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The establishment of the stromal microenvironment in the thymus and its implications for T-cell development

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The establishment of the stromal microenvironment in the thymus and its implications for T-cell development.

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Summary

The thymus coordinates the generation of a diverse pool of immunologically competent and self-tolerant T cells. Given that thymic function is not constant throughout life, it is of fundamental and clinical relevance to understand how to harness T cell development. This process is tightly regulated by stromal cells that include thymic epithelial cells (TECs), thymic mesenchymal cells (TMCs), and thymic endothelial cells, among others. TECs are classically divided into two functionally distinct cortical (cTECs) and medullary (mTECs) subsets, which arise from common bipotent TEC progenitors (TECp), and represent key functional components of thymic stroma. While cTECs control T cell lineage commitment and positive selection, mTECs regulate negative selection and T regulatory (T_{reg}) cell generation. We began by critically reviewing the changes that occur in progenitors and mature TEC subsets during the postnatal life, integrating the timely coordination between those alterations and the decline in thymic function (Chapter II).

Understanding the molecular principles regulating TEC development is essential to decipher the intricacies underlying T cell-mediated immunity and tolerance. In this regard, several studies suggest that post-transcriptional modifications control TEC differentiation and function, and therefore, RNA-binding proteins (RBPs) arise as potential regulators of the transcriptional program of TECs. Based on previous genome-wide transcriptomic analysis of TEC sub-populations, we examined the role of Zinc Finger Protein (ZFP36) RBP family in TEC biology. Analysis of novel cKO mice revealed that TEC-specific deletion of *Zfp36* and *Zfp36l1* reduced TEC cellularity without affecting thymopoiesis. Strikingly, the combined deletion of *Zfp36* and *Zfp36L1* induced a similar reduction in TEC cellularity, but that instead led to a noticeable decrease in thymopoietic activity. These results suggest a cooperative role for ZFP36 proteins in TEC homeostasis and function (chapter III).

We also focused our studies on TMCs, which have important regulatory roles in thymus organogenesis, function and regeneration. Nevertheless, the mechanisms underlying the development of TMCs remain elusive. Here, we identified two novel thymic fibroblast subsets (TF^A and TF^B) with distinct developmental features. While TF^A were more abundant in the embryonic thymus, TF^B predominated in postnatal life. Lineage analysis showed that TF^A had the potential to generate TF^B. Lastly, the homeostasis of TF subsets was perturbed in *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ immunodeficient mice models, indicating that TMC differentiation depends on signals provided by developing thymocytes (chapter IV).

Collectively, our results provide new insights into the molecular networks and the developmental trajectories underlying the differentiation of TECs and TMCs. The enhanced

comprehension of these processes will contribute with basic knowledge to be integrated in novel therapeutics to reverse or correct thymic involution.

Resumo

O timo coordena o desenvolvimento de um reportório de linfócitos T imunologicamente competentes e tolerantes contra os nossos próprios componentes. Tendo em consideração que a função tímica não é constante ao longo da vida, é crucial em termos de conhecimento fundamental e aplicação clínica o entendimento de como controlar o desenvolvimento de células T. Este processo é regulado minuciosamente pelas células estromais do timo que ocupam os nichos corticais e medulares tímicos e incluem células epiteliais tímicas (CETs), células mesenquimais tímicas (CMTs) e células endoteliais tímicas, entre outras. As CETs são classicamente divididas em dois subgrupos funcionalmente distintos, CETs corticais (cCETs) e medulares (mCETs), que são originadas por progenitores bi-potentes comuns e representam componentes fundamentais do estroma tímico. Enquanto as cCETs controlam a especialização de linhagem em células T e a seleção positiva, as mCETs regulam a seleção negativa e a geração de células T reguladoras. Nós começamos por providenciar uma revisão crítica da literatura relativamente às mudanças que ocorrem nos progenitores e em subgrupos de CETs maduras durante o período pós-natal, integrando a coordenação temporal entre essas alterações e o declínio da função tímica (capítulo II).

O conhecimento dos princípios moleculares que regulam o desenvolvimento das CETs é essencial para decifrar os complexos mecanismos subjacentes à imunidade e tolerância mediada por células T. Neste sentido, vários estudos sugeriram que modificações pós-transcricionais controlam a diferenciação e função de CETs e por isso proteínas de ligação ao RNA surgem como potenciais reguladores do seu programa transcricional. Baseado em análises anteriores do transcriptoma de subpopulações de CETs, nós examinamos o papel da família de proteínas Zinc Finger Protein (ZFP36) na sua biologia. As análises de novos modelos de ratinho mutantes revelaram que a deleção específica em CETs de *Zfp36* ou *Zfp36l1* provocou uma redução na sua celularidade sem alterações detetáveis na timopoiése. Surpreendentemente, a deleção combinada de *Zfp36* e *Zfp36l1* provocou uma redução similar na celularidade das CETs, mas desta vez foi observada uma diminuição da actividade timopoiética. Estes resultados sugerem que as proteínas ZFP36 tem uma função cooperativa na homeostase e função das CETs (capítulo III).

Nós também nos focamos nas CMTs que tem um importante papel regulador na organogénese, função e regeneração do timo. No entanto, os mecanismos subjacentes ao desenvolvimento das CMTs permanecem por clarificar. Nesta tese, nós identificamos duas novas populações de fibroblastos tímicos (TF^{A} and TF^{B}) que possuem um padrão de

desenvolvimento distinto. Enquanto TF^A são mais abundantes no timo embrionário, as TF^B predominam na vida pós-natal. Análises de potencial de linhagem demonstraram que as TF^A tinham capacidade de gerar TF^B. Por último, a homeostase de fibroblastos tímicos está alterada em modelos de ratinho imunodeficientes (*Rag2*$^{-/}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$), indicando que a diferenciação de CMTs depende de sinais providenciados por timócitos em desenvolvimento (capítulo IV).

Globalmente, os nossos resultados providenciam novas pistas acerca das redes moleculares e trajetórias de desenvolvimento subjacentes à diferenciação de CETs e CMTs. O conhecimento mais completo destes processos vai contribuir para o conhecimento fundamental da biologia do timo, podendo ser integrado em terapias inovadoras para reverter ou corrigir a involução tímica.

List of Abbreviations

Aire - Autoimmune regulator;
AGM – Aorta-gonad-mesonephros;
AMP – Antimicrobial peptides;
APS-1 – Autoimmune polyglandular syndrome type 1;
BCR - B cell receptor;
BM - Bone marrow;
BMP - Bone morphogenic protein;
BMT - Bone marrow transplant;
CCL – Chemokine (C-C motif) ligand;
CCR – C-C Chemokine receptor;
CDA – Cytidine deaminases;
CD - Cluster of differentiation;
CD40 - Cluster of differentiation 40;
CD40L - Cluster of differentiation 40 ligand;
cKO – Conditional knockout;
Cld - Claudin;
CLP – Common lymphoid progenitor;
CMJ - Cortico-medullary junction;
cTEC - Cortical thymic epithelial cell;
CTR - Control;
CXCL – Chemokine (C-X-C motif) ligand;
CXCR – C-X-C Chemokine receptor;
DC - Dendritic cell;
DETC – Dendritic epidermal T cells;
dGuo - 2-deoxyguanosine;
Dll4 - Delta-like 4;
DN - Double negative;
DP - Double positive;
Dscam – Down syndrome cell adhesion molecule;
E - Embryonic day;
ECM – Extracellular matrix;
EpCAM - Epithelial cell adhesion molecule;
ETP – Early thymic progenitor;
Eya1 – Eyes absent 1 homologue;
Fezf2 – FEZ family zing finger 2;
FGF - Fibroblast growth factor;
Foxp3 - Forkhead box P3;
Foxn1 - Forkhead box N1;
FREP – Fibrinogen-related proteins;
FTOC - Fetal thymic organ culture;
GATA – GATA-binding protein;
Gcm2 - Glial cells missing homolog 2;
GO – Gene ontology;
H3K4 – Histone 3 lysine 4;
HDAC3 – Histone deacetylase 3;
Hoxa3 – Homeobox A3;
ICAM-1 – Intercellular Adhesion Molecule 1
IGF - Insulin growth factor;
IL - Interleukin;
iNKT – Invariant natural killer T cell;
ISP - Immature single positive;
jTECs – Junctional thymic epithelial cells;
K - Cytokeratin;
KO – Knockout;
LPS – lipopolysaccharide;

Lti - Lymphoid tissue inducer;
LTβR - Lymphotoxin beta-receptor;
MAIT – Mucosal-associated invariant T
MHC - Major histocompatibility complex;
mTEC - Medullary thymic epithelial cell;
MTS - Mouse thymic stroma;
NC - Neural crest;
NF-κB - Nuclear factor kappa B;
NIK - NF-κB-inducing kinase;
NK - Natural killer;
NLR – Nod-like receptors;
OPG - Osteoprotegerin;
pGE - Promiscuous gene expression;
PAMP – Pathogen-associated molecular patterns;
Pax – Paired box gene;
PDPN – Podoplanin;
PGRP – Peptidoglycan recognition proteins;
Plet1 – Placenta-expressed transcript- 1;
PRR – Pattern recognition receptors;
pMHC - self-peptide-MHC complexes;
PP – Pharyngeal pouch;
qPCR - Quantitative polymerase chain reaction;
RA - Retinoic acid;
RAG - Recombination activating gene;
RANK - Receptor activator of nuclear factor kappa B;
RANKL - Receptor activator of nuclear factor kappa B ligand;
RE – Responsive element;
RelB - Reticuloendotheliosis viral oncogene related B;
RLR – RIG-I-like receptors;
RTE – Recent thymic emigrants;
RTOC - Reaggregate thymic organ culture;
Runx – Runt-related transcription factor;
S1P - Sphingosine-1-phosphate;
S1P1 - Sphingosine-1-phosphate receptor 1;
SCF - Stem cell factor;
SCID - Severe combined immunodeficiency;
SL-TBI – Sub-lethal total body irradiation;
SP - Single positive;
SSEA-1 – Stage-specific embryonic antigen;
TCR - T cell receptor;
Th-POK – T-helper inducing POZ/Kruppel-like factor;
TECs - Thymic epithelial cells;
TECp - Thymic epithelial cell progenitor;
TLR – Toll-like receptor;
TNFRSF - Tumour necrosis factor receptor super family;

TRA - Tissue restricted antigen;
TRAF - Tumour necrosis factor receptor-associated factor;
T_{reg} - Regulatory T cell;
TSSP - Thymus-specific serine protease;
TSP – Thymic seeding progenitors;
TZF – Tandem zinc finger
UEA - *Ulex europaeus* agglutinin;
VCAM-1 – Vascular cell adhesion molecule 1;
VLR – Variable lymphocyte receptor;
Wnt - Wingless-related MMTV integration;
Wt - Wild Type;
XCL1 – Chemokine (X-C motif) ligand 1;
YFP - Yellow fluorescence protein;
γ^c - gamma chain;

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Chapter I

Introduction

1.The hurdles of innate immunity

The evolutionary need for immune defense mechanisms might be related with a self-discrimination problem. A living organism is an organized demarcation of organic matter that operates autonomously at the individual level for its perpetration by a complex plethora of biochemical reactions, counteracting the natural state of entropy. In this context, everything that subverts the harmony of this organized structure must be readily identified and either tolerated or possibly eliminated in order to preserve the identity of their structural organization. From prokaryotes to the more complex multicellular eukaryotes, strategies have been developed throughout evolution to fulfill the requirement of biologic preservation of an individual or population. Comparative immunology has gathered multiple examples suggesting that one of the first strategies implemented during evolution was the acquisition by the host of germline-encoded molecules that targeted specific constituents of pathogens [1, 2].

Even simple microorganisms lacking the degree of structural complexity are equipped with immune mechanisms to cope with foreign components. The ancestral CRISPR-Cas system becomes activated in response to bacteriophage infections in bacteria and archaea, representing a primitive defense mechanism [3]. In the class of invertebrates, some orthopods such as some species of drosophila, encode Down Syndrome cell adhesion molecules (Dscam), which are secreted upon infection and facilitate phagocytosis, working presumably as an opsonin [1, 4, 5]. Insects, for example, express Peptidoglycan recognition proteins (PGRPs) which recognize peptidoglycans widely present in the cell wall of Gram-positive [1]. They can be secreted or transmembrane proteins that upon ligand binding induce the expression of antimicrobial peptides (AMPs) [1]. Snails' hemocytes secrete fibrinogen-related proteins (FREPs) in response to trematode parasitic worms infection [1]. These proteins recognize sporocysts and their secretory/excretory products (SEPs) contributing to their clearance through the promotion of phagocytosis by hemocytes and the release of oxygen radicals [1]. The generation of diversity of these pathogen recognition receptors (PRR) includes processes such as alternative splicing and homologous recombination, which can expand the range of different types of pathogens that can be recognized [1].

Regarding vertebrates, the arsenal of germline-encoded PRRs capable of recognizing pathogen-associated molecular patterns (PAMPs) are better characterized, particularly in mammalians. Toll-like receptors (TLRs) are a family of extracellular and intracellular receptors capable of recognizing components of the bacterial cell wall and flagella such as lipoteichoic acids, lipopolysaccharide (LPS) and flagellin [6, 7]. Moreover, intracellular TLRs can also recognize foreign nucleic acids from virus and bacteria engulfed

by endocytosis [6, 7]. RIG-I-like receptors (RLRs) are cytoplasmatic receptors which also recognize viral RNAs [8, 9]. Another class of PRRs are represented by Nod-like receptors (NLR), which are located in the cytoplasm and bind to digestion products derived from bacterial cell-wall [10]. These receptors are expressed by the innate immune cells, such as macrophages, neutrophils, and DCs, and are critical for the first line of defense rapidly activated upon infection. The complement system discovered in 1980 by Jules Bordet also comprises soluble proteins that are activated in microbial surfaces and participate in pathogen elimination either directly or by the recruitment of cells of innate immunity [11]. Indeed, many of these receptors are present in the most primitive multicellular invertebrates [2, 12], however in some cases playing additional non-immunological functions or unknown roles such as the case of the *Drosophila* TLR homolog, Toll-1, that binds to spätzle defining dorsal-ventral polarity during embryogenesis [13]. Therefore, during the course of evolution some of these receptors present in invertebrates acquired different features (*e.g.*, binding mechanism) and functions [14]. One possible explanation for this functional diversification might rely on the successive rounds of genome duplication, which enabled that certain copies of those genes acquired different functions by random mutation [15].

Thus, innate immunity in both vertebrates and invertebrates is dependent on a panoply of different germ-line encoded receptors binding to pathogen constituents. However, pathogens have simultaneously evolved mechanisms to evade innate immune recognition. This would imply the almost virtually impossible need to develop a broader number of germ-line encoded receptors that can recognize the new changes in pathogens. In this context, the acquisition of an adaptive immune system by the gnathostomes decreased the dependency on a continual development of new PRRs. The general concept relies on the customization of receptors that are randomly assembled from a diverse set of gene segments, enabling the specific recognition of elements from pathogens, without the need of encoding them previously on the genome in their final configuration. This personalized strategy came at the cost of a slower immune response compared to the one provided by innate immunity. Yet, this is further compensated by the development of an immune memory, which upon a subsequent encounter initiates a faster and stronger response. Therefore, this evolutionary mechanism, “adaptive immunity”, equipped vertebrates with a new capacity to respond to pathogens and foreign elements in a customized and specific manner, whose initiation still depends on collaborative interactions with innate immune components.

2.The discovery of the adaptive immune system

Lymphocytes constitute the arm of adaptive immune response, and are suggested to emerge in a vertebrate ancestor more than 500 million years ago [16]. Contrarily to cells of innate immune system, these cell types equipped vertebrates with the capacity to mount targeted responses to different kinds of pathogens, counteracting the selective pressure that pathogens are constantly subjected to evade immune recognition. This strategy implies that lymphocytes must be virtually capable of recognizing antigens derived from every possible pathogen or other foreign entity with which the organism is confronted with during their lifetime. The ability to selectively recognize pathogen-derived antigens (mostly peptides) resides on the very peculiar receptors expressed in T and B lymphocytes, including the T cell receptor (TCR) and B cell receptor (BCR), respectively. The seminal discoveries of these two cell types occurred during the 50s and 60s. In 1956, Bruce Glick *et al* described for the first time the immunological function of a structure called Bursa of Fabricius in birds, which is responsible for the production of antibody secreting cells [17]. However, at that time the separation in B and T cells was yet ignored, being their identification formalized in the next decade (60s). Jacques Miller and Max Cooper were critical for these major breakthroughs in the field. In 1961, Jacques Miller showed that the thymus was essential for the immune response, demonstrating that neonatal thymectomized mice were incapable of rejecting skin grafts [18]. Interestingly, in 1965, Max Cooper proposed for the first time the separation of lymphocytes in two different lineages in birds, B cells produced in the Bursa of Fabricius and T helper cells produced in the thymus [19]. Nonetheless, the mammalian equivalent of Bursa of Fabricius was yet unknown and only in 1968 did Jacques Miller propose that antibody-producing cells were generated in the bone marrow whereas T cells developed in the thymus [20]. Subsequent findings have further suggested that the B-T cell cooperation was vital for the correct function of the immune response, establishing one of the most basic concepts in immunology [21].

The fascinating capacity of the immune system to generate the diversity of BCR and TCR repertoires puzzled immunologists in the following decades. In 1976, Susumu Tonegawa proposed for the first time that a set of inherited gene segments were irreversibly and randomly recombined to generate millions of different BCRs with different specificities [22]. Follow-up studies during the 80's showed that TCR loci displayed a similar organization and shared the same mechanisms to generate TCR variability [23-25]. These studies established how B and T cells can produce a broad BCR and TCR repertoire from a limited number of gene segments (V, D and J). Thus, this genetic configuration allows vertebrates to encode only the essential building blocks of these receptors, which upon

genetic recombination as well as random nucleotide addition/deletion construct a varied BCR and TCR repertoire [26, 27]. The developmental advantage of this mechanism is to compress the critical genetic information for these receptors in a concise bit of information, from where the system can form a functional competent and self-tolerant pool of B and T cells. Importantly, the randomness of the process comes at the cost of generating non-functional and non-coding BCR/TCRs as well as specificities that might be harmful, namely recognizing the self-constituents. Hence, it is crucial that the process of B and T cell development is tightly regulated. While the progression in the bone marrow does not definitively determines the BCR specificity, since activated B cells can pass through the affinity maturation process in the germinal centers, the development of T cells in the thymus permanently defines their TCR identity. This thesis will focus on the study of thymus biology and aims to understand the fundaments that turn this organ so relevant for immunity and self-tolerance.

3.Thymus: the orchestrator of T cell development

The thymus is considered to have evolved in the first jawed vertebrates, which appeared around 500 million years ago and provided the basis for the primordial adaptive immune system [16]. Interestingly, lampreys that belong to agnathans, a clade composed by jawless vertebrates, have a rudimentary structure localized in the gill baskets, called thymoid, possessing some functional similarity with thymus [28]. Thymoid resident cells express the orthologue of *Foxn1* (*Foxn4l*), a crucial transcription factor for thymus function, and *Dll-b* the orthologue of *Dll4*, an important driver of T cell lineage commitment [28]. Additionally, this structure contains two additional cell types characterized by the expression of VLRA or VLRC, which are transmembrane proteins that are not germline encoded [28-30]. Similarly to the TCR, VLR can be diversified by the differential inclusion of Leucine-rich repeats (LRRs) rendering lampreys the ability to “customize” these receptors [31, 32]. This mechanism is operated by cytidine deaminases (CDA), particularly CDA1, which are specifically expressed by those cells in thymoid, suggesting a parallelism to RAG proteins involved in the genetic recombination of TCRs [1, 33]. Finally, these cells express homologs of *Gata2/3* and *Bcl11b*, which are important transcription factors involved in T cell development, supporting the notion that they might represent ancestors of a T-like lineage [29]. These observations are compatible with studies indicating that lampreys can mount an adaptive immune response [34]. Thus, the thymus in jawed vertebrates might have evolved from the lymphoid-like organ existent in agnathans. Interestingly, although in gnathostomes the existence of the thymus is a common feature, the ontogeny of this organ

differs between species [35]. In sharks, the thymus is originated from the 2-6 pharyngeal pouches (PPs), whereas in teleost, 2-4 PPs are involved in its formation [35, 36]. Yet, the thymus ontogeny only involves the 3 PP in mammals. Presumably related with that, the number of the thymus in the organisms is also different. Sharks possess 5 different thymus, one per PP [35]. However, this is not a general rule, since in chickens only 2 PPs (3 and 4) contribute to the thymus formation but they have 7 different thymus distributed to the neck [35]. Interestingly, the occasional presence of a cervical thymus in some mammals has been described [37, 38]. Despite this heterogeneity in thymus organogenesis, the cortical and medullary compartmentalization is a common feature of the thymus in gnathostomes, suggesting that this architecture was preserved throughout evolution.

The critical importance of the thymus in T cell development can be appreciated in patients with mutations in the Forkhead box protein N1 (*Foxn1*) gene, a master regulator of thymic epithelial cell (TEC) development and function, discussed more in detail in the next sections [39]. *Foxn1* mutations lead to a pronounced immunodeficiency, caused by a premature arrest in thymus development which impacts the production of T lymphocytes [39-41]. There are other genetic alterations in TECs that can still disturb thymus function. One of these examples is Autoimmune regulator (*Aire*) mutations that provoke the autoimmune polyendocrine syndrome type-1 (APS-1), characterized by the immune attack of organs, mainly of endocrine origin [42-44]. The two classical examples mentioned above, and further discussed below, illustrate that defects or alterations in thymus function impact not only the quantity of T lymphocytes generated, but also their quality (self-reactivity). In this regard, the physiologic thymic involution starting early on in life has been associated with more susceptibility to infections, increased incidence of cancer, and poor response to vaccines in the elderly [45, 46]. Presumably related with that, the decline in thymus export of recent thymic emigrants (RTEs) conditions the renewal of the naïve T cell pool, narrowing TCR repertoire and contributing to its functional exhaustion [45]. Thus, the thymus is critical for the production, diversification and maintenance of TCR clonotypes capable of responding virtually to all the foreign antigens.

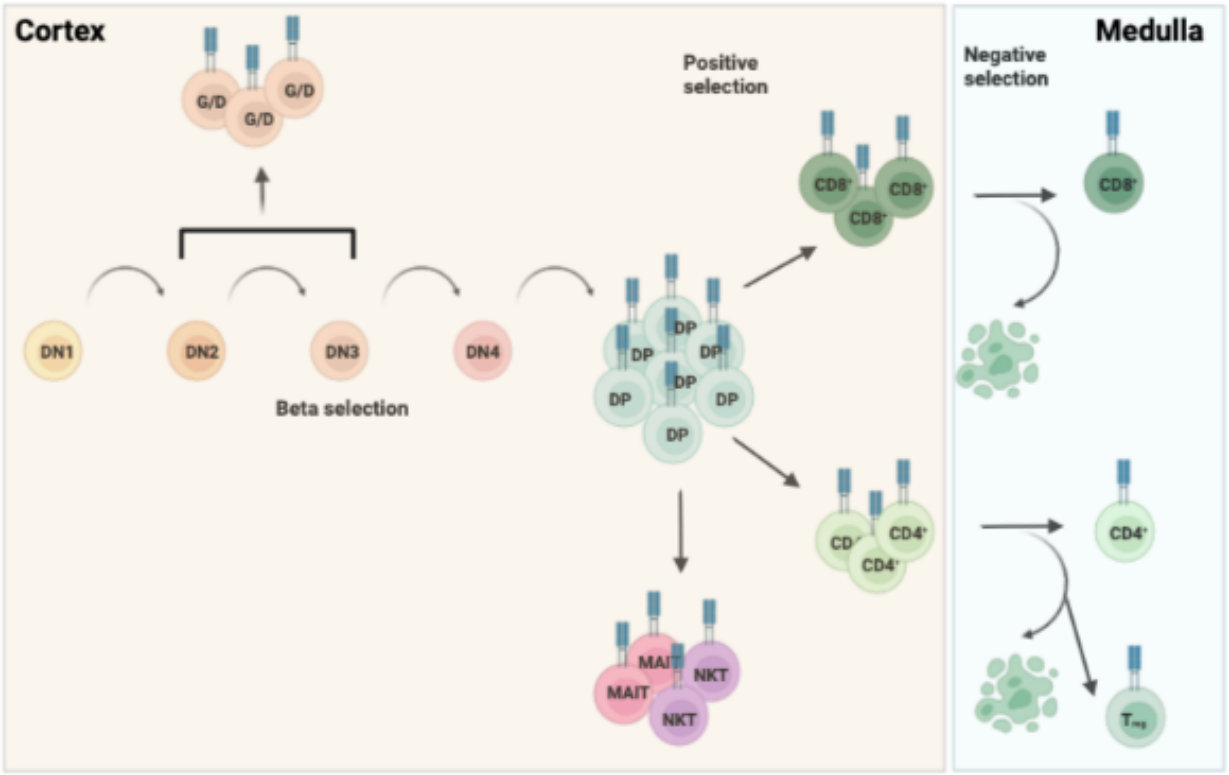

Figure 1 - Schematic representation of T cell development. Thymopoiesis is a multistage process that proceeds in distinct cortical and medullary microenvironments and can be monitored by the differential expression of several markers. Double-negative (DN) stage can be subdivided in: DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). During DN2-DN3 stage TCRβ, TCRγ, and TCRδ gene loci are recombined and the majority of developing thymocytes commit to αβ T cell lineage upon beta selection checkpoint, while others differentiate into γδ T cells. After DN stage, thymocytes upregulate CD4 and CD8 co-receptors and progress to the double-positive (DP) stage, where they initiate the recombination of V_α and J_α gene segments of the TCRα chain. DP thymocytes expressing TCRαβ are 'probed' for their capacity to interact with self-peptide:MHC complexes during 'positive selection' and commit to SP4 or SP8 T cell lineage. A small fraction of DP thymocytes differentiate into unconventional T cell lineages (NKT and MAIT cells) due to their capacity to interact with unconventional MHC molecules (CD1 and MR1). The small fraction of thymocytes that pass positive selection checkpoint migrates to the medulla where thymocytes expressing potentially autoreactive TCR are eliminated during ´negative selection' or deviated, in the case of SP4 cells, into the T regulatory cells.

The development of T lymphocytes in the thymus is a multistage process that includes critical quality checkpoints and ensures the adequate maturation of conventional (αβ T cells) and unconventional T cells (γδ T cells, NKT cells and MAIT cells) [47, 48] (**Figure 1**). In the first place, T cell development requires the CCR7- and CCR9-dependent migration of thymus seeding precursors (TSPs) from the bone marrow to the thymus [49, 50]. Recent data in mice identified two waves of TSP, the first starting at E12 and the second at E16 [51, 52]. While the first wave of TSPs predominantly generates specific γδT cell subsets (e.g., DETCs) and also displays a different kinetic of T cell commitment, the second

wave generates T cells, but retains a broader lineage potential to generate B cells and myeloid cells [52, 53]. Although the identity of the thymus seeding progenitor (TSP) is not completely understood, one possibility is that TSPs comprise distinct populations of progenitor cells with different lineage potential [53, 54]. Then, the early steps of T cell development occur in the cortex and progress through four developmental stages: DN1, DN2, DN3 and DN4, which can be mapped in mice by the differential expression of *Cd44* and *Cd25* and the absence of *Cd4* and *Cd8* co-receptor expression [47]. During DN1-DN2 stages, Notch signaling induces the expression of *Tcf7* and *Gata3*, which in turn will initiate the expression of *Bcl11b*, playing a key role in T cell lineage commitment [55, 56]. In the transition from DN2 to DN3, thymocytes initiate the expression of recombinant activating gene (RAG) proteins and recombine their TCRβ, TCRγ and TCRδ genes [47, 57]. Two main models have been postulated to explain the mechanism underlying αβ/γδ lineage choice at the first TCR-driven checkpoint during DN3 stage [58]. One argues that the differentiation into γδ or αβ T cells is predetermined before the rearrangement of TCR loci, whereas the other proposes that the strength of the signal perceived by thymocytes is the critical cue for the lineage decision, with strong signaling favoring γδ T cell differentiation. Still, the ligands that select γδ T cells remain largely unknown [59]. In this thesis, we will mainly focus on αβ T cell lineage differentiation. The newly recombined TCRβ chain will be first probed in the beta selection checkpoint [60], whereby it pairs with the pre-T cell receptor alpha (pTα) and forms the pre-TCR [47, 60]. Concomitantly with Interleukin 7 receptor-mediated signals, the correct signaling initiated by this provisory receptor allows thymocytes to survive, proliferate and proceed to DN4 stage [60]. Whether pre-TCR signaling is dependent on antigen recognition remains controversial, although recent studies suggest the involvement of ligands presented by TECs [61-64]. Subsequently, thymocytes upregulate *Cd4* and *Cd8* expression and initiate the rearrangement of V_α and J_α gene segments, assembling the α and β chains of the TCR. At this stage, a small group of thymocytes with a very restricted TCR repertoire capable of recognizing molecules presented by the unconventional MHC molecules Cd1 and MR1 are diverted to the natural killer T (NKT) cell and mucosal-associated invariant T (MAIT) cell lineage, respectively [48, 65, 66]. However, the majority of the $CD4^+CD8^+$ double positive (DP) thymocytes are probed for the capacity of their TCR to interact with conventional self-peptide:MHC-complexes presented by TECs, a process known as positive selection that takes place in the cortex. It is considered that only a minor fraction of DP (2-5%) expresses TCRs capable of interacting with an intermediate range of affinities with selecting ligands, and differentiate into conventional single positive (SP) $CD4^+$ or $CD8^+$ thymocytes [67]. The vast majority of DP thymocytes die by neglect because they fail to receive survival signals from the interaction of their TCR with self-peptide:MHC-

complexes [67]. SP thymocytes that complete positive selection then migrate to the medulla to complete their maturation process [68]. Here, thymocytes bearing potentially autoreactive TCRs are further selected, being either eliminated in a process denominated negative selection or deviated for the T regulatory (T_{reg}) cell lineage [69]. Although negative selection is more common in medullary SP thymocytes, it can also take place at DP stage in the cortex [67]. In summary, the thymus ensures the development of immunologically competent T cells expressing TCRs that recognize foreign peptides, while eliminating T cell clones expressing non-functional TCR, either by their incapacity to interact with MHC complexes or their autoreactivity.

4.Thymic stroma

Importantly, T cell development and selection is not a cell-autonomous process, being tightly regulated by the complex thymic stromal microenvironment, composed by thymic epithelial cells (TECs), thymic mesenchymal cells (TMCs), hematopoietic cells and thymic endothelial cells (**Figure 2**). In the next sections, I will cover the contribution of each of these stromal populations to thymic organogenesis and function, providing a wide overview of their contribution to the development of this pivotal organ in the adaptive immune system.

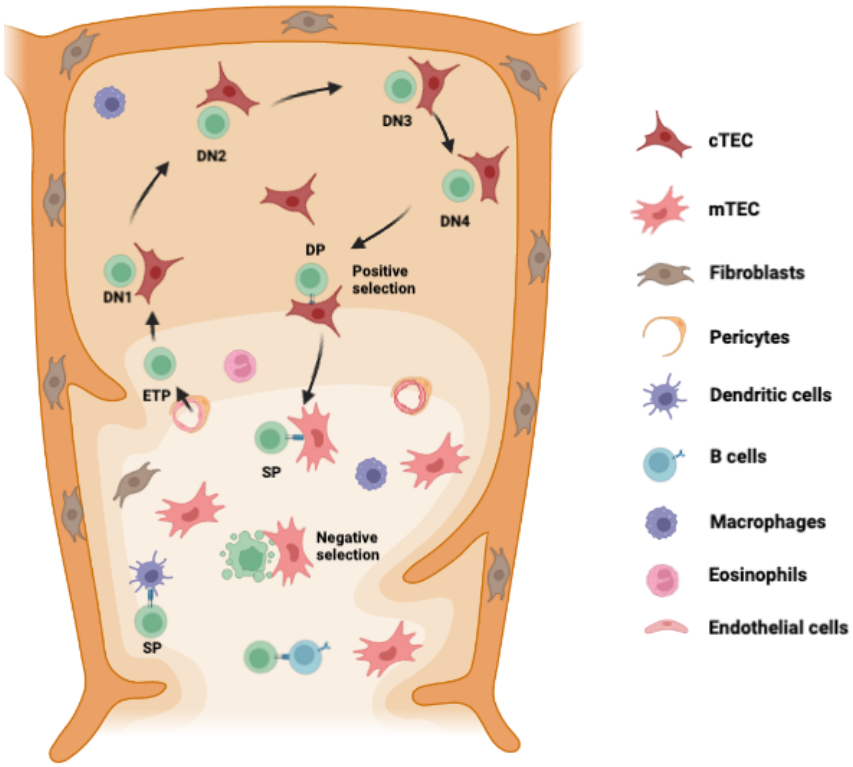

Figure 2 – The diverse composition of the thymic stroma compartment. The thymic microenvironment includes a variety of stromal cells, including thymic epithelial cell (TECs), thymic mesenchymal cells (TMCs), hematopoietic cells and thymic endothelial cells. Together, they form a specialized inductive microenvironment for T cell development and selection.

4.1 Thymic epithelial cells (TECs)

Thymic epithelial cells (TECs) are a critical component of thymic stroma with key functions in T cell development [70]. The first reports on their phenotypic characterization date back to the 80's and coincide with the first studies suggesting that the thymus shaped TCR repertoire [71, 72]. In this regard, experiments using bird models showed that chick hosts transplanted with quailed thymus tolerated quail-derived tissues, which were otherwise rejected in a normal situation. These results suggested that the thymic microenvironment was a key agent imposing immunological tolerance [73]. At that time, cortical TECs (cTECs) and medullary TECs (mTECs) were described based on their distinct anatomical location, morphology and expression of specific markers [74]. The progressive increase in available monoclonal antibodies revealed new subsets within the two main lineages. More recently, the advent of single-cell RNA sequencing (scRNAseq) has shed further light on new layers of heterogeneity inside TECs (discussed below). Concerning their function, an earlier study in 1994 provided evidence that the cortical (cTECs) and medullary (mTECs) microenvironments have distinct roles in T cell development, as indicated by the large amount of thymocytes undergoing apoptosis in the cortex relatively to the medulla [75]. In the following sections, I will specify how their distinct anatomical compartmentalization relates to their different roles in thymopoiesis. Additionally, I will discuss the different models describing TEC development throughout life. Finally, I will outline the signaling pathways involved in their differentiation and maintenance.

4.1.1 Composition and function of Cortical TEC (cTEC) compartment

Some past studies suggested that postnatal cTECs present a heterogeneous expression of *Dll4*, *Il7* and *Ackr4,* suggesting the existence of different cortical subpopulations [76, 77]. More recently, scRNAseq analysis identified two subsets of cTECs that were dynamically regulated during life. One with features associated with proliferative cells that predominates during perinatal period and another with mature and inflammageing gene signature that accumulates with age [78]. Still, the heterogeneity in cTECs remains poorly understood, in part due to their rarity in adult thymus and the lack of cTEC-stage specific markers. Indeed, recent microscopy-based studies of the whole thymus have suggested that the common isolation methods underestimate TEC cellularity [79]. One attempted approach to overcome their reduced availability has been the development of transgenic models with an enlarged TEC compartment such as cyclin D1 transgenic mice,

which possess around 10 times more TECs and reproduce with high fidelity the normal TEC and thymocyte development [80]. Therefore, combining optimized protocols for TEC isolation and new transgenic mice with expanded TEC compartment might improve cTEC recovery, allowing a better phenotypic, genetic and proteomic characterization of cTEC compartment.

Concerning their function, cTECs are largely responsible for driving early stages of T cell development (**Figure 3**). The key expression of two critical chemokines, *Cxcl12* and *Ccl25*, by cTECs is essential for the homing of thymus seeding progenitors (TSPs) and their correct cortical positioning [81, 82]. Moreover, the expression of delta-like 4 (Dll4) by cTECs will activate notch signaling in T cell precursors, inducing the early expression of Gata3 and Tcf7 [55]. Mice deficient in *Dll4* specifically in TECs, lacked T cells and showed an aberrant differentiation of B cells on the thymus [76]. Equally important, cTECs also contribute to the survival and proliferation of early thymocytes by providing IL-7 and stem cell factor (KIT-L) [83, 84]. Mice in which *Il7* and *Kitlg* expression was inactivated on TECs showed a drastic contraction on thymic cellularity [84, 85]. Lastly, another critical function of cTECs is their role in positive selection (discussed below).

The pioneer studies of Rolf Zinkernagel and Peter Doherty in the 70's demonstrated that T cell responses were restricted to self-MHC molecules [86, 87]. Hence, the integration of TCR-mediated signals induced by the interaction with peptide:self-MHC complexes will modulate T cell function. Some studies have shown that there are conserved amino acids in the TCR and MHC that favor TCR-MHC interaction [88, 89], supporting the hypothesis proposed by Niels Jerne in 1971 that these molecules have co-evolved to maximize their recognition [90]. Yet, the random rearrangements of TCR segments inevitability end up producing TCRs incapable of interacting with MHC molecules [67, 91]. In this regard, the laboratory of Alfred Singer has shown that rare T cells educated in MHC deficient mice can still develop and respond independently of MHC presentation, displaying antibody-like recognition properties [92, 93]. Thus, positive selection is essential to select T cell precursors bearing functional TCRs capable of interacting with self-peptide;MHC molecules within an intermediate range of affinities. Besides MHC recognition, the nature of self-peptides presented by cTECs is also critical for positive selection. Cortical cells possess a peculiar machinery of antigen processing and presentation, which render them the capacity to present a private self-peptide repertoire critical for positive selection [94]. The most well studied case is the essential role of thymoproteasome in the selection of CD8 T cells [95, 96]. Proteasomes are large protein complexes, which possess the capacity to cleave proteins in small peptides due to their catalytic core composed by 2 rings of seven subunits (β1-7) [97]. They are involved in protein homeostasis as well as in antigen presentation. According to the inclusion of different β1, β2 and β5 subunits in their catalytic core, they can

be classified in: constitutive (β1/β2/β5) immuno (β1i/β2i/β5i) or thymo-proteasome (β1i/β2i/β5t)[97]. β5t subunit is exclusively expressed in cTECs [95] and in striking contrast with the constitutive proteasome and immunoproteasome, the thymoproteasome possesses a weak chymotrypsin-like activity presumably due to the increased composition of hydrophilic amino acids in the pocket of β5t [95]. It is considered that the thymoproteassome confers to cTECs the ability to produce a unique peptide repertoire critical for positive selection of CD8 T cells [98]. This notion is corroborated by the block in SP8 selection in *Psmb11* deficient mice, which seem to be independent to a certain extent of medullary selection [95, 96, 99, 100]. Moreover, the few peripheral OT-I TCR transgenic $CD8^+$ T cells that develop in the absence of β5t show a defective TCR responsiveness [101]. These results suggest that thymoproteasome is not only important for SP8 selection *per se* but also to the fitness of $CD8^+$ T cells to respond. Regarding the positive selection of MHC-class II-restricted CD4 T cells, cTECs express a distinct machinery of antigen processing, including thymus-specific serine protease (TSSP) and cathepsin L (*Ctsl*) that are two important enzymes in this process [94]. TSSP is involved in the cleavage of proteins in small peptides inside MHC II compartment (MIIC). Mice deficient in this protein exhibit alterations in CD4 TCR repertoire, as demonstrated by the poor responses to egg lysozyme vaccination as well as by their resistance to the development of autoimmune diabetes in NOD background [102, 103]. CTSL belongs to the cathepsin family that comprises proteases generally associated to Ii chain degradation, an important step for MHC II maturation [104]. Germline deletion of this protease in mice significantly impairs the generation of SP4 in the thymus [105]. Interestingly, this block in SP4 differentiation seems to not only involve the role of CTSL in Invariant chain (Ii) chain degradation but also suggests that CTSL might have other roles in antigen presentation [106]. However, cTECs in addition to special machinery of antigen presentation, also possess a non-conventional mechanism of antigen processing. In this context, they display high levels of macroautophagy which confers them the capacity to capture and degrade cytoplasmic self-proteins in the autophagolysosome and then presents them by MHC II complexes [67]. Indeed, this route of protein acquisition is critical for thymic selection as demonstrated by the impairment in the positive selection of some MHC II-restricted TCR specificities in nude mice transplanted with *Atg5* deficient thymus which exhibit a defective autophagy [107]. Moreover, two recent studies showed that LAMP2 and PIK3C3, two proteins implicated in autophagy, were required for positive selection of SP4 cells, reinforcing the idea that the autophagic route contributes for the generation and presentation of self-peptides:MHC II complexes [108, 109]. Future studies should dissect the nature of MHC I- and II-bound peptides, in order to understand what makes them special to promote positive selection.

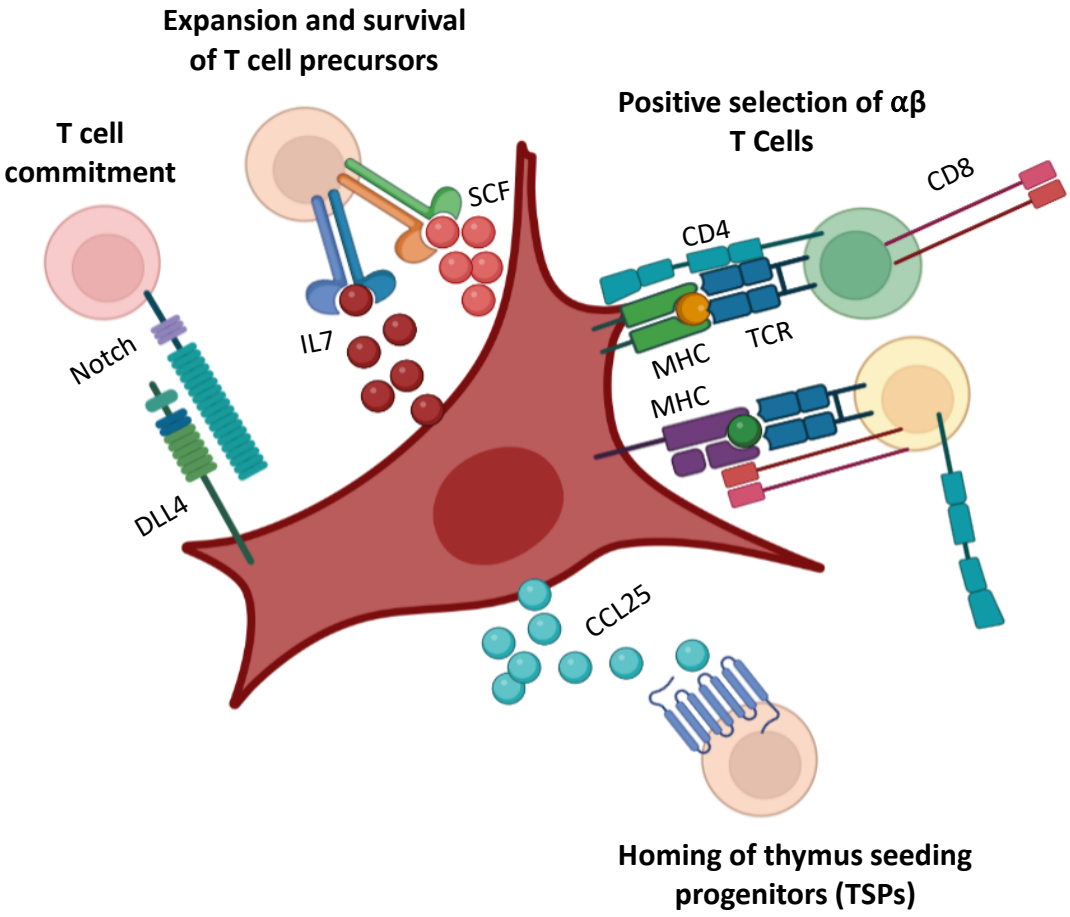

Figure 3: Functional diversity of cortical epithelium in thymopoiesis. cTECs have a large contribution for T cell development, coordinating important developmental stages of this process. First, the production of CCL25 by TECs promotes the homing of thymus seeding progenitors (TSPs) from the bone marrow to the thymus. Then, the DLL4-mediated activation of notch signalling in thymocytes by TECs initiates the transcriptional program of T cells. Simultaneously, they also provide key factors (IL-7 and SCF) that promote the survival and proliferation of T cell precursors. Lastly, cTECs regulate positive selection, which selects a population of DP thymocytes capable of interacting with MHC-self-peptides complexes within an intermediate range of affinities.

4.1.2 The heterogeneity and function of Medullary TECs (mTECs)

In contrast with cTECs, our understanding of the heterogeneity and functional complexity of mTECs is far vaster. In the past, mTECs were classically subdivided in $mTEC^{lo}$ and $mTEC^{hi}$ on the basis of lower and higher levels of MHC II and CD80, respectively [110]. It has been initially shown that the $mTEC^{lo}$ cells give rise to $mTEC^{hi}$ cells, indicating a possible precursor-product lineage relationship between these subtypes [111]. However, we now know that the two categories of mTECs rather than representing two homogeneous populations enclose functionally different subsets, a topic that will be further discussed. One example of heterogeneity within $mTEC^{lo}$ is $CCL21^{+}$ mTECs, which attract $CCR7^{+}$ positively selected thymocytes towards the medulla to complete their maturation and selection (negative selection/T_{reg} differentiation) [112-114]. Consistent with this idea, mice deficient in *Ccl21a* gene showed an accumulation of thymocytes in the cortex, suggesting an arrest in their migration, and defects in thymus architecture and central tolerance induction [68]. Regarding $mTEC^{hi}$, the expression of autoimmune regulator (*Aire*) and *Fezf2* defines two subsets: $AIRE^{+}FEZF2^{+}$ and $AIRE^{-}FEZF2^{+}$ mTECs [115]. Still, $FEZF2^{+}$ cells are also found in $mTEC^{lo}$ [115]. These different mTEC populations ensure the expression of the majority of TRAs (discussed more in detail in the next section) that are critical to eliminate or deviate into T_{reg} cell lineage thymocytes expressing potential autoreactive TCRs (**Figure 4**) [115, 116]. According to the "terminal differentiation" model it was initially postulated that $AIRE^{+}$ mTECs were terminally differentiated cells [117]. However, fate-mapping analysis identified a post-AIRE stage, composed of cells that downregulated AIRE, MHC II and CD80 and acquired features akin to terminally differentiated keratinocytes [118, 119]. More recently, RNA sequencing analysis corroborated their existence, identifying an mTEC cluster enriched in genes of the soft cornified epithelial pathway (e.g., *Krt10* and *Ivl*) [120, 121]. Worth noting, post-AIRE cells resemble the Hassall's corpuscles described in the human thymus, although its exact function remains elusive [119]. More recent studies exposed a further degree of complexity, with the identification of thymic tuft cells, which shared molecular traits with tuft cells at mucosal barriers and follow an AIRE-dependent and independent differentiation pathway [120, 121]. Although their biological role is barely defined, they appear to control the homeostasis of ILC2s and NKT2 cells [120-122].

Critical to the role of mTECs in central tolerance is their capacity to promiscuously express tissue-restricted antigens (TRA), which enables them to anticipate the presentation of peripheral self-peptides to thymocytes expressing autoreactive TCRs [116]. This property

was initially described as a “leaky” expression of several tissue-specific genes in the thymus with investigators interpreting these observations as experimental artifacts [123-125]. However, in 1998, the laboratory of Bruno Kyewski provided earlier evidence that mTECs expressed c-reactive protein (*Crp*), a gene specifically expressed in the liver [126]. Moreover, the same laboratory in a subsequent study in 2001 performed a broader characterization of TRA expression, confirming the special ability of mTECs to promiscuously express genes from peripheral tissues [127]. Along with these discoveries, Diane Mathis and colleagues showed that *Aire* deficient mice, which possess defects in the expression of TRAs, exhibited signs of autoimmunity, suggesting that promiscuous gene expression (pGE) was essential for central tolerance induction [43]. Supporting this idea, the targeted disruption of *Ins2*, an AIRE-dependent TRA, in TECs by itself resulted in autoimmune diabetes [128]. These findings supported the original idea first proposed in 1989 by Richard Lenski, Max Gottesman and Benvenuto Pernis that ectopic expression of genes in the thymus could contribute to central tolerance [129].

Subsequent studies tried to characterize in depth the degree by which TECs, particularly mTECs, can cover the full catalogue of TRAs. In 2014, the laboratory of Georg Hollander estimated that mTECs express 89% of the protein-coding genes [116]. However, this analysis may be obscured by the fact that one gene can give rise to different transcripts due to alternative splicing [130]. In this regard, a recent analysis of the total number of transcripts expressed in mTECs showed that the percentage ranged around 60%. Although the expression coverage was lower than previously estimated, it was still higher than in all the other tissues analysed [131]. Notably, several reports have shown that only 1-3% of total TRAs are expressed in mTECs at the single-cell level [116, 132, 133]. As mentioned above, AIRE and FEZF2 are essential to coordinate the expression of the TRA repertoire of mTECs. While AIRE-induced gene expression requires the binding to super-enhancers, that can then be recruited to inactive chromatin regions enriched in unmethylated lysine 4 of histone 3 (H3K4me0) chromatin marks [134-136], FEZF2 seems to recognize active transcriptional sites [136]. However, the exact mechanism involved in the coordination at the single-cell level of a specific set of AIRE- or FEZF2-dependent TRAs remains to be determined. In this context, the first studies dedicated to understanding how TRA expression was established obtained conflicting results. While in some studies the TRA repertoire in a single mTEC was characterized by an “ordered stochasticity”, meaning that each single mice possesses its own constellations of defined TRA clusters [133], others showed that TRA expression was organized in different modules according to their genomic positions, suggesting a coordinated process common to different mice [132]. However, these initial studies employed scRNAseq analysis of a very reduced number of mTECs (150-200 cells). To overcome this limitation, a more recent study from the laboratory of

Georg Hollander significantly increased the number of analysed mTECs, and reached a different conclusion [137]. Indeed, TRA expression was organized in different modules that appeared constant across different mice, but at the same time stochastic because there was not any correlation between the different TRAs expressed in each group [137]. Interestingly, a recent paper has shown that post-AIRE mTECs form mimetic modules that can recapitulate the genetic programs of peripheral cell types, including chromatin conformation, transcription profiles and expression of specific transcription factors [138]. Yet, the mechanism behind the activation of these pre-determined TRA modules remains unknown. In the future, it would be interesting to test whether the target disruption of specific mimetic models will result in tissue-restricted autoimmunity.

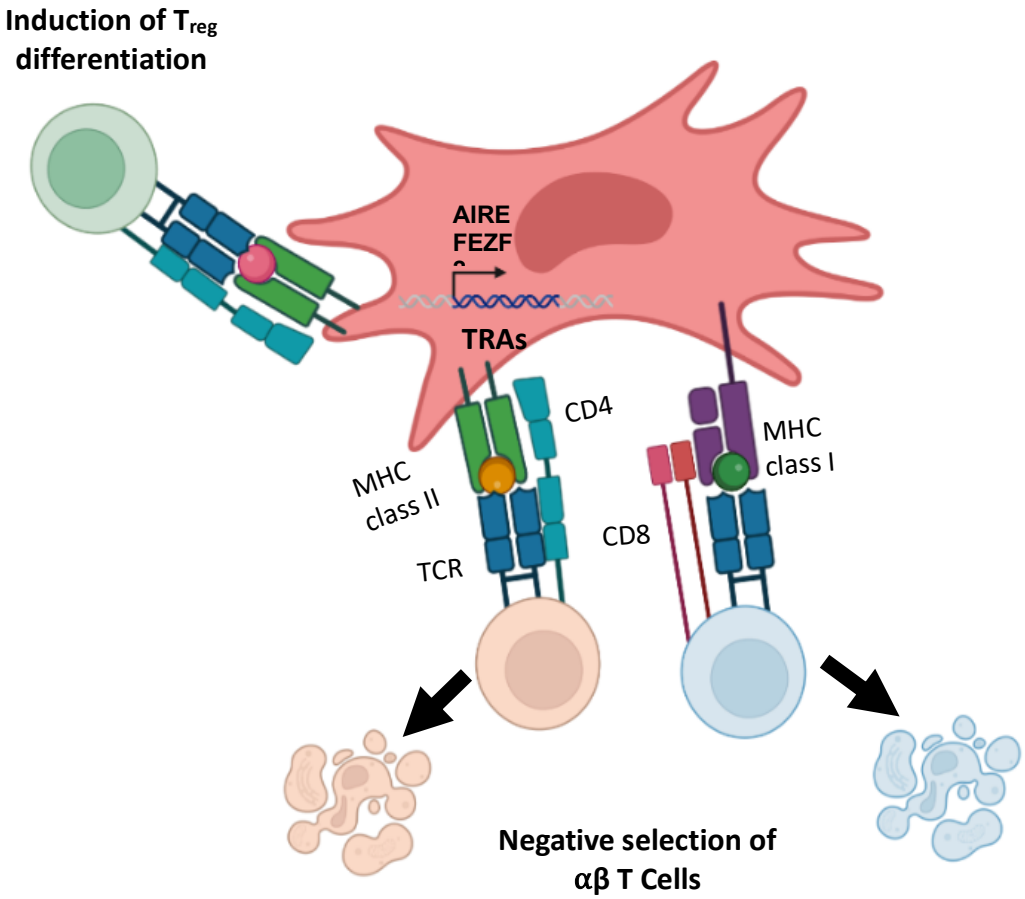

Figure 4: mTECs contribute to central tolerance induction. mTECs regulate negative selection and the differentiation of T_{reg} cells. This special ability relies on their unparalleled capacity to express tissue restricted antigens (TRAs), which enables them to anticipate the presentation of peripheral self-peptides to thymocytes expressing autoreactive TCRs. Promiscuous gene expression (pGE) has been estimated to cover up to 89% of the protein-coding genes. Nevertheless, at the single cell level only 3% of the TRA repertoire is expressed by single mTEC, suggesting that medullary microenvironment provides a mosaic of different TRA modules. This ability depends on the expression of *Aire* and *Fezf2* by mTECs through mechanisms yet to be clarified.

4.1.3 TEC developmental models during embryonic and postnatal period

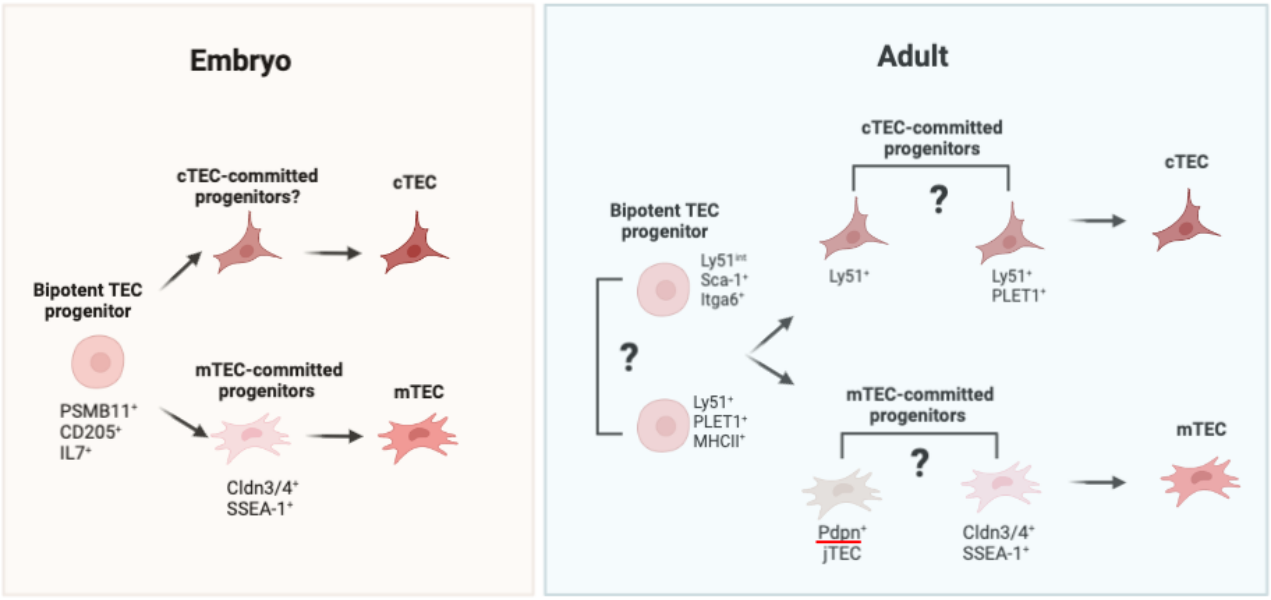

Figure 5 – Models of TEC development in the embryonic and postnatal thymus. The embryonic and adult thymus contain bipotent TEC progenitors and lineage-committed precursors with the potential to differentiate in mature c/mTECs. The identity of cTEC progenitors remains to be defined at the single cell-level.

The search for the origin of the different types of cells in the thymus was launched in the early 60's. At that time, the main school of thought argued that T cell precursors were derived from the transdifferentiation of either epithelial cells or mesenchymal cells. In 1967, the pioneer work of John Owen based on parabiosis experiments in chick embryos demonstrated that TSPs were recruited from the circulation [139]. Moreover, in mid-70's the seminal work of Le Douarin showed that pericytes and capsular mesenchymal cells derived from the neural crest [140]. Regarding TEC ontogeny, the path was more complex. Initially, the laboratory of Cardier postulated that ectoderm and endoderm participated in thymic epithelium generation, leading to the so-called "dual-origin" model [141, 142]. In 2004, a report by the laboratories of Nancy Manley and Clare Blackburn disputed this idea and provided experimental evidence that only endoderm contributes to thymic epithelium, leading to the currently accepted endodermic-centric model. They showed that transplantation of definitive endoderm from the second and third pharyngeal pouches obtained at E8-9 was capable of generating a functional thymus, providing earlier evidence that cTECs and mTECs derived from a common endoderm-derived progenitor [143].

Although it is still not fully understood how thymus and parathyroid co-develop during early stages of embryogenesis [144], it is conceptually accepted that the primordial

embryonic thymus contains bipotent TEC progenitors capable of giving rise to cTEC/mTEC lineages (**Figure 5**). Eric Jenkinson's laboratory in 2006 showed that E12-derived single-cell TEC precursors injected into age-matched developing thymus originated both populations [145]. This work was in line with two previous reports showing that MTS24$^+$ TECs harbor bipotent TEC precursors [146, 147]. Although MTS24 was considered a putative marker for the identification of bipotent TEC progenitors, a subsequent study demonstrated that embryonic TECs expressing or lacking MTS24 generated cTEC and mTEC lineages [148]. Several groups subsequently attempted to identify and phenotypically characterize those progenitors. Currently, there is a large body of evidence showing that embryonic bipotent progenitors may present cTEC-associated traits (e.g., IL7, β5t, CD205 and ACKR4)[77, 149-151]. These observations led to the serial progression model for TEC differentiation, which argues that bipotent TEC progenitors transverse through a cell state that shares similar features with cTEC lineage before committing to the mTEC lineage [152]. This model is corroborated by recent scRNAseq studies showing that the genetic program detected in the earliest embryonic TECs is more closely related with cTECs than with mTECs [153]. Interestingly, a recent study indicates that early bipotent progenitor activity is compromised early on in life, suggesting that they may become senescent with time and compromise TEC maintenance [78]. Still, TEC development does not rely exclusively on bipotent progenitors, but also presumably in downstream lineage-restricted precursors. Concerning embryonic mTEC compartment, it was identified an mTEC-committed progenitor expressing Claudin3/4 and SSEA-1$^+$ that contribute to the maintenance of mTEC compartment [154, 155] and share a lineage relationship with β5t$^+$ bipotent progenitors [150]. Similarly with what occurs with bipotent TEC progenitors, the progenitor activity of these mTEC precursors is suggested to decline with life, in a process dependent on TEC-thymocyte crosstalk [155]. In contrast, the existence of dedicated progenitors for cTEC compartment in the embryonic thymus is yet to be demonstrated. A recent study intending to characterize the initial stages (E11,5-13,5) of TEC differentiation did not find evidence of a cTEC-committed progenitors within thymus anlagen [153]. Instead, this study identified the presence of a bi-potent progenitor which by pseudo-time analysis can give rise to both cTECs and mTECs, in accordance with what was previously mentioned [153]. Hence, future studies are required to unveil the differentiation steps required for cTEC-lineage commitment.

Studies showing that cTECs and mTECs have a limited half-life and can recover after total body irradiation indicate that the adult thymus retains a reservoir of TEC progenitor cells capable of contributing to the maintenance and regeneration of both TEC lineages [150, 156-158]. In line with this hypothesis, the laboratories of Ann Chidgey and Richard Boyd described the identification of a bipotent progenitor, called TEC^{lo}, defined as

MHCII^{lo}UEA-1^{-}Sca-1^{+}Itga6^{+} [159]. A complementary study by the laboratory of Clare Blackburn suggested that bipotent cells were found within Ly51^{+}MHCII^{+}PLET1^{+}[160]. It still remains to be determined whether these two subsets share or not a direct lineage-relationship. However, to determine the lineage potential of these purported TEC progenitors, both reports employed transplanted thymic organ cultures (RTOCs) in the kidney capsule, a system that can only partly mimic the physiological thymic microenvironment. The future development of advanced thymus organoid systems might represent a better alternative to recreate thymic anatomical structures, providing a more faithful niche for TEC differentiation. In addition to bipotent progenitors, TEC homeostasis is also maintained through downstream progenitors. The above mentioned Claudin3/4^{+}SSEA-1^{+} mTEC-restricted progenitors can still contribute, although less efficiently, to the adult medulla maintenance [155]. Moreover, another subset of purported mTEC precursors included the so called junctional TECs (jTECs), which reside in cortical-medullary regions, express *Pdpn* and *Ccl21a* [161] and also share a lineage relationship with β5t^{+} bipotent TEC progenitors [162]. Recent scRNAseq studies have found computational predictions compatible with the idea of jTECs being mTEC precursors [78]. It is worth mentioning that a more recent study using single-cell RNA velocity identified a highly proliferative TEC subset (TAC-TEC) that can presumably give rise to CCL21^{+} and AIRE^{+} mTECs [163]. Further studies are required to test the direct or indirect developmental links between Claudin3/4^{+}SSEA-1^{+}, jTECS and TAC-TEC subsets. Similarly to the embryonic period, the scenario is more obscured regarding the homeostasis of the adult cortical epithelium. There is evidence demonstrating that the postnatal cTEC compartment recovers after its selective ablation [164]. This idea seems to be compatible with the existence of bipotent and/or cTEC precursors residing within the cTEC compartment that may contribute for cTEC recovery [160]. Interestingly, cTEC regeneration seems to be compromised specifically in 2-3 months old male adult mice [164]. Although the reasons for the sex-bias cTEC regeneration remains elusive, the increased percentage of proliferating cTECs found in females might offer a reasonable explanation for the differences in progenitors' fitness[165]. The future identification of new molecular markers for TEC progenitors as well as the development of better experimental systems to test progenitor activity will facilitate our understanding of the regulatory mechanism underlying thymic epithelium homeostasis.

4.1.4 Transcription regulators of thymus morphogenesis

The thymus organogenesis develops from a common primordium, which also generates the parathyroid [144]. Several studies uncovered the molecular determinants involved in the early stages of thymic development [166]. One of the known major regulators of this process is HOXA3, which is expressed in the mesenchyme throughout the third and fourth arches and in the endodermal cells of the third and fourth pouches at E10,5 [167]. *Hoxa3* deficient mice presented an athymia, which might result from defects in NC cells and endoderm tissue of the third pharyngeal pouch, however the seemingly normal migration and number of NC cells suggest that HOXA3 has a direct role in the early patterning of the endoderm [167]. Interestingly, HOXA3 may be linked to other downstream transcription factor such as PAX1, which started to be expressed in the third pharyngeal pouch at E10,5 [168]. *Pax1* deficient mice also display an hypoplastic thymus, with an early defect in the correct definition of thymus/parathyroid domains at E11,5, or in some cases an impairment in the detachment of common primordium from pharyngeal endoderm [169]. Demonstrating that HOXA3 acts upstream in this pathway, continued expression of *Pax1* seem to be dependent on HOXA3 [167]. *Pax9* is also expressed in all the pharyngeal pouches, with *Pax9* mutant mice showing a defect in the detachment from pharyngeal endoderm and a severe thymic hypoplasia [170]. *Pax1* and *Pax9* double KO mice do not show alterations of *Hoxa3* expression at E10, consistent with the idea that they act downstream of HOXA3 [171]. The subsequent activation of *Eya1* is also crucial for thymus organogenesis, since the thymus anlagen is not detected in *Eya1* deficient mice [172]. The lack of *Foxn1* and *Gmc2* expression, two major regulators of thymus and parathyroid domains, together with a defective separation of the 3rd pharyngeal pouch from pharyngeal endoderm might explain failures in common primordium formation [172]. The normal expression of *Hox3*, *Pax1* and *Pax9* at E9,5-10,5 found in *Eya1*$^{-/-}$ mice further corroborates the notion that EYA1 acts as a downstream transcription factor of this axis[172]. Finally, the expression of *Six1* and *Six4* was shown to be also important for thymus morphogenesis. Analysis of the double *Six1* and *Six4* KO mice showed an initial formation of thymus/parathyroid primordium with demarked expressing of *Foxn1* and *Gmc2,* but then regress and disappear at E18,5 [171]. These results suggest that SIX1 and SIX4 are not necessary for the first steps of endoderm patterning, but required in later stages of this process [171]. Moreover, the regular expression of *Eya1* in double mutant mice indicates that SIX1 and SIX4 are downstream targets on this cascade [171]. Nevertheless, the activation of these different transcriptional factors is not unidirectional. An illustrative example is the fact that *Eya1*$^{-/-}$ *Six1*$^{-/-}$ mutant mice show a reduced expression of *Pax1*, a purported upstream transcriptional factor [171]. This

indicates that sequential activation of different transcriptional regulators might reinforce the activation of their predecessors, forming a complex network of multiple and bidirectional interactions controlling endoderm patterning. Lastly, *Tbx1* was also suggested to be involved in thymus organogenesis. Patients with DiGeorge syndrome, which is caused by a deletion in the human chromosome 22 that, among others, affects the *Tbx1* gene, exhibit in some cases a severe immunodeficiency provoked by athymia [173]. In line with the important role of this transcription factor in thymus morphogenesis, *Tbx1*$^{-/-}$ mice recapitulate the athymic phenotype found in humans [174]. However, in *Tbx1* deficient mice the 3rd pharyngeal pouch is not formed at E9,5, indicating that the athymia may be a result of previous defects on pouch development [175]. Moreover, *Tbx1* expression is circumscribed to the parathyroid domain [176]. Supporting this notion, forced expression of *Tbx1* in thymic epithelium led to a downregulation of *Foxn1* and an impairment of TEC differentiation [177]. Thus, the role of TBX1 might not be important for thymus formation *per se* but it is rather crucial for the normal development of pharyngeal pouches at earlier stages of embryonic development.

4.1.5 Foxn1: The master regulator of TEC lineage specification and differentiation

One of the key steps in thymic organogenesis is the initiation of *Foxn1* expression by the primitive TEC precursors, which represent the building blocks for the construction of the three-dimensional network supporting T cell development [144]. The discovery of *Foxn1* dates back to the 60's when an autosomal recessive mutation, referred as 'Nude' was described to provoke a condition of congenital alopecia and absence of the thymus [178, 179]. Furthermore, these animals were highly susceptible and exhibited a severe lymphopenia accompanied by liver necrosis, with more than half of them dying prematurely before the weaning period [178, 179]. Nevertheless, the identification of *Foxn1* as the gene affected by this mutation was only achieved in 1994 by Thomas Boehm [40]. FOXN1 belongs to the forkhead box (FOX) family of transcription factors, binding to the consensus sequence GACGC through the Forkhead domain (FKH), a DNA binding domain characteristic of these family of proteins [41, 180]. Consistent with the idea that this gene was conserved during evolution, paralogues of *Foxn1* gene have been found in the more distant metazoans such as cnidarians that express *Foxn4b* [41]. However, *Foxn4*, the paralogue sharing the highest homology with *Foxn1,* only appeared in cephalochordates and urochordates [41]. Despite the similarities between the two genes, the replacement of *Foxn1* by *Foxn4* in mice led to the generation of a lymphoid organ capable of supporting T and B cell development, suggesting that FOXN1 in vertebrates allowed the specialization of the thymus as an exclusive site for T cell production [181]. The initial characterization of

Foxn1$^{-/-}$ (nude) mice, showed an athymic phenotype, characterized by the presence of a rudimentary thymus anlagen containing epithelial cells arrested at a premature stage of TEC differentiation, suggesting that the initial stages of TEC commitment are independent of *Foxn1* expression [182, 183]. This was further supported by the observed compartmentalization in K8^{+} and K5^{+} areas at E13,5 in nude mice which is associated with cortex and medulla delimitation, respectively [184]. Moreover, the thymus rudiment of Nude mice contains Claudin3/4^{+}SSEA-1^{+} cells, indicating that the early mTEC-committed precursors might not require *Foxn1* induction for their specification[184, 185]. However, TEC precursors remain blocked at this developmental stage due to *Foxn1* mutation, abolishing thymus formation[182]. The pioneer work from the laboratory of Thomas Boehm in 2006 showed that reactivation of *Foxn1* in the postnatal thymus is sufficient to rescue the nude thymic phenotype, indicating that TEC precursors are maintained in the adult nude thymus in a developmental arrested stage [186]. Corroborating this idea, the reactivation of a hypomorphic *Foxn1* allele, which express only 15% of the normal levels of wt mice, promoted the generation of a functional thymus [187]. Although the exact molecular signaling initiated in TECs is not completely understood, the work of the laboratory of Georg Hollander has shown that FOXN1 induces the activation of a complex genetic program in cTECs, which regulates the expression of many genes involved in the migration, commitment and selection of T-cell precursors (e.g. *Ccl25*, *Dll4*, *Prss16* and *Psmb11*) [188]. Therefore, FOXN1 is an important orchestrator of TEC development and function.

Several reports have linked thymus involution to the progressive downregulation of *Foxn1* expression. While in the embryonic period more than 90% of TECs express *Foxn1*, this percentage drops to 56% in 10-week-old mice, and further declines with aging [189], consistent with the reduction in the *Foxn1* promotor activity [190]. The association between the decrease in *Foxn1* expression and thymus atrophy is consistent with the idea that FOXN1 is necessary for TEC homeostasis and function. Using an inducible mice model that overexpressed *Foxn1* upon tamoxifen treatment, the laboratory of Clare Blackburn showed that increased *Foxn1* expression led to a striking thymic rejuvenation and an augmented thymic output in aged mice, presumably due to an expansion and functionalization of TECs [191]. Indeed, the size of TEC compartment is a limiting factor for thymus growth, as demonstrated by K5-CyclinD1 transgenic mice that possess a ten-fold increase in thymus cellularity promoted by a non-physiological expansion of TECs [80]. Moreover, the decline in the expression of cell cycle-related genes in TECs has been associated with the reduced size of TEC niches, and thymus involution [192]. Thus, the modulation of *Foxn1* expression levels might be a promising approach to recover thymic function in patients undergoing anti-cancer treatments as well as in elderly people, which are highly susceptible to opportunistic infections and respond poorly to vaccines.

4.1.6 Signaling pathways underlying TEC development and differentiation

Transforming growth factor-beta (TGF-β) signaling

Several reports have described the role of TGF-β signaling pathway in TEC development and differentiation [70]. The TGF-β superfamily members BMP4 and NOG are expressed in the 3rd pharyngeal pouch endoderm around E10,5 and have been implicated in thymic development [193]. First, the conditional deletion of *Bmp4* in pharyngeal endoderm led to an ectopic and smaller thymus, while the conditional deletion of *Bmp4* in TECs did not provoke any major alteration [194]. These results suggest that BMP4 has a specific role in the early stages of thymus organogenesis prior *Foxn1* expression. Secondly, FTOC treated with BMP4 led to an increased expression of *Foxn1* by TECs, indicating that BMP4 might be (directly or indirectly) involved in the induction of *Foxn1* expression [195]. Nevertheless, transgenic mice expressing noggin, a Bmp antagonist, under the control of *Foxn1* promotor displayed an hypoplastic and ectopic thymus and an abnormal TEC differentiation, suggesting that Bmp signaling must be tightly regulated during development [196]. Follow up studies are still necessary to examine whether other Bmp ligands are effectively involved in thymus organogenesis and TEC development. TGF-β has also been described to have a negative impact in TEC homeostasis, as demonstrated by the attenuation of thymic involution upon the deletion of TGF-β type II receptor in TECs [197]. Moreover, it was shown that TGF-β signaling restrains mTEC compartment expansion by counteracting the non-classical NF-κB pathway, which is essential for mTEC differentiation (discussed below) [198]. As a result, mice with TEC-specific deficiency in *Tgfrbr2* presented enlarged medullary compartments and a more efficient negative selection and T_{reg} cell generation, which led to a higher resistance to develop autoimmunity[198]. Further studies are required to investigate whether the increased negative selection in the situation of *Tgfrbr2* deficiency compromises the antitumor response, in a similar fashion to *Tnfrsf11b*-deficient thymus stroma, which also displays an augmented mTEC differentiation and T_{reg} cell production [199].

Sonic hedgehog signaling

The sonic hedgehog (Shh) pathway is also involved during initial stages of TEC development. The specific deletion of *Shh*, one of the three ligands for Patched1 (*Ptch1*) receptor, provoked an early defect in mTEC differentiation at E15,5, which was maintained throughout adulthood, leading to alterations in T cell development, including an augment in mature SP4 and SP8 thymocytes [200]. However, this experimental approach does not exclude the possibility for the role of other ligands of Ptch1 receptor, Indian hedgehog (*Ihh*) and Desert Hedgehog (*Dhh*). In order to fully comprehend the biological impact of Shh, future approaches should attempt to block the entire signaling pathway either by targeting all the ligands or the receptor.

Wnt signaling

One of the first evidence pointing to the role of Wnt signaling in TEC development arose from the observation that *Wnt4* and *Wnt5b* were detected in the 3rd pharyngeal pouch at E10.5. Moreover, overexpression of *Wnt4* and *Wnt5b* in a cTEC1-2 cell line upregulated *Foxn1*, suggesting that Wnt signaling controls *Foxn1* expression [201]. A following study showed that the TEC-specific deletion of β-Catenin, a downstream target of Wnt signaling activation, mediated by *Foxn1*-driven Cre provoked a drop in thymic cellularity, particularly in cTECs and thymocytes, at E15,5 [202]. Interestingly, the deletion of the same protein when mediated by *Psmb11* delayed the reported phenotype, with alterations in TEC and thymocyte cellularity being found only in the postnatal thymus [203]. Since the initiation of *Foxn1* expression (E11,25) [204] precedes β5t (PSMB11) expression (E12,5) [205], these results suggest that Wnt signaling might have a prominent role in TEC biology at early stages of thymic development, consistent with the early expression of *Wnt4* and *Wnt5b* [201]. While these results showed the relevance of Wnt signaling for thymus development, other studies have further highlighted the importance of fine-tuning this signaling pathway. In this context, constitutive activation of β-Catenin in TECs altered thymus positioning, reduced *Foxn1* expression, led to abnormalities in TEC differentiation, and an impairment in T cell development during the embryonic period [202, 206]. Furthermore, transgenic expression of *Wnt4* in TEC also produced defects in thymus migration and TEC differentiation [202]. Once more, the dissimilarities found between *Foxn1*-Cre and *Psmb11*-Cre systems in the loss-of-function of β-Catenin were also verified in these two mice lines when used to constitutively activate Wnt signaling [202, 203], emphasizing that depending

on the window of time when certain genes operate, the usage of these two mice models to conditionally delete genes in TECs might not induced the exactly equivalent consequences.

Notch signaling

The role of Notch signaling in T cell lineage commitment is widely acknowledged [47]. Nevertheless, this signaling pathway also contributes to TEC differentiation. Indeed, TECs express some of the notch receptors and downstream signaling molecules of this pathway [207], being required to repress the genetic program of cTECs for the lineage commitment of mTEC precursors [208, 209]. Nonetheless the constitutive activation of Notch signaling disrupted thymic epithelium architecture, indicating that the correct silencing of this signaling pathway is important for TEC differentiation [208-210].

NF-κB signaling

NF-κB pathway is one of the most well studied signaling pathways in TEC development, particularly in the context of mTEC differentiation [211]. It can be subdivided in canonical and noncanonical according to the participation of distinct receptors, adaptor molecules and protein complexes. There is however some interdependence between the two pathways [212] that ultimately activated NF-κB factors, upon successive steps of phosphorylation and ubiquitination of intermediate agents [212]. These NF-κB elements comprise 5 members (p50, p52, RelA, c-Rel and RelB) that form specific dimers and bind to specific DNA sequences, promoting the transcription of target genes [212].

The first evidence suggesting the pivotal importance of this signaling pathway for TEC differentiation was provided by the analysis of *Relb* deficient mice that were almost devoid of mTEC microenvironments [213] and exhibited several autoimmune manifestations [214]. Since RELB is one of the NF-κB elements activated by the non-canonical pathway, this suggested the involvement of this pathway in mTEC differentiation. In line with these results, mice deficient in adaptor protein TRAF6, display a severe block in mTEC maturation with the transplantation of *Traf6* deficient thymus to a nude mice provoking autoimmune manifestations similar to the ones observed in the *Relb* mutant mice [215]. Subsequent studies targeting other components of this signaling pathway (e.g. NIK and IKKα) reached similar results [216-218]. In accordance with the important role of this signaling pathway in mTEC differentiation, members of the tumor necrosis factor receptor superfamily (TNFRSF), including *Ltbr*, *Tnfrsf11a* (RANK), *Tnfrsf11b* (OPG) and *Cd40*, are

expressed by mTECs and upon ligand binding are the main regulators of the non-canonical NF-κB signaling [211]. Concordantly, *Ltbr* deficient mice show an aberrant mTEC differentiation accompanied by defects in T cell development and self-tolerance induction [219-222]. Supporting the specific role on mTEC development, TEC-specific deletion of *Ltbr* significantly impaired the differentiation and location of multiple mTEC subsets, including $AIRE^+$ mTECs, $CCL21^+$ mTECs, terminally differentiated mTECs and thymic tuft cells, although autoimmunity was not observed on these mice [223]. Interestingly, LTBR-mediated signaling seems also important for DCs homeostasis, thymic endothelium and thymic mesenchyme, suggesting that the phenotype of *Ltbr* deficient mice might result from the cumulative defects in different cell types [223-225]. Indeed, deletion of *Ltbr* in thymic mesenchyme led to defects in central tolerance, supporting the notion that Ltβr has a wide impact in thymic stroma [225]. The relevance of RANK for mTEC differentiation is shown by the defects in mature mTECs, including $AIRE^+$ subset, in *Tnfrsf11a* deficient mice [226, 227]. Indeed, NF-κB binds a non-coding DNA region upstream of *Aire* and this region is required for its expression, suggesting that NF-κB signaling is involved in AIRE induction [228, 229]. Reciprocally, deficiency in *Tnfsf11* (RANK ligand) resulted in a reduction of mTECs [230, 231]. Moreover, the transplantation of RANK or RANKL deficient thymic lobes into nude mice promoted autoimmunity [226, 231]. Importantly, the intensity of RANK signaling in mTECs needs to be properly calibrated. In this regard, the regulation of RANK activation is controlled by OPG, a soluble decoy receptor for RANK ligand, predominantly expressed in the postnatal period [199]. The engagement of RANK induces the Spi-B-mediated expression of OPG, which in turn operates as a negative feedback loop to balance RANK signaling [199]. Mice deficient in *Tnrfs11b* display a substantial enlargement of mTEC compartment, which seems to augment negative selection and decrease the anti-tumor responses, due to a presumably more refined elimination of autoreactive T cell clones (including cancer specific) [199]. These findings indicate that there is a cost-benefit compromise between central tolerance induction and autoreactivity [199]. Regarding the role of CD40, the evidence suggests that it is less critical for mTEC development than the other members [231]. Moreover, transplantation of *Cd40* deficient thymic lobes for a nude mouse did not have a clear impact in self-tolerance induction [231]. Notwithstanding, the simultaneous deletion of *Tnfsf11* and *Cd40* has a synergistic negative repercussion in mTEC differentiation, affecting in greater extent the mTEC cellularity rather than the deletion of *Tnfsf11* alone [231]. Hence, CD40 signaling might have a cooperative role with the other TNFRSF members during mTEC development.

The TNFRSF members are activated in mTECs and their precursors upon interaction with ligands provided by distinct populations of thymocytes [230]. Indeed,

CD3etg26 and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice, which have a premature block in early stages of T cell development, are almost depleted of mTECs, demonstrating the critical importance of lymphoepithelial interactions between thymocytes and TECs, usually defined as thymus crosstalk [232, 233]. While during the embryonic period RANK ligand is provided by lymphoid tissue inducer (LTi) cells and Vγ5^{+} dendritic epidermal T cells (DETCs) [226, 227], in the postnatal thymus, SP thymocytes, particularly SP4, are the main source of RANK ligand, Ltα, Ltβ and CD40 ligand [230]. In accordance, mice deficient in SP4 thymocytes showed an impairment in mTEC differentiation, presumably due to an ineffective induction of NF-κB signaling in mTECs [234]. Thymic NKT cells may also be an alternative source of RANK ligand implicated in mTEC differentiation, since *Cd1* deficient mice have decreased mTEC cellularity [235]. Interestingly, the strength of TEC-thymocyte interaction is also an important regulator of mTEC differentiation, with augmented negative selection overstimulating mTEC differentiation and the expression of TRAs [234, 236]. Therefore, thymus crosstalk is crucial for medullary microenvironment construction with a dynamic interaction between different stromal and hematopoietic cell types during this process.

4.1.7 Epigenetic and post-transcriptional regulators of TEC differentiation

The previously described signaling pathways activate distinct transcription regulatory networks, which will promote TEC maintenance and differentiation. Besides the binding of transcription factors to promotor regions, gene expression is also modulated by epigenetic and post-transcriptional modification, which shape chromatin conformation or mRNA maturation/stability, respectively.

Epigenetic modifications alter the accessibility of molecular complexes to chromatin, thereby regulating the transcription of target genes. Recent studies have shed light on the importance of Polycomb Repressive Complexes (PRC) in TEC development. This group is composed by two major complexes, PRC1 and PRC2, which are constituted by different proteins [237]. PRC1 can be classified in canonical and non-canonical complexes according with their protein composition, repressing gene transcription through several mechanisms such as direct chromatin compaction as well as monoubiquitylation of histone H2A-K119, which stabilizes the activities of PRC1 and PRC2 maintaining the repressive state of chromatin [237-239]. The impact of disrupting the canonical PRC1 was examined by transplanting thymic lobes deficient in *Bmi1*, one of the components of canonical PRC1, into the kidney capsule of Wt mice. The transplanted thymus exhibited an impairment growth and regeneration, presumably due to a reduced TEC proliferation [240]. In a subsequent study, the TEC-specific deletion of *Cbx4*, which is part of the canonical and noncanonical PRC1, provoked thymus hypoplasia accompanied by defects in TEC

proliferation and differentiation, leading to a severe thymic atrophy and an abnormal differentiation of B cells within the thymus [241]. Further studies are required to better dissect the individual impact of each PRC1 variants in the differentiation and role of TECs. Regarding PRC2, this complex deposits methyl groups in lysine 27 on histone H3 protein (H3K27), leading to chromatin condensation and consequently to the repression of gene transcription [237]. TEC-specific deletion of *Eed*, which disrupts the PRC2 complex, severely affected mTEC differentiation and TRA expression, while the cTEC compartment was seemingly normal [242, 243]. These defects led to failures in many stages of thymocyte maturation and an alteration in T cell repertoire on the thymus [242], suggesting that PRC2 plays a role in mTEC differentiation and function, impacting T cell generation. Besides histone methylation, histone deacetylation has also been described to be important in the regulation of TEC biology [210, 244, 245]. This histone modification is mediated by histone deacetylases (HDAC) and increases the interaction between chromatin and histones, therefore decreasing the accessibility to transcription factors and silencing gene expression [246]. The laboratory of Jakub Abramson studied the role of HDAC by inactivating *Hdac1*, *Hdac2* and *Hdac3* in TECs [210]. The most striking phenotype was observed in *Hdac*3$^{f/f}$ *Foxn1*-cre mice, which showed a severe reduction in mTEC compartment [210]. The increased expression of notch signaling components in these cKO mice suggests that deacetylation by HDAC3 might regulate notch signaling and mTEC differentiation [210]. Indeed, although being important for the early diversification of mTEC-committed precursors, Notch signaling requires proper regulation in order to allow an adequate TEC differentiation, as observed by the complete disruption of thymic architecture in mice with constitutive activation of notch signaling [208, 209]. Taking in consideration that HDACs might have redundant roles, it would be interesting to analyse the combined role of the three members in TECs. Another deacetylase involved in TEC development is Sirtuin 6 (*Sirt6)* which belong to the III class of HDAC [246]. TEC-specific *Sirt6* deficiency affected the cellularity and the differentiation of mTEC compartment, due to the hyperactivation of NF-κB pathway [245]. As a consequence, *Sirt6* cKO mice showed several signs of autoimmunity, suggesting a role for SIRT6 in tolerance induction [245]. In line with these results, the removal from TECs of another member of the same class of HDAC, Sirtuin 1 (*Sirt1*), also provoked defects in central tolerance induction, however in this case the phenotype was mainly due to a disruption in TRA expression with no major alterations found in TEC composition [244].

Besides epigenetic regulation, gene expression can be modulated by post-transcriptional modifications that control several processes related with mRNA maturation and stability [247]. One of the key regulators in this process are MicroRNAs (miRNAs), which are short non-coding RNAs of 22 nucleotides that associate with Argonaute proteins,

forming the miRNA-induced silencing complex (miRISC) [248]. miRNAs promote the decay or translation repression of the target transcripts through complementary binding, thus inhibiting gene expression [248]. In this context, TEC-specific deletion of Dicer or DiGeorge syndrome critical region 8 (DGCR8), two important components that catalyze the production of miRNAs, led to severe impairment in mTEC differentiation, accompanied by alterations in T cell development and defects in self-tolerance induction [249-251]. Moreover, the expression of many miRNAs has been described to be dynamically regulated in TECs [250, 252], raising the possibility that miRNAs are important for TEC biology. In this regard, mIR-29a was identified as one of the candidate targets implicated in TEC homeostasis and function, being involved in the regulation of TRA expression [252]. In addition, mIR-29a was described to have a protective role against infection-mediated thymic involution upon pathogen infection. The postulated mechanism involves the downregulation of *Ifnar1*, as suggested by the partial recovery of thymic cellularity upon IFNAR1 blockade in mIR-29a deficient mice treated with dsRNA molecule poly(I:C) (a synthetic PAMP) [249]. Future research should focus in identifying new miRNAs and their role in TEC development and differentiation.

4.2 Non-epithelial components of thymic stroma

Beside TECs, thymic microenvironment is composed by other stromal cells, including mesenchymal cells, hematopoietic cells and endothelial cells. In the next sections, I will cover these other populations and their roles in thymopoiesis.

4.2.1 Thymic mesenchymal cells (TMCs)

TMC heterogeneity and differentiation

Thymic mesenchymal cells (TMCs) are derived from neural crest (NC) cells that migrate and surround the thymus anlagen during early development [144]. Taking advantage of interspecific grafts between chicks and quails, the seminal work of Le Douarin laboratory in the 70´s demonstrated the contribution of neural tube cells to TMCs that form the capsule and surround the vessels [140]. These early results were later confirmed by fate-mapping analysis which evaluated NC progeny [253-255].

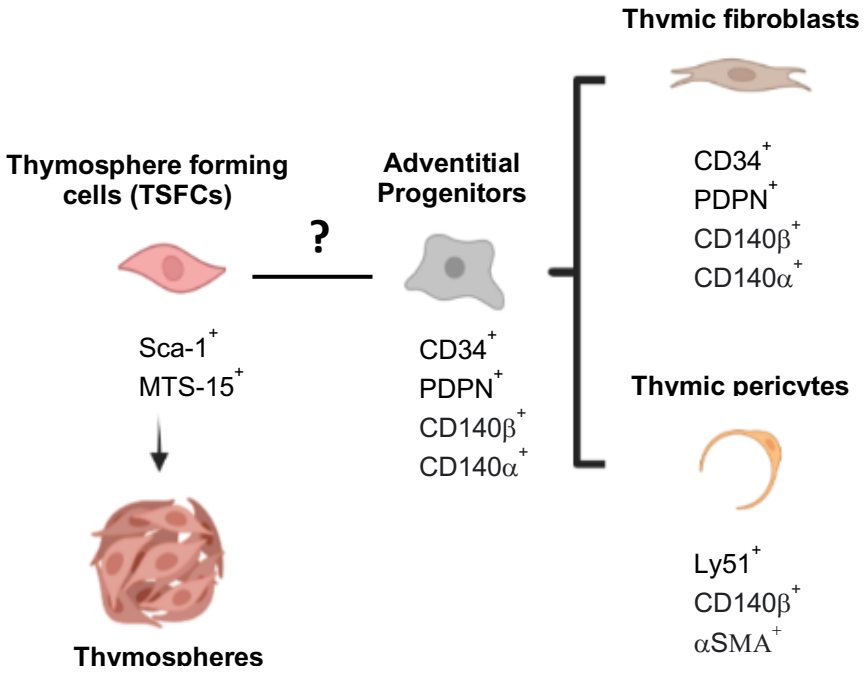

Figure 6 – Thymic mesenchymal progenitors and their progeny. The presence of thymic mesenchymal progenitors was suggested by the identification of thymosphere-forming cells (TSFCs) that arise from mesenchymal cells expressing the markers SCA-1 and MTS-15. The description of 'adventitial progenitors´ capable of originating both thymic fibroblasts and pericytes within the thymus strongly corroborates this hypothesis.

The phenotypic characterization of TMC subsets has been challenging due to its dynamic nature and complex composition. Nevertheless recent discoveries have contributed to expand our comprehension about thymic mesenchyme [256]. The simultaneous expression of CD140β, CD140α and PDPN within $CD45^{-}EpCAM^{-}CD31^{-}$ cells of the thymus identifies thymic fibroblasts [255] that can be further subdivided in $DPP4^{+}$ capsular fibroblasts (CapFb) and $DDP4^{-}$ medullary fibroblasts (MFb) [225]. CapFbs are detected in thymus anlagen at E13,5 and increase their representation throughout the embryonic period, while MFb appear and differentiate in a *Ltbr*-dependent manner concomitantly with the establishment of medullary microenvironments [225]. Thus, SP thymocytes might play a pivotal role in the promotion of MFb maturation and homeostasis since they are the main providers of lymphotoxin ligands in the adult thymus [230]. These results emphasize the importance of thymic crosstalk also for TMC differentiation [230]. Interestingly, a recent study has shown that both CapFb and MFb fibroblasts can be segmented in multiple subclusters peaking at different stages, suggesting that TMCs might

harbor a more complex dynamic and heterogeneous composition [257]. Pericytes represent another subset of TMCs that surround endothelial cells and regulate the development and function of thymic vasculature [258]. They are typically found within CD140β$^+$CD140a$^-$Ly51$^+$ TMC compartment which is further subdivided based on the expression of alpha smooth muscle actin (αSMA) into contractile (αSMA$^+$) and non-contractile (αSMA$^-$) subsets [255]. Their differentiation starts around E14,5 consistent with the identification of a TMC cluster with a gene signature of mature pericytes at E16,5 [255, 257]. This developmental window coincides with the initiation of angiogenesis within the thymus, which is essential for the homing of T cell precursors [259].

Despite the aforementioned knowledge, the differentiation of different TMCs is not well understood (**Figure 6**). The existence of TMC progenitor in the thymus is suggested by the presence of SCA-1$^+$MTS-15$^{+/-}$ mesenchymal cells with clonogenic potential as demonstrated by their capacity to form thymospheres [260]. Moreover, the identification in the adult thymus of an adventitial progenitor cell (CD34$^+$PDPN$^+$CD140β$^+$CD140α$^+$) residing in close proximity to thymic vasculature and capable of generating pericytes and fibroblasts corroborates this hypothesis [255]. It remains to be tested whether adventitial progenitors represent a homogeneous population of bipotent TMC progenitors or a mixture of lineage-committed precursor cells for thymic fibroblasts and pericytes.

The distinct functions of TMCs

The role of TMCs in thymus function has gained more attention in the last 25 years, with several studies demonstrating their involvement in thymus morphogenesis, regulation of thymic epithelium expansion, T cell development and thymus regeneration [256]. The importance of TMCs in thymus organogenesis can be appreciated in *Pax3* mutant embryos, which have a severe depletion of NC-derived perythymic cells and showed an abnormal definition of thymus/parathyroid domains as well as an ectopic thymus [261]. In line with their important role in thymus migration, the partial disruption of ephrin signaling pathway through the deletion of ephrin-B2 in NC-derived cells impaired the correct thymus positioning in the thoracic cavity [262]. Lastly, TMCs also provide retinoic acid during the embryonic period that is essential for the development of thymic vasculature and the adequate thymus morphogenesis [263]. Supporting this notion, mice with a hylomorphic allele for the enzyme involved in RA generation (*Raldh2*), which is specifically expressed by TMCs in thymic microenvironment [263], showed a hypoplasia or aplasia of the thymus [264]. Hence, TMCs contribute to the definition of thymus/parathyroid domains and the correct positioning and growth of the thymus during early organogenesis.

Concerning the TMC role in the growth and maintenance of TECs, it can be segmented in two different periods: embryonic and postnatal stage. Earlier studies showed that enzymatic dissection of perithymic mesenchymal cells from the embryonic thymus reduced the proliferation of TECs and thymus growth [265, 266]. Their function is mediated by the production of growth factors, such as fibroblast growth factors (FGF-7 and FGF-10), insulin growth factors (IGF-1 and IGF-2), retinoic acid, and epidermal growth factor (EGF), which together regulate the size of TEC compartment [263, 265, 266]. Concordantly, mice deficient in *FgfR2-IIIb* presented a hypoplastic thymus and an impairment in T cell differentiation [267]. Thus, TMCs contribute for the accelerated TEC growth during embryonic life [158]. However, the role of TMCs extends to the postnatal life. The expression of the growth factors above mentioned is maintained in adult thymus, indicating that TMCs may regulate the maintenance of the TEC compartment throughout life [255]. Indeed, provision of exogenous IGF-1 increased TEC proliferation and growth [268]. Moreover, a study which blocked the capacity of TECs to respond to RA, mainly produced by TMCs, induced a burst in cTEC proliferation, indicating that TMCs might have a negative impact in the growth of cortical epithelium in adult thymus [269]. Lastly, thymic fibroblast-specific protein 1 (FSP1)-expressing fibroblasts was shown to contribute to the homeostasis of mature mTECs [270]. These findings indicate that TMCs are active players in the regulation of thymic epithelium size at different stages of thymus development.

In striking contrast with well-known role of TECs in T cell development [70], the contribution of TMCs for thymopoiesis is poorly understood. In this context, the laboratory of Jason Cyster in 2010 found that perivascular TMCs generate a S1P gradient controlling the egress of T cells from the thymus [271]. Additionally, the production of heparan sulfate by TMCs during the neonatal period was shown to be important for thymic egress via the retention of CCL21 around the vessels [272]. Recently, an interesting report suggested that thymic MFb express TRAs in a LTBR signaling-dependent manner and participate in central tolerance induction [225]. However, the lack of MHC II expression makes MFb incapable of directly presenting TRAs to developing $CD4^+$ thymocytes, suggesting that they first need to transfer these antigens to professional APCs (such as dendritic cells or mTECs) in order to induce tolerance. Notably, the special capacity of MFb to express TRAs was questioned in a single-cell RNA sequencing-based study that did not find any particular enrichment in TRA expression when compared to other nonepithelial stromal cells [257]. Thus further studies are required to reconcile these conflicting results.

Recent evidence has also shown that TMCs contribute to thymus recovery upon damage. Mice depleted of $FSP1^+$ thymic fibroblasts displayed a delayed thymic regeneration after cyclophosphamide (Cy) treatment [270]. Moreover, a study showed that Flt3 ligand (FL) expression in perivascular fibroblasts was associated with a better thymic

recovery after irradiation [273]. More recently, it was suggested that the early expression of *MafB* upon irradiation by thymic mesenchyme was important for the recovery of thymus function [274], although the molecular mechanism needs to be further explored in future studies.

4.2.2 Hematopoietic cells

Thymic dendritic cells (DCs)

Besides thymocytes, other hematopoietic cells reside in the thymus playing a critical role in T cell development. One of the most well-studied cases are DCs, which participate in central tolerance induction. Generally thymic DCs can be subdivided in plasmacytoid DCs (pDCs) and classical DCs (cDCs)[275], being the latter further segmented in two functionally distinct subsets, SIPRα^-CD8α^+ (cDC1) and SIPRα^+CD8α^- (cDC2). While pDCs and cDC2 originate outside the thymus and reside within the thymic medulla [275], cDC1 arise intrathymically from immature precursors in a CCR7-dependent manner [276, 277]. Recently, a new subset of DCs positive for Sirpα and CD14, called monocyte-derived DCs (moDCs), was identified through single-cell RNA sequencing [278]. DC migration to the thymus was shown to be dependent on CCR2, CCR9 and CX3CR1 signaling [279-281]. Interestingly, a recent study has shown that thymocyte-TEC crosstalk was capable to fine-tune the recruitment of migratory DCs [282]. The proposed mechanism postulates that upon engagement of Ltα-Ltβr, mTECs downregulate CCL2, CCL8 and CCL12, which in turn dampens CCR2-dependent homing of DCs [282].

The initial concept that dendritic cells were specialized in the negative selection was supported by the observation that DCs were more efficient than TECs killing thymocytes through superantigen presentation in RTOCs [283]. This hypothesis was further rejected by several reports showing that indeed mTECs are crucial for negative selection and T_{reg} cells generation [284, 285]. Nevertheless, DCs also play a non-redundant role in this process. One of the first pieces of evidence showed in 1997 that mice reconstituted with MHC class I and II deficient bone marrow displayed a two-fold increase in the number of thymocytes generated, suggesting a key role of DCs in thymocyte elimination [286]. Although at that time the contribution of other hematopoietic-derived APC (monocytes/macrophages and B cells) could not be formally excluded, the specific contribution of DCs for this process was then elegantly demonstrated by the impairment in SP4 selection and self-tolerance induction observed upon the selective depletion of DCs [287]. However, DCs are also involved in T_{reg} induction as demonstrated by the *in vitro* co-culture of DCs with thymocytes [288] as well as through bone marrow reconstitution with MHC class II deficient cells which

led to a reduction in the frequency of T_{reg} cells compared to control counterpart [289, 290]. Altogether these showed that DCs participate in negative selection as well as in T_{reg} cell differentiation.

The functions of DCs in T cell development are linked to their capacity to capture antigens from mTECs through several mechanisms, such as exosome transfer, uptake of apoptotic bodies and membrane exchange [291-293]. Interestingly, different DC subsets appear to be specialized in acquiring antigens by specific routes such as the case of thymic cDC1 that can phagocytize apoptotic bodies through the scavenging receptor CD36 [291]. Apart from the different mechanism of antigen uptake, they also exhibit distinct proficiencies in this process. While some studies have highlighted that cDC1 are the most efficient subset in antigen uptake [291, 294], others showed that cDC2 [295] or even moDCs can perform better in this task [278]. These differences might be related to the different reporter or Cre-based transgenic mice (*Foxn1*, *Aire* and Rat insulin promoter (RIP)), used to evaluate the transfer of fluorescent-based antigens which display distinct antigen availability within the mTEC compartment. Furthermore, each DC subset might have a preferential cellular partner to interact with [296] . Follow-up studies should further explore the implications of the preferential acquisition of antigens from specific mTEC populations, dissecting whether distinct DC subsets are specialized in the coordination of central tolerance against different sets of TRAs.

Other hematopoietic cells

Thymic B cells were first identified in patients with myasthenia gravis and lymphoproliferative disorders [297], being further reported to exist in the human thymus of healthy individuals [298]. The analysis of RAG-GFP mice together with parabiosis experiments suggested that the majority of thymic B cells are generated intrathymically [299]. While the mechanisms involved in their differentiation are illusive, CD40-CD40l interactions appear to be required for their maintenance [300]. Regarding their role, the observation that MOG-specific thymocytes were eliminated in a mouse model that ectopically expresses the MOG peptide in B cells [301] together with the observation that the frequency of SP4 thymocytes was increased in mice deficient in B cells, suggested the contribution of thymic B cells for T cell selection [302]. Moreover, CD40-dependent signaling enhanced the capacity of thymic B cells for antigen presentation, inducing the expression of MHC II, CD80 and AIRE [302]. This process is named “licensing” and program thymic B cells to express some TRAs, although future investigation is required to better understand the extent of TRA repertoire expression.

Other hematopoietic subsets include macrophages that have been described to be scattered throughout the thymus parenchyma [75, 303, 304]. Concordantly to a previous study that identified two subsets of thymic macrophages [75], a recent report described a population of yolk sack-derived Timd4$^+$ residing in the cortex, and a distinct subset of Cx3cr$^+$ macrophages located in the medulla and cortical-medullary region that accumulated in the aged mice [305]. Besides their role in the clearance of thymocytes undergoing apoptosis [75, 305], thymic macrophages seem to activate NKT2 cells to produce IL-4 and IL-13, an important axis involved in the regulation of thymocyte emigration and thymus recovery [306, 307]. This special ability might be related with the upregulation of genes involved in lipid metabolism that ultimately allow macrophages to present lipid derivatives to NKT cells [306]. Further studies are required to better characterize thymic macrophage function.

Recently it was demonstrated that eosinophils are readily recruited to the irradiated thymus, in a process that depends on CCR3. Their migration involves the action of radioresistant NKT2 cells and ILC2s and enhances thymus recovery [307]. Nevertheless, the relevance of eosinophils accumulation in the thymus is yet unclear. The increased frequency of AnexinV$^+$ thymocytes in eosinophil-deficient ΔdblGATA mice might indicate that they are involved in the clearance of apoptotic cells [307]. This would be in line with the recent work of the laboratory of Dudakov showing that the elimination of dying cells derepresses the release of pro-regenerative factors, BMP4 and IL23, by thymic endothelial cells [308].

4.2.3 Thymic endothelial cells

The development of thymus vasculature is a dynamic process initiated in the primordium of thymus organogenesis, being remodeled throughout the postnatal period until reaching the configuration found in the adult thymus [309]. The first CD31$^+$ endothelial cells are detected in thymus parenchyma at E13,5 [259], followed by CD140β^+ cells and αSMA$^+$ cells, presumably pericytes, which surround thymic endothelial cells and indicate a progression in thymic vasculature maturation [259].

The initial characterization of thymic endothelial cells revealed some heterogeneity at the populational level. In particular, thymic endothelial cells were subdivided in three different subsets, defined by the differential expression of *Ly6c* and P-selectin (*Selp*) and with distinct transcriptomic profiles [224]. This heterogeneity was further extended by the identification of different clusters of thymic endothelial cells in scRNAseq analysis of the thymus of humans and mice [153, 257, 310]. Moreover, it was demonstrated that thymic vasculature permeability is not homogeneous, being reduced in blood vessels spread throughout the cortex in comparison with the ones localized in the medulla [311]. The

decreased permeability of the cortical vessels was correlated with the expression of Claudin 5 (*Cld5*) by endothelial cells, which presumably forms a tighter barrier against the paracellular transport [311]. Nevertheless, thymic endothelial cells are not only passive players in thymic microenvironment with the single function of creating a physical barrier. Instead, several reports have shown that the expression of *Selp* and *Ccl25* by thymic endothelial cells contributed to the homing of TSPs, working as a sensor of thymus occupancy and regulating TSP import and T cell export [312]. The interactions between TSP and thymic endothelial cells are mediated by the binding of integrin VLA-4 and LFA-1 expressed by TSP to VCAM-1 and ICAM-1, respectively, adhesion molecules expressed by thymic endothelium [313]. Recently, it was also demonstrated that the expression of SIRPα in thymic endothelial cells promotes the transendothelial migration of TSPs in a CD47 dependent manner [314]. Apart from this important function in the regulation of thymocyte migration and emigration, thymic endothelial cells also play a role in the chemotaxis and the correct positioning of thymic DCs, through their production of CCL25 and CX3CL1 [280, 315]. Nevertheless, the molecular bases underlying thymic endothelium homeostasis are barely defined. In this context, the activation of NF-κB signaling via Ltβr was shown to be important for their development and function [224, 316].

Thymic endothelial cells appear also to play a role in thymus regeneration under non-homeostatic conditions. Supporting this notion, a recent work from the laboratory of Dudakov has shown that the production of BMP4 and IL-23 by thymic endothelial cells contributed to thymic recovery after total body irradiation [308]. The proposed mechanism postulated that thymic endothelial cells act as sensors for the apoptotic thymocyte level through the TAM-Rac1 axis, being activated upon radiation-induced thymocyte depletion [308]. Further studies are required to examine whether there is a crosstalk between thymic endothelial cells and other cells, such as TEC, TMCs and eosinophils, during thymic recovery [270, 274, 307].

Since the discovery of thymus function in 1961 our comprehension about the mechanisms regulating T cell development grew exponentially. However, there are still many gaps in our understanding regarding the development and differentiation of the heterogenous population of thymic resident cells as well as how their interactions can shape thymic function. In the next chapters, I will detail how our work provides important insights about the molecular networks and the developmental trajectories underlying the differentiation of TECs and TMCs.

Aims

In this thesis, we aimed to understand the molecular and cellular principles underlying thymic epithelial cell (TEC) and thymic mesenchymal cell (TMC) development. The preservation of a regular thymic function across life depends on the renewal and maintenance of TEC microenvironments. In chapter II, we reviewed the most recent data on TEC homeostasis during the first weeks of the murine postnatal life and integrated how these alterations anticipate processes associated with thymic involution. In chapter III, we investigated the role of the post-transcriptional regulators, ZFP36 and ZFP36L1, belonging to tristetraprolin (TTP) family in TEC biology and function. Lastly, in chapter IV, we examined the development pathway of TMCs.

In order to answer these questions, we took a holistic approach, proceeding from the study of molecular processes taking place at the cellular level to the *in vivo* analysis at the organismal level. To study the role of TEC and TMC in the thymus, we integrated the use of several mice models, including new TEC-specific conditional knockout mice (cKO), thymic organotypic cultures, genome-wide RNA sequencing, multicolour flow cytometry and fluorescence microscopy analyses. Deciphering the mechanisms involved in the differentiation and function of these key stromal components is essential to increase our comprehension on thymus development, maintenance and regeneration.

Chapter II

The early postnatal life: A dynamic period in thymic epithelial cell differentiation

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Abstract

The microenvironments formed by cortical (c) and medullary (m) thymic epithelial cells (TECs) play a non-redundant role in the generation of functionally diverse and self-tolerant T cells. The role of TECs during the first weeks of the murine postnatal life is particularly challenging due to the significant augment in T cell production. Here, we critically review recent studies centered on the timely coordination between the expansion and maturation of TECs during this period and their specialized role in T cell development and selection. We further discuss how aging impacts on the pool of TEC progenitors and maintenance of functionally thymic epithelial microenvironments, and the implications of these chances in the capacity of the thymus to sustain regular thymopoiesis throughout life.

Introduction

The current pandemic caused by the SARS-CoV-2 virus underscores the importance of maintaining a pool of immunologically competent T cells, which are capable of responding to virtually any new foreign threats while tolerant to the host own tissues. The establishment of a diverse T cell receptor (TCR) repertoire arises from the random recombination of V(D)J gene segments during T cell development in the thymus. Yet, the arbitrariness underlying this process can also produce autoreactive T lymphocytes. The thymus has developed several control mechanisms to simultaneously establish T cell immunity against non-self elements and impose self-tolerance. Particularly important in the choreography of T cell selection are thymic epithelial cells (TECs), which represent a key component of the thymic stromal microenvironment. TECs are typically subdivided into functionally distinct cortical (cTEC) and medullary (mTEC) lineages[317]. While cTECs primarily mediate T cell lineage commitment and positive selection, mTECs fine-tune the negative selection of autoreactive thymocytes or promote their deviation into the T regulatory cell lineage [152]. It is conceptually accepted that cTECs and mTECs differentiate from thymic epithelial progenitors (TEPs) present within the embryonic and postnatal thymus [152]. Deficits in the function of TECs arise with aging, cytoablative regimens and infection, leading to a lower naïve T cell output. These thymic failures are pertinent in the elderly and patients undergoing bone marrow transplantations (BMT), contributing to their poor T-cell responses to new pathogens or predisposing to autoimmunity [318]. Thus, the preservation of a regular thymic function also depends on the maintenance and differentiation potential of bipotent or lineage restricted TEPs. In this review, we focus on critical changes in the molecular traits

of TECs that occur during the first weeks of the murine postnatal life, and integrate how these alterations might precede events coupled with thymic involution.

The build-up of TEC microenvironments

The initiation of TEC development coincides with the onset of thymus organogenesis, which starts around day 9-10 of the murine embryonic gestation (E9-10) [35]. The expression of Forkhead box protein N1 (Foxn1) in the ventral area of the common thymus and parathyroid primordium marks a critical step in TEC specification [204]. Still, Foxn1 expression needs to be continuously maintained during the differentiation of c/mTEC, wherein it imposes a complex genetic program that confers them the capacity to support distinct stages of thymopoiesis [188]. TEPs formed during early thymus ontogeny constitute the primordial building blocks for the establishment and maintenance of c/mTEC microenvironments [146, 147, 319]. Our comprehension about the mechanisms underlying TEC differentiation has considerably advanced with the identification of distinct populations containing bipotent or lineage-restricted progenitor activity [77, 151, 155, 159, 320-327] (further detailed below and reviewed in [70, 328]). These studies led to the proposal of different refined models of TEC differentiation, whereby TEPs traverse through transitional stages that share a closer or distinct relationship with cTEC- or mTEC-unipotent precursors, prior to the commitment in mature c/mTEC subsets (reviewed in [152, 329, 330]). Yet, it remains unclear the trajectories and molecular elements governing the differentiation of TEC progenitors into mature c/mTEC lineages.

The expansion and functionalization of c/mTEC compartment during early postnatal stages generates a supportive microenvironment that increases thymopoiesis, reaching its peak during young adulthood. Thereafter, T cell production progressively declines with aging, becoming residual in the aged thymus [331]. During these periods, TECs undergo concomitant alterations in their composition and differentiation program. Although the density of TECs based on flow cytometry analysis might be underestimated [79], the number of TECs vigorously expands during postnatal life and early adulthood, followed by a progressive decline with age [332, 333]. Changes in the size of TEC microenvironment appears to relate with the function of the thymus. While a reduction in the TEC compartment below a certain threshold restrains thymopoiesis [334, 335], the expansion of the thymic epithelial niche, for example via transgenic expression of Foxn1 or Cyclin D1, increases T cell generation [336, 337]. Along this line, the frequency of cycling TECs is elevated during fetal life, progressively declines during the postnatal life and become a rare fraction in the aged mouse thymus [332]. Transcriptomic analysis revealed that the expression of cell-

cycle regulators is downregulated in TECs as early as 1 month [192]. Moreover, the enforced expression of cMyc in TECs promotes the expansion of the TEC compartment, via the engagement of a genetic program akin to the one found in embryonic TECs [338]. These results suggest that the loss in the proliferative rate of TECs, together with other alterations such as changes in cell survival and rate of differentiation, may contribute to a reduction in the size of TEC compartments with age. In the next sections, we outline specific cellular and molecular alterations that take place in c/mTEC during early postnatal life, and conjecture how those changes may anticipate subsequent functional losses in the capacity of TECs to sustain regular thymopoiesis in the long-term.

The assembly of functionally dedicated cTEC and mTEC compartments

The first weeks of the postnatal life marks a period of intense turnover and functional diversification in the TEC niche, wherein key mature subsets in tolerance induction are generated or expanded [70]. During this period, the changes in the cellularity and functionality of cTECs appear to unfold concomitant with the expansion and diversification of mTECs [77, 151, 164, 232, 339]. This leads to a conspicuous inversion in the cTEC/mTEC ratio within the first 2 weeks after birth, which correlates with the intensification of thymopoiesis [77, 151, 332]. In this regard, the consequent rise in the number of positive and negative selection events, will impose an increase demand on TEC compartments. Given that mature cTECs and mTECs have a limited life-span, the maintenance and specialization of their microenvironment seem to depend on the continual differentiation of their progenitors. These functional requirements are in part met by a symbiotic relationship with thymocytes (discussed further below) that stimulate specific proliferative and differentiation programs in TECs [340].

It remains surprising how little we know about the molecular program that underlies the differentiation of cTECs. Despite these gaps, several studies highlight that cTECs undergo molecular and functional changes during neonatal and puberty periods. In particular, cTECs downregulate the expression of key thymopoietic factors, such as Dll4 and IL-7, during the first weeks of postnatal life, which result from continual lymphoepithelial interactions [76, 232, 339, 341]. These quantitative and qualitative disruptions in cTECs appear to anticipate the bona fide hallmarks that characterize TECs in the involuted thymus. In contrast to cTECs, our understanding of the cartography of mTEC differentiation is more complete [328]. This process depends on reciprocal signals provided by several types of hematopoietic cells [317]. These lymphoepithelial interactions, commonly referred as thymic crosstalk, engage specific members of the tumour necrosis factor receptor superfamily (TNFRSF), including receptor activator of NF-κB (RANK), CD40 and

lymphotoxin β receptor (LTβR), in mTECs and their progenitors, leading to the activation of a nuclear factor kappa B (NF-κB)-dependent maturation program (reviewed in [317, 328]). The cooperative action of TNFRSF members is not only important for the expansion of mTEC niches but also for their functional diversification. Upon the initial subdivision in $mTEC^{low}$ and $mTEC^{high}$ [342], the discovery of Autoimmune regulator (AIRE)-, CCl21- and forebrain embryonic zinc finger-like protein 2 (FEZfF)-expressing cells revealed that mTECs harbours a variety of functionally distinct mature subsets [317, 328]. Although $AIRE^{+}$ and $FEZF2^{+}$ cells emerge during embryonic life [317, 328], their abundance significantly increases in the first weeks of life. In this regard, RANK-mediated signalling is essential to the expansion of $AIRE^{+}$ mTECs, whereas CD40 also contributes to this process [230, 231]. Although LTβR signalling was initially coupled to the development of $AIRE^{+}$ [220] and $FEZF2^{+}$ lineages [115], subsequent studies indicated its involvement in the architecture of postnatal medullary compartment [223]. AIRE and FEZF2 regulate the capacity of mTECs to express large sets of non-overlapping tissue restricted antigens (TRAs), which are randomly organized in patterns of gene expression at the single cell level [116, 133, 343] and are reported to decrease their levels with age [191, 344, 345]. In this regard, an earlier study underscore the importance of AIRE expression in mTECs during neonatal period [346], which corelates with their capacity to control the generation of a unique population of T_{reg} cells [347]. It remains to be determined whether Aire expression during this temporal window particularly impacts on the quantity or quality of TRAs expression by mTECs.

The role of mTECs in tolerance induction extends beyond their promiscuous gene expression capacity. CCL21-producing cells represent a prototypical example of alternative roles of mTECs. CCL21-expressing mTEC represent a subset of $mTEC^{lo}$ and control the migration of positively selected thymocytes towards the medulla [68, 348]. $CCl21^{+}$ cells emerge during embryogenesis and their numbers also undergo a marked increase during the first weeks of life [348]. Recent scRNAseq analysis suggests that *Aire*- and *Ccl21a*-expressing mTEC subsets do not share a direct lineage relationship [349]. Moreover, the discoveries that $AIRE^{+}$mTECs differentiate into Post-AIRE cells [350, 351] further extended our view on the heterogeneity within thymus medulla. Post-AIRE mTECs shutdown the expression of AIRE, certain TRAs, CD80 and MHCII, while acquiring traits of terminally differentiated keratinocytes [118, 119]. Two reports identified a highly differentiated mTECs that share molecular traits with tuft cells found at mucosal barriers. Fate-mapping analysis suggests that this subset can develop via an AIRE-dependent and AIRE-independent pathway [120, 121]. Although their complete functional relevance remains elusive, tuft-like mTECs appear to regulate the development of invariant NKT cells and ILCs [120, 121]. Future studies may uncover new specialized mTEC subsets and their role in imposing the limits of tolerance, or alternative processes in thymus biology.

The thymic epithelial cell progenitor reservoir

The diversification of TECs during the first weeks of life is dictated by the intricate balance between the rate of proliferation and differentiation of mature subsets. The rapid turnover of TEC microenvironments, with an estimated replacement time of one to two weeks to mTECs [332, 350], implicates the requirement for a regular generation of mature TECs from their upstream progenitors. One possibility is that bipotent TEPs continually produce lineage-committed precursors lacking long-term self-renewal capacity. Alternatively, and not mutually exclusive, the abundance of bipotent TEPs might decrease with age, being the maintenance of cortical and medullary epithelial niches assured by downstream compartment-restricted precursors. In the last years, several studies provide evidence for the existence of an arsenal of subsets enriched in purported bipotent TEC progenitors in the postnatal thymus [159, 320-322]. One approach has employed *in vitro* 2D-clonogenic [320] or spheroids [321] assays to respectively isolate TEC progenitors that reside within $EpCAM^{+}Ly51^{+}$cTECs or $EpCAM^{-}$ cells, which were expanded *in vitro* and revealed the capacity to give rise to c/mTEC. Nonetheless, a more recent study indicate that cells isolated from $EpCAM^{-}$derived spheroids represent mesenchymal progenitors [260]. Other methodologies resolved bipotent progenitor activity within defined subsets of $UEA\text{-}1^{-}MHII^{lo}Sca\text{-}1^{+}$ TECs [159] and $MHCII^{hi}$ $Ly51^{+}Plet1^{+}$ cTECs [322]. Both strategies employ reaggregate organ cultures (RTOCS) to determine the precursor-product lineage relationship to mature cells. Despite the advances, it remains to be determined the physiological contribution of these cells to the TEC microenvironment in the adult thymus. Thus, we still lack experimental evidence that demonstrates the existence of bona-fide bipotent TEC progenitors in the postnatal thymus, and their identification at the single cell level

Downstream of TEC progenitors, complementary studies documented how mTEC compartments evolved from bipotent TEP and mTEC-restricted precursors (mTEPs), including mTEC-restricted $SSEA\text{-}1^{+}$ and $podoplanin^{+}$ (PDPN) mTEPs [155, 324]. Fate-mapping studies show that the adult mTEC network arise from fetal- and newborn-derived TEPs expressing beta5t (β5t), a prototypical cTEC marker. Yet, the contribution of $\beta5t^{+}$ TEPs to the adult mTEC niche decreases with age [325, 326], suggesting that the maintenance of the adult medullary epithelium is assured by mTEPs. Although bipotent TEPs might lose the expression of some traits found in the embryo (e.g. β5t), it is also possible that the abundance and/or the self-renewal properties of bipotent TEPs and/or lineage-restricted progenitors decline with time. Supporting this view, the clonogenic activity

of purported bipotent TEPs that reside within the cortex decrease with age [320] and Cld3,4^{+}SSEA1^{+} mTEC-restricted cells become rare in the adult thymus [155]. Given that the numbers of embryonic TEPs dictates the size of functional TEC microenvironments [334], we infer that the loss in the TEC network that takes place with age may result from the decrease in the bioavailability and self-renewal capacity of TEPs early in life.

The advent of scRNAseq analysis have also contributed to our understanding of the heterogeneity and dynamic of TEC progenitors. This approach has emerged as a new unbiased method to identify novel subsets, providing a valuable platform to analyze their developmental trajectories and determine their relationships with progenitor subsets identified by conventional methodologies. In this regard, new clusters termed “pre-AIRE mTEC 1 and 2” [352] appear to present molecular traits similar to the ones found in podoplanin^{+} (PDPN) mTEPs [324]. A subsequent study identified a novel cluster of “intertypical TECs” [344] that harbors traits akin to the ones found in podoplanin^{+} (PDPN) mTEPs [324], UEA-1^{-}MHII^{lo}Sca-1^{+} [159] and MHCII^{hi} Ly51^{+}Plet1^{+}[322] TECs. Since “intertypical TECs” are further segmented in distinct 4 subclusters, it would be interesting to determine if they associate to a particular bipotent or unipotent subset. Moreover, scRNAseq analysis reveal the existence of a previously unrecognized cluster of “perinatal cTECs”. Interestingly, this subset harbors cells with a highly proliferative status and their abundance declines with age [344]. Moreover, the combination of scRNAseq and fate mapping analysis revealed that β5t^{+} TEPs acquire senescent-like properties with age, potentially explaining their failure to contribute to mTEC lineage beyond the neonatal stage [325, 326]. Together, these findings indicate that the integration of multiple experimental approaches provides a more complete strategy to resolve the intricacies of the TEC compartment. Future studies should attempt to identify specific markers to resolve the newly characterized populations at a single level.

Concluding remarks

The aforementioned studies underscore that the period between birth and early adulthood is a time of intense alterations in TEC microenvironments, which prepares them to the highly demand role of choreographing the selection of growing number of T cell precursors. In this sense, it is remarkable to appreciate the synchronous coordination between TEC differentiation and the requisites imposed by T cell development. Yet, the erosion of the pool of TEC progenitors seem to accompany the generation of specialized subsets with key roles in tolerance induction. We reason that an in-depth molecular analysis of TEC differentiation during early postnatal may provide insights on how TEC niches are

maintained, and can be repaired in the aged thymus. Despite recent advances, it remains unclear how changes in the bioavailability of TEPs impact on the maintenance of TEC microenvironment across life, and ultimately on thymic output. Another unexplored area pertains to the physiological causes underlying the presumed age-dependent decrease and/or senescence of TEPs. Knowledge in these areas will not only permit to comprehend the basic principles that governs thymic function, but also target pathways for the treatment of disorders coupled to dysfunctional thymic/T cell responses.

Chapter III

The cooperative role of RNA-binding proteins ZFP36 and ZFP36L1 in TEC development and function

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Abstract

Thymic epithelial cells (TECs) are critical for the coordination of T cell development. While cTECs participate in the early stages of thymopoiesis, mTECs have an important contribution for the mechanisms of central tolerance. However, aged-dependent thymic involution is associated with a progressive decline in the size of TEC compartment which compromises thymopoietic activity and consequently the functional competence of the immune system. Thus, understanding the molecular principles that govern TEC differentiation and maintenance is essential to comprehend the requirements for the generation of competent and self-tolerant T cells. Here, based on previous genome-wide transcriptomic analysis of TEC sub-populations as well as on the important role of post-transcriptional modifications in the regulation of TEC genetic program, we examined the biological role of ZFP36 RBP family. The specific deletion of ZFP36 or ZFP36L1 in thymic epithelium led to a size contraction of TEC compartment and alterations in its composition with no major consequences in T cell development. Notably, combined deletion of both proteins had a similar deleterious impact in thymic epithelium but this time led to defects in T cell production accompanied by an increase predisposition to develop autoimmune manifestations. Collectively, our results demonstrate the cooperative role of ZFP36 and ZFP36L1 in TEC biology.

Introduction

The generation of functionally diverse and self-tolerant T cells in the thymus is regulated by thymic epithelial cells (TECs), which form specialized three-dimensional cortical and medullary microenvironments [110, 353]. While cortical TECs (cTECs) are involved in early stages of T cell development, including T cell lineage commitment and positive selection, medullary TECs (mTECs) have a more specialized role in negative selection and T_{reg} cell differentiation [110, 353]. Both TEC subsets are maintained via the differentiation of bipotent and lineage-committed TEC progenitors present in the embryonic and adult thymic microenvironment [149, 150, 154, 159-161]. Therefore, it is considered that the decrease in the bioavailability and function of TEC progenitors correlates with age-associated thymic involution [78, 155, 165]. The decline in thymic function is particularly important in the elderly and immunosuppressed patients, contributing to poor T-cell responses to vaccines and infections [45]. Hence, the dissection of the molecular mechanisms regulating TEC differentiation is important to understand the foundations underpinning T cell generation and facilitate the design of strategies to restore thymic function.

The genetic program that drives the development and function of TEC depends on complex epigenetic, transcriptional and post-transcriptional mechanisms. In this regard, histone modifications mediated by Polycomb Repressive Complexes (PRC), which promote chromatin compaction and consequently repress gene expression, have been shown to regulate TEC development. While the disruption of PRC1 complex in TECs provokes thymic hypoplasia and defects in T cell generation [240, 241], deficiency in PRC2 complex severely affects mTEC differentiation and TRA expression, demonstrating their important role in mTEC development and function [242, 243]. Moreover, changes in the histone acetylation status mediated by Histone deacetylase 3 (HDAC3), Sirtuin 6 (Sirt6) and Sirtuin 1 (Sirt1) also control mTEC development and self-tolerance induction [210, 244, 245].

The induction of a particular genetic program can also be modulated by post-transcription modifications that interfere with the maturation and stability of mRNA. Relevant in this context, RNA interference (RNAi)-mediated gene silencing has emerged as an active process in TEC development. In particular, micro-RNAs (miRNAs) have been implicated in TEC differentiation and T cell development, and the deregulation of its function is associated with defects in self-tolerance induction [249, 250]. Moreover, miRNAS are also involved in the attenuation of infection-induced thymic involution [249]. Apart of microRNAs, RNA-binding proteins (RBPs) represent another class of chief regulators of gene expression, controlling mRNA bioavailability and translation of master regulatory genes [354]. In this context, genome-wide transcriptomic analysis of postnatal TECs previously conducted by the host laboratory found that the expression of members of tristetraprolin (TTP), also known as Zinc Finger Protein 36 (ZFP36), family of RBPs were enriched in particular subsets of embryonic day 14 TECs and cTECs of the postnatal thymus (unpublished data). The ZFP36 family includes three members in man and most other mammals: ZFP36/TTP, ZFP36L1 and ZFP36L2. Some rodents have an extra member named ZFP36L3, expressed in the placenta during embryonic development [355]. Structurally, ZFP36 proteins have two main domains, a CCCH Tandem Zinc Finger (TZF) domain that binds AU-rich elements (AREs) in the 3' untranslated region of the target mRNAs; and a C-terminal domain that interacts with NOT1, the scaffold of a large multi-protein complex containing deadenylases, which progressively remove the adenosine residues from the poly(A) tail [355]. The three ZFP36 RBPs share a high level of homology within the TZF RNA-binding domain, raising the possibility of overlapping, but not necessarily interchangeable, functions. Analysis of individual targeted gene knockout mice revealed that ZFP36 RBPs regulate a wide range of biological processes such as lymphocyte development, inflammation, muscle repair, keratinocyte and mammary epithelial cell differentiation [356-360]. To test their intrinsic role in TEC biology and thymus function, we generated conditional knockout mice of ZFP36 RBPs in the thymic epithelium. Our results suggest that TEC-specific deficiency in *Zfp36* or

Zfp36l1 induced a decrease in the number of TECs, without substantially affecting thymopoiesis. Interestingly, double conditional KO (dcKO) mice presented a similar reduction in TEC cellularity, but that led to a conspicuous failure in thymic function.

Material and methods

Mice

Zfp36$^{fl/fl}$, *Zfp36l1*$^{fl/fl}$, *Zfp36*$^{fl/fl}$*Zfp36l1*$^{fl/fl}$, *Foxn1*-cre:*Zfp36*$^{fl/fl}$, *Foxn1*-cre:*Zfp36l1*$^{fl/fl}$ and *Foxn1*-cre:*Zfp36*$^{fl/fl}$*Zfp36l1*$^{fl/fl}$ were all bred on a C57BL/6 background and housed under specific pathogen-free conditions at I3S' animal facility. Experiments were performed under the European guidelines for animal experimentation.

TEC and hematopoietic cell isolation

TECs were isolated as described [157]. Briefly, the thymus was cut into small pieces and subjected to a gentle mechanical dissociation to liberate thymocytes. Thymic fragments were digested for 30 minutes at 37º C with agitation in PBS containing 20mg/ml of collagenase D (Roche) and passed through 100-μm filter to remove debris. Further stromal cell enrichment was carried out by incubation with anti-CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (Miltenyi) according to manufacturer's instructions. 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 ammo- nium chloride (Sigma-Aldrich, A9434), 10 mM potassium bicarbonate (Sigma-Aldrich, 237,205).

Flow cytometry

Cell suspensions were stained as described with Alexa Fluor 488/FITC-conjugated anti-CD44 (clone IM7; Biolegend, 103,022), anti-15G4 (Santa Cruz Biotechnology, sc-53,946), anti-CD5 (clone 53–7.3; Biolegend, 100,605) and anti-CCL21 (clone 59,106; R&D Systems, IC457G-100UG); PE-conjugated anti-TCRB (clone H57-597; eBioscience, 12–5961-82) and anti-ENPEP/LY51 (clone 6C3; eBioscience, 12–5891-82); PerCP-Cy5.5-conjugated anti-PTPRC/CD45.2 (clone 104; Biolegend, 109,828) and anti-TCRB (clone H57-597; eBioscience, 45–5961-80); 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-CD40 (clone 1C10; eBioscience, 17–0401-81), anti-Ly6G (clone RB6-8CS; eBioscience 50-5931-82), anti-γδ TCR (eBioGL3; eBioscience 17-5711-82), anti-CD11c (N418; eBioscience 50-0114-82), anti-CD19 (eBio1D3; eBioscience 50-0193-82), anti-NK1.1 (PK136; eBioscience 17-5941-82), anti-TER-119 (TER-119; eBioscience 17-5921-82) and anti-CD11b (M1/70; eBiosceince 50-0112-82); APC-eFluor780-conjugated anti-I-A/I-E (clone M5/114-15-2; eBioscience, 47– 5321-82) and anti-CD117 (2B8; eBiosceince 47-1171-82);eFluor450-conjugated anti-CD24 (clone M1/69; eBioscience, 48–0242-82); BV421 conjugated anti-EPCAM (clone G8.8; Biolegend, 118,225); BV605 conjugated anti-SELL/CD62L (clone MEL-14; Biolegend, 104,437); 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) were revealed by 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).

Histology

The indicated organs were harvested and incubated 48 hours in 4% paraformaldehyde (PFA). After the fixation, the tissues were embedded in Paraffin, sectioned and stained with hematoxylin and eosin (H&E).

Autoantibody detection

Organs were fixed in 4% paraformaldehyde in PBS solution, washed in PBS solution, incubated in a solution of 30% sucrose, embedded in OCT compound, and frozen at -80ºC. Frozen sections (10 μm) were cut with an Cryostat Leica CM 3050S and collected onto Superfrost/Plus slides. After blocking (10% BSA in PBS solution), samples were incubated with 1/50 sera from the indicated mouse strains at room temperature followed by detection with goat anti–mouse IgG(H+L) (Alexa Fluor 568, Invitrogen). The slides were stained with DAPI, and mounted with antifade mounting medium. Images were acquired with a Zeiss axio imager Z1 microscope and processed using Fiji software. Quantification of

autoantibodies was performed according with the staining intensity on an arbitrary scale of low, intermediate (int) and high.

Anti-nuclear antibodies detection

Serum was collected from 6 to 8-months-old mice and stored at -20ºC. The detection of Anti-nuclear antibodies was performed using the Mouse anti-nuclear antibodies (ANA) total Ig ELISA Kit provided by Alpha Diagnostic according to the supplier's protocol.

Statistical analysis

Statistical analyses were performed using GraphPad software, Version 9. Column graphs are represented showing the mean plus one standard error of the mean (SEM). Statistical analysis was performed by using a two-tailed t-test.

Results

TEC-specific deletion of *Zfp36* or *Zfp36l1* alters the size and composition of TEC microenvironment without affecting thymopoiesis.

In order to examine the role of ZFP36 family members in TEC biology, we generated novel conditional knockout (cKO) mice of the three ZFP36 family members. To do so, we crossed *Zfp36*-, *Zfp36l1*- and *Zfp36l2*-floxed mice to *Foxn1*-cre mice (Fig. S1A). In these cKO mice, the expression of Cre recombinase is under the control of *Foxn1* promoter leading to the disruption of targeted genes specifically in TECs within the thymus [361]. Although we have generated single, double and triple cKO forms, my thesis focused on the biological role of ZFP36 and ZFP36L1, which are the ZFP36 members mostly expressed in TECs (unpublished). We selected to analyze TECs from *Zfp36* and *Zfp36l1* cKO mice at two developmental stages, the early postnatal (2 weeks) and young adult (10 weeks) life, as they defined stages of expansion and establishment of the thymic microenvironment [362]. The number of TECs was reduced in *Zfp36* and *Zfp36l1* cKO mice at both time points (Fig. 1A). At 2 weeks of age, only *Zfp36* cKO thymus exhibited alterations in cTEC/mTEC proportions, due to a specific reduction in the numbers of mTECs (Fig S1B). Although cTECs/mTECs ratios changed in *Zfp36* and *Zfp36l1* cKO mice relative to controls at 10 weeks, both cTEC and mTEC numbers decreased in *Zfp36* cKO thymus, while the mTECs subset was the most affected subset in *Zfp36l1* cKO mice (Fig. 1B).

The more significant impact of *Zfp36*- and *Zfp36l1*-deficiency in mTECs led us to investigate possible alterations in their differentiation. To do so, we started by analysing the mTEC^{lo} and mTEC^{hi} subsets, defined based on the differential expression of MHC II and CD80 [353]. While mTEC^{lo} (MHC II^{lo}CD80^{lo}) contains diverse subsets, including immatures cells, CCL21^{+} cells and a minor subset of terminally differentiated post-AIRE cells, mTEC^{hi} includes mostly mature cells, including the AIRE^{+} subset [353]. At 2 weeks, the frequency of mTEC^{lo} and mTEC^{hi} was comparable to their control counterparts (Fig. S1C). Still, an altered mTEC^{hi}/mTEC^{lo} ratio with an accumulation of mTEC^{hi} was noted in the *Zfp36* and *Zfp36l1* cKO adult thymus, (Fig. 1C). These results suggested that the deficiency of *Zfp36* and *Zfp36l1* impacted the composition and differentiation of TECs, mostly of the mTEC lineage, from the early postnatal period to adulthood.

Next, we studied whether the defects in thymic epithelium found in *Zfp36* and *Zfp36l1* cKO mice led to alterations in T cell development. The frequency and numbers of the main

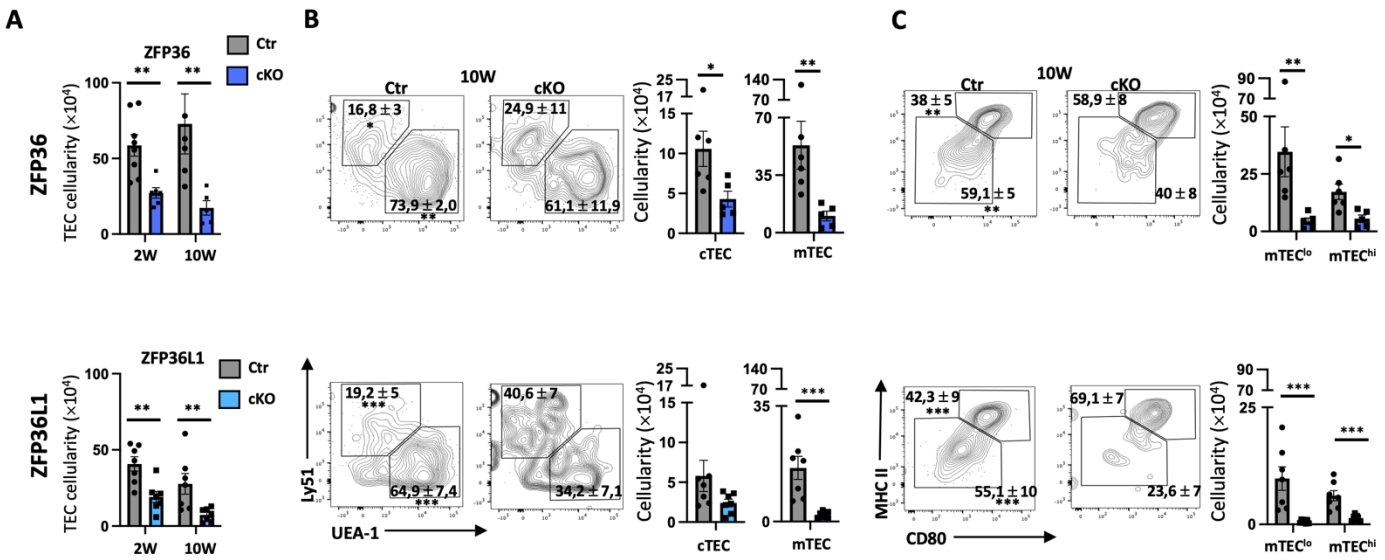


Figure 1: Deletion of *Zfp36* or *Zfp36l1* impacts the composition and differentiation of TECs. The composition of TECs was analysed in *Zfp36* (*Foxn1*Cre:*Zfp36*$^{fl/fl}$) cKO mice (upper row) and *Zfp36l1* (*Foxn1*Cre:*Zfp36l1*$^{fl/fl}$) cKO mice (lower row) relatively to respective controls (*Zfp36*$^{fl/fl}$ and *Zfp36l1*$^{fl/fl}$) littermates. A) The cellularity of TECs (CD45$^-$EpCAM$^+$) was analysed in 2- and 10-week-old mice. B) The composition of cTECs and mTECs was determined at 10 weeks of age. Dot plots show representative Ly51/UEA-1 staining in TECs and gates to define cTEC (Ly51$^+$) and mTEC (UEA-1$^+$) subsets. C) Dot plots show representative analysis of mTEC^{lo} (MHII^{lo}CD80^{lo}) and mTEC^{hi} (MHII^{hi}CD80^{hi}) subsets based on the expression of MHC II and CD80 at 10 weeks. Graphs in A, B and C represent the average cellularity ± SEM of each respective TEC population (2 independent experiments, n=5 to 7 per group). ***P<0.001, **P<0.01, *P<0.05.

thymocyte subsets, including double-negative (DN), double-positive (DP) and single-positive (SP) CD4 and CD8 cells, found in both mutants were comparable to control mice. We subsequently focused on more defined stages of T cell development, including the early differentiation of DN thymocytes, positive selection, SP maturation and T_{reg} differentiation. Analysis of the progression through DN1-DN4 stages, which can be mapped by the differential expression of CD44 and CD25 [47], revealed a reduction in the frequency of DN1 thymocytes in both cKO lines, without impacting on the progression of the subsequent DN stages (Fig. S2A, Fig. S3A). To evaluate positive selection, we analyzed the differential expression of CD69 and TCRβ on thymocytes, which defined 4 populations; TCRβ$^{-/lo}$ CD69$^-$ cells comprise essentially pre-selected thymocytes (population 0); TCRβ^{int} CD69^{int} cells contain TCR-signaled thymocytes initiating positive selection (population I); TCRβ^{hi} CD69^{hi} cells harbor post-positively selected thymocytes (population II); and TCRβ^{hi} CD69$^-$ cells represent more mature thymocytes (population III)[363]. The frequency of these different subsets was unaltered in both mutant mice, suggesting that positive selection was seemingly normal in the absence of ZFP36 and ZFP36L1(Fig. S2B, Fig. S3B). Following

positive selection, SP thymocytes complete their maturation in the medulla before leaving the thymus [364]. We found minor alterations in proportions of immature ($CD24^+$ $CD62L^-$) and mature ($CD24^-$ $CD62L^+$) thymocytes in *Zfp36l1* cKO mice, but that did not impact on the cellularity of these subsets in both mutants (Fig S2C and Fig S3C). Given that mTECs are critical for the generation of T_{reg} cells [284] and mTEC differentiation was affected by the deletion of *Zfp36* or *Zfp36l1*, we studied whether their differentiation was impaired in the respective single cKO mice. The frequencies of T_{reg} cells ($Foxp3^+CD25^+$), and their $Foxp3^+CD25^-$ or $Foxp3^-CD25^+$ precursors [365, 366], were comparable to their control counterparts (Fig. S2D, Fig. S3D), indicating that the *Zfp36* or *Zfp36l1* deficient mTEC microenvironment appeared to retain the capacity to promote normal T_{reg} cell development. We also did not find differences in the abundance of γδ T cells between cKO and control mice (Fig. S2E, Fig. S3E). Hence, despite having a disturbed TEC differentiation, *Zfp36* and *Zfp36l1* cKO thymus appear to promote a normal program of T cell development.

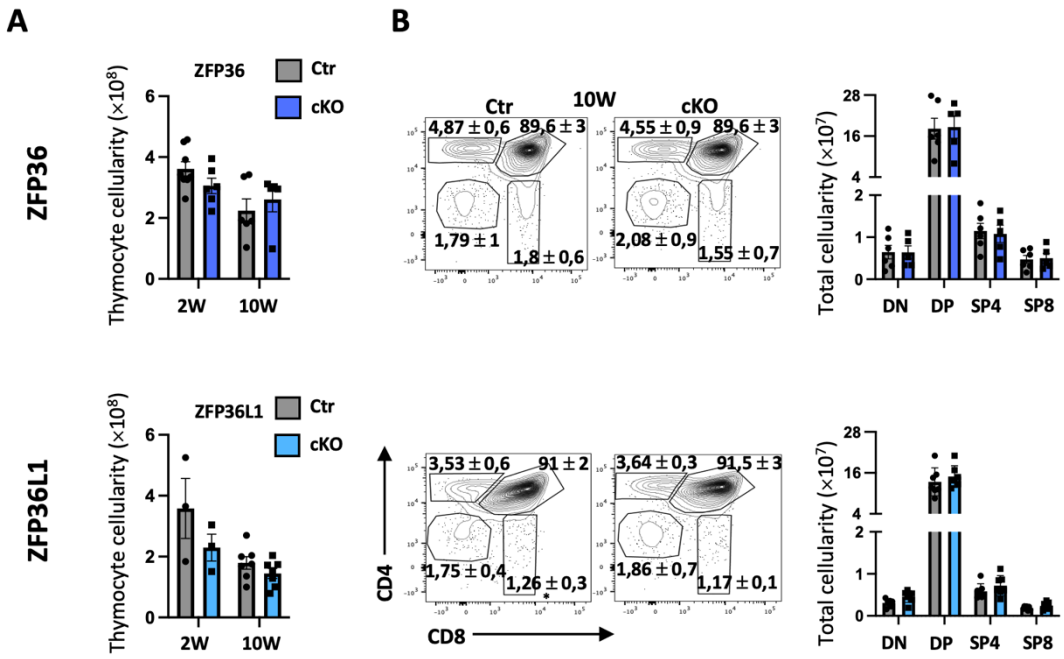


Figure 2: Analysis of thymopoiesis in *Zfp36* and *Zfp36L1* cKO mice. Total thymocyte cellularity ($CD45^+$) and T cell development were analysed in *Zfp36* cKO mice (upper row) and *Zfp36l1* cKO mice (lower row) relatively to respective control litter mates. A) Thymocyte cellularity was determined in 2- and 10-week-old mice. B) Dot plots show representative analysis of CD4/CD8 expression in total thymocytes: $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), $CD4^+CD8^-$ single positive 4 (SP4) and $CD4^-CD8^+$ single positive 8 (SP8). Graphs in A and B represent the average cellularity ± SEM of the indicated populations (2 independent experiments, n=3 to 7 per group). ***P<0.001, **P<0.01, *P<0.05.

Combined deletion of *Zfp36* and *Zfp36l1* in thymic epithelium disturbs the cellularity and composition of mTEC in the adult thymus

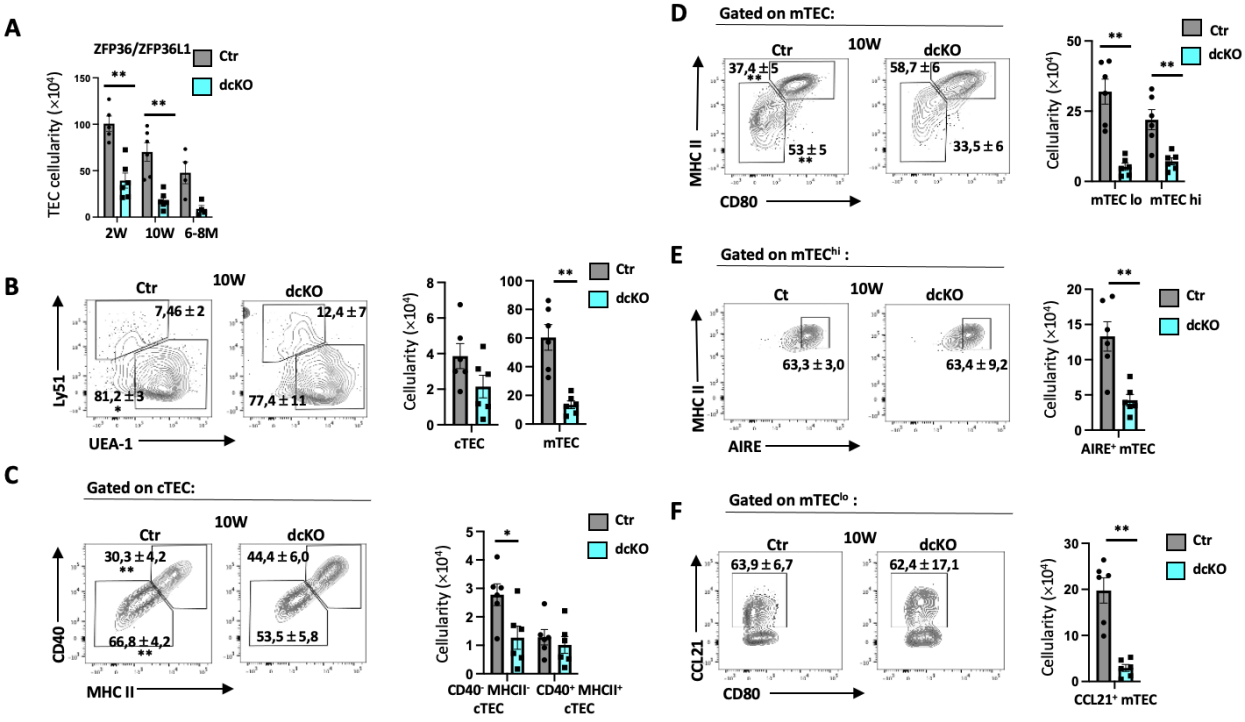


Figure 3: Combined deletion of *Zfp36* and *Zfp36l1* affects the size and composition of mTEC compartment in the adult thymus. A) The composition of TECs ($CD45^{-}EpCAM^{+}$) was analysed in *Zfp36* and *Zfp36l1* double conditional knockout (dcKO) (*Foxn1:Cre-Zfp36*$^{fl/fl}$*Zfp36l1*$^{fl/fl}$*)* and control (*Zfp36*$^{fl/fl}$*Zfp36l1*$^{fl/fl}$*)* mice at 2 weeks, 10 weeks and 6 to 8 months of age. B) The composition of cTECs and mTECs was determined in 10-week-old dcKO mice. Dot plots show representative Ly51/UEA-1 staining in TECs and gates to define cTECs ($Ly51^{+}$) and mTECs ($UEA\text{-}1^{+}$) subsets. C) Dot plots show representative analysis of cTECs for the expression of MHC II and CD40 at 10 weeks of age; D) Dot plots show representative analysis of $mTEC^{lo}$ ($MHII^{lo}CD80^{lo}$) and $mTEC^{hi}$ ($MHII^{hi}CD80^{hi}$) subsets based on the expression of MHC II and CD80 at 10 weeks. E) Analysis of AIRE-expressing cells gated in $mTEC^{hi}$ ($MHII^{hi}CD80^{hi}$) subset. F) Analysis of CCL21-expressing cells gated in $mTEC^{lo}$ ($MHII^{lo}CD80^{lo}$) subset. Graphs in A, B, C, D, E and F represent the average cellularity ± SEM of each respective TEC populations (3 independent experiments n=5 to 6 per group). ***P<0.001, **P<0.01, *P<0.05.

To address the redundant role of ZFP36 family members in TEC biology, we generated *Zfp36* and *Zfp36l1* dcKO mice. We found a profound reduction in the numbers of TECs in the thymus of dcKO mice at 2 and 10 weeks of age (Fig. 3A), without majorly affecting the proportions of c/mTECs (Fig. 3B, Fig. S4A). Concerning the maturation of cTECs, we analyzed the expression of CD40 and MHC-II expression and observed an increase in the ratio of mature ($CD40^{hi}$MHC-II^{hi}) vs immature ($CD40^{lo}$ MHC-II^{lo}) cortical subsets in dcKO mice, mainly due to a decrease in the numbers of immature cTECs (Fig.

3C). Relatively to mTECs, we found an accumulation of $mTEC^{hi}$ cells in the dcKO adult thymus, suggesting possible alterations in mTEC differentiation (Fig. 3D). Moreover, the levels of MHC II were increased in *Zfp36*:*Zfp36l1* double deficient cTECs and mTECs (Fig. S5A). To evaluate MHC II processing, we used 15G4 antibody that detects $I\text{-}A^b$ occupied by the CD74/Ii degradation intermediates small leupeptin induced protein or CLIP[367]. Although not statistically significant, mTECs from dcKO mice exhibited a mild decrease in 15G4 staining (Fig. S5). Further analysis of specific mature mTECs subsets revealed that the frequencies of $AIRE^+$ and $CCL21^+$ within $mTEC^{hi}$ and $mTEC^{lo}$, respectively, were unaltered in mutant mice. Still, the numbers of $AIRE^+$ and $CCL21^+$ mTECs were significantly reduced in the thymus of dcKO mice, with the decline being more prominent in $CCL21^+$ mTECs (Fig. 3E,F). Although mTEC differentiation was still operational, the combined deletion of *Zfp36* and *Zfp36l1* appeared to differentially affect the diversification of particular functional mTEC populations.

Double conditional KO mice present a premature thymic atrophy.

We determined how the dual deficiency in *Zfp36* and *Zfp36l1* in TECs affected T cell generation. In contrast with single cKO mice, the thymus of dcKO mice displayed a marked reduction in total thymocyte cellularity at 2 weeks of age, which become more pronounced in adult and aged mice (Fig. 4A,B). The numbers of the main thymocyte populations decreased (Fig. 4C), suggesting a premature decline in thymopoietic activity. Although the proportion of the most immature DN1 stage (DN1) cells were mildly reduced, the frequencies of DN2-4 populations (Fig. 4D), pre- and post-positively selected subsets (Fig. 4E), mature SP4 cells (Fig. 4F), T_{reg} cells (Fig. 4G) and $\gamma\delta$ T cells (Fig. 4H) were comparable between control and double mutant thymus, with the exception of the proportion of immature/mature SP8 thymocytes (Fig. 4F). Except a possible defect in SP8 maturation, our results suggest that dual deficiency in *Zfp36* and *Zfp36l1* in TECs reduced the overall capacity of the thymus to orchestrate the development and selection of T cells.

To determine whether the reduction in thymopoietic activity affected the peripheral T cell compartment, we analysed the splenic T cells of adult dcKO mice, and found that the number of CD4 and CD8 T cells was not statistically different to the control group. Yet, a moderate decrease in the frequency of CD8 T cells was detected in dcKO mice (Fig. 5A). The proportions of naïve $CD62L^+CD44^-$ and effector/memory ($CD62L^-CD44^+$) CD4 T cells were slightly decreased in dcKO, but with no significant alterations in their absolute numbers (Fig. 5B). The cellularity of $\gamma\delta$ T cells and T_{reg} cells remained unaltered in the spleen of dcKO

mice (Fig. 5C,D).

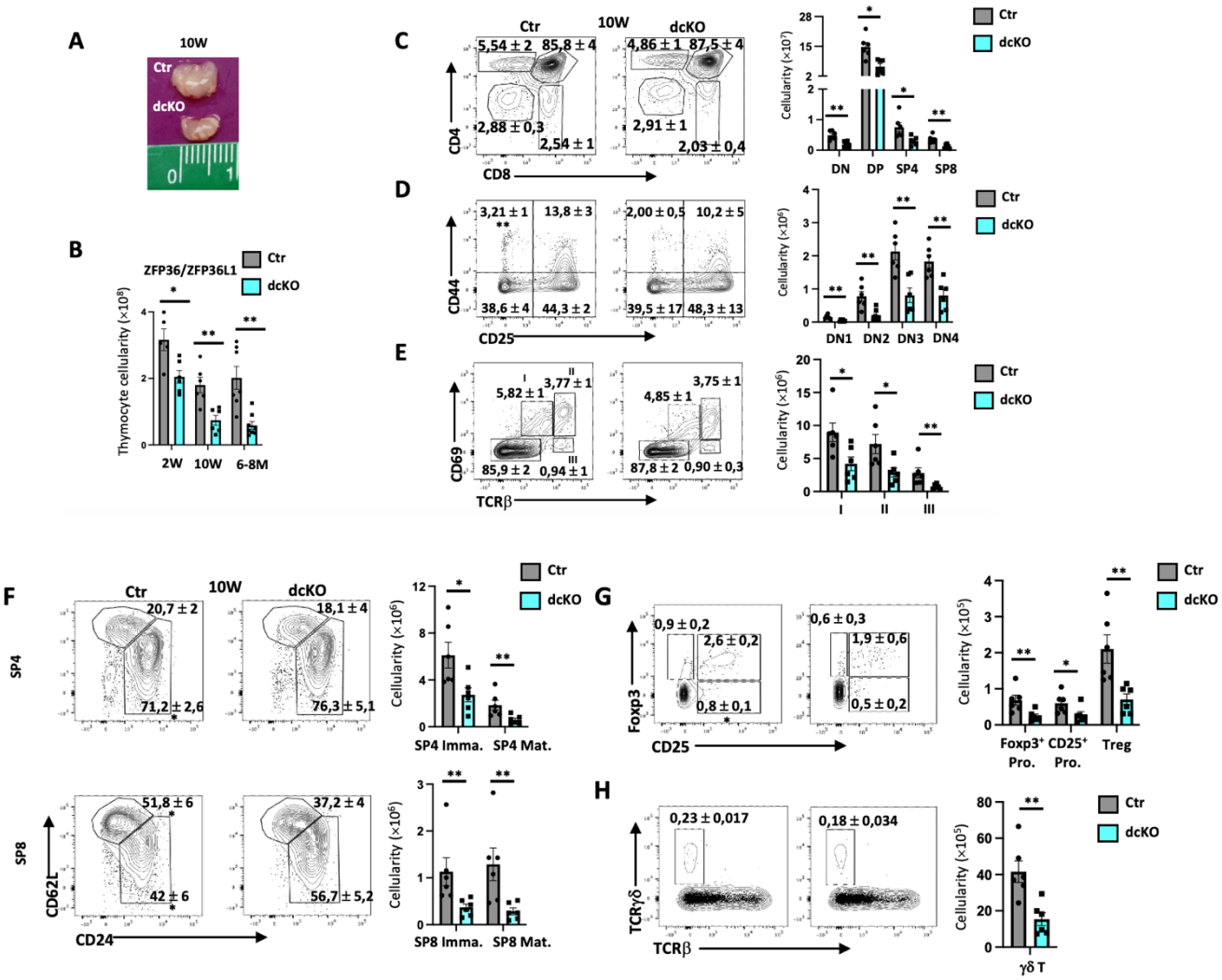


Figure 4: T cell generation is compromised in *Zfp36*/*Zfp36l1* dcKO mice. A) Representative picture of thymi isolated from 10-week-old dcKO (*Foxn1*:Cre-*Zfp36*$^{fl/fl}$/*Zfp36l1*$^{fl/fl}$, Bottom) and control (Ctr, *Zfp36*$^{fl/fl}$/*Zfp36l1*$^{fl/fl}$, Top) littermates. B) Quantification of total thymocyte cellularity in 2-weeks, 10-weeks and 6 to 8-month-old mice. C) Dot plots show representative analysis of CD4/CD8 expression in total thymocytes: CD4$^-$CD8$^-$ double negative (DN), CD4$^+$CD8$^+$ double positive (DP), CD4$^+$CD8$^-$ single positive 4 (SP4) and CD4$^-$CD8$^+$ single positive 8 (SP8). D) Analysis of CD44 and CD25 expression in DN cells gated on CD45$^+$Lin$^-$CD4$^-$CD8$^-$ thymocytes where Lin represents GR, NK1.1, CD11b, CD11c, CD19, Ter119 and γδ TCR. E) Analysis of CD69 and TCRβ expression in total thymocytes. F) SP4 (CD8$^-$CD4$^+$TCRβ$^+$) and SP8 (CD8$^-$CD4$^+$TCRβ$^+$) cells were analysed for the expression of CD24 and CD62L at the indicated time points. G) SP4 (CD8$^-$CD4$^+$TCRβ$^+$) were analysed for the expression of CD25 and FOXP3 and subdivided in FOXP3$^+$CD25$^-$ and FOXP3$^-$CD25$^+$ T regulatory cell precursors and FOXP3$^+$CD25$^+$ T regulatory cells. H) Quantification of thymic γδ T cells. Graphs in B-H represent the average cellularity ± SEM of the respective thymocyte subset (3 independent experiments, n=6 per group).***P<0.001, **P<0.01, *P<0.05.

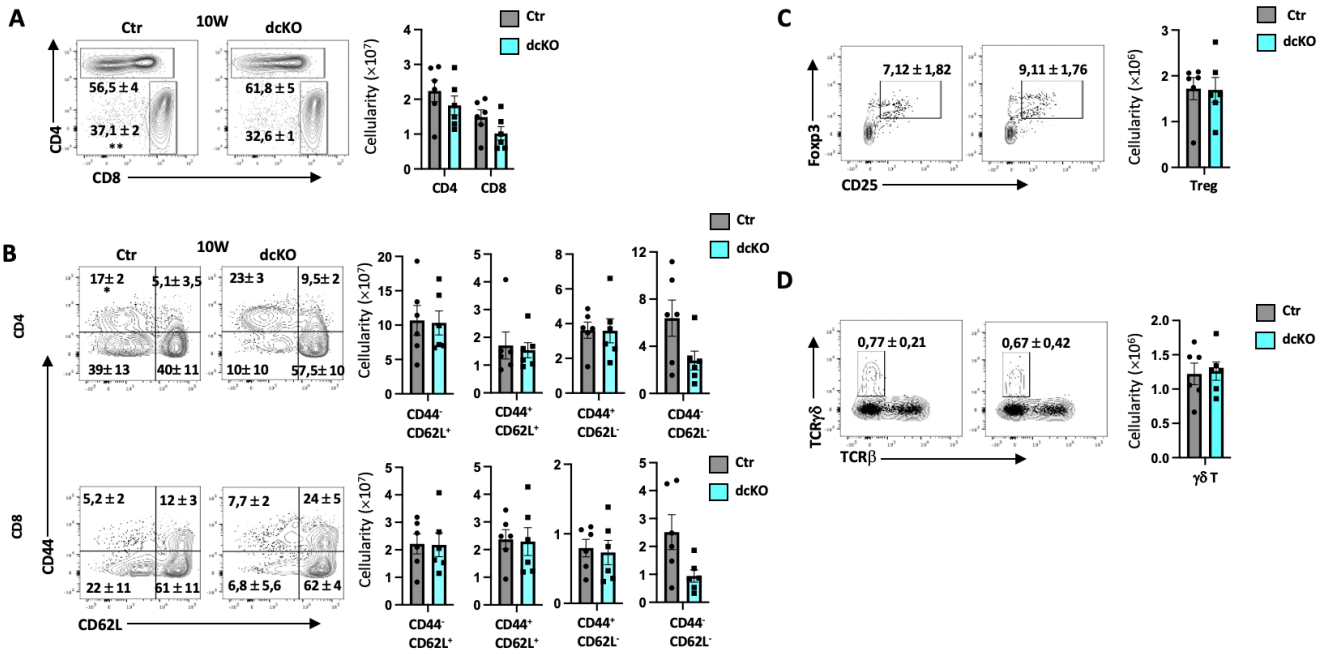


Figure 5: Analysis of the peripheral T cell compartment of *Zfp36*/*Zfp36l1* dcKO mice. A) Dot plots show representative analysis of $CD4^+$ and $CD8^+$ splenic T ($TCR\beta^+$) cells in 10-week-old control and dcKO mice. B) Splenic $CD4^+$ T cells ($CD4^+TCRC\beta^+$, top) and $CD8^+$ T cells ($CD8^+TCRC\beta^+$, bottom) were analysed for the expression of CD44 and CD62L C) Splenic $CD4^+$ T cells ($CD4^+TCRC\beta^+$) were analysed for the expression of CD25 and FOXP3; D) Quantification of splenic γδ T cells. Graphs in A-D represent the average numbers ± SEM of each T cell population (3 independent experiments, n= 6 per group). ***P<0.001, **P<0.01, *P<0.05.

We investigated whether thymic and peripheral phenotypes were associated to a dysregulation in peripheral T cell tolerance, analyzing 6- to 8-months-old double mutant mice for the presence of autoantibodies and cell infiltrates. First, several organs from a *Rag*$^{-/-}$ mice were sequentially probed with sera from control and dcKO aged mice, and anti-mouse IgG, being scored as low, intermediated or high following fluorescence microscopy analysis. Although not penetrant in all animals, the proportion of individuals with intermediate and high detection of autoantibodies was higher in dcKO mice (Fig. 6 A). Moreover, we analysed antinuclear antibodies (ANA), predicting the release of antigens from damaged cells, such as nuclear antigens, in the context of autoimmunity, that were otherwise hidden from immune cell recognition**.** Yet, the level of ANA was comparable between control and dcKO mice (Fig. 6B). Moreover, the determination of lymphocytic infiltrates in different tissues showed a slight increase in the proportion of dcKO animals with infiltrates in the liver and pancreas (Fig. 6C). Lastly, the composition of naïve, effector/memory subsets in the spleen of aged mice did not reveal any major alterations (Fig. S6 A, B, C). Hence, these results suggest that dcKO mice may have a slight increase susceptibility to develop autoimmune manifestations.

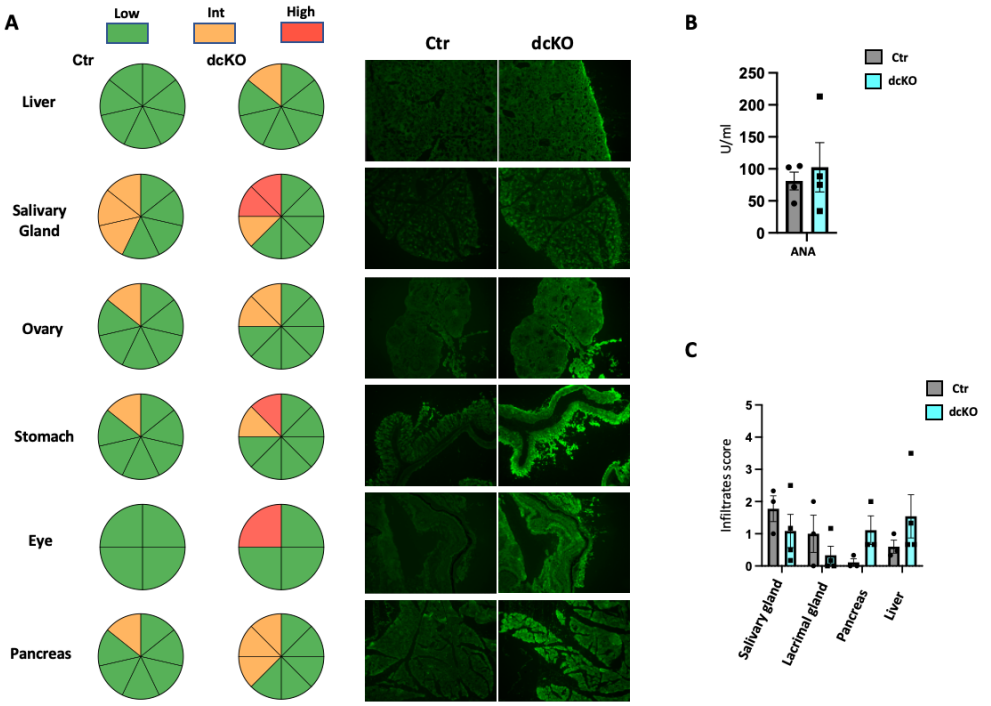


Figure 6: Analysis of autoimmune manifestations in *Zfp36*/*Zfp36l1* dcKO mice. A) Analysis of autoantibody production in *Zfp36*/*Zfp36l1* dcKO mice. Representative images from sections of the indicated organs of *Rag2* KO mice probed with sera from 6 to 8-months-old control (Ctr) and dcKO mice. Samples were subdivided in low, intermediate (Int) and high based on the intensity of signal measure by fluorescence microscopy (data from two independent experiments, n= 4-8 per group); Pie graphs represent the incidence of auto-antibodies detection in Ctr and dcKO mice. B) Anti-nuclear antibodies (ANA) detection in dcKO mice. Graph B represents the average concentration of ANA ± SEM in units (U)/mL of Ctr and dcKO mice, where U represent an arbitrary unit (1 independent experiment n= 4 per group); C) Quantification of lymphocytic infiltrates in the indicated organs harvested from 6 to 8-months-old Ctr and dcKO mice. Graph C represents the average score ± SEM of inflammatory infiltrates (1 independent experiment n=4 per group).

Discussion

It is known that several post-transcriptional modifications shape genetic program of TEC, regulating their lineage differentiation [249-252]. In this study, we investigated the biological role of the ZFP36 RBPs in TEC homeostasis and function. Our findings demonstrated that the combined conditional deletion of *Zfp36* and *Zfp36l1* disrupted the number of TECs, predominantly mTECs. A previous study showed that the growth and maintenance of the thymic epithelium depends on the size of TEC progenitor pool [368]. Thus, the reduction of immature cTECs (CD40 ^{lo}MHC-II ^{lo}) in dcKO mice, which presumably contains the TEC ^{lo} bi-potent progenitors reported to exist in the adult thymus [159], might contribute to the conspicuous reduction in TEC cellularity. To decipher the molecular basis underlying the thymic phenotype, future analysis should focus on examining the impact of *Zfp36* and *Zfp36l1* deficiency in the transcriptional program of mutant TECs by scRNAseq.

This approach will allow us to identify at the single-cell level clusters that are dependent on *Zfp36* and *Zfp36L1* expression and potential candidate targets regulated by these RBPs. It has been previously shown that ZFP36L1 and ZFP36L2 directly downregulate NOTCH1 receptor in developing thymocytes upon beta selection [360]. As mTEC differentiation requires the downregulation of Notch signaling [208, 209], this pathway emerges as an appealing target affected in TECs from dcKO mice. This possibility is in line with our results showing that mTEC lineage was the most affected by the deletion of ZFP36 proteins. Future studies combining transcriptomic data and CLIP or TRIBE assays should validate the identity of ZFP36-binding targets.

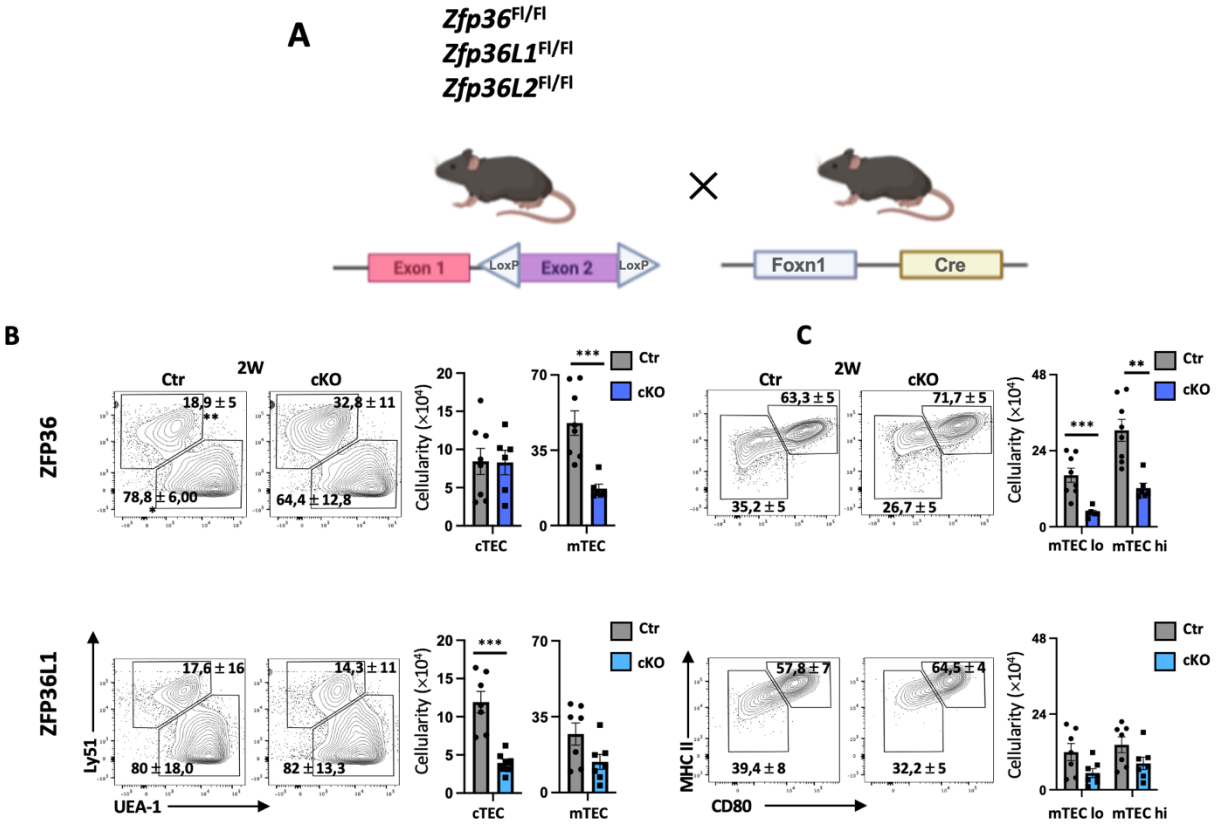
Considering the high homology between the tandem zinc finger (TZF) domains of ZFP36 family members [355], it was curious to observe that the phenotype of the two single cKO mice concerning c/mTEC differentiation was distinct. Still, these RBPs display subtle differences in this domain that perhaps might be sufficient to slightly shift their affinity for different transcripts [355]. It is also important to take into consideration that differences outside TZF domain might also lead to the recruitment of different proteins complexes, contributing for the differential role of ZFP36 proteins [355]. Lastly, different members might be expressed in different TEC subsets at distinct developmental stages. This possibility is supported by the observation that ZFP36 proteins are differentially expressed by the distinct TEC subclusters identified in recent scRNAseq-based studies [78, 153]. All of these hypotheses are not mutually exclusive, and together can contribute to explain the phenotypes observed in single and dual cKO mice.

Our results also demonstrate that the combined deletion of *Zfp36* and *Zfp36l1* provoked a premature thymic atrophy. This reduction in the capacity of the thymus to generate T cells were not found in single *Zfp36* and *Zfp36l1* cKO mice, despite the similar decrease in the size of TEC compartment compared to the one found in dcKO mice. The reasons for this difference are unclear but might suggest that while ZFP36 and ZFP36L1 have distinct and yet cooperative roles in the regulation of TEC developmental program, these two proteins might have redundant functions concerning the regulation of key factors involved in T cell development. Given the possible increased propensity to develop autoimmune manifestations of dcKO mice, one can consider TRAs as potential targets of ZFP36 RBPs. Future population and scRNAseq might allow us to examine whether the expression of the TRA repertoire is altered in mutant mTECs. Moreover, future analysis should evaluate whether the reduction in thymopoietic activity may be associated with alterations in antigen processing and presentation in dcKO TECs. In this regard, the increased levels of MHC class II in TECs from dcKO mice, together with the reported role of ZFP36 in the downregulation of MHC class I molecules [369], might indicate a more stringent thymic selection in mutant thymus. Our initial analysis of MHC II processing based

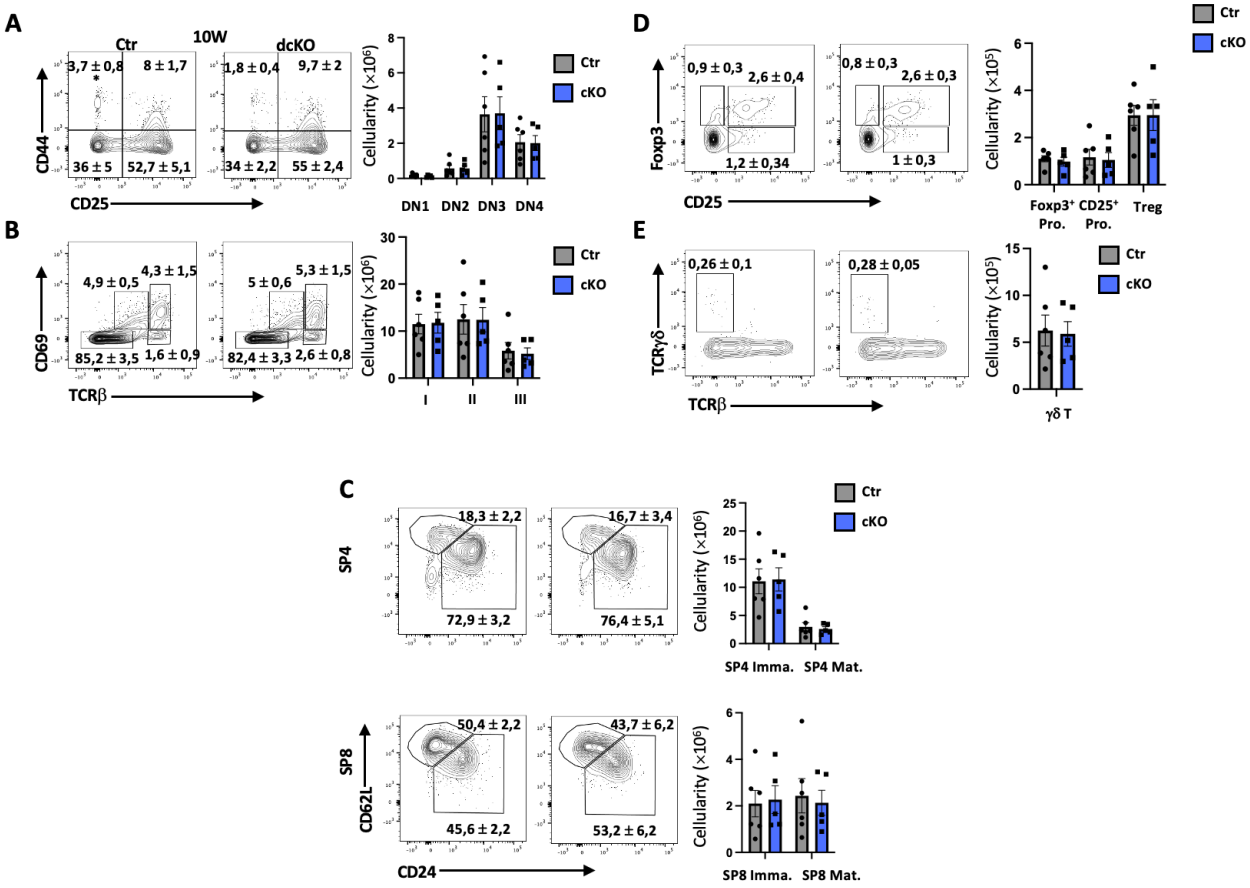
on the analysis 15G4 may suggest increase availability of MHC: self-peptide complexes, but further studies are needed to confirm this possibility. If true, these results could implicate a higher proficiency for antigen presentation by mutant TECs, which although appearing paradoxical with the increase in autoimmunity in dcKO mice, might simply mean that repertoire of peptides presented by TECs is altered. Interestingly, despite the frequency of SP4 and SP8 appeared normal, SP8 mature thymocytes were reduced in adult thymus, consistent with the slight reduction in the frequency of $CD8^+$ T cells in the spleen. Future TCR repertoire analysis is required to address qualitative and quantitative differences in TCR diversity account for changes in thymic selection. This hypothesis can be tested in the future using in parallel the analysis of mutant mice on a TCR transgenic background. This approach may overcome the limitation of the polyclonal setting, wherein changes in some TCR specificities might be diluted by the huge diversity of TCRs generated in the thymus.

The immunological consequences of the defective T cell generation in dcKO mice should also be more broadly explored. In this context, although we have analysed the production of thymic $\gamma\delta$ T cells, future studies can determine if the differentiation of other innate-like cells (e.g. NKT and MAIT) is altered in these mutants. Additionally, the colonization of different tissues (e.g., skin and gut) by specialized T cell subsets can be evaluated, in order to clarify the implications of the reduced thymic activity in peripheral T cell homing to their anatomic niches [370-372]. The immune competence of T cells generated by dcKO thymus should also be assessed either *in vitro* (*e.g.,* PMA and ionomycin stimulation) or in a more physiological context, for example using infection mice models. Moreover, considering the fact that thymic involution has been associated with the increased susceptibility to infections in the elderly [45] and the dcKO mice show a premature thymic atrophy, it would be interesting to follow the age-associated decline in the immune response, evaluating the ability to resolve an infection at different ages. Therefore, our results paved way for future studies aiming to decipher the exact biological role of ZFP36 family members, and contributing to elucidate the complex network of transcriptional regulators of TEC development and function.

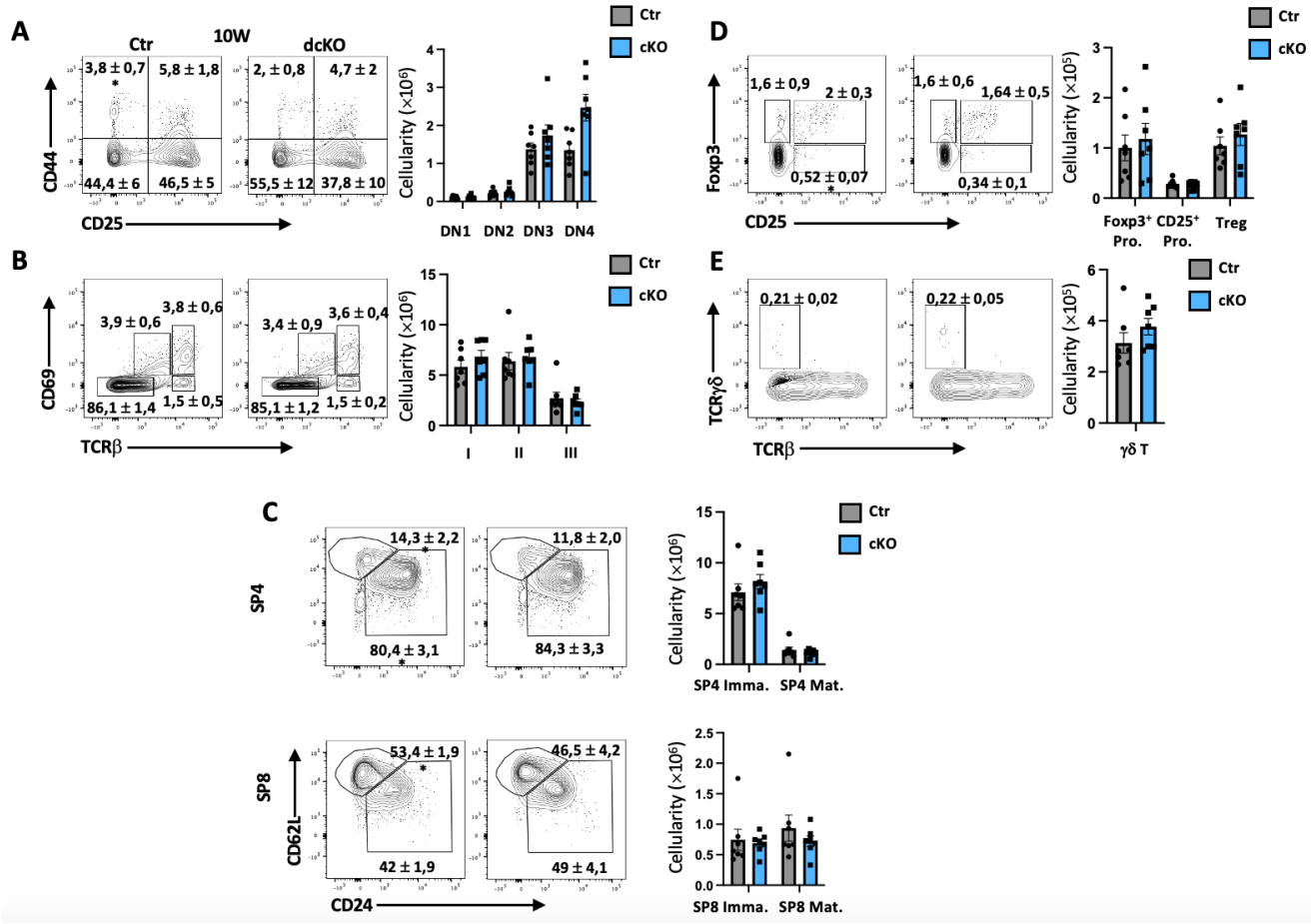
Supplementary information



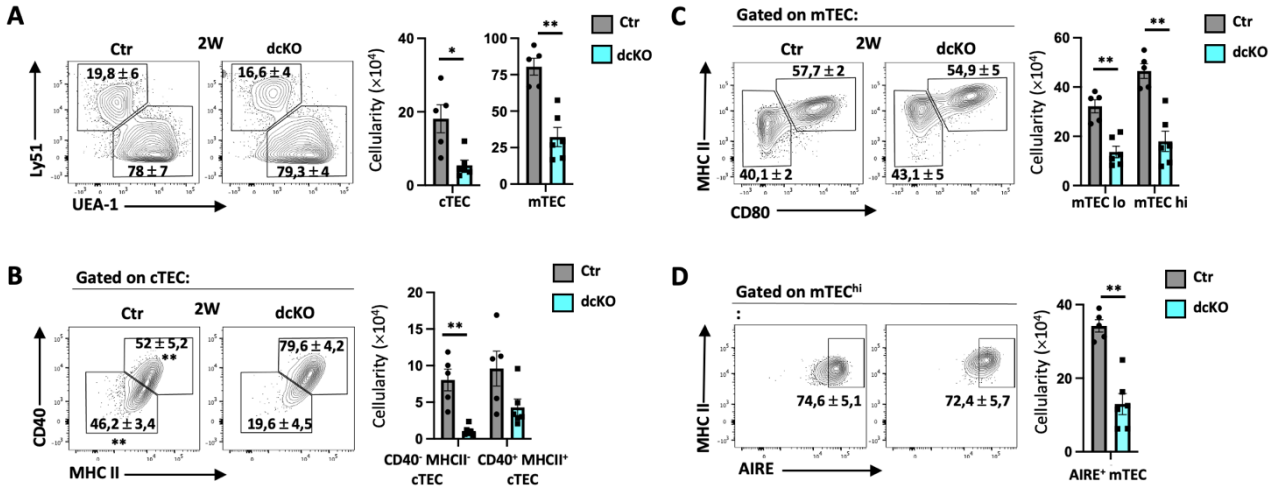
Supplementary figure 1: Impact of *Zfp36* or *Zfp36l1* deficiency in TEC differentiation in the early postnatal period. The composition of TECs was analysed in *Zfp36* (*Foxn1*Cre:*Zfp36*$^{fl/fl}$) cKO mice (upper row) and *Zfp36l1* (*Foxn1*Cre:*Zfp36l1*$^{fl/fl}$) cKO mice (lower row) relatively to respective control (*Zfp36*$^{fl/fl}$ and *Zfp36l1*$^{fl/fl}$) littermates A) Schematic representation of the generation of cKO mice for ZFP36 proteins. B) The composition cTECs and mTECs was determined in 2-week-old mice. Dot plots show representative Ly51/UEA-1 staining in TECs and gates to define cTEC (Ly51^{+}) and mTEC (UEA-1^{+}) subsets. C) Dot plots show representative analysis of mTEC^{lo} (MHII^{lo}CD80^{lo}) and mTEC^{hi} (MHII^{hi}CD80^{hi}) subsets based on the expression of MHC II and CD80 in 2-week-old thymus. Graphs in B and C represent the average cellularity ± SEM of each respective TEC populations (2 independent experiments, n=6 to 7 per group). ***P<0.001, **P<0.01, *P<0.05.



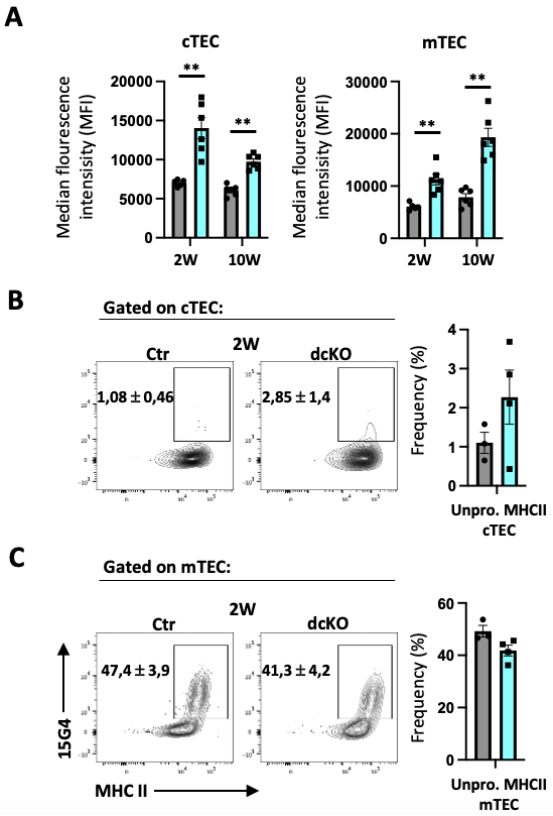
Supplementary figure 2: Analysis of T cell development in adult *Zfp36* cKO mice. A) Analysis of CD44 and CD25 expression in DN cells gated on $CD45^+Lin^-CD4^-CD8^-$ thymocytes where Lin represents GR, NK1.1, CD11b, CD11c, CD19, Ter119 and γδ TCR. B) Analysis of CD69 and TCRβ expression on total thymocytes. C) SP4 ($CD8^-CD4^+TCR\beta^+$) and SP8 ($CD8^-CD4^+TCR\beta^+$) cells were analysed for the expression of CD24 and CD62L at the indicated time point D) SP4 ($CD8^-CD4^+TCR\beta^+$) cells were analysed for the expression of CD25 and FOXP3 and subdivided in $FOXP3^+CD25^-$ and $FOXP3^-CD25^+$ T_{reg} cell precursors and $FOXP3^+CD25^+$ T_{reg} cells; E) Quantification of thymic γδ T cells. Graphs in A-E represent the average cellularity ± SEM of the respective thymocyte subset (2 independent experiments, n=5 to 6 per group). ***P<0.001, **P<0.01, *P<0.05.



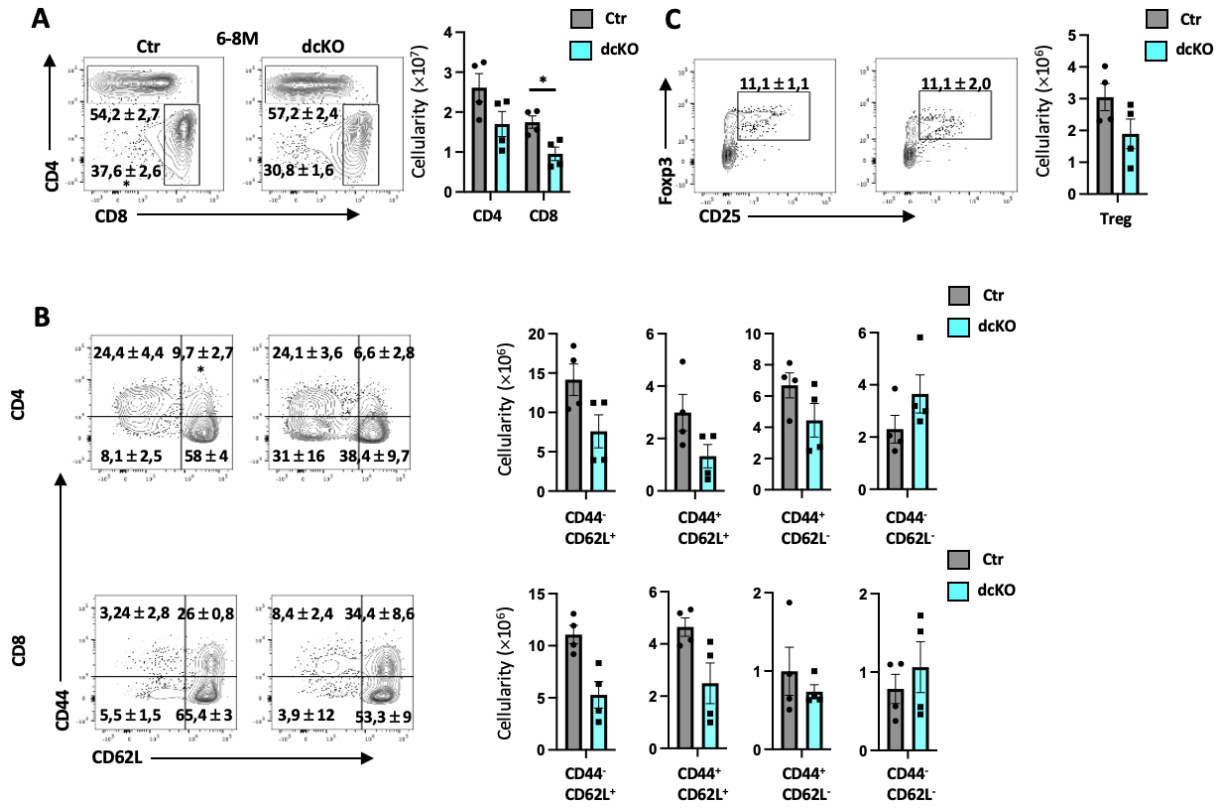
Supplementary figure 3: Analysis of T cell development in adult *Zfp36l1* cKO mice. A) Analysis of CD44 and CD25 expression in DN cells gated on $CD45^+Lin^-CD4^-CD8^-$ thymocytes where Lin represents GR, NK1.1, CD11b, CD11c, CD19, Ter119 and γδ TCR. B) Analysis of CD69 and TCRβ expression on total thymocytes. C) SP4 ($CD8^-CD4^+TCR\beta^+$) and SP8 ($CD8^-CD4^+TCR\beta^+$) cells were analysed for the expression of CD24 and CD62L at the indicated time points. D) SP4 ($CD8^-CD4^+TCR\beta^+$) cells were analysed for the expression of CD25 and FOXP3 and subdivided in $FOXP3^+CD25^-$ and $FOXP3^-CD25^+$ T_{reg} cell precursors and $FOXP3^+CD25^+$ T_{reg} cells; E) Quantification of thymic γδ T cells. Graphs in A-E represent the average cellularity ± SEM of the respective thymocyte subset (2 independent experiments, n=7 per group). ***P<0.001, **P<0.01, *P<0.05.



Supplementary figure 4: *Zfp36*/*Zfp36l1* dcKO mice display an overall reduction in TEC cellularity in the early postnatal period. A) The composition of cTECs and mTECs was determined in the 2-week-old control (Ctr) and dcKO mice. Dot plots show representative Ly51/UEA-1 staining in TECs and gates to define cTEC (Ly51^{+}) and mTEC (UEA-1^{+}) subsets. B) Dot plots show representative analysis of cTECs for the expression of MHC II and CD40 at 2 weeks. C) Dot plots show representative analysis of mTEC^{lo} (MHII^{lo}CD80^{lo}) and mTEC^{hi} (MHII^{hi}CD80^{hi}) subsets based on the expression of MHC II and CD80 at 2 weeks. D) Analysis of AIRE-expressing cells gated in mTEC^{hi} subset. Graphs in A, B, C and D represent the average cellularity ± SEM of each respective TEC populations (2 independent experiments n=5 to 6 per group). ***P<0.001, **P<0.01, *P<0.05.



Supplementary figure 5: The abundance of MHCII-complexes is altered in dcKO mice. A) Quantification of MHCII MFI in cTECs ($CD45^-Epcam^+Ly51^+$) and mTECs ($CD45^-Epcam^+UEA\text{-}1^+$) of control (Ctr) and dcKO mice at the indicated time points (2 and 10 weeks). Graphs A represent the average MFI ± SEM of MHCII in cTECs and mTECs (2 independent experiments n=6 per group). Dot plots show representative analysis of 15G4 staining in cTECs B) and mTECs C). Graphs B and C represent the average frequency ± SEM of $15G4^+$ cells in cTEC and mTEC compartment, respectively (1 experiment n= 3 to 4 per group). ***P<0.001, **P<0.01, *P<0.05.



Supplementary figure 6: Analysis of periphery T cell homeostasis in aged dcKO mice. A) Dot plots show representative analysis of $CD4^+$ and $CD8^+$ splenic T ($TCR\beta^+$) cells in 6 to 8-months-old control (Ctr) and dcKO mice. B) Splenic $CD4^+$ T cells ($CD4^+TCRC\beta^+$) and $CD8^+$ T cells ($CD8^+TCRC\beta^+$) were analysed for the expression of CD44 and CD62L. C) Splenic $CD4^+$ T cells ($CD4^+TCRC\beta^+$) were analysed for the expression of CD25 and FOXP3. Graphs A, B and C represent the average numbers ± SEM of each T cell population (2 independent experiments n= 4 per group). ***P<0.001, **P<0.01, *P<0.05.

Chapter IV

Identification of fibroblast progenitors in the developing thymus

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Abstract

The thymus stroma constitutes a fundamental microenvironment for T cell generation. Despite the chief contribution of thymic epithelial cells, recent studies emphasize the regulatory role of mesenchymal cells in thymic function. Mesenchymal progenitors are suggested to exist in the postnatal thymus, nonetheless our understanding of their nature and the mechanism controlling their homeostasis in vivo remain elusive. We resolved two new thymic fibroblast subsets with distinct developmental features. While CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{-} cells prevailed in the embryonic thymus and declined thereafter, CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{+} cells emerged in the late embryonic period and predominated in the postnatal life. The fibroblastic-associated transcriptional program was upregulated in CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{+} cells, suggesting that they represent a mature subset. Lineage analysis showed that CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{+} maintained their phenotype in thymic organoids. Strikingly, CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{-} generated CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{+}, inferring that this subset harboured progenitor cell activity. Moreover, the abundance of CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{+} fibroblasts was gradually reduced in Rag2-/- and Rag2-/-Il2rg-/- thymi, indicating that fibroblast maturation depends on thymic crosstalk. Our findings identify CD140$\alpha\beta^{+}$GP38^{+}SCA-1^{-} as a source of fibroblast progenitors and define SCA-1 as a marker to map a developmental stage in thymic fibroblast differentiation.

Introduction

The thymic microenvironment offers a unique inductive site for the generation of functionally diverse and self-tolerant T cells. The thymic stroma is formed by cells of non-hematopoietic origin, such as thymic epithelial cells (TECs), endothelial cells and thymic mesenchymal cells (TMCs), and cells of hematopoietic origin, including dendritic cells and monocytes/macrophages [256]. The development of this heterogeneous microenvironment starts in the embryo and continues during postnatal life, involving the participation of cells from all three embryonic germ layers: endoderm-derived epithelium, neuroectoderm-derived neural-crest (NC) mesenchyme and mesoderm-derived hematopoietic and endothelial cells [144]. Given the non-redundant role of TECs in T cell development, there has been considerable interest in studying the mechanisms that control TEC differentiation and function. Yet, several studies underscore the contribution of other non-epithelial stromal cells in shaping TEC and T cell differentiation [373].

In particular, TMCs, which includes fibroblasts, vascular-supporting pericytes and smooth muscle cells, exert a pleiotropic role in thymus biology [373]. At an early stage of

thymus organogenesis, NC-derived mesenchymal cells surround the thymic primordia and provide Fibroblast Growth Factor 7 (FGF7), FGF10, epidermal growth factor (EGF) and insulin-like growth factor (IGF), which contribute to the growth of the TEC microenvironment [265, 266]. Interestingly, FGF7/10-producing cells also express retinoic acid, which suppresses the proliferation of cortical TECs [263, 269]. Thus, TMCs have the functional capacity to positively and negatively control the size of the TEC compartment. Thymic fibroblasts also produce a range of extracellular matrix (ECM) components, which can capture and present critical thymopoietic factors (e.g. IL-7 and CCL21) to the developing T cells [272, 374]. Moreover, vascular-associated pericytes and smooth muscle cells surrounding the endothelium regulate thymic vasculature and T cell egress[255, 271]. Particularly, TMCs create sphingosine-1-phosphate (S1P) gradients that promote the egress of mature T cells from the thymus [271]. More recently, medullary fibroblasts have been implicated in T cell tolerance [225]. Despite the aforementioned functional diversity, distinct TMC subsets share a precursor-product relationship with NC cells [253-255]. Still, our understanding of the mechanisms that control the differentiation and the turnover of mature TMCs remains elusive. Moreover, although thymic mesenchymal progenitors are considered to exist in the adult thymus [255], their nature and functional competence remain poorly characterized in vivo.

Herein, we resolved a novel population of thymic fibroblast progenitors and uncovered a novel checkpoint in mesenchymal differentiation that depends on thymic crosstalk. Our findings offer a novel roadmap to monitor TMC homeostasis in aging and regeneration.

Material and methods

Mice

WT, $Rag2^{-/-}$, $Rag2^{-/-}Il2rg^{-/-}$ and Actin-RFP mice [77, 151], were all bred on a C57BL/6 background and housed under specific pathogen-free conditions at I3S' animal facility. Experiments were performed under the European guidelines for animal experimentation.

Isolation of thymic stromal cells

Thymic stromal cells were isolated using a modified protocol previously described to obtain TECs [320]. Briefly, the thymus was cut into small pieces and subjected to a gentle mechanical dissociation to liberate thymocytes. Thymic fragments were digested for 30 minutes at 37º C with agitation in PBS containing 20mg/ml of collagenase D (Roche) and

passed through 100-μm filter to remove debris. Further stromal cell enrichment was carried out by incubation with anti-CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (Miltenyi) according to manufacturer's instructions.

Flow cytometry

TMCs were isolated as described [320]. Cell suspensions were stained with the following antibodies: PerCP-Cy5-conjugated anti-CD45.2 (clone 104, Cat#: 45-0454-82), PE-conjugated anti-Ly51 (clone 6C3, Cat#: 12-5891-82), Alexa eFluor 647-conjugated anti-EpCAM (clone G8.8, Cat#: 14-5791-81), APC-conjugated anti-Ter-119 (clone TER-119, Cat#: 17-5921-82), all from eBioscience; BV421-conjugated anti-EpCAM (clone G8.8, Cat#: 118225), BV786-conjugated anti-Sca1 (clone D7, Cat#: 108139), Alexa 488-conjugated anti-Sca1 (clone D7, Cat#: 108111), PE-Cy7-conjugated anti-GP38 (clone 8.1.1, Cat#: 127411), APC-conjugated anti-DPP4 (clone H194-112, Cat#: 137807), BV605-conjugated anti-CD140α (clone APA5, Cat#: 135916), all from Biolegend; Biotinylated anti-CD140β (clone APB5, Cat#: 136009, Biolegend) was revealed with BV711-conjugated (Cat#: 405241, Biolegend) or PE-Cy7-conjugated streptavidin (Cat#: SA1012, eBioscience). Intracellular staining with eFlour660-conjugated anti-αSMA (clone 1A4, Cat#: 50-9760-82, eBioscience) was performed following cell fixation and permeabilization using the Foxp3/Transcription factor staining buffer set (eBioscience) according to manufacturer's instructions. Flow cytometry analyses were performed on a LSRFortessa and cells sorted on a FACS ARIA II (both from BD Bioscience) with purities above 95%. Data were analysed on FlowJo software (Tree Star Inc).

RNA sequencing

Total RNA library preparation and high-throughput sequencing of sorted postnatal (P3-5) TF^{A-B} and MC subsets were performed at the EMBL Genomics Core facility (Germany). Nine sequencing libraries, three for TF^{A}, three for TF^{B} and three for MC were prepared using NEB Next RNA ultra protocol (#E7530 NEB). Obtained libraries were quantified fluorimetrically, pooled in equimolar amounts and sequenced on the Illumina NextSeq 500 sequencer in single-end mode (75 bases), following the manufacturer's instructions (Illumina). The reads were mapped to the mouse genome (GRCm38) using STAR (version 2.4.2a) with GRCm38.99 GTF annotation. The number of reads per gene was generated during the alignment step (quantMode GeneCounts) and gene counts were then analysed with the DESeq2 package. Genes with FDR < 10% are considered as differentially expressed. Enriched Gene Ontology (GO) terms (biological processes and molecular

functions) in the differentially expressed genes have been identified using the model-based gene set analysis (MGSA)[375]. The analysis was performed with 10 independent runs of the Markov chain of 1.10^8 steps each. The parameters p, alpha, and beta were used as default. Functional categories with a marginal posterior probability estimate higher than 0.65 were retained for further analysis. The hierarchical clustering, represented as a dendrogram, of TEC populations was performed using the hclust function in R on euclidean distances between the variance of the rlog-transformed read counts for each gene across samples.

Fetal thymus organ culture (FTOC)

Fetal thymus organ cultures (FTOCs) were established as previously described [77, 320] by placing isolated thymic lobes obtained from E14 C57BL/6 embryos. On the indicated days, FTOCs were dissociated and analysed by flow cytometry as previously described.

Reaggregate thymus organ culture (RTOC)

Reaggregate thymus organ cultures (RTOCs) were established as previously described[77, 320] by combining $7x10^5$ total thymic cells obtained from WT C57BL/6 thymus and $3.5\text{-}4x10^4$ sorted $TF^{A\text{-}B}$ subsets obtained from newborn Actin-RFP C57BL/6 thymic lobes. After 7 days in culture, RTOCs were dissociated and analysed by flow cytometry as previously described.

Statistical analysis

Statistical analyses were performed using GraphPad software, Version 9. Column graphs are represented showing the mean plus one standard deviation (SD). Statistical analysis was performed by using a two-tailed t-test.

Results

Analysis of thymic fibroblast differentiation during development.

Several markers, including CD140α, CD140β, GP38, ER-TR7, MTS-15, SCA-1, αSMA, CD146, CD34, Ly51, Itga7 and DPP4 have been used to phenotypically identify specific populations of TMCs [225, 253, 255, 260, 263, 265, 266, 376, 377]. Nonetheless,

as some of these markers are also expressed by other cell types, they cannot per se define distinct differentiation states of TMCs when employed in a restrictive manner. To dissect the heterogeneity within TMCs, we sought for cells expressing progenitor hallmarks within the entire postnatal mesenchymal compartment. We selected the postnatal day 7 thymus, as a period when the main hematopoietic, epithelial and mesenchymal subsets were present. Employing multiparameter flow cytometry, we analyzed the expression of 10 well-known cell surface markers. To discriminate hematopoietic, epithelial, endothelial and erythroid lineages, we included CD45, EpCAM, CD31 and Ter119 respectively. For the

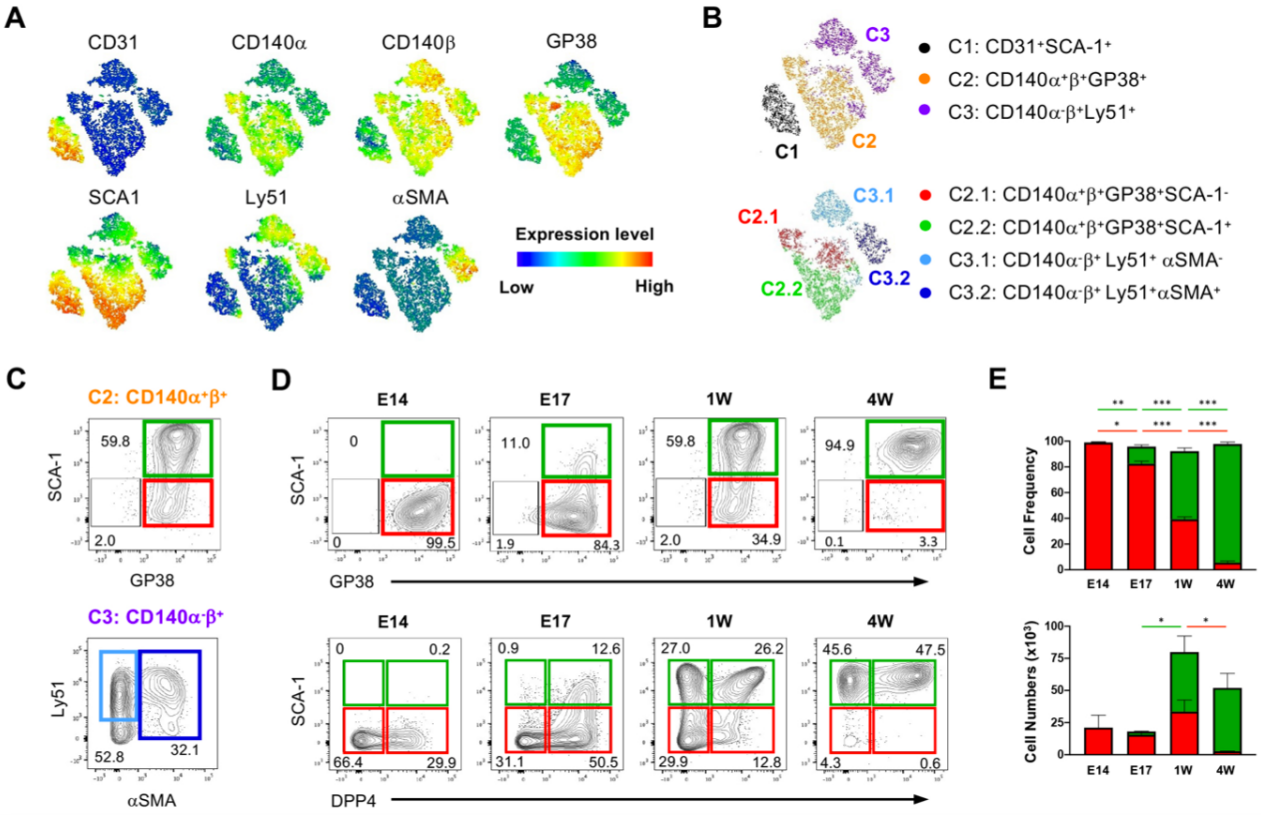


Figure 1: GP38 and SCA1 expression on TMC subsets. A) Total thymi cells from 1 week-old mice were isolated, and total TMCs ($CD45^-EpCAM^-$) were analysed by flow cytometry. tSNE representation of the expression of CD31, CD140α, CD140β, GP38, SCA-1, Ly51 and αSMA. B) Three main clusters were identified: Cluster 1 ($CD31^+$), Cluster 2 ($CD140\alpha^+\beta^+$) and Cluster 3 ($CD140\alpha^-\beta^+$). Clusters 2 and 3 were respectively subdivided into cluster 2.1 ($CD140\alpha^+\beta^+GP38^+SCA1^-$) and 2.2 ($CD140\alpha^+\beta^+GP38^+SCA1^+$); 3.1 ($CD140\alpha^-\beta^+Ly51^+\alpha SMA^-$) and 3.2 ($CD140\alpha^-\beta^+Ly51^+\alpha SMA^+$). C) TMCs ($CD45^-EpCAM^-CD31^-$) were analysed for the indicated markers, and sub-cluster 2.1 (red gate), 2.2 (green gate), 3.1 and 3.2 (Light and dark blue gates) were identified. D) Analysis of GP38, SCA-1 and DPP4 expression in TF^A (red gate) and TF^B (green gate) populations at the day of embryonic development E) 14, E17, 1 week-old (W) and 4W. Numbers in plots indicate the frequency of cells found within each gate. Plots are of a representative analysis per timepoint. E) Bar graphs correspond to the mean plus SD of the frequency and cellularity of TF^A and TF^B subsets, of three independent analyses per timepoint. Differences between TF subsets, CD140 $CD140\alpha^+\beta^+GP38^+SCA1^-$(red) and CD140 $CD140\alpha^+\beta^+GP38^+SCA1^+$ (green), were statistically analysed throughout age: *** p<0.001, ** p<0.01, * p<0.05.

analysis of TMCs, we initially considered the following markers: CD140α, CD140β, GP38, SCA-1, Ly51 and αSMA. Flow cytometry data of non-hematopoietic and non-epithelial cells was analysed by nonlinear dimensionality reduction algorithms, producing maps that clustered cells based on their phenotypic similarity (t-distributed stochastic neighbor embedding [t-SNE]) (Fig. 1A). This unsupervised approach revealed three main clusters within CD45$^-$EpCAM$^-$ cells. Cluster 1 was formed by CD31$^+$SCA-1$^+$ cells, cluster 2 comprised CD140α$^+$β$^+$GP38$^+$ cells, and cluster 3 contained CD140α$^-$β$^+$Ly51$^+$ cells (Fig. 1B). Changes on SCA-1 and αSMA expression respectively showed an additional layer of heterogeneity within clusters 2 and 3: while the differential expression of SCA-1 identified sub-clusters 2.1 (CD140α$^+$β$^+$GP38$^+$SCA-1$^-$) and 2.2 (CD140α$^+$β$^+$GP38$^+$SCA-1$^+$), alterations in αSMA expression distinguished sub-clusters 3.1 (CD140α$^-$β$^+$Ly51$^+$αSMA$^-$) and 3.2 (CD140α$^-$β$^+$Ly51$^+$αSMA$^+$) (Fig. 1B). Employing a directed gating strategy, we identified the same TMC subsets: CD140α$^+$β$^+$GP38$^+$SCA-1$^-$ (2.1), CD140α$^+$β$^+$GP38$^+$SCA-1$^+$ (2.2), CD140α$^-$β$^+$Ly51$^+$αSMA$^-$ (3.1) and CD140α$^-$β$^+$Ly51$^+$αSMA$^+$ (3.2) (Fig. 1C and Fig. S1). These results suggested that Cluster 1 defined endothelial cells, Cluster 2 included fibroblasts and Cluster 3 identified endothelial-supporting mesenchymal cells, which can be further subdivided into pericytes (3.1) and smooth muscle cells (3.2). Our observations further showed that the differential expression of CD140α can be used to distinguish fibroblasts (CD140α$^+$β$^+$) from pericyte-like cells (CD140α$^-$β$^+$). Moreover, SCA-1-expressing thymic fibroblasts (2.2) have been previously reported [377]. Yet, the segregation of CD140α$^+$β$^+$GP38$^+$ in SCA-1- (2.1) and SCA-1+ (2.2) was intriguing and led us to direct our attention to these subsets. We referred hereafter to cells within cluster 2.1 (CD140α$^+$β$^+$GP38$^+$SCA-1$^-$) and cluster 2.2 (CD140α$^+$β$^+$GP38$^+$SCA-1$^+$) as thymic fibroblast A (TF^A) and B (TF^B), respectively.

To examine whether TF^A and TF^B defined two distinct subsets, we analysed their development during thymic ontogeny and postnatal life. TF^A cells predominated at embryonic day 14 (E14) and their numbers were relatively constant up to the first week of postnatal life, followed by a decrease in the 4-weeks-old thymus. Contrarily, TF^B cells arose around E17 and expanded in frequency and number during the perinatal period (E17-4wk) (Fig. 1 D-E). We further addressed how the differentiation of TF^A and TF^B related to recently described medullary (DDP4$^-$) and capsular (DPP4$^+$) fibroblasts [225]. At E14.5, a period wherein TF^B were virtually absent, TF^A contained DPP4$^+$ and DPP4$^-$ cells. The first TF^B (SCA-1$^+$) cells appeared at E17 and were mostly DPP4$^+$, suggesting that their immediate precursors could be within TF^A DPP4$^+$. From the postnatal period onwards, TF^B contained both DPP4$^-$ and DPP4$^+$ cells (Fig. 1 D). A population of TF^A expressing low levels of DPP4 persisted in 1-week-old thymi (Fig. 1 D). In line with previous reports [225], the observation

that DPP4$^-$ and DPP4$^+$ cells appeared in the early embryonic TF^A subset may suggest that segregation of capsular and medullary sub-lineages may occur early in thymic development. Moreover, our results indicate that SCA-1 expression was acquired firstly by capsular (DDP4$^+$) fibroblast followed by medullary (DDP4$^-$) counterparts. As such, the acquisition of SCA-1 expression appears to represent a maturation marker commonly acquired by capsular and medullary thymic fibroblasts and does not by itself discriminate these subsets. The developmental kinetic of TF^A and TF^B led us to consider that they could represent distinct stages of the same differentiation pathway. In this scenario, TF^A should contain precursors with the potential to differentiate into TF^B. Alternatively, TF^A and TF^B could define unrelated thymic mesenchymal cells. We conducted genome-wide transcriptional and lineage tracing experiments to further investigate the precursor-product relationship between these subsets.

TF^A and TF^B subsets have distinct transcriptional programs.

To examine whether TF^A and TF^B identified different states of fibroblast differentiation, we characterized their genome-wide transcriptional profile by employing RNA sequencing analysis. TF^A and TF^B were purified by cell sorting from the 1-week-old thymus, a period wherein these subsets were equally represented. Additionally, we purified endothelial-supporting mural cells (MC) (cluster 3) and included them as a complementary reference population in the transcriptional analysis.

Principal component analysis showed that the biological replicates of each subset clustered together, demonstrating that these populations had low intrapopulation variability. Moreover, TF^A and TF^B were more closely related to each other relatively to MC (Fig. 2A, Fig. S2A and Table S1). Employing available transcriptomic data sets from other studies [225, 255, 377], we extracted sets of genes associated with fibroblasts, vascular-supporting cells, and cross-examined their expression pattern in TMC subsets. Firstly, the expression of genes used as phenotypic markers to define TF^A, TF^B and MC subsets followed the expected pattern, validating the accuracy of the purified samples. Secondly, most fibroblasts-associated genes were upregulated in TF^A to TF^B, while transcripts linked to vascular-supporting cells were specifically enriched in MC (Fig. 2B and Table S2). Moreover, an unsupervised cross-analysis of genes linked to capsular and medullary fibroblasts [225], revealed that these transcripts were majorly increased in TF^B (Figure S2B and Tables S3-4). These observations were in line with the representation of capsular and medullary subsets within TF^A and TF^B in the 1-week-old thymus (Fig. 1E) and support their fibroblastic identity. Further bioinformatic analysis identified 470 and 721 uniquely

upregulated genes in TF^{A} and TF^{B}, respectively (Fig. S2C, Tables S5-6). Gene ontology enrichment analysis of these sub-lineage specific sets revealed a stringent association to

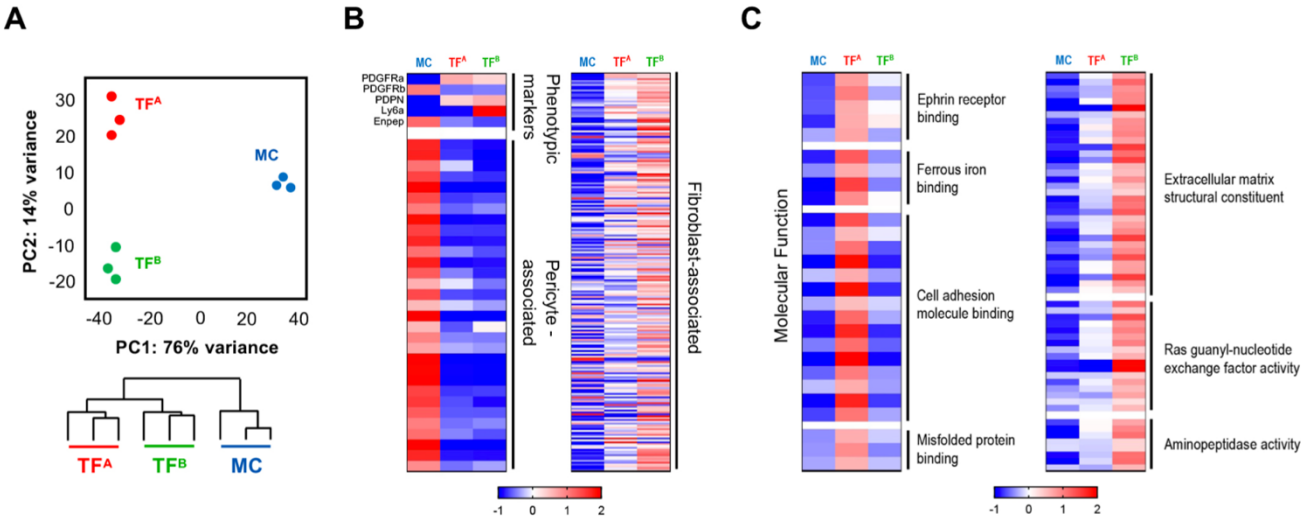


Figure 2: Genome-wide transcriptomic analysis of TF subsets identifies stages with distinctive gene expression profiles. A) Principal component analysis plot and dendrogram, detailing the hierarchical clustering between the biological samples, performed with data obtained from total RNA-sequencing analysis of sorted TF^{A} (CD45^{-}EpCAM^{-}GP38^{+}SCA-1^{-}) (n=3), TF^{B} (CD45^{-}EpCAM^{-}GP38^{+}SCA-1^{+}) (n=3) and MC (CD45^{-}EpCAM^{-}GP38^{-}SCA-1^{-}Ly51^{+}) (n=3) populations. B) Heat maps representing the deviation from average expression of the phenotypic markers used to identify TMC populations, of genes previously associated with pericytes and of genes previously associated with thymic fibroblasts. C) Heat maps representing the deviation from average expression of the uniquely upregulated genes identified for populations TF^{A} and TF^{B} and the associated molecular functions identified by GO analysis. Genes with FDR<10% were considered as differentially expressed. Enriched GO terms (molecular functions) were identified using MGSA. Represented categories had a marginal posterior probability estimate higher than 0.65.

diverse functional categories. Specifically, TF^{A}-enriched genes were linked to broad cellular processes, including ephrin receptor signaling, cell adhesion, binding to iron and misfolded protein. Contrarily, genes upregulated in TF^{B} were associated with more restricted processes, including extracellular matrix (ECM) components, GTPase signaling and aminopeptidase activity (Fig. 2C and Tables S7-8). Several collagen genes were upregulated in TF^{B}, consistent with the association with ECM constituents (Fig. S2D and Table S9). Recent findings implicated LTβR-mediated signaling in the thymic medullary fibroblast differentiation [225]. Detailed analysis of members of the TNFRSF family showed that *Ltbr*, *Tnfrsf1b*, *Tnfrsf12a* and *Tnfrsf23* were specifically upregulated in TF^{B} (Fig. S2E and Table S6). Therefore SCA-1 acquisition might identify a developmental stage where medullary fibroblasts are more responsive to TNFRSF family members which is an important signaling pathway for their maturation and functional competence. Together, our

results suggest that TF^A may contain more immature cells, while TF^B appear to define mature thymic fibroblasts.

TF^A can give rise to TF^B and their homeostasis is altered in the alymphoid thymus.

The observations that TF^B developed at E17 presumably from TF^A suggested a possible precursor-product lineage relationship between these populations. To assess this hypothesis, we first established Fetal Thymic Organs Cultures (FTOC) with E14 thymi, a stage wherein TF^B were virtually absent. TF^B emerged upon 4-day culture, partially phenocopying the composition of TF subsets in the E17 thymus (Fig. S3A). These results suggested that TF^B precursors already existed in the E14 thymus and that subsequent intrathymic interactions may promote their differentiation. To determine the lineage potential of TF^{A-B} in the postnatal thymus, we purified (FACS sorting) these populations from 1-week-old-thymus and established reaggregate thymus organ cultures (RTOCs). TF subsets were isolated from the thymus of $Actin^{RFP}$ reporter mice [320] and mixed with WT-derived embryonic thymic cells (carriers). In this system, RFP expression is constitutively active in "spiked" cells ($TF^{A/B}$), providing an intrinsic label for lineage tracing analysis of TF subsets (Fig. 3A and Fig. S3B). The differentiation potential of TF subsets was analyzed upon 7 days of culture. Whereas TF^B largely maintained their phenotype, TF^A gave rise to TF^B (Fig. 3B). None of the two subsets originated vascular-supporting cells ($CD140\alpha^-\beta^+Ly51^+$) (data not shown) which might be attributed to the limitations of RTOC to recapitulate normal angiogenesis. In both RTOCs, embryonic carrier cells (RFP^-), which are mostly composed of TF^A, followed the same differentiation trajectory (Fig. S3B-C). These results suggested that TF^B represented a more committed fibroblast population, whereas TF^A contains cells with fibroblast progenitor activity.

It is well recognized that the establishment of epithelial microenvironments depends on functional bidirectional interactions between hemopoietic cells and TECs [378]. A recent study showed that the differentiation of thymic medullary fibroblasts also depends on signals provided by developing thymocytes [225]. Thymic organotypic cultures allow the normal program of T cell and TEC differentiation [77, 320]. Thus, the observations that TF^A gave rise to TF^B in FTOC and RTOC led us to consider whether there was a stage-specific requirement for thymocyte crosstalk during thymic fibroblast differentiation. To evaluate this possibility, we analysed TF development in mutant mice in which thymocyte development is inhibited at different stages. While in *Rag2*-/- mice T cell development is blocked at the double negative (DN) 3 stage, *Rag2*-/-*Il2rg*-/- mice display a premature and more severe arrest in thymocyte development [77, 320]. Relatively to the WT thymus, the proportion of TF^B was profoundly affected in the 1- and 4-week-old *Rag2*-/-I*l2rg*-/- thymus, leading to an

accumulation of GP38$^{-/low}$ and an overall reduced GP38 expression at 1 and 4 weeks of age (Fig. 3C). The frequency of TF^{B} in *Rag2-/-* thymus was also reduced in the 1-week-old-thymus relatively to WT counterparts, although to a lesser extent compared to *Rag2-/-Il2rg-/-*. Yet, the representation TF^{B} in *Rag2-/-* thymus at 4 weeks normalized to the ones obtained in the WT thymus. Strikingly, the numbers of TF^{B} were markedly reduced in both 1- and 4-week-old *Rag2-/-* and *Rag2-/-Il2rg-/-* thymus when compared to WT counterparts (Fig. 3C). Thus, these results suggests that fibroblast maturation depends on thymic crosstalk.

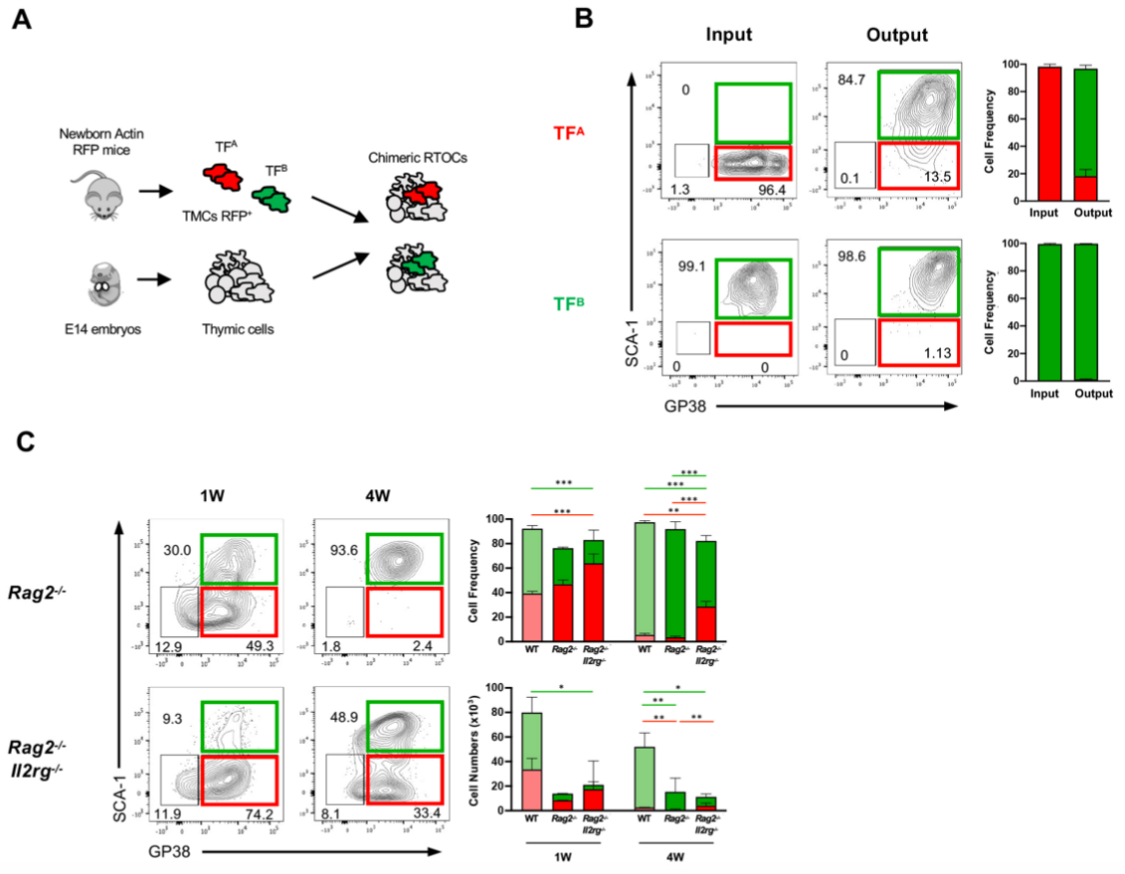


Figure 3: TF^{A} contains progenitor cells capable of generating TF^{B}, in a process dependent on thymic crosstalk. A) Chimeric RTOCs were established with E14 cells from WT thymus and mixed with TF^{A} or TF^{B} cells isolated from the postnatal day 1-3 Actin-RFP mice. B) Flow cytometry analysis of the chimeric RTOC at day 0 (input) and after 7 days in culture (output). Data presented and bar graphs correspond to mean+s.d. of two independent analyses. C) Analysis of GP38 and SCA-1 expression within TF populations from 1- and 4-week-old Rag2$^{-/-}$ and Rag2$^{-/-}$Il2rg$^{-/-}$ mice. Numbers in plots indicate the frequency of cells found within each gate. Flow cytometry plots are of a representative analysis. Bar graphs correspond to mean+s.d. of two (1-week-old Rag2$^{-/-}$) and three (1-week-old Rag2$^{-/-}$Il2rg$^{-/-}$ and 4-week-old Rag2$^{-/-}$ and Rag2$^{-/-}$Il2rg$^{-/-}$) independent experiments per time point. Each experiment contains a pool of two to four mice per analysis. The numbers of TF subsets found in the WT thymus are co-represented as a reference and were originally described in Fig. 1. Differences between WT and Rag2$^{-/-}$Il2rg$^{-/-}$ TF subsets at 1 week and between WT, Rag2$^{-/-}$ and Rag2$^{-/-}$Il2rg$^{-/-}$ at 4 weeks were statistically analysed: ***P<0.001, **P<0.01, *P<0.05.

Discussion

Here, we identify a novel fibroblast progenitor within the developing thymus, whose bioavailability is controlled by thymic crosstalk. However, the results in the *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ thymus cannot formally exclude an additional role for γ_c-mediated signaling in thymic fibroblast differentiation. Few reports indicate that γ_c cytokine family may also affect the function of non-hematopoietic stromal cells, such as endothelial cells [379]. However, the observation that TF^B differentiation was also impaired in the *Rag2*$^{-/-}$ thymus, wherein γ_c-mediated signaling was intact, supported the hypothesis that thymic fibroblast maturation is controlled by cooperative signals provided by thymocytes passing the β selection checkpoint. In this regard, the maturation of MFbs also required cellular interactions with mature TCRαβ-expressing thymocytes [225]. Moreover, it remains unknown whether mature thymic fibroblasts are replaced by dedicated progenitors or a multilineage precursor. A mesenchymal progenitor population referred to as CD34$^+$ adventitial cells (CD34$^+$GP38$^+$) has been previously reported to exist in the adult thymus with capacity to generate fibroblast and pericytes [255]. Strikingly, TF^A isolated within the postnatal thymus revealed a more fibroblastic-restricted progenitor activity. Nevertheless, the complete lineage potential of the precursor cells residing in TF^A population might be hindered in RTOCs done in vitro. In this regard, the transplantation of thymic organoids, for example into the kidney capsule, might provide a more physiological microenvironment, allowing the system to be connected to systemic circulation and consequently favoring the development of thymic vasculature. Further studies should determine whether CD34$^+$ adventitial cells and TF^A are developmentally unrelated or define distinct stages of the same TMC differentiation process. Moreover, future analysis should resolve whether DDP4$^-$ and DDP4$^+$ existing within TF$^{A/B}$ at different stages of life represent unipotent or bipotent precursors of thymic capsular and medullary fibroblasts. The decline of TF^A with age within the normal thymus, and their maintenance in *Rag*$^{-/-}$*Il2rg*$^{-/-}$, suggests that the pool of TF progenitors is negatively regulated by thymic crosstalk. Interestingly, a similar feedback mechanism has been reported for distinct progenitor TEC subsets. In particular, the maturation of mTECs depends on the cooperative role of TNFR superfamily members, including receptor activator of NF-κB (RANK), lymphotoxin β receptor (LTβR), and CD40, which are stimulated by their respective ligands expressed in several hematopoietic cells, namely lymphoid tissue inducer cells, γδ T cells, positively selected double-positive (DP) thymocytes and αβ CD4$^+$ single-positive thymocytes (SP4) [227, 230, 231, 380-382]. Our results suggest that cooperative signals derived from thymocytes that passed the β-selection checkpoint control

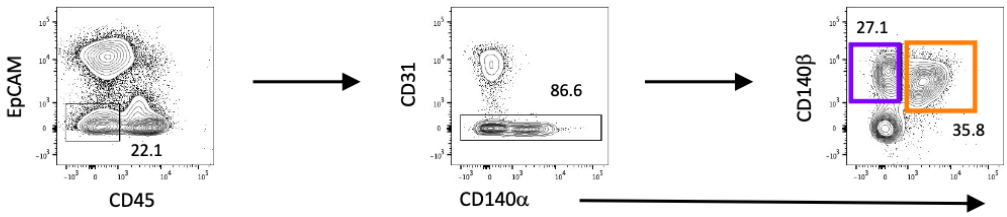
thymic fibroblast differentiation. These findings implicate that thymocyte-derived signals have a dual effect on thymic stromal differentiation, promoting the differentiation of mature lineage while depleting the bioavailability of the pool of distinct progenitor cells. Further studies are required to elucidate the signals that control the turnover of thymic fibroblasts *in vivo* and whether this process entails direct thymocyte-fibroblast interactions or is mediated by other cell-cell contacts.

The understanding of the homeostasis of thymic fibroblast under development and steady-state circumstances is relevant to non-homeostatic conditions, such as ageing, disease and regeneration. In this context, aged-related thymic atrophy is accompanied by the accumulation of adipocytes. Yet, it is still unclear the molecular mechanisms as well as the cell source involved in adipogenesis. Fate-mapping mice models revealed that adipocytes in abdominal white adipose tissue (WAT) are derived from progenitor cells expressing platelet-derived growth factor receptor alpha (PDGFRa), CD34, and SCA-1 [383]. Thus, TF^B might harbor the ability to (trans)differentiate into adipocytes upon receiving specific environmental cues. Indeed, thymospheres formed with SCA-1^+ fibroblasts give rise to adipocytes, corroborating the idea that thymic fibroblasts might serve as adipocytic progenitors [260]. Further studies should explore the molecular mechanisms involved in the reprogramming mechanism underlying the differentiation of adipocytes in the aged thymus. One possibility can be examining the impact of blocking adipogenesis in thymic atrophy. Concerning the role of thymic fibroblasts in disease, it was recently shown that *Tbx1*$^{+/-}$ and *Crkl*$^{+/-}$ mice, which mimic some of the hallmarks of DiGeorge syndrome, exhibit alterations in the composition of TMC compartment together with modifications in the transcriptional program of TFs conducing with an accelerated ageing [257]. Therefore, TFs might contribute actively in some diseases, opening new avenues to better characterize their exact role as well as to find new therapeutic approaches that specifically target thymic mesenchyme. Finally, the notion that TMCs play a role in thymus recovery after an insult is compatible with previous studies [273, 274]. In this context, it was interesting to notice in our RNA-sequencing data that both TF^A and TF^B express IL-33. This cytokine has been described to be produced by mesenchymal cells in WAT in order to regulate the pool of ILC2 cells [384]. Taking in consideration that ILC2 and eosinophils were linked to thymus recovery upon irradiation [307], one can speculate that thymic fibroblasts sustain a pool of thymic ILC2, which are hardwire to be activated upon injury. Consistent with this hypothesis thymic ILC2 remain constant from birth to the adult life, in contrast with ILC3 that are highly prevalent during neonatal period but then rapidly disappear from thymic microenvironment [385]. Therefore, the role of TFs may extend beyond homeostatic physiology, potentially regulating thymic function under distinct pathophysiological conditions.

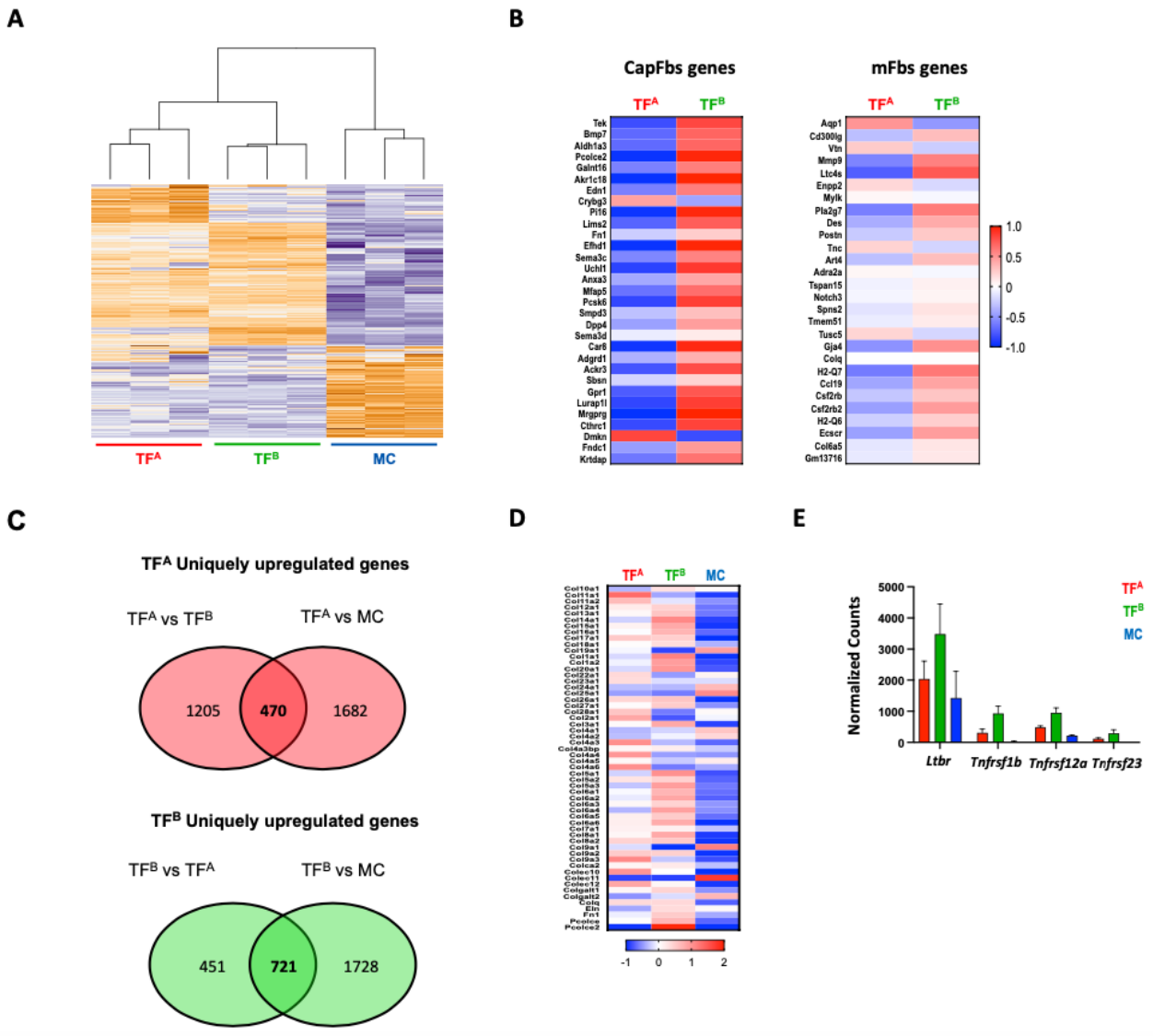
In sum, our study resolves the identity of novel thymic fibroblast precursors and exposes a checkpoint in TF differentiation that is controlled by thymic crosstalk *in vivo*. These findings represent a novel roadmap to understand the processes underlying the establishment of thymic mesenchymal cells in regular and deficient thymopoiesis.

Supplementary information

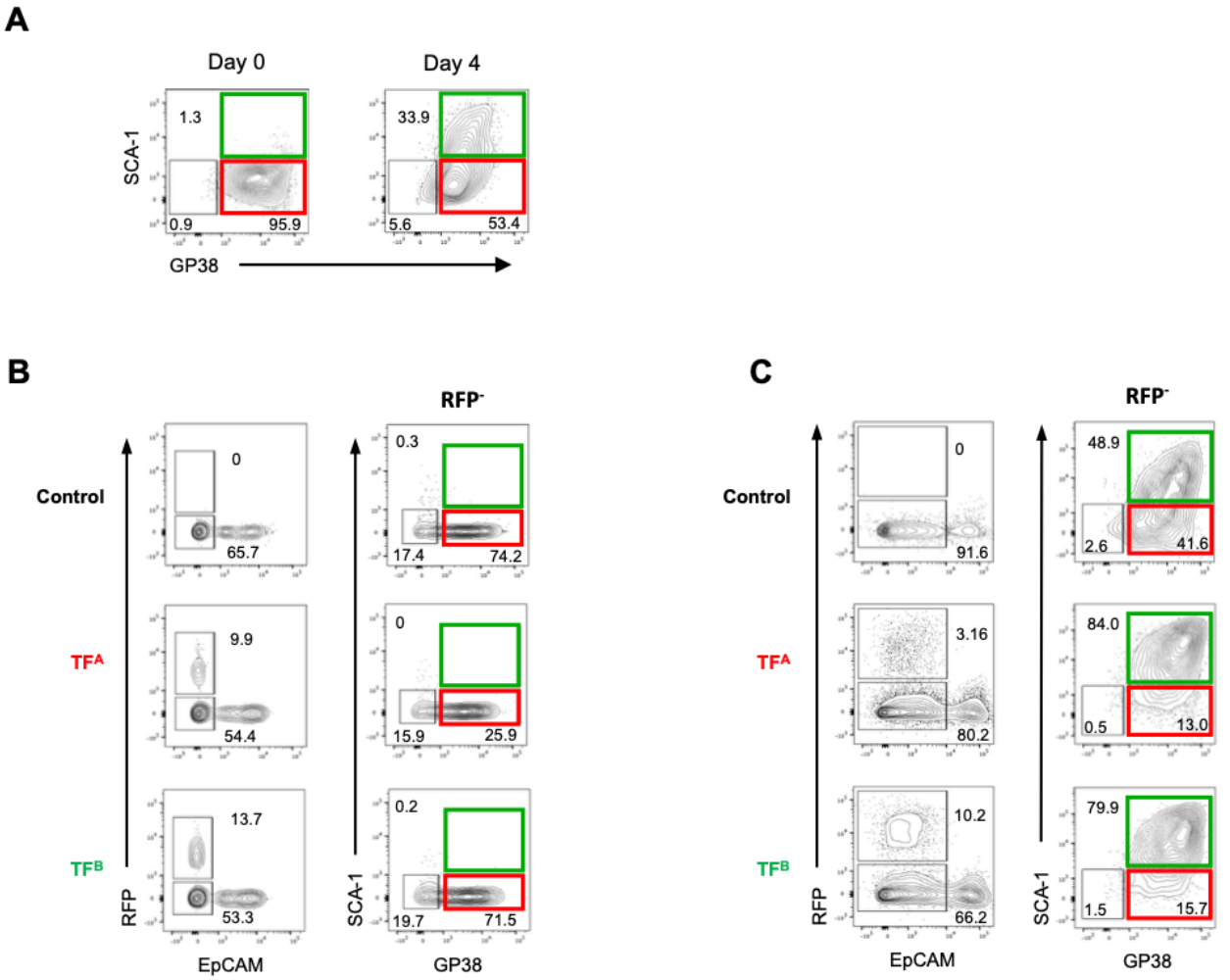
A



Supplementary figure 1: Gating strategy for flow cytometry analysis of thymic mesenchymal cells. A) Representative analysis of cells obtained from 1 week-old thymus depicting the gating strategy used to identify TMCs defined as clusters 2 and cluster 3. Numbers in plots indicate the frequency of cells found within each gate.



Supplementary figure 2: RNA sequencing analysis of TMC subsets. A) Heat map representing the 1000 most expressed genes in the assessed TMC populations and associated dendrogram detailing the hierarchical clustering between the biological samples. (B) Heat maps represent the deviation from the average expression of the top expressed genes associated with capsular and medullary fibroblasts. C) Venn diagrams represent the identification of the 470 and 721 uniquely upregulated genes in TF^A red) and TF^B green) populations, respectively. Genes with FDR < 10% were considered as differentially expressed. D) Heat maps represent the deviation from the average expression of the different collagen and collagen associated genes in the different TMC populations. E) Bar graph representing the mean plus SD expression value of the TNFRSF family genes upregulated in TF^B.



Supplementary figure 3: Precursor-Product relationship between TF subsets. A) Flow cytometry analysis of the expression pattern of GP38 and SCA-1 at day 0 and after 4 days in culture, from TMCs obtained from fetal thymic organ cultures (FTOC) established with thymic lobes collected from E14 C57BL/6 mice. B) Flow cytometry analysis of day 0 (input) and day 7 (output) RTOC established by combining cells obtained from disaggregated E14 thymus cells from C57BL/6 mice alone (Control) or co-cultured with either TF^A or TF^B cells isolated from postnatal day P1-P3 Actin-RFP C57BL/6 mice. C) Representative analysis of cells obtained from 1 week-old thymus depicting the gating strategy used to identify TMCs defined as clusters 2 and cluster 3 in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$. Numbers in plots indicate the frequency of cells found within each gate.

Chapter V

General discussion and future perspectives

General discussion and future perspectives

In this thesis, we focused in two main components of thymic stroma, TECs and TMCs, which provide specialized microenvironments for T cell development. In the second chapter, we set the general scope of this thesis discussing in a review article how the expansion and maturation of TECs during the early postnatal period set the foundations for their role in T cell development throughout life. In the third chapter, we examined the role of RBPs belonging to the ZFP36 family in TEC differentiation and function. Lastly, we investigated the development of TMCs, shedding light into their differentiation trajectories and demonstrating that thymic crosstalk regulates thymic fibroblast maturation. In this closing section, I will discuss the implications of our results in the context of the minimal required conditions to the establishment of central tolerance. Lastly, I will revisit the concept of thymus crosstalk and cover how our recent findings on thymic fibroblasts might help solving the puzzle of TMC development.

Revisiting the role of the thymus in tolerance induction

The development of autoimmunity is a multifactorial process that ultimately will result in the activation of the immune system against our own cells. In this context, the thymus plays a central role because it eliminates thymocytes expressing potential autoreactive TCRs from the peripheral TCR repertoire or alternatively deviates them to the T_{reg} cell lineage [69]. As described in the general introduction, mTECs are key players in this process, in part due to its unique ability to express and present TRAs [116]. Our results showed that *Zfp36* and *Zfp36l1* dcKO mice exhibited a slight increase in the development of autoimmune manifestations, despite the severe contraction in mTEC cellularity (~3-fold reduction). This observation is consistent with studies showing that a reduction in the mTEC compartment does not always translate into major defects in self-tolerance [223, 242], and raises the question: how many mTECs are needed to impose central tolerance induction? The analysis of pGE expression at the single-cell level indicates that 200-500 mTECs are sufficient to cover the complete TRA repertoire [67]. This estimation suggests that the same TRA is presented by multiple mTECs across medullary microenvironment. Thus, it is still possible that a reduction in the mTEC compartment does not completely deplete the expression of all TRAs, enabling an efficient negative selection and/or T_{reg} cell differentiation. The picture is more complex because several findings indicate that central tolerance in the thymus is not completely efficient, as demonstrated by the presence of autoreactive T cell clones found in the peripheral blood of healthy individuals [386]. This observation suggests that central tolerance displays a natural intrinsic inefficacy, which can

be explained by several mechanisms, including the exclusion of peripheral-restricted splice isoforms from the TRA repertoire expressed in TECs [131].

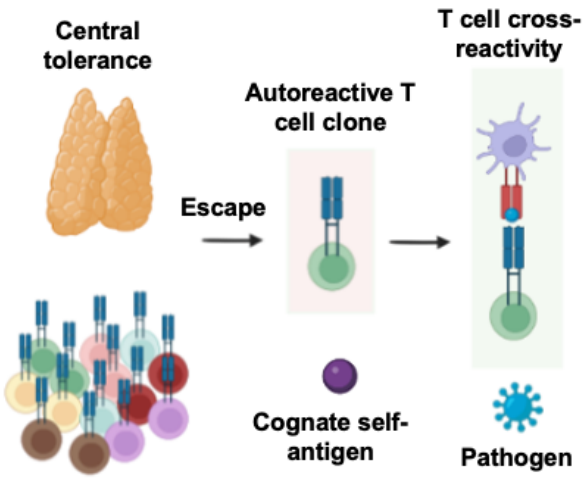


Figure 5 – Central tolerance is permissive with autoreactivity in order to increase the breadth of the TCR repertoire. Central tolerance eliminates autoreactive thymocytes or alternatively deviate them to the T_{reg} cell lineage. Nevertheless, taking in consideration the discrepancy between the theoretical diversity of different epitopes (10^{10}-10^{18}) generated during antigen processing and the estimated size of TCR repertoire in mice spleen (10^{5}-10^{6}), it is plausible to consider that some TCRs may be cross-reactive in order to increase the scope of antigen recognition. Since some autoreactive TCRs might also be cross-reactive against pathogen-derived epitopes there may exist a fine equilibrium between negative selection and self-reactivity in order to prevent holes in TCR repertoire required to recognize pathogen-derived antigens.

What can then be the advantage of developing a system that allows the escape of autoreactive T cells for the function of the immune system? In the first place, several studies suggest that a certain degree of basal self-reactivity must exist between the TCR and self-peptide:MHC complexes, as this interaction is essential to the peripheral maintenance of T cells [387, 388]. The second one is related to the difference between the potential number of different peptides that T-cells might encounter displayed by MHC molecules and the number of different TCRs that they express. It is estimated that 10^{10}-10^{18} different peptides can be generated during antigen processing [389]. Assuming that only 1% of those peptides can effectively bind to, and be presented by, MHC molecules at different moments, the number of potential presented antigens ranges from 10^{8} -10^{16} [389]. Despite the TCR diversity potential (10^{15}) [390], the estimated number of total peripheral murine T cells is around 10^{8} total T lymphocytes [391]. Even if all these T lymphocytes would represent

unique T cell clones, which it is not the case, TCR repertoire in any given moment may not cover the entire antigen diversity that can be virtually presented, assuming the initially proposed view that one TCR recognizes one specificity. Yet, TCR recognition appears to be more complex. Several evidence shows that a single TCR possess cross-reactivity to different antigens, as indicated by the fact that one specific TCR can recognize up to 10^6 different peptides [392, 393]. Moreover, it was demonstrated that the vaccination against flu expanded T cell clones expressing TCRs with cross-reactivity against antigens from other pathogens [394]. Finally, some studies suggested that autoreactive T cell clones can recognize pathogen-derived antigens [395-397]. Therefore, it is tempting to speculate that the complete elimination of autoreactive thymocytes may favor the elimination of T cells that although possessing self-reactivity also recognize pathogen-derived epitopes, creating holes in the TCR repertoire that would make the system more susceptible to infections.

Another aspect to be considered is the definition of a failure in central tolerance. First, it is necessary to emphasize that T cell function is modulated by the integration of signals induced by the interaction of the TCR with peptide:self-MHC complexes. In this context, if the MHC haplotypes of a given organism cannot accommodate the self-peptides recognized by the autoreactive cells in the thymus, it is possible that those clones can escape negative selection, increasing the TCR repertoire but without representing an effective danger to the system. This problem can arise when an epitope not covered by the TRA repertoire of TECs is presented by MHC-expressing peripheral APCs to autoreactive T cells that were not eliminated. An example of this possibility is the distinct regulation of proteolipid protein (PLP) expression in the thymus and periphery. PLP is expressed in the brain and it's recognition by autoreactive T cells has been implicated in the development of experimental autoimmune encephalomyelitis (EAE). Yet, TEC express a short splice variant of this protein that lacks a specific loop of 35 amino acids. Therefore, T cells reactive against antigens derived from this region can escape negative selection and colonize the periphery [398]. Interestingly, the development of EAE is also influenced by the MHC haplotype and the capacity of MHC molecules to present self-antigens, and not merely by the presence of those autoreactive T cells in the periphery. While SJL/J and Balb/s mice are highly susceptible because their MHC molecules present in the periphery the epitopes from the region that is absent in the thymus, C57BL/6 mice are resistant because their MHC haplotypes cannot present those epitopes [398]. These results suggest that the autoreactive T cell clones in the C57BL/6 background do not represent a risk because in the periphery those T cells are "blind" for these antigens. The observations that Aire deficient mice with different genetic backgrounds present distinct autoimmune manifestations [398] further suggests that differences in MHC composition and antigen presentation capacity could be an important factor in the complex process of tolerance

induction. In line with this hypothesis, there is a strong association between autoimmune diseases and MHC haplotypes [399]. We can speculate that perhaps MHC loading constraints and TRA coverage might have evolved together in a Darwinian sense, with the selection pressure selecting the ones that reach an optimal balance between these two parameters in order to maximize the diversity of TCR repertoire without compromising self-tolerance. Worth mentioning, there are also mechanisms of peripheral tolerance that control autoreactive T cells, including the induction of anergy and clonal deletion [400]. Furthermore, some organs (*e.g.,* brain, testis and eye) are immune-privileged sites that either restrain the entry of immune cells or provide an immunosuppressive milieu that inhibits T cell activation [401-403]. These peripheral mechanisms would also contribute to control autoreactive T cell clones, ensuring self-tolerance without compromising immune reactivity to foreign threats.

Attempting to draw a model for TMC development

The characterization of TMC heterogeneity has been improved over the last years [225, 255, 257, 377]. Our results defined two main populations of TMCs distinguished on the basis of the differential expression of CD140α and CD140β: thymic fibroblasts (TFs) (CD140α^+ CD140β^+) and thymic pericytes (CD140α^- CD140β^+). While TFs can be further subdivided in Sca-1$^-$ and Sca-1$^+$ populations, a trajectory common to capFbs (DPP4$^+$) and mFbs (DPP4$^-$), thymic pericytes enclose contractile (αSMA$^+$) and non-contractile (αSMA$^-$) pericytes. Therefore, our results provide a better guide to categorize thymic mesenchyme for future studies. Nevertheless, the cellular and molecular mechanism underlying TMC maintenance *in vivo* remains poorly characterized. In our study, we identified a potential source of progenitor cells in the developing thymus that is capable of giving rise to mature fibroblasts but not pericytes *in vitro*. Further *in vivo* studies should define whether these progenitor cells have a fibroblast-restricted potential or represent cells with a multipotent capacity to generate several TMC lineages. Indeed, the notion that thymic mesenchymal compartment is sustained by a pool of TMC progenitors is supported by the existence of adventitial progenitor cells (CD34$^+$ CD140α^+PDPN$^+$) in the adult thymus, which are capable of giving rise to both thymic fibroblasts and pericytes [255]. Corroborating the existence of TMC progenitors, adult Sca-1$^+$ MTS-15$^{+/-}$ TMCs are enriched in thymosphere-forming cells (TSFCs), a feature of cells with stemness properties [260]. Although the frequency of TSFCs was higher in the Sca-1$^+$MTS-15$^-$ TMCs, Sca-1$^+$ MTS-15$^+$ population still possesses an elevated frequency of those cells, supporting the notion that there might exist more than one type of thymic mesenchymal progenitors residing in the adult thymus [260]. In this

regard, MTS-15^{+} TMCs were found scattered throughout the subcapsular zone, corticomedullary junction and trabeculae, whereas adventitial progenitors are more widely distributed across thymic parenchyma and capsule [255, 376]. This suggests that adventitial progenitors might have a larger contribution to TMC replenishment within the thymus, while MTS-15^{+} precursors a more compartmentalized role. Whether these two purported progenitors can give rise to both lineages (fibroblasts and pericytes) or instead have different lineage potential remains to be addressed. In order to answer these questions thymic organotypic or advanced organoid systems might be used to test the individual capacity of these progenitors to generate both lineages *in vivo*.

Lastly, another aspect raised by our study is related to the lineage relationship between the progenitors identified in the developing thymus and the ones reported in the adult period. Our results demonstrated that TF^{A} subset is almost absent from adult thymus and contains cells capable of giving rise to TF^{B} population. Future studies should address the lineage-relationship between adventitial cells in the adult thymus and TF subsets. Concerning MTS-15^{+} precursor cells, the observation that MTS-15^{+} TMCs emerge for the first time in the thymus around E15 may indicate that they reside within TF^{A} subset since at this stage thymic microenvironment is essentially devoid of TF^{B} cells. Nonetheless, future experiments are required to establish the lineage relationship between the recently identified TMC progenitors.

Thymus crosstalk: It takes more than two to tango

Thymus crosstalk between TECs and thymocytes is reported to be essential for mTEC differentiation and therefore for the construction of medullary microenvironments. Our results showed that thymocyte-derived signals are also required for the differentiation of thymic fibroblasts (TFs), suggesting that thymus crosstalk might be extended to other thymic stromal populations. Our findings are consistent with previous observations showing that TCR$\alpha^{-/-}$ mice display a defect in the differentiation of mFbs [225]. In the same study, it was shown that deletion of *Ltbr* from TMCs impaired the differentiation and function of mFbs, suggesting that the Ltα and Ltβ ligands expressed majorly by SP thymocytes in adult thymus might contribute to their development and functional maturation [225]. However, as *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and *Rag2*$^{-/-}$ mice, used in our study as well as TCR$\alpha^{-/-}$ mice, used in [225], display a severe block in TEC development, one cannot exclude a direct role of TECs in TF differentiation. Analysis of TF compartment in mice with an impairment in mTEC differentiation (*Foxn1*-Cre *Tnfrsf11a*$^{fl/fl}$ *Cd40*$^{-/-}$) showed that the proportion of mFbs and capFbs was not significantly changed [225], possibly arguing against the hypothesis that interactions with mTECs are important for TF development. Further studies are required to

better characterize TMC compartment under conditions of severe atrophy of thymic epithelium. Moreover, the analysis of TMC development in mice with TEC-specific deficiency in *Traf3*, which bypass the requirement of lymphoepithelial interactions and display a seemingly regular mTEC differentiation in alymphoid mice, might be very insightful to investigate the specific contribution of thymocytes and TECs in this process. In this context, the checkpoint in TF differentiation, defined by the Sca-1 acquisition might enable a better dissection of TMC maturation in future works. Notably, although there is not clear evidence on the role of epithelial mesenchymal interactions in TMC differentiation, the disruption of TF homeostasis impacted on the size and composition of mTEC compartment, suggesting that TFs contribute for the establishment and maintenance of medullary microenvironments [225, 270]. Follow up studies are necessary to explore the molecular basis underlying TEC-mesenchyme interactions.

Concluding remarks

In this thesis we uncovered the biological role of ZFP36 family of RBP in thymic epithelium. ZFP36 and ZFP36L1 were shown to be important for the development and function of TECs. Moreover, the combined deletion of both proteins compromised the production of T cells leading to a slight predisposition to develop autoimmune manifestations.

In chapter IV we described the identification of a novel source of TF progenitors abundant in the embryonic thymus but rapidly decline in the early postnatal period. Furthermore, we found that the expression of SCA-1 maps a developmental checkpoint in their differentiation into mature fibroblasts that is dependent on thymus crosstalk.

Collectively our results contribute to enlarge our understanding about the molecular and cell mechanisms involved in the development and maintenance of key thymic stromal populations (TECs and TMCs). These findings are of fundamental and clinical relevance to understand how to restore thymic function.

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Appendix

frontiers in Immunology

MINI REVIEW
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The Early Postnatal Life: A Dynamic Period in Thymic Epithelial Cell Differentiation

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The microenvironments formed by cortical (c) and medullary (m) thymic epithelial cells (TECs) play a non-redundant role in the generation of functionally diverse and self-tolerant T cells. The role of TECs during the first weeks of the murine postnatal life is particularly challenging due to the significant augment in T cell production. Here, we critically review recent studies centered on the timely coordination between the expansion and maturation of TECs during this period and their specialized role in T cell development and selection. We further discuss how aging impacts on the pool of TEC progenitors and maintenance of functionally thymic epithelial microenvironments, and the implications of these chances in the capacity of the thymus to sustain regular thymopoiesis throughout life.

Keywords: thymus, thymic epithelial cells, tolerance, early postnatal life, aging

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INTRODUCTION

The current pandemic caused by the SARS-CoV-2 virus underscores the importance of maintaining a pool of immunologically competent T cells, which are capable of responding to virtually any new foreign threats while tolerant to the host own tissues. The establishment of a diverse T cell receptor (TCR) repertoire arises from the random recombination of V(D)J gene segments during T cell development in the thymus. Yet, the arbitrariness underlying this process can also produce autoreactive T lymphocytes. The thymus has developed several control mechanisms to simultaneously establish T cell immunity against non-self elements and impose self-tolerance. Particularly important in the choreography of T cell selection are thymic epithelial cells (TECs), which represent a key component of the thymic stromal microenvironment. TECs are typically subdivided into functionally distinct cortical (cTEC) and medullary (mTEC) lineages (1). While cTECs primarily mediate T cell lineage commitment and positive selection, mTECs fine-tune the negative selection of autoreactive thymocytes or promote their deviation into the T regulatory cell lineage (2). It is conceptually accepted that cTECs and mTECs differentiate from thymic epithelial progenitors (TEPs) present within the embryonic and postnatal thymus (2). Deficits in the function of TECs arise with aging, cytoablative regimens and infection, leading to a lower naïve T cell output. These thymic failures are pertinent in the elderly and patients undergoing bone marrow transplantations (BMT), contributing to their poor T-cell responses to new pathogens or predisposing to autoimmunity (3). Thus, the preservation of a regular thymic function also depends on the maintenance and differentiation potential of bipotent or lineage restricted TEPs.

In this review, we focus on critical changes in the molecular traits of TECs that occur during the first weeks of the murine postnatal life, and integrate how these alterations might precede events coupled with thymic involution.

THE BUILD-UP OF TEC MICROENVIRONMENTS

The initiation of TEC development coincides with the onset of thymus organogenesis, which starts around day 9-10 of the murine embryonic gestation (E9-10) (4). The expression of Forkhead box protein N1 (Foxn1) in the ventral area of the common thymus and parathyroid primordium marks a critical step in TEC specification (5). Still, Foxn1 expression needs to be continuously maintained during the differentiation of c/mTEC, wherein it imposes a complex genetic program that confers them the capacity to support distinct stages of thymopoiesis (6). TEPs formed during early thymus ontogeny constitute the primordial building blocks for the establishment and maintenance of c/mTEC microenvironments (7–9). Our comprehension about the mechanisms underlying TEC differentiation has considerably advanced with the identification of distinct populations containing bipotent or lineage-restricted progenitor activity (10–21) [further detailed below and reviewed in (22, 23)]. These studies led to the proposal of different refined models of TEC differentiation, whereby TEPs traverse through transitional stages that share a closer or distinct relationship with cTEC- or mTEC-unipotent precursors, prior to the commitment in mature c/mTEC subsets [reviewed in (2, 24, 25)]. Yet, it remains unclear the trajectories and molecular elements governing the differentiation of TEC progenitors into mature c/mTEC lineages.

The expansion and functionalization of c/mTEC compartment during early postnatal stages generates a supportive microenvironment that increases thymopoiesis, reaching its peak during young adulthood. Thereafter, T cell production progressively declines with aging, becoming residual in the aged thymus (26). During these periods, TECs undergo concomitant alterations in their composition and differentiation program. Although the density of TECs based on flow cytometry analysis might be underestimated (27), the number of TECs vigorously expands during postnatal life and early adulthood, followed by a progressive decline with age (28, 29). Changes in the size of TEC microenvironment appears to relate with the function of the thymus. While a reduction in the TEC compartment below a certain threshold restrains thymopoiesis (30, 31), the expansion of the thymic epithelial niche, for example *via* transgenic expression of Foxn1 or Cyclin D1, increases T cell generation (32, 33). Along this line, the frequency of cycling TECs is elevated during fetal life, progressively declines during the postnatal life and become a rare fraction in the aged mouse thymus (28). Transcriptomic analysis revealed that the expression of cell-cycle regulators is downregulated in TECs as early as 1 month (34). Moreover, the enforced expression of cMyc in TECs promotes the expansion of the TEC compartment, *via* the engagement of a genetic program akin to the one found in embryonic TECs (35). These results suggest that the loss in the proliferative rate of TECs, together with other alterations such as changes in cell survival and rate of differentiation, may contribute to a reduction in the size of TEC compartments with age. In the next sections, we outline specific cellular and molecular alterations that take place in c/mTEC during early postnatal life, and conjecture how those changes may anticipate subsequent functional losses in the capacity of TECs to sustain regular thymopoiesis in the long-term.

THE ASSEMBLY OF FUNCTIONALLY DEDICATED CTEC AND MTEC COMPARTMENTS

The first weeks of the postnatal life marks a period of intense turnover and functional diversification in the TEC niche, wherein key mature subsets in tolerance induction are generated or expanded (23). During this period, the changes in the cellularity and functionality of cTECs appear to unfold concomitant with the expansion and diversification of mTECs (11, 12, 36–38). This leads to a conspicuous inversion in the cTEC/mTEC ratio within the first 2 weeks after birth, which correlates with the intensification of thymopoiesis (11, 12, 28). In this regard, the consequent rise in the number of positive and negative selection events, will impose an increase demand on TEC compartments. Given that mature cTECs and mTECs have a limited life-span, the maintenance and specialization of their microenvironment seem to depend on the continual differentiation of their progenitors. These functional requirements are in part met by a symbiotic relationship with thymocytes (discussed further below) that stimulate specific proliferative and differentiation programs in TECs (39).

It remains surprising how little we know about the molecular program that underlies the differentiation of cTECs. Despite these gaps, several studies highlight that cTECs undergo molecular and functional changes during neonatal and puberty periods. In particular, cTECs downregulate the expression of key thymopoietic factors, such as Dll4 and IL-7, during the first weeks of postnatal life, which result from continual lymphoepithelial interactions (37, 38, 40, 41). These quantitative and qualitative disruptions in cTECs appear to anticipate the bona fide hallmarks that characterize TECs in the involuted thymus. In contrast to cTECs, our understanding of the cartography of mTEC differentiation is more complete (22). This process depends on reciprocal signals provided by several types of hematopoietic cells (1). These lymphoepithelial interactions, commonly referred as thymic crosstalk, engage specific members of the tumor necrosis factor receptor superfamily (TNFRSF), including receptor activator of NF-κB (RANK), CD40 and lymphotoxin β receptor (LTβR), in mTECs and their progenitors, leading to the activation of a nuclear factor kappa B (NF-κB)-dependent maturation program [reviewed in (1, 22)]. The cooperative action of TNFRSF members is not only important for the expansion of mTEC niches but also for their functional diversification. Upon the initial subdivision in

mTEC^{low} and mTEC^{high} (42), the discovery of Autoimmune regulator (Aire)-, Ccl21- and forebrain embryonic zinc finger-like protein 2 (Fezf2)-expressing cells revealed that mTECs harbors a variety of functionally distinct mature subsets (1, 22). Although Aire$^+$ and Fezf2$^+$ cells emerge during embryonic life (1, 22), their abundance significantly increases in the first weeks of life. In this regard, RANK-mediated signaling is essential to the expansion of Aire$^+$ mTECs, whereas CD40 also contributes to this process (43, 44). Although LTβR signaling was initially coupled to the development of Aire$^+$ (45) and Fezf2$^+$ lineages (46), subsequent studies indicated its involvement in the architecture of postnatal medullary compartment (47). Aire and Fezf2 regulate the capacity of mTECs to express large sets of non-overlapping tissue restricted antigens (TRAs), which are randomly organized in patterns of gene expression at the single cell level (48–50) and are reported to decrease their levels with age (51–53). In this regard, an earlier study underscore the importance of Aire expression in mTECs during neonatal period (54), which corelates with their capacity to control the generation of a unique population of T regulatory cells (55). It remains to be determined whether Aire expression during this temporal window particularly impacts on the quantity or quality of TRAs expression by mTECs.

The role of mTECs in tolerance induction extends beyond their promiscuous gene expression capacity. CCL21-producing cells represent a prototypical example of alternative roles of mTECs. CCL21-expressing mTEC represent a subset of mTEC^{lo} and control the migration of positively selected thymocytes towards the medulla (56, 57). CCl21$^+$ cells emerge during embryogenesis and their numbers also undergo a marked increase during the first weeks of life (57). Recent single cell RNA sequencing analysis suggests that Aire- and Ccl21a-expressing mTEC subsets do not share a direct lineage relationship (58). Moreover, the discoveries that Aire$^+$mTECs differentiate into Post-Aire cells (59, 60) further extended our view on the heterogeneity within thymus medulla. Post-Aire mTECs shutdown the expression of Aire, certain TRAs, CD80 and MHCII, while acquiring traits of terminally differentiated keratinocytes (61, 62). Two reports identified a highly differentiated mTECs that share molecular traits with tuft cells found at mucosal barriers. Fate-mapping analysis suggests that this subset can develop *via* an AIRE-dependent and AIRE-independent pathway (63, 64). Although their complete functional relevance remains elusive, tuft-like mTECs appear to regulate the development of invariant NKT cells and ILCs (63, 64). Future studies may uncover new specialized mTEC subsets and their role in imposing the limits of tolerance, or alternative processes in thymus biology.

THE THYMIC EPITHELIAL CELL PROGENITOR RESERVOIR

The diversification of TECs during the first weeks of life is dictated by the intricate balance between the rate of proliferation and differentiation of mature subsets. The rapid turnover of TEC microenvironments, with an estimated replacement time of one to two weeks to mTECs (28, 59), implicates the requirement for a regular generation of mature TECs from their upstream progenitors. One possibility is that bipotent TEPs continually produce lineage-committed precursors lacking long-term self-renewal capacity. Alternatively, and not mutually exclusive, the abundance of bipotent TEPs might decrease with age, being the maintenance of cortical and medullary epithelial niches assured by downstream compartment-restricted precursors. In the last years, several studies provide evidence for the existence of an arsenal of subsets enriched in purported bipotent TEC progenitors in the postnatal thymus (10, 13–15). One approach has employed *in vitro* 2D-clonogenic (10) or spheroids (13) assays to respectively isolate TEC progenitors that reside within EpCAM$^+$Ly51$^+$cTECs or EpCAM$^-$ cells, which were expanded *in vitro* and revealed the capacity to give rise to c/mTEC. Nonetheless, a more recent study indicate that cells isolated from EpCAM$^-$derived spheroids represent mesenchymal progenitors (65). Other methodologies resolved bipotent progenitor activity within defined subsets of UEA-1$^-$MHII^{lo} Sca-1$^+$ TECs (14) and MHCII^{hi} Ly51$^+$Plet1$^+$ cTECs (15). Both strategies employ reaggregate organ cultures (RTOCS) to determine the precursor-product lineage relationship to mature cells. Despite the advances, it remains to be determined the physiological contribution of these cells to the TEC microenvironment in the adult thymus. Thus, we still lack experimental evidence that demonstrates the existence of bona-fide bipotent TEC progenitors in the postnatal thymus, and their identification at the single cell level.

Downstream of TEC progenitors, complementary studies documented how mTEC compartments evolved from bipotent TEP and mTEC-restricted precursors (mTEPs), including mTEC-restricted SSEA-1+ and podoplanin+ (PDPN) mTEPs (16, 18). Fate-mapping studies show that the adult mTEC network arise from fetal- and newborn-derived TEPs expressing beta5t (β5t), a prototypical cTEC marker. Yet, the contribution of β5t+ TEPs to the adult mTEC niche decreases with age (19, 20), suggesting that the maintenance of the adult medullary epithelium is assured by mTEPs. Although bipotent TEPs might lose the expression of some traits found in the embryo (e.g. β5t), it is also possible that the abundance and/or the self-renewal properties of bipotent TEPs and/or lineage-restricted progenitors decline with time. Supporting this view, the clonogenic activity of purported bipotent TEPs that reside within the cortex decrease with age (10) and Cld3,4$^+$SSEA1$^+$ mTEC-restricted cells become rare in the adult thymus (16). Given that the numbers of embryonic TEPs dictates the size of functional TEC microenvironments (30), we infer that the loss in the TEC network that takes place with age may result from the decrease in the bioavailability and self-renewal capacity of TEPs early in life.

The advent of single cell RNA sequencing (scRNAseq) analysis have also contributed to our understanding of the heterogeneity and dynamic of TEC progenitors. This approach has emerged as a new unbiased method to identify novel subsets, providing a valuable platform to analyze their developmental

trajectories and determine their relationships with progenitor subsets identified by conventional methodologies. In this regard, new clusters termed "pre-Aire mTEC 1 and 2" (66) appear to present molecular traits similar to the ones found in podoplanin+ (PDPN) mTEPs (18). A subsequent study identified a novel cluster of "intertypical TECs" (51) that harbors traits akin to the ones found in podoplanin+ (PDPN) mTEPs (18), UEA-1^{-}MHII^{lo}Sca-1^{+} (14) and MHCII^{hi} Ly51^{+}Plet1^{+} (15) TECs. Since "intertypical TECs" are further segmented in distinct 4 subclusters, it would be interesting to determine if they associate to a particular bipotent or unipotent subset. Moreover, scRNAseq analysis reveal the existence of a previously unrecognized cluster of "perinatal cTECs". Interestingly, this subset harbors cells with a highly proliferative status and their abundance declines with age (51). Moreover, the combination of scRNAseq and fate mapping analysis revealed that $\beta5t^{+}$ TEPs acquire senescent-like properties with age, potentially explaining their failure to contribute to mTEC lineage beyond the neonatal stage (19, 20). Together, these findings indicate that the integration of multiple experimental approaches provides a more complete strategy to resolve the intricacies of the TEC compartment. Future studies should attempt to identify specific markers to resolve the newly characterized populations at a single level.

CONCLUDING REMARKS

The aforementioned studies underscore that the period between birth and early adulthood is a time of intense alterations in TEC microenvironments, which prepares them to the highly demand role of choreographing the selection of growing number of T cell precursors. In this sense, it is remarkable to appreciate the synchronous coordination between TEC differentiation and the requisites imposed by T cell development. Yet, the erosion of the pool of TEC progenitors seem to accompany the generation of specialized subsets with key roles in tolerance induction. We reason that an in-depth molecular analysis of TEC differentiation during early postnatal may provide insights on how TEC niches are maintained, and can be repaired in the aged thymus. Despite recent advances, it remains unclear how changes in the bioavailability of TEPs impact on the maintenance of TEC microenvironment across life, and ultimately on thymic output. Another unexplored area pertains to the physiological causes underlying the presumed age-dependent decrease and/or senescence of TEPs. Knowledge in these areas will not only permit to comprehend the basic principles that governs thymic function, but also target pathways for the treatment of disorders coupled to dysfunctional thymic/T cell responses.

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NA and RP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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RESEARCH REPORT

Identification of fibroblast progenitors in the developing mouse thymus

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ABSTRACT

The thymus stroma constitutes a fundamental microenvironment for T-cell generation. Despite the chief contribution of thymic epithelial cells, recent studies emphasize the regulatory role of mesenchymal cells in thymic function. Mesenchymal progenitors are suggested to exist in the postnatal thymus; nonetheless, an understanding of their nature and the mechanism controlling their homeostasis *in vivo* remains elusive. We resolved two new thymic fibroblast subsets with distinct developmental features. Whereas CD140αβ$^+$GP38$^+$SCA-1$^-$ cells prevailed in the embryonic thymus and declined thereafter, CD140αβ$^+$GP38$^+$SCA-1$^+$ cells emerged in the late embryonic period and predominated in postnatal life. The fibroblastic-associated transcriptional programme was upregulated in CD140αβ$^+$GP38$^+$SCA-1$^+$ cells, suggesting that they represent a mature subset. Lineage analysis showed that CD140αβ$^+$GP38$^+$SCA-1$^+$ maintained their phenotype in thymic organoids. Strikingly, CD140αβ$^+$GP38$^+$SCA-1$^-$ generated CD140αβ$^+$GP38$^+$SCA-1$^+$, inferring that this subset harboured progenitor cell activity. Moreover, the abundance of CD140αβ$^+$GP38$^+$SCA-1$^+$ fibroblasts was gradually reduced in *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ thymi, indicating that fibroblast maturation depends on thymic crosstalk. Our findings identify CD140αβ$^+$GP38$^+$SCA-1$^-$ as a source of fibroblast progenitors and define SCA-1 as a marker for developmental stages of thymic fibroblast differentiation.

KEY WORDS: Thymic mesenchymal cells, Thymic stroma, Thymus, Progenitors, Mouse

INTRODUCTION

The thymic microenvironment offers a unique inductive site for the generation of functionally diverse and self-tolerant T cells. The thymic stroma is formed by cells of non-haematopoietic origin, such as thymic epithelial cells (TECs), endothelial cells and thymic mesenchymal cells (TMCs), and cells of haematopoietic origin, including dendritic cells and monocytes/macrophages (James et al., 2021a). The development of this heterogeneous microenvironment starts in the embryo and continues during postnatal life, involving the participation of cells from all three embryonic germ layers: endoderm-derived epithelium, neuroectoderm-derived neural-crest (NC) mesenchyme and mesoderm-derived haematopoietic and endothelial cells (Gordon and Manley, 2011). Given the non-redundant role of TECs in T-cell development, there has been considerable interest in studying the mechanisms that control TEC differentiation and function. However, several studies underscore the contribution of other non-epithelial stromal cells in shaping TEC and T-cell differentiation (Nitta et al., 2021).

In particular, TMCs, including fibroblasts, vascular-supporting pericytes and smooth muscle cells, exert a pleiotropic role in thymus biology (Nitta et al., 2021). At an early stage of thymus organogenesis, NC-derived mesenchymal cells surround the thymic primordia and provide fibroblast growth factor 7 (FGF7), FGF10, epidermal growth factor (EGF) and insulin-like growth factor (IGF), which contribute to the growth of the TEC microenvironment (Jenkinson et al., 2003; Jenkinson et al., 2007). Interestingly, FGF7/10-producing cells also express retinoic acid, which suppresses the proliferation of cortical TECs (Sitnik et al., 2012; Wendland et al., 2018). Thus, TMCs have the functional capacity to positively and negatively control the size of the TEC compartment. Thymic fibroblasts also produce a range of extracellular matrix (ECM) components, which can capture and present crucial thymopoietic factors (e.g. IL7 and CCL21) to the developing T cells (Banwell et al., 2000; James et al., 2021b). Moreover, vascular-associated pericytes and smooth muscle cells surrounding the endothelium regulate thymic vasculature and T-cell egress (Zachariah and Cyster, 2010; Sitnik et al., 2016). Particularly, TMCs create sphingosine-1-phosphate (S1P) gradients that promote the egress of mature T cells from the thymus (Zachariah and Cyster, 2010). More recently, medullary fibroblasts have been implicated in T-cell tolerance (Nitta et al., 2020). Despite the aforementioned functional diversity, distinct TMC subsets share a precursor-product relationship with NC cells (Müller et al., 2008; Foster et al., 2008; Sitnik et al., 2016). Still, our understanding of the mechanisms that control the differentiation and the turnover of mature TMCs remains incomplete. Moreover, although thymic mesenchymal progenitors are considered to exist in the adult thymus (Sitnik et al., 2016), their nature and functional competence remain poorly characterized *in vivo*.

Herein, we resolved a previously unidentified population of thymic fibroblast progenitors and uncovered a checkpoint in mesenchymal differentiation that depends on thymic crosstalk. Our findings offer a roadmap to monitor TMC homeostasis in ageing and regeneration.

RESULTS AND DISCUSSION

Analysis of thymic fibroblast differentiation during development

Several markers, including CD140α (PDGFRA), CD140β (PDGFRB), GP38 (PDPN), ER-TR7, MTS-15, SCA-1 (Ly6a), αSMA (ACTA2), CD146 (MCAM), CD34, Ly51 (ENPEP), Itga7

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and DPP4 have been used to phenotypically identify specific populations of TMCs (Jenkinson et al., 2003; Jenkinson et al., 2007; Gray et al., 2007; Foster et al., 2008; Sitnik et al., 2012; Patenaude and Perreault, 2016; Sitnik et al., 2016; Sheridan et al., 2017; Nitta et al., 2020). Nonetheless, as some of these markers are also expressed by other cell types, they cannot specifically define distinct differentiation states of TMCs when employed in a restrictive manner. To dissect the heterogeneity within TMCs, we sought to identify cells expressing progenitor hallmarks within the entire postnatal mesenchymal compartment. We selected the postnatal day 7 thymus, as a period when the main haematopoietic, epithelial and mesenchymal subsets were present. Employing multiparameter flow cytometry, we analysed the expression of ten well-known cell-surface markers. To discriminate haematopoietic, epithelial, endothelial and erythroid lineages, we included CD45 (PTPRC), EpCAM, CD31 (PECAM1) and Ter119 (Ly76), respectively. For the analysis of TMCs, we initially considered the following markers: CD140α, CD140β, GP38, SCA-1, Ly51 and αSMA. Flow cytometry data of non-haematopoietic and non-epithelial cells was analysed by nonlinear dimensionality reduction algorithms, producing maps that clustered cells based on their phenotypic similarity [t-distributed stochastic neighbour embedding (t-SNE)] (Fig. 1A). This unsupervised approach revealed three main clusters within CD45$^-$EpCAM$^-$ cells. Cluster 1 was formed by CD31$^+$SCA-1$^+$ cells, cluster 2 comprised CD140α$^+$β$^+$GP38$^+$ cells, and cluster 3 contained CD140α$^-$β$^+$Ly51$^+$ cells (Fig. 1B). Changes in SCA-1 and αSMA expression, respectively, showed an additional layer of heterogeneity within clusters 2 and 3: whereas the differential expression of SCA-1 identified sub-clusters 2.1 (CD140α$^+$β$^+$GP38$^+$SCA-1$^-$) and 2.2 (CD140α$^+$β$^+$GP38$^+$SCA-1$^+$), alterations in αSMA expression distinguished sub-clusters 3.1 (CD140α$^-$β$^+$Ly51$^+$αSMA$^-$) and 3.2 (CD140α$^-$β$^+$Ly51$^+$αSMA$^+$) (Fig. 1B). Employing a directed gating strategy, we identified the same TMC subsets: CD140α$^+$β$^+$GP38$^+$SCA-1$^-$ (2.1), CD140α$^+$β$^+$GP38$^+$SCA-1$^+$ (2.2), CD140α$^-$β$^+$Ly51$^+$αSMA$^-$ (3.1) and CD140α$^-$β$^+$Ly51$^+$αSMA$^+$ (3.2) (Fig. 1C, Fig. S1). These results suggested that cluster 1 defined endothelial cells, cluster 2 included fibroblasts and cluster 3 identified endothelial-supporting mesenchymal cells, which can be further subdivided into pericytes (3.1) and smooth muscle cells (3.2) (Sitnik et al., 2016). Our observations further showed that the differential expression of CD140α can be used to distinguish fibroblasts (CD140α$^+$β$^+$) from pericyte-like cells (CD140α$^-$β$^+$). Moreover, SCA-1-expressing thymic fibroblasts (2.2) have been

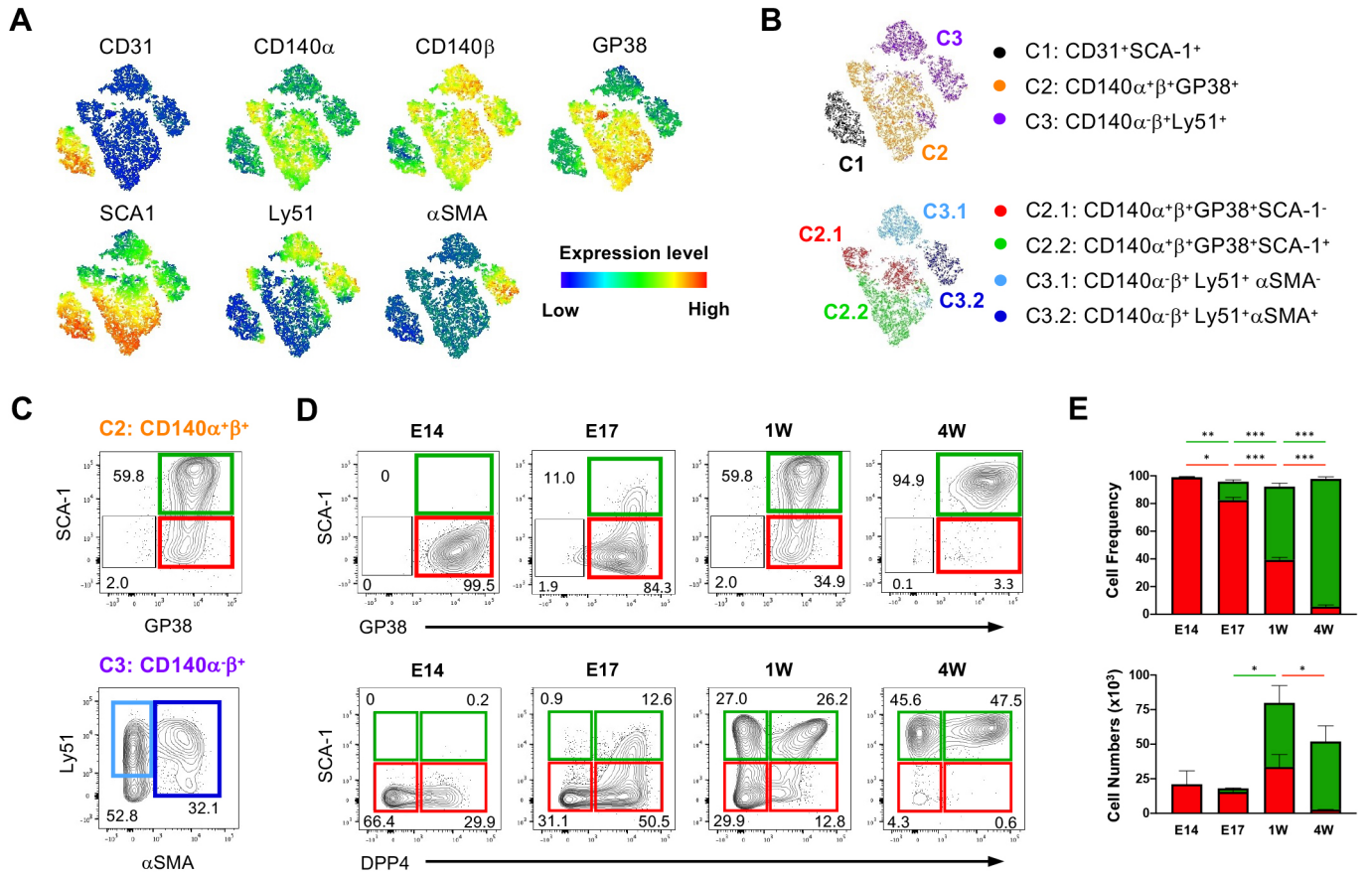

Fig. 1. GP38 and SCA1 expression on TMC subsets. (A) Total thymi cells from 1-week-old mice were isolated, and total TMCs (CD45$^-$EpCAM$^-$) were analysed by flow cytometry. t-SNE representation of the expression of CD31, CD140α, CD140β, GP38, SCA-1, Ly51 and αSMA. (B) Three main clusters were identified: cluster 1 (CD31$^+$), cluster 2 (CD140α$^+$β$^+$) and cluster 3 (CD140α$^-$β$^+$). Clusters 2 and 3 were respectively subdivided into cluster 2.1 (CD140α$^+$β$^+$GP38$^+$SCA1$^-$) and 2.2 (CD140α$^+$β$^+$GP38$^+$SCA1$^+$); and 3.1 (CD140α$^-$β$^+$Ly51$^+$αSMA$^-$) and 3.2 (CD140α$^-$β$^+$Ly51$^+$αSMA$^+$). (C) TMCs (CD45$^-$EpCAM$^-$CD31$^-$) were analysed for the indicated markers, and sub-cluster 2.1 (red gate), 2.2 (green gate), 3.1 and 3.2 (light- and dark-blue gates) were identified. (D) Analysis of GP38, SCA-1 and DPP4 expression in TF^A (red gate) and TF^B (green gate) populations at E14, E17, 1 week old (W) and 4W. Numbers in plots indicate the frequency of cells found within each gate. Plots are of a representative analysis per time point. (E) Bar graphs showing mean+s.d. of the frequency and cellularity of TF^A and TF^B subsets, of three independent analyses per time point. Differences in TF subsets, CD140α$^+$β$^+$GP38$^+$SCA1$^-$ (red) and CD140α$^+$β$^+$GP38$^+$SCA1$^+$ (green), were statistically analysed at different ages: ***P<0.001, **P<0.01, *P<0.05.

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previously reported (Patenaude and Perreault, 2016; Sheridan et al., 2017). Yet, the segregation of CD140α$^+$β$^+$GP38$^+$ in SCA-1$^-$ (2.1) and SCA-1$^+$ (2.2) was intriguing and led us to direct our attention to these subsets. We refer hereafter to cells within cluster 2.1 (CD140α$^+$β$^+$GP38$^+$SCA-1$^-$) and cluster 2.2 (CD140α$^+$β$^+$GP38$^+$SCA-1$^+$) as thymic fibroblast A (TF^A) and B (TF^B), respectively.

To examine whether TF^A and TF^B defined two distinct subsets, we analysed their development during thymic ontogeny and postnatal life. TF^A predominated at embryonic day (E) 14 and their numbers were relatively constant up to the first week of postnatal life, followed by a decrease in the 4-week-old thymus. Contrarily, TF^B cells arose around E17 and expanded in frequency and number during the perinatal period (E17 to 4 weeks old) (Fig. 1D,E). We further addressed how the differentiation of TF^A and TF^B related to recently described medullary (DPP4$^-$) and capsular (DPP4$^+$) fibroblasts (Nitta et al., 2020). At E14.5, a period wherein TF^B were virtually absent, TF^A contained DPP4$^+$ and DPP4$^-$ cells. The first TF^B (SCA-1$^+$) appeared at E17 and were mostly DPP4$^+$, suggesting that their immediate precursors could be within the TF^{A}DPP4$^+$ population. From the postnatal period onwards, TF^B contained both DPP4$^-$ and DPP4$^+$ cells (Fig. 1D). A population of TF^A expressing low levels of DPP4 persisted in 1-week-old thymi (Fig. 1D). In line with a previous report (Nitta et al., 2020), the observation that DPP4$^-$ and DPP4$^+$ cells appeared in the early embryonic TF^A subset may suggest that segregation of capsular and medullary sub-lineages occurs early in thymic development. Moreover, our results indicate that SCA-1 expression was acquired firstly by capsular (DPP4$^+$) fibroblast followed by medullary (DPP4$^-$) counterparts. As such, the acquisition of SCA-1 expression appears to represent a maturation marker commonly acquired by capsular and medullary thymic fibroblasts and does not by itself discriminate these subsets. The developmental kinetic of TF^A and TF^B led us to consider that they could represent distinct stages of the same differentiation pathway. In this scenario, TF^A should contain precursors with the potential to differentiate into TF^B. Alternatively, TF^A and TF^B could define unrelated thymic mesenchymal cells. We conducted genome-wide transcriptional and lineage-tracing experiments to investigate further the precursor-product relationship between these subsets.

TF^A and TF^B subsets have distinct transcriptional programmes

To examine whether TF^A and TF^B identified different states of fibroblast differentiation, we characterized their genome-wide transcriptional profile by employing RNA-sequencing analysis. TF^A and TF^B were purified by cell sorting from the 1-week-old thymus, a period wherein these subsets were equally represented. Additionally, we purified endothelial-supporting mural cells (MCs) (cluster 3) and included them as a complementary reference population in the transcriptional analysis. Principal component analysis showed that the biological replicates of each subset clustered together, demonstrating that these populations had low intrapopulation variability. Moreover, TF^A and TF^B were more closely related to each other than to MCs (Fig. 2A, Fig. S2A, Table S1). Employing available transcriptomic data sets from other studies (Patenaude and Perreault, 2016; Sitnik et al., 2016; Nitta et al., 2020), we extracted sets of genes associated with fibroblasts, vascular-supporting cells, and cross-examined their expression pattern in TMC subsets. First, the expression of genes used as phenotypic markers to define TF^A, TF^B and MC subsets followed the expected pattern, validating the accuracy of the purified samples. Second, most fibroblasts-associated genes were upregulated in TF^A to TF^B, whereas transcripts linked to vascular-supporting cells were specifically enriched in MCs (Fig. 2B, Table S2). Moreover, an unsupervised cross-analysis of genes linked to capsular and medullary fibroblasts (Nitta et al., 2020), revealed that these

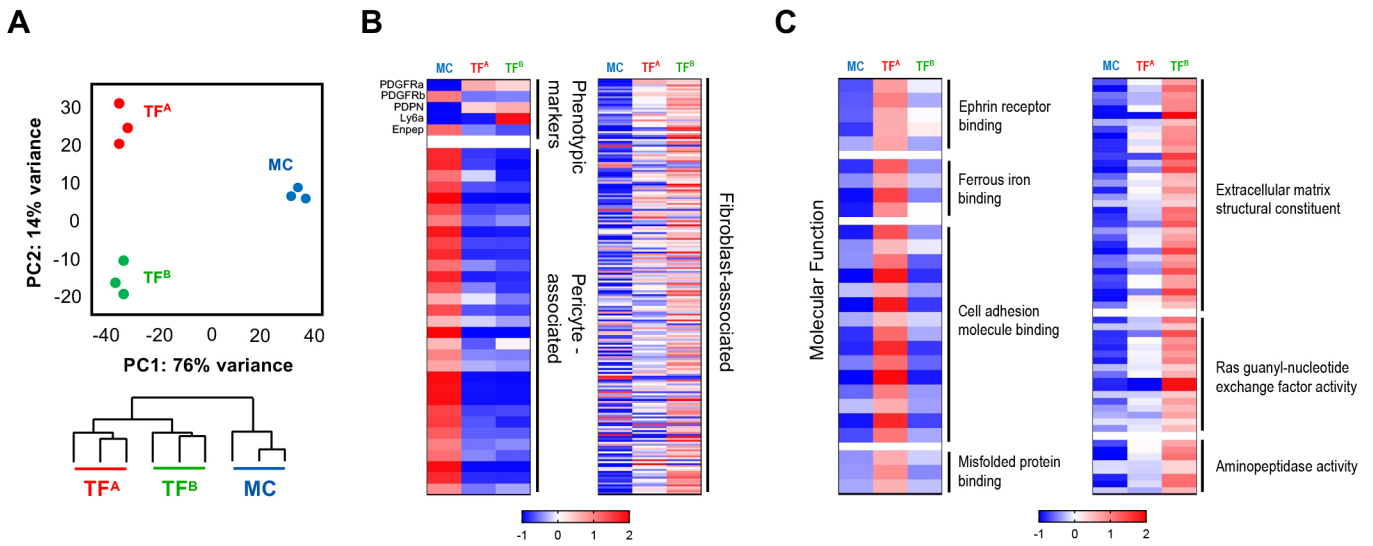

Fig. 2. Genome-wide transcriptomic analysis of TF subsets identifies stages with distinctive gene expression profiles. (A) Principal component analysis plot and dendrogram, detailing the hierarchical clustering between the biological samples, performed with data obtained from total RNA-sequencing analysis of sorted TF^A (CD45$^-$EpCAM$^-$GP38$^+$SCA-1$^-$) (*n*=3), TF^B (CD45$^-$EpCAM$^-$GP38$^+$SCA-1$^+$) (*n*=3) and MC (CD45$^-$EpCAM$^-$GP38$^-$SCA-1$^-$Ly51$^+$) (*n*=3) populations. (B) Heat maps representing the deviation from average expression of the phenotypic markers used to identify TMC populations, of genes previously associated with pericytes and of genes previously associated with thymic fibroblasts. (C) Heat maps representing the deviation from average expression of the uniquely upregulated genes identified for populations TF^A and TF^B and the associated molecular functions identified by GO analysis. Genes with FDR<10% were considered as differentially expressed. Enriched GO terms (molecular functions) were identified using MGSA. Represented categories had a marginal posterior probability estimate higher than 0.65.

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transcripts were greatly increased in TF^B (Fig. S2B, Tables S3, S4). These observations were in line with the representation of capsular and medullary subsets within TF^A and TF^B in the 1-week-old thymus (Fig. 1E) and support their fibroblastic identity. Further bioinformatic analysis identified 470 and 721 uniquely upregulated genes in TF^A and TF^B, respectively (Fig. S2C, Tables S5, S6). Gene ontology (GO) enrichment analysis of these sub-lineage specific sets revealed a stringent association to diverse functional categories. Specifically, genes enriched in TF^A were linked to broad cellular processes, including ephrin receptor signalling, cell adhesion, binding to iron and misfolded protein. By contrast, genes upregulated in TF^B were associated with more restricted processes, including ECM components, GTPase signalling and aminopeptidase activity (Fig. 2C, Tables S7, S8). Several collagen genes were upregulated in TF^B, consistent with the association with ECM constituents (Fig. S2D, Table S9). Recent findings implicated LTβR-mediated signalling in thymic medullary fibroblast differentiation (James et al., 2018; Nitta et al., 2020). Detailed analysis of members of the TNFRSF family showed that *Ltbr*, *Tnfrsf1b*, *Tnfrsf12a* and *Tnfrsf23* were specifically upregulated in TF^B (Fig. S2E, Table S6). Together, our results suggest that TF^A may contain more immature cells, whereas TF^B appear to define mature thymic fibroblasts.

TF^A can give rise to TF^B and their homeostasis is altered in the alymphoid thymus

The observations that TF^B developed at E17 presumably from TF^A suggested a possible precursor-product lineage relationship between these populations. To assess this hypothesis, we first established fetal thymic organ cultures (FTOCs) with E14 thymi, a stage at which TF^B were virtually absent. TF^B emerged after 4 days of culture, partially phenocopying the composition of TF subsets in the E17 thymus (Fig. S3A). These results suggested that TF^B precursors already existed in the E14 thymus and that subsequent intrathymic interactions may promote their differentiation. To determine the lineage potential of $TF^{A/B}$ in the postnatal thymus, we purified (by fluorescence-activated cell sorting) these populations from 1-week-old-thymus and established reaggregate thymus organ cultures (RTOCs). TF subsets were isolated from the thymus of $Actin^{RFP}$ reporter mice (Meireles et al., 2017) and mixed with wild type (WT)-derived embryonic thymic cells (carriers). In this system, RFP expression is constitutively active in 'spiked' cells ($TF^{A/B}$), providing an intrinsic label for lineage-tracing analysis of TF subsets (Fig. 3A, Fig. S3B). The differentiation potential of TF subsets was analysed after 7 days of culture. Whereas TF^B largely maintained their phenotype, TF^A gave rise to TF^B (Fig. 3B). None of the two subsets originated vascular-supporting cells ($CD140\alpha^-\beta^+Ly51^+$) (data not shown). In both RTOCs, embryonic carrier cells (RFP^-), which are mostly composed of TF^A, followed the same differentiation trajectory (Fig. S3B,C). These results suggested that TF^B represents a more committed fibroblast population, whereas the TF^A population contains cells with fibroblast progenitor activity.

It is well recognized that the establishment of epithelial microenvironments depends on functional bidirectional interactions between haematopoietic cells and TECs (Rodrigues et al., 2018). A recent study showed that the differentiation of thymic medullary fibroblasts also depends on signals provided by developing thymocytes (Nitta et al., 2020). Thymic organotypic cultures allow the normal programme of T-cell and TEC differentiation (Ribeiro et al., 2013; Meireles et al., 2017). Thus, the observations that TF^A gave rise to TF^B in FTOC and RTOC led us to consider whether there was a stage-specific requirement for thymocyte crosstalk during thymic fibroblast differentiation. To evaluate this possibility, we analysed TF development in mutant mice in which thymocyte development is inhibited at different stages. Whereas in $Rag2^{-/-}$ mice T-cell development is blocked at the double negative (DN) 3 stage, $Rag2^{-/-}Il2rg^{-/-}$ mice display a premature and more severe arrest in thymocyte development (Ribeiro et al., 2013; Meireles et al., 2017). Relative to the WT thymus, the proportion of TF^B was profoundly affected in the 1- and 4-week-old $Rag2^{-/-}Il2rg^{-/-}$ thymus, leading to an accumulation of $GP38^{-/low}$ cells and an overall reduced GP38 expression at 1 and 4 weeks of age (Fig. 3C). The frequency of TF^B in $Rag2^{-/-}$ thymus was also reduced in the 1-week-old-thymus relative to WT counterparts, although to a lesser extent compared with $Rag2^{-/-}Il2rg^{-/-}$. However, the representation TF^B in $Rag2^{-/-}$ thymus at 4 weeks was similar to that observed in the WT thymus. Strikingly, the numbers of TF^B were markedly reduced in both 1- and 4-week-old $Rag2^{-/-}$ and $Rag2^{-/-}Il2rg^{-/-}$ thymus compared with WT counterparts (Fig. 3C). The results in the $Rag2^{-/-}Il2rg^{-/-}$ thymus cannot formally exclude an additional role for γ_c-mediated signalling in thymic fibroblast differentiation. Some reports indicate that γ_c cytokine family may also affect the function of non-haematopoietic stromal cells, such as endothelial cells (Leonard et al., 2019). However, the observation that TF^B differentiation was also impaired in the $Rag2^{-/-}$ thymus, wherein γ_c-mediated signalling was intact, supports the hypothesis that thymic fibroblast maturation is controlled by cooperative signals provided by thymocytes passing the β selection checkpoint. In this regard, the maturation of medullary fibroblast also required cellular interactions with mature TCRαβ-expressing thymocytes (Nitta et al., 2020). Moreover, it remains unknown whether mature thymic fibroblasts in the adult thymus are replaced by dedicated progenitors. A mesenchymal progenitor population referred to as $CD34^+$ adventitial cells ($CD34^+GP38^+$) has been previously reported to exist in the adult thymus (Sitnik et al., 2016), and adult-derived $CD34^+$ adventitial cells presented bipotent mesenchymal potential capable of generating fibroblast and pericytes (Sitnik et al., 2016). TF^A isolated within the postnatal thymus revealed a more fibroblastic-restricted progenitor activity. Further studies should determine whether $CD34^+$ adventitial cells and TF^A are developmentally unrelated or define distinct stages of the same TMC differentiation process. Moreover, future analysis should resolve whether $DPP4^-$ and $DPP4^+$ existing within $TF^{A/B}$ at different stages of life represent unipotent or bipotent precursors of thymic capsular and medullary fibroblasts. The decline of TF^A with age within the normal thymus, and their maintenance in $Rag^{-/-}Il2rg^{-/-}$, suggests that the pool of TF progenitors is negatively regulated by thymic crosstalk. Interestingly, a similar feedback mechanism has been reported for distinct progenitor TEC subsets. In particular, the maturation of medullary TEC depends on the cooperative role of TNFR superfamily members, including receptor activator of NF-κB (RANK), lymphotoxin β receptor (LTβR) and CD40, which are stimulated by their respective ligands expressed in several haematopoietic cells, namely lymphoid tissue inducer cells, γδ T cells, positively selected double-positive (DP) thymocytes and αβ $CD4^+$ single-positive (SP4) thymocytes (Rossi et al., 2007; Hikosaka et al., 2008; Akiyama et al., 2008; Mouri et al., 2011; Desanti et al., 2012; Roberts et al., 2012). Our results suggest that cooperative signals derived from thymocytes that passed the β selection checkpoint control thymic fibroblast differentiation. These findings indicate that thymocyte-derived signals have a dual effect on thymic stromal differentiation,

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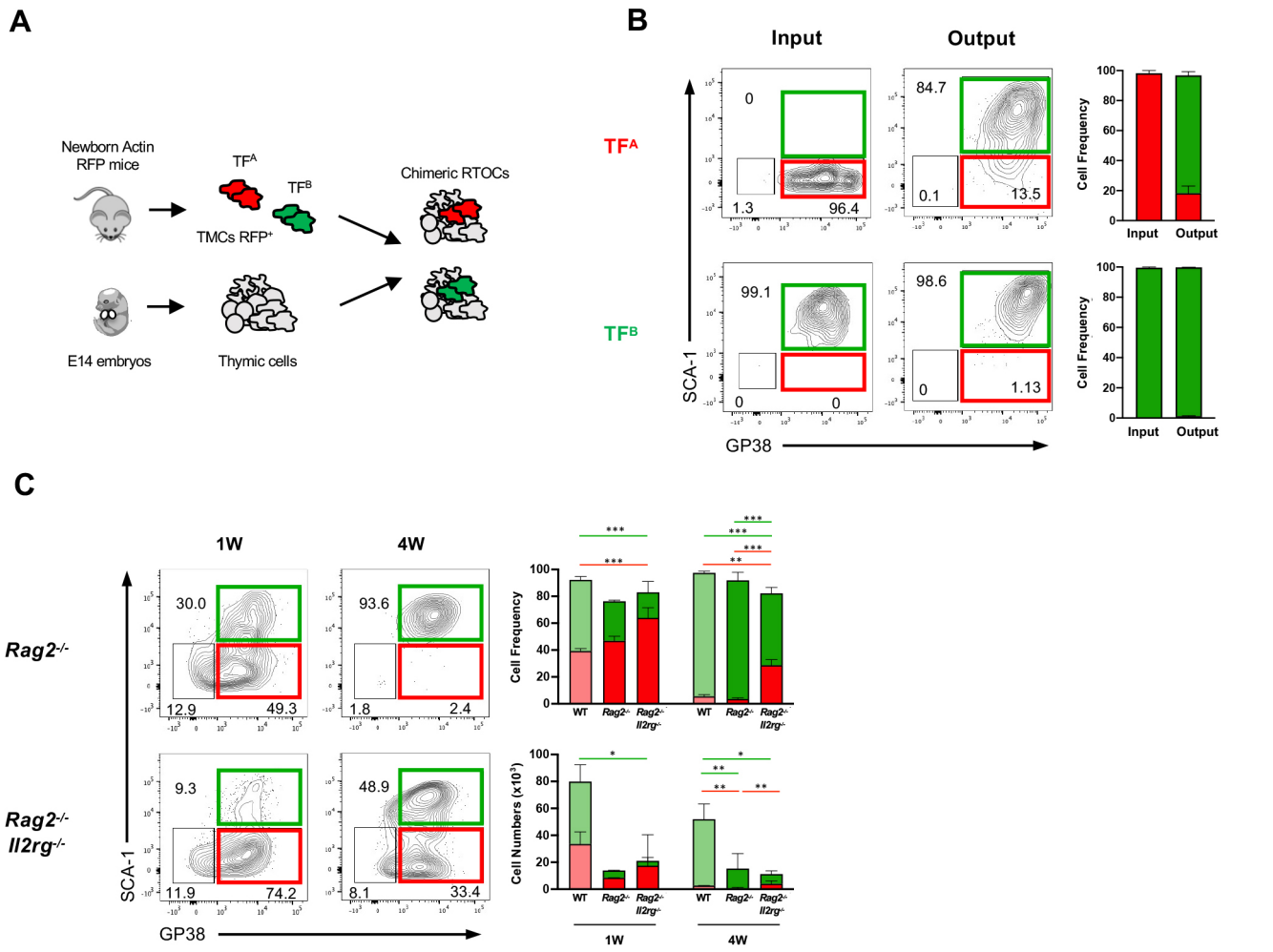

Fig. 3. TF^A contains progenitor cells capable of generating TF^B, in a process dependent on thymic crosstalk. (A) Chimeric RTOCs were established with E14 cells from WT thymus and mixed with TF^A or TF^B cells isolated from the postnatal day 1-3 Actin-RFP mice. (B) Flow cytometry analysis of the chimeric RTOC at day 0 (input) and after 7 days in culture (output). Data presented and bar graphs correspond to mean+s.d. of two independent analyses. (C) Analysis of GP38 and SCA-1 expression within TF populations from 1- and 4-week-old *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Numbers in plots indicate the frequency of cells found within each gate. Flow cytometry plots are of a representative analysis. Bar graphs correspond to mean+s.d. of two (1-week-old *Rag2*$^{-/-}$) and three (1-week-old *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and 4-week-old *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$) independent experiments per time point. Each experiment contains a pool of two to four mice per analysis. The numbers of TF subsets found in the WT thymus are co-represented as a reference and were originally described in Fig. 1. Differences between WT and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ TF subsets at 1 week and between WT, *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ at 4 weeks were statistically analysed: ***P<0.001, **P<0.01, *P<0.05.

promoting the differentiation of mature lineage while depleting the bioavailability of the pool of distinct progenitor cells. Further studies are required to elucidate the signals that control the turnover of thymic fibroblasts *in vivo* and whether this process entails direct thymocyte-fibroblast interactions or is mediated by other cell-cell contacts.

In summary, our study resolves the identity of previously unidentified populations of thymic fibroblast precursors and exposes a checkpoint in TF differentiation that is controlled by thymic crosstalk *in vivo*. These findings represent a roadmap to understanding the processes underlying the establishment of thymic mesenchymal cells in regular and deficient thymopoiesis.

MATERIALS AND METHODS

Mice

WT, *Rag2*$^{-/-}$, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and Actin-RFP mice (Ribeiro et al., 2013; Ribeiro et al., 2014) were all bred on a C57BL/6 background and housed under specific pathogen-free conditions at the I3S animal facility. Experiments were performed under the European guidelines for animal experimentation.

Isolation of thymic stromal cells

Thymic stromal cells were isolated using a protocol previously described to obtain TECs (Meireles et al., 2017), with modifications. Briefly, the thymus was cut into small pieces and subjected to a gentle mechanical dissociation to liberate thymocytes. Thymic fragments were digested for 30 min at 37°C with agitation in PBS containing 20 mg/ml of collagenase D (Roche) and passed through 100-µm filter to remove debris. Further stromal cell enrichment was carried out by incubation with anti-CD45 microbeads (Miltenyi Biotec) according to the manufacturer's instructions.

Flow cytometry

TMCs were isolated as described (Meireles et al., 2017). Cell suspensions were stained with the following antibodies: PerCP-Cy5-conjugated anti-CD45.2 (clone 104, 45-0454-82), PE-conjugated anti-Ly51 (clone 6C3, 12-5891-82), Alexa eFluor 647-conjugated anti-EpCAM (clone G8.8, 14-5791-81), APC-conjugated anti-Ter-119 (clone TER-119, 17-5921-82), all from eBioscience; BV421-conjugated anti-EpCAM (clone G8.8, 118225), BV786-conjugated anti-Sca1 (clone D7, 108139), Alexa 488-conjugated anti-Sca1 (clone D7, 108111), PE-Cy7-conjugated anti-GP38 (clone 8.1.1, 127411), APC-conjugated anti-DPP4 (clone H194-112, 137807), BV605-conjugated anti-CD140α (clone APA5, 135916), all from BioLegend;

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biotinylated anti-CD140β (clone APB5, 136009, BioLegend) was revealed with BV711-conjugated (405241, BioLegend) or PE-Cy7-conjugated streptavidin (SA1012, eBioscience). Intracellular staining with eFluor 660-conjugated anti-αSMA (clone 1A4, 50-9760-82, eBioscience) was performed following cell fixation and permeabilization using the Foxp3/Transcription factor staining buffer set (eBioscience) according to the manufacturer's instructions. Flow cytometry analyses were performed on a LSRFortessa and cells sorted on a FACS ARIA II (both from BD Bioscience) with purities above 95%. Data were analysed using FlowJo software (Tree Star Inc).

RNA sequencing

Total RNA library preparation and high-throughput sequencing of sorted postnatal (P3-5) $TF^{A/B}$ and MC subsets were performed at the EMBL Genomics Core facility (Germany), as previously described (23). Nine sequencing libraries, three for TF^{A}, three for TF^{B} and three for MCs, were prepared using NEB Next RNA ultra protocol (E7530 NEB). Obtained libraries were quantified fluorometrically, pooled in equimolar amounts and sequenced on an Illumina NextSeq 500 sequencer in single-end mode (75 bases), following the manufacturer's instructions (Illumina). The reads were mapped to the mouse genome (GRCm38) using STAR (version 2.4.2a) with GRCm38.99 GTF annotation. The number of reads per gene was generated during the alignment step (quantMode GeneCounts) and gene counts were then analysed with the DESeq2 package (24). Genes with FDR <10% were considered as differentially expressed. Enriched GO terms (biological processes and molecular functions) for the differentially expressed genes were identified using model-based gene set analysis (MGSA) (Bauer et al., 2010). The analysis was performed with ten independent runs of the Markov chain of 1.10^8 steps each. The parameters p, alpha and beta were used as default. Functional categories with a marginal posterior probability estimate higher than 0.65 were retained for further analysis. The hierarchical clustering, represented as a dendrogram, of TEC populations was performed using the hclust function in R on Euclidean distances between the variance of the rlog-transformed read counts for each gene across samples.

FTOCs

FTOCs were established as previously described (Ribeiro et al., 2013; Meireles et al., 2017) by placing isolated thymic lobes obtained from E14 C57BL/6 embryos on a 0.8 mm Isopore membrane filter (Millipore, ATTP01300) over a submerged foam sponge in DMEM medium supplemented with 10% FCS, 1% L-glutamine 200mM (Gibco). On the indicated days, FTOCs were dissociated and analysed by flow cytometry as previously described.

RTOCs

RTOCs were established as previously described (Ribeiro et al., 2013; Meireles et al., 2017) by combining 7×10^5 total thymic cells obtained from WT C57BL/6 thymus and $3.5\text{-}4\times10^4$ sorted $TF^{A/B}$ subsets obtained from newborn Actin-RFP C57BL/6 thymic lobes. After 7 days in culture, RTOCs were dissociated and analysed by flow cytometry as previously described.

Statistical analysis

Statistical analyses were performed using GraphPad software, Version 9. Column graphs show mean+s.d. Statistical analysis was performed using two-tailed *t*-tests.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.F., R.G.R.P., N.L.A.; Methodology: P.F., R.G.R.P.; Software: J.J.M.L.; Validation: P.F., R.G.R.P., J.J.M.L., N.L.A.; Formal analysis: P.F., R.G.R.P., J.J.M.L.; Investigation: P.F., R.G.R.P., N.L.A.; Writing - original draft: P.F., R.G.R.P., N.L.A.; Writing - review & editing: P.F., R.G.R.P., N.L.A.; Visualization: N.L.A.; Supervision: N.L.A.; Project administration: N.L.A.; Funding acquisition: N.L.A.

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Data availability

RNA-sequencing data have been deposited in European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession number PRJEB50163.

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Supplementary Figure 1

A

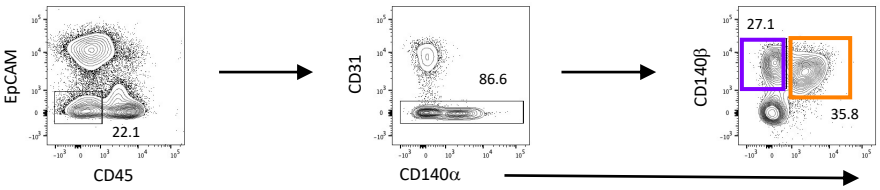

Fig. S1. Gating strategy for flow cytometry analysis of thymic mesenchymal cells. **(A)** Representative analysis of cells obtained from 1 week-old thymus depicting the gating strategy used to identify TMCs defined as clusters 2 and cluster 3. Numbers in plots indicate the frequency of cells found within each gate.

Supplementary Figure 2

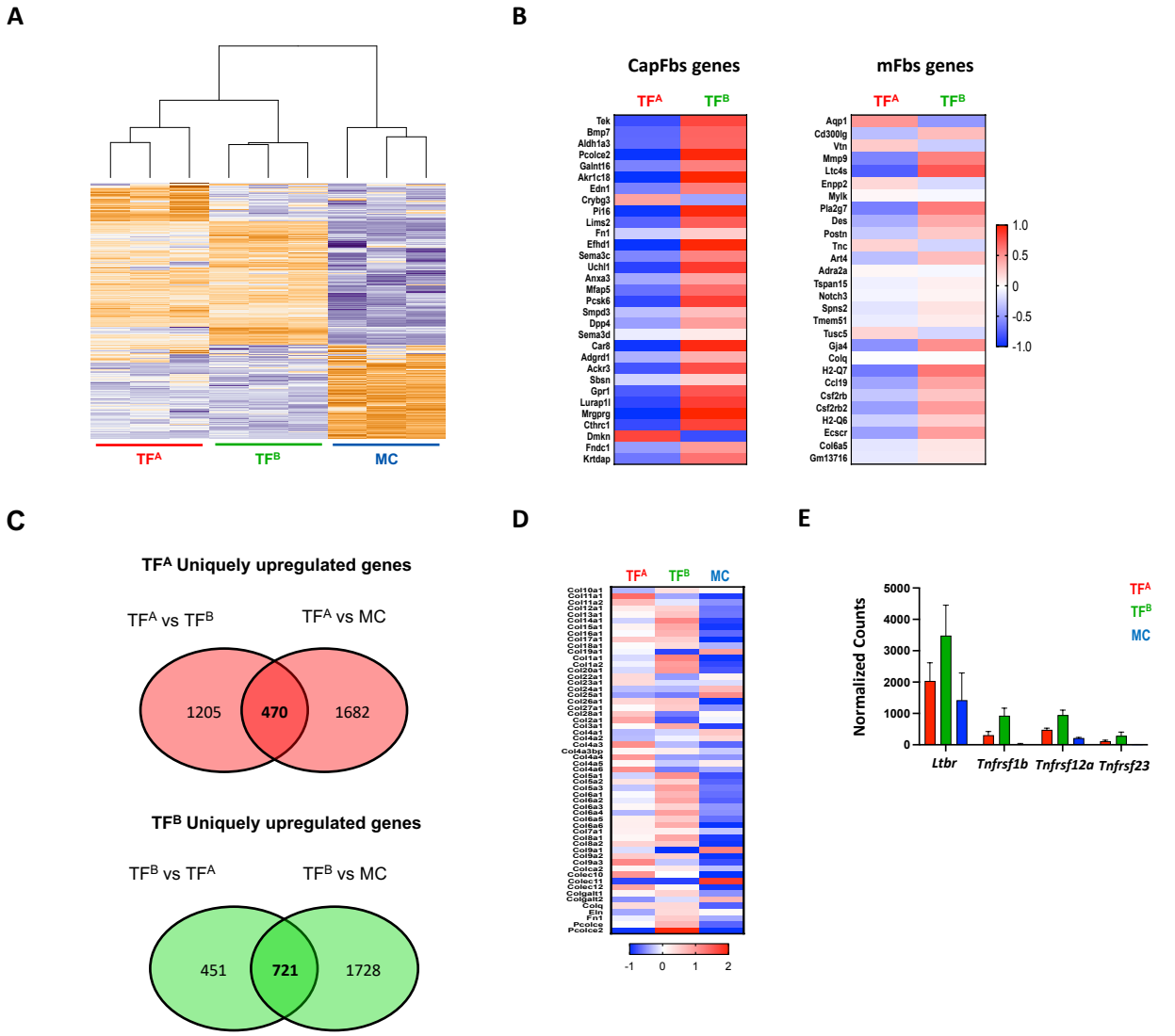

Fig. S2. RNA sequencing analysis of TMC subsets. (A) Heat map representing the 1000 most expressed genes in the assessed TMC populations and associated dendrogram detailing the hierarchical clustering between the biological samples. **(B)** Heat maps represent the deviation from the average expression of the top expressed genes associated with capsular and medullary fibroblasts as described in (Meireles et al., 2017). **(C)** Venn diagrams represent the identification of the 470 and 721 uniquely upregulated genes in TF^A (red) and TF^B (green) populations, respectively. Genes with FDR < 10% were considered as differentially expressed. **(D)** Heat maps represent the deviation from the average expression of the different collagen and collagen associated genes in the different TMC populations. **(E)** Bar graph representing the mean plus SD expression value of the TNFRSF family genes upregulated in TF^B.

Supplementary Figure 3

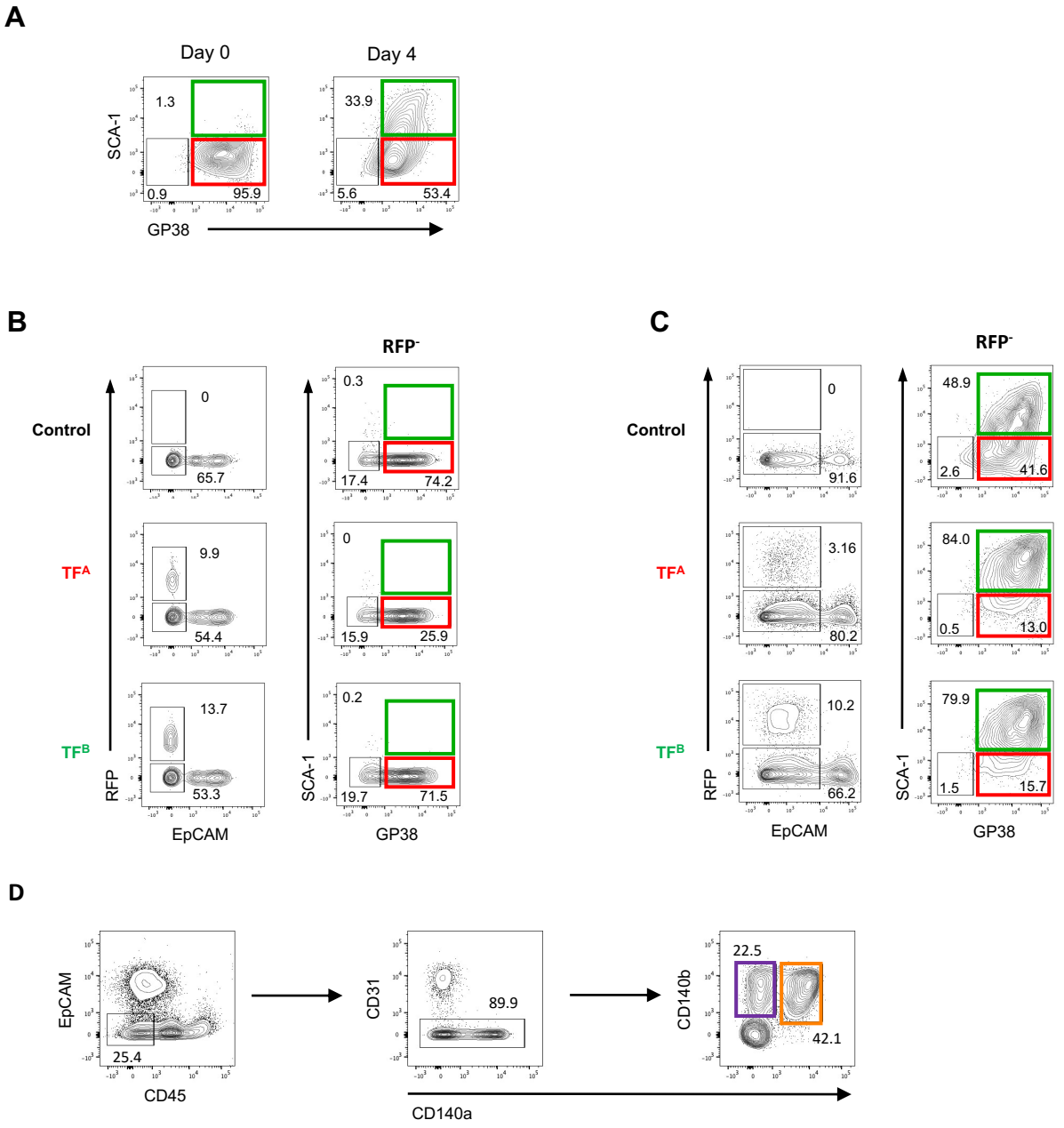

Fig. S3. Precursor-Product relationship between TF subsets. (A) Flow cytometry analysis of the expression pattern of GP38 and SCA-1 at day 0 and after 4 days in culture, from TMCs obtained from fetal thymic organ cultures (FTOC) established with thymic lobes collected from E14 C57BL/6 mice. **(B)** Flow cytometry analysis of day 0 (input) and day 7 (output) RTOC established by combining cells obtained from disaggregated E14 thymus cells from C57BL/6 mice alone (Control) or co-cultured with either TF^A or TF^B cells isolated from Post-natal day P1-P3 Actin-RFP C57BL/6 mice. **(C)** Representative analysis of cells obtained from 1 week-old thymus depicting the gating strategy used to identify TMCs defined as clusters 2 and cluster 3 in *RAG2*$^{-/-}$ *Il2rg*$^{-/-}$. Numbers in plots indicate the frequency of cells found within each gate.

Table S1. Total normalized counts for all detected genes in the RNAseq analysis of populations TF^A, TF^B and MC.

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Table S2. Total normalized counts and deviation to the mean expression value obtained for genes used as phenotypic markers of populations TF^A, TF^B and MC and for genes previously associated with pericyte and fibroblast cells.

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Table S3. Total normalized counts and deviation to the mean expression value obtained for genes previously associated with capsular fibroblast cells in our TF^A and TF^B cell populations.

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Table S4. Total normalized counts and deviation to the mean expression value obtained for genes previously associated with medullar fibroblast cells in our TF^A and TF^B cell populations.

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Table S5. Total normalized counts of the uniquely upregulated genes of population TF^A in relation to populations TF^B and MC.

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Table S6. Total normalized counts of the uniquely upregulated genes of population TF^B in relation to populations TF^A and MC.

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Table S7. Total normalized counts of the gene ontology analysis of the uniquely upregulated genes of population TF^{A}.

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Table S8. Total normalized counts of the gene ontology analysis of the uniquely upregulated genes of population TF^{B}.

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Table S9. Total normalized counts and deviation to the mean expression value obtained for collagen and ECM associated genes in our TF^{A} and TF^{B} cell populations.

Click here to download Table S9