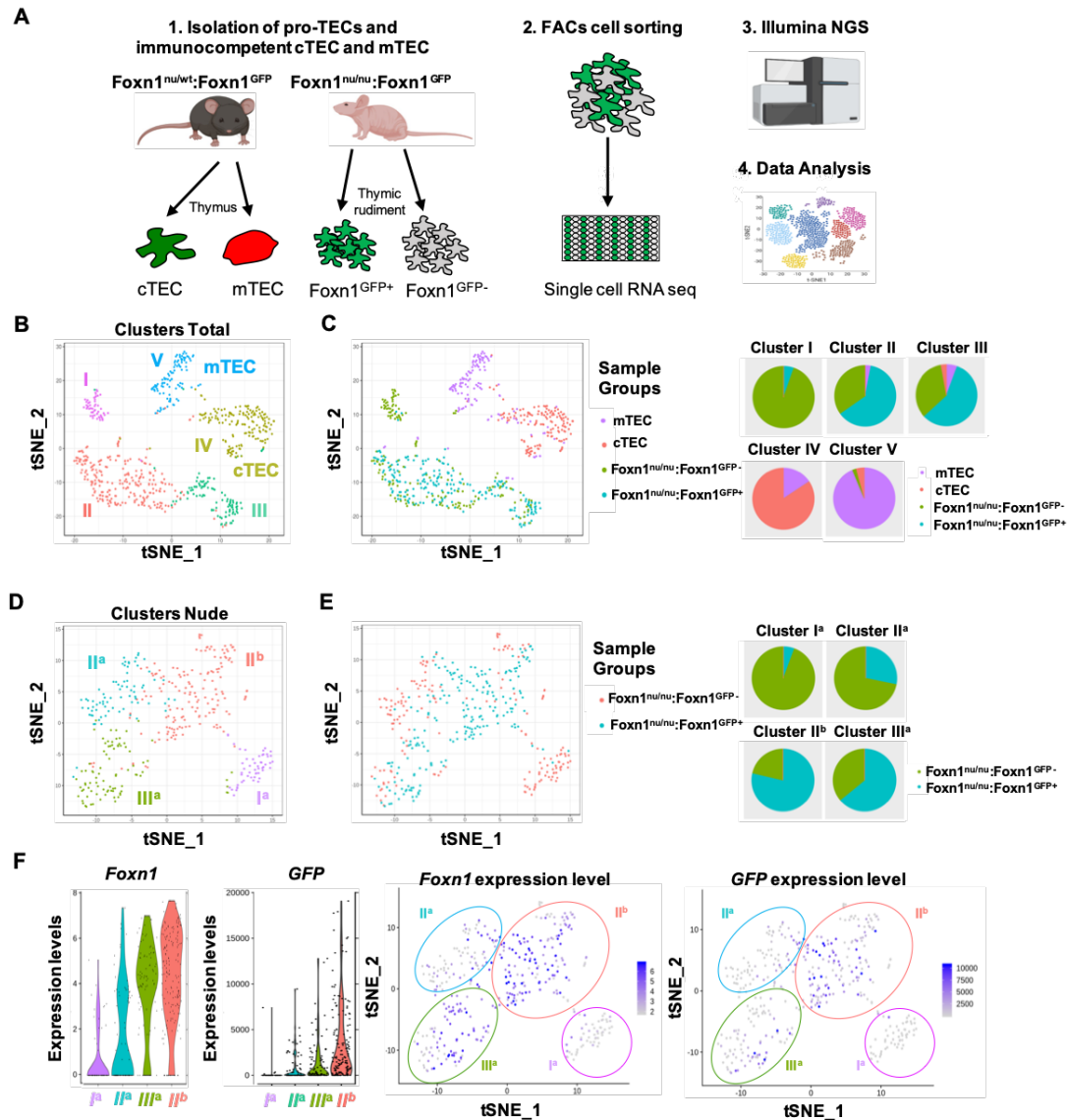


Figure 5. Single cell RNA sequencing of nude cells that reside within the thymic rudiment shows unappreciated heterogeneity. (A) CD45⁺ EpCAM⁺ Foxn1^{nu/nu}:Foxn1^{GFP+} and Foxn1^{nu/nu}:Foxn1^{GFP-}, Foxn1^{wt}:Foxn1^{GFP+} Ly51⁺ CD80⁻ cTECs and Foxn1^{wt}:Foxn1^{GFP+} Ly51⁻ CD80⁺ mTECs were isolated from mice at embryonic day (E)16 and analyzed by single cell RNA sequencing (using Smart-Seq2 protocol). (B-C) Two-dimensional representation of cells via t-distributed Stochastic Neighbor Embedding (tSNE). Colored graphs highlight either cluster identity (B) or distinct TEC subpopulations (C). Each dot represents one cell. Pie graphs represent the distribution of the different subpopulations across the total clusters. (D-E) Cells isolated from Foxn1^{nu/nu}:Foxn1^{GFP} mice were exclusively represented via tSNE. Graphs highlight either cluster identity (D) or the distribution of GFP⁺ or GFP⁻ cells (E). Each dot represents one cell. Pie graphs represent the distribution of the different subpopulations across the nude clusters. (F) The transcript levels of Foxn1 and GFP were analyzed across Foxn1^{nu/nu} cells in each cluster.

First, we applied an unsupervised analysis using t-distributed stochastic neighbor embedding (t-SNE) as a tool that allows cells that share a similar transcriptional profile to be clustered together. Overall, TECs (EpCAM⁺) from Foxn1^{nu/nu} and Foxn1^{nu/wt} samples grouped into five distinct clusters (referred to as I-V) (**Figure 5B**). We found that pro-TECs derived from Foxn1^{nu/nu}:Foxn1^{GFP} mice segregated from Foxn1^{nu/wt}-derived cTECs and mTECs, indicating that these cells display distinct transcriptional programs (**Figure 5B and C**). As expected, cTECs (cluster IV) and mTECs (cluster V) formed two distinct clusters as these represent established mature subpopulations. Strikingly, we found that Foxn1^{nu/nu}:Foxn1^{GFP}-derived cells showed unexpected heterogeneity, with cells grouping into three separate clusters (referred to as clusters I, II and III), each with different proportions of Foxn1^{GFP}⁻ and Foxn1^{GFP}⁺ cells (**Figure 5B and C**). Next, we performed a similar approach wherein we excluded mature Foxn1^{nu/wt}:Foxn1^{GFP}-cTECs and mTECs from the unsupervised analysis and surprisingly found a further degree of heterogeneity within Foxn1^{nu/nu}:Foxn1^{GFP} pro-TECs (**Figure 5D and E**). These cells were further subdivided into four distinct clusters, with cluster II now segregating into clusters II^a and II^b (referred to as I^a, II^a, II^b and III^a). These observations suggest the presence of an unrecognized heterogeneity within the pro-TEC compartment of the nude thymic rudiment.

Although under Foxn1-deficient conditions a functional protein is not produced, TECs from Foxn1^{nu/nu} mice still initiate *Foxn1* transcription. Hence, we sought to compare whether Foxn1^{GFP} expression correlated with the detection of *Foxn1* transcripts within each cluster from Foxn1^{nu/nu}:Foxn1^{GFP}-derived pro-TECs. Notably, we found an association between the levels of *GFP* and *Foxn1* transcripts in each cluster (**Figure 5F**). While clusters I^a and II^a co-expressed low levels of *Foxn1* and *GFP* transcripts, clusters III^a and II^b represented the subsets with the highest levels of both *Foxn1* and *GFP* transcripts. The correlation found between the levels of *GFP* and *Foxn1* transcripts reinforces the notion that Foxn1^{GFP} expression can be used as a proxy to survey Foxn1 expression, and further supports that pro-TECs within Foxn1^{nu/nu}:Foxn1^{GFP} mice remain capable of activating the endogenous *Foxn1* promoter and transcribe the *Foxn1* gene. Together, these results suggested that the Foxn1^{nu/nu} thymic rudiment might contain distinct subpopulations and it validates the usefulness of our generated mouse model to survey the induction of *Foxn1*.

Discussion

The transcription factor Foxn1 represents one of the most significant regulators of thymic function. This gene appears to have arisen from its paralog Foxn4 due to gene duplication as result of the evolutionary pressure to establish a dedicated niche for the specific differentiation of T cells (Bajoghli et al., 2009). Yet, despite the acknowledgment of Foxn1

as a pivotal driver of TEC differentiation, little advances have been made regarding the signaling pathways that drive its expression *in vivo*. While Foxn1 downstream targets have been identified and partially characterized, the complex signaling pathways induced by Foxn1 once expressed have precluded the precise identification of its upstream regulators (Vaidya et al., 2016). Some studies have proposed the role of several molecules in the modulation of Foxn1 expression. As an example, recent studies revealed that increased activity of the members of the E2F family of transcription factors E2F3 and E2F4 correlated with the augmented expression of Foxn1 in TECs. The expression of these E2Fs is constrained by the Rb family of proteins that, therefore, appear to negatively regulate Foxn1 expression (Garfin et al., 2013). Furthermore, T box transcription factor 1 (Tbx1) might also repress the transcription of Foxn1 and consequent TEC differentiation, if ectopically expressed at the early thymic domain during organogenesis (Reeh et al., 2014). Yet, it is still not entirely clear whether these molecules affect the induction of Foxn1 directly or indirectly. Furthermore, BMP4 and Wnt proteins were proposed to promote the upregulation of Foxn1, although the mechanisms remain undetermined. Additionally, these assays were mostly conducted *in vitro*, therefore lacking further *in vivo* physiological confirmation (Balciunaite et al., 2002; Soza-Ried et al., 2008; Tsai et al., 2003). To further contribute to this conundrum, Foxn1 has been suggested to regulate its own expression, through a positive feedback loop (Zook et al., 2011). As a result, the characterization of this molecule within the thymic microenvironment has been extremely complex as, once expressed, it is involved in the activation of several pathways and exerts a myriad of functions.

In this chapter, we intended to establish a model that aids the identification of potential upstream regulators of Foxn1 expression. To do so, we made use of Foxn1^{GFP} reporter mice as a means to closely monitor the transcription of Foxn1. We validated the usefulness of this mouse model, as it was able to recapitulate the expected dynamics of Foxn1 expression throughout life (O'Neill et al., 2016; Terszowski et al., 2006). We and others have analyzed Foxn1 reporter animals and found that the levels of Foxn1 vary within each TEC subpopulation (O'Neill et al., 2016). Although the levels of Foxn1^{GFP} expression between cTECs and mTECs were distinct (**Supplemental Figure 1E**), these did not completely overlap with what was previously described with a different Foxn1 reporter mouse model (O'Neill et al., 2016). These discrepancies might be correlated with the distinct approaches conducted in the design of the mouse model itself. Nonetheless, we have found that within the mTEC compartment, mTEC^{hi} expressed higher Foxn1^{GFP} levels than their mTEC^{lo} counterparts, which was in line with previous observations (O'Neill et al., 2016). Moreover, we observed that thymic crosstalk is not required for the induction of Foxn1 in the young adult thymus, which might be expected as Foxn1-inductive mechanisms take place before

developing thymocytes even start colonizing the thymus. Nonetheless, it is still possible that these signals might have a role in the maintenance of *Foxn1* expression across life. Conversely, the presence of the N-terminal domain of the *Foxn1* protein seems to be required for thymocyte-dependent TEC differentiation (Su et al., 2003). The authors describe that, in the absence of the N-terminal domain of *Foxn1*, TECs are arrested in an immature phenotype that might arise from the inexistence of thymocyte-derived signals. These results, nonetheless, may be explained by the fact that an intact *Foxn1* protein is required to exert its role in promoting TEC differentiation and function, which in turn impacts T cell development and indirectly affects thymic crosstalk. As *Foxn1* expression *per se* is a pre-requisite for TEC differentiation, our results suggest that thymic crosstalk is a downstream event with no apparent effect on induction and maintenance *Foxn1* expression in the adult thymus.

We have generated a novel mouse model that allows us to monitor *Foxn1* induction by crossing *Foxn1*^{GFP} reporter mice into *Foxn1*^{nu/nu} background and found that they contained a fraction of *Foxn1*^{GFP}-reporting cells. These results demonstrate that *Foxn1*-deficient pro-TECs contain the specific transcriptional machinery that is required to activate the expression of *Foxn1* through the engagement of the *Foxn1* promoter. Important to note, that the analysis of *Foxn1*^{nu/wt} mice revealed a dose-dependent effect of *Foxn1*, which has been previously reported (Chen et al., 2009; Scheiff et al., 1978). Although we did not observe major alterations in their overall cTEC/mTEC profile, there was a moderate reduction in the overall total thymic and TEC cellularity across distinct timepoints analyzed. Nonetheless, there were no striking alterations in the frequency of *Foxn1*^{GFP} cells across time.

Our lineage-tracing model has revealed that at least a fraction of cells with a history of engaging *Foxn1* promoter persists in the adult thymic rudiment of *Foxn1*^{nu/nu} mice, indicating an active loss in the capacity to sustain the activity the *Foxn1* promoter. Future studies should try to elucidate the precise mechanisms behind the loss in their capacity to activate the *Foxn1* promoter and respond to *Foxn1*-inductive external signals. Furthermore, the analysis of dual reporter mice, wherein we would be capable of dissecting simultaneously both *Foxn1*^{GFP} and *Foxn1*R26^{YFP} expression (*Foxn1*^{nu/nu}: *Foxn1*^{GFP};*Foxn1*^{Cre}:R26^{EYFP}, resulting from the crossing of *Foxn1*^{nu/nu}:*Foxn1*^{GFP} mice with *Foxn1*^{Cre}:ROSA26^{loxP-stop-loxP-EYFP} animals), would unequivocally allow for the identification of cells actively reporting *Foxn1*^{GFP+} *Foxn1*R26^{EYFP+} and the identification of potential subpopulations of pro-TECs that either shared history of *Foxn1* expression (*Foxn1*^{GFP+} *Foxn1*R26^{EYFP+}) or instead might represent a *Foxn1*-independent lineage (*Foxn1*^{GFP-} *Foxn1*R26^{EYFP-}).

Single-cell transcriptomics has arisen as a useful method to study the transcriptional profile at the individual level and unravel novel cellular heterogeneity within otherwise poorly characterized tissues (Adil et al., 2021). Moreover, it is particularly useful to study the transcriptomic dynamics of rare populations of cells such as those within the nude thymic rudiment. Strikingly, our pilot single cell RNA sequencing analysis revealed that the embryonic thymic rudiment of nude mice contains clusters of pro-TECs that possess distinct transcriptomic profiles. Such heterogeneity has never been appreciated elsewhere before. Furthermore, the variations on the levels of *Foxn1* transcripts might suggest that cells within clusters I-II-II share a possible precursor-product lineage relationship, with immature pro-TECs displaying first lower levels of *Foxn1* transcripts and then acquiring higher levels of these transcripts, although these cells never fully differentiate into mature TECs. Hence, while it is believed that *Foxn1*-deficient cells are arrested in a rather homogeneous progenitor-like state (Nehls et al., 1996), these cells appear to form distinct and yet uncategorized subpopulations, at least to what regards their capacity to drive and sustain *Foxn1* expression. The definition of cluster-specific transcriptional signatures will allow us to potentially identify a panel of unique cell-surface markers that would permit for the isolation and characterization of distinct subpopulations within the *Foxn1*^{nu/nu} thymus. Our data is being further subjected to bioinformatic analysis in order to define the differentially expressed genes (Kumar et al.) between the different subpopulations. The list of DEG between *Foxn1*^{GFP}-reporting cells and the remaining non-reporting pro-TECs represents a particularly useful tool to identify genes with a potential role in *Foxn1* induction. Furthermore, transmission electron microscopy studies have revealed that the thymic rudiment of *Foxn1*^{nu/nu} mice does not contain the typical organized epithelial layer that is commonly associated with the thymus but instead, contains cystic structures with undifferentiated cells (Cordier, 1974). Hence, a more in-depth histological analysis of the thymus rudiments of our mouse models would represent a valuable tool to further study the spatial localization of *Foxn1* reporting cells within the nude thymic microenvironment. It would be also interesting to complement our pilot transcriptomic analysis that was conducted at E16 with the analysis of *Foxn1*-deficient pro-TECs at earlier embryonic (around E11) timepoints as it would provide a reliable snapshot of the genetic circuit that immediately precede the induction of *Foxn1* expression. The complementary analysis should also be performed at later timepoints in life, which would provide valuable insights regarding the genetic network that controls the maintenance of *Foxn1* in TECs.

Our results can also be further submitted to bioinformatic analysis to infer gene ontology (GO) enrichment analyses and ultimately identify their genetic regulatory circuits. Furthermore, pseudo-temporal analysis will offer the opportunity to infer potential

developmental trajectories within the distinct clusters (Song & Li, 2021). Collectively, these analyses may provide a platform to unravel the genetic machinery that arises in pro-TECs during the embryonic period and identify specific regulators of *Foxn1* expression. Recent studies have delimited the critical region that is required for the specific expression of Foxn1 in the thymus (Kadouri et al., 2022; Larsen et al., 2019). The first demonstrated the presence of a 1.6 kb cis regulatory element region within the first intron of Foxn1 that is critical to support thymus development (Larsen et al., 2019). Analysis of mice lacking this region virtually phenocopied the athymia that is observed in nude mice, without compromising hair follicle development. The second study further delimited these regulatory regions within the first intron of Foxn1 and explored the role of some transcription factors on Foxn1 expression, such as Meis1, Six1 and Sox9 (Kadouri et al., 2022). Specifically, the authors describe a role for Six1 in TEC development at later stages of life using Six1 conditional knockout (cKO) mice. These observations, together with the bioinformatic analysis of DEG specifically enriched in primitive TECs, offers us an unprecedented opportunity to identify potential candidate genes that critically affect Foxn1 expression and consequently, thymic function. These candidate genes can be tested through a series of *in vitro* functional validation studies and their role can be further characterized through *in vivo* studies. We are currently establishing an *in vitro* system that allows us to differentiate mouse embryonic stem cells (mESCs) into TEC progenitors. We aim at generating mESCs derived from Foxn1^{GFP} reporter animals, wherein the detection of Foxn1^{GFP} expression function as a proxy to monitor Foxn1 induction during TEC commitment. As soon as the system is established, we can assess the impact of deleting or overexpressing our candidate genes on Foxn1 induction. Furthermore, once their role is validated *in vitro*, we will then move to physiological *in vivo* studies through the generation of conditional KO animals. Our innovative approach will therefore contribute to elucidating one of the most challenging and still unanswered questions in thymus biology.

Foxn1 represents a remarkable molecule in TEC biology as it is involved in numerous processes, spanning from differentiation to maintenance. The identification of two putative binding sites for Foxn1 itself within the intronic region that was pinpointed as critical for thymic development has raised the possibility that it might regulate its own expression through a positive autoregulatory loop (Bredenkamp et al., 2014). This observation demonstrates that the regulation of Foxn1 expression might be even more complex than previously appreciated. A recent study wherein these two Foxn1 putative binding sites were mutated has proposed that, although this feedback loop appears to be dispensable at later stages of life, it may be required during the embryonic and the neonatal periods (Kadouri et al., 2022). Together, these observations demonstrate that the temporal requirement for the

Foxn1-dependent autoregulatory loop further increases the complexity of the mechanisms behind Foxn1 regulation. Nevertheless, whether the loss of Foxn1 expression over time ensues as a consequence of a faulty self-regulatory loop remains to be determined. It would be interesting to understand whether Foxn1 induction and maintenance either comprise two independent or instead two interdependent processes. Collectively, this chapter demonstrated that our innovative approach offers the possibility to explore the Foxn1^{nu/nu} thymic rudiment as an experimental tool to comprehend the details controlling the induction of Foxn1 expression. The elucidation on the mechanisms that control the induction of this transcription factor in TECs would represent an unprecedented opportunity to clinically restore thymic function.

Material and Methods

Mice

We used Foxn1^{GFP}, Foxn1^{Cre}, *Rag2*^{-/-}/*Il2rg*^{-/-}, ROSA26^{loxP-stop-loxP-EYFP} and Foxn1^{nu/nu} (nude) mice that have been described previously and are all in a C57BL/6 background (Colucci et al., 1999; Soza-Ried et al., 2008; Srinivas et al., 2001; Terszowski et al., 2006). Mice were housed under specific pathogen-free conditions, and all animal experiments were performed in accordance with European guidelines (Directive 2010/63/EU). For fetal studies, the day of the vaginal plug detection was designated as embryonic day (E) 0.5.

TEC isolation

TECs were isolated as described (Meireles et al., 2017; Rodrigues et al., 2017; Rodrigues et al., 2018). Briefly, cells from the thymus were prepared by mechanical disruption and enzymatic digestion using 0.05% trypsin-EDTA (Gibco-Invitrogen), followed by fetal bovine serum (FBS) to stop the reaction. Single-cell suspensions were then stained with a different panel of specific antibodies.

Flow cytometry

Cells were pre-treated with FC block (anti-FCGR3/CD16-FCGR2B/CD32 antibodies TruStain fcX; Biologend, Biolegend,101,320). Cell suspensions were stained as described (Crotzer et al., 2010; Tanaka et al., 2000; Zhou et al., 2005) with PE-conjugated anti-ENPEP/LY51 (clone 6C3; eBioscience, 12–5891-82), PerCPCy5.5-conjugated anti-PTPRC/CD45.2 (clone 104; Biolegend,109,828); APC/eFluor660-conjugated anti-CD80 (clone 16–10A1; eBioscience, 17–0801-82), anti-EPCAM (clone G8.8; Biolegend,118,212); APC-eFluor780-conjugated anti-I-A/I-E (clone M5/114-15-2; eBioscience, 47–5321-82); BV421 conjugated anti-EPCAM (clone G8.8; Biolegend, 118,225). Flow cytometry was performed on a LSRFortessa, with data analyzed on FlowJo software (BD). Cell sorting was performed on a FACS Aria I (BD), with purities >96%.

Single cell RNA sequencing and data analysis

Single cells were sorted into 96-well plates containing lysis buffer (RNeasy kit from Qiagen). Samples were shipped to our collaborators at the European Molecular Biology Laboratory, in Germany. Briefly, for Smart2 Seq protocol, cells are lysed in a lysis buffer

containing free dNTPs and oligo(dT)-tailed oligonucleotides with a universal 5'-anchor sequence. Reverse transcription is performed to produce cDNA and a tagmentation step is used to generate sequencing libraries in a quick and efficient way from the amplified cDNA. Single-cell libraries were sequenced using a NovaSeq 6000 (Illumina). STAR aligner (version STAR_2.6.1d) was used to align the reads back to the GRCh38 reference genome downloaded from ENSEMBL. STAR aligner (version STAR_2.6.1d) was also used to generate the gene counts with the GRCh38 gtf. Cells with 2000–9000 detected genes and expressing <15% mitochondrial genes were retained. Counts were log transformed and total counts per cell were normalized. The JackStrawPlot function was used to compare the distribution of p-values for each PC with a uniform distribution. Significant PCs will show a strong enrichment of genes with low p-values. Clustering results were visualized using tSNE and UMAP.

Statistical analysis

Analysis was performed using Prism 9.1.1 software (GraphPad Software). The two-tailed Mann-Whitney test was used for statistical differences between groups. For multiple comparisons, a two-way ANOVA was used. $p < 0.05$ was considered significant.

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Supplemental Information

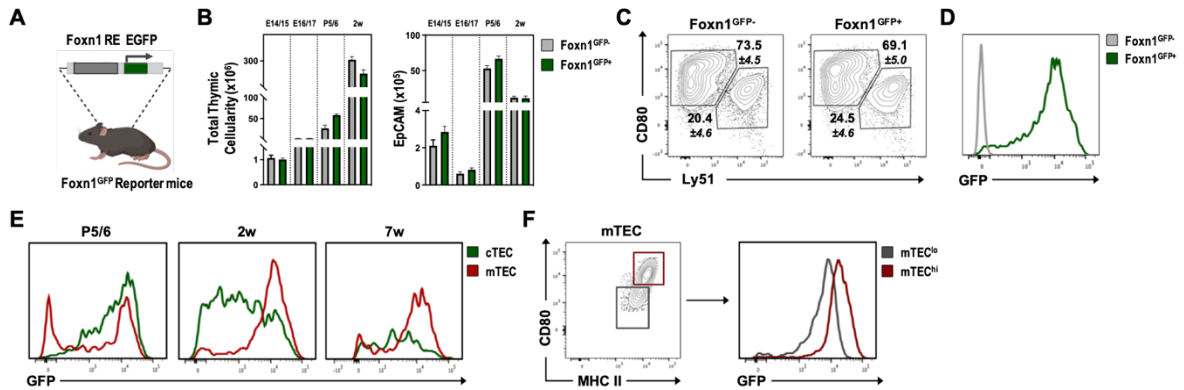


Figure S1. GFP expression within the thymus of Foxn1^{GFP} reporter mice. (A) Schematic representation of Foxn1^{GFP} reporter mice. In these animals, the necessary Foxn1 regulatory elements (RE) drive the expression of the enhanced green fluorescent protein (EGFP). (B) Graphs represent the average total thymic cellularity (left graph) and TEC (CD45⁻ EpCAM⁺) cellularity (right graph) in Foxn1^{GFP} (in grey) and Foxn1^{GFP} reporter mice (in green) at distinct timepoints: from left to right bars represent embryonic days 14/15 (E14/15), E16/17, postnatal days 5/6 (P5/6), 2 weeks old (2w) and 7w, from 7 independent experiments (n = 5–12 animals/group). (C) Flow cytometric profile of cTEC (CD45⁻EpCAM⁺ Ly51⁻ CD80⁻) and mTEC (CD45⁻EpCAM⁺ Ly51⁺ CD80⁺) composition in 2 weeks old Foxn1^{GFP} and Foxn1^{GFP} reporter thymus. Dot plots show a representative Ly51 and CD80 staining from 2 independent experiments (n = 5–6 animals/group). (D) Representative GFP expression (by flow cytometry) in Foxn1^{GFP} (in grey) and Foxn1^{GFP} reporter mice (in green) at 2w of age. Data representative of 2 independent experiments (n= 5–6 animals/group). (E) Representative GFP expression (by flow cytometry) in cTEC (CD45⁻ EpCAM⁺ Ly51⁺ CD80⁺), in green, and mTEC (CD45⁻ EpCAM⁺ Ly51⁻ CD80⁻), in red, from Foxn1^{GFP} reporter mice at postnatal day 2/5 (P2/5), 2 weeks (2w) and 7w of age. Data representative of 12 independent experiments (n= 1–7 animals/group). (F) Representative flow cytometric profile of mTEC^{lo} (CD45⁻ EpCAM⁺ Ly51⁻ CD80^{lo} MHC II^{lo}) and mTEC^{hi} (CD45⁻ EpCAM⁺ Ly51⁻ CD80^{hi} MHC II^{hi}) composition and respective GFP expression at 2 weeks of age. Dot plots show a representative CD80 and MHC II profile from 2 independent experiments (n=5–6 animals/group). Results in B are shown as mean ± SEM.

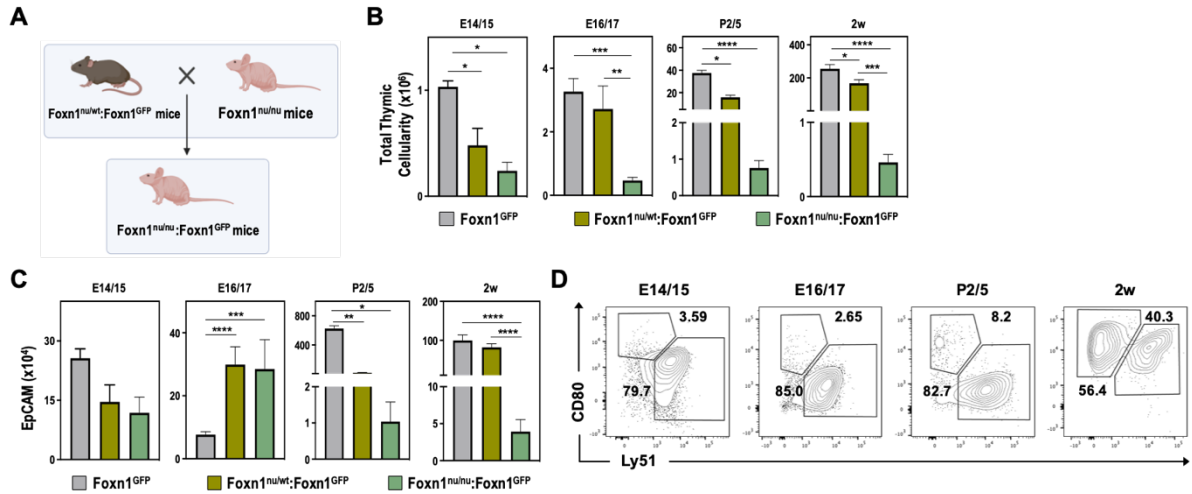


Figure S2. GFP expression within the thymus of Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice. (A) Foxn1^{GFP} reporter mice were crossed to athymic Nude (Foxn1^{nu/nu}) mice to generate a Nude Foxn1 reporter mice (Foxn1^{nu/nu}:Foxn1^{GFP}). (B) Total thymic cellularity of Foxn1^{GFP} reporter mice (in grey), Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice (in dark yellow) and Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice (in dark green) at distinct timepoints: embryonic days 14/15 (E14/15), E16/17, postnatal days 2/5 (P2/5) and 2 weeks old (2w) thymus, from 6 independent experiments (n = 2–18 animals/group). (C) TEC (CD45⁺ EpCAM⁺) cellularity of Foxn1^{GFP} reporter mice (in grey), Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice (in dark yellow) and Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice (in dark green) at distinct timepoints: embryonic days 14/15 (E14/15), E16/17, postnatal days 2/5 (P2/5) and 2 weeks old (2w) thymus, from 6 independent experiments (n = 2–18 animals/group). (D) Representative flow cytometric profile of cTEC (CD45⁺ EpCAM⁺ Ly51⁺ CD80⁺) and mTEC (CD45⁺ EpCAM⁺ Ly51⁻ CD80⁺) composition of Foxn1^{nu/nu}:Foxn1^{GFP} reporter mice at 2 weeks old thymus. Dot plots show a representative Ly51 and CD80 profile in the indicated timepoints from 2 independent experiments (n=12-15 animals/group). Results in B and C are shown as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

Chapter III

LAMP2 regulates autophagy in the thymic epithelium and thymic stroma-dependent CD4 T cell development

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Abstract

Within the thymus, thymic epithelial cells (TECs) provide dedicated thymic stroma microenvironments for T cell development. Because TEC functionality is sensitive to aging and cytoablative therapies, unraveling the molecular elements that coordinate their thymopoietic role has fundamental and clinical implications. Particularly, the selection of CD4 T cells depends on interactions between TCRs expressed on T cell precursors and self-peptides:MHC II complexes presented by cortical TECs (cTECs). Although the macroautophagy/ autophagy-lysosomal protein degradation pathway is implicated in CD4 T cell selection, the molecular mechanism that controls the generation of selecting MHC II ligands remains elusive. LAMP2 (lysosomal-associated membrane protein 2) is a well-recognized mediator of autolysosome (AL) maturation. We showed that LAMP2 is highly expressed in cTECs. Notably, genetic inactivation of *Lamp2* in thymic stromal cells specifically impaired the development of CD4 T cells that completed positive selection, without misdirecting MHC II-restricted cells into the CD8 lineage. Mechanistically, defects in autophagy in *lamp2*-deficient cTECs were linked to alterations in MHC II processing, which was associated with a marked reduction in CD4 TCR repertoire diversity selected within the *lamp2*-deficient thymic stroma. Together, our findings suggest that LAMP2 interconnects the autophagy-lysosomal axis and the processing of selecting self-peptides:MHC II complexes in cTECs, underling its implications for the generation of a broad CD4 TCR repertoire.

Introduction

The thymus generates T cells expressing $\alpha\beta$ T cell receptors (TCRs) that recognize foreign antigens presented by self-major histocompatibility complex (MHC) molecules and are tolerant to self-components. This process is developmentally regulated by interactions between TCR expressed on T cell precursors (thymocytes) and self-peptide-MHC complexes presented by thymic epithelial cells (TECs) (Abramson & Anderson, 2017). TECs express a myriad of soluble and cell-surface ligands that coordinate all stages of T cell differentiation. Importantly, the thymopoietic role of TECs is sensitive to aging and cytoablative therapies, which compromise T-cell responses to pathogens and vaccination in the elderly and immunocompromised patients (Baran-Gale et al., 2020). Therefore, the identification of novel regulators of TEC functionality is vital to comprehend the foundations of T cell-immunity and to restore thymopoiesis in disorders associated with defective T cell responses. TECs are divided into functionally distinct cortical (cTECs) and medullary (mTECs) subtypes. While cTECs guide T cell lineage commitment and positive selection,

mTECs regulate negative selection and T regulatory cell differentiation (Anderson & Takahama, 2012). The functional segregation between cTECs and mTECs is, in part, determined by their unique antigen processing and presentation properties, which are controlled by specific protein degradation machineries and transcriptional programs (Klein et al., 2014; Takada et al., 2017). However, how these proteolytic machineries coordinate the presentation of selective self-peptide-MHC complexes remains elusive at the molecular level. In this regard, mTECs employ conventional proteolytic pathways to generate “public” MHC I- and MHC II-bound peptides that negatively select autoreactive thymocytes or drive their deviation into regulatory T cells (Klein et al., 2014). These key steps for tolerance induction depend on the distinctive ability of mTECs to express tissue-restricted antigens, a process that is mediated by AIRE and FEZ family zinc finger 2 (Abramson & Anderson, 2017). Alternatively, cTECs harbor unique antigen processing properties that generate sets of “private” MHC I- and MHC II-bound self-peptides, which are critical for the positive selection of double-positive (DP) thymocytes and their commitment into CD4 or CD8 T cells. The generation of MHC I-bound peptides that select CD8⁺ T cells depends on the distinct proteolytic activity of PSMB11/β5t (proteasome(prosome, macropain) subunit, beta type 11)-containing thymoproteasome (Murata et al., 2007; Takada et al., 2017). For the generation of MHC II-bound peptides, cTECs employ unconventional endogenous MHC II-loading pathways that involve the autophagy-mediated transport of cytosolic proteins into lysosomes (Munz, 2016; Nedjic, Aichinger, & Klein, 2008). Lysosomal proteases, including CTSL (cathepsin L) and PRSS16/TSSP (protease, serine 16 (thymus)) contribute to the generation of specific self-peptides that select CD4⁺ T cells (Gommeaux et al., 2009; Honey et al., 2002; Nakagawa et al., 1998; Viret et al., 2011). Although autophagy has been coupled to the control of thymic selection (Munz, 2016, 2021; Nedjic, Aichinger, & Klein, 2008), the molecular mechanism that links this catabolic pathway to the generation of selective self-peptides in the lysosomes remains elusive. Our study identifies a role for LAMP2 (lysosomal-associated membrane protein 2) in the thymic stroma as a determinant regulator of CD4 T cell development and TCR repertoire formation.

Results

LAMP2 is dispensable for the establishment of major TEC Microenvironments

To identify new candidates involved in the antigen processing capacity of cTECs, we analyzed our transcriptomic data from postnatal cTECs and mTECs (Rodrigues et al., 2017), together with available data sets from other studies (Sansom et al., 2014; St-Pierre et al., 2013). Given that MHC II-bound peptide generation involves the endosomal–lysosomal pathway, we sought genes associated with these vesicular compartments that

were selectively enriched in cTECs relatively to mTECs. Ctsl (cathepsin L) and Prss16 were expectedly among the top genes upregulated in cTECs. We selected to analyze LAMP2, which was the only member of the LAMP family upregulated (4-fold) in cTECs (**Figure 1A**, **Figure S1A**). LAMP2 is a lysosomal membrane protein that regulates macroautophagy and chaperone-mediated autophagy (Kaushik & Cuervo, 2012), promoting the fusion of autophagic vacuoles with lysosomes (Eskelinen et al., 2002; Tanaka et al., 2000). LAMP2 has been also implicated in the regulation of MHC II presentation in human B cells (Crotzer et al., 2010; Zhou et al., 2005). Yet, its involvement in T cell development and selection is unknown.

In man, genetic mutations in LAMP2 are associated with Danon Disease, a fatal X-linked syndrome caused by lysosomal dysfunction (Nishino et al., 2000). Mice deficient for *Lamp2* (*lamp2* knockout; *lamp2* KO) recapitulate key aspects of the human condition, including the accumulation of autophagic vacuoles in several cell types (Eskelinen et al., 2002; Saftig et al., 2001; Tanaka et al., 2000). Despite the increased mortality in early adulthood, *lamp2* KO mice survive the first weeks of life (Tanaka et al., 2000). Hence, to study the function of LAMP2 in the establishment of major TEC subsets, we analyzed the *lamp2* KO thymus during embryonic and postnatal life. We began by analyzing the expression of LAMP2 protein in embryonic wild-type (WT) TECs, and using cells from *lamp2* KO mice as a control for the antibody staining (**Figure S1B**). Within the postnatal thymus, TECs represented the highest LAMP2-expressing subset, relatively to thymocytes and other non-TEC stromal cells, including endothelial and mesenchymal cells. The corresponding *lamp2* KO subsets were included to further validate antibody staining. In line with the transcriptomic data, cTECs significantly expressed higher levels of LAMP2 relatively to mTEC^{lo}/hi subtypes (**Figure 1B**). The deficiency in *Lamp2* did not alter the generation of primordial cTECs and mTECs at embryonic day 15 nor affected the cellularity of the main cTEC and mTEC subsets in the 2-week old thymus (**Figure 1C**). Further analysis of the expression of cTEC-associated markers (LY75/CD205, CD40) or mTEC subpopulations, including mTEC^{lo} (MHC II^{lo} CD40^{lo} CD80^{lo}), mTEC^{hi} (MHC II^{hi} CD40^{hi} CD80^{hi}), CCL21⁺ and AIRE⁺ cells, revealed no differences between WT and *lamp2* KO TECs (**Figure S1C**). Additionally, the spatial organization of cortical and medullary areas was normal in the *lamp2* KO thymus (**Figure 1D**). Our results indicate that LAMP2 is not essential for the establishment of global cTEC and mTEC compartments.

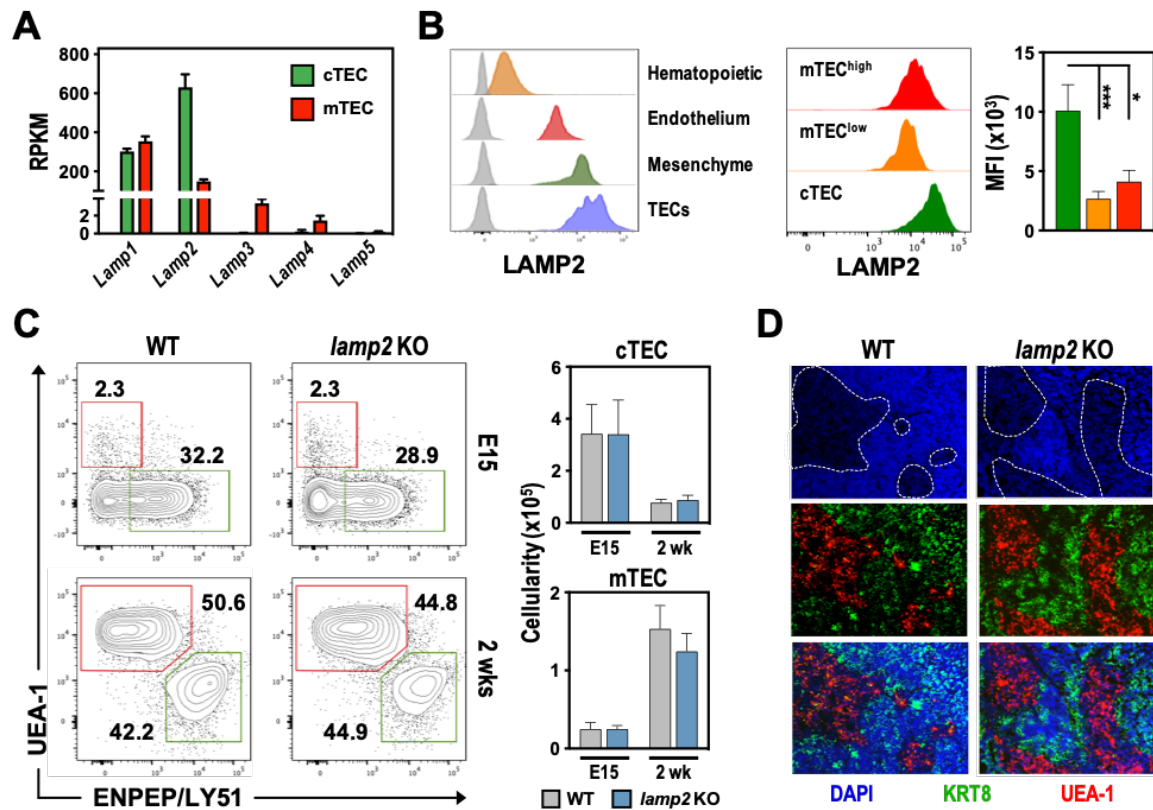


Figure 1. The deficiency in Lamp2 does not affect global cTEC/mTEC differentiation. (A) Expression (RNAseq analysis) of LAMP family members in cTECs (green) and mTECs (red) from WT mice. Error bars represent mean $\pm$ SEM. (B) LAMP2 expression in distinct thymic cell populations in 2-week-old WT and *lamp2* KO mice. Left histogram: Hematopoietic (PTPRC/CD45⁺), endothelial (PECAM1/CD31⁺), mesenchymal (PDGFRA-PDGFRB [$\alpha\beta$]⁺) and TEC (EPCAM⁺) cells ($n = 3$), (WT and *lamp2* KO subsets in color and gray, respectively); Right histogram: cTECs (ENPEP/Ly51⁺ UEA-1⁻), mTEC^{lo} (ENPEP/Ly51⁻ UEA-1⁺ CD80^{lo}) and mTEC^{hi} (ENPEP/Ly51⁻ UEA-1⁺ CD80^{hi}). Bars graphs represent mean fluorescence intensity (MFI) of LAMP2 expression. Data representative of 3 independent experiments ($n = 12$ animals). (C) cTEC and mTEC composition in embryonic day 15.5 (E15.5) and 2-week-old WT and *lamp2* KO thymus. Dot plots show a representative ENPEP/Ly51 and UEA staining and graphs represent the average cellularity of c/mTECs in the indicated timepoints from 3 independent experiments ($n = 11-14$ animals/group). (D) Immunofluorescence analysis of thymic sections from 2-week-old WT and *lamp2* KO thymus stained for DAPI, UEA-1 (red) and KRT8 (keratin 8; green). Results in A-C are shown as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

LAMP2 in thymic stromal cells specifically controls the development of CD4 T cells

The pleiotropic defects of *lamp2* KO mice and the broader expression of LAMP2 in several tissues hampered a direct assessment of its role in TEC-mediated T cell selection. To circumvent this aspect, we employed a well-established thymic transplantation model wherein WT or *lamp2* KO embryonic thymi were grafted under the kidney capsule of nude adult recipients, referred to hereafter as WT-Nude and *lamp2* KO-Nude. In these chimeras, the deficiency in *Lamp2* was restricted to TECs, endothelial and mesenchymal cells, allowing the assessment of how recipient-derived T cell precursors (from WT origin)

developed and were selected within WT and *lamp2* KO thymic stroma, in the absence of confounding effects of *lamp2*-deficiency in other tissues. Although TECs have a non-redundant role in T cell commitment and selection (Klein et al., 2014), this approach did not formally exclude a potential contribution for LAMP2 in endothelial and mesenchymal cells in these processes.

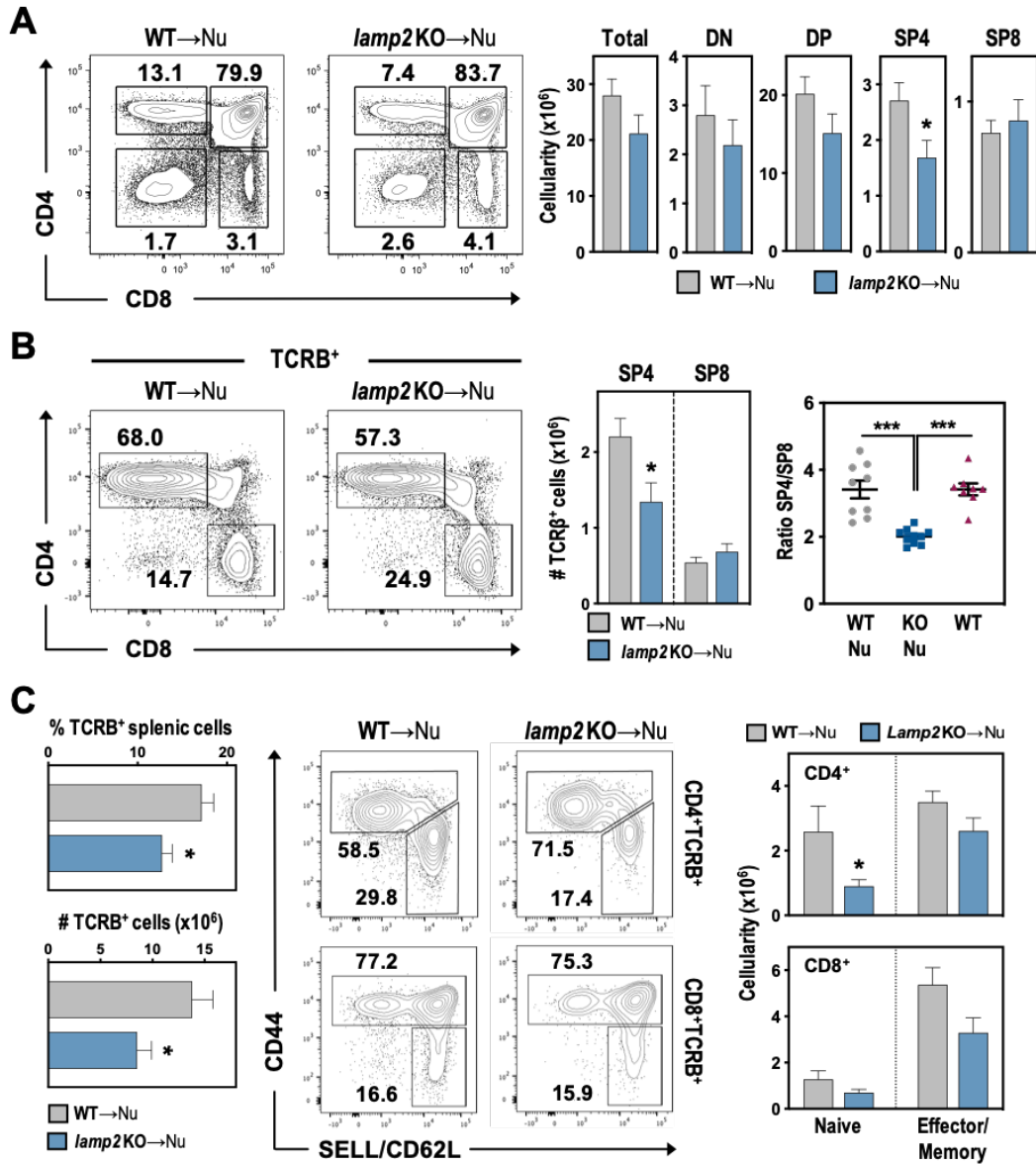


Figure 2. T cell development in WT and *lamp2* KO thymic grafts. Representative dot plots of CD4 and CD8 expression within (A) total thymocytes and (B) TCRB⁺ thymocytes in thymic grafts of Nude recipients transplanted with WT (WT-Nu) and *lamp2* KO (*lamp2* KO-Nu) ectopic thymus, 8–10 weeks after transplantation. Numbers on the plots indicate the frequencies of the different subsets. Bar graphs in A and B depict the average absolute cellularity of the thymus and the indicated thymocyte subsets in WT-Nu (gray) and *lamp2* KO-Nu. In B, ratio of the frequency of SP4:SP8 is shown in WT-Nu and *lamp2* KO-Nu, as well in the endogenous thymus. (C) Analysis of peripheral T cells in spleens from WT-Nu and *lamp2* KO-Nu mice. Graphs (left) represent the average % and numbers of TCRB⁺ T cells in the spleen. Dot plots show CD44 and SELL/CD62L expression within CD4 and CD8 T cells. Graphs (right) show average cellularity of naïve (SELL/CD62L⁺ CD44^{lo}) and effector/memory (SELL/CD62L⁺ CD44^{hi}) CD4 and CD8 T cells. Data represent an average of 3 independent experiments (n = 10 WT and *lamp2* KO ectopic thymi). Results in A–C are shown as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001.

Analysis of T cell development 8–10 weeks post-transplantation showed that the percentages of immature double-negative (DN) and DP thymocytes were similar in WT-Nude and *lamp2* KO-Nude thymic grafts. While the numbers of total, DN and DP thymocytes appeared slightly reduced, these differences were not statistically significant (**Figure 2A**, **Figure S2A**), indicating that LAMP2 is dispensable for β -selection and DN-DP transition. Notably, the frequencies of single-positive 4 (SP4) and SP8 thymocytes were altered in *lamp2* KO thymi. These changes resulted in a specific and statistically significant, reduction in the number of SP4 but not SP8 cells (**Figure 2A**). Analysis of T cells expressing high levels of TCRB (T cell receptor beta chain) (TCRB^{hi}) confirmed the decrease of circa 40% in the average number of SP4 cells in the *lamp2* KO thymus (1.3×10^6 cells) relatively to the cellularity found in the WT counterparts (2.2×10^6 cells). These changes led to a deviation in SP4:SP8 ratios in the mutant thymi as compared to controls, and endogenous thymi of WT mice (**Figure 2B**). The thymic alterations coincided with changes in the peripheral T-cells, with a statistically significant reduction in the number of *lamp2* KO thymus-derived splenic naïve CD4⁺ T cells. The numbers of effector/memory CD4, and naïve and effector/memory CD8 T cells were moderately reduced, but these changes were not statistically significant (**Figure 2C**). Together, these results pointed to an impaired CD4 T cell development within the *lamp2*-deficient thymic microenvironment.

To identify the stage at which CD4⁺ T cell development was altered in *lamp2* KO thymus, we examined distinct stages of pre- and post-positive selection, based on the differential expression of TCRB and CD69 expression on thymocytes: Population I (TCRB^{neg/int} CD69^{neg}) contains mostly pre-selected DP thymocytes; Population II (TCRB^{int} CD69^{int}) harbors TCR-signaled cells initiating positive selection; Population III (TCRB^{hi} CD69^{hi}) represents post-positively selected SP thymocytes; Population IV (TCRB^{hi} CD69^{neg}) consists of more mature SP cells (Yamashita et al., 1993). Although the frequency of populations II, III and IV within total thymocytes were similar, we found a noticeable alteration in the frequency of SP4 and SP8 in post-positively selected cells (TCRB^{hi} CD69^{hi}, III) of the *lamp2* KO-Nude thymi (**Figure 3A-B**). This led to a significant reduction in the numbers of post-positively selected SP4, but not SP8 thymocytes, in *lamp2* KO-Nude thymi (**Figure 3B**). We further analyzed the expression of CD5 in thymocyte subsets as this correlates with TCR affinity for self-peptide-MHC ligand (Daley et al., 2013; Mandl et al., 2013). Stage II DP thymocytes from WT and *lamp2* KO thymus expressed comparable CD5 levels, suggesting normal TCR signaling during the early stages of positive selection (**Figure 3C**, **Figure S2B**).

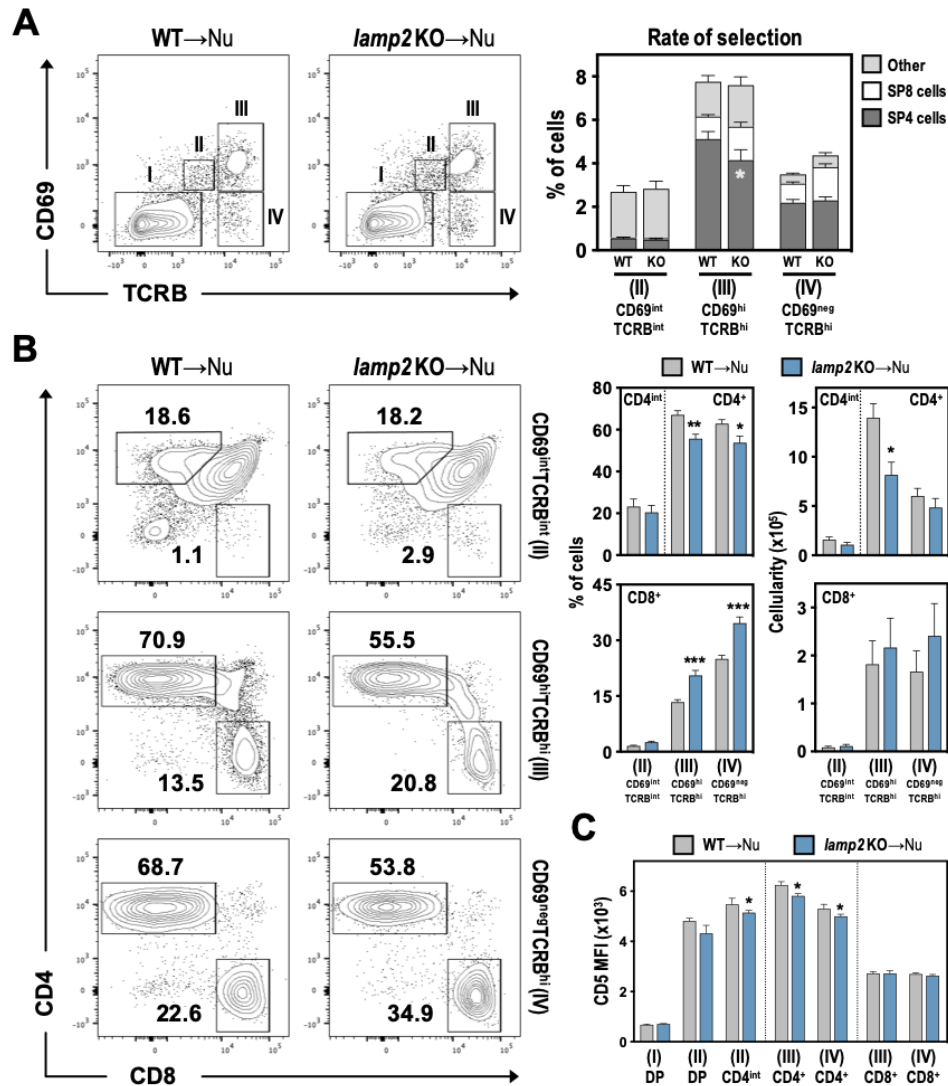


Figure 3. Pre- and post-positive T cell selection in WT and *lamp2* KO thymic grafts. (A) Flow cytometry analysis of pre- and post-positive selection in WT (WT-Nu) and *lamp2* KO (*lamp2* KO-Nu) ectopic thymus grafted into Nude recipients. Dot plots show the analysis of CD69 and TCRB expression on total thymocytes from WT-Nu and *lamp2* KO-Nu ectopic thymus. Stage I (TCRB^{neg/int} CD69^{neg}), II (TCRB^{int} CD69^{int}), III (TCRB^{hi} CD69^{hi}) and IV (TCRB^{hi} CD69^{neg}) defined pre-, recently-, post-selected and mature stages, respectively. Graph represents the average percentages of total populations II, III and IV. In each bar (populations II, III and IV bar) is shown the corresponding frequency of SP4 and SP8 cells, which were calculated based on the frequencies of SP4 and SP8 shown in panel B. (B) CD4/CD8 expression within stages II (TCRB^{int} CD69^{int}), III (TCRB^{hi} CD69^{hi}) and IV (TCRB^{hi} CD69^{neg}), which were defined on total thymocytes (A). Graphs represent the average percentages and absolute numbers of the indicated thymocyte subsets in WT-Nu (gray) and *lamp2* KO-Nu. (C) Graphs represent the MFI of CD5 expression in the indicated subsets (defined as shown in Figure S2). Data shown represent an average of 10 ectopic WT and 10 ectopic *lamp2* KO thymi from 3 independent experiments. Results in A-C are shown as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001.

Early after positive selection, DP thymocytes transverse through a CD4⁺ CD8^{int} (CD4^{int}) transitional stage prior to their commitment into CD4 and CD8 lineage (Singer et al., 2008). The frequency and numbers of stage II CD4^{int} cells in *lamp2* KO-Nude mice were comparable to the WT-Nude setting (Figure 3B). Yet, the levels of CD5 in stage II CD4^{int}

and III–IV SP4 thymocytes were moderately reduced within the *lamp2* KO-Nude thymus, suggesting an attenuation of TCR signaling in cells that completed positive selection (**Figure 3C, Figure S2B**). Additionally, stage IV SP4 thymocytes did not accumulate in the *lamp2* KO thymus nor presented elevated CD5 levels (**Figure 3B-C, Figure S2B**), arguing against excessive TCR signaling. To study whether *lamp2*-deficiency could affect other steps of CD4 T cell development that depend on mTECs, we analyzed SP4 maturation and the regulatory T cell differentiation (Abramson & Anderson, 2017). The proportion of immature (CD24⁺ SELL/CD62L⁻) and mature (CD24⁻ SELL/CD62L⁺) SP4 thymocytes were comparable within WT and *lamp2* KO thymus. Additionally, the frequency of immature (IL2RA/CD25⁺ Forkhead box P3 (FOXP3)⁻ and IL2RA/CD25⁻ FOXP3⁺) and mature CD4 T regulatory (IL2RA/CD25⁺ FOXP3⁺) cells was similar in WT and *lamp2* KO thymi, suggesting that the maturation of T reg was not affected by *lamp2*-deficiency. Although moderately reduced, the numbers of mature CD4 T regulatory cells were not statistically different (**Figure S2C-D**). Contrarily, the cellularity of immature (IL2RA/CD25⁺ FOXP3⁻) T reg, immature and mature SP4 was statistically diminished in *lamp2* KO thymus. The reduction in these subsets may arise from the reduction in SP4 that completed positive selection, which was the subset mostly affected by the deficiency in *Lamp2* and included immature T reg and SP. Together, our results suggested a more prominent function for LAMP2 in regulating the development of CD4⁺ T cells that complete positive selection.

The selection of CD4⁺ T cells depends on “strong” and persistent TCR-mediated signals (Gascoigne et al., 2016). The decreased CD5 levels on SP4 cells could suggest that the reduction in SP4 cells in *lamp2* KO thymus was functionally linked to a defective CD4 commitment. In this scenario, MHC II-restricted thymocytes were misdirected toward the CD8 lineage due to an attenuated TCR signaling. To examine this possibility and prevent MHC-I-driven CD8 selection, we generated mice double deficient for *Lamp2* and *B2m* (beta-2 microglobulin) (*lamp2 b2m* dKO mice). Thymic transplantations were set with *b2m* KO or *lamp2 b2m* dKO embryonic thymus grafted into WT mice. Owing to the deficiency in *b2m*, and consequently MHC-I expression, the selection of MHC-I-restricted SP8 thymocytes was expectedly abolished in *b2m* KO thymic grafts (Koller et al., 1990), so that mature TCRB⁺ cells mostly comprised SP4 thymocytes (**Figure 4A-B**). Notably, the frequency and numbers of SP4 thymocytes were reduced in *lamp2 b2m* dKO thymus, without a concomitant increase in SP8 thymocytes (**Figure 4A-B, Figure S3A**). Moreover, recently post-selected (TCRB^{hi} CD69^{hi}) and mature (TCRB^{hi} CD69^{neg}) SP4 cells were significantly reduced in frequency and numbers in *lamp2 b2m* dKO thymic grafts (**Figure 4C, Figure S3A**). To evaluate the possible contribution of LAMP2 in cortical and medullary negative selection at a polyclonal level, we analyzed the expression of IKZF2/HELIOS (IKAROS

family zinc finger 2) on DP (TCRB^{lo/int} IL2RA/ CD25⁻ FOXP3⁻) and CD4SP (TCRB^{hi} IL2RA/CD25⁻FOXP3⁻) thymocytes (Daley et al., 2013). The frequency of IKZF2/ HELIOS⁺ cells was similar in both WT- and *lamp2* KO-derived thymocyte subsets (Figure 4D, Figure S3B). These observations support that deficiency in *Lamp2* does not redirect MHC II-restricted cells into CD8 lineage, but instead prevents the development of CD4 T cells that completed positive selection.

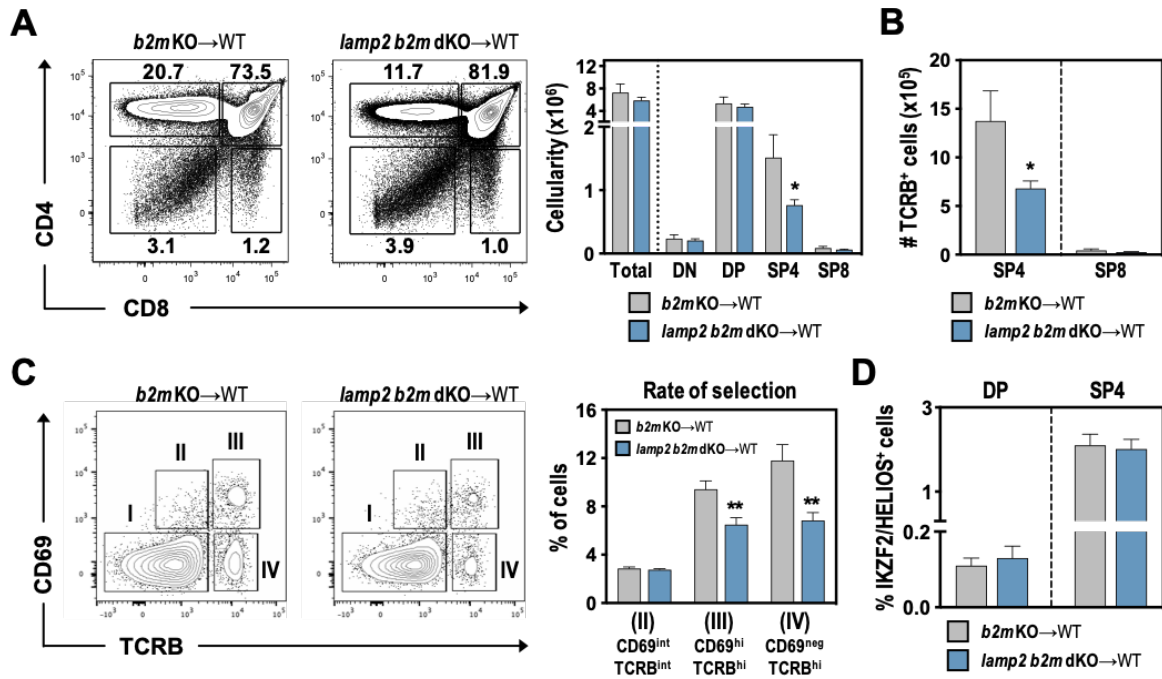


Figure 4. Combined deficiency in *Lamp2* and *B2m* specifically reduces CD4 T cell development, without redirecting MHC II-restricted cells into the CD8 lineage. (A-B) Flow cytometry analysis of T cell development in *b2m* KO (*b2m* KO-WT) and in *lamp2* and *b2m* double-KO (*lamp2 b2m* dKO-WT) thymus grafted into WT recipients. (A) Representative dot plots of CD4 and CD8 expression on total thymocytes, with numbers indicating the frequencies of the different subsets. Graphs represent the cellularity of total thymocytes and the indicated subsets in *b2m* KO (gray) and *lamp2 b2m* dKO. (B) Graph represents the absolute numbers of SP4 and SP8 within mature TCRB⁺ thymocytes. Data shown represent an average of 9 *b2m* KO and 10 *lamp2 b2m* dKO ectopic thymi from 3 independent experiments. (C) Dot plots show the flow cytometric profile for CD69 and TCRB on total thymocytes from *b2m* KO-WT and *lamp2 b2m* dKO-WT ectopic thymi. Stage I (TCRB^{neg/int} CD69^{neg}), II (TCRB^{int} CD69^{int}), III (TCRB^{hi} CD69^{hi}) and IV (TCRB^{hi} CD69^{lo/neg}) were defined as represented. Graphs represent the average percentages of cells in the indicated gates in *b2m* KO-WT (gray) and *lamp2 b2m* dKO-WT ectopic thymi. (D) Graphs represent the average frequency of IKZF2/HELIOS⁺ cells in DP and SP4 from *b2m* KO-WT (gray) and *lamp2 b2m* dKO-WT (defined as shown in Figure S3B). Data include an average of 2 independent experiments (n = 6 *b2m* KO and 8 *lamp2 b2m* dKO-WT). Results in A-D are shown as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001.

Alterations in autophagy in *lamp2* KO cTECs are associated with changes in MHC II processing

To determine whether the defect in CD4 T cell development could be mechanistically linked to changes in autophagy provoked by the deficiency in *Lamp2*, we evaluated the autophagic flux in TECs. *Ex vivo* isolated *lamp2* KO cTECs displayed an increased level of

Cyto-ID, a tracer that marks AP and AL (Chan et al., 2012) (**Figure 5A**), indicative of altered autophagy dynamic. Moreover, *lamp2* KO mice were crossed with transgenic mice that ubiquitously expressed a RFP-GFP-MAP1LC3A/LC3 in a pH-dependent manner (RFP-GFP-LC3). In RFP-GFP-LC3 mice, RFP is stable and GFP is quenched in the acidic pH of lysosomal vesicles, allowing the distinction between AP (GFP⁺ RFP⁺) and AL (RFP⁺) (Li et al., 2014). The RFP-GFP-LC3 expression was predominantly detected in TECs relatively to the hematopoietic and non-TEC stroma of WT and *lamp2* KO thymus (**Figure S4A**). Moreover, the proportion of RFP⁺-GFP⁺-LC3 TECs was specifically increased in *lamp2* KO cTECs, but not mTECs (**Figure 5B** and **Figure S4B**). The accumulation of RFP⁺-GFP⁺-LC3 cells resulted from an augmented expression of GFP-LC3, but not RFP-LC3, in *lamp2* KO cTECs (**Figure 5B**). We further analyzed the autophagic flux at the single-cell level by employing imaging flow cytometry. *lamp2* KO cTECs contained cells with a reduced number of RFP⁺-LC3 puncta/cell compared to WT counterparts, being the *lamp2* KO subset mostly composed of cells with dual RFP⁺-GFP⁺-LC3 puncta (**Figure 5C**). As the status of autophagy flux correlates with the number of autophagic vesicles, these results indicated that AP-lysosome fusion was markedly affected in *lamp2* KO cTECs, as reported in other cells deficient in *Lamp2* (Eskelinen et al., 2002; Saftig et al., 2001; Tanaka et al., 2000).

We next studied whether changes in LAMP2-mediated autophagy were associated with alterations in self-peptide: MHC II processing in cTECs, which in turn could condition their capacity to select CD4 T cells. MHC II molecules are stabilized in the endoplasmatic reticulum by their binding to the CD74 antigen/li (invariant polypeptide of major histocompatibility complex, class II antigen-associated). Subsequently, CD74/li is degraded into a fragment called CLIP (class II-associated invariant chain peptides) in the MIIC (MHC II processing compartment) to permit antigen binding (Honey & Rudensky, 2003; Roche & Furuta, 2015). To evaluate MHC II processing and maturation, we employed 15G4 antibody that recognizes I-Ab occupied by the CD74/li degradation intermediates small leupeptin induced protein or CLIP (Hsieh et al., 2002; Nakagawa et al., 1999). Strikingly, 15G4 staining was substantially increased in *lamp2* KO cTECs, but not in mTECs, relative to WT counterparts (**Figure 5D**). In line with previous studies (Nakagawa et al., 1998), mTECs displayed higher 15G4 staining as compared to cTECs (**Figure 5D**). Control and mutant cTECs, as well as mTECs, expressed comparable levels of MHC II and I molecules (**Figure S4C**), suggesting a specific role of LAMP2 in the processing of MHC II, but not in its trafficking to the cell membrane. Exploring the fact that *Lamp2* resides on X chromosome, we analyzed TECs from *lamp2* WT/KO heterozygous thymus, which due to random X-inactivation in females included WT and *lamp2* KO TECs (**Figure S4D**). *lamp2* KO cTECs presented an augmented 15G4 staining, even within a microenvironment containing WT

cells, suggesting that LAMP2 controls MHC II processing in a cell-autonomous manner (Figure 5E).

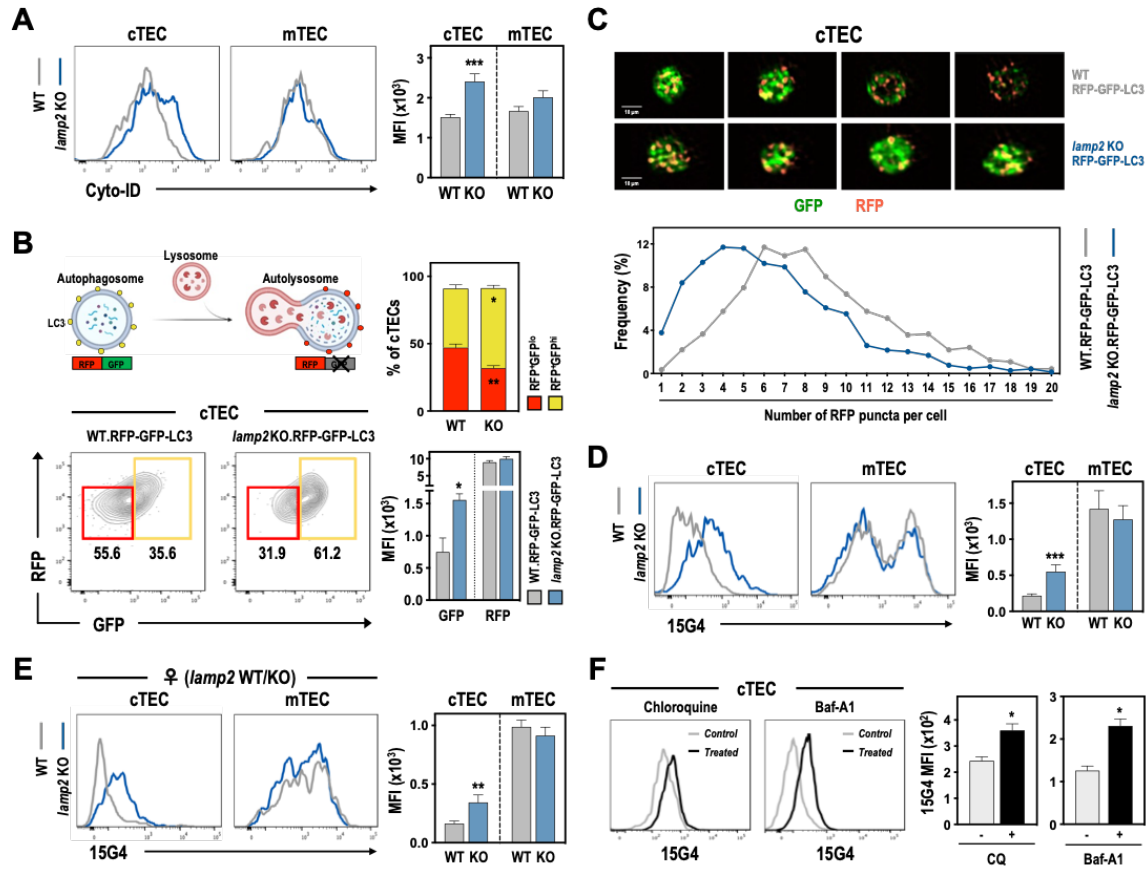


Figure 5. Defective autophagy and MHC II processing in *lamp2* KO cTECs. (A-B) Autophagic flux in cTECs from 12 days old WT and *lamp2* KO mice was analyzed by flow cytometry. (A) Histograms show representative Cyto-ID analysis in cTEC and mTEC from WT and *lamp2* KO. Graphs represent the MFI of Cyto-ID in the indicated subsets. (B) Scheme (top left) represents autophagic flux using RFP-GFP-LC3 mice. Differential detection of GFP and RFP allows the distinction between AP (GFP⁺ RFP⁺) and AL (RFP⁺). Dot plot (bottom left) represents GFP and RFP expression in cTECs of WT and *lamp2* KO RFP-GFP-LC3 mice. Graphs (top right) represent the average frequency of RFP⁺ GFP^{hi} (yellow) and RFP⁺ GFP^{low} (red) cells in WT and *lamp2* KO RFP-GFP-LC3 cTECs. Graphs (bottom right) represent the MFI of GFP and RFP expression in WT (gray) and *lamp2* KO RFP-GFP-LC3. (C) WT and *lamp2* KO RFP-GFP-LC3 cTECs (EPCAM⁺ ENPEP/LY51⁺) were analyzed by imaging flow cytometry. Representative images are shown. Graph show the distribution of the number of RFP puncta in WT (n = 1345 cells) and *lamp2* KO (n = 1422 cells) RFP-GFP-LC3 cTECs. Data in A-C include an average of 2–3 independent experiments (n = 4–6 WT and n = 4–6 *lamp2* KO). (D) Flow cytometry analysis of 15G4 staining on cell surface of cTECs (ENPEP/LY51⁺ UEA-1⁻) and mTECs (ENPEP/LY51⁻ UEA-1⁺) from 12 days old WT and *lamp2* KO mice. Histograms show representative staining and graphs represent the MFI of 15G4 staining in the indicated subsets. Data include an average of 4 independent experiments (n = 6 WT and 7 *lamp2* KO). (E) Histograms show representative 15G4 staining on the cell surface of WT and *lamp2* KO c/mTECs from *lamp2* WT/KO heterozygous mice. Graphs represent the MFI found in indicated subsets in control (gray) and mutant cells (n = 8 from 4 experiments). (F) Postnatal day 3–5 WT thymus were treated overnight with chloroquine (50 μ M) or with Baf-A1 (0.5 μ M) for 5 h. Histograms show the representative staining with 15G4 staining in control (light gray) and chloroquine-treated (black) or Baf-A1-treated (black) in WT cTECs. Graphs represent the MFI in control (gray) and treated (dark gray/ black) cTEC (n = 4–6 animals from 3–4 independent experiments). Results in A-F are shown as mean $\pm$ SEM. * p < 0.05; ** p < 0.01; *** p < 0.001.

Given the role of CTSL in the degradation of CD74/li in cTECs (Honey et al., 2002; Nakagawa et al., 1998), we studied if altered MHC II processing resulted from a decrease in CTSL or lysosomal functions. The proteolytic function of CTSL and global lysosomal activity were increased in *lamp2* KO cTECs (**Figure S4E-F**). These results suggest that the augmented levels of MHC II-bound to CD74/li degradation intermediates did not result from a decrease in CTSL activity or lysosomal processing capacities. Lastly, we evaluated whether changes in LAMP2-mediated autophagy could be functionally linked to the alterations in MHC II processing. To do so, postnatal day 3–5 WT thymi were treated with two inhibitors, chloroquine and bafilomycin A1 (Baf-A1), of AP-lysosome fusion. Interestingly, chloroquine and Baf-A1 treatment increased 15G4 staining in WT cTECs (**Figure 5F**). The elevated 15G4 staining of WT mTECs was not substantially altered in Baf-A1-treated cells (**Figure S4G**). Worth noting, mTECs are less abundant in the postnatal thymus (Rodrigues et al., 2017) and were sensitive to apoptosis following culture. Although the results with pharmacological inhibitors should be considered with caution due to additional specific or off-target effects, our data suggest that inhibition of AP-lysosome fusion in WT cTECs mimicked the phenotype observed on *lamp2* KO cTECs, and establish a presumable causality between changes in autophagy flux and MHC II processing.

LAMP2 in thymic stroma controls the generation of a broad CD4 TCR repertoire

Lastly, we determined whether thymic stroma deficient in *Lamp2* altered the generation of TCR repertoire diversity in developing CD4 T cells, possibly by affecting the MHC II-processing and the generation of positive selecting self-peptides in cTECs. Despite considerable efforts to identify the nature of self-peptides that promote positive selection (Takada et al., 2017), it remains challenging to conduct this biochemical characterization with sensitivity in rare populations, such as TECs. Due to this limitation, we determined the TCR clonal composition of SP4 thymocytes generated in WT or *lamp2* KO thymi. The assessment of TCR diversity could serve as an indirect indicator of the quality of the MHC II-peptide ligandome presented by *lamp2* KO cTECs. Given the vast combinations of TCRA/ α -TCRB β chains in SP4 thymocytes in a broad polyclonal setting, we used mice expressing a fixed transgenic TCR V β 6 (beta6) chain (Hassler et al., 2019) as recipients of thymic grafts. This model referred as TCR:V β 6Tg, reduced the complexity of the TCR repertoire to the diversity of TCRA chains and favored CD4⁺ T cell selection, as confirmed by the accumulation of SP4 thymocytes in WT thymic grafts (**Figure 6A, Figure S5A**). The bias toward CD4 lineage may result from the fact that the V β 6 chain is derived from a MHC II-restricted TCR (Hassler et al., 2019). Consistent with the results in the fully polyclonal

TCR repertoire of Nude and WT recipients, *lamp2*-deficiency restrained the number of SP4 TCR:V β 6Tg thymocytes (**Figure 6A**). The cellularity of DP cells was also reduced in TCR:V β 6Tg model (**Figure S5A**), possibly due to the ubiquitous expression of MHC-II restricted V β 6 chain that may anticipate the effects provoked by *lamp2*-deficiency in the DP stage.

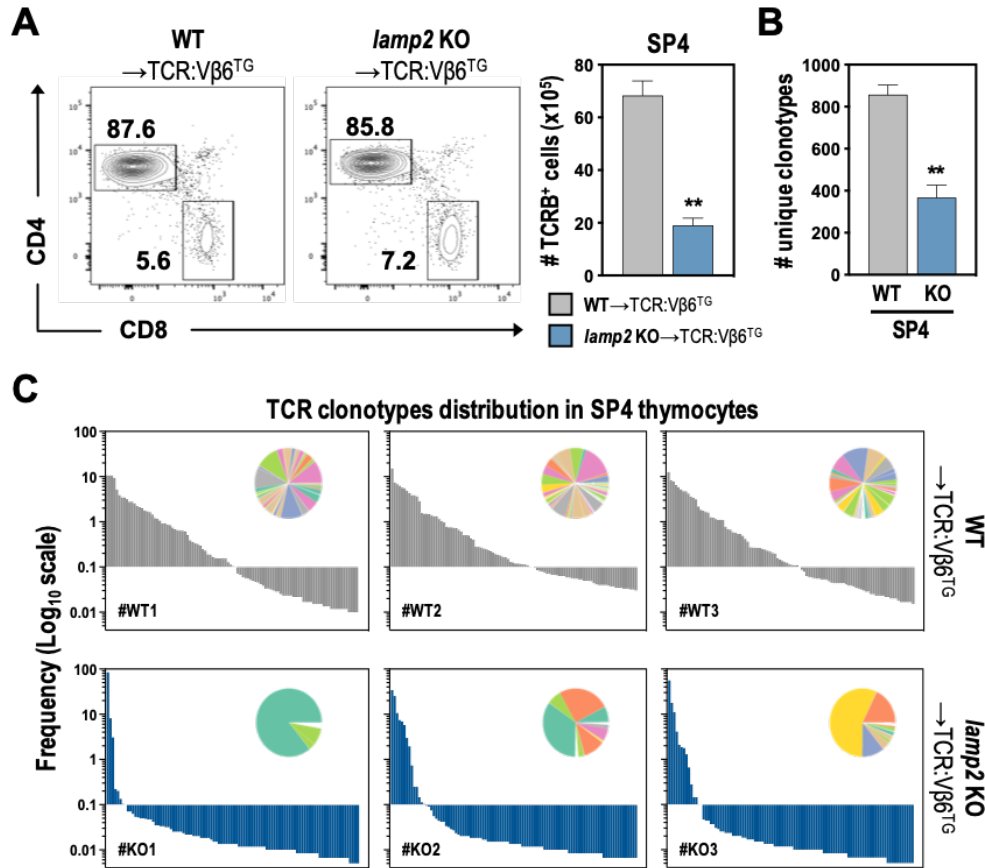


Figure 6. Analysis of SP4⁺ TCR repertoire in WT and *lamp2* KO thymus. (A) T cell development in WT (WT-TCR:V β 6Tg) and *lamp2* KO (*lamp2* KO-TCR:V β 6Tg) ectopic thymus grafted into fixed TCRB/TCR β Tg recipients. CD4 and CD8 expression within mature TCRB⁺ thymocytes. Graphs represent the absolute number of the indicated thymocyte subsets in WT-TCR:V β 6Tg (gray) and *lamp2* KO-TCR:V β 6Tg thymi. Data shown represent an average of 2 independent experiments (n = 6 WT and 6 *lamp2* KO). Graphs indicated (B) the number and (C) frequency of unique sequences of *Tcr* clones found in SP4 thymocytes developing within WT and *lamp2* KO ectopic thymus. Pie graphs in C depict a colorimetric representation of the most abundant clonotypes found in each thymi. Results in A-B are shown as mean $\pm$ SEM. * p < 0.05; ** p < 0.01; *** p < 0.001.

For repertoire analysis, we isolated RNA from 3 samples of 1.10⁶ (FASC sorted) SP4 (CD4⁺ TCRB⁺) thymocytes from WT and *lamp2* KO thymus. The *Tcra* gene was specifically amplified and the generated libraries were subjected to deep sequencing analysis of *Tcra* transcripts. We recovered 0.7–1.2.10⁶ sequences in SP4 cells derived from WT and *lamp2* KO grafts, from which 0.6–2.10⁵ corresponded to specific TCRs (**Figure S5B**, first two columns). Further analysis identified 1,3–1,5.10³ unique TCRs in the WT setting. This

number ($0,3\text{--}0,4 \cdot 10^3$) was notably reduced in SP4 cells from *lamp2* KO thymi (**Figure S5B**, third column). Given the variable number of retrieved TCR sequences, the analysis was normalized to $6 \cdot 10^5$ randomized TCR sequences/sample. This threshold was defined by the lower number of sequences identified on one sample (KO2) (**Figure S5B**). Following unbiased normalization, the number of unique clonotypes remained significantly lower in *lamp2* KO-derived SP4 thymocytes relatively to the ones generated from WT thymus (**Figure 6B**, **Figure S5B**). Additionally, the distribution of TCRA sequences was distinctively different. While clonotypes of WT-derived thymus presented the stereotypical broad distribution expected to be found in a polyclonal repertoire, *lamp2* KO counterparts displayed a clear skewing in the TCR catalog (**Figure 6C**). Further analysis revealed that the clonality index was markedly increased in mutant samples (**Figure S5C**).

These results led us to evaluate whether the restricted thymic SP4 TCR repertoire affected the function of peripheral T cells generated within *lamp2* KO thymic stroma. To do so, we resorted to the fully polyclonal TCR repertoire model wherein WT and *lamp2* KO thymi were grafted in Nude recipients, as described in **Figure 2**. We probed the proliferative response of splenic CD4 and CD8 T cells of WT- or *lamp2* KO-nu mice to polyclonal TCR stimulation *in vitro*, and found no differences between groups as measured by CFSE analysis (**Figure S6A**). These results suggest that CD4 and CD8 T cells developing in *lamp2* KO thymic stroma respond to a polyclonal TCR-mediated stimulation. Lastly, we determined whether thymic defects in TCR repertoire formation influence the outcome of an immune response to a live intracellular pathogen. We employed a well-defined model of T-cell dependent recall response to *Listeria monocytogenes* (Lm) (Neighbors et al., 2001). Recipient mice were inoculated with $2 \cdot 10^3$ CFU (colony-forming units) of Lm at 12 weeks post thymic transplantation, to guarantee peripheral T cell reconstitution, and then rechallenged with $1 \cdot 10^6$ CFU 4 weeks later (**Figure S6B**). Non-thymic grafted nude recipients did not resist the infection protocol (**Figure S6C**), highlighting the requirement for a T cell-mediated response in this model. We assessed the T cell reconstitution in the spleen, and the clearance of Lm in the liver of infected WT-nu and *lamp2* KO-nu animals 3 days after the secondary challenge. Although reduced, the numbers of naïve and effector/memory splenic CD4 and CD8 T cells were not significantly altered in WT-nu or *lamp2* KO-nu (**Figure S6D**). All recipient mice were reconstituted. Despite the variability in CFU counts and the absence of complete bacterial clearance in both genotypes, the average CFU counts were slightly elevated in *lamp2* KO-nu mice, which presented an increase in the number of animals with a higher bacterial burden ($\geq \log_{10}^4$) (**Figure S6E**). Interestingly, the CFU counts inversely correlated with the amount of IFNG/IFN γ (interferon gamma)-producing CD4 found in *lamp2* KO-nude after the recall response. These cells

have been shown to provide protection against Lm (Neighbors et al., 2001). A similar association was observed in IFNG/IFN γ -producing CD8, but this correlation was not significant (**Figure S6E**). Although antigen-specific responses were not monitored, *lamp2* KO-nu appeared to present a compromised *in vivo* T-cell response to secondary infection by Lm. Together, our results suggest that thymic stroma deficient in *Lamp2* restricts not only the quantity of selected CD4⁺ T cells, but also the quality of their TCR repertoire.

Discussion

Following the discoveries on the contributions of CTSL and PRSS16/TSSP to the generation of selecting MHC II:peptides complexes (Gommeaux et al., 2009; Honey et al., 2002; Nakagawa et al., 1998; Viret et al., 2011), there have been few advances in the mechanism that regulates CD4⁺ T cell selection. Our findings suggest that LAMP2 may represent a functional liaison that interconnects autophagy and processing of self-peptides-MHC II for selection in cTECs, which in turn regulates the development of CD4 T cells and the diversity of their TCR repertoire. LAMP2 is expressed in TECs, but it was also detected in mesenchymal and endothelial cells of the thymic stroma. As non-TEC subsets do not have a major role in thymocyte selection (Klein et al., 2014), it is possible to conjecture that the defects in CD4 T cell development in *lamp2* KO thymic stroma may arise from LAMP2-dependent failures in TECs. Future studies with TEC-specific conditional knockout mice will provide more precise details on the cell-intrinsic role of LAMP2 in TECs. The strength and duration of the interaction between TCR and MHC II:self-peptide ligandome have implications in thymic selection (Gascoigne et al., 2016; Singer et al., 2008). Thymic stroma deficient in *Lamp2* led to a particular reduction in CD4 T cells that completed positive selection. While changes in TCRB^{int} CD69^{int} and TCRB^{hi} CD69^{hi} suggest defects in positive selection, alterations in TCRB^{hi} CD69^{hi} and TCRB^{hi} CD69^{neg} may reflect survival defects following positive selection, altered lineage commitment or negative selection. Our findings indicate that the development of CD4 T cells was not mechanistically associated with an abnormal redirection of MHC II-restricted cells into CD8 T cell lineage. Moreover, the reduced CD5 levels in TCRB^{int} CD69^{int} and TCRB^{hi} CD69^{hi} SP4s and the lack of alterations in IKZF2/HELIOS in DP and SP4 collectively suggested that TCR signaling and negative selection of SP4 thymocytes were not augmented. Instead, they may support the hypothesis that LAMP2 regulates the development of MHC II-restricted CD4 T cells that completed positive selection in the thymic cortex.

The observation that deficiency in *Lamp2* particularly affected autophagy flux in cTECs, but not mTECs nor other non-TEC thymic stromal cells, are in line with the reported high levels of autophagy in cTECs (Aichinger et al., 2013; Munz, 2016; Nedjic, Aichinger,

Emmerich, et al., 2008; Nedjic, Aichinger, & Klein, 2008) and the role of LAMP2 in lysosome-AP fusion (Eskelinen et al., 2002; Tanaka et al., 2000). Moreover, autophagic vesicles gain access to the MHC II compartment in cTECs (Kasai et al., 2009) and autophagy has been linked with the generation of the peptide repertoire presented by MHC I (Aichinger et al., 2013; Munz, 2016; Nedjic, Aichinger, Emmerich, et al., 2008; Nedjic, Aichinger, & Klein, 2008). We found that CTSL and lysosomal activities were elevated in *lamp2* KO cTECs, arguing against the possibility that alterations in CD74/Ii-MHC II processing results from a failure in its proteolytic function. The increased lysosomal activity was also reported in the hippocampus of *lamp2* KO mice (Michelle Rothaug, 2015), suggesting that LAMP2-mediated autophagy impacts lysosomal function. Several non-mutually exclusive possibilities might explain the reduction in SP4 selection by *lamp2*-deficient thymic stroma. The inhibition of AP-lysosome fusion in *lamp2* KO cTECs can compromise the shuttling of autophagy-derived cytoplasmatic selective antigens into the late endosomal MIIC loading pathway. Under these conditions, CTSL (Honey et al., 2002; Nakagawa et al., 1998), PRSS16/TSSP (Gommeaux et al., 2009; Viret et al., 2011) and other lysosomal proteases may not have access to substrates to produce MHC II-restricted self-peptides specialized for positive selection of CD4 T cells. This can lead to a compensatory upregulation of lysosomal activities as typically reported in models of lysosomal dysfunction or lysosomal storage disorders (Eskelinen et al., 2002). Additionally, the alteration in autophagic flux might also disturb CD74/Ii-MHC II trafficking to MIIC compartment or H2M function, thereby preventing the complete degradation of CD74/Ii or self-peptide loading. Furthermore, CTSL also contributes to the generation of MHC II-bound self-peptides presented by cTECs (Honey et al., 2002; Hsieh et al., 2002; Nakagawa et al., 1998). Thus, the increase in CTSL activity in *lamp2* KO cTECs could also interfere with the quality of MHC II-peptide ligandome presented by these cells during positive selection. Hence, the incomplete degradation of CD74/Ii-MHC II in *lamp2* KO cTECs may result from altered trafficking of CD74/Ii-MHC II to MIIC and/or from a reduction in MHC II-loaded with positive selection-inducing self-peptides, due to an altered antigen processing and peptide repertoire generation. The consequent change in the composition of selective MHC II ligands may lead to a reduction of CD4 T cells that are completing positive selection and a skewing of their TCR repertoire. It will be important to elucidate the biochemical mechanism by which LAMP2 potentially regulates MHC class II-peptide generation in cTECs. Under regular conditions, endogenous antigens may access MHC II molecules through several LAMP2-regulated autophagic routes (Eskelinen et al., 2002; Kaushik & Cuervo, 2012). Future studies should also aim at investigating whether LAMP2-dependent macroautophagy delivers cytosolic antigens into autolysosomes for processing before loading to MHC II, and/or antigens are directly routed into the lysosome via CMA. In this regard, ATG5-dependent autophagy has been linked to

positive selection of CD4 T cells (Nedjic, Aichinger, Emmerich, et al., 2008; Nedjic, Aichinger, & Klein, 2008), but it also contributes to mTEC-mediated tolerance induction (Aichinger et al., 2013; Schuster et al., 2015). Worth noting, *lamp2*-deficiency did not cause major defects in mTEC-dependent function nor disturbed tolerance, as measured by the presence of autoantibodies/lymphocytic infiltrates at 12 weeks post-transplantation (data not shown). Future studies should address whether breaks in tolerance can unfold in aged settings. Alternatively, autophagy may have distinct roles in c/mTEC function, whereby the specificity of the route and its consequence in thymic selection, can also be imposed by distinct downstream elements. While ATG5 participates in early phases of autophagy and may have a broader effect in cTEC- and mTEC-mediated thymic selection (Nedjic, Aichinger, Emmerich, et al., 2008; Nedjic, Aichinger, & Klein, 2008), LAMP2 mostly regulates later stages of the autophagic process and MHC II processing in cTECs. Future analysis should examine whether changes provoked by *lamp2*-deficiency in cTECs account for a more dedicated role in the control of CD4 T cell positive selection.

The observations that *lamp2*-deficiency in thymic stroma induced a skewing, but not a complete block, in CD4 TCR repertoire diversity, suggests that LAMP2 may regulate the generation of particular sets of autophagy-dependent MHC II-bound positive selecting peptides in cTECs. This hypothesis is in line with previous studies (Wong et al., 2000) supporting that positive selection is limited by the bioavailability of specific self-peptide/MHC ligands expressed by cTECs. Still, previous studies showed that a single MHC-peptide ligand can positively select multiple TCRs (Fukui et al., 1997; Ignatowicz et al., 1996), indicating that positive selection is relatively permissive regarding the composition of MHC-peptide ligands in cTECs. Assuming that LAMP2 contributes to the generation of particular peptide:MHC II ligands, its deficiency may have variable effects depending on the plasticity of a given TCR. Future analysis employing TCR transgenics will allow defining LAMP2-dependent specificities and the contribution of LAMP2 in positive and negative selection. Additionally, post-selected thymocytes expressing high-affinity TCR intrathymically proliferate in response to their self-peptide/MHC ligands (Le Campion et al., 2002). Thus, the dominance of particular clonotypes within the *lamp2*-deficient thymus may result from the expansion of SP4 T cells bearing favorable TCR affinities for overrepresented self-peptide/MHC ligands. Importantly, the TCR repertoire analysis was conducted in total SP4 cells, without segregating conventional and regulatory CD4 T cells. Although T reg comprises a small fraction of thymic SP4, one can only presume that their TCR repertoire is also affected. Future analysis is required to clarify the impact of LAMP2 in TCR repertoire formation and the function of T regulatory cells. Lastly, the defect in thymic production imposed by *lamp2*-deficiency impacted on the peripheral T cell pool, mostly on the numbers

of naïve CD4 T cells, and to a less extent on naïve CD8 T cells and effector/memory CD4 and CD8 T cells. In the thymic transplant of Nude mice, the establishment of the peripheral T cell pool depends on thymic output and LIP (lymphopenia-induced proliferation). As thymic CD8 T cell development was not affected, the reduced peripheral CD8 T cell pool may arise indirectly from insufficient CD4 T cell help. In this regard, it was shown that the LIP of CD8 T cells depends on CD4 T cells (Hamilton & Jameson, 2008; Hamilton et al., 2006; Le Saout et al., 2008). Additionally, our results indicate that CD4 and CD8 T cells, which developed in *lamp2*-deficient thymic stroma, respond to polyclonal TCR-mediated signaling. Yet, *lamp2* KO-nude recipients appear to hold a compromised T cell-dependent recall immune response to Lm infection. Future studies should clarify the peripheral consequences of these thymic defects, including a more in-depth analysis of T cell homeostasis, antigen-specific responses and tolerance, as well as the role of CD4 T cell in B cell responses and CD8 T cell memory.

In sum, our findings suggest that *lamp2*-deficiency in thymic stroma conditions the development and TCR repertoire formation of CD4 T cells. These findings provide a novel framework to further investigate how LAMP2 functionally interconnects autophagy and lysosomal generation of selecting self-peptides in cTEC, and its implications for CD4 T cell positive selection. Moreover, our results may also open new avenues for the identification of the “peptidic-self” in the thymus, which remains one of the crucial gaps in our understanding of thymus biology.

Material and Methods

Mice

We used *lamp2* KO, *b2m* KO, RFP-GFP-LC3 Tg, TCR:V β 6 Tg and Nude mice that have been described previously and are all in a C57BL/6 background (Hassler et al., 2019; Koller et al., 1990; Li et al., 2014; Tanaka et al., 2000). Mice were housed under specific pathogen-free conditions, and all animal experiments were performed in accordance with European guidelines (Directive 2010/63/EU). For fetal studies, the day of the vaginal plug detection was designated as embryonic day (E) 0.5.

Thymic kidney transplants

Thymi from WT and *lamp2* KO mice were transplanted under the kidney capsule of recipient mice as described (Meireles et al., 2017). Briefly, thymic lobes from E15.5 WT and *lamp2* KO mice were cultured for 2 days with 1.35 mM 2'-deoxyguanosine (SIGMA, D0901) to deplete lymphoid cells. Prior to transplantation recipient mice were pre-treated with the analgesic buprenorphine (0.05 mg/kg of body weight) and anesthetized with a gaseous mixture of 3% isoflurane and oxygen.

TEC and hematopoietic cell isolation

TECs were isolated as described (Meireles et al., 2017; Rodrigues et al., 2017; Rodrigues et al., 2018). Hematopoietic cells from thymus and spleen were prepared by mechanical disruption of the respective tissues. Splenic red blood cells were lysed using erythrocyte lysis solution: 155 mM ammonium chloride (Sigma-Aldrich, A9434), 10 mM potassium bicarbonate (Sigma-Aldrich, 237,205).

Flow cytometry

Cells were pre-treated with FC block (anti-FCGR3/CD16-FCGR2B/CD32 antibodies TruStain fcX; Biolegend, Biolegend, 101,320). Cell suspensions were stained as described (Crotzer et al., 2010; Tanaka et al., 2000; Zhou et al., 2005) with Alexa Fluor 488/FITC-conjugated anti-CD8A (clone 53-6.7; eBioscience, 11-0081-82), anti-CD44 (clone IM7; Biolegend, 103,022), anti-15G4 (Santa Cruz Biotechnology, sc-53,946), anti-CD5 (clone 53-7.3; Biolegend, 100,605), anti-PECAM1/CD31 (clone MEC13.3; Biolegend, 102,514) and anti-CCL21 (clone 59,106; R&D Systems, IC457G-100UG); PE-conjugated

anti-CD4 (clone GK1.5; eBioscience, 12–0041-82), anti-SELL/CD62L (clone MEL-14; eBioscience, 12–0621-81), anti-TCRB (clone H57-597; eBioscience, 12–5961-82), anti-CD80 (clone 16–10A1; eBioscience, 12–0801-82), anti-ENPEP/LY51 (clone 6C3; eBioscience, 12–5891-82), anti-LAMP2 (clone M3/84; Biolegend, 108,505), anti-CD40 (clone 3/23; BD Pharmingen, 553,791) and anti-MHCIDb (clone 28–14-8; eBioscience, 12–5999-82); PerCPy5.5-conjugated anti-PTPRC/CD45.2 (clone 104; Biolegend, 109,828), anti-TCRB (clone H57-597; eBioscience, 45–5961-80) and anti-MHCIKb (clone AF6-88.5; Biolegend, 116,515); PerCPeFluor710 anti-CD4 (clone GK1.5; eBioscience, 46–0041, 82); PECy7-conjugated anti-IL2R/CD25 (clone PC.61.5; eBioscience, 25–0251-82) and anti-CD69 (clone H1.2F3; eBioscience, 25–0691-81); APC/eFluor660-conjugated anti-CD8 (clone 53–6.7; eBioscience, 50–0081-82), anti-CD80 (clone 16–10A1; eBioscience, 17–0801-82), anti-EPCAM (clone G8.8; Biolegend, 118,212), anti-CD40 (clone 1C10; eBioscience, 17–0401-81) and anti-CD74 (In1/CD74, Biolegend, 151,003); APC-eFluor780-conjugated anti-I-A/I-E (clone M5/114-15-2; eBioscience, 47–5321-82); eFluor450-conjugated anti-CD24 (clone M1/69; eBioscience, 48–0242-82); BV421 conjugated anti-EPCAM (clone G8.8; Biolegend, 118,225) and anti-CD69 (clone H1.2F3; Biolegend, 104,527); BV510 conjugated anti-CD24 (clone M1/69; Biolegend, 101,831); BV605 conjugated anti-SELL/CD62L (clone MEL-14; Biolegend, 104,437); anti-PDGFR α (clone APA5; Biolegend, 135,916); BV650 conjugated anti-CD80 (clone 16–10A1; Biolegend, 104,731) and anti-CD8A/CD8 α (clone 53–6.7; Biolegend, 100,741); BV785 conjugated anti-CD4 (clone GK1.5; Biolegend, 100,453). The binding of biotinylated Ulex europaeus agglutinin-1 (UEA-1) (Vector Laboratories, B-1065-2), anti-ENPEP/LY51 (clone 6C3; BD Pharmingen, 553,159), anti-LY75/CD205 (clone NLDC-145; Biolegend, 138,211), and anti-PDGFR β (clone APB5; Biolegend, 136,009) were revealed by either PE-Cy7-conjugated (eBioscience, 25–4317.82) or BV711-conjugated (Biolegend, 405,241) streptavidin. For intracellular staining, cells were prepared according to the supplier's protocol (FOXP3 staining kit, eBioscience, 00–5523-00) and stained with FITC-conjugated anti-IL2F2/HELIOS (clone 22F6; Biolegend, 137,204), APC/eFluor660-conjugated anti-FOXP3 (clone FJK-16s; eBioscience, 2,059,207) and anti-Aire (clone 5H12; eBioscience, 1,929,296) antibodies. Flow cytometry was performed on a LSRFortessa, with data analyzed on FlowJo software (BD). Cell sorting was performed on a FACS Aria I (BD), with purities >96%.

Immunohistological analysis

Thymi were prepared for immunofluorescence as described (Rodrigues et al., 2017). Briefly, samples were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), and

8-µm sections were stained with biotinylated-UEA-1 (Vector Laboratories, B-1065-2) and anti-rat KRT8/cytokeratin 8 (Troma-1; DSHB, AB 531826) as primary antibodies, with Alexa Fluor 647 anti-rat (Invitrogen, A21247) and streptavidin Alexa Fluor 555 (Invitrogen, S21381) as secondary antibodies. Vectashield mounting medium with DAPI (Vector Laboratories, H-1200-10) was used to prepare the slides. Analysis was performed in an AxioImager.Z1 (Zeiss). Images were processed with and Fiji Software.

Autophagy analysis

We stained cells with Cyto-ID autophagy detection kit (Enzo, ENZ-51031-0050) according to the manufacturer's protocol. In brief, cells were first washed with PBS, mixed with Cyto-ID staining solution and incubated at 37°C for 30 min (Cyto-ID) and subjected to flow cytometry analysis. To block autophagy, thymi were cultured with chloroquine (50 µM; Enzo, ENZ-51031-0050) for 16 h and Baf-A1 (0.5 µM; SIGMA, B1793) for 5 h.

CTSL and lysosomal activity assays

CTSL and lysosome activities were respectively monitored using Magic Red CTSL (ImmunoChemistry Technologies, 941) and Lysosome-Specific (Biovision, K448) detection kits, according to the manufacture's protocol. Briefly, cells were either incubated with a magic red staining solution containing the CTSL fluorogenic substrate or Lysosome-Specific Self-Quenched Substrate for 1 h at 37°C in DMEM (10% FBS). Cells were then washed and stained with TEC markers for flow cytometry analysis. In cells with active CTSL, the substrate fluoresces red upon proteolytic cleavage. The fluorescent signal is proportional to the intracellular lysosomal activity in cells with active lysosomes.

TCR sequencing

SP4 thymocytes (1.0×10^6 cells) derived from TCR:Vβ6Tg hematopoietic progenitors and developing in WT or *lamp2* KO thymus grafts were purified by cell sorting. RNA was extracted using RNeasy Mini kit (Qiagen, 74,106) following the manufacturer's instructions. Full-cDNA library was prepared using Mint-2 kit (Evrogen, SK005), which introduces 5'-adapters to cDNA fragments, according to the manufacturer's instructions. The *Tcra* gene was then specifically amplified using Platinum™ Taq DNA polymerase high fidelity (Invitrogen, 11,304,011) and a primer pair specific for the 5'-adapter and the C region of the *Tcra* gene. The sequencing 436 P. M. RODRIGUES ET AL. library was prepared using the

Nextera kit (Illumina, FC-131-1024), in which each sample was barcoded, and sequenced using 250 bp paired-end illumina MiSeq technology. High-throughput sequencing were performed at Gene Core facility (EMBL, Germany).

Deep sequencing data analysis

Paired-end 250 bp illumina sequencing data were initially trimmed using Trimmomatic and subsequently merged using PEAR. clonotypeR toolkit was then used to perform TCR sequence annotation and quantitative analysis in R (Bolger et al., 2014; Maceiras et al., 2017; Zhang et al., 2014). Out of the X raw reads obtained from the 6 samples (3 WT and 3 *lamp2* KO), we identified X-Y TCR clonotypes from X-Z productive TCR sequences. For the samples to be comparable, the analyses were performed on 60,000 randomly selected TCR sequences for each data set as it was the lowest number of TCR sequences found in a data set. The presented clonality metric is 1 – Pielou's evenness index and can vary from 0 to 1 (more diverse to less diverse). The Pielou's evenness corresponds to the Shannon's entropy (using log 2) for each sample divided by the number of unique clonotypes (in log 2) of the same sample (Maceiras et al., 2017; Stewart et al., 1997).

***In vitro* T cell activation**

WT and *lamp2* KO-derived CD4 and CD8 T cells were sorted, labeled with 1 μ M of CellTrace™ CFSE (carboxyfluorescein diacetate succinimidyl ester; ThermoFisher Scientific, C34554) and cultured in the presence of 1 μ g/ml anti-CD3 mAb (clone 145–2C11; BD Pharmingen, 553,057) and 5 μ g/ml anti-CD28 mAb (clone 37.51; BD Pharmingen, 553,294). The precursor frequency of dividing cells (percentage of cells in the initial population that undergone one or more divisions after culture) was calculated as follows: $[\sum n \geq 1 (P_n/2^n)] / [\sum n \geq 0 (P_n/2^n)]$, where n is the division number that cells have gone through and P_n is the number of cells in division, as described (Alves et al., 2007).

Listeria infection

Mice were inoculated with Lm reference strain EGDe as described in (Glaser et al., 2001). Thymic grafted Nude recipients were intravenously infected 12 weeks post-transplantation, through the tail vein with 2×10^3 colony-forming units (CFUs) of Lm. Mice were rechallenged with 1×10^6 CFUs of Lm 28 days after the first priming. Three days after secondary infection, mice were sacrificed, livers were aseptically removed, homogenized in PBS, and

homogenates were serially diluted and plated on BHI-agar (Fisher Scientific, 11,708,872). Lm colonies were enumerated after 24 h incubation at 37°C. For the intracellular IFNG/IFN- γ analysis, total spleen cells were stimulated for 5 h at 37°C, using the Cell Activation Cocktail, containing phorbol 12-myristate 13-acetate, ionomycin and brefeldin A (Biolegend, 423,304) according to the manufacturer's instructions. After cell surface staining, cells were prepared according to the supplier's protocol (FOXP3 staining kit; eBioscience, 00-5523-00), stained with PE-labeled anti-IFNG/IFN γ Ab (clone XMG1.2; Biolegend, 505,807) and analyzed by flow cytometry.

Statistical analysis

Analysis was performed using Prism 9.1.1 software (GraphPad Software). The two-tailed Mann-Whitney test was used for statistical differences between groups. For multiple comparisons, a two-way ANOVA was used. $p < 0.05$ was considered significant.

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Supplemental Information

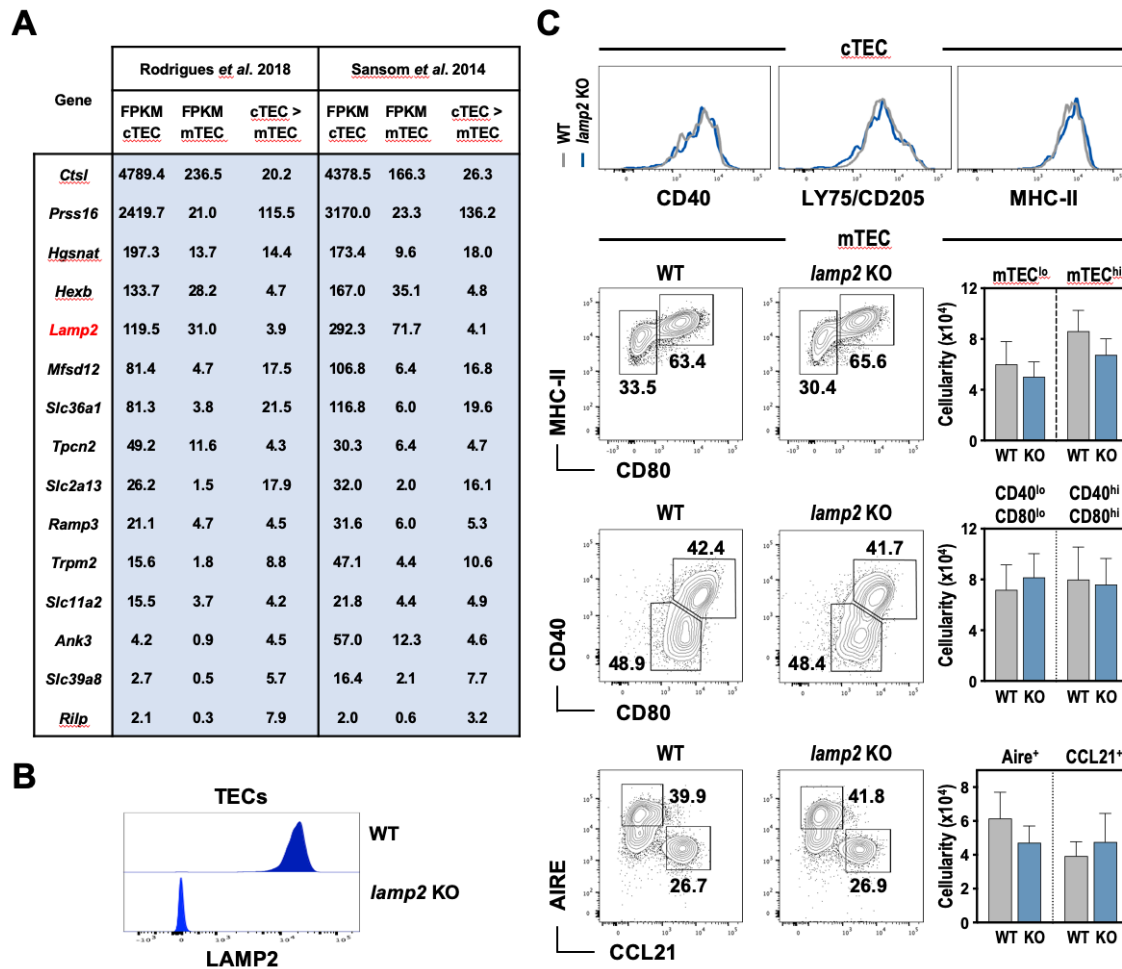


Figure S1. LAMP2 expression in the thymus. (A) The gene list shows the top, most highly expressed genes that are associated with the gene ontology term "lysosomes", obtained from RNA sequencing analysis of cTECs and mTEC of postnatal thymus [21-25]. cTEC/mTEC columns represent fold change. (B) LAMP2 expression (flow cytometry) in TECs (PTPRC/CD45⁺ EPCAM⁺) from embryonic day 14.5 WT and *lamp2* KO mice. (C) Analysis of TECs from WT and *lamp2* KO thymus from 2-week-old mice for a broad panel of cTEC- and mTEC-specific markers. Top row: Histograms show the expression of CD40, LY75/CD205 and MHC II in WT and *lamp2* KO cTECs. Dot plots show the expression of CD80 and MHC II (2nd row), CD80 and CD40 (3rd row) and AIRE and CCL21 (bottom row) in WT and *lamp2* KO mTECs. Graphs represent the average cellularity of respective mTEC subsets (mTEC^{lo/hi}, CCL21⁺ and AIRE⁺). Results in C are shown as mean ± SEM.

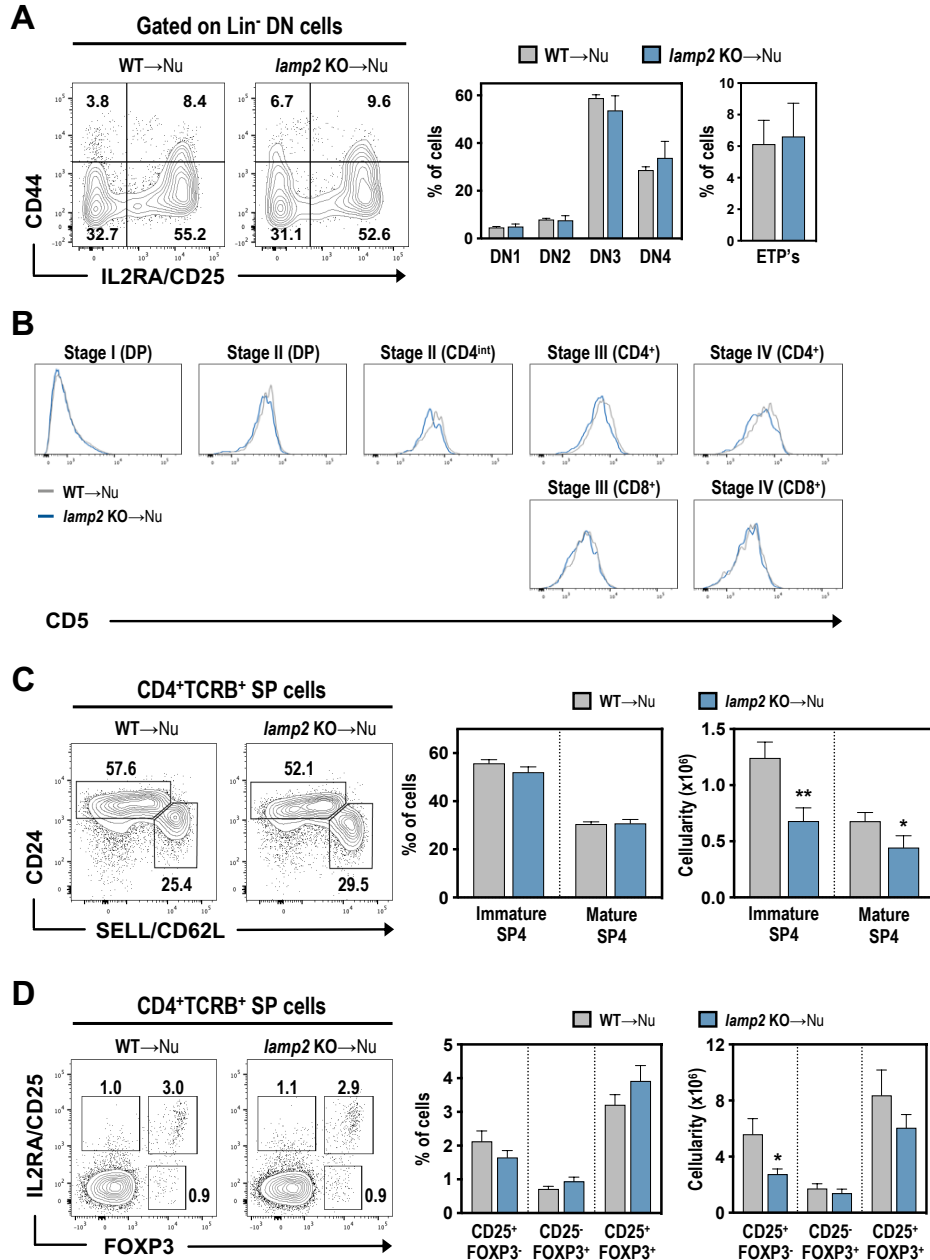


Figure S2. Thymic T cell development in WT- and *lamp2* KO-Nude mice. (A) Flow cytometry analysis of early stages of T cell development in thymic grafts of Nude recipients transplanted with WT (WT-Nu) and *lamp2* KO (*lamp2* KO-Nu) ectopic thymus, 8-10 weeks after thymic transplantation. Representative plots of CD44 and IL2RA/CD25 expression on DN thymocytes (gated on Lin⁻ cells). ETPs are defined as Lin⁻ CD177⁺ CD44⁺ IL2RA/CD25⁻. (B) Histograms show representative CD5 expression in DPs from TCRB^{neg} CD69^{neg} (Stage I), DPs and CD4^{int} from TCRB^{int} CD69^{int} (stage II), SP4 and SP8 from TCRB^{hi} CD69^{hi} (stage III) and TCRB^{hi} CD69^{neg} (stage IV) thymocytes derived from WT-Nu (gray) and *lamp2* KO-Nu thymus. (C, D) Dot plots show flow cytometry analysis of (C) SP4 maturation (CD24 and SELL/CD62L expression) and (D) T regulatory cell differentiation (IL2RA/CD25 and FOXP3 expression) in CD4 SP cells (CD4⁺ TCRB⁺). Graphs represent the mean ± SEM of the frequency and cellularity of indicated subsets. * p < 0.05, ** p < 0.01.

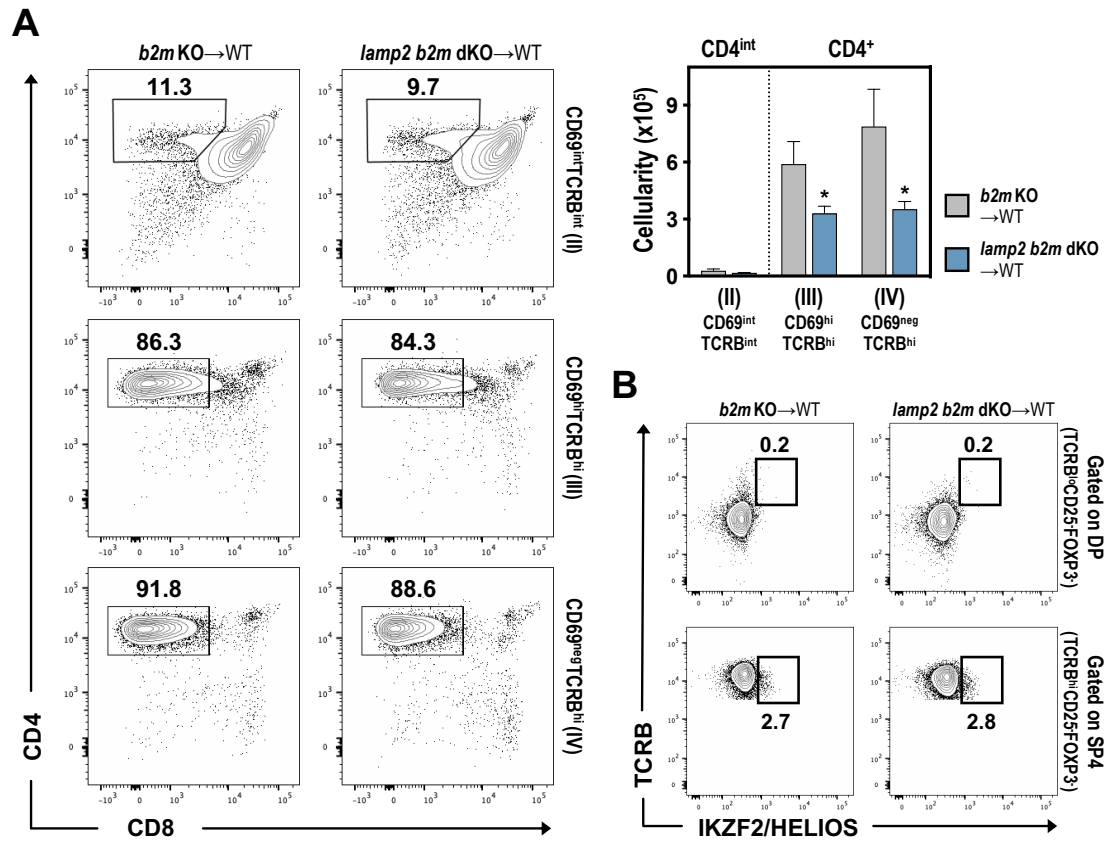


Figure S3. T cell development in *b2m* KO and *lamp2b2m dKO* thymus. (A) CD4/CD8 expression within stages II (TCRB^{int} CD69^{int}), III (TCRB^{hi} CD69^{hi}) and IV (TCRB^{hi} CD69^{neg}) is defined on total thymocytes. Bar graphs represent mean ± SEM absolute numbers of the indicated thymocyte subsets in *b2m* KO-WT (gray) and *lamp2b2m dKO*-WT ectopic thymi. * P < 0.05. **(B)** Dot plots show representative data of TCRB and IKZF2/HELIOS expression within DP (TCRB^{lo} IL2RA/CD25⁺ FOXP3⁻) and SP4 (TCRB^{hi} IL2RA/CD25⁺ FOXP3⁻) in *b2m* KO-WT and *lamp2b2m dKO*-WT ectopic thymi.

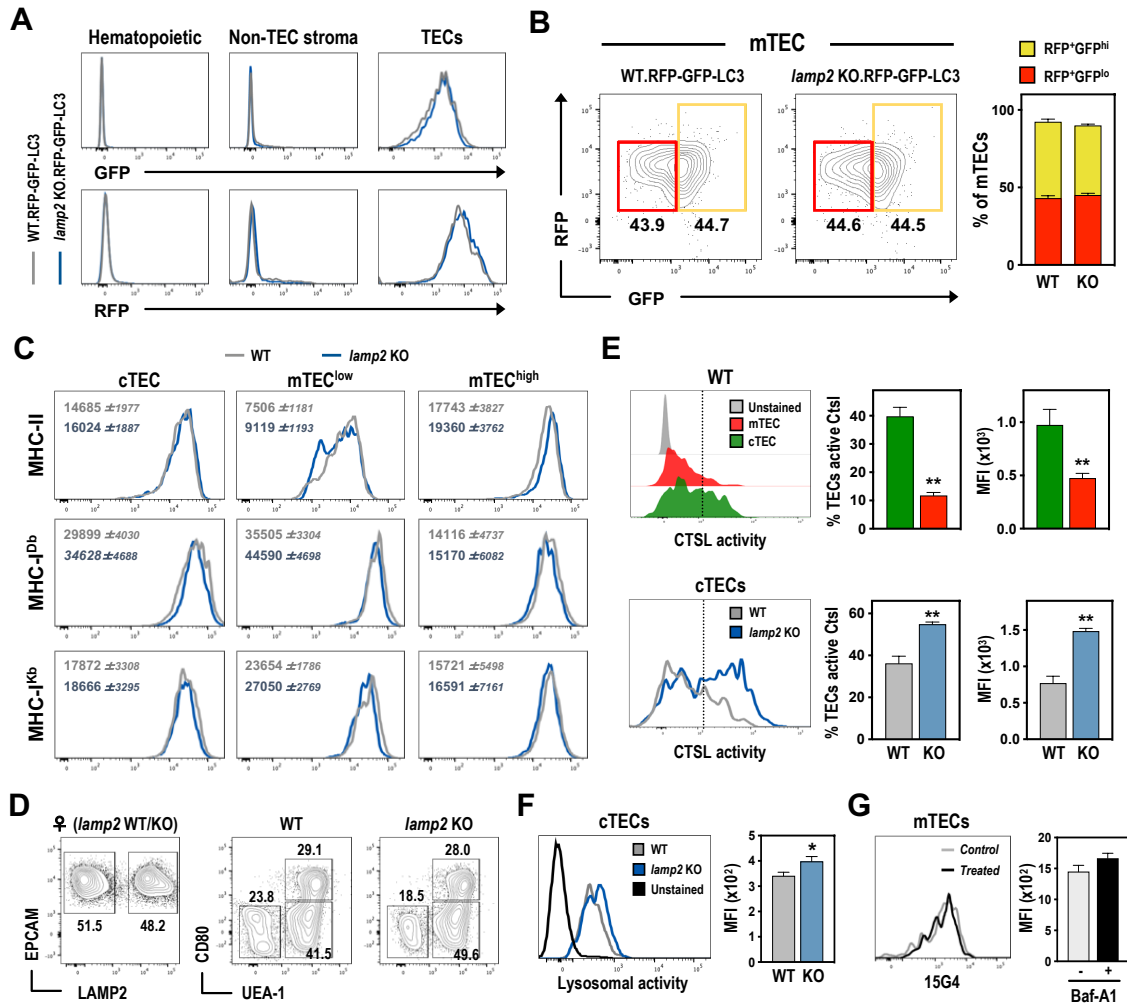


Figure S4. Analysis of MHC II processing and autophagy in *lamp2* KO TECs. Flow cytometry analysis of (A) hematopoietic (PTPRC/CD45⁺ EPCAM⁺), non-TEC stromal (PTPRC/CD45⁺ EPCAM⁺) and TECs (PTPRC/CD45⁺ EPCAM⁺) of WT (gray) and *lamp2* KO RFP-GFP-LC3 mice. (B) TECs isolated from WT and *lamp2* KO RFP-GFP-LC3 mice. Dot plot represents GFP and RFP expression on mTECs. (C) TECs from 12 days old WT and *lamp2* KO mice. Histograms show representative MHC-II (I-Ab) and MHC-I (H2-Kb and H2-Db) staining in cell surface of cTEC (ENPEP/Ly51⁺ UEA-1⁺), mTEC^{low} (ENPEP/Ly51⁺ UEA-1⁺ CD80^{low}) and mTEC^{hi} (ENPEP/Ly51⁺ UEA-1⁺ CD80^{hi}) in WT (gray) and *lamp2* KO mice. Numbers represent the MFI (mean fluorescent intensity) in the indicated subsets. Data shown in A represents an average of 2 independent experiments with 5 WT and 6 *lamp2* KO thymus. (D) LAMP2 expression in TECs from 12 days old *lamp2* WT/KO heterozygous mice. Dot plots represent CD80/UEA-1 analysis in WT and *lamp2* KO TECs from *lamp2* WT/KO heterozygous thymus (n=6). (E) Histograms show representative assessments of CTSL activity in cTECs (green) and mTECs (red) from WT thymus, and histograms show cTECs from WT (gray) and *lamp2* KO (bottom), as measured by the usage of Magic Red cathepsin L detection kit. (F) Histograms show representative assessments of lysosomal activity in WT (gray) and *lamp2* KO cTECs. (G) Postnatal day 3-5 WT thymus were treated with Baf-A1 (0.5 μ M) for 5h. Histograms show the representative staining with 15G4 staining in control (light gray) and Baf-A1-treated (black) in WT mTECs. Graphs represent the MFI in control (gray) and treated (dark black) mTEC (n= 3-4 animals). Data shown in (B-E) represents an average of 2 independent experiments with 4 WT and 7 *lamp2* KO thymus. Graphs represent mean $\pm$ SEM frequency of TEC subsets. * P < 0.05; ** P < 0.01.

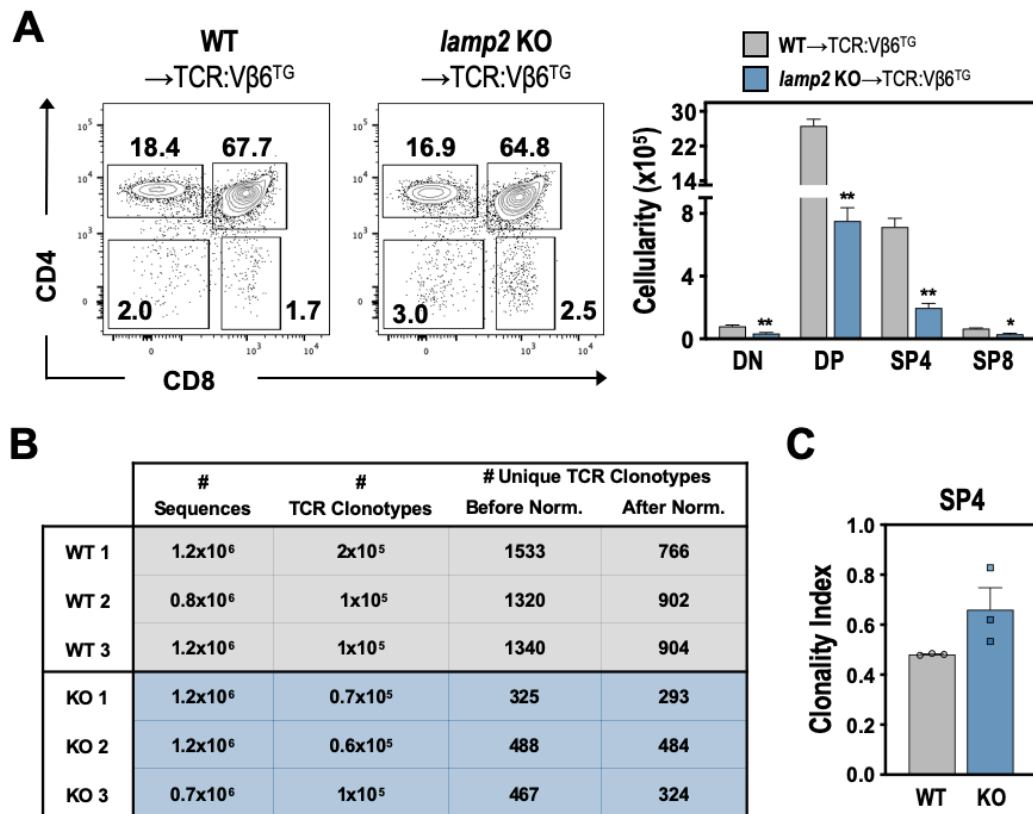


Figure S5. Analysis of CD4⁺ TCR repertoire in *lamp2* KO thymus. (A) Dot plots show flow cytometry analysis of CD4 and CD8 expression on total thymocytes from WT (WT-TCR:VB6^{TG}) and *lamp2* KO (*lamp2* KO-TCR:VB6^{TG}) ectopic thymus transplanted under the kidney capsule of TCR:VB6^{TG} transgenic mice. Bar graphs are average absolute numbers of the indicated thymocyte subsets in control (gray) and mutant ectopic thymus. (B) Table shows the total number of sequences, total and unique (before and after normalization), TCRA clonotypes in SP4 thymocytes generated in WT (n=3) and *lamp2* KO (n=3) ectopic thymus. (C) Clonality index of WT- and *lamp2* KO-derived SP4 cells. Results are shown as mean $\pm$ SEM. * P < 0.05; ** P < 0.01.

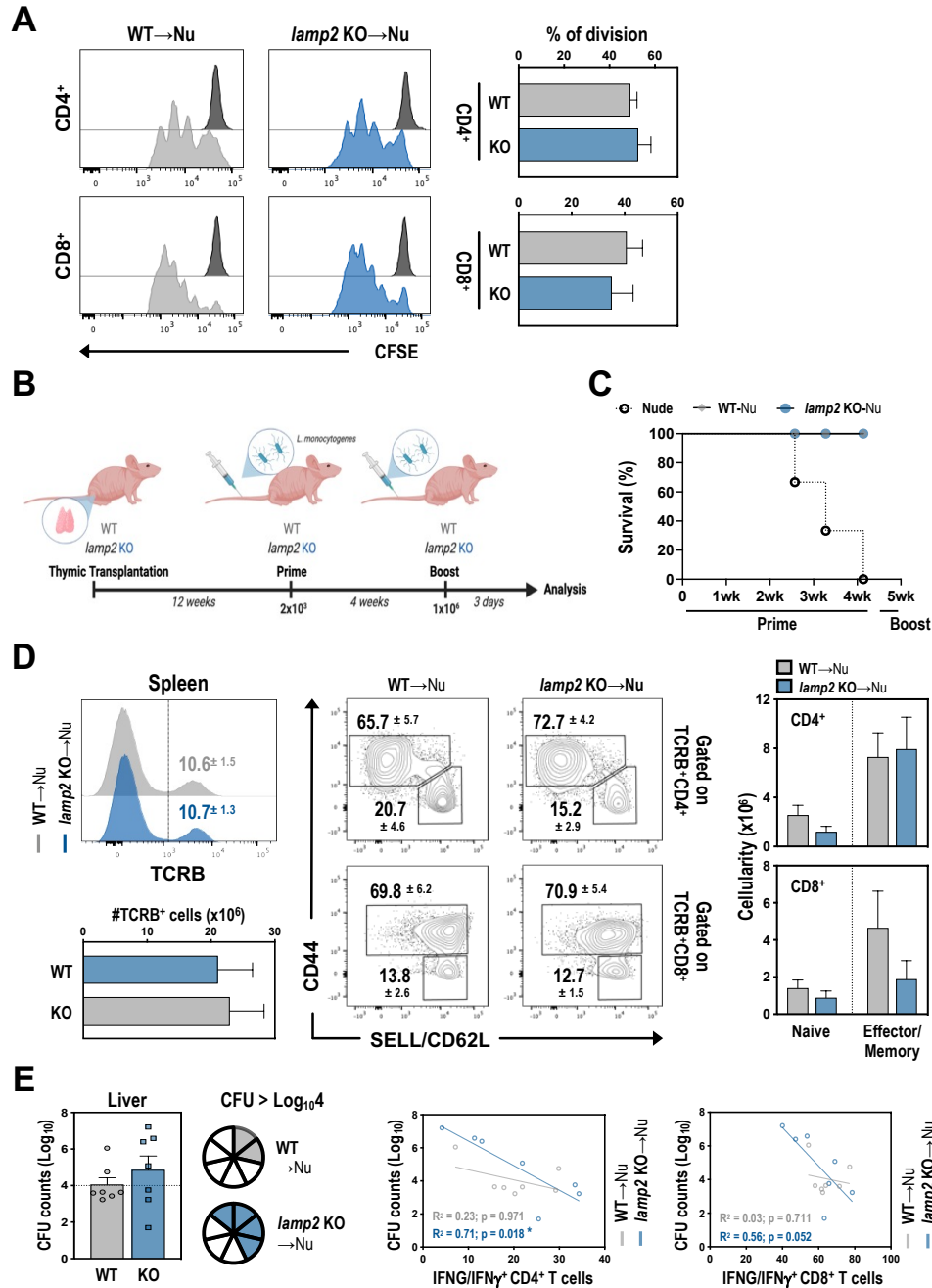


Figure S6. Analysis of peripheral T cell compartment and immune response of WT- and *lamp2* KO-Nude mice. (A) Sorted and CFSE-labeled CD4⁺ and CD8⁺ T cells were stimulated *in vitro* with anti-CD3/anti-CD28 for 3 days. Histograms show representative experiments. Graph represents the precursor frequency of dividing T cells. Results are presented as mean ± SEM of 2 independent experiments (n=4). **(B)** Scheme of recall immune response protocol with Lm. Recipient mice were immunized with 2 × 10³ colony forming units (CFU) of Lm at 12 weeks post thymic transplantation and then rechallenge with 1 × 10⁶ CFU of Lm 4 weeks later. **(C)** Survival curve of Nude (n=3), WT-Nu (n=7) and *lamp2* KO-Nu (n=7). **(D)** Analysis of peripheral T cells in spleens from infected WT- Nu and *lamp2* KO-Nu mice. Graphs (left) represent the numbers of TCRB⁺ T cells in the spleen. Representative dot plots show CD44 and SELL/CD62L expression within CD4⁺ and CD8⁺ T cells. Graphs (right) show average cellular density of naïve (SELL/CD62L⁺ CD44^{lo}) and effector/memory (SELL/CD62L⁺ CD44^{hi}) CD4⁺ and CD8⁺ T cells. **(E)** Bacterial CFUs in the liver were determined by plating serial dilutions of organ homogenates onto BHI agar. Graphs represent the correlation between CFU counts in the liver and the percentage of IFNG producing CD4 and CD8 T cells in individual WT-Nu and *lamp2* KO-Nu mice. Data are representative of an experiment with 7 mice per group. Results are shown as mean ± SEM.

Chapter IV

General Discussion and Concluding Remarks

General discussion

The thymus represents a unique site for the generation of T lymphocytes, one of the crucial effectors of adaptive immunity (Miller, 2011). Its particular function is mostly regulated by two main types of specialized epithelial subsets, namely cortical and medullary TECs. These distinct subsets provide instructive cues to developing thymocytes that promote their proliferation, survival, selection and maturation (Kadouri et al., 2020). Thus, understanding the molecular mechanisms that control TEC differentiation and function is of absolute importance in order to harness the foundations of T cell biology. In this thesis, we explore the early molecular events that control the establishment of the thymic epithelium during organogenesis (**Chapter II**) as well as study the antigen processing features of cTECs and their impact in the generation of a broad T cell repertoire (**Chapter III**).

Section 1

The multifaceted role of Foxn1 is crucial for the establishment of an intact and functional thymic stromal microenvironment

The expression of Foxn1 is indispensable to promote TEC differentiation in the embryo and TEC homeostasis in the postnatal life (Romano et al., 2013). Additionally, it controls the expression of key genes involved in thymopoiesis (Zuklys et al., 2016). In its absence, TEC differentiation does not occur and consequently, the thymus becomes a small alymphoid structure that is unable to nurture T cell development (Blackburn et al., 1996). Nevertheless, in mice bearing mutations on Foxn1, the early stages of thymus organogenesis do still take place until the formation of the common thymic and parathyroid domain (Nehls et al., 1996). Therefore, one might consider that thymic organogenesis can be subdivided into Foxn1-independent and Foxn1-dependent events. Yet, the events that take place before the induction of this transcription factor remain puzzling, as well as the effective role of Foxn1 in the establishment of the TEC microenvironment.

While the precise details of the events that take place prior to Foxn1 expression remain unclear, some molecules were demonstrated to be expressed in a Foxn1-independent fashion. Curiously, the expression of IL-7, a cytokine that is vital for thymopoiesis, also ensues in a Foxn1-independent fashion (Zamisch et al., 2005). Its expression is restricted to the thymic domain and begins immediately before the induction of Foxn1, suggesting that IL-7 might serve as an early marker to identify thymic-fated cells. Indeed, IL-7 transcripts are detected within the thymic rudiments of nude mice, which demonstrates that the induction of this cytokine occurs independently of Foxn1-mediated signaling (Zamisch et al., 2005).

It is intriguing to consider that a molecule that is described to be fundamental for thymopoiesis appears to be induced so early during thymic ontogeny, prior to the arrival of the first wave of T cell precursors (Zamisch et al., 2005). Whether IL-7 might be required during early stages to functionally prepare the thymic epithelium to support subsequent T cell development before hematopoietic colonization or if it plays other roles during this developmental window remains unclear. In this regard, it would also be interesting to explore the dynamics of IL-7 expression within the distinct clusters of Foxn1^{GFP}:Foxn1^{nu/nu} pro-TECs that we have identified in our single-cell transcriptomic analysis.

Although the early events that control the induction of Foxn1 remain undetermined, it has been proposed that, in a Foxn1-deficient setting, TECs are sustained in a progenitor stage that can be reversed once Foxn1 expression is restored (Bleul et al., 2006). Despite the fact that it has been proposed that the transcriptional network that lies upstream of Foxn1 is stable and Foxn1-deficient cells retain their thymic epithelial lineage specification, our data actually proposes that Foxn1-inducing machinery is dynamically regulated. Nonetheless, the ectopic transplantation of E9 isolated endoderm tissue from mouse embryos into the kidney capsule of syngeneic recipients revealed the appearance of both cortical- and medullary-like compartments (Gordon et al., 2004). These observations suggest that the early embryonic endoderm tissue contains cells with the capacity to commit and develop towards the TEC lineage. The fact that TEPs retain the capacity to differentiate into mature TECs once Foxn1 is expressed offers the possibility to regenerate TEC microenvironments via manipulation of Foxn1 levels and thus restore thymic function. Yet, the inability to isolate TEPs has precluded the assessment of their *in vitro* or *in vivo* stemness potential. Studies have reported the existence of a rare population of epithelial stem cells in the adult thymus that displays a low-cycling rate and self-renewal properties as well as bipotent abilities, by giving rise to both cTEC and mTEC subpopulations (Ucar et al., 2014; Wong et al., 2014). Moreover, other studies have suggested the existence of a self-renewal population within the embryonic TEC compartment that expresses the claudins 3 and 4 and preferentially gives rise to mature mTECs throughout life (Sekai et al., 2014). Within this population, they have also identified a fraction of stage specific embryo antigen 1 (SSEA-1)-expressing cells, which is a marker commonly associated with stem cells. They propose that these cells represent a population of mTEC-restricted stem cells that sustain the mTEC population with age and therefore ensure the establishment of central tolerance. Along with these potential mTEC-restricted stem cells, a population of podoplanin-expressing precursors have been proposed to represent lineage-committed mTEC progenitors (Onder et al., 2015). Yet, whether these cells represent bonafide stem cells is arguable. In parallel, the identification of stem cells within the human thymus has also been a desire to accomplish. A recent study

has explored the postnatal human thymus at the single-cell level and it has identified a population of stem cells that express a panel of cytokeratins (Ragazzini et al., 2023). These reside within the subcapsular and perivascular regions of the thymus and display clonogenic properties. The identification of cells with stemness potential has major implications for the clinic, with the possibility to rejuvenate thymic function and ameliorate thymic-associated disorders. With our available transcriptomic data on Foxn1^{GFP}:Foxn1^{nu/nu} pro-TECs, it would be interesting to explore the expression of these progenitor-associated markers within the distinct clusters to understand whether these would be retained in a Foxn1-deficient environment. Pro-TECs may retain the expression of some of these molecules, as these might represent progenitor cells that are arrested in their differentiation. This would mean that the maintenance of their progenitor identity would not require the expression of Foxn1, as it has been proposed (Bleul et al., 2006; Nehls et al., 1996), and it would allow us to further explore the transcriptomic and phenotypical properties of these cells. Furthermore, studies have suggested that Foxn1 regulates not only the differentiation of TECs, but it also promotes TEC proliferation (Itoi et al., 2001). Using a mouse model of Foxn1 overexpression, the authors observed that there was an expansion of a population of Plet-1⁺ cells, which represents a subpopulation of bipotent TEPs (Li et al., 2023). Yet, it remains undetermined whether the lack of proliferation is a direct or an indirect consequence of the absence of this transcription factor.

The role of Foxn1 in TECs appears to influence also other thymic populations. In the absence of Foxn1 expression, thymic vasculature-associated endothelial and mesenchymal cells are scarce, suggesting that Foxn1 might also be required for the intact vascularization of the organ (Mori et al., 2010). However, it remains unclear whether it is a direct effect of Foxn1 itself in TECs or if these alterations are a result of the absence of downstream pathways activated in Foxn1-dependent mature TEC compartment. Nonetheless, there is an overall failure of vascular organization, with few detectable capillaries and leaky blood vessels (Bryson et al., 2013). To complement these observations, a study has evaluated the role of TEC-derived vascular endothelial growth factor (VEGF) in the vascularization of the organ (Muller et al., 2005). The authors describe that, in the absence of this angiogenic molecule, the thymus displays an altered thymic vasculature, which points to a specific role of TECs for the intact vascularization of the organ. It would be interesting to understand whether this is a Foxn1-dependent process. Foxn1 represents, therefore, a complex molecule that is required to promote the overall establishment of an intact thymic structure as well as a functional TEC microenvironment.

Section 2**The intricate network that governs the role of cTECs in T cell positive selection**

The mechanisms that control the positive selection events by cTECs remain elusive. Positive selection represents one of the most crucial quality-control checkpoints of T cell development, with developing DP thymocytes being screened for the ability of their newly generated TCRs to recognize with the proper affinity self-peptides that are presented via MHC molecules on the surface of cTECs (Klein et al., 2014). This process is extremely stringent, as only a small fraction of cells is allowed to further differentiate into SP thymocytes. In the medulla, positively selected thymocytes expressing TCRs with high affinity for self-peptide:MHC complexes presented by mTEC and/or DCs are eliminated or, in the case of SP4 thymocytes, diverted into T regulatory cells (Klein et al., 2014; Takada et al., 2017). Hence, thymic selection is essential to assure the capacity of newly generated T cells to recognize foreign antigens in the periphery while tolerant to our own tissue. The fate of developing thymocytes is dictated not only by the cell-autonomous properties of their newly rearranged TCR but also by the distinct strength and duration of TCR-mediated signals. Interestingly, these signals depend on the specialized self-peptide:MHC complexes presented by cTECs and mTECs. These two subsets display complementary and yet non-overlapping functions during thymic selection, due to their specialized protein degradation machineries and transcriptional programs (Klein et al., 2014; Takada et al., 2017). It is well established that mTECs possess the unique ability to express TRAs, in a process that is partially dependent on Aire and Fezf2, known as promiscuous gene expression (pGE) (Abramson & Anderson, 2017; Klein et al., 2014). The pGE capacity of mTECs allows them to serve as a library of ubiquitous and tissue-specific antigens, which are expressed in a stochastic yet randomly organized fashion (Brennecke et al., 2015; Dhalla et al., 2020; Meredith et al., 2015). This distinctive pattern of expression maximizes the effectiveness of TRA presentation. In sharp contrast to mTECs that use the more conventional antigen processing machineries, cTECs possess unique antigen processing capacities that promote the generation of a panel of distinct selective peptides (Klein et al., 2014). It is considered that cTECs produce “private” sets of MHC I- or MHC II-bound self-peptides to select either CD8 or CD4 T cells, respectively. Interestingly, this implies that positive selecting peptides do not overlap with the ones that are being presented in the medulla by either mTECs or thymic DCs. While the precise nature of the self-peptides generated by cTECs remains unknown, recent studies, including ours, have identified novel molecular pathways that control the selection of CD4 T cells (Rodrigues et al., 2023).

The generation of self-peptides that will promote the positive selection of CD8 T cells critically depends on the peculiar expression of a distinct type of proteasome by cTECs, known as the thymoproteasome, which includes the $\beta 5t$ subunit into the structure of the proteasome and therefore possess exclusive catalytic activities (Takada et al., 2017). Two distinct models have been proposed to explain how thymoproteasome-derived peptides control the positive selection of these cells. The low-affinity motif model proposes that the thymoproteasome produces unique MHC I-bound self-peptides that specifically promote low-affinity interactions with TCR-expressing DP thymocytes and their selection towards the CD8 lineage (Ohigashi et al., 2021; Xing et al., 2013). The peptide-switch model complements the first model by adding that the presentation of unique peptides by cTECs prevents the overlap with peptides derived from other forms of proteasome. The identification of the MHC I-bound self-peptides generated by cTECs may help to understand whether the two models are mutually exclusive or instead compatible in regards of positive selection. The $\beta 5t$ -mediated selection prevents T cells from undergoing clonal deletion when they arrive at the medulla (Kincaid et al., 2016). This idiosyncrasy is a reflection of the specialized protein-degradation and antigen-processing machineries in these cells that can generate specific sets of MHC I-bound selective ligands. The unique peptidome generated by cTECs promotes the positive selection of CD8 T cells that will likely encounter the same self-peptide in the peripheral organs.

On the other hand, the generation of peptides that promote the selection of CD4 T cells is not yet fully understood. The lysosomal endopeptidase cathepsin L and the endosomal thymus-specific serine protease have been acknowledged to have distinct but complementary roles in the establishment of a polyclonal CD4 T cell compartment (Gommeaux et al., 2009; Honey et al., 2002; Nakagawa et al., 1998; Viret et al., 2011). Regarding the MHC II processing pathways, cTECs appear to have developed unconventional processing routes that include the autophagic-mediated shuttling of cytoplasmatic proteins into the lysosomes, as a mechanism to overcome their apparent inefficiency in the capturing and processing of extracellular proteins using the classical endocytic pathway (Klein et al., 2014). Interestingly, cTECs display a high constitutive level of macroautophagy at steady state, suggesting that this process might be required for their proper function, especially to optimize the process of positive selection (Glaser et al.). Initial studies on the Autophagy related 5 (Atg5) or in the C-Type Lectin Domain Containing 16A (Clec16a) genes validated the role of autophagy in the positive selection of some MHC II-restricted TCR specificities (Nedjic et al., 2008; Schuster et al., 2015). Furthermore, autophagy in mTECs appears also to be important to impose tolerance induction, although the precise mechanisms are still not entirely resolved (Aichinger et al., 2013; Nedjic et al.,

2008). In our study, we position the lysosomal protein LAMP2 as a novel player that interconnects autophagy to the MHC II compartment and the generation of self-peptides provided by cTECs during positive selection (**Figure 1**) (Rodrigues et al., 2023). We propose that this protein is required for the generation of a fully established catalogue of polyclonal TCRs that are positively selected by cTECs. Defects in self-peptide MHC II processing reflect a reduction in the number of CD4 T cells that complete positive selection and a restricted panoply of CD4 TCR specificities. As LAMP2 is required for distinct autophagy-related processes such as macroautophagy and chaperone-mediated autophagy (Cooper et al.), whether these two processes are equally active in these cells and if they represent similarly critical molecular pathways for the selection of CD4 T cells requires further investigation. As the isoform LAMP2A has been demonstrated to play a dedicated role in CMA (Qiao et al., 2023), the generation of a mouse model carrying a specific deletion of LAMP2A in TECs (using Foxn1^{Cre} animals) would provide a valuable tool to assess its cell-intrinsic effect. These targeted studies would also allow us to assess whether the generation of positively selecting self-peptide:MHC depends on distinct intracellular routes, which might partially overlap or/and play complementary roles. Interestingly, a recent study has described a role for the lipid kinase Vps34 in the positive selection of CD4 T cells and in their TCR repertoire formation (Postoak et al., 2022). This molecule is known to be important for both autophagy and endocytic vesicle trafficking and this study supported the notion that autophagy-related molecular pathways support the selection of CD4 T cells. Understanding the molecular determinants that control the generation of CD4-selective peptides would therefore provide novel insights on the intricacies that compose the MHC-II ligandome of cTECs.

Several studies have tried to understand the phenomenon of positive selection and how it regulates peripheral T cell responses, in which some focused their attention on studying the specific effects of the $\beta 5t$ subunit and the acquisition of CD5 during positive selection. During such selection events, developing T cells acquire the expression of this molecule as a reflection of the intensity of TCR signaling, thus rendering thymocytes that recognize with low affinity MHC:self-peptide complexes with correspondent lower levels of CD5 (CD5^{lo}) (Azzam et al., 1998). Studies in TCR transgenic mice have revealed that the selection of CD5^{lo} CD8⁺ clones appears to be dependent on $\beta 5t$, as the removal of this subunit promoted the generation of T cell clones with high levels of CD5 (Nitta et al., 2010; Xing et al., 2013). The generation of specific peptides by $\beta 5t$ appears to be biased towards a selection of CD5^{lo} T cell clones. These CD5^{lo} CD8 T cells display defective homeostatic maintenance when comparing with their CD5^{hi} counterparts as well as exhibiting lower levels of basal TCR phosphorylation (Mandl et al., 2013). The fact that cTECs incorporate

a unique protein degradation system that would, apparently, select CD8 T cell clones displaying weak interactions with self-peptide:MHC complexes is intriguing. One could envision that a CD5^{hi} T cell would be more equipped to respond and mount a more efficient anti-pathogen response. However, these cells with higher self-reactivity represent a potential menace as they might incite autoimmune reactions. Nonetheless, there is a spectrum of T cells with distinct CD5 levels that are capable of mounting an effective immune response (Persaud et al., 2014). These observations demonstrate that β 5t-containing proteasomes are essential to promote the optimal selection of CD8 T cells and to establish an immunocompetent and self-protective CD8 T cell repertoire (Sasaki et al., 2015; Xing et al., 2013). The apparent bias towards a low TCR signaling intensity might encompass a tight and controlled set of affinities that are required for the generation of an optimized CD8 T cell repertoire.

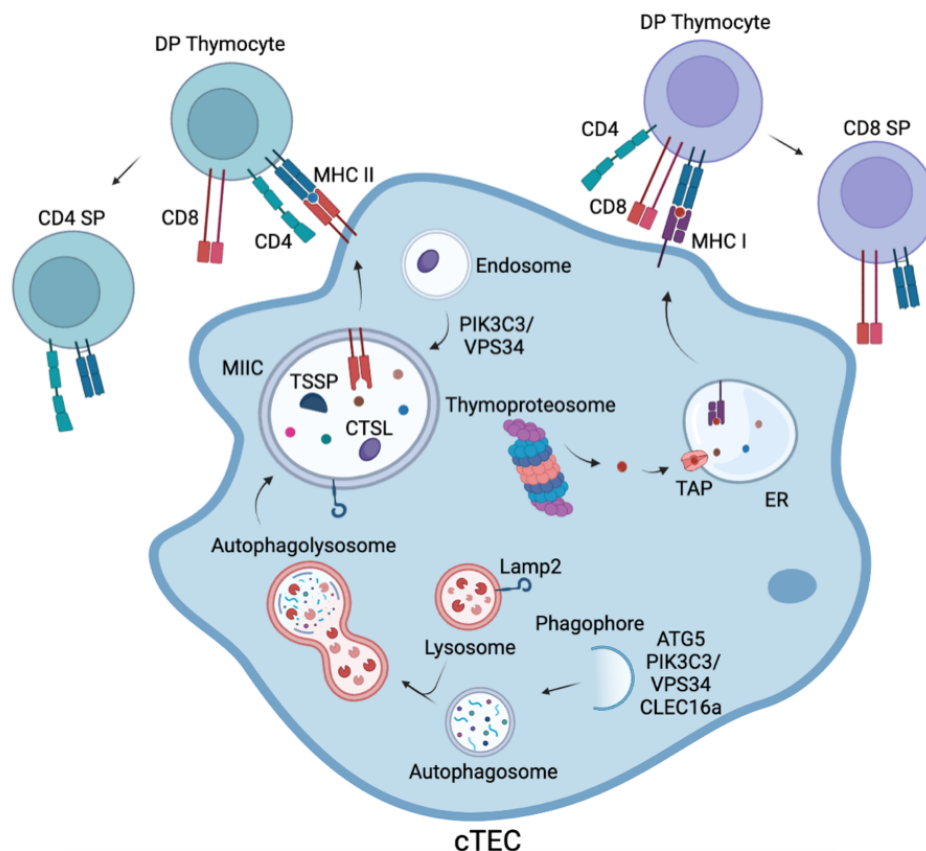


Figure 1. Model depicting the molecular basis for the generation and presentation of self-peptide complexes by cTECs. Left: MHC II-bound self-peptides required for positive selection of CD4 T cell derived from the autophagic route. During the autophagic process, LAMP2 mediates the fusion of the intracellular components of autophagosome with lysosomes. The maturation of the autophagolysosome ultimately culminates in the formation of the MHCII compartment (MIIC). Generated peptides are loaded in mature MHC II molecules and the MHC II:peptide complex presented to developing double positive (DP) thymocytes. DP thymocytes expressing MHC II restricted-TCR will commit toward the CD4 lineage upon interaction with self-peptides/MHC II complexes. Right: MHC I-bound self-peptides required for positive selection of CD8 T cell derived from cytosolic antigens degraded by the thymoproteasome. Following transport via transporter associated with antigen presentation (TAP) into the lumen of the endoplasmic reticulum (ER) and loading into MHC I molecules, self-peptides are presented by MHC I to developing DP thymocytes. Thymocytes expressing MHC I restricted-TCR will commit toward the CD8 lineage upon interaction with self-peptides/MHC I complexes. Adapted from(Sousa et al., 2023).

Similar to CD8 T cells, there is a direct correlation between the affinity a TCR from a CD4 T cell displays to a self-peptide during positive selection and its affinity to a foreign antigen (Mandl et al., 2013). The positive selection of CD4 T cells, in sharp contrast with what was abovementioned for CD8 T cells, exhibits a preference for the retention of TCRs that display a higher degree of affinity for self-peptides, within a certain range of controlled reactivities (Mandl et al., 2013). Yet, a study has revealed that, although thymic education is critical to ensure the effector capacities of CD4 T cells in the periphery and the higher affinity of their TCR reflects their overall response in the peripheral organs. Similarly to CD8 T cells, there is a spectrum of peripheral T cells that exhibit distinct levels of CD5 and were shown to be equally able to mount an effective immune response (Persaud et al., 2014). Nonetheless, it remains puzzling the contrast between the details governing the selection of CD4 over CD8 T cells regarding the range of affinities that promote the differential upregulation of CD5, which might correlate with their helper vs effector functions. Moreover, the higher range of affinities in the selection of CD4 thymocytes possibly reflects a need for the generation of T regulatory cells, which are described to be cells that are selected in the borders of higher reactivity, therefore representing a dedicated pool of CD5^{hi} cells.

Taken together, cTECs display unusual antigen processing machineries and, despite not being entirely understood, these unique features assure the diversification of the T cell repertoire and their maximal versatility. The elucidation of novel molecules that have an impact in antigen presentation and processing incites the research field toward the identification of the “peptidic self” within the thymus.

The text included in Section 2 was adapted from a review article herein we made an overview of the most recent studies on the role of TECs in T cell selection (Sousa *et al*, *Eur. J. Immunol.* 2023 – attached file).

Concluding Remarks

In this thesis, we focused on distinct aspects that regulate the induction and the maintenance of thymic function. Specifically, in **Chapter II** we have established that the *Foxn1*^{nu/nu} thymic microenvironment might be composed of distinct subpopulations, with these cells actively downregulating the transcription of *Foxn1* over time. In **Chapter III** we described the novel role of the lysosomal protein LAMP2 particularly in the autophagy-lysosomal axis of cTECs. We propose that this lysosomal protein is required for the intact presentation of self-peptide:MHC II complexes by cTECs and for their role in positive selection and the generation of a broad CD4 TCR repertoire.

In conclusion, this thesis provided innovative insights on the molecular basis of TEC development and function, which will contribute to the elucidation of some of the foundations of both basic and clinical aspects of thymus biology.

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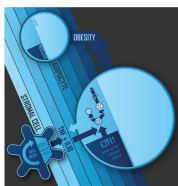
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Chapter V

Appendix



LAMP2 regulates autophagy in the thymic epithelium and thymic stroma-dependent CD4 T cell development

Pedro M. Rodrigues, Laura G. Sousa, Chiara Perrod, Ana R. Maceiras, Pedro Ferreirinha, Rita Pombinho, Gema Romera-Cárdenas, María Gomez-Lazaro, Meryem Senkara, Jelena Pistolic, Didier Cabanes, Ludger Klein, Paul Saftig & Nuno L. Alves

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RESEARCH PAPER



LAMP2 regulates autophagy in the thymic epithelium and thymic stroma-dependent CD4 T cell development

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ABSTRACT

Within the thymus, thymic epithelial cells (TECs) provide dedicated thymic stroma microenvironments for T cell development. Because TEC functionality is sensitive to aging and cytoablative therapies, unraveling the molecular elements that coordinate their thymopoietic role has fundamental and clinical implications. Particularly, the selection of CD4 T cells depends on interactions between TCRs expressed on T cell precursors and self-peptides:MHC II complexes presented by cortical TECs (cTECs). Although the macroautophagy/autophagy-lysosomal protein degradation pathway is implicated in CD4 T cell selection, the molecular mechanism that controls the generation of selecting MHC II ligands remains elusive. LAMP2 (lysosomal-associated membrane protein 2) is a well-recognized mediator of autolysosome (AL) maturation. We showed that LAMP2 is highly expressed in cTECs. Notably, genetic inactivation of *Lamp2* in thymic stromal cells specifically impaired the development of CD4 T cells that completed positive selection, without misdirecting MHC II-restricted cells into the CD8 lineage. Mechanistically, defects in autophagy in *lamp2*-deficient cTECs were linked to alterations in MHC II processing, which was associated with a marked reduction in CD4 TCR repertoire diversity selected within the *lamp2*-deficient thymic stroma. Together, our findings suggest that LAMP2 interconnects the autophagy-lysosomal axis and the processing of selecting self-peptides:MHC II complexes in cTECs, underling its implications for the generation of a broad CD4 TCR repertoire.

Abbreviations: AIRE: autoimmune regulator (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy); AL: autolysosome; AP: autophagosome; Baf-A1: bafilomycin A₁; B2M: beta-2 microglobulin; CTSL: cathepsin L; CD74/II: CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated); CFSE: carboxyfluorescein succinimidyl ester; CFU: colony-forming unit; CLIP: class II-associated invariant chain peptides; cTECs: cortical TECs dKO: double knockout; DN: double negative; DP: double positive; ENPEP/LY51: glutamyl aminopeptidase; FOXP3: forkhead box; P3 IFNG/IFN γ : interferon gamma; IKZF2/HELIOS: IKAROS family zinc finger 2; IL2RA/CD25: interleukin 2 receptor, alpha chain; KO: knockout; LAMP2: lysosomal-associated membrane protein 2; LIP: lymphopenia-induced proliferation; Lm: *Listeria monocytogenes*; MAP1LC3/LC3: microtubule-associated protein 1 light chain 3; MHC: major histocompatibility complex; mTECs: medullary TECs; PRSS16/TSSP: protease, serine 16 (thymus); SEL/CD62L: selectin, lymphocyte; SP: single positive; TCR: T cell receptor; TCRB: T cell receptor beta chain; TECs: thymic epithelial cells; UEA-1: *Ulex europaeus* agglutinin-1; WT: wild-type.

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Introduction

The thymus generates T cells expressing $\alpha\beta$ T cell receptors (TCRs) that recognize foreign antigens presented by self-major histocompatibility complex (MHC) molecules and are tolerant to self-components. This process is developmentally regulated by interactions between TCR expressed on T cell precursors (thymocytes) and self-peptide-MHC complexes presented by thymic epithelial cells (TECs) [1]. TECs express a myriad of soluble and cell-surface ligands that coordinate all stages of

T cell differentiation. Importantly, the thymopoietic role of TECs is sensitive to aging and cytoablative therapies, which compromise T-cell responses to pathogens and vaccination in the elderly and immunocompromised patients [2]. Therefore, the identification of novel regulators of TEC functionality is vital to comprehend the foundations of T cell-immunity and to restore thymopoiesis in disorders associated with defective T cell responses. TECs are divided into functionally distinct cortical (cTECs) and medullary (mTECs) subtypes. While

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cTECs guide T cell lineage commitment and positive selection, mTECs regulate negative selection and T regulatory cell differentiation [3]. The functional segregation between cTECs and mTECs is, in part, determined by their unique antigen processing and presentation properties, which are controlled by specific protein degradation machineries and transcriptional programs [4,5]. However, how these proteolytic machineries coordinate the presentation of selective self-peptide-MHC complexes remains elusive at the molecular level.

In this regard, mTECs employ conventional proteolytic pathways to generate “public” MHC I- and MHC II-bound peptides that negatively select autoreactive thymocytes or drive their deviation into regulatory T cells [4]. These key steps for tolerance induction depend on the distinctive ability of mTECs to express tissue-restricted antigens, a process that is mediated by AIRE and FEZ family zinc finger 2 [1]. Alternatively, cTECs harbor unique antigen processing properties that generate sets of “private” MHC I- and MHC II-bound self-peptides, which are critical for the positive selection of double-positive (DP) thymocytes and their commitment into CD4 or CD8 T cells. The generation of MHC I-bound peptides that select CD8⁺ T cells depends on the distinct proteolytic activity of PSMB11/β5t (proteasome (prosome, macropain) subunit, beta type 11)-containing thymoproteasome [5,6]. For the generation of

MHC II-bound peptides, cTECs employ unconventional endogenous MHC II-loading pathways that involve the autophagy-mediated transport of cytosolic proteins into lysosomes [7,8]. Lysosomal proteases, including CTSL (cathepsin L) and PRSS16/TSSP (protease, serine 16 (thymus)) contribute to the generation of specific self-peptides that select CD4⁺ T cells [9–12]. Although autophagy has been coupled to the control of thymic selection [7,8,13], the molecular mechanism that links this catabolic pathway to the generation of selective self-peptides in the lysosomes remains elusive. Our study identifies a role for LAMP2 (lysosomal-associated membrane protein 2) in the thymic stroma as a determinant regulator of CD4 T cell development and TCR repertoire formation.

Results

LAMP2 is dispensable for the establishment of major TEC microenvironments

To identify new candidates involved in the antigen processing capacity of cTECs, we analyzed our transcriptomic data from postnatal cTECs and mTECs [14], together with available data sets from other studies [15,16]. Given that MHC II-bound peptide generation involves the endosomal–lysosomal pathway, we sought genes associated with these vesicular compartments

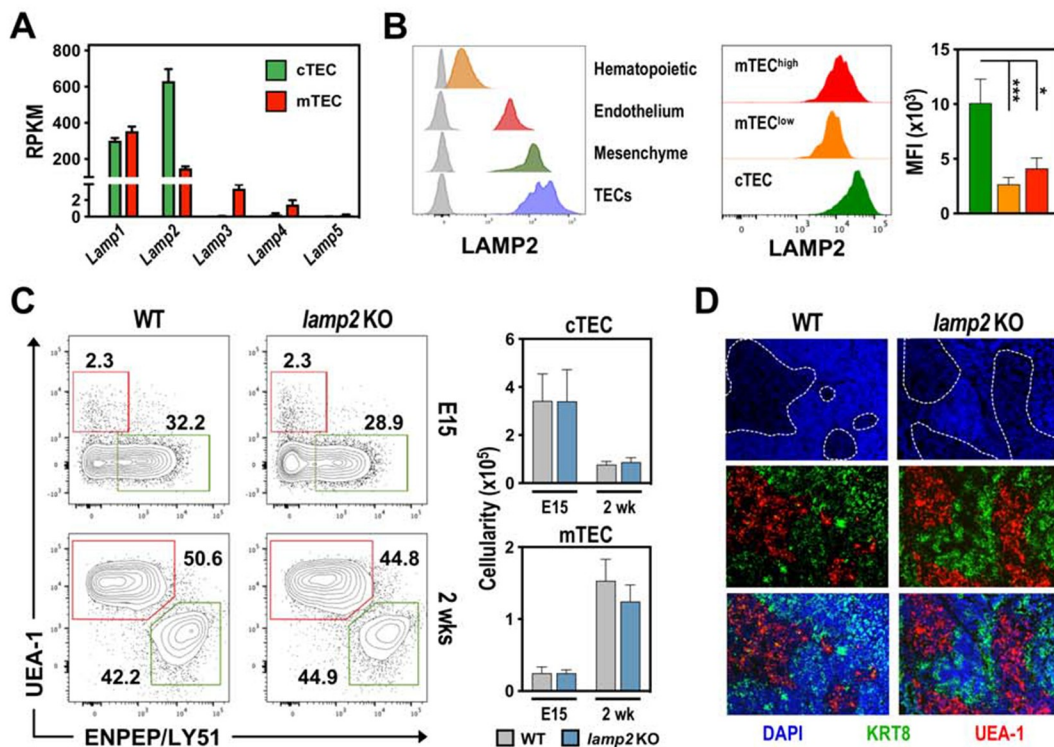


Figure 1. The deficiency in *Lamp2* does not affect global cTEC/mTEC differentiation. (A) Expression (RNAseq analysis) of LAMP family members in cTECs (green) and mTECs (red) from WT mice. Error bars represent mean $\pm$ SEM. (B) LAMP2 expression in distinct thymic cell populations in 2-week-old WT and *lamp2* KO mice. Left histogram: Hematopoietic (PTPRC/CD45⁺), endothelial (PECAM1/CD31⁺), mesenchymal (PDGFRA-PDGFRB [$\alpha\beta$]⁺) and TEC (EPCAM⁺) cells ($n = 3$), (WT and *lamp2* KO subsets in color and gray, respectively); Right histogram: cTECs (ENPEP/Ly51⁺ UEA-1⁻), mTEC^{low} (ENPEP/Ly51⁻ UEA-1⁺ CD80^{low}) and mTEC^{hi} (ENPEP/Ly51⁻ UEA-1⁺ CD80^{hi}). Bars graphs represent mean fluorescence intensity (MFI) of LAMP2 expression. Data representative of 3 independent experiments ($n = 12$ animals). (C) cTEC and mTEC composition in embryonic day 15.5 (E15.5) and 2-week-old WT and *lamp2* KO thymus. Dot plots show a representative ENPEP/Ly51 and UEA staining and graphs represent the average cellularity of c/mTECs in the indicated timepoints from 3 independent experiments ($n = 11$ –14 animals/group). (D) Immunofluorescence analysis of thymic sections from 2-week-old WT and *lamp2* KO thymus stained for DAPI (blue), UEA-1 (red) and KRT8 (keratin 8; green). Results in **A–C** are shown as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

that were selectively enriched in cTECs relatively to mTECs. *Ctsl* (cathepsin L) and *Prss16* were expectedly among the top genes upregulated in cTECs. We selected to analyze LAMP2, which was the only member of the LAMP family upregulated (4-fold) in cTECs (Figure 1(A), Figure S1A). LAMP2 is a lysosomal membrane protein that regulates macroautophagy and chaperone-mediated autophagy (CMA) [17], promoting the fusion of autophagic vacuoles with lysosomes [18,19]. LAMP2 has been also implicated in the regulation of MHC II presentation in human B cells [20,21]. Yet, its involvement in T cell development and selection is unknown.

In man, genetic mutations in *LAMP2* are associated with Danon Disease, a fatal X-linked syndrome caused by lysosomal dysfunction [22]. Mice deficient for *Lamp2* (*lamp2* knockout; *lamp2* KO) recapitulate key aspects of the human condition, including the accumulation of autophagic vacuoles in several cell types [18,19,23]. Despite the increased mortality in early adulthood, *lamp2* KO mice survive the first weeks of life [19]. Hence, to study the function of LAMP2 in the establishment of major TEC subsets, we analyzed the *lamp2* KO thymus during embryonic and postnatal life. We began by analyzing the expression of LAMP2 protein in embryonic wild-type (WT) TECs, and using cells from *lamp2* KO mice as a control for the antibody staining (Figure S1B). Within the postnatal thymus, TECs represented the highest LAMP2-expressing subset, relatively to thymocytes and other non-TEC stromal cells, including endothelial and mesenchymal cells. The corresponding *lamp2* KO subsets were included to further validate antibody staining. In line with the transcriptomic data, cTECs significantly expressed higher levels of LAMP2 relatively to mTEC^{lo/hi} subtypes (Figure 1(B)). The deficiency in *Lamp2* did not alter the generation of primordial cTECs and mTECs at embryonic day 15 nor affected the cellularity of the main cTEC and mTEC subsets in the 2-week-old thymus (Figure 1(C)). Further analysis of the expression of cTEC-associated markers (LY75/CD205, CD40) or mTEC subpopulations, including mTEC^{lo} (MHCII^{lo} CD40^{lo} CD80^{lo}), mTEC^{hi} (MHCII^{hi} CD40^{hi} CD80^{hi}), CCL21⁺ and AIRE⁺ cells, revealed no differences between WT and *lamp2* KO TECs (Figure S1C). Additionally, the spatial organization of cortical and medullary areas was normal in the *lamp2* KO thymus (Figure 1(D)). Our results indicate that LAMP2 is not essential for the establishment of global cTEC and mTEC compartments.

LAMP2 in thymic stromal cells specifically controls the development of CD4 T cells

The pleiotropic defects of *lamp2* KO mice and the broader expression of LAMP2 in several tissues hampered a direct assessment of its role in TEC-mediated T cell selection. To circumvent this aspect, we employed a well-established thymic transplantation model wherein WT or *lamp2* KO embryonic thymi were grafted under the kidney capsule of nude adult recipients, referred to hereafter as WT-Nude and *lamp2* KO-Nude. In these chimeras, the deficiency in *Lamp2* was restricted to TECs, endothelial and mesenchymal cells, allowing the assessment of how recipient-derived T cell precursors (from WT origin) developed and were selected within WT and *lamp2* KO thymic stroma, in the absence of confounding effects of *lamp2*-deficiency in other tissues. Although TECs

have a non-redundant role in T cell commitment and selection [4], this approach did not formally exclude a potential contribution for LAMP2 in endothelial and mesenchymal cells in these processes.

Analysis of T cell development 8–10 weeks post-transplantation showed that the percentages of immature double-negative (DN) and DP thymocytes were similar in WT-Nude and *lamp2* KO-Nude thymic grafts. While the numbers of total, DN and DP thymocytes appeared slightly reduced, these differences were not statistically significant (Figure 2(A), Figure S2A), indicating that LAMP2 is dispensable for β -selection and DN-DP transition. Notably, the frequencies of single-positive 4 (SP4) and SP8 thymocytes were altered in *lamp2* KO thymi. These changes resulted in a specific and statistically significant, reduction in the number of SP4 but not SP8 cells (Figure 2(A)). Analysis of T cells expressing high levels of TCRB (T cell receptor beta chain) (TCRB^{hi}) confirmed the decrease of circa 40% in the average number of SP4 cells in the *lamp2* KO thymus (1.3×10^6 cells) relatively to the cellularity found in the WT counterparts (2.2×10^6 cells). These changes led to a deviation in SP4:SP8 ratios in the mutant thymi as compared to controls, and endogenous thymi of WT mice (Figure 2(B)). The thymic alterations coincided with changes in the peripheral T-cells, with a statistically significant reduction in the number of *lamp2* KO thymus-derived splenic naïve CD4⁺ T cells. The numbers of effector/memory CD4, and naïve and effector/memory CD8 T cells were moderately reduced, but these changes were not statistically significant (Figure 2(C)). Together, these results pointed to an impaired CD4 T cell development within the *lamp2*-deficient thymic microenvironment.

To identify the stage at which CD4⁺ T cell development was altered in *lamp2* KO thymus, we examined distinct stages of pre- and post-positive selection, based on the differential expression of TCRB and CD69 expression on thymocytes: Population I (TCRB^{neg/int} CD69^{neg}) contains mostly pre-selected DP thymocytes; Population II (TCRB^{int} CD69^{int}) harbors TCR-signaled cells initiating positive selection; Population III (TCRB^{hi} CD69^{hi}) represents post-positively selected SP thymocytes; Population IV (TCRB^{hi} CD69^{neg}) consists of more mature SP cells [24]. Although the frequency of populations II, III and IV within total thymocytes were similar, we found a noticeable alteration in the frequency of SP4 and SP8 in post-positively selected cells (TCRB^{hi} CD69^{hi}, III) of the *lamp2* KO-Nude thymi (Figure 3(A-B)). This led to a significant reduction in the numbers of post-positively selected SP4, but not SP8 thymocytes, in *lamp2* KO-Nude thymi (Figure 3(B)). We further analyzed the expression of CD5 in thymocyte subsets as this correlates with TCR affinity for self-peptide-MHC ligand [25,26]. Stage II DP thymocytes from WT and *lamp2* KO thymus expressed comparable CD5 levels, suggesting normal TCR signaling during the early stages of positive selection (Figure 3(C), Figure S2B). Early after positive selection, DP thymocytes transverse through a CD4⁺CD8^{int} (CD4^{int}) transitional stage prior to their commitment into CD4 and CD8 lineage [27]. The frequency and

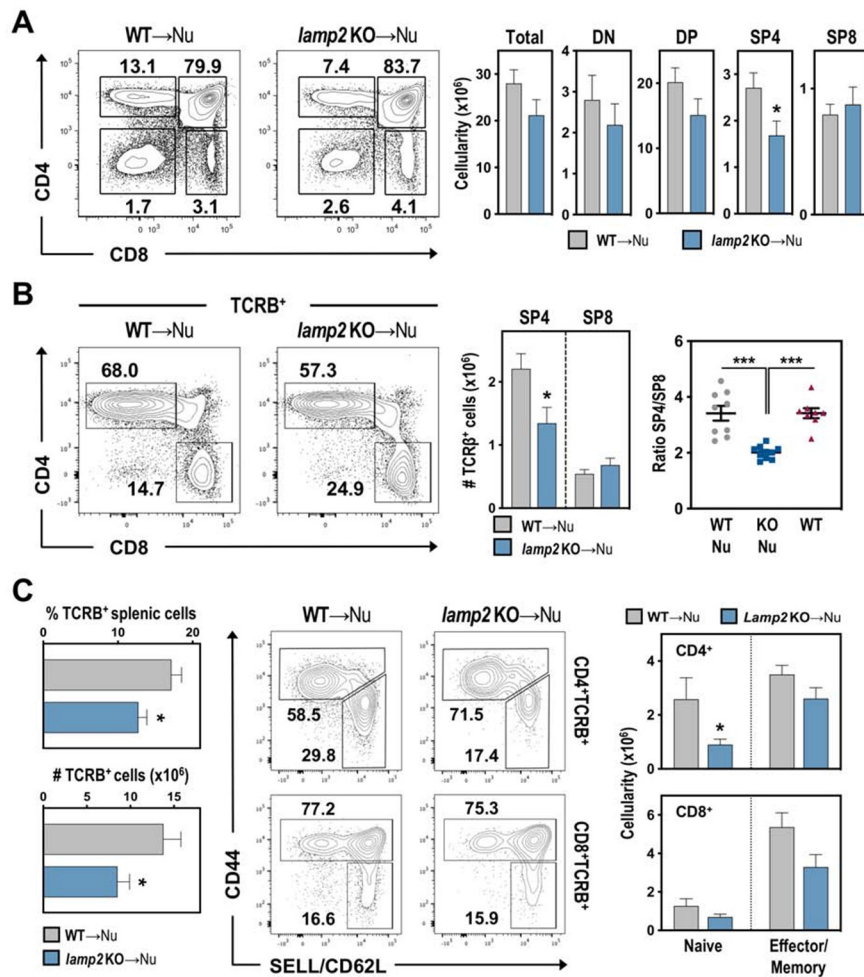


Figure 2. T cell development in WT and *lamp2* KO thymic grafts. Representative dot plots of CD4 and CD8 expression within (A) total thymocytes and (B) TCRB⁺ thymocytes in thymic grafts of Nude recipients transplanted with WT (WT→Nu) and *lamp2* KO (*lamp2* KO→Nu) ectopic thymus, 8–10 weeks after transplantation. Numbers on the plots indicate the frequencies of the different subsets. Bar graphs in A and B depict the average absolute cellularity of the thymus and the indicated thymocyte subsets in WT→Nu (gray) and *lamp2* KO→Nu (blue). In B, ratio of the frequency of SP4:SP8 is shown in WT→Nu and *lamp2* KO→Nu, as well in the endogenous thymus. (C) Analysis of peripheral T cells in spleens from WT→Nu and *lamp2* KO→Nu mice. Graphs (left) represent the average % and numbers of TCRB⁺ T cells in the spleen. Dot plots show CD44 and SELL/CD62L expression within CD4 and CD8 T cells. Graphs (right) show average cellularity of naïve (SELL/CD62L⁺ CD44^{lo}) and effector/memory (SELL/CD62L⁺ CD44^{hi}) CD4 and CD8 T cells. Data represent an average of 3 independent experiments (n = 10 WT and *lamp2* KO ectopic thymi). Results in A–C are shown as mean ± SEM. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

numbers of stage II CD4^{int} cells in *lamp2* KO-Nude mice were comparable to the WT-Nude setting (Figure 3(B)). Yet, the levels of CD5 in stage II CD4^{int} and III–IV SP4 thymocytes were moderately reduced within the *lamp2* KO-Nude thymus, suggesting an attenuation of TCR signaling in cells that completed positive selection (Figure 3(C), Figure S2B). Additionally, stage IV SP4 thymocytes did not accumulate in the *lamp2* KO thymus nor presented elevated CD5 levels (Figure 3(B–C), Figure S2B), arguing against excessive TCR signaling. To study whether *lamp2*-deficiency could affect other steps of CD4 T cell development that depend on mTECs, we analyzed SP4 maturation and the regulatory T cell differentiation [1]. The proportion of immature (CD24⁺ SELL/CD62L[−]) and mature (CD24[−] SELL/CD62L⁺) SP4 thymocytes were comparable within WT and *lamp2* KO thymus. Additionally, the frequency of immature (IL2RA/CD25⁺ Forkhead box P3 (FOXP3)[−] and IL2RA/CD25[−] FOXP3⁺) and mature CD4 T regulatory (IL2RA/CD25⁺ FOXP3⁺) cells was similar in WT and

lamp2 KO thymi, suggesting that the maturation of T reg was not affected by *lamp2*-deficiency. Although moderately reduced, the numbers of mature CD4 T regulatory cells were not statistically different (Figure S2C–D). Contrarily, the cellularity of immature (IL2RA/CD25⁺ FOXP3[−]) T reg, immature and mature SP4 was statistically diminished in *lamp2* KO thymus. The reduction in these subsets may arise from the reduction in SP4 that completed positive selection, which was the subset mostly affected by the deficiency in *Lamp2* and included immature T reg and SP. Together, our results suggested a more prominent function for LAMP2 in regulating the development of CD4⁺ T cells that complete positive selection.

The selection of CD4⁺ T cells depends on “strong” and persistent TCR-mediated signals [28]. The decreased CD5 levels on SP4 cells could suggest that the reduction in SP4 cells in *lamp2* KO thymus was functionally linked to a defective CD4 commitment. In this scenario, MHC II-restricted thymocytes were misdirected toward the CD8

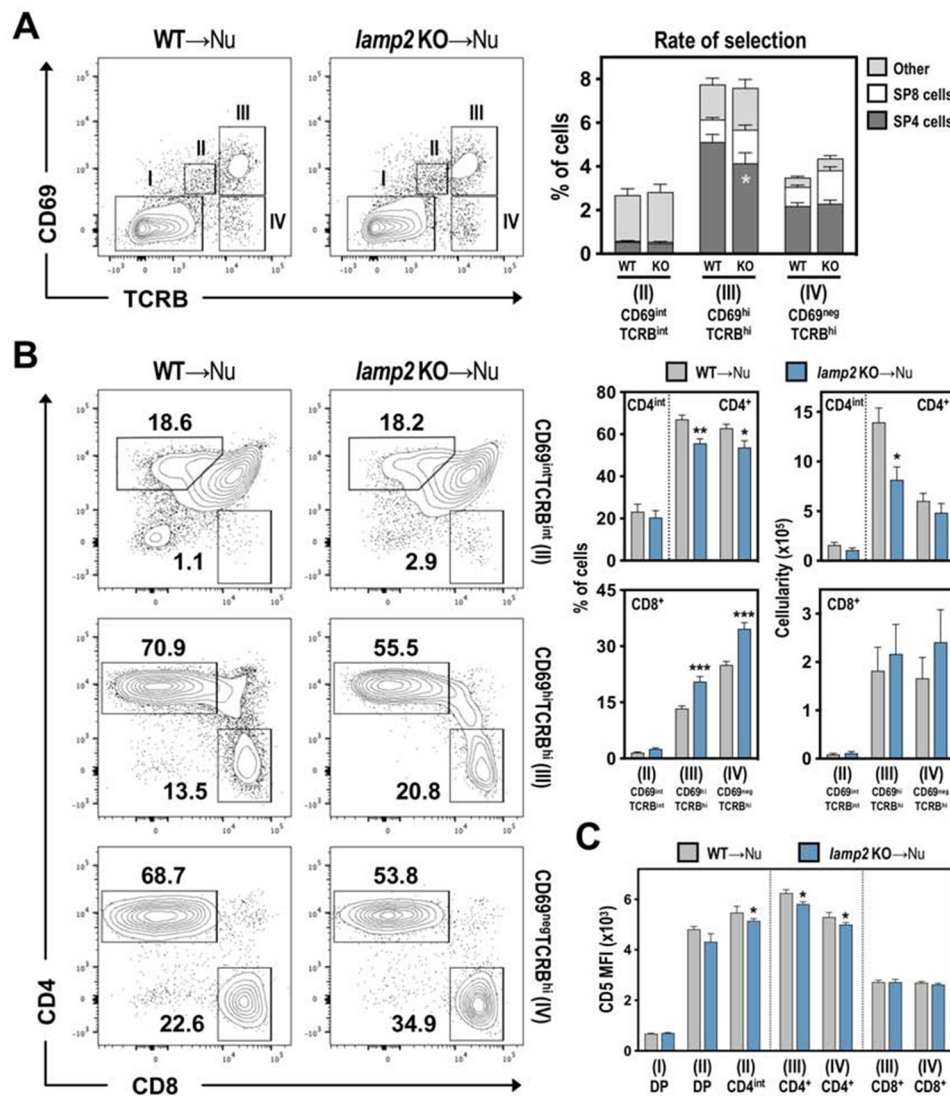


Figure 3. Pre- and post-positive T cell selection in WT and *lamp2* KO thymic grafts. (A) Flow cytometry analysis of pre- and post-positive selection in WT (WT→Nu) and *lamp2* KO (*lamp2* KO→Nu) ectopic thymus grafted into Nude recipients. Dot plots show the analysis of CD69 and TCRB expression on total thymocytes from WT→Nu and *lamp2* KO→Nu ectopic thymus. Stage I (TCRB^{neg/int} CD69^{neg}), II (TCRB^{int} CD69^{int}), III (TCRB^{hi} CD69^{hi}) and IV (TCRB^{hi} CD69^{neg}) defined pre-, recently-, post-selected and mature stages, respectively. Graph represents the average percentages of total populations II, III and IV. In each bar (populations II, III and IV bar) is shown the corresponding frequency of SP4 and SP8 cells, which were calculated based on the frequencies of SP4 and SP8 shown in panel B. (B) CD4/CD8 expression within stages II (TCRB^{int} CD69^{int}), III (TCRB^{hi} CD69^{hi}) and IV (TCRB^{hi} CD69^{neg}), which were defined on total thymocytes (A). Graphs represent the average percentages and absolute numbers of the indicated thymocyte subsets in WT→Nu (gray) and *lamp2* KO→Nu (blue). (C) Graphs represent the MFI of CD5 expression in the indicated subsets (defined as shown in Figure S2). Data shown represent an average of 10 ectopic WT and 10 ectopic *lamp2* KO thymi from 3 independent experiments. Results in **A-C** are shown as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

lineage due to an attenuated TCR signaling. To examine this possibility and prevent MHC-I-driven CD8 selection, we generated mice double deficient for *Lamp2* and *B2m* (beta-2 microglobulin) (*lamp2 b2m* dKO mice). Thymic transplantations were set with *b2m* KO or *lamp2 b2m* dKO embryonic thymus grafted into WT mice. Owing to the deficiency in *b2m*, and consequently MHC-I expression, the selection of MHC-I-restricted SP8 thymocytes was expectedly abolished in *b2m* KO thymic grafts [29], so that mature TCRB⁺ cells mostly comprised SP4 thymocytes (Figure 4(A-B)). Notably, the frequency and numbers of SP4 thymocytes were reduced in *lamp2 b2m* dKO thymus, without a concomitant increase in SP8 thymocytes

(Figure 4(A-B), Figure S3A). Moreover, recently post-selected (TCRB^{hi} CD69^{hi}) and mature (TCRB^{hi} CD69^{neg}) SP4 cells were significantly reduced in frequency and numbers in *lamp2 b2m* dKO thymic grafts (Figure 4(C), Figure S3A). To evaluate the possible contribution of LAMP2 in cortical and medullary negative selection at a polyclonal level, we analyzed the expression of IKZF2/HELIOS (IKAROS family zinc finger 2) on DP (TCRB^{lo/int} IL2RA/CD25⁻ FOXP3⁻) and CD4SP (TCRB^{hi} IL2RA/CD25⁻ FOXP3⁻) thymocytes [26]. The frequency of IKZF2/HELIOS⁺ cells was similar in both WT- and *lamp2* KO-derived thymocyte subsets (Figure 4(D), Figure S3B). These observations support that deficiency in *Lamp2* does not

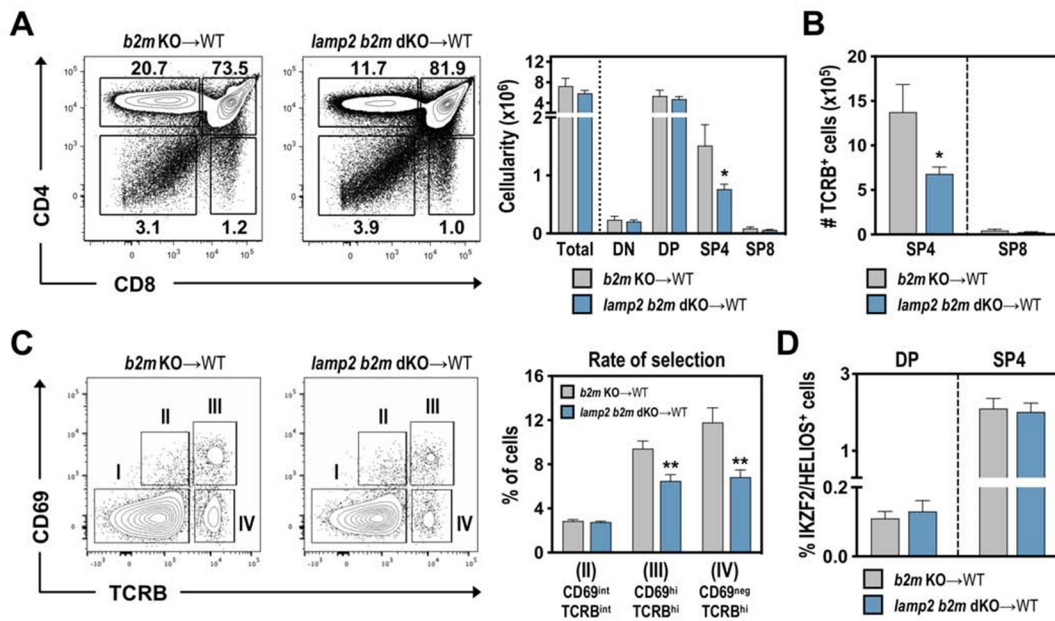


Figure 4. Combined deficiency in *Lamp2* and *B2m* specifically reduces CD4 T cell development, without redirecting MHC II-restricted cells into the CD8 lineage. (A–B) Flow cytometry analysis of T cell development in *b2m* KO (*b2m* KO→WT) and in *lamp2* and *b2m* double-KO (*lamp2 b2m* dKO→WT) thymus grafted into WT recipients. (A) Representative dot plots of CD4 and CD8 expression on total thymocytes, with numbers indicating the frequencies of the different subsets. Graphs represent the cellularity of total thymocytes and the indicated subsets in *b2m* KO (gray) and *lamp2 b2m* dKO (blue). (B) Graph represents the absolute numbers of SP4 and SP8 within mature TCRB⁺ thymocytes. Data shown represent an average of 9 *b2m* KO and 10 *lamp2 b2m* dKO ectopic thymi from 3 independent experiments. (C) Dot plots show the flow cytometric profile for CD69 and TCRB on total thymocytes from *b2m* KO→WT and *lamp2 b2m* dKO→WT ectopic thymi. Stage I (TCRB^{neg/int} CD69^{neg}), II (TCRB^{int} CD69^{int}), III (TCRB^{hi} CD69^{hi}) and IV (TCRB^{hi} CD69^{lo/neg}) were defined as represented. Graphs represent the average percentages of cells in the indicated gates in *b2m* KO→WT (gray) and *lamp2 b2m* dKO→WT (blue) ectopic thymi. (D) Graphs represent the average frequency of IKZF2/HELIOS⁺ cells in DP and SP4 from *b2m* KO→WT (gray) and *lamp2 b2m* dKO→WT (blue) (defined as shown in Figure S3B). Data include an average of 2 independent experiments (n = 6 *b2m* KO and 8 *lamp2 b2m* dKO→WT). Results in **A–D** are shown as mean ± SEM. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001.

redirect MHC II-restricted cells into CD8 lineage, but instead prevents the development of CD4 T cells that completed positive selection.

Alterations in autophagy in *lamp2* KO cTECs are associated with changes in MHC II processing

To determine whether the defect in CD4 T cell development could be mechanistically linked to changes in autophagy provoked by the deficiency in *Lamp2*, we evaluated the autophagic flux in TECs. *Ex vivo* isolated *lamp2* KO cTECs displayed an increased level of Cyto-ID, a tracer that marks AP and AL [30] (Figure 5(A)), indicative of altered autophagy dynamic. Moreover, *lamp2* KO mice were crossed with transgenic mice that ubiquitously expressed a RFP-GFP-MAP1LC3A/LC3 in a pH-dependent manner (RFP-GFP-LC3). In RFP-GFP-LC3 mice, RFP is stable and GFP is quenched in the acidic pH of lysosomal vesicles, allowing the distinction between AP (GFP⁺ RFP⁺) and AL (RFP⁺) [31]. The RFP-GFP-LC3 expression was predominantly detected in TECs relatively to the hematopoietic and non-TEC stroma of WT and *lamp2* KO thymus (Figure S4A). Moreover, the proportion of RFP⁺-GFP⁺-LC3 TECs was specifically increased in *lamp2* KO cTECs, but not mTECs (Figure 5(B) and Figure S4B). The accumulation of RFP⁺-GFP⁺-LC3 cells resulted from an augmented expression of GFP-LC3, but not RFP-LC3, in *lamp2* KO cTECs (Figure 5(B)). We further analyzed the autophagic flux at the single-cell level by employing imaging flow cytometry. *lamp2* KO cTECs contained cells with a reduced number of RFP⁺-LC3 puncta/cell compared

to WT counterparts, being the *lamp2* KO subset mostly composed of cells with dual RFP⁺-GFP⁺-LC3 puncta (Figure 5(C)). As the status of autophagy flux correlates with the number of autophagic vesicles, these results indicated that AP-lysosome fusion was markedly affected in *lamp2* KO cTECs, as reported in other cells deficient in *Lamp2* [18,19,23].

We next studied whether changes in LAMP2-mediated autophagy were associated with alterations in self-peptide: MHC II processing in cTECs, which in turn could condition their capacity to select CD4 T cells. MHC II molecules are stabilized in the endoplasmatic reticulum by their binding to the CD74 antigen/Ii (invariant polypeptide of major histocompatibility complex, class II antigen-associated). Subsequently, CD74/Ii is degraded into a fragment called CLIP (class II-associated invariant chain peptides) in the MIIC (MHC II processing compartment) to permit antigen-binding [32,33]. To evaluate MHC II processing and maturation, we employed 15G4 antibody that recognizes I-A^b occupied by the CD74/Ii degradation intermediates small leupeptin induced protein or CLIP [34,35]. Strikingly, 15G4 staining was substantially increased in *lamp2* KO cTECs, but not in mTECs, relative to WT counterparts (Figure 5(D)). In line with previous studies [10], mTECs displayed higher 15G4 staining as compared to cTECs (Figure 5(D)). Control and mutant cTECs, as well as mTECs, expressed comparable levels of MHC II and I molecules (Figure S4C), suggesting a specific role of LAMP2 in the processing of MHC II, but not in its trafficking to the cell membrane. Exploring the fact that *Lamp2* resides on X chromosome, we analyzed TECs from

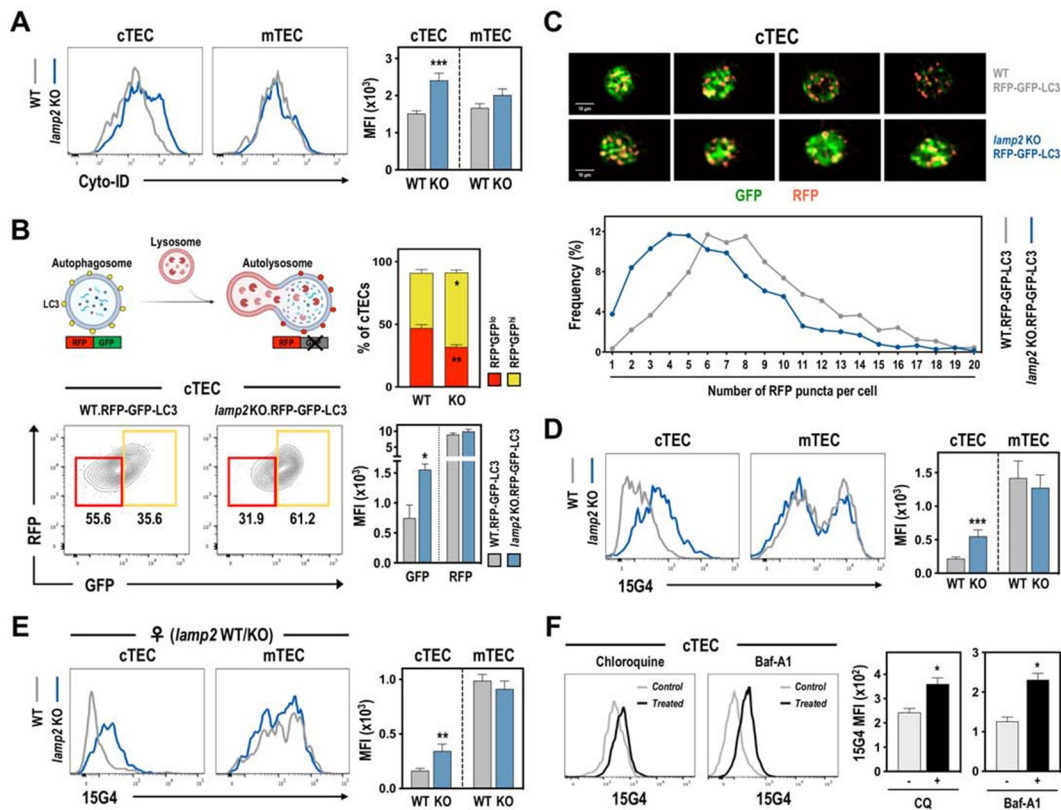


Figure 5. Defective autophagy and MHC II processing in *lamp2* KO cTECs. (A–B) Autophagic flux in cTECs from 12 days old WT and *lamp2* KO mice was analyzed by flow cytometry. (A) Histograms show representative Cyto-ID analysis in cTEC and mTEC from WT and *lamp2* KO. Graphs represent the MFI of Cyto-ID in the indicated subsets. (B) Scheme (top left) represents autophagic flux using RFP-GFP-LC3 mice. Differential detection of GFP and RFP allows the distinction between AP (GFP⁺ RFP⁺) and AL (RFP⁺). Dot plot (bottom left) represents GFP and RFP expression in cTECs of WT and *lamp2* KO RFP-GFP-LC3 mice. Graphs (top right) represent the average frequency of RFP⁺ GFP^{hi} (yellow) and RFP⁺ GFP^{low} (red) cells in WT and *lamp2* KO RFP-GFP-LC3 cTECs. Graphs (bottom right) represent the MFI of GFP and RFP expression in WT (gray) and *lamp2* KO (blue) RFP-GFP-LC3. (C) WT and *lamp2* KO RFP-GFP-LC3 cTECs (EPCAM⁺ ENPEP/LY51⁺) were analyzed by imaging flow cytometry. Representative images are shown. Graph shows the distribution of the number of RFP puncta in WT (n = 1345 cells) and *lamp2* KO (n = 1422 cells) RFP-GFP-LC3 cTECs. Data in A–C include an average of 2–3 independent experiments (n = 4–6 WT and n = 4–6 *lamp2* KO). (D) Flow cytometry analysis of 15G4 staining on cell surface of cTECs (ENPEP/LY51⁺ UEA-1⁺) and mTECs (ENPEP/LY51⁺ UEA-1⁺) from 12 days old WT and *lamp2* KO mice. Histograms show representative staining and graphs represent the MFI of 15G4 staining in the indicated subsets. Data include an average of 4 independent experiments (n = 6 WT and 7 *lamp2* KO). (E) Histograms show representative 15G4 staining on the cell surface of WT and *lamp2* KO c/mTECs from *lamp2* WT/KO heterozygous mice. Graphs represent the MFI found in indicated subsets in control (gray) and mutant (blue) cells (n = 8 from 4 experiments). (F) Postnatal day 3–5 WT thymus were treated overnight with chloroquine (50 μ M) or with Baf-A1 (0.5 μ M) for 5 h. Histograms show the representative staining with 15G4 staining in control (light gray) and chloroquine-treated (black) or Baf-A1-treated (black) in WT cTECs. Graphs represent the MFI in control (gray) and treated (dark gray/ black) cTEC (n = 4–6 animals from 3–4 independent experiments). Results in A–F are shown as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

lamp2 WT/KO heterozygous thymus, which due to random X-inactivation in females included WT and *lamp2* KO TECs (Figure S4D). *lamp2* KO cTECs presented an augmented 15G4 staining, even within a microenvironment containing WT cells, suggesting that LAMP2 controls MHC II processing in a cell-autonomous manner (Figure 5(E)). Given the role of CTSL in the degradation of CD74/Ii in cTECs [9,10], we studied if altered MHC II processing resulted from a decrease in CTSL or lysosomal functions. The proteolytic function of CTSL and global lysosomal activity were increased in *lamp2* KO cTECs (Figure S4E–F). These results suggest that the augmented levels of MHC II-bound to CD74/Ii degradation intermediates did not result from a decrease in CTSL activity or lysosomal processing capacities. Lastly, we evaluated whether changes in LAMP2-mediated autophagy could be functionally linked to the alterations in MHC II processing. To do so, postnatal day 3–5 WT thymi were treated with two inhibitors, chloroquine and bafilomycin A₁

(Baf-A1), of AP-lysosome fusion. Interestingly, chloroquine and Baf-A1 treatment increased 15G4 staining in WT cTECs (Figure 5(F)). The elevated 15G4 staining of WT mTECs was not substantially altered in Baf-A1-treated cells (Figure S4G). Worth noting, mTECs are less abundant in the postnatal thymus [14] and were sensitive to apoptosis following culture. Although the results with pharmacological inhibitors should be considered with caution due to additional specific or off-target effects, our data suggest that inhibition of AP-lysosome fusion in WT cTECs mimicked the phenotype observed on *lamp2* KO cTECs, and establish a presumable causality between changes in autophagy flux and MHC II processing.

LAMP2 in thymic stroma controls the generation of a broad CD4 TCR repertoire

Lastly, we determined whether thymic stroma deficient in *Lamp2* altered the generation of TCR repertoire diversity in

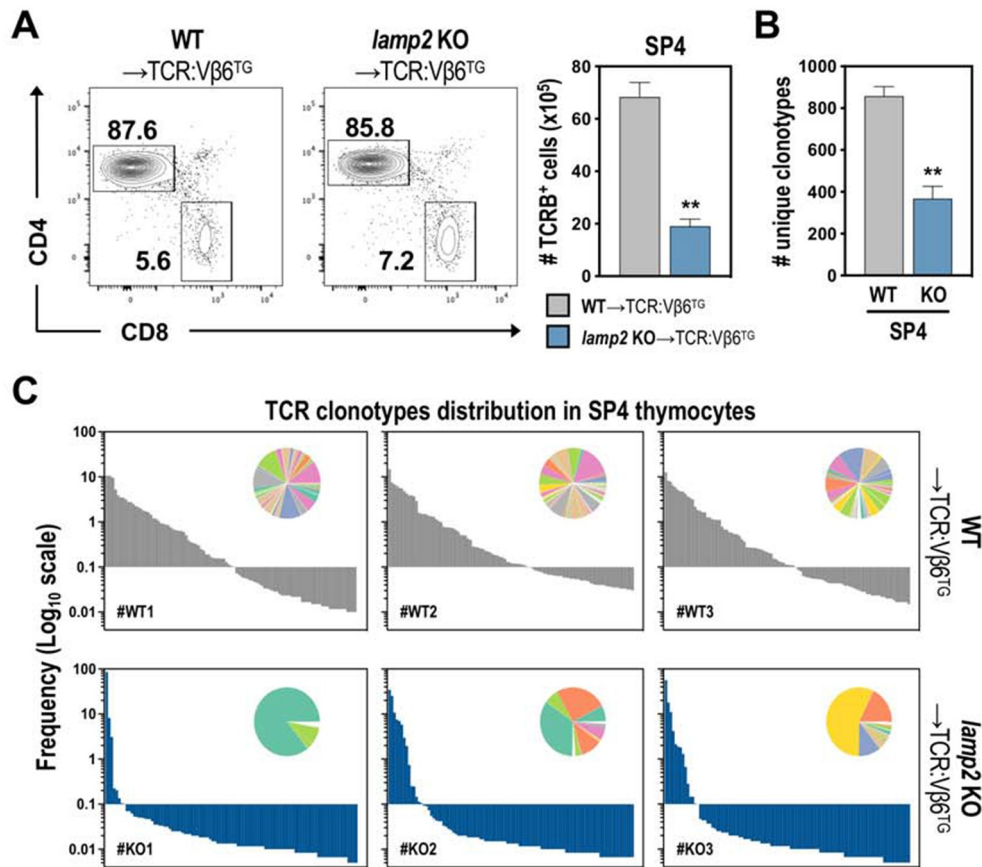


Figure 6. Analysis of SP4⁺ TCR repertoire in WT and *lamp2* KO thymus. (A) T cell development in WT (WT-TCR:Vβ6^{Tg}) and *lamp2* KO (*lamp2* KO-TCR:Vβ6^{Tg}) ectopic thymus grafted into fixed TCRβ/TCRβ^{Tg} recipients. CD4 and CD8 expression within mature TCRβ⁺ thymocytes. Graphs represent the absolute number of the indicated thymocyte subsets in WT-TCR:Vβ6^{Tg} (gray) and *lamp2* KO-TCR:Vβ6^{Tg} (blue) thymi. Data shown represent an average of 2 independent experiments (n = 6 WT and 6 *lamp2* KO). Graphs indicated (B) the number and (C) frequency of unique sequences of *Tcr* clones found in SP4 thymocytes developing within WT and *lamp2* KO ectopic thymus. Pie graphs in C depict a colorimetric representation of the most abundant clonotypes found in each thymi. Results in **A–B** are shown as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

developing CD4 T cells, possibly by affecting the MHC II-processing and the generation of positive selecting self-peptides in cTECs. Despite considerable efforts to identify the nature of self-peptides that promote positive selection [5], it remains challenging to conduct this biochemical characterization with sensitivity in rare populations, such as TECs. Due to this limitation, we determined the TCR clonal composition of SP4 thymocytes generated in WT or *lamp2* KO thymi. The assessment of TCR diversity could serve as an indirect indicator of the quality of the MHC II-peptide ligandome presented by *lamp2* KO cTECs. Given the vast combinations of TCRA/α-TCRBβ chains in SP4 thymocytes in a broad polyclonal setting, we used mice expressing a fixed transgenic TCR Vβ6 (beta6) chain [36] as recipients of thymic grafts. This model referred as TCR:Vβ6Tg, reduced the complexity of the TCR repertoire to the diversity of TCRA chains and favored CD4⁺ T cell selection, as confirmed by the accumulation of SP4 thymocytes in WT thymic grafts (Figure 6(A), Figure S5A). The bias toward CD4 lineage may result from the fact that the Vβ6 chain is derived from a MHC II-restricted TCR [36]. Consistent with the results in the fully polyclonal TCR repertoire of Nude and WT recipients, *lamp2*-deficiency restrained the number of SP4 TCR:Vβ6Tg thymocytes (Figure 6(A)). The cellularity of DP cells was also reduced in TCR:Vβ6Tg model (Figure S5A),

possibly due to the ubiquitous expression of MHC-II-restricted Vβ6 chain that may anticipate the effects provoked by *lamp2*-deficiency in the DP stage. For repertoire analysis, we isolated RNA from 3 samples of 1.10⁶ (FACS sorted) SP4 (CD4⁺ TCRβ⁺) thymocytes from WT and *lamp2* KO thymus. The *Tcra* gene was specifically amplified and the generated libraries were subjected to deep sequencing analysis of *Tcra* transcripts. We recovered 0.7–1.2.10⁶ sequences in SP4 cells derived from WT and *lamp2* KO grafts, from which 0.6–2.10⁵ corresponded to specific TCRs (Figure S5B, first two columns). Further analysis identified 1.3–1.5.10³ unique TCRs in the WT setting. This number (0.3–0.4.10³) was notably reduced in SP4 cells from *lamp2* KO thymi (Figure S5B, third column). Given the variable number of retrieved TCR sequences, the analysis was normalized to 6.10⁵ randomized TCR sequences/sample. This threshold was defined by the lower number of sequences identified on one sample (KO2) (Figure S5B). Following unbiased normalization, the number of unique clonotypes remained significantly lower in *lamp2* KO-derived SP4 thymocytes relatively to the ones generated from WT thymus (Figure 6(B), Figure S5B). Additionally, the distribution of TCRA sequences was distinctively different. While clonotypes of WT-derived thymus presented the stereotypical broad distribution expected to be found in a polyclonal repertoire, *lamp2*

KO counterparts displayed a clear skewing in the TCR catalog (Figure 6(C)). Further analysis revealed that the clonality index was markedly increased in mutant samples (Figure S5C).

These results led us to evaluate whether the restricted thymic SP4 TCR repertoire affected the function of peripheral T cells generated within *lamp2* KO thymic stroma. To do so, we resorted to the fully polyclonal TCR repertoire model wherein WT and *lamp2* KO thymi were grafted in Nude recipients, as described in Figure 2. We probed the proliferative response of splenic CD4 and CD8 T cells of WT- or *lamp2* KO-nu mice to polyclonal TCR stimulation *in vitro*, and found no differences between groups as measured by CFSE analysis (Figure S6A). These results suggest that CD4 and CD8 T cells developing in *lamp2* KO thymic stroma respond to a polyclonal TCR-mediated stimulation. Lastly, we determined whether thymic defects in TCR repertoire formation influence the outcome of an immune response to a live intracellular pathogen. We employed a well-defined model of T-cell dependent recall response to *Listeria monocytogenes* (*Lm*) [37]. Recipient mice were inoculated with 2.10^3 CFU (colony-forming units) of *Lm* at 12 weeks post thymic transplantation, to guarantee peripheral T cell reconstitution, and then rechallenged with 1.10^6 CFU 4 weeks later (Figure S6B). Non-thymic grafted nude recipients did not resist the infection protocol (Figure S6C), highlighting the requirement for a T cell-mediated response in this model. We assessed the T cell reconstitution in the spleen, and the clearance of *Lm* in the liver of infected WT-nu and *lamp2* KO-nu animals 3 days after the secondary challenge. Although reduced, the numbers of naïve and effector/memory splenic CD4 and CD8 T cells were not significantly altered in WT-nu or *lamp2* KO-nu (Figure S6D). All recipient mice were reconstituted. Despite the variability in CFU counts and the absence of complete bacterial clearance in both genotypes, the average CFU counts were slightly elevated in *lamp2* KO-nu mice, which presented an increase in the number of animals with a higher bacterial burden ($\geq \log_{10}4$) (Figure S6E). Interestingly, the CFU counts inversely correlated with the amount of IFNG/IFN γ (interferon gamma)-producing CD4 found in *lamp2* KO-nude after the recall response. These cells have been shown to provide protection against *Lm* [37]. A similar association was observed in IFNG/IFN γ -producing CD8, but this correlation was not significant (Figure S6E). Although antigen-specific responses were not monitored, *lamp2* KO-nu appeared to present a compromised *in vivo* T-cell response to secondary infection by *Lm*. Together, our results suggest that thymic stroma deficient in *Lamp2* restricts not only the quantity of selected CD4⁺ T cells, but also the quality of their TCR repertoire.

Discussion

Following the discoveries on the contributions of CTSL and PRSS16/TSSP to the generation of selecting MHC II:peptides complexes [9–12], there have been few advances in the mechanism that regulates CD4⁺ T cell selection. Our findings suggest that LAMP2 may represent a functional liaison that interconnects autophagy and processing of self-peptides-MHC II for selection in cTECs, which in turn regulates the

development of CD4 T cells and the diversity of their TCR repertoire. LAMP2 is expressed in TECs, but it was also detected in mesenchymal and endothelial cells of the thymic stroma. As non-TEC subsets do not have a major role in thymocyte selection [4], it is possible to conjecture that the defects in CD4 T cell development in *lamp2* KO thymic stroma may arise from LAMP2-dependent failures in TECs. Future studies with TEC-specific conditional knockout mice will provide more precise details on the cell-intrinsic role of LAMP2 in TECs. The strength and duration of the interaction between TCR and MHC II:self-peptide ligandome have implications in thymic selection [27,28]. Thymic stroma deficient in *Lamp2* led to a particular reduction in CD4 T cells that completed positive selection. While changes in TCRB^{int} CD69^{int} and TCRB^{hi} CD69^{hi} suggest defects in positive selection, alterations in TCRB^{hi} CD69^{hi} and TCRB^{hi} CD69^{neg} may reflect survival defects following positive selection, altered lineage commitment or negative selection. Our findings indicate that the development of CD4 T cells was not mechanistically associated with an abnormal redirection of MHC II-restricted cells into CD8 T cell lineage. Moreover, the reduced CD5 levels in TCRB^{int} CD69^{int} and TCRB^{hi} CD69^{hi} SP4s and the lack of alterations in IKZF2/HELIOS in DP and SP4 collectively suggested that TCR signaling and negative selection of SP4 thymocytes were not augmented. Instead, they may support the hypothesis that LAMP2 regulates the development of MHC II-restricted CD4 T cells that completed positive selection in the thymic cortex.

The observation that deficiency in *Lamp2* particularly affected autophagy flux in cTECs, but not mTECs nor other non-TEC thymic stromal cells, are in line with the reported high levels of autophagy in cTECs [7,8,38,39] and the role of LAMP2 in lysosome-AP fusion [18,19]. Moreover, autophagic vesicles gain access to the MHC II compartment in TECs [40] and autophagy has been linked with the generation of the peptide repertoire presented by MHC II [7,8,38,39]. We found that CTSL and lysosomal activities were elevated in *lamp2* KO cTECs, arguing against the possibility that alterations in CD74/Ii-MHC II processing results from a failure in its proteolytic function. The increased lysosomal activity was also reported in the hippocampus of *lamp2* KO mice [41], suggesting that LAMP2-mediated autophagy impacts lysosomal function. Several non-mutually exclusive possibilities might explain the reduction in SP4 selection by *lamp2*-deficient thymic stroma. The inhibition of AP-lysosome fusion in *lamp2* KO cTECs can compromise the shuttling of autophagy-derived cytoplasmatic selective antigens into the late endosomal MHC II loading pathway. Under these conditions, CTSL [9,10], PRSS16/TSSP [11,12] and other lysosomal proteases may not have access to substrates to produce MHC II-restricted self-peptides specialized for positive selection of CD4 T cells. This can lead to a compensatory upregulation of lysosomal activities as typically reported in models of lysosomal dysfunction or lysosomal storage disorders [18]. Additionally, the alteration in autophagic flux might also disturb CD74/Ii-MHC II trafficking to MHC II compartment or H2M function, thereby preventing the complete degradation of CD74/Ii or self-peptide loading. Furthermore, CTSL also contributes to the generation of MHC II-bound self-peptides presented by cTECs [9,10,35]. Thus, the increase in CTSL activity in *lamp2* KO cTECs could also interfere

with the quality of MHC II-peptide ligandome presented by these cells during positive selection. Hence, the incomplete degradation of CD74/Ii-MHC II in *lamp2* KO cTECs may result from altered trafficking of CD74/Ii-MHCII to MIIC and/or from a reduction in MHC II-loaded with positive selection-inducing self-peptides, due to an altered antigen processing and peptide repertoire generation. The consequent change in the composition of selective MHC II ligands may lead to a reduction of CD4 T cells that are completing positive selection and a skewing of their TCR repertoire. It will be important to elucidate the biochemical mechanism by which LAMP2 potentially regulates MHC class II-peptide generation in cTECs. Under regular conditions, endogenous antigens may access MHC II molecules through several LAMP2-regulated autophagic routes [17,18]. Future studies should also aim at investigating whether LAMP2-dependent macroautophagy delivers cytosolic antigens into autolysosomes for processing before loading to MHC II, and/or antigens are directly routed into the lysosome via CMA. In this regard, ATG5-dependent autophagy has been linked to positive selection of CD4 T cells [8,39], but it also contributes to mTEC-mediated tolerance induction [38,42]. Worth noting, *lamp2*-deficiency did not cause major defects in mTEC-dependent function nor disturbed tolerance, as measured by the presence of autoantibodies/lymphocytic infiltrates at 12 weeks post-transplantation (data not shown). Future studies should address whether breaks in tolerance can unfold in aged settings. Alternatively, autophagy may have distinct roles in c/mTEC function, whereby the specificity of the route and its consequence in thymic selection, can also be imposed by distinct downstream elements. While ATG5 participates in early phases of autophagy and may have a broader effect in cTEC- and mTEC-mediated thymic selection [8,39], LAMP2 mostly regulates later stages of the autophagic process and MHC II processing in cTECs. Future analysis should examine whether changes provoked by *lamp2*-deficiency in cTECs account for a more dedicated role in the control of CD4 T cell positive selection.

The observations that *lamp2*-deficiency in thymic stroma induced a skewing, but not a complete block, in CD4 TCR repertoire diversity, suggests that LAMP2 may regulate the generation of particular sets of autophagy-dependent MHC II-bound positive selecting peptides in cTECs. This hypothesis is in line with previous studies [43] supporting that positive selection is limited by the bioavailability of specific self-peptide/MHC ligands expressed by cTECs. Still, previous studies showed that a single MHC-peptide ligand can positively select multiple TCRs [44,45], indicating that positive selection is relatively permissive regarding the composition of MHC-peptide ligands in cTECs. Assuming that LAMP2 contributes to the generation of particular peptide:MHC II ligands, its deficiency may have variable effects depending on the plasticity of a given TCR. Future analysis employing TCR transgenics will allow defining LAMP2-dependent specificities and the contribution of LAMP2 in positive and negative selection. Additionally, post-selected thymocytes expressing high-affinity TCR intrathymically proliferate in response to their self-peptide/MHC ligands [46]. Thus, the dominance of particular clonotypes within the *lamp2*-deficient thymus may result from the expansion of SP4 T cells bearing favorable TCR affinities for overrepresented self-peptide/MHC ligands. Importantly, the TCR repertoire analysis was conducted in total SP4 cells,

without segregating conventional and regulatory CD4 T cells. Although T reg comprises a small fraction of thymic SP4, one can only presume that their TCR repertoire is also affected. Future analysis is required to clarify the impact of LAMP2 in TCR repertoire formation and the function of T regulatory cells. Lastly, the defect in thymic production imposed by *lamp2*-deficiency impacted on the peripheral T cell pool, mostly on the numbers of naïve CD4 T cells, and to a less extent on naïve CD8 T cells and effector/memory CD4 and CD8 T cells. In the thymic transplant of Nude mice, the establishment of the peripheral T cell pool depends on thymic output and LIP (lymphopenia-induced proliferation). As thymic CD8 T cell development was not affected, the reduced peripheral CD8 T cell pool may arise indirectly from insufficient CD4 T cell help. In this regard, it was shown that the LIP of CD8 T cells depends on CD4 T cells [47–49]. Additionally, our results indicate that CD4 and CD8 T cells, which developed in *lamp2*-deficient thymic stroma, respond to polyclonal TCR-mediated signaling. Yet, *lamp2* KO-nude recipients appear to hold a compromised T cell-dependent recall immune response to *Lm* infection. Future studies should clarify the peripheral consequences of these thymic defects, including a more in-depth analysis of T cell homeostasis, antigen-specific responses and tolerance, as well as the role of CD4 T cell in B cell responses and CD8 T cell memory.

In sum, our findings suggest that *lamp2*-deficiency in thymic stroma conditions the development and TCR repertoire formation of CD4 T cells. These findings provide a novel framework to further investigate how LAMP2 functionally interconnects autophagy and lysosomal generation of selecting self-peptides in cTEC, and its implications for CD4 T cell positive selection. Moreover, our results may also open new avenues for the identification of the “peptidic-self” in the thymus, which remains one of the crucial gaps in our understanding of thymus biology.

Material and methods

Mice

We used *lamp2* KO, *b2m* KO, RFP-GFP-LC3 Tg, TCR:V β 6 Tg and Nude mice that have been described previously and are all in a C57BL/6 background [19,29,31,36]. Mice were housed under specific pathogen-free conditions, and all animal experiments were performed in accordance with European guidelines (Directive 2010/63/EU). For fetal studies, the day of the vaginal plug detection was designated as embryonic day (E) 0.5.

Thymic kidney transplants

Thymi from WT and *lamp2* KO mice were transplanted under the kidney capsule of recipient mice as described [50]. Briefly, thymic lobes from E15.5 WT and *lamp2* KO mice were cultured for 2 days with 1.35 mM 2'-deoxyguanosine (SIGMA, D0901) to deplete lymphoid cells. Prior to transplantation recipient mice were pre-treated with the analgesic buprenorphine (0.05 mg/kg of body weight) and anesthetized with a gaseous mixture of 3% isoflurane and oxygen.

TEC and hematopoietic cell isolation

TECs were isolated as described [14,50,51]. Hematopoietic cells from thymus and spleen were prepared by mechanical disruption of the respective tissues. Splenic red blood cells were lysed using erythrocyte lysis solution: 155 mM ammonium chloride (Sigma-Aldrich, A9434), 10 mM potassium bicarbonate (Sigma-Aldrich, 237,205).

Flow cytometry

Cells were pre-treated with FC block (anti-FCGR3/CD16-FCGR2B/CD32 antibodies TruStain fcX; Biolegend, Biolegend, 101,320). Cell suspensions were stained as described [19–21] with Alexa Fluor 488/FITC-conjugated anti-CD8A (clone 53–6.7; eBioscience, 11–0081-82), anti-CD44 (clone IM7; Biolegend, 103,022), anti-15G4 (Santa Cruz Biotechnology, sc-53,946), anti-CD5 (clone 53–7.3; Biolegend, 100,605), anti-PECAM1/CD31 (clone MEC13.3; Biolegend, 102,514) and anti-CCL21 (clone 59,106; R&D Systems, IC457G-100UG); PE-conjugated anti-CD4 (clone GK1.5; eBioscience, 12–0041-82), anti-SELL/CD62L (clone MEL-14; eBioscience, 12–0621-81), anti-TCRB (clone H57-597; eBioscience, 12–5961-82), anti-CD80 (clone 16–10A1; eBioscience, 12–0801-82), anti-ENPEP/LY51 (clone 6C3; eBioscience, 12–5891-82), anti-LAMP2 (clone M3/84; Biolegend, 108,505), anti-CD40 (clone 3/23; BD Pharmingen, 553,791) and anti-MHCI^{Db} (clone 28–14-8; eBioscience, 12–5999-82); PerCP-Cy5.5-conjugated anti-PTPRC/CD45.2 (clone 104; Biolegend, 109,828), anti-TCRB (clone H57-597; eBioscience, 45–5961-80) and anti-MHCI^{Kb} (clone AF6-88.5; Biolegend, 116,515); PerCP-eFluor710 anti-CD4 (clone GK1.5; eBioscience, 46–0041, 82); PE-Cy7-conjugated anti-IL2R/CD25 (clone PC.61.5; eBioscience, 25–0251-82) and anti-CD69 (clone H1.2F3; eBioscience, 25–0691-81); APC/eFluor660-conjugated anti-CD8 (clone 53–6.7; eBioscience, 50–0081-82), anti-CD80 (clone 16–10A1; eBioscience, 17–0801-82), anti-EPCAM (clone G8.8; Biolegend, 118,212), anti-CD40 (clone 1C10; eBioscience, 17–0401-81) and anti-CD74 (In1/CD74, Biolegend, 151,003); APC-eFluor780-conjugated anti-I-A/I-E (clone M5/114-15-2; eBioscience, 47–5321-82); eFluor450-conjugated anti-CD24 (clone M1/69; eBioscience, 48–0242-82); BV421 conjugated anti-EPCAM (clone G8.8; Biolegend, 118,225) and anti-CD69 (clone H1.2F3; Biolegend, 104,527); BV510 conjugated anti-CD24 (clone M1/69; Biolegend, 101,831); BV605 conjugated anti-SELL/CD62L (clone MEL-14; Biolegend, 104,437); anti-PDGFRα (clone APA5; Biolegend, 135,916); BV650 conjugated anti-CD80 (clone 16–10A1; Biolegend, 104,731) and anti-CD8A/CD8α (clone 53–6.7; Biolegend, 100,741); BV785 conjugated anti-CD4 (clone GK1.5; Biolegend, 100,453). The binding of biotinylated *Ulex europaeus* agglutinin-1 (UEA-1) (Vector Laboratories, B-1065-2), anti-ENPEP/LY51 (clone 6C3; BD Pharmingen, 553,159), anti-LY75/CD205 (clone NLDC-145; Biolegend, 138,211), and anti-PDGFRβ (clone APB5; Biolegend, 136,009) were revealed by either PE-Cy7-conjugated (eBioscience, 25–4317.82) or BV711-conjugated (Biolegend, 405,241) streptavidin. For intracellular staining, cells were prepared according to the supplier's protocol (FOXP3 staining kit, eBioscience, 00–5523-00) and stained with FITC-conjugated anti-IKZF2/HELIOS (clone 22F6; Biolegend, 137,204), APC/eFluor660-conjugated anti-FOXP3 (clone FJK-

16s; eBioscience, 2,059,207) and anti-Aire (clone 5H12; eBioscience, 1,929,296) antibodies. Flow cytometry was performed on a LSRFortessa, with data analyzed on FlowJo software (BD). Cell sorting was performed on a FACSaria I (BD), with purities >96%.

Immunohistological analysis

Thymi were prepared for immunofluorescence as described [14]. Briefly, samples were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), and 8-μm sections were stained with biotinylated-UEA-1 (Vector Laboratories, B-1065-2) and anti-rat KRT8/cytokeratin 8 (Troma-1; DSHB, AB 531826) as primary antibodies, with Alexa Fluor 647 anti-rat (Invitrogen, A21247) and streptavidin Alexa Fluor 555 (Invitrogen, S21381) as secondary antibodies. Vectashield mounting medium with DAPI (Vector Laboratories, H-1200-10) was used to prepare the slides. Analysis was performed in an AxioImager.Z1 (Zeiss). Images were processed with and Fiji Software.

Autophagy analysis

We stained cells with Cyto-ID autophagy detection kit (Enzo, ENZ-51031-0050) according to the manufacturer's protocol. In brief, cells were first washed with PBS, mixed with Cyto-ID staining solution and incubated at 37°C for 30 min (Cyto-ID) and subjected to flow cytometry analysis. To block autophagy, thymi were cultured with chloroquine (50 μM; Enzo, ENZ-51031-0050) for 16 h and Baf-A1 (0.5 μM; SIGMA, B1793) for 5 h.

CTSL and lysosomal activity assays

CTSL and lysosome activities were respectively monitored using Magic Red CTSL (ImmunoChemistry Technologies, 941) and Lysosome-Specific (Biovision, K448) detection kits, according to the manufacture's protocol. Briefly, cells were either incubated with a magic red staining solution containing the CTSL fluorogenic substrate or Lysosome-Specific Self-Quenched Substrate for 1 h at 37°C in DMEM (10% FBS). Cells were then washed and stained with TEC markers for flow cytometry analysis. In cells with active CTSL, the substrate fluoresces red upon proteolytic cleavage. The fluorescent signal is proportional to the intracellular lysosomal activity in cells with active lysosomes.

TCR sequencing

SP4 thymocytes (1.0×10^6 cells) derived from TCR:Vβ6Tg hematopoietic progenitors and developing in WT or *lamp2* KO thymus grafts were purified by cell sorting. RNA was extracted using RNeasy Mini kit (Qiagen, 74,106) following the manufacturer's instructions. Full-cDNA library was prepared using Mint-2 kit (Evrogen, SK005), which introduces 5'-adapters to cDNA fragments, according to the manufacturer's instructions. The *Tcra* gene was then specifically amplified using PlatinumTM Taq DNA polymerase high fidelity (Invitrogen, 11,304,011) and a primer pair specific for the 5'-adapter and the C region of the *Tcra* gene. The sequencing

library was prepared using the Nextera kit (Illumina, FC-131-1024), in which each sample was barcoded, and sequenced using 250 bp paired-end illumina MiSeq technology. High-throughput sequencing were performed at Gene Core facility (EMBL, Germany).

Deep sequencing data analysis

Paired-end 250 bp illumina sequencing data were initially trimmed using Trimmomatic and subsequently merged using PEAR. clonotypeR toolkit was then used to perform TCR sequence annotation and quantitative analysis in R [52–54]. Out of the X raw reads obtained from the 6 samples (3 WT and 3 *lamp2* KO), we identified X-Y TCR clonotypes from X-Z productive TCR sequences. For the samples to be comparable, the analyses were performed on 60,000 randomly selected TCR sequences for each data set as it was the lowest number of TCR sequences found in a data set. The presented clonality metric is 1–Pielou's evenness index and can vary from 0 to 1 (more diverse to less diverse). The Pielou's evenness corresponds to the Shannon's entropy (using log 2) for each sample divided by the number of unique clonotypes (in log 2) of the same sample [54,55].

In vitro T cell activation

WT and *lamp2* KO-derived CD4 and CD8 T cells were sorted, labeled with 1 μ M of CellTrace™ CFSE (carboxyfluorescein diacetate succinimidyl ester; ThermoFisher Scientific, C34554) and cultured in the presence of 1 μ g/ml anti-CD3 mAb (clone 145–2C11; BD Pharmingen, 553,057) and 5 μ g/ml anti-CD28 mAb (clone 37.51; BD Pharmingen, 553,294). The precursor frequency of dividing cells (percentage of cells in the initial population that undergone one or more divisions after culture) was calculated as follows: $[\sum n \geq 1(Pn/2^n)]/[\sum n \geq 0(Pn/2^n)]$, where n is the division number that cells have gone through and Pn is the number of cells in division, as described [56].

Listeria infection

Mice were inoculated with *Lm* reference strain EGDe as described in [57]. Thymic grafted Nude recipients were intravenously infected 12 weeks post-transplantation, through the tail vein with 2.10^3 colony-forming units (CFUs) of *Lm*. Mice were rechallenged with 1.10^6 CFUs of *Lm* 28 days after the first priming. Three days after secondary infection, mice were sacrificed, livers were aseptically removed, homogenized in PBS, and homogenates were serially diluted and plated on BHI-agar (Fisher Scientific, 11,708,872). *Lm* colonies were enumerated after 24 h incubation at 37°C. For the intracellular IFNG/IFN- γ analysis, total spleen cells were stimulated for 5 h at 37°C, using the Cell Activation Cocktail, containing phorbol 12-myristate 13-acetate, ionomycin and brefeldin A (Biolegend, 423,304) according to the manufacturer's instructions. After cell surface staining, cells were prepared according to the supplier's protocol (FOXP3 staining kit; eBioscience, 00–5523-00), stained with PE-labeled anti-IFNG/IFN γ Ab (clone XMGI.2; Biolegend, 505,807) and analyzed by flow cytometry.

Statistical analysis

Analysis was performed using Prism 9.1.1 software (GraphPad Software). The two-tailed Mann-Whitney test was used for statistical differences between groups. For multiple comparisons, a two-way ANOVA was used. $p < 0.05$ was considered significant.

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MINI-REVIEW

T-cell selection in the thymus: New routes toward the identification of the self-peptide ligandome presented by thymic epithelial cells

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Within the thymus, thymic epithelial cells (TECs) provide a dedicated niche for the selection of functional T cells expressing a highly variable and self-tolerant T-cell receptor (TCR) repertoire. In this minireview, we start by summarizing recent studies that have improved our understanding on the composition of cortical TEC and medullary TEC microenvironments. Next, we focus on the molecular processes that control the function of TECs in T-cell selection. In particular, we discuss the role of cortical TECs in positive selection and the pathways employed by these cells to generate and present selecting self-peptides:MHC II complexes. Several studies have underscored the role of the $\beta 5t$ -containing thymoproteasome in the production of unique MHC I-bound peptides critical for CD8 T-cell selection. Contrarily, the identity of the molecular determinants that regulate the generation of MHC II-bound self-peptides capable of positive selecting CD4 T cells is far more uncertain. We highlight recent advances that interconnect the autophagy-lysosomal pathway, the presentation of specific sets of self-peptide:MHC II complexes, and the diversification of CD4 TCR repertoire. Lastly, we discuss how these findings may open up new avenues for deciphering the identity of the MHC I and MHC II ligandome in the thymus.

Keywords: Autophagy · Positive selection · T-cell selection · TECs · Thymus

Introduction

Within the thymus, the thymic stroma creates an essential three-dimensional microenvironment for the development and selection of immunological competent T cells. The thymic stroma includes thymic epithelial cells (TECs), mesenchymal and endothelial cells, dendritic cells (DC) and B cells, which together promote T-cell development. In particular, TECs are fundamental for the generation of a pool of T cells expressing functionally diverse, MHC-restricted and self-tolerant T-cell receptors (TCR) [1]. Therefore, understanding the molecular mechanisms that under-

lie the function of the thymic epithelium is crucial to comprehend the basis of T-cell development and harness T-cell-mediated responses. TECs are conventionally divided into cortical TEC (cTEC) and medullary TEC (mTEC) subtypes that occupy distinct anatomic areas of the thymus and regulate distinct stages of thymopoiesis. While cTECs promote T-cell lineage commitment and positive selection, mTECs orchestrate later stages of T-cell development, namely negative selection and regulatory T-cell (Treg) differentiation [1]. In this review, we begin by resuming recent studies that improve our understanding of the mechanisms underlying the establishment of TEC microenvironments. We then cover the molecular processes that control the function of TECs in T-cell selection, with a particular focus on the role of cTECs in positive selection.

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New insights on the development of cortical and medullary TEC compartments

Cortical and medullary TECs arise from common bipotent TEC progenitors (TEPs) that reside within the thymic microenvironment [2]. Earlier studies demonstrated the existence of TEPs in the embryonic thymus [3–8]. These discoveries led to refined models of TEC differentiation, whereby progenitors progress through transitional stages that are associated with cTEC lineage, prior to their commitment into mature c/mTEC subsets [9]. Several reports described various TEC subsets enriched in bipotent TEPs in the postnatal thymus [10–12] (reviewed in detail in [2, 9]). More recently, Boehm and colleagues revealed the existence of adult bipotent TEPs that contributed to mature cTEC and mTEC microenvironments in vivo [13]. Combining single-cell RNA sequencing (scRNAseq) and bar-code-based fate mapping analyses, the authors further showed that embryonic and adult TEPs partially shared a developmental relationship and exhibited distinct activity in vivo. In line with previous studies [12, 14], these results indicate that the bioavailability of TEPs is influenced by aging and continual lymphoepithelial interactions. In the recent years, advances in high-content scRNAseq analysis have expanded our knowledge on the intricate landscape of c/mTEC differentiation. Although our understanding of the developmental pathways that control the establishment of cTECs remains incomplete, Hollander and colleagues described a new subset of proliferative cTEC, referred as perinatal TECs, whose representation declined with age [15]. The age-dependent decrease in perinatal cTECs mimicked the dynamics of $\beta 5t^+$ TEPs, which gradually lose the capacity to contribute to mTEC lineage early in life [16, 17]. It remains to be determined whether these subsets represent a pool of unipotent precursors of mature cTECs. While a better characterization of the cortical epithelium is needed, new reports have revealed a constellation of specialized mTEC populations that add up to the previously identified precursor (Claudin-3/4⁺, Claudin-3/4⁺, Podoplanin⁺) and mature (chemokine (C-C motif) ligand 21 [CCL21]⁺, autoimmune regulator [Aire]⁺, Post-Aire⁺) subsets [2]. Two recent studies identified a population of proliferating mTECs (also termed transit-amplifying TAC) that may include distinct precursors of Aire⁺ and CCL21⁺ cells [15, 18]. Future fate-mapping analysis should address whether Aire⁺ and CCL21⁺ lineages share a direct precursor–product lineage relationship. The identification that Aire⁺mTECs further differentiate into Post-Aire cells [2] provided initial indications that the composition of mTECs was more complex than initially appreciated. Two earlier studies reported specialized populations of tuft-like mTECs that are partially dependent on AIRE. Tuft-like mTECs appear to regulate the development of invariant natural killer T (NKT) cells and innate lymphoid cells [19, 20]. More recently, additional post-Aire mTECs have been identified based on the expression of lineage-specific traits that mimic keratinocyte-, microfold-, enterocyte, muscle, and ciliated-like cells [21]. Despite the unquestionable advances in deciphering the complexity of TEC niches, our comprehension of the molecular basis that underlies the differentiation of these emerging subsets remains incomplete. Future

studies are needed to uncover the role of new specialized mTEC subsets in immunological tolerance, or alternative processes in thymus biology. Moreover, the identification of many of the new progenitors or mature subsets depends so far on complex scRNAseq approaches, which are incompatible with their isolation for functional analysis. Therefore, there is a need to develop easier methodologies to prospectively identify these populations based on the expression of specific cell-surface markers [16, 17, 21, 22]. Lastly, complementary genome-wide studies have provided valuable insights into the cellular heterogeneity and transcriptomic diversity of human TECs [23–25], which appear to mimic in part the data obtained in the mouse models. Future efforts should also concentrate in studying how this molecular complexity contributes to advance our comprehension of human TEC differentiation and to study the expression of tissue-restricted antigen (TRA) associated with human autoimmune diseases.

cTEC/mTEC: The yin and yang of T-cell selection

The understanding of the molecular mechanisms that control positive selection, CD4/CD8 lineage choice, negative selection and T-regulatory selection remains a topic of intense scrutiny. During positive selection, CD4⁺ CD8⁺ double positive thymocytes expressing TCRs that weakly interact with self-peptide:MHC complexes presented by cTECs survive and differentiate into CD4 or CD8 single positive cells. During negative/regulatory T-cell selection, thymocytes expressing TCRs with high affinity for self-peptide:MHC complexes typically presented by mTEC and DCs are eliminated or, in case of SP4 thymocytes, can also be converted into T regulatory cells [1, 26]. The spatiotemporal control of these multistep selection checkpoints depends on the interaction between $\alpha\beta$ TCR expressed by developing T cells and unique sets of self-peptides bound to MHC molecules expressed by TECs [1]. Considerable efforts have been made in studying how the fate of developing thymocyte is dictated by the cell-autonomous integration of TCR-mediated signals with distinct strength and duration [27]. Yet, an equal important variable in thymic selection is the generation, and presentation, of specialized selective self-peptide-MHC complexes by cTECs/mTECs. The complementary functions of cTECs and mTECs in regulating T-cell selection are determined by their specific antigen processing and presentation features, which are in turn controlled by specialized protein degradation machineries and transcriptional programs [1, 26]. It is recognized that mTECs have distinctive (epi)genetic abilities to express TRA, in a process that is partially dependent on Aire and forebrain embryonic zinc finger 2 (Fezf2) [1, 2]. Apart from direct control of TRA expression, Aire regulates alternative processes in mTEC function that cooperates to ensure tolerance induction and prevent autoimmunity [22, 28, 29]. The promiscuous gene expression capacity of mTECs allows them to function as a library of ubiquitous antigens and TRAs, which at a single cell level are presented in an ordered but randomly organized expression pattern [30–32]. This distinctive pattern of expression maximizes

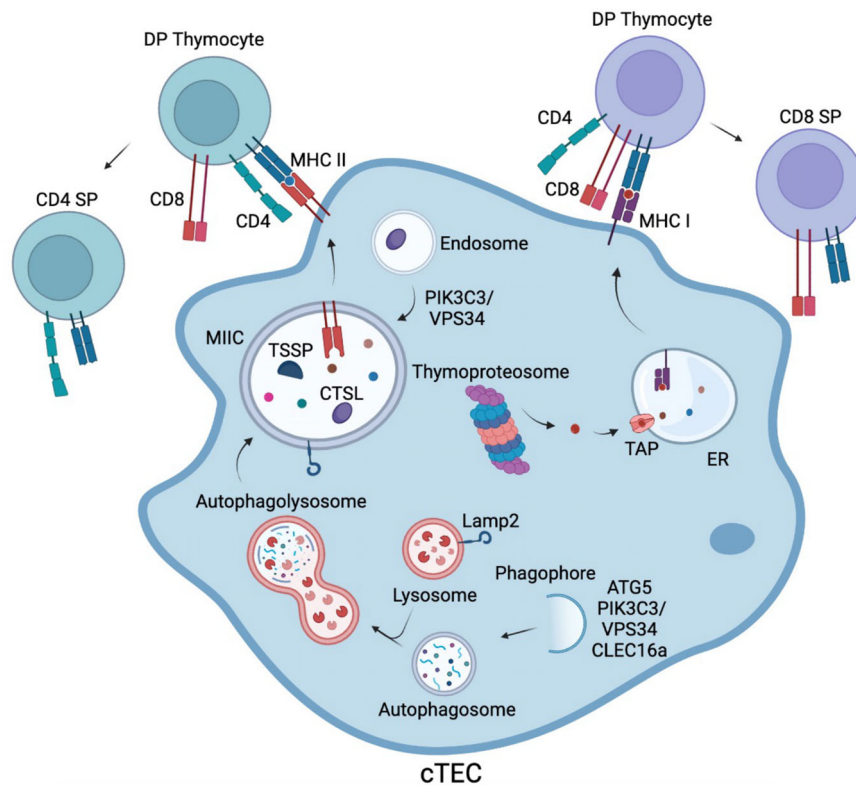


Figure 1. Model depicting the molecular basis for the generation and presentation of self-peptide complexes by cTECs. Left: MHC II-bound self-peptides required for positive selection of CD4 T cell derived from the autophagic route. During the autophagic process, LAMP2 mediates the fusion of the intracellular components of autophagosome with lysosomes. The maturation of the autophagolysosome ultimately culminates in the formation of the MHC II compartment (MIIC). Generated peptides are loaded in mature MHC II molecules and the MHC II:peptide complex presented to developing double positive (DP) thymocytes. DP thymocytes expressing MHC II restricted-TCR will commit toward the CD4 lineage upon interaction with self-peptides/MHC II complexes. Right: MHC I-bound self-peptides required for positive selection of CD8 T cell derived from cytosolic antigens degraded by the thymoproteasome. Following transport via transporter associated with antigen presentation (TAP) into the lumen of the endoplasmic reticulum (ER) and loading into MHC I molecules, self-peptides are presented by MHC I to developing DP thymocytes. Thymocytes expressing MHC I restricted-TCR will commit toward the CD8 lineage upon interaction with self-peptides/MHC I complexes

the effectiveness of TRA presentation. mTECs employ conventional proteolytic pathways to present the so-called TRA-derived “public” MHC-bound peptides that are critical to negatively select autoreactive thymocytes and/or driving their clonal deviation into regulatory T-cell lineage [1]. Interestingly, new findings suggest that TRA expression is dynamically controlled across life, a process that may have implications for the maintenance of tolerance in the older people [15, 30, 33].

How do cTECs coordinate positive selection?

Our comprehension on how cTECs regulate thymic selection is far more uncertain. It is conceptually accepted that cTECs possess unique antigen processing properties to generate sets of “private” MHC-bound self-peptides to select CD8 or CD4 T cells. The most accepted current view indicates that cTECs present unique sets of MHC-bound self-antigens, also referred as “private,” which are distinct from those that are generated by other thymic APCs, including mTECs or DC, or peripheral APCs found elsewhere in the body [1, 26]. This idiosyncrasy is associated with the role of specialized protein-degradation and antigen-processing machineries in cTECs and that can generate specific sets of MHC I- or MHC II-bound selective ligands. Although the identity of MHC-self-peptide ligandome remains unknown, recent reports have shed new light on the molecular mechanisms that control the generation of thymic selective ligands for positive selection (Fig. 1).

The generation of MHC I-associated selective self-peptides

The positive selection of CD8 T cells is critically dependent on a specific type of proteasome, thymoproteasome, which is exclusively found in cTECs. This form of the proteasome harbors the unique $\beta 5t$ (*Psmb11*) catalytic component that confers to the thymoproteasome specific substrate proteolytic activities [34]. *Psmb11*-deficient mice exhibit a compromised CD8 T-cell development, altered TCR repertoire, and defective T-cell responses [35, 36]. Since proteasomes generate peptides for MHC I presentation, these data indicated that $\beta 5t$ -containing proteasomes produce unique sets of self-peptides for optimal CD8 T-cell selection [37, 38]. Two models have been proposed to explain how thymoproteasome-derived peptides contribute to CD8 positive selection. In the low-affinity motif model, the thymoproteasome produces unique MHC I-bound self-peptides that specifically promote low-affinity interactions with TCR-expressing double positive thymocytes and their selection to the CD8 lineage [38, 39]. The peptide-switch model complements that the presentation of unique peptides by cTECs is switched in other thymic APCs found in the medulla, which do not express $\beta 5t$. In this setting, the $\beta 5t$ -mediated selection avoids the overlap with peptides derived from other forms of proteasome (conventional and immunoproteasome), preventing that positively selected CD8 T cells undergo clonal deletion by encountering the same peptide on other thymic medullary APC [40]. The two models have been reviewed in detail in Ref. [26, 34]. The elucidation of the biochemical identity of MHC I-bound self-peptides may help to clarify whether the two

models are mutually exclusive or congruent relatively to the function of thymoproteasome in positive selection.

The generation of MHC II-associated selective self-peptides

The master determinants that regulate the generation of MHC II-bound self-peptides capable of positively selecting CD4 T cells remain elusive. Earlier studies showed a critical role for the lysosomal endopeptidase cathepsin L in the positive selection of CD4 T cells. Cathepsin L is highly abundant in cTECs and controls the processing of MHC-II invariant chain (Ii) and the generation of selecting MHC-II-associated self-peptides for CD4⁺ T-cell development [41, 42]. Subsequent studies identified the contribution of the endosomal thymus-specific serine protease (TSSP). Although *Tsssp*-deficient mice displayed an overall normal polyclonal CD4⁺ T-cell compartment, the selection of certain MHC-II restricted transgenic TCRs was affected by the absence of TSSP [43, 44]. These results could indicate that TSSP controls the generation of restricted sets of positively selecting self-peptides bound to MHC II molecules in cTECs. cTECs appear inefficient in presenting extracellular proteins through the classical endocytic pathway on MHC II molecules [1]. Instead, cTECs rely on unconventional endogenous MHC II-loading routes that involve the autophagic-mediated shuttling of cytoplasmatic proteins into the lysosomes [1]. TECs possess a high constitutive level of macroautophagy at the steady state, being the autophagic flux highly detected in cTECs [45]. The functional role of autophagy in the thymic epithelium was initially determined by the analysis of the thymus of *Atg5*-deficient mice transplanted into athymic recipients. While *Atg5* deficiency in the thymic stroma did not impair the regular program of T-cell development, it affected positive selection of some MHC-II restricted TCR specificities and induced severe colitis and multiorgan inflammation in lymphopenic recipients [45]. Complementary studies revealed that *Clec16a* deficiency, a gene associated with multiple autoimmune disorders, disrupted autophagy in TECs, which in turn lead to changes in both positive and negative selection of developing thymocytes, and promoted autoimmunity in a nonobese diabetic model for type 1 diabetes [46]. These results indicate that autophagy in TECs is important for their role on tolerance induction. Yet, a subsequent study revealed that targeted deletion of ATG5 in the thymic epithelium did not lead to overt tissue inflammation under normal conditions in 6- to 12-month-old mice [47]. This apparent discrepancy may be related to differences in the genetic background, insufficient aging of ATG5 conditional KO mice in the steady state, and/or the effect of a peripheral lymphopenic microenvironment in experimental settings based on thymic transplantations, a factor known to promote the development of autoimmunity. Although autophagy has been linked to the generation of thymic-selecting ligands, the molecular liaisons that connect autophagy to the MHC II compartment were not known until recent. Complementary studies from our laboratory and others provide new exciting evidence that establishes the link between the autophagy-lysosomal pathway and the

generation of positively selecting self-peptides by cTECs, and how this impact CD4 T cell development. We reported that *Lamp2*-deficiency blocks autophagy dynamics and augments the abundance of invariant chain intermediates bound to surface MHC class II molecules specifically in cTECs. These alterations suggest defects in self-peptide MHC II processing, leading to a reduction in CD4 T cells that complete positive selection and a restricted catalogue of TCR specificities [48]. Lysosomal-associated membrane protein 2 (LAMP2) is a lysosomal membrane protein that controls distinct autophagic routes, including macroautophagy and chaperone-mediated autophagy [49]. Because autophagy is particularly augmented in cTECs and LAMP2 controls autophagy dynamics, we conjecture that LAMP2 controls the generation of positively selecting self-peptides by cTECs. Interestingly, complementary studies by Postoak et al. showed that deletion of *Pik3c3/Vps34* in TECs induced similar changes in positive selection of CD4 T cells and TCR repertoire formation. Although PIK3C3/VPS34 controls autophagy in cTECs, its role in CD4 T-cell selection appears to involve the regulation of vesicle trafficking required for cathepsin L activity [50]. Given the similarities in the phenotypes provoked by *Lamp2* and *Pik3c3/Vps34* deficiency, one possibility is that autophagy and vesicle trafficking participate in distinct steps of a broader pathway involved in CD4 T-cell selection (Fig. 1). Yet, both proteins have alternative functions besides autophagy, including cholesterol homeostasis (LAMP2) and endocytosis (PIK3C3/VPS34) [50]. Therefore, understanding the molecular role of these proteins in the processing of self-peptide MHC II processing should be investigated in the future. Moreover, it remains to be further investigated whether distinct autophagic routes are active in cTEC, and if so, whether they allow compartment-specific self-peptides to gain access to the MHC-II loading compartment. This would open the possibility that the generation of positively selecting MHC II:self-peptides at the lysosome/MHC II compartment arises from the protein cargo degradation from different intracellular routes, including macroautophagy and chaperone-mediated autophagy. The development of new mouse models to dissect these possibilities may allow to determine the MHC-II ligandome that derives from different autophagic pathways. This concept could implicate that the selection of a functionally diverse CD4 TCR repertoire is imposed by unique sets of self-antigens that are integrated in the lysosome/MHC II compartment from distinct intracellular sources. Whether the specific trafficking of substrates for selective self-peptide/MHC II ligands influence the formation of TCR repertoires and the pattern of recognition of those TCRs remains to be investigated.

Conclusions

These new results provide an important framework in the understanding on the role of cTECs in the choreography of CD4 and CD8 T-cell positive selection. The identification of new molecules and pathways involved in antigen presentation and processing may open new research avenues toward the identification of

the “peptidic self” in the thymus. The achievement of this goal remains technically challenging due to the low abundance of cTECs and the sensitivity threshold for the biochemical characterization of MHC I- and MHC II-bound peptides. The development of new mouse models and more sensitive analytic methods will facilitate the identification of the MHC I and II ligandome. Knowledge in these areas would close an important gap in our understanding of TCR repertoire formation and thymus function. Understanding how cTECs perform the selection of immunological competent and self-tolerant CD4 and CD8 T cells will ultimately lead to a better comprehension on the basis of immune responses and tolerance induction.

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- Abbreviations:** Aire: autoimmune regulator · CCL21: chemokine (C-C motif) ligand 21 · cTECs: cortical TECs · LAMP2: lysosomal-associated membrane protein 2 · mTECs: Medullary TECs · scRNAseq: single cell RNA sequencing · TECs: thymic epithelial cells · TEPs: TEC progenitors · TRA: tissue-restricted antigen · TSSP: thymus-specific serine protease
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