Thymic Stroma and T Cell Development. Impacts of Retinoic Acid Signaling

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Impacts of Retinoic Acid Signaling

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Abstract
The development of functionally competent, self-tolerant T cells in the thymus is an essential prerequisite for the formation of adaptive immune responses against foreign pathogens. Thymic epithelial cells (TECs) form specialized stromal microenvironments in the cortex (cTEC) and medulla (mTEC) of the thymus that support all stages of T cell development, from the entry of thymocyte progenitors to the exit of mature naive T cells. Conversely, TECs require crosstalk with developing thymocytes and surrounding mesenchymal stromal cells (TMCs) for their own differentiation and maturation. However, the signals governing TEC homeostasis and functions remain incompletely understood.

The aim of the work presented in this thesis was to understand the role of the vitamin A metabolite retinoic acid (RA), a known regulator of immune responses and epithelial homeostasis, in TEC functionality and T cell development (Paper I) as well as for the generation of cytotoxic $CD8^+$ T cell responses (Paper II). Additionally, we set out to investigate the poorly characterized heterogeneity within the TMC compartment and to better define the ontogeny and developmental origin of mesenchymal stromal cell subsets in lymphoid organs (Paper III).

In Paper I, we show that *in vivo* RA signaling in TECs regulates their homeostasis and controls gene expression associated with distinct biological pathways in cTEC and mTEC. Consequently, inability to respond to RA due to expression of a dominant-negative RA receptor (dnRAR) resulted in increased cTEC proliferation and the accumulation of cTECs expressing an immature precursor-like phenotype, while mTEC numbers were reduced during the early postnatal phase of TEC expansion. Additionally, RA signaling in TECs impacted on thymopoiesis with impaired generation of CD4 single-positive (SP) and CD8SP thymocytes.

In Paper II, we addressed the role of RA signaling in $CD8^+$ T cell development and function directly, by inducing expression of the dnRAR in thymocytes and peripheral T cells. Using this model, we showed that absence of RA signaling in developing thymocytes severely perturbs thymopoiesis, leading to an accumulation of phenotypically mature CD8SP cells displaying alterations in T cell receptor expression levels and repertoire. Moreover, naive peripheral $CD8^+$ T cells were skewed towards an activated phenotype in the absence of RA signaling and while these cells displayed enhanced survival and expansion upon TCR stimulation, they were impaired in mediating cytotoxic effector functions and displayed reduced expression of various RA-controlled effector genes.

In Paper III, we performed comparative analysis of mesenchymal stromal cells from thymus and lymph nodes and identified a population of $CD34^+$ adventitial cells as a conserved component of the vascular niche in lymphoid tissues. Furthermore, we demonstrated that adult $CD34^+$ cells contain progenitors that have the potential to give rise to lymphoid stromal populations in a context-dependent manner in *in vivo* grafting experiments.

Collectively, the work included in this thesis identified RA signaling as an important regulator of thymic epithelium and T cell functionality and broadens our understanding of mesenchymal stromal cell functions and development in lymphoid organs.

Key words
Thymus, Thymic Epithelial Cells, T Cell Development, Retinoic Acid, $CD8^+$ T Cell function, Stromal Cells

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Für meine Eltern

"A smooth sea never made a skilled sailor"
Franklin D. Roosevelt

Preface

"Like dwarfs standing on the shoulders of giants" - Bernard de Chartres

There is no doubt among immunologists today regarding the importance of the thymus for the establishment and maintenance of our adaptive immune system. The notion however that the thymus is not only relevant in immunity but the key organ of T cell development came about only in the 1960s. Until then, the thymus had been considered an evolutionary relict, an organ that did no longer fulfill any biological functions - at best, it was considered a graveyard for dying lymphocytes.

Surprising as it may seem today to deny the thymus any immunological function, based on the state of knowledge at that time this conclusion made sense. Upon immunization, the thymus of mice did not show any signs of an ongoing immune response that were known from spleen and lymph nodes. Moreover, removing the thymus of adult mice did not lead to impaired immune functions in these mice. How important could something possibly be if you can remove it without any consequences? As it turned out: very!

The key to discovering the immunological function of the thymus was timing. While aiming to study the development of virus-induced leukemia, the Australian scientist Jacques Miller removed the thymus of newborn mice and was surprised to find these mice being more susceptible to infections and cancer and, most importantly, unable to reject grafts of foreign skin tissue. These findings, together with the dramatically reduced numbers of peripheral lymphocytes, prompted Miller to suggest that *"the thymus at an early stage in life plays a very important part in the development of immunological response"*.

Although met with a lot of skepticism at first, it was ultimately proven that Miller was right in suggesting the presence of two types of lymphocytes, T and B cells, and that T cells emerged from the thymus as *"specially selected cells"*.

T cells and the stromal networks they develop and mature in are at the core of this thesis and I hope by reading it you will find a new appreciation of the thymus and its discoverer Jacques Miller, whose work laid the foundation for generations of immunologists to follow.

Kerstin Wendland
Lund, September 2018

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PAPER II

The Role of Retinoic Acid Signaling in CD8$^+$ T Cell Development and Function

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Context-Dependent Development of Lymphoid Stroma from Adult CD34$^+$ Adventitial Progenitors

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*Cell Reports 2016 March 15; 14(10): 2375-2388**

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Abbreviations

ADH	alcohol dehydrogenase
Aire	autoimmune regulator
APC	antigen-presenting cell
BAFF	B cell activating factor
BCR	B cell receptor
BM	bone marrow
Bmp	bone morphogenic protein
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
CLP	common lymphoid progenitor
CMJ	cortico-medullary junction
CRABP	cellular retinoic acid-binding protein
cTEC	cortical thymic epithelial cell
CTL	cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DC	dendritic cell
DLL4	delta-like 4
DN	double-negative
DNA	deoxyribonucleic acid
dnRAR	dominant-negative retinoic acid receptor
DP	double-positive
DR	direct repeat
E	embryonic day
ECM	extracellular matrix
EpCAM-1	epithelial cell adhesion molecule 1
ETP	early thymic progenitor
Eya1	eyes absent homolog 1
Fas	first apoptosis signal receptor
FasL	first apoptosis signal receptor ligand

FDC	follicular dendritic cell
Fezf2	FEZ family zinc-finger 2
Fgf	fibroblast growth factor
Flt3	receptor-type tyrosine-protein kinase
Foxn1	forkhead box N1
Foxo1	forkhead box O1
Foxp3	forkhead box P3
FRC	fibroblastic reticular cell
FTOC	fetal thymic organ culture
Gcm2	glial cell missing 2
GluR1	glutamate receptor 1
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1
H3/H4	histone 3/histone 4
HDAC	histone deacetylase
Hoxa3	homeobox A3
HSC	hematopoietic stem cell
HSV	herpes simplex virus
ICAM-1	intercellular adhesion molecule 1
Igf	insulin growth factor
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
Klf2	Krüppel-like factor 2
LCMV	lymphocytic choriomeningitis virus
LMPP	lymphoid-primed multipotent progenitor
LN	lymph node
LNMC	lymph node mesenchymal cell
LT	lymphotoxin
LTβR	lymphotoxin β receptor
LTi	lymphoid tissue inducer
LTo	lymphoid tissue organizer
MAdCAM-1	mucosal vascular addressin cell adhesion molecule 1
MAMP	microbe-associated molecular pattern
MAP	mitogen-activated protein
MHC	major histocompatibility complex
mLN	mesenteric lymph node

MPEC	memory precursor effector cell
MPP	multipotent progenitor
MRC	marginal reticular cell
mTEC	medullary epithelial cell
NCC	neural-crest derived cell
NCoR	nuclear receptor corepressor
NF-κB	nuclear factor kappa-B
NK	natural killer
Pax	Paired box
PDGFR	platelet derived growth factor receptor
PDPN	podoplanin
Plet1	placenta expressed transcript 1
PP3	third pharyngeal pouch
PPAR	peroxisome proliferator-activated receptor
PRR	pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand 1
PSMB	proteasome subunit beta
RA	retinoic acid
Rag	recombination-activating gene
RALDH	retinaldehyde dehydrogenase
RANK	receptor activator of nuclear factor kappa-B
RANKL	receptor activator of nuclear factor kappa-B ligand
RAR	retinoic acid receptor
RARE	retinoic acid responsive element
RBP	retinol binding protein
RDH	retinol dehydrogenase
RNA	ribonucleic acid
RORγt	retinoic acid receptor-related orphan receptor gamma t
RTE	recent thymic emigrant
Runx3	runt-related transcription factor
RXR	retinoid X receptor
S1P	sphingosine-1-phosphate
S1P1	sphingosine-1-phosphate receptor 1
Sca-1	stem cell antigen 1
SCF	stem cell factor
SCID	severe combined immunodeficiency

SCS	subcapsular sinus
SI	small intestine
SIRPα	signal regulatory protein alpha
Six	sine oculis homeobox homolog
SLEC	short-lived effector cell
SLO	secondary lymphoid organ
SOCS	suppressor of cytokine signaling
SP	single-positive
Sphk	sphingosine kinase
SSEA-1	stage-specific embryonic antigen 1
STRA6	stimulated by retinoic acid receptor 6
Tbx1	T-box 1
TCR	T cell receptor
TEC	thymic epithelial cell
TEPC	thymic epithelial progenitor cell
TGF-β	transforming growth factor beta
Th	T helper
Thpok	T helper-inducing POZ/Krueppel-like factor
TLR	toll-like receptor
TNC	thymic nurse cell
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNFRSF	tumor necrosis factor receptor super family
TMC	thymic mesenchymal cell
TRA	tissue-restricted antigen
TRAF6	tumor necrosis factor receptor-associated factor 6
Treg	regulatory T cell
TSP	thymus-seeding progenitor
TSSP	thymus-specific serine protease
UEA-1	ulex europeaus agglutinin 1
VAD	vitamin A deficient
VCAM-1	vascular cell adhesion molecule 1
Wnt	wingless-int
XCL	X-C motif chemokine ligand
XCR	X-C motif chemokine receptor
γ_c	common gamma chain

Introduction

On a daily basis our immune system is faced with the monumental task of protecting us from infections and cancer, caused by invading pathogens of various kinds or cell-intrinsic malignancies. To achieve this, an intricate network of specialized cells is dedicated to recognize and eliminate foreign, potentially harmful, components in a rapid and efficient manner. At the same time, cells of the immune system must remain tolerant to self and be able to terminate an immune response upon clearance of the infectious agent. Any imbalance in the activation and regulation of the immune system can have severe consequences, ranging from increased susceptibility to infections to the development of allergies, autoimmunity and chronic inflammation.

The vertebrate immune system is generally divided into an innate and adaptive side that are characterized by different means of recognition and response to pathogens, as well as the ability to generate immunological memory. Despite those differences, the interplay between innate and adaptive immunity is essential for mounting a successful immune response.

As a first line of defense the innate immune system consists of physical barriers such as the skin or the epithelium of mucosal surfaces. If these barriers are breached, cells of the innate immune system can recognize invading pathogens through conserved structures termed microbe-associated molecular patterns (MAMPs). Binding of MAMPs by so-called pattern recognition receptors (PRRs) triggers different rapid defense mechanisms in innate immune cells, depending on the cell type. Macrophages respond to PRR activation with increased phagocytosis while granulocytes are prompted to release anti-microbial factors stored in their granules. Notably, PRR activation on innate cells also induces secretion of inflammatory mediators such as cytokines and chemokines, which reinforce and modify the immune response through recruitment of both innate and adaptive immune cells to the site of infection.

Unlike the immediate actions of the innate immune system, adaptive immune responses involve the activation and differentiation of T and B cells into effector cells and can take several days to be generated. However, the advantage of the adaptive

immune system is the virtually unlimited repertoire of receptors that T and B cells can generate to recognize antigens. The highly diverse T cell receptors (TCRs) and B cell receptors (BCRs) result from random recombination of gene segments during the development of these cells in the thymus and bone marrow, respectively. In contrast to B cells, that can recognize native antigen, T cells require antigen-presenting cells (APCs) to process and present antigen to them in the context of major histocompatibility complex (MHC) molecules. Cells that can act as professional APCs include macrophages, B cells and dendritic cells (DCs). Of note, epithelial cells in the thymus are also specialized in presenting antigen to developing thymocytes to ensure the generation of a functional self-tolerant T cell compartment. The processes underlying T cell development in the thymus and the involvement of thymic epithelial cells will be discussed in detail in Chapter 1.

There are two main lineages of T cells, defined by expression of the co-receptors CD4 or CD8, which recognize antigen presented in the context of MHC class II or MHC class I, respectively. $CD4^+$ T cells are helper cells that upon recognition of their cognate antigen start to produce cytokines and directly act on other cells to shape the immune response. They enhance the phagocytic activity of macrophages and are essential for antibody production and class switching of B cells. Importantly, $CD4^+$ T cell help is also relevant for the proliferation and activation of their $CD8^+$ counterparts. The main function of cytotoxic $CD8^+$ T cells is the killing of target cells that present their cognate antigen, either through release of soluble mediators or through direct engagement of receptors on the target cell surface. The details of $CD8^+$ T cell activation and effector function will be further discussed in Chapter 2.

A hallmark of the adaptive immune system is the ability to generate immunological memory through the generation of long-lived memory T and B cells during the initial immune response. These cells ensure a faster and improved response to a previously encountered pathogen and are the essential mediators of long lasting immunity upon vaccination. Different subsets of memory T cells can be found in various locations of the body and their respective features will be discussed at the end of Chapter 2 for the $CD8^+$ T cell population.

Chapter 1. Thymopoiesis and the Thymic Microenvironment

The thymus is the primary lymphoid organ of T cell development and supports the generation of TCRαβ-expressing $CD4^+$ and $CD8^+$ T cells, so-called conventional T cells, and various subsets of unconventional T cells, such as $TCR\gamma\delta^+$ T cells and natural killer (NK) T cells. For the purpose of this thesis, the following chapter will focus on the development of conventional T cells.

T Cell Development

Despite being the major site of T cell development, the thymus does not harbor any hematopoietic stem cells (HSCs) but instead relies on the regular recruitment of bone marrow (BM)-derived progenitors. Upon entry of the thymus, these progenitors follow a highly regulated pathway of commitment, differentiation and selection that leads to the generation and release of naive T cells into the periphery. The developmental route of $CD4^-$ $CD8^-$ double-negative (DN), $CD4^+$ $CD8^+$ double-positive (DP) and $CD4^+$ single-positive (SP) and CD8SP thymocytes is shown in **Figure 1**.

In the subsequent sections I will discuss the distinct steps of T cell development before focusing on the ontogeny and functions of thymic epithelial cells (TECs), the most important component of the thymic stromal microenvironment.

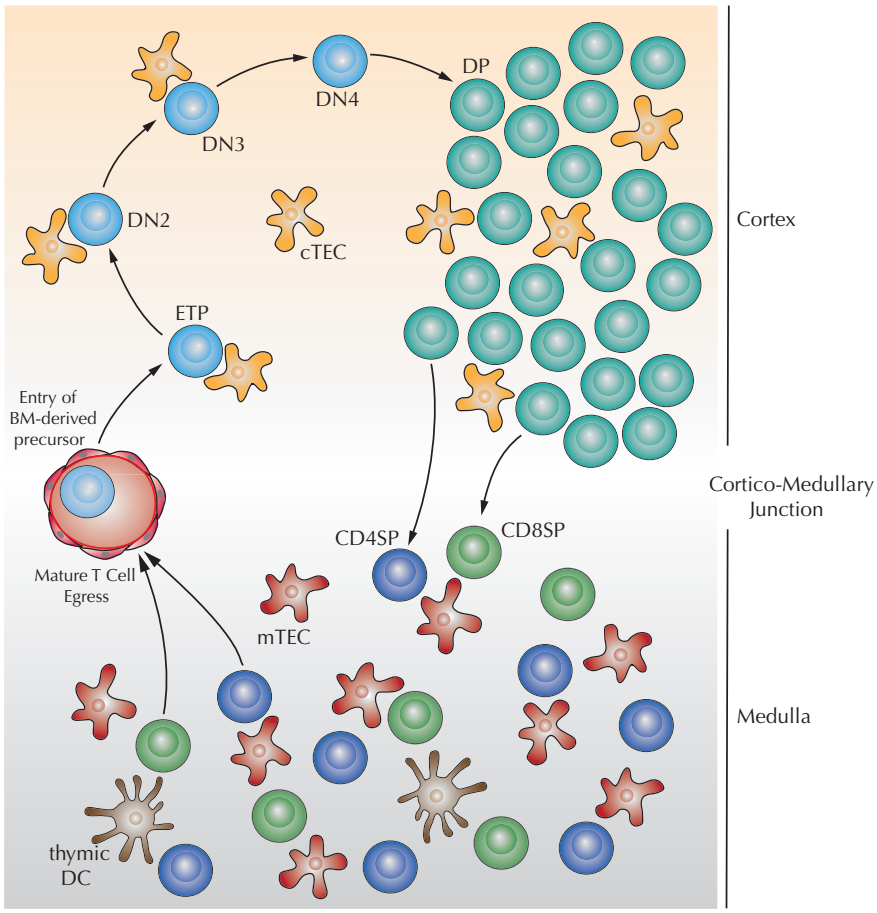

Figure 1 - Development of conventional T cells in the thymus. Bone marrow (BM)-derived precursors enter the thymus through blood vessels at the cortico-medullary junction from where they progress through various $CD4^-$ $CD8^-$ double-negative (DN) stages before proliferating heavily at the $CD4^+$ $CD8^+$ double-positive (DP) stage. Survival and selection of DN and DP thymocytes are supported through signals provided by cortical thymic epithelial cells (cTEC). Upon commitment to either the CD4 or the CD8 lineage, $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes migrate to the medulla where interactions with medullary thymic epithelial cells (mTEC) and thymic DCs eliminate thymocytes bearing potentially autoreactive TCR specificities. ETP, early thymic progenitor.

Thymus Colonization

The absence of a hematopoietic stem cell pool in the thymus requires the import of BM-derived thymus seeding progenitors (TSPs) via the blood to ensure continuous T cell development. The identity of the TSP population has been debated over the years and different experimental approaches have been used to determine the contribution of HSCs, multipotent progenitor (MPP) subsets and common lymphoid progenitor (CLP) subsets in thymus seeding.

While T cell lineage potential has been demonstrated for all three of these BM populations, both by *in vitro*[1] and *in vivo* approaches[2], this alone is not sufficient to confer TSP status. In addition, TSPs must be able to leave the bone marrow into the circulation and have the capacity to enter the thymus through the vascular endothelium. Populations of lymphoid-primed MPPs (LMPPs) and CLPs have been identified in the blood[3, 4] and depletion-based transfers of bone marrow-derived precursors revealed TSPs to be a heterogeneous population of (L)MPPs and CLPs that are characterized by shared expression of CD27 and CD135 (also known as receptor-type tyrosine-protein kinase Flt3)[5, 6]. In line with this, recent single cell analysis of the earliest thymic progenitors in neonates confirmed their close functional and molecular relationship to CLP and LMPP populations from the bone marrow[7]. Notably, MPPs and CLPs show distinct kinetics of T cell development, with the more restricted CLP population progressing faster towards an SP thymocyte stage[5], and it might be this heterogeneity within the TSP population that ensures the continuous generation of T cells, as the thymus is only periodically receptive to progenitor seeding[8].

The wave-like colonization of the thymus by progenitors has been attributed to alternating thymic levels of P-selecting and CCL25 that interact with P-selectin glycoprotein ligand (PSGL-1) and the chemokine receptor CCR9, respectively, to mediate adhesion and entry of TSPs[9]. Additionally, the availability of TSP niches is limited by the earliest thymocyte populations, most notably early thymic progenitors (ETP) and DN2 thymocytes[10]. Of note, the estimated life span of ETPs of 10 to 12 days[11] correlates with the periodicity of 9 to 12 days for thymus seeding, as demonstrated in sequential transfer experiment of TSPs[10].

Apart from the above-mentioned P-selectin/PSGL-1 and CCR9/CCL25 axis several other adhesion molecules and chemokine receptors have been implicated in homing of TSPs to the thymus, including CD44[12], CCR7[13, 14] and CXCR4[15]. Furthermore, expression of the integrins α4β1 and αLβ2 is required for TSPs to interact with vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) on the thymic vasculature[16]. Importantly, blocking of any of these individual selectin, integrin and chemokine receptor interactions usually has little to no effects on thymus colonization and the numbers of ETPs, suggesting a high level of functional redundancy among TSP surface molecules[17]. In fact, the most striking phenotype concerning loss of thymus homing is only observed in CCR7/CCR9 double-deficient mice where ETPs and DN2 thymocytes are almost absent[13, 14], underscoring the importance of these two chemokine receptors together for progenitor colonization of the thymus. Interestingly, total thymic cellularity is essentially normal in CCR7/CCR9 double-deficient mice[13], suggesting mechanisms of compensatory proliferation at later DN stages in the cortex.

Recently it has been shown that lymphotoxin β receptor (LTβR) signaling is involved in thymus homing, demonstrated by a significant reduction of ETPs in LTβR-deficient mice[18]. The impaired progenitor entry in the absence of LTβR has been linked to lower ICAM-1 and VCAM-1 expression in the thymic mesenchyme and endothelium[18] and the loss of a specific subsets of thymic endothelial cells (termed thymic portal endothelium) in LTβR-deficient mice further supports the importance for LTβR signaling in the thymic endothelium in regulating progenitor entry[19].

Differentiation and Positive Selection

Once TSPs have successfully entered the thymus at the cortico-medullary junction (CMJ) they become part of the most immature intrathymic progenitor population, the ETPs, and develop progressively through various DN stages while migrating towards the outer cortex (**Figure 1**).

Whereas ETPs and DN2 thymocytes still harbor non-T cell potential[7, 20], commitment to the T cell lineage is completed at the transition to the DN3 stage[21]. The key requirement for adopting T cell fate is signaling through Notch-1 on DN progenitors mediated by cortical Notch ligands, the most important being Delta-like 4 (Dll4)[22]. Consequently, conditional ablation of the DLL4 gene in the cortex

completely abrogates T cell development and instead leads to B cell development in the thymus[23, 24].

Apart from promoting T cell lineage commitment, the cortical environment also provides essential growth factors for the survival and proliferation of DN thymocytes, including stem cell factor (SCF; also known as Kit ligand) and interleukin (IL)-7 that serve as ligands to c-kit (CD117) and the IL-7Rα (CD127), respectively[25, 26, 27]. Whereas SCF- or c-kit-deficient mice display severely reduced DN1 numbers but normal further T cell development[28], IL-7 deficiency causes dramatic reduction in overall thymus cellularity attributed to an incomplete halt of T cell development at the DN3 stage[29, 30]. Furthermore, disruptions of the IL-7Rα gene result in substantial lymphopenia in mice[31] and the development of severe combined immunodeficiency (SCID) in humans, as a consequence of impaired T cell development[32].

Further expansion and differentiation of DN3 thymocytes to the DP stage is mediated by signaling through the pre-TCR (consisting of a rearranged TCRβ chain and an invariant α chain), in a process called β-selection[33]. DN3 thymocytes with a successfully assembled TCRβ chain are signaled to undergo gene arrangement of the TCRα chain and progress to the DP stage, characterized by low-level expression of a complete TCRαβ. The random assembly of the different variable (V), diversity (D) and joining (J) gene segments at the TCR loci under the control of recombination-activating gene (Rag)-encoded enzymes allows for the generation of over 10^{15} possible TCR specificities[34]. While this elaborate mechanism of receptor generation maximizes the chance of recognizing virtually any kind of peripheral antigen, not all of these combinations will eventually be functional. Thus, only those DP thymocytes with useful TCR specificities receive the required survival signals, selected on their ability to recognize self-peptide/MHC complexes with low or intermediate affinity/avidity. Importantly, DP thymocytes have a short lifespan of 3 to 4 days and any cell that does not receive survival signals through their TCR during that time, due to lack of interaction with MHC complexes, undergoes death by neglect[35]. This process of positive selection is the prerequisite for the generation of a functional T cells that will be able to recognize their cognate antigens presented to them by APCs in the periphery.

Positive selection of $CD4^+$ and $CD8^+$ T cells depends on different proteases involved in antigen processing in the cortical epithelium. These include cathepsin L and thymus-specific serine protease (TSSP) for MHC class II presentation regarding optimal $CD4^+$ T cell selection[36, 37], as well as the thymic-specific proteasome subunit

β5t required for MHC class I presentation during the selection of $CD8^+$ T cells[38]. The details of antigen processing and presentation in the cortex will be further discussed in the section concerning cTEC functionality.

CD4 and CD8 Lineage Choice

As mentioned earlier, the fate decision of positively selected DP thymocytes to undergo either CD4 or CD8 lineage commitment is determined by the interactions of their TCR with MHC class II or class I molecules, respectively. How exactly these interactions confer lineage choice has been a matter of debate for decades and two classical models have been proposed to explain this phenomenon in the past. The 'instructive' model suggested that engagement of the TCR by MHC class II or class I ligands induce distinct intracellular signaling signatures and that these unique signals are the determining factors for loss of either of the two co-receptors. In contrast, the 'stochastic' model proposed random downregulation of one co-receptor by DP thymocytes right after positive selection, irrespective of the TCR interactions they received. In this model, a later signal would be required to promote survival of only those cells that still express the correct co-receptor that matches the MHC specificity of their TCR[39].

However, several lines of evidence arguing against either one of these models have led to the emergence of the 'kinetic signaling' model that is currently the most widely accepted model of CD4/CD8 fate decisions[40]. The 'kinetic signaling' model suggests that lineage choice is based on TCR signal duration and the contribution of common γ chain (γ_c) cytokines such as IL-7. Recently positively selected thymocytes terminate CD8 expression, thus acquiring a transitional $CD4^+CD8^{lo}$ phenotype[41] that provides a window for the determination of correct lineage fate. MHC class II-mediated TCR signaling will be maintained at the $CD4^+CD8^{lo}$ stage, as it is independent of CD8, and stronger and persistent TCR signals will thus induce a $CD4^+$ phenotype. Conversely, as signaling through MHC class I-restricted TCRs is terminated in the absence of CD8 expression an interrupted shorter signal allows for IL-7 and other γ_c cytokines to act as sensors of TCR signal duration to induce $CD8^+$ fate[41, 42].

Irrespective of the exact nature of the initial signals that instruct CD4 or CD8 lineage commitment, the molecular mechanisms of co-receptor expression and stability are beginning to emerge. A complex interplay of transcription factors and epigenetic regulation enforces lineage commitment of DP thymocytes, most notably controlled

by the transcription factors Runx3 and Thpok, whose expression is mutually exclusive and induces a reciprocal inhibitive feedback loop. More specifically, Runx3 is induced by γ_c cytokine signals[42] and subsequently suppresses Thpok transcription by directly binding to silencer elements in the Thpok locus[43, 44]. In contrast, Thpok induced by persisting TCR signals[43] represses Runx3 expression indirectly, through transcriptional activation of suppressor of cytokine signaling (SOCS) genes that inhibit γ_c signaling-dependent Runx3 expression[45].

Consequently, expression of Thpok is essential for CD4 lineage commitment and spontaneous mutations or targeted deletion of Thpok redirect MHC class II-restricted thymocytes towards the CD8 lineage[46, 47]. Several additional transcription factors are involved in CD4 lineage specification by controlling the expression of Thpok, such as Lef-1, Tcf-1 and Gata3[48, 49]. On the other hand, Runx3 is the key transcription factor to seal CD8 lineage fate and impaired Runx activity (achieved only by combined deletion of Runx3 and Runx1, due to functional redundancy between the two) leads to redirection of MHC class I-restricted thymocytes to the CD4 lineage[44, 50]. Accordingly, it has been shown that Thpok and Runx3 exert their function as lineage decisive transcription factors by directly binding and repressing either Cd8 or Cd4 gene loci, respectively[49, 51].

Negative Selection and Central Tolerance

Positively selected thymocytes home to the thymic medulla where they are further screened for any autoreactive potential, characterized by TCR specificities that present a strong affinity/avidity for self-peptide MHC complexes. These cells could potentially attack the body's own tissues when released into the periphery, which is why they need to be eliminated during their development. This process of negative selection is essential to the establishment of central tolerance and any interference with it through inherited deficiencies or induced mutations commonly results in severe autoimmunity. It is important to note that negative selection does not only occur in the medulla, thymocytes that display too strong TCR engagements during their time in the cortex will be equally eliminated. However, the cells in the thymic medulla are highly specialized in presenting a broad array of self-antigens, thereby extending the ability to identify autoreactive T cell clones.

Clonal deletion

Different forms of negative selection have been described for high affinity TCR-bearing thymocytes, including receptor editing of the TCRα chain[52], developmental diversion into functionally anergic TCRαβ$^+$ DN thymocytes[53] and, most importantly, clonal deletion through apoptosis[54]. Regarding the proximal TCR signaling events that determine clonal deletion fates, several mitogen-activated protein (MAP) kinases, including Jnk1, Jnk2 and p38, have been implicated in the upregulation of the pro-apoptotic Bcl-2 family member Bim[55, 56]. Through antagonizing the anti-apoptotic function of Bcl-2, Bim mediates cytochrome *c* release from mitochondria and promotes caspase-mediated cell death. The importance of Bim/Bcl-2 interactions for negative selection has been demonstrated by impaired clonal deletion observed in Bim-deficient mice[57]. However, while overexpression of Bcl-2 was sufficient to rescue other forms of Bim-mediated cell death, such as death by neglect, it did not abrogate clonal deletion in Bcl-2 transgenic mice[58], arguing for additional mechanisms of apoptosis-induction during negative selection.

Another key mediator of TCR signaling-induced cell death is the orphan nuclear receptor Nur77 that exerts its function in two major ways. Firstly, it acts as a transcription factor for several pro-apoptotic genes[59, 60] and secondly, it can translocate to mitochondria where it converts Bcl-2 into a pro-apoptotic form[61, 62]. Of note, this interference with the anti-apoptotic properties of Bcl-2 not only represents another mechanisms of clonal deletion, but also provides a possible explanation for the inability to inhibit Bim-mediated apoptosis by Bcl-2 overexpression during negative selection specifically. In line with the described functions of Nur77, blocking its activity by expression of a dominant-negative form averts clonal deletion while constitutive expression in the thymus promotes massive apoptosis of developing thymocytes[63].

Peripheral antigen presentation in the medulla

In order to purge the developing T cell pool from thymocytes harboring TCRs with self-reactive potential, the process of negative selection relies on the presentation of an extensive repertoire of self-antigens that might be encountered on tissues in the periphery. In this regard, mTECs and thymic DCs are the key autonomous and non-redundant mediators of peripheral antigen presentation and defective APC potential of either of the two subsets, due to impaired MHC class II expression, compromises clonal deletion of CD4SP thymocytes[64].

The hallmark of mTECs is their ability to express a large variety of tissue-restricted antigens (TRAs) through elaborate mechanisms of promiscuous gene expression, most notably controlled by the transcription factors autoimmune regulator (Aire)[65] and FEZ family zinc-finger 2 (Fezf2)[66]. While the details of mTEC-specific TRA expression will be further discussed in the section concerning mTEC functionality, it is important to note that mTECs express between 18.000 and 19.000 genes, thus covering about 85- 89% of the entire coding genome[67, 68]. While on a cellular level the frequency of mTECs expressing any given TRA is with 1-3% rather low[69], the mosaic gene expression pattern created by individual mTECs expressing distinct TRA clusters is sufficient for the presentation of the whole known TRA repertoire on a population level[70, 71].

In contrast to mTECs, thymic DCs can acquire peripheral antigen in several ways. Owing to their preferred location around the vasculature at the CMJ and in the perivascular space they can sample circulating antigens directly from the blood[72, 73]. Secondly, they can obtain and cross-present both native and MHC-bound antigens from mTECs[74] and, finally, subsets of extrathymically derived thymic DCs can bring in antigen captured in the peripheral tissues upon homing to the thymus[75, 76].

Regulatory T cell development

Despite the intricate mechanisms described above, negative selection in the thymus is not 100% efficient and there will always be some self-reactive T cells released into the periphery. Limiting the tissue-damaging effects of these cells is the main responsibility of regulatory T cells (Tregs), a specialized population of T cells that possesses immunosuppressive potential. The development and function of these cells is critically dependent on the transcription factor forkhead box P3 (Foxp3)[77] and absence of Tregs due to Foxp3-deficiency results in fatal multi-organ autoimmunity in both mice[78] and humans[79]. The vast majority of all Tregs developing in the thymus are $CD4^+$ (with only 3-4% being $CD8^+$)[80] and their differentiation is believed to occur during a specific developmental window, as Treg potential decreases with longer medullary residency of CD4SP cells[81]. Of note, Tregs can also develop from naive peripheral $CD4^+$ T cells upon TCR stimulation in combination with transforming growth factor β (TGF-β) and IL-2 signals[82]. These peripherally induced Tregs (pTregs) are mostly found at intestinal tissues and carry TCR specificities for microbial and dietary antigens[83]. For the purpose of this thesis, I will focus solely on the development of thymic-derived Tregs (tTreg).

In the affinity model of T cell development where low affinity TCR engagement promotes positive selection while high affinity agonists lead to negative selection, tTreg development occurs in a window between the two over a broad range of affinity interactions, from intermediate conditions to those strong enough to induce negative selection[84]. The partly stochastic overlap between clonal diversion and clonal deletion has been demonstrated in TCR-transgenic mouse models where the same agonist ligand can induce both tTreg differentiation and apoptosis[85, 86, 87]. The signals determining the final fate of the developing thymocyte are not fully understood, although it has been suggested that additive signaling effects of multiple TCR interactions or alterations in thymocyte sensitivity might play a role[88]. Interestingly, the transcription factor Nur77 that has an important role in clonal deletion is also required for functional tTreg development[60, 89], further underscoring the shared molecular complexity of negative selection and tTreg development.

Apart from TCR signaling strength, additional essential cues for tTreg differentiation are provided by signals downstream of the co-stimulatory receptor CD28 such as c-Rel[90], with CD28- and c-Rel-deficient mice displaying dramatically reduced numbers of $CD4^+CD25^+$ thymocytes[91, 92]. Furthermore, tTreg development is critically dependent on several γ_c cytokines such as IL-2, IL-7 and IL-15[93], as demonstrated by the complete absence of Treg development in γ_c-deficient[94] and IL-2/IL-5 double-deficient mice[95]. With regards to IL-2 it has recently been shown that both existing tTregs and recirculating pTregs can limit the niche for *de novo* tTreg development by sequestering IL-2, providing an additional role for IL-2 signaling in controlling the generation of tTregs[96, 97].

Thymic egress

SP thymocytes have been shown to reside in the medulla for 4 to 5 days before exiting the thymus at the CMJ [98], which allows them to undergo the necessary molecular and phenotypic changes required for thymic egress during post-selection maturation. Immature and mature SP thymocytes can be identified based on the expression of several surface molecules, some of which are known to play important roles in regulating thymic egress.

Thus, $CD69^{+}Qa2^{-}CD24^{high}CD62L^{low}$ expression defines immature SP thymocytes that are sensitive to clonal deletion upon engagement of the TCR, while mature $CD69^{-}Qa2^{+}CD24^{low}CD62L^{high}$ SP thymocytes functionally resemble naive T cells in the periphery in the sense that they respond to agonistic TCR signaling by proliferation and cytokine production[98]. Furthermore, mature SP thymocytes upregulate the transcription factors forkhead box O1 (Foxo1) and Krüppel-like factor 2 (Klf2) that induce expression of CD62L and the sphingosine-1-phosphate receptor 1 (S1P1)[99, 100, 101]. While CD62L is required on naive T cells for entering lymph nodes (LNs) and gut-associated lymphoid tissues through interactions with its ligands glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1)[102, 103], the G-protein coupled receptor S1P1 has a direct role in thymic emigration. Expression of S1P1 allows mature thymocytes to migrate towards an S1P gradient provided by thymic pericytes[104] and thymic endothelial cells lining the blood vessels of the CMJ[105]. Accordingly, the critical role of S1P1 in thymic output has been demonstrated by the impaired egress and intrathymic accumulation of S1P1-deficent thymocytes, causing a subsequent reduction in peripheral T cell numbers[106]. Importantly, S1P1 expression is suppressed by CD69, as binding of CD69 to the receptor induces its internalization and degradation[107]. This function as a negative regulator of S1P1 explains the absence of CD69 expression on mature SP thymocytes to enable their exit from the thymus.

Other regulators of thymic egress independent of the S1P-S1P1 axis have been suggested to include LTβR[108] and IL-4Rα[109], and mice with LTβR- or IL-4Rα-deficiency both display intrathymic accumulation of mature $CD69^{-}$ $CD62L^{high}$ SP thymocytes[108, 109], although further work is required to determine the exact role of these signaling pathways in thymic egress.

Development of Thymic Epithelial Cells

TECs are the main component of the thymic microenvironment and together with other stromal cells such as thymic mesenchymal cells (TMC) and thymic DCs they provide the essential cues for the different stages of T cell development. The following section focuses on the development of cTEC and mTEC populations and the identity of TEC progenitors in the embryonic and adult thymus. Since cTEC and mTEC share a common bipotent origin during thymic development, I will first provide an overview of the molecular mechanisms regulating thymus organogenesis.

Thymus organogenesis

The developmental process of thymus formation includes the hallmarks of general organogenesis, such as positioning, initiation, outgrowth and patterning and finally, separation and differentiation (**Figure 2**). Regulation and control of these carefully coordinated steps is mediated by a network of transcription factors and growth factors, including the key regulator of TEC development, forkhead box N1 (Foxn1). Consistent with its critical role in maintaining TEC identity and cTEC/mTEC differentiation[110], Foxn1 is indispensable for thymus organogenesis. Spontaneous homozygous loss-of-function mutation of Foxn1 occurring in *nude* mice, a hairless mouse mutant first described in the late 1960s[111, 112], or directed disruption of the Foxn1 gene lead to formation of a hypoplastic thymus that is unable to support thymopoiesis[113, 114, 115]. Consequently, these mice, as well as humans carrying mutations in the Foxn1 gene, are severely immunocompromised due to lack of a functional T cell compartment[114, 115, 116].

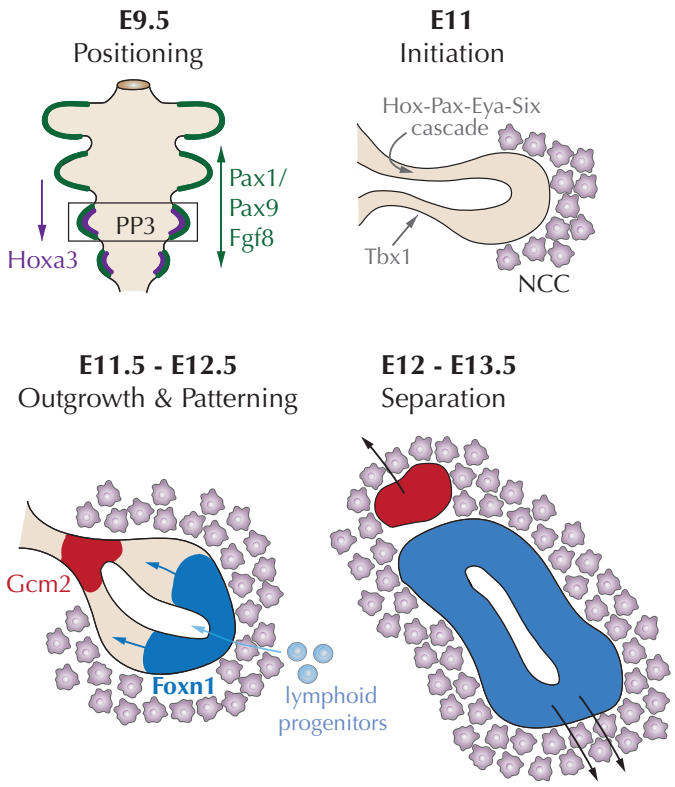

Figure 2 - Overview of thymus organogensis. The thymus develops from the third pharyngeal pouch (PP3) whose formation and axial identity are controlled by Pax1, Pax9, Fgf8 and Hoxa3. At E11, initiation of rudiment budding is under the control of the Hox-Pax-Eya-Six cascade as well as Tbx1. At the same time, neural crest-derived cells (NCC) start to encapsulate the primordium. From E11.5 further outgrowth and patterning of the shared thymus-parathyroid primordium are defined by Foxn1 expression in the ventral part marking the thymus anlagen while Gcm2 expression in the dorsal part identifies the prospective parathyroid domain. The first lymphoid progenitors enter the thymic domain at this time. At E12 separation of the shared primordia from the pharynx is complete and the physical association between thymus and parathyroid is lost by E13.

The fact that Foxn1-deficient mice are not entirely athymic but possess a developmentally arrested thymic rudiment indicates that the earliest steps of thymus organogenesis do not require Foxn1 expression[114]. Thus, thymus organogenesis can be divided into an initial Foxn1-independent phase and a later Foxn1-dependent phase.

Foxn1-independent events (E9 - E11.5)

The thymus arises from the third pharyngeal pouch (PP3) endoderm and its development is tightly linked to that of the parathyroid glands. Given the importance of the PP3 for thymus development, the genes controlling its formation (paired box gene 1 (Pax1), Pax9[117, 118], Fibroblast growth factor 8 (Fgf8[119]) and axial identity (homeobox A3 (Hoxa3[120]) are of key importance for the positioning of the thymus anlagen around embryonic day (E)9.5 (**Figure 2**). Together with eyes absent homolog 1 (Eya1) and sine oculis homeobox homolog 1/4 (Six1, Six4), Hoxa3 and Pax1/9 form a transcriptional network, termed Hox-Pax-Eya-Six cascade, that mediates initiation of the thymus rudiment around E11 (**Figure 2**) and mutations in any of the genes involved in this regulatory cascade leads to defective PP3 formation and failure to initiate thymus organogenesis[117, 118, 121, 122].

Apart from the Hox-Pax-Eya-Six cascade expression of the transcription factor T-box 1 (Tbx1) in the pharyngeal pouch endoderm is required for the formation and outgrowth of the PP3[123] and its deletion results in several clinical manifestations referred to as DiGeorge syndrome in humans, including absence or hypoplasia of the thymus and parathyroid glands, congenital heart disease and abnormal facies[124].

Around E11, the outgrowing rudiment starts to be surrounded by neural crest-derived cells (NCCs) (**Figure 2**), a migratory cell population of ectodermal origin that represent the embryonic precursors of the thymic mesenchymal cell (TMC) compartment[125]. Eventually these cells will give rise to the mesenchymal thymic capsule and pericytes associated with the thymic vasculature[126, 127]. The presence of NCCs is critically required for thymus organogenesis and ablation of this cell population results in defective thymus development[128]. Interestingly however, it has been shown that, despite their early accumulation around the thymic rudiment, NCCs are not required for initial organ formation from the PP3[129]. Instead, NCCs play a critical role later in development, during the establishment of thymus- and parathyroid-specific domains and for the separation from the pharynx[130].

Foxn1-dependent events (from E11.5)

Following positioning and initial outgrowth of the organ rudiment, patterning of the thymus- and parathyroid domain as well as differentiation of the initial TEC precursors are required to complete thymus organogenesis. Both of these processes are critically dependent on Foxn1, whose expression can be detected from E11.5 onwards in the epithelial cells of the ventral part of the shared primordium[131], marking the

prospective thymic domain. On the other hand, expression of the transcription factor glial cell missing 2 (Gcm2) in the dorsal part is required for parathyroid development[132] (**Figure 2**). Although it is not fully understood what regulates Foxn1-expression in the thymus domain, several signaling pathways including sonic hedgehog (Shh), bone morphogenic protein (Bmp), wingless-int (Wnt) and Fgf signaling have been implicated in the initial patterning of the thymus[130], with NCCs being suggested as the source of key signaling molecules such as Bmp4 and Wnt4/Wnt5b[133, 134, 135].

Expression of Foxn1 promotes the differentiation of the epithelium in the thymus domain into functional TECs and enables them to recruit and support the development of the first lymphoid progenitors entering at E11.5[136] (**Figure 2**), in part through expression of the Foxn1-target genes CCL25 and DLL4[137]. Accordingly, initial colonization of the thymus by lymphocyte precursors is regulated via CCR7- and CCR9-mediate cytokine signals, through production of the ligands CCL21 and CCL25 in the shared organ primordia[136, 138]. As the thymus rudiment is not vascularized at this point, the first lymphoid progenitors have to cross the surrounding mesenchyme before entering through the basement membrane[126]. From E13.5 (but not earlier), the presence of developing thymocytes is also required for continuous TEC differentiation and formation of the medulla, suggesting that TEC development relies on epithelial-epithelial and/or epithelial-mesenchymal interactions initially but requires lympho-stromal crosstalk eventually to generate a functional competent thymus[139, 140].

The final steps of organogenesis include detachment of the shared primordia from the pharynx via apoptotic cell death by E12.0 and the subsequent separation of parathyroid and thymus domains by E12.5 (**Figure 2**), with both processes requiring the presence of neural crest-derived mesenchyme[141]. Consequently, pharyngeal detachment and parathyroid-thymus separation is delayed or completely absent in NCC-deficient mice[129]. Upon separation from their parathyroid counterparts, the two thymic lobes that were generated in parallel, each from one PP3 structure on either side of the pharynx, move towards their final anatomical location just above the heart where they come together by E16-17[130]. This final migration process concluding thymus organogenesis is also under the control of the surrounding NCCs, mediated at least in part by ephrin B2 signals[142].

Ontogeny of thymic epithelial cells

Adult cTEC and mTEC compartments can be clearly distinguished based on the expression of signature surface molecules, co-stimulatory molecules and transcription factors[143]. In the embryonic thymus however, phenotypic definition of cTEC/mTEC lineages and, even more so, of their common bipotent progenitor, remains challenging. In the following section I will first discuss what is known about TEC differentiation in the embryonic and neonatal thymus before focusing on the recent findings concerning adult TEC progenitors (TEPCs) that are important for maintenance of the postnatal TEC compartment.

Early stages of TEC development

During thymus organogenesis both cTECs and mTECs derive from a common bipotent progenitor of endodermal origin that requires Foxn1 for further differentiation into distinct cTEC and mTEC lineages[144, 145]. While the phenotypic identity of this initial precursor population remains elusive, the downstream developmental progression towards mature cTEC and mTEC compartments is thought to occur via lineage-committed progenitors, a notion first supported by the finding that clonally derived 'islets' of mTEC-committed progenitors give rise to the thymic medulla[146].

Using the cell surface markers CD205 and CD40, commonly used to identify cTEC and mTEC lineage in the adult, respectively[147], it has been shown that the majority of embryonic TEC are $CD205^{+}CD40^{-}$ around E12-13 and further progress through a $CD205^{+}CD40^{+}$ intermediate state before eventually giving rise to $CD205^{-}CD40^{+}$ cells[148]. Thus, embryonic TEC initially display a cTEC-like phenotype before subsequently acquiring mTEC-specific features, with $CD205^{+}CD40^{-}$ TECs being able to give rise to both cTEC and mTEC lineages[149]. Together with the finding that mTECs are derived from progenitor cells that have expressed the cTEC-specific protease subunit β5t during embryonic development[150], these results support a 'serial progression model' of TEC development with a bipotent progenitor displaying cTEC-like features upstream of mTEC lineage-committed precursors[151]. In line with this, an $IL\text{-}7^{+}$ embryonic TEC population, defining a specialized subset of cTEC during ontogeny, could give rise to mTECs in reaggregate thymic organ cultures (RTOC)[152]. Interestingly however, the fact that $IL\text{-}7^{-}$ cells showed enhanced propensity to differentiate into mature mTECs compared to the $IL\text{-}7^{+}$ subset suggests the presence of direct mTEC precursors within the $IL\text{-}7^{-}$ TEC population, further

supporting the idea that both cTEC-like bipotent progenitors and more committed mTEC precursors contribute to the development of the mTEC lineage[152].

Along these lines, a self-renewing embryonic subset of claudin-3/4 (Cld 3,4) ^{high} stage-specific embryonic antigen 1 (SSEA-1)$^{+}$ mTEC stem cells has recently been described to sustain long-term generation of mature mTECs capable of maintaining self-tolerance[153]. Further analysis of these mTEC stem cells in regard to receptor activator of nuclear factor (NF)-κB (RANK) expression, a member of the tumor necrosis factor receptor super family (TNFRSF) critical for thymus medulla formation[154], revealed a population of RANK^{+} Cld3,4^{high} SSEA-1^{-} mTEC-committed progenitors downstream of RANK^{-} mTEC stem cells[155]. Of note, RANK^{+} mTEC progenitors were absent in mice deficient for the transcription factor RelB, another known regulator of medulla formation[156], while mTEC stem cells were not affected[155]. Taken together, these findings suggest a possible regulatory mechanism controlling the generation of mTEC-committed progenitors from the mTEC stem cell pool in a RelB-dependent manner, with a subsequent requirement for RANK signaling.

In another study, embryonic precursors of Aire^{+} mTEC (termed pMEC) were identified that expressed RANK in combination with the known cTEC markers Ly51 and cytokeratin (CK)-8[157], and differentiation of these cells into Aire^{+} mTEC required TNF receptor-associated factor 6 (TRAF6)-dependent RANK signaling[157]. Furthermore, pMECs were shown to develop from an earlier RANK^{low} progenitor (pro-pMEC) upon non-classical NF-κB activation by RANK and LTβR signaling[157]. While the relationship between pMEC/pro-pMEC and mTEC stem cells remains unclear, these findings collectively underscore the importance of several TNFRSF members in controlling early mTEC lineage fate.

Compared to the progress made in understanding the differentiation of the mTEC lineage in the embryonic thymus, very little is known about the developmental stages of cTEC. So far it has proven difficult to identify lineage-committed cTEC progenitors, mainly owing to the cTEC-like phenotype of bipotent progenitors described above. Embryonic development of cTEC is thought to involve upregulation of CD40 and MHC class II by immature CD205^{+} β5t-expressing progenitors in a Foxn1-dependent manner[148]. Additionally, complete developmental progression to mature CD205^{+} CD40^{high} MHCII^{high} cTEC has been shown to require the presence of DN1-3 thymocytes[148], although the exact mechanisms through which thymocyte crosstalk promotes early cTEC development remain to be identified.

TEC progenitors in the postnatal thymus

The existence of TEC progenitors that continue to give rise to mature cTEC and mTEC in the postnatal thymus is strongly suggested by the fact that the thymus maintains the capacity to regenerate even in the adult[158, 159]. Moreover, the turnover rate for mature CD80$^+$ mTECs of 2 to 3 weeks indicates that this compartment must be continuously replenished by progenitor cells[160, 161]. In recent years, efforts have been made to identify putative TEC progenitors with stem-like characteristics in the adult, and while these studies have improved our understanding of how the postnatal TEC compartment might be maintained, there is still no consensus regarding their defining phenotype.

Importantly, the existence of adult bipotent TEC progenitors (TEPC) able to give rise to both cTEC and mTEC lineage has been demonstrated in *ex vivo* generated thymospheres of clonal origin[162, 163] and in *in vivo* studies using reaggregate thymic organ grafts and thymus transplants[164, 165]. TEPCs identified in these studies comprised a small population of self-renewing and largely quiescent cells, present at least up to 8 weeks of age[162, 165] and mature cTEC and mTEC progeny of bipotent TEPCs could be observed for as long as 9 months[164]. Taken together, these findings provide evidence for long-term generation of cTECs and mTECs from a rare subset of precursors displaying a stem/progenitor-phenotype present in the adult thymus.

Despite the overall agreement in the field regarding the existence of bipotent TEPCs in the adult, considerable uncertainty remains in terms of the phenotypic characteristics of these cells. While Wong et al. identified bipotent TEPCs as a subset of epithelial cell adhesion molecule 1 (EpCAM-1)$^+$ MHCII^{low} cells that expressed high levels of stem cell-associated markers stem cell antigen 1 (Sca-1) and α6 integrin[165], Ulyanchenko et al. described these cells as being EpCAM$^+$ MHCII^{high} and expressing high levels of placenta expressed transcript 1 (Plet1)[164], known to mark bipotent progenitors in the embryonic thymus[144]. Even more surprising, thymosphere-forming TEPCs were negative for both EpCAM-1 and Foxn1[162, 163], key markers of TEC identity expressed in the other TEPC population[164, 165]. However, whether this particular population of EpCAM-1$^-$ Foxn1$^-$ cells is able to sustain TEC progenitor potential under more physiological conditions remains to be seen. Regarding the spatial location of TEPCs, the CMJ has been proposed as a site enriched for bipotent TEPC populations within in the postnatal thymus[165, 166], supporting the notion that progenitors replenishing both cortical and medullary areas would most likely reside at the interface between the two.

In addition to bipotent TEPCs, more restricted cTEC-[164] or mTEC-lineage committed progenitor [167, 168, 169] have also been identified in the postnatal thymus. In this regard it is noteworthy that, in contrast to mTEC-lineage generation in the embryonic and neonatal phase, bipotent progenitors with cTEC-like features do not contribute significantly to mTEC generation in the adult[168]. Instead, maintenance and injury-induced regeneration of the adult mTEC compartment is mediated by lineage restricted mTEC-committed progenitors[168, 169]. In line with this, a small fraction of Cld3,4^{high} SSEA-1^{+} cells, corresponding to embryonic mTEC stem cells described above, could be detected in the adult thymus and was capable of giving rise to mature MHCII^{high} mTECs[153]. Interestingly, the availability and clonogenic activity of these mTEC-committed precursors was negatively regulated by thymocyte development[153], suggesting ongoing thymopoiesis as the most likely explanation for the observed decrease in mTEC stem cells after birth, a time in which thymocyte numbers expand rapidly. While the exact signals controlling this negative feedback loop are unknown, it is conceivable that the same thymocyte-derived signals driving mTEC maturation, such as RANK, CD40 and LTβR signaling[108, 154, 170], are also involved in limiting TEC precursor frequency and activity.

Functions of thymic epithelial cells

The thymic epithelium plays a vital role in all phases of T cell development, from the recruitment of early thymocyte progenitors to the egress of functionally mature T cells in to the periphery. As outlined above, thymocytes undergo differentiation and maturation while passing through the thymus and cTECs and mTECs express a range of surface molecules, cytokines, chemokines and transcription factors that support the distinct developmental events occurring in the cortex and medulla, respectively. The details of cTEC and mTEC functionality are discussed below, together with an overview of their phenotype in the adult thymus.

Phenotype and function of cortical epithelial cells

As a whole, TECs are defined as $CD45^-$ $EpCAM^+$ with cTECs further expressing CD205, Ly51 and the cytokeratins CK-8 and CK-18, all of which are markers commonly used to identify cTECs by flow cytometry or confocal microscopy[171].

Supporting the early events of T cell development is a main function of cTECs (**Figure 3**). Consequently, cTECs produce several chemokines that regulate thymus seeding, including CCL25 and CXCL12, to attract thymocyte progenitors expressing the respective chemokine receptors CCR9 and CXCR4[172]. Although cTECs do not produce the CCR7-ligands CCL19 and CCL21, the CCR7/CCR9 axis of thymus homing might further be regulated by cTECs through expression of the atypical chemokine receptor 4 (ACKR4, also known as CCRL1). ACKR4 acts as a scavenger receptor for CCL19, CCL21 and CCL25, which limits the availability of CCR7 and CCR9 ligands in the thymus and thereby negatively controls thymic entry[173, 174]. However, later stages of T cell development seem to occur independent of ACKR4 as ACKR4-deficient mice display normal thymopoiesis and thymic output[173, 174].

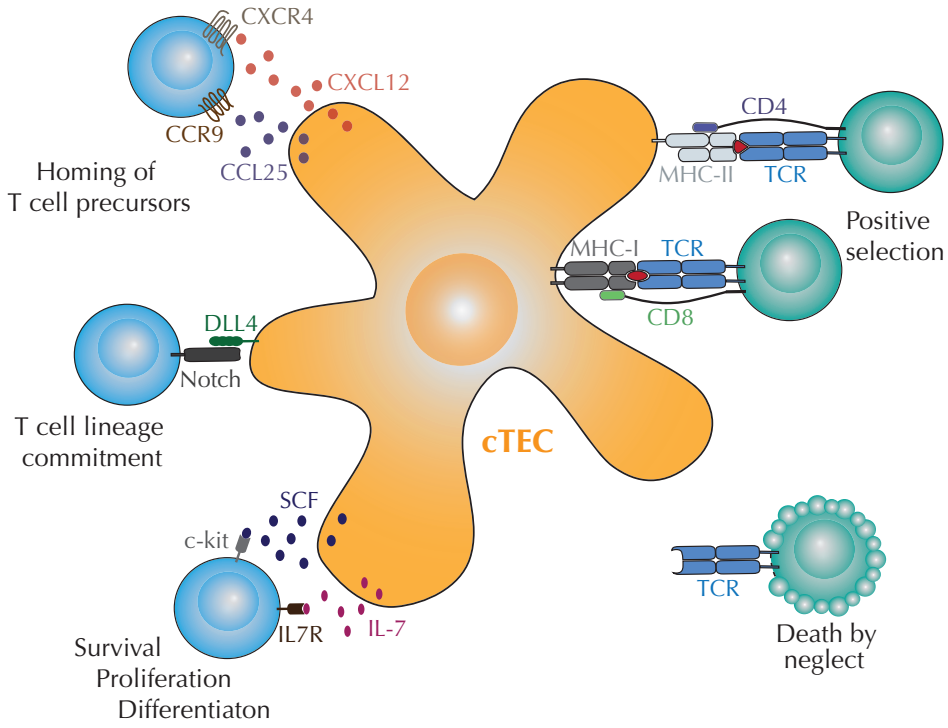

Figure 3 - Functions of cortical thymic epithelial cells. cTECs provide the essential cues to mediate homing of thymic precursors, commitment and differentiation of DN thymocytes (light blue) and positive selection of DP thymocytes (turquoise).

Apart from regulating colonization of the thymus, cTECs provide essential survival factors to the incoming progenitors and their immature thymocyte progeny (**Figure 3**). Production of the cytokines IL-7 and SCF by cTECs is required for the survival and proliferation of DN thymocytes[25, 152] and the expression of the membrane-bound Notch ligand Dll4 is critically required for the differentiation and T lineage commitment of thymocyte progenitors[22, 23, 175]. Of note, ongoing thymopoiesis induces downregulation of Dll4 on cTECs during ontogeny, suggesting a mechanism of control for early thymocyte development through negative regulation by DP thymocytes[175].

The second key function of cTECs is to mediate and regulate positive selection of DP thymocytes bearing functional TCRs that are capable of recognizing self-peptide/MHC complexes (**Figure 3**). An intricate machinery of protein degradation and antigen presentation allows cTECs to present a unique array of self-peptides in

the context of MHC class I and II molecules. In contrast to most other cell types, cTECs display high levels of constitutive macroautophagy, enabling the constant degradation of intracellular proteins to be loaded on MHC class II complexes[176]. Impaired autophagy due to disruption of the autophagy-related gene 5 (ATG5) leads to alterations in the generation and selection of MHC class II-restricted CD4SP thymocytes, suggesting a role for autophagy in shaping the TCR repertoire of $CD4^+$ T cells[177].

Regarding positive selection of $CD4^+$ T cells, two cTEC-specific lysosomal proteases, cathepsin L and TSSP, have been shown to be critically involved in MHC class II antigen processing and presentation. In addition to its role in MHC class II heterodimer assembly, cathepsin L is important for the generation of positively selecting MHC class II peptide ligands, with cathepsin L-deficiency resulting in impaired CD4SP selection and reduced peripheral $CD4^+$ T cell numbers[36]. By contrast, mice deficient for TSSP have normal levels of $CD4^+$ T cells, despite reduced frequencies of $MHCII^{high}$ cTECs[37]. However, a near complete absence of CD4SP thymocytes in TSSP-deficient MHC class II-restricted TCR transgenic mouse models indicated that TSSP is required for positive selection of $CD4^+$ T cells in a monoclonal setting[37]. Along these lines, it was later shown in a polyclonal setting that TSSP is required for the selection of certain CD4SP TCR specificities, but not for the majority of generated CD4SP thymocytes[178]. Collectively, these results suggest that TSSP in cTECs contributes to the diversification of the positively selected CD4SP TCR repertoire.

In terms of MHC class I antigen processing required for $CD8^+$ T cell selection cTECs are unique in the expression of the thymoproteasome, a specialized type of proteasome where the regular β5 subunit (encoded by the gene PSMB5) is replaced by the β5t subunit (encoded by the gene PSMB11)[179]. Incorporation of the β5t subunit allows for the generation of unique MHC class I-associated peptide motifs that represent primarily low affinity TCR ligands, thus facilitating the positive selection of CD8SP thymocytes[180]. Accordingly, positive selection is severely impaired in the absence of the β5t subunit, leading to reduced generation and functional impairment of CD8SP thymocytes that display a limited TCR repertoire[38]. Moreover, peripheral $CD8^+$ T cells of β5t-deficient mice display reduced TCR responsiveness, altered response to viral and bacterial infection and defects in maintaining the naive T cell pool[38, 181], further supporting the important role of β5t in cTECs for the selection and functionality of $CD8^+$ T cells.

Apart from the expression of protease systems that directly regulate positive selection, cTECs also influence positive selection events in their function as morphologically specialized thymic nurse cells (TNCs). These complexes consist of one individual cTEC enclosing several DP thymocytes and represent about 10% of the cTEC compartment[182, 183]. Importantly, TNCs provide a microenvironment for optimizing positive selection via receptor editing, as the enclosed DP thymocytes within the TNC complex are undergoing secondary rearrangement of the TCRα chain[183].

Phenotype and function of medullary epithelial cells

Common markers for postnatal mTEC populations include CK-5 and CK-14 as well ERTR5, MTS10 and the lectin UEA-1. Additionally, and in contrast to cTECs for which no clearly defined subsets have been described, mTECs can be further subdivided based on expression levels of CD80 and MHC class II. Thus, two main subsets of $CD80^{-}MHCII^{low}$ mTEC ($mTEC^{lo}$) and $CD80^{+}MHCII^{high}$ mTEC ($mTEC^{hi}$) exist in the medulla, with the $mTEC^{hi}$ subset supposed to originate from progenitors within the $mTEC^{lo}$ compartment[184].

The key function of mTECs is to establish central tolerance by eliminating self-reactive TCR specificities within the newly generated SP thymocyte pool during negative selection (**Figure 4**). Additionally, mTECs are critical for the development of tTregs that represent an important mechanism of control for autoreactive T cells that have managed to escape into the periphery. Essential to both these processes is the mTEC-specific ability to present an extensive array of TRAs by several means of promiscuous gene expression[185].

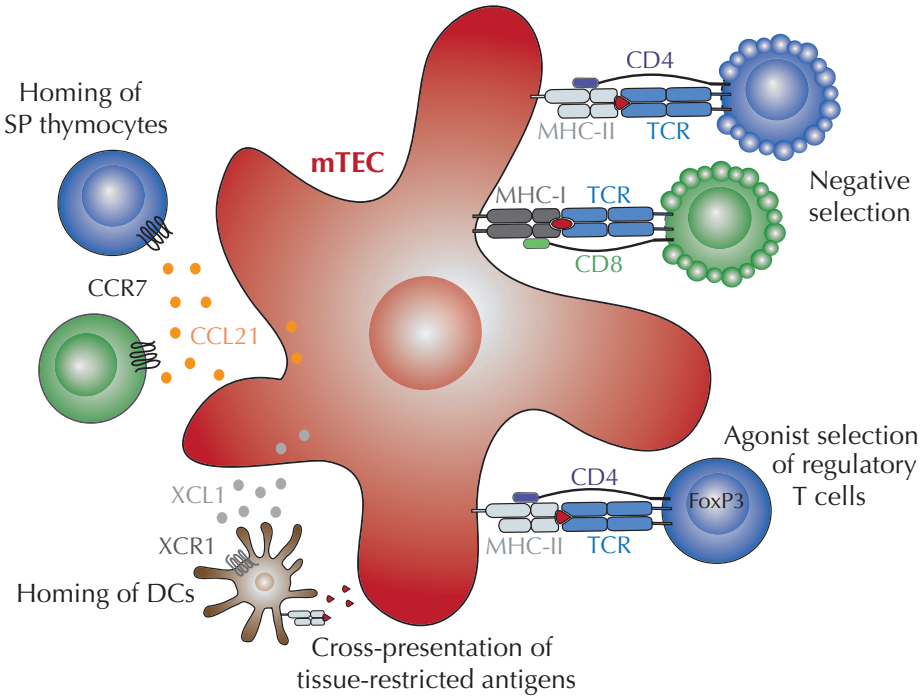

Figure 4 - Functions of medullary thymic epithelial cells. mTECs produce cytokines to mediate homing of CD4SP (blue) and CD8SP (green) thymoyctes to the medulla where they eliminate potentially autoreactive SP thymocytes via negative selection or by inducing Treg development. Homing and positioning of thymic DCs is also controlled by mTECs and DCs further support negative selection as they can cross-present mTEC-derived tissue-restricted antigens.

The first transcription identified to control promiscuous gene expression of TRAs in mTECs was Aire, whose expression is restricted to a subset of mTEC^{hi} cells[65]. Loss-of-function mutations of Aire cause autoimmune polyendrocrine syndrome type 1 (APS-1) in humans, a disease associated with formation of autoantibodies against several endocrine targets and complex autoimmunity affecting multiple organs[186]. A similar organ-specific autoimmune phenotype has been observed in mice deficient for Aire and these mice have been of use in understanding the extend of Aire-dependent promiscuous gene expression for central tolerance induction[65, 187, 188].

On a molecular level, Aire employs several mechanisms of epigenetic regulation and cooperates with other transcriptional regulators to initiate expression of tissue-specific genes that are typically silenced in other cells of the body. Of note, Aire target genes are enriched for repressive histone 3 (H3) marks such as H3K4me0, H3K27me3 and

H3K9me3 that indicate transcriptionally inactive chromatin sites[189]. Aire can bind to these silencing marks and induce local histone modifications and chromatin remodeling that allow for activation of gene transcription, possibly through H3K4 methylation and/or H3/H4 acetylation[190, 191]. Additionally, Aire can interact with several cofactors such as cyclin T and cyclin-dependent kinase 9, to initiate release of stalled RNA polymerase II at target gene promoter sites[192, 193, 194]. Recently, the protein deacetylase sirtuin-1 (Sirt1) that is highly expressed in mTEC^{hi} cells has been identified as a direct regulator for Aire-dependent expression of TRA genes. Sirt1 controls Aire activity through regulation of its acetylation status and Sirt1-deficiency leads to an autoimmune phenotype that resembles that of Aire-deficient mice[195].

As mentioned previously, mTECs are thought to express more than 18.000 genes[68], significantly more than any other cell types in the body, and the fact that Aire is only accounting for the expression of about 3.000 to 4.000 of these genes suggests additional Aire-independent mechanisms of promiscuous gene expression[67, 68, 69]. In this regard, the transcription factor Fezf2 has been shown to control the expression of a number of Aire-independent TRA transcripts that are essential for maintaining central tolerance[66]. Importantly, and in contrast to Aire, Fezf2 expression is not limited to a subset of mTEC^{hi} cells but is detectable in mTEC^{lo} and mTEC^{hi} cells alike[66], indicating that the mTEC compartment as a whole might be capable of promiscuous gene expression of TRAs via Aire-independent mechanisms, many of which are yet to be identified.

Apart from mTECs, thymic DCs are also involved in negative selection and tTreg development and their homing to the medulla is controlled by mTECs (**Figure 4**). Production of the cytokine XCL1 by mTECs ensures the correct positioning of CD8α$^+$ conventional DCs (cDCs) that express the relevant chemokine receptor, XCR1[196, 197]. Medullary accumulation of CD8α$^+$ cDCs is abrogated in XCL1-deficient mice, leading to the development of thymocytes with enhanced autoimmune potential and reduced tTreg numbers[197], suggesting that intrathymically derived CD8α$^+$ cDCs might play a non-redundant role in maintaining central tolerance and tTreg development. While extrathymically derived SIRPα$^+$ cDCs and plasmacytoid DCs also contribute to establishing central tolerance in the thymus, notably by capturing and presenting peripheral and blood-borne antigen[72, 75], it is unclear whether mTECs also play a role in the homing of these thymic DCs subsets.

In terms of thymocyte homing, mTECs provide the chemokine CCL21, a ligand for CCR7 expressed by newly generated CD4SP and CD8SP thymocytes that guides their migration to the medulla (**Figure 4**). In mice displaying abrogated CCR7 interactions, caused by CCR7-deficiency or loss of CCR7 ligands, thymocytes that have completed positive selection cannot home to the medulla[198]. While conventional T cell development can still progress in the absence of mTEC interactions[199], the emerging peripheral T cell pool will only undergo incomplete negative selection and thus lack tolerance to several TRAs[199, 200, 201].

Lastly, mTECs are also involved in controlling thymic egress of mature SP thymocytes. As outlined above, expression of S1P1 and downregulation of its negative regulator CD69 enables SP thymocytes to follow an S1P gradient towards the site of exit at the CMJ. Maintaining low levels of S1P in medullary areas distant from the vascular exit sites is essential for facilitating thymic egress and mTECs regulate upkeep of the S1P gradient by expressing enzymes that inactivate and degrade S1P, such as lipid phosphate phosphatase 3 (LPP3)[202] and S1P lyase[203]. Importantly, the targeted deletion of LPP3 expression specifically in mTECs leads to increased intrathymic levels of S1P and an accumulation of mature thymocytes within the medulla, indicative of impaired thymic egress[202]. Thus, mTECs can directly control medullary residence of mature SP thymocytes by influencing S1P levels.

Chapter 2. Peripheral T Cell Functions

Having completed the T cell development program described in chapter 1, $CD4^+$ and $CD8^+$ T cells leave the thymus and enter the peripheral circulation. Newly generated T cells are referred to as recent thymic emigrants (RTEs), representing a subset of the naive T cell pool that continues phenotypic and functional maturation in the periphery for about three weeks[204]. Entry into secondary lymphoid organs (SLOs) such as spleen and LNs is essential for RTE maturation and SLOs are also the place where mature naive T cells become activated once they encounter their cognate antigen presented to them by APCs. Activation of naive T cells leads to the production of effector T cells that fulfill various functions during the ongoing immune response. Whereas $CD8^+$ effector T cells are generally cytotoxic and can directly mediate killing of their target cell, $CD4^+$ effector T cells are more diverse and are commonly divided into different T helper (Th) subsets based on their cytokine secretion profile and transcription factor expression. The best-characterized Th subsets include Th1, Th2 cells and Th17 cells that are implicated in the immune response against intracellular pathogens, parasitic helminthes and extracellular bacteria and fungi, respectively.

As $CD8^+$ T cell responses are a major focus of Paper II of this thesis, the following chapter will focus on the activation of naive $CD8^+$ T cells, their effector functions as CTLs and the formation of various $CD8^+$ memory subsets.

Priming of naive $CD8^+$ T cells

The activation of naive $CD8^+$ T cells upon initial antigen encounter is referred to as priming and drives clonal expansion and differentiation events that initiate the $CD8^+$ T cell effector response. Apart from interactions between the TCR and cognate peptide-MHC class I complexes presented by the APC (most likely to be a DC), co-stimulation by the same APC is a key prerequisite for the priming of naive $CD8^+$ T cells. The requirement for a simultaneous second signal ensures that no effector

responses are being generated against self-antigens that naive $CD8^+$ T cells might encounter on any MHC class I-bearing tissue cell. Importantly, in cases where antigen recognition occurs in the absence of co-stimulatory signals the naive $CD8^+$ T cell becomes anergic, which means it does not proliferate but instead enters a long-term hyporesponsive state[205] that cannot be overcome, even if a secondary antigen encounter occurs in the context of co-stimulation[206]. Thus, clonal anergy represents an important mechanism of control during $CD8^+$ T cell priming and is essential for maintaining peripheral tolerance.

In terms of the co-stimulatory signals involved in $CD8^+$ T cell priming, CD28 engagement by B7 molecules (CD80 and CD86) expressed on activated DCs is crucial for the expression of IL-2 and the high affinity IL-2R complex by naive $CD8^+$ T cells (**Figure 5**). Consequently, increasing levels of available IL-2 and enhanced IL-2 responsiveness promote proliferation and differentiation into effector $CD8^+$ T cells. Whereas CD28 is constitutively expressed on naive $CD8^+$ T cells, B7 molecules are only induced on mature DCs that must undergo activation themselves before they can activate $CD8^+$ T cells. While in some settings the inflammatory signals elicited during the early infection phase can be strong enough to induce co-stimulatory activity in DCs, most $CD8^+$ effector responses require the help of $CD4^+$ T cells for DC maturation[207]. In the latter case, binding of a $CD4^+$ effector cell specific for the antigen presented by the DC initiates a two-sided activation of both partners via CD40-CD40L interactions that results in upregulation of co-stimulatory B7 molecules on the DC and the production of IL-2 by the $CD4^+$ T cell (**Figure 5**).

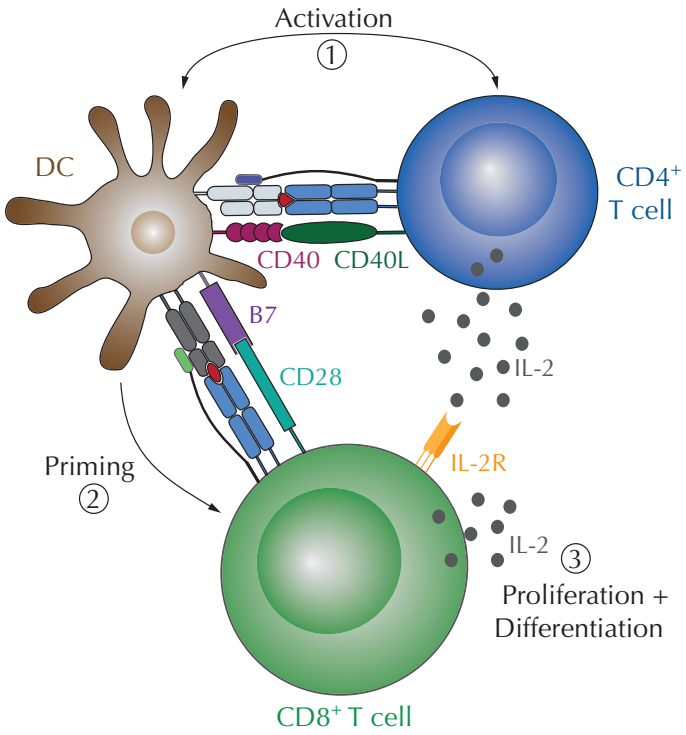

Figure 5 - Mechnism of CD4$^+$ T cell help during DC-mediated CD8$^+$ T cell priming. Recognition of MHCII-bound antigen on the DC by a CD4$^+$ T cell induces CD40-CD40L interactions between the two, leading to activation of both DC and CD4$^+$ T cell. As a result if this, the CD4$^+$ T cell produces IL-2 and the DC starts to express high levels of co-stimulatory B7 molecules required for optimal priming of a naive CD8$^+$ T cell that expresses CD28 and the antigen-specific TCR. Integration of proximal TCR signaling and co-stimulatory signals induces the expression of the high affinity IL-2R and autocrine production of IL-2 to enhance IL-2-mediated proliferation and differentiation into CD8$^+$ effector T cells. Inflammatory cytokines (not pictured) can further enhance the CD8$^+$ effector response.

Various virus infection models have been used to study CD8$^+$ T cell responses and while CD8$^+$ T cell priming in response to viral antigen often requires help from CD4$^+$ T cells, for example during herpes simplex virus (HSV)[208, 209] and vaccinia virus infections[210], it can occur in the absence of CD4$^+$ T cells during infections with lymphocytic choriomeningitis virus (LCMV)[211] and influenza virus[212]. The mechanism of CD4$^+$ T cell help is thought to compensate for a weak inflammatory milieu during antigen acquisition[207] and indeed the amount of interferon (IFN)-α/β produced during the innate response against viral infections has been shown to influence the requirement for CD4 T cell help[210, 211, 213]. Thus, abrogating IFN-α/β secretion can render the normally helper-independent CD8$^+$ T cell response to

LCMV infection helper-dependent[211], while increased IFN-α/β levels can circumvent helper-dependency in vaccinia virus infections[210, 213].

In addition to type I interferons, other inflammatory cytokines have been shown to play an important role during $CD8^+$ T cell priming, most notably IL-12 and IL-15, and $CD4^+$ T cell help has been shown in different settings to be required for the provision of these inflammatory cytokines by DCs[210, 214, 215]. A recent study investigating how the same mechanism of $CD4^+$ T cell help, i.e. CD40 signaling, can result in various cytokine secretion profiles, proposed that rather than considering $CD4^+$ T cell help as a substitute for innate signaling, it appears to act as an amplifier of said signals, thereby promoting pathogen-specific cytokine release by DCs[216]. In line with this, increasing innate signaling strength through high concentrations of toll-like receptor (TLR)-stimulating adjuvants was sufficient to induce inflammatory cytokine production by DCs at levels required for optimal $CD8^+$ T cell priming[216].

With regards to the downstream molecular effects of cytokine signaling received by naive $CD8^+$ T cell during the priming phase, IL-2 and IL-12 both mediate expression of the transcriptional repressor Blimp1[217], which together with the transcription factor T-bet, a target of Blimp1 that is further induced by IL-12[218], is essential during terminal effector differentiation[219, 220]. Importantly, during the generation of the primary effector response the expanding $CD8^+$ T cell population is functionally and phenotypically heterogeneous and can be divided into short-lived effector cells (SLEC), representing terminally differentiated cells with optimal effector functions that will undergo apoptosis once the infectious agent has been cleared; and memory precursor effector cells (MPEC) that are long-lived and contribute to the formation of the antigen-specific memory population that remains after clearance of the infection[221, 222]. Whereas Blimp1 and T-bet, together with additional transcription factors such as Id2 and Zeb2[223, 224], have been reported to be essential for SLEC differentiation, the formation of MPECs is promoted by Eomes, Id3, Bcl6 and Tcf1[223, 225, 226, 227]. Consequently, deficiency of Blimp1 or T-bet leads to impaired generation and functionality of the SLEC compartment with a concomitant skewing towards the MPEC lineage[218, 219, 220], while IL-12-dependent suppression of Tcf1 during T cell priming promotes SLEC functions[228].

How exactly SLEC and MPEC populations are being generated during clonal expansion of a single naive $CD8^+$ T cell is not completely understood, although asymmetrical partitioning of key transcription factors during the first rounds of cell

division is thought to play a role[229, 230], most likely in combination with additional modulation through the cytokine environment[228].

$CD8^+$ T cell effector functions

A strong $CD8^+$ T cell effector response is usually observed in infections with intracellular pathogens such as viruses and certain bacteria that live and replicate within host cells. To avoid further spread of the pathogen the preferred way to clear the infection is by eliminating the infected cell by inducing apoptosis to ensure the coordinated breakdown of all cellular compartments, including any infectious pathogen-derived material. $CD8^+$ effector T cells (termed CTL hereafter, for cytotoxic T lymphocyte) have several means to induce apoptosis once they encounter a target cell, depicted in **Figure 6** and further discussed below. Importantly, CTL activity does not require co-stimulation through CD28-B7 interactions, meaning the presentation of cognate antigen on MHC class I molecules, that are ubiquitously expressed, is enough to induce cytotoxicity. This is important as virtually any body cell, not only APCs, can be infected or show signs of malignant transformation and thus need to be targetable for CTLs. In fact, during the course of the effector response CTLs upregulate inhibitory co-stimulatory molecules such as CTLA-4 which are crucial in limiting the proliferative capacities and mediating the eventual termination of the immune response.

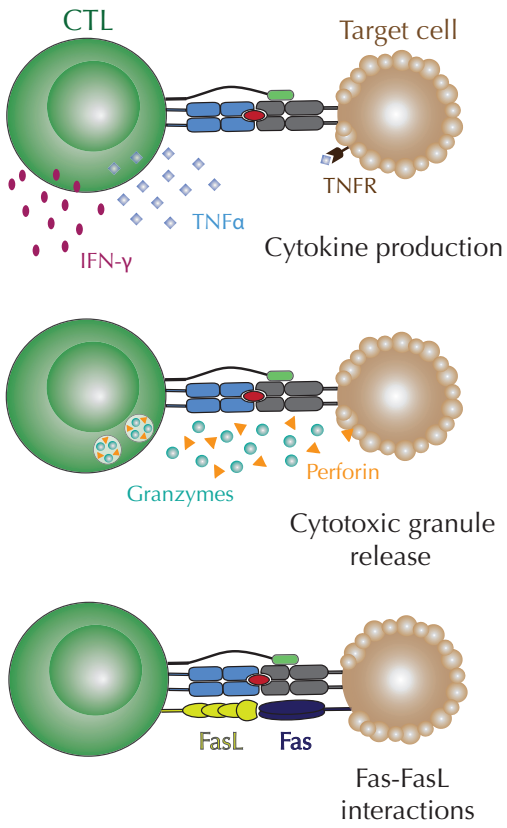

Figure 6 - Effector functions of cytotoxic CD8$^+$ T lymphocytes (CTLs) upon target cell encounter.

Upon encounter and recognition of a target cell, CTLs increase their cytokine production, most notably of IFN-γ and TNF-α. Whereas TNF-α occurs both in membrane-bound and soluble form and induces apoptosis through binding of the TNFR1 on the surface of target cells, IFN-γ can directly inhibit viral replication and activate macrophages. Furthermore, IFN-γ induces enhanced antigen processing and presentation by MHC class I molecules which increases the chance of recognition of infected cells by other CTLs[231]. Cytokine production by CTLs is one effector mechanism that acts globally and affects more cells than the initial target cell itself.

A more directed mode of action is the concentrated release of cytotoxic granules containing perforin and several serine proteases called granzymes. Cytotoxic granules are synthesized already during the priming phase and subsequent TCR engagement of the CTL induces initial degranulation and *de novo* synthesis of perforin and

granzymes to ensure continuous release of cytotoxic granules. Moreover, in order to avoid bystander killing and limit tissue damage, the release of these granules is highly directed, with the CTL orientating its secretory domain, including the Golgi network, towards the site of contact with the target cell marked by clusters of TCR:MHC complexes[232, 233]. This directed release of cytotoxic granules is crucial as the proteins within are non-specific and will trigger apoptosis in any cell they encounter. While perforin is essential in mediating the entry of granzymes into the cytosol by forming pores in the plasma membrane, granzymes themselves are responsible for inducing apoptosis of the target cell by activating caspase-3 cascade and causing mitochondrial cytochrome *c* release[234, 235]. CTLs themselves are protected from the cytotoxic proteins they release by lysosomal enzymes, notably cathepsin B, that are brought to the cell surface during degranulation. Surface cathepsin B maintains its proteolytic function even extracellularly and can inactivate perforin, thereby ensuring CTL membrane integrity and avoiding entry of granzymes into the cytosol[236]. However, mice deficient for cathepsin B display normal CTL survival upon degranulation[237], suggesting a certain level of redundancy regarding membrane-bound proteases and/or perforin inhibitors involved in CTL protection.

Lastly, CTLs can induce apoptosis through engagement of the first apoptosis signal receptor (Fas), a death receptor of the TNFR super family whose binding induces caspase-mediated apoptosis. Importantly, the fact that Fas expression is upregulated in activated T and B cells while CTLs themselves express both Fas ligand[187] and the Fas receptor, provides an important role for Fas-FasL interactions in regulating the numbers of CTLs and other activated lymphocytes[238]. This negative feedback function of Fas-mediated apoptosis by CTLs is crucial in the contraction phase of the immune response, after the pathogen has been cleared and the effector response needs to be shut down. Consequently, mutations in Fas- or FasL-encoding genes cause autoimmune lymphoproliferative syndrome (ALPS) in humans, a disease characterized by massive accumulation of activated T cells and high levels of autoantibodies due to insufficient control of the adaptive effector response[239].

In addition to the key effector functions described above that mainly have the purpose to induce target cell killing, CTLs have also been shown to be major producers of the immunosuppressive cytokine IL-10 during acute viral infections affecting the lung and brain[240, 241, 242]. Of note, IL-10 production at the peripheral sites of infection was enriched in CTLs with superior killing capacities (increased production of granzyme B, IFN-γ and TNF-α), suggesting that IL-10 produced by

highly cytotoxic effector cells plays an important role in minimizing inflammation in the surrounding tissue, especially in areas with limited regenerative potential such as the brain[240]. In this regard, IL-10 production by CTLs represents an additional key effector function that is essential for maintaining a balance between cytotoxicity directed against infected cells and protection of healthy tissue from inflammatory effects.

$CD8^+$ T cell memory

The massive clonal expansion of individual naive $CD8^+$ T cells upon primary antigen encounter is estimated to produce up to 10^7 cytotoxic effector $CD8^+$ T cells within one week after the infection[243] that serve to eliminate pathogen-infected cells. Following resolution of the infection, the vast majority of CTLs will undergo apoptosis, leaving only between 5% and 10% of the expanded effector pool to survive and differentiate into long-lived memory $CD8^+$ T cells[243]. One obvious benefit of $CD8^+$ memory T cell generation is the long-lasting increase in the number of antigen-specific cells that can react to a recurring infection of the same pathogen. Additionally, $CD8^+$ memory T cells possess certain qualities that allow them to respond in a faster and stronger manner to an infection, compared to their naive counterparts.

Before discussing the details of memory $CD8^+$ T cell functionality, I will give a brief overview of the three major subsets of antigen-experienced $CD8^+$ memory T cells and introduce the concept of 'virtual memory' cells whose development occurs independent of prior antigen encounter.

Subsets of $CD8^+$ memory T cells

$CD8^+$ memory T cells can be distinguished into central memory (T_{CM}), effector memory (T_{EM}) and tissue-resident memory (T_{RM}) subsets that differ in regards to surface receptor expression and anatomical location within the body.

Central memory and effector memory cells

T_{CM} and T_{EM} cells represent recirculating $CD8^+$ memory subsets, with $CD62L^{high}$ $CCR7^{high}$ T_{CM} cells preferentially residing in SLOs and $CD62L^{low}$ $CCR7^{low}$ T_{EM} cells mostly recirculating throughout the whole body. Both of these subsets can be differentiated from naive cells based on their expression of CD44, IL-7Rα (CD127) and IL-2Rβ/IL-15Rβ (CD122), however they differ slightly in their response to re-encountered antigen. While T_{EM} cells can rapidly produce inflammatory cytokines and release cytotoxic granules, T_{CM} cells lack these immediate effector functions and require more time to eventually differentiate into effector cells[244]. However, T_{CM} cells are important producers of IL-2 and have increased proliferative potential compared to T_{EM} cells, including self-renewal capacities[245]. Their preferred anatomical location provides the prerequisite for T_{EM} and T_{CM} functions as early responders at peripheral sites of infection and controllers of systemic infections, respectively, although their ability to recirculate enables them to functionally complement each other at any site in the body.

Tissue-resident memory cells

More recently, a third subset of $CD8^+$ memory cells has been identified that is not found in circulation but permanently resides within peripheral tissues; accordingly this subset is referred to as T_{RM} cells[246, 247]. While T_{RM} cells have been identified in various lymphoid and non-lymphoid organs (including brain[248, 249], kidneys[250, 251], liver[252] and pancreas[250]), they are mostly recognized for their role in maintaining protection of mucosal barrier surfaces in the intestine, the lungs, the female reproductive tract and the skin[253]. Within mucosal tissues T_{RM} cells are often found in association with the epithelium, which represents the common route of infection for many pathogens. Consequently, T_{RM} cells are characterized by a number of surface molecules that are important in confining their distinct localization, including CD69 and the integrins αE (CD103) and α1 (CD49a)[253]. In its role as S1P1-antagonist CD69 generally restricts egress of T_{RM} cells from the tissue[254], while CD103 mediates accumulation and maintenance within the epithelium through binding of its ligand E-cadherin[250, 255, 256], a cell adhesion molecule expressed by various types of epithelial cells[257]. Similarly, CD49a, in its function as part of the collagen IV-binding integrin dimer VLA-1, acts to position T_{RM} cells in close proximity to collagen-rich areas of intestinal and lung epithelium[258, 259]. Collectively, their exclusive tissue-residence and preferential localization to epithelial sites within mucosal tissues specifically indicates the importance of T_{RM} cells in responding to recurring local infections.

Virtual memory cells

A common feature of all three memory subsets described above is the fact that their development is initiated during a primary immune response that includes clonal expansion of antigen-specific effector cells. Hence, these memory cells are antigen-experienced and continue to survey the peripheral circulation or previously infected tissue for recurrence of their cognate antigen. Interestingly however, $CD8^+$ T cells displaying a $CD44^{high}CD122^{high}$ memory phenotype have been shown to exist in healthy wild type mice that have never be challenged with foreign antigen[260]. While it remains possible that some of these memory-like cells arise in response to certain environmental antigens such as food- or microbiota-derived antigens, the fact that memory-like $CD8^+$ T cells were found in in pathogen-free and germ-free mice[260], as well as in mice kept on a food antigen-free diet[261] strongly indicates that their development is independent of antigen recognition.

In order to discriminate them from 'true' memory subsets generated in response to a particular antigen, these antigen-inexperienced memory cells were termed virtual memory (T_{VM}) cells[262]. The T_{VM} phenotype bears a striking resemblance to that of $CD8^+$ T cells undergoing homeostatic proliferation in lymphopenic hosts, a process that is mostly cytokine-driven and requires low-level stimulation by MHC molecules[263, 264, 265]. In line with these similarities, it was shown that T_{VM} development is critically dependent on the cytokine IL-15, as these cells are absent from IL-15 deficient mice and mice lacking the IL-15Rβ chain CD122 on $CD8^+$ T cells[266]. Additionally, the expansion of the T_{VM} pool is further controlled by IL-4 and IFN-γ that stimulate proliferation and lead to increased IL-15 sensitivity, respectively[267, 268]. Regarding the cellular source of the signals controlling T_{VM} generation and expansion it has been shown that $CD8\alpha^+$ cDCs are key mediators of T_{VM} development, largely due to their ability to *trans*-present IL-15[266].

T_{VM} cells are generated in the periphery from naive $CD8^+$ T cells that display high levels of CD5, a marker indicative of TCR signaling strength during positive selection, suggesting that naive cells with particularly strong affinities for self-peptides are most likely to develop into T_{VM} cells[269]. Given the fact that T_{VM} cells primarily respond to inflammatory cytokines such as IL-12, IL-15 and IL-18, rather than cognate TCR stimulation, it might be beneficial to promote the differentiation of high affinity TCR-bearing naive $CD8^+$ T cells into T_{VM} cells to limit the risk of self-reactive T cell responses[269].

Functions of CD8$^+$ memory T cells

In order to fulfill their purpose of providing long lasting antigen-specific protection, memory T cells formed during the primary response need to be maintained in the periphery over an extensive period of time. In contrast to the survival of naive cells, homeostasis of the CD8$^+$ memory pool does not require contact with self-MHC complexes and is instead regulated by a combination of IL-7 and IL-15 that promote the survival and intermittent proliferation of CD8$^+$ memory T cells[270]. Consequently, IL-7- or IL-15-deficienct mice display markedly reduced numbers of CD8$^+$ memory T cells due to failed memory formation and/or impaired long-term maintenance after infection[271, 272, 273].

Reactivation of antigen-experienced memory CD8$^+$ T cells does not only occur in response to antigenic signals but also, similar to activation of T_{VM} cells, in response to the inflammatory milieu established by innate sentinel cells upon pathogen encounter. Production of IFN-γ, IL-12, IL-15 and IL-18 by distinct subsets of cDCs, inflammatory monocytes and tissue-resident macrophages rapidly initiates cell-intrinsic activation and cell cycle entry and can induce IFN-γ release and differentiation of memory CD8$^+$ T cells into cytotoxic effector cells[274, 275, 276, 277, 278]. Additionally, sensing of chemokines produced by myeloid sentinel cells initiates the recruitment of circulating CD8$^+$ memory T cells to the site of infection via chemotaxis, notably through CXCR3-dependent migration in response to IFN-γ-induced CXCL9 and CXCL10[279].

While antigen-independent mechanisms such as cytokine and chemokine sensing play a vital role in initiating and boosting memory CD8$^+$ T cell activation, recognition of cognate antigen is necessary to drive full effector cell expansion, especially in the case of T_{RM} cells[280, 281, 282]. Furthermore, antigen recognition promotes sustained IFN-γ release and the production of chemokines such as CCL3, CCL4 and CCL5 by activated memory CD8$^+$ T cells which in turn mediates the recruitment of additional innate effector cells including NK cells, macrophages, monocytes and neutrophils[283, 284]. This key role of CD8$^+$ memory T cells as orchestrators of secondary immune responses has been further demonstrated in a model of *Listeria monocytogenes* infection where protection of vaccinated mice is fully dependent on the presence of CD8$^+$ memory cells but not on their ability to express perforin or mediate Fas-induced apoptosis[285]. Instead, clearance of the bacterium was mediated by TNF-α

produced by activated monocytes and neutrophils whereas activated $CD8^+$ memory cells were required for the initial recruitment of these cells[286].

Taken together, $CD8^+$ memory T cells are activated by a combination of both antigen-independent and antigen-dependent signals and the interplay between the two ensures a faster and improved response to secondary infections. Moreover, the functions of $CD8^+$ memory T cells are more multi-faceted than previously thought and comprise several effector mechanisms in addition to their reinstated cytotoxic capacities, including the recruitment of various innate immune cells that contribute to the rapid clearance of a recurring pathogen.

Chapter 3. Mesenchymal Stromal Cells in Lymphoid Organs

Stromal cells fulfill essential immunoregulatory functions in lymphoid organs that support the lymphocyte development and activation events described in chapter 1 and 2. As discussed previously, epithelial stromal cells are of particular importance for thymic function and while they represent the major stromal subset in the thymus they do require crosstalk with another subset of mesenchymal stromal cells for their own expansion and maintenance. Similarly, while lacking an epithelial stroma compartment, LNs contain distinct subsets of mesenchymal stromal cells that mediate lymphocyte survival and facilitate the interactions of naive T and B cells with APCs. Despite their significant role in the regulation of adaptive immune cell development and functionality, little is known about the ontogeny and developmental relationship of thymic and LN mesenchymal cells. In Paper III of this thesis we investigate these questions and further define the heterogeneous and previously poorly characterized thymic mesenchymal compartment in detail. Therefore, the following chapter aims to provide an overview of the state of knowledge regarding thymic and LN mesenchymal cells prior to the publication of Paper III.

Functions of thymic mesenchyme

The thymic mesenchymal cell (TMC) compartment is primarily derived from neural crest cells, a multipotent population of ectodermal origin that is indispensable for the correct development and migration of the embryonic thymus (see also the section **Thymus Organogenesis**). During embryogenesis, NC-derived mesenchymal cells initially form the capsule wrapping around the emerging thymus rudiment at E12 and continue to colonize the inner core where they associate with the developing vasculature, with a subset of those cells acquiring pericyte- and vascular smooth muscle cell-characteristics by E15[126, 127]. The presence of TMCs during early thymic

development is essential for the proliferation of TECs, with removal of the TMC compartment by E12 leading to the formation of a hypoplastic thymus[287]. The positive regulation of TEC turnover by TMCs has been attributed to the provision of several growth factors including Fgf7 and Fgf10[288, 289], as well as insulin growth factors (Igf)1 and Igf2[287]. However, embryonic TMCs are also a major intrathymic source of the vitamin A metabolite retinoic acid (RA) that limits TEC expansion[290], indicating that TMCs can both positively and negatively control TEC proliferation. Importantly, the fact that TMCs continue to express the above-mentioned growth factors and RA also in the adult thymus[290, 291], suggests that TMCs may represent important regulators of TEC homeostasis throughout life.

In addition to the TEC compartment, TMCs also directly influence thymopoiesis by providing signals to developing thymocytes. Thus, the expression of DN thymocyte survival factors such as Flt3 ligand[292, 293] and SCF[291] by TMCs suggests a role in maintaining the early thymocyte progenitor pool. Furthermore, TMCs have been shown to produce CXCL12 and CCL19[291], indicating a potential role of these cells in controlling homing of thymocyte precursors and migration of positively selected thymocytes. Lastly, the pericyte-like subset of the TMC compartment is instrumental in regulating thymic egress, due to the expression of S1P-generating enzymes sphingosine kinase (Sphk)1 and Sphk2 that maintain high levels of S1P around the blood vessels where mature thymocytes exit the thymus[104].

Several surface markers have been used to identify and characterize the TMC compartment, including platelet derived growth factor receptors PDGFRα and PDGFRβ[287, 294], podoplanin (PDPN)[290], Ly51[127] and the antibody MTS-15 (recognizing the glycolipid Forssman antigen)[291]. Whereas some of these markers overlap with mesenchymal stromal cell markers in other organs (PDGFRα, PDGFRβ, PDPN), others seem to be thymus-specific (Ly51, MTS-15). However, it remains unclear to what extent the heterogeneity within the TMC compartment represents functionally distinct subsets and how these subsets relate to mesenchymal cells residing in other lymphoid organs.

Mesenchymal cell subsets in the lymph node

In contrast to TMCs, LN mesenchymal cells (LNMCs) have been studied in more detail, with several specialized subsets being identified that play non-redundant roles in maintaining LN functionality. Adult LMNC subsets are thought to derive from lymphoid tissue organizer (LTo) cells, a multipotent embryonic mesenchymal precursor population that is essential in mediating the formation and development of LN anlagen[295]. More specifically, LN organogenesis relies on crosstalk between LTo cells and hematopoietic lymphoid tissue inducer[117] cells and critically requires IL-7R-[296, 297], RANK-[298] and LTβR signaling[299], with deficiency in any of those receptors leading to partial or complete absence of peripheral LNs[298, 300, 301]. Although dispensable for initial LN anlage formation, LTβR signaling in particular is important for the maturation of LTo cells that involves the gradual upregulation of ICAM-1 and VCAM-1[301]. Moreover, it is believed that functionally mature LTo cells originate from pre-adipocytes in the fat pads surrounding the LN anlagen[302] and both embryonic and postnatal PDGFRα$^+$ PDPN$^+$ pre-adipocytes have been shown to support LN anlagen growth and the generation of LNMC subsets in grafting experiments[303].

As mentioned above, the adult LNMC compartment comprises functionally specialized subsets that can be found in distinct sites of the lymph node, respective to their main functions in supporting T and B cell responses (**Figure** 7). The following sections aim to discuss the functions of the three main LMNC subsets, T cell zone-associated fibroblastic reticular cells (FRCs), B cell follicle-resident follicular dendritic cells (FDCs) and marginal reticular cells (MRCs).

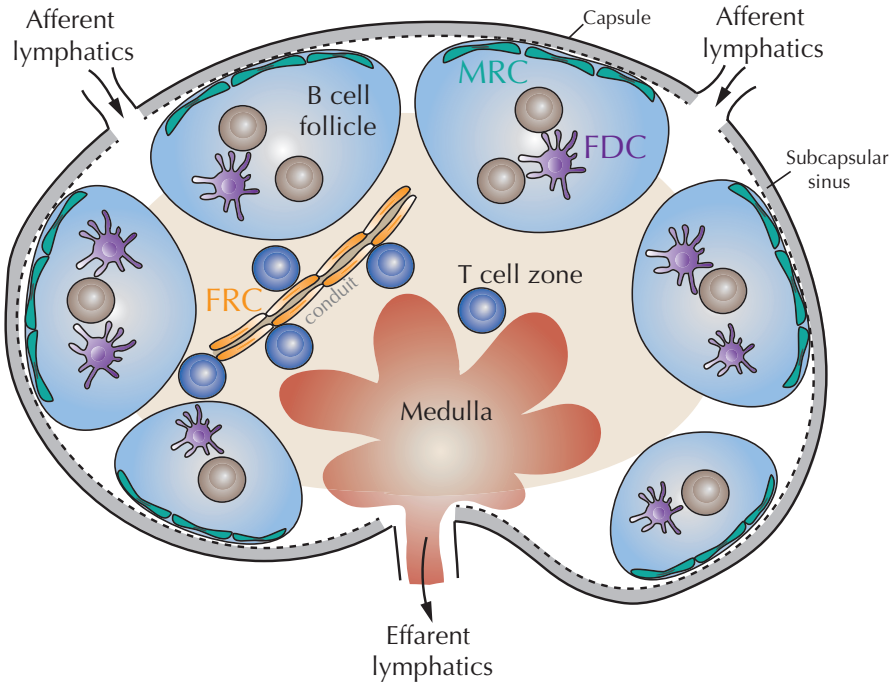

Figure 7 - Localization of mesenchymal stromal cells in the lymph node. Fibroblastic reticular cells (FRCs) are located in the T cell zone where they consitute a 3D network along which T cells and APCs migrate and engage in antigen presentation. Furthermore, FRCs generate a conduit system that mediates the transport of soluble antigen deep into the LN. In contrast, follicualr dendritic cells (FDCs) and marginal reticular cells (MRCs) reside in distinct parts of B cell follicles to support B cell homeostasis and the formation of germinal centers upon initiation of an immune response.

Fibroblastic reticular cells (FRCs)

FRCs located in the T cell zone produce and ensheath extracellular matrix (ECM) proteins, thereby forming a 3D reticular network that physically supports the migration and interaction of T cells and APCs[304] (**Figure** 7). Additionally, FRCs produce chemokines such as CCL19 and CCL21 that attract and control the localization of T cells and DCs within the T cell zone[305, 306, 307]. Apart from mediating spatial organization of immune cells, FRCs also directly support the survival and homeostasis of naive T cells through the production of IL-7[308]. Moreover, the 3D reticular network provided by ECM and FRCs creates a conduit system for the lymph to transport soluble low molecular weight antigens throughout the T cell zone[309]. Collectively, the FRCs possess essential properties to facilitate antigen presentation in the T cell zone and promote T cell survival, thereby maximizing the chances of naive

T cells to encounter their cognate antigen. Interestingly, while mostly seen as indirect supporters of T cell priming, FRCs have also been suggested to promote peripheral tolerance through direct presentation of peripheral tissue antigen[310, 311].

Follicular dendritic cells (FDCs)

FDCs are found in the center of B cell follicles where they create a cellular scaffold for migrating B cells and support B cell follicle homeostasis via production of the survival factor B cell activating factor (BAFF) and the B cell follicle homing chemokine CXCL3[312, 313, 314]. In addition, FDCs express the complement receptors (CR)-1 and CR-2 (also known as CD35/CD21) enabling them to retain and present soluble antigen and immune complexes to B cells[315]. Upon antigen encounter, FDCs aid in the formation and maturation of germinal centers, specialized structures that support B cell proliferation, somatic hypermutation of BCR genes and subsequent affinity maturation to optimize the humoral immune response[316]. Differentiation of mesenchymal precursors and MRCs into FDCs is thought to contribute to the expansion of FDCs during the germinal center response[317, 318]. Furthermore, differentiation and maintenance of FDCs has been shown to require TNFα and LT signals from the surrounding B cells[319, 320], with the absence of B cells leading to impaired FDC development[321].

Marginal reticular cells (MRCs)

In addition to centrally located FRCs, B cell follicles also contain MRCs that form a layer in the outer follicle beneath the subcapsular sinus (SCS) (**Figure** 7). This particular location enables MRCs to funnel small soluble antigen arriving with the afferent lymph at the SCS into the inner follicle via a conduit system similar to that established by FRCs in the T cell zone[322, 323]. Detailed knowledge regarding additional specific functions of MRCs remains elusive, however, MRCs have been suggested to represent the adult counterparts of LTo cells[324] and can differentiate into FDCs following immunization (see above). In line with a potential FDC-precursor function, MRCs share expression of CXCL3 and MAdCAM-1 with mature FDCs, but do lack other functional FDC markers such as CR-1/CR-2[325].

Chapter 4. Retinoic Acid Signaling

Retinoic acid is a metabolite of vitamin A, or retinol, that controls a variety of biological processes via its function as a ligand for nuclear receptors. Thus, RA signaling is crucial for early developmental processes such as embryonic patterning and organogenesis and continues to control cell proliferation and differentiation during adulthood. Moreover, RA is essential for establishing and maintaining a functional immune system and the increased susceptibility to infections caused by vitamin A deficiency still represents an important health issue in developing countries.

RA signaling is a central part of the work presented in Paper I and Paper II of this thesis, where we addressed its role in TECs, T cell development and $CD8^+$ T cell functionality. In order to put these results in context, I will first provide an overview of the biology of RA, including its metabolism and molecular function, before focusing on its role in shaping adaptive immune responses.

Metabolism of vitamin A and RA

Prior to the generation of RA, retinol has to be obtained from the diet, as the body is unable to produce this vitamin itself. Uptake of dietary retinol is mediated by epithelial cells in the small intestine (SI) from where it needs to be brought to the liver for long-term storage in the form of retinyl esters[326] (**Figure 8**). As a fat-soluble vitamin, retinol is transported to the liver via chylomicrons, specialized lipoprotein particles that carry phospholipids and fatty acids through the circulation[327]. Importantly, SI epithelial cells are also capable of directly metabolizing retinol into RA, which is then released into the intestinal microenvironment[328]. Other target cells receive retinol via the blood stream upon its release from the liver in the form of retinol binding protein (RBP)-bound complexes[329]. Retinol enters the cytosol of target cells via surface receptors such as the stimulated by RA 6 receptor (STRA6)[330] and is subsequently metabolized into RA via a two-step oxidative process (**Figure 8**).

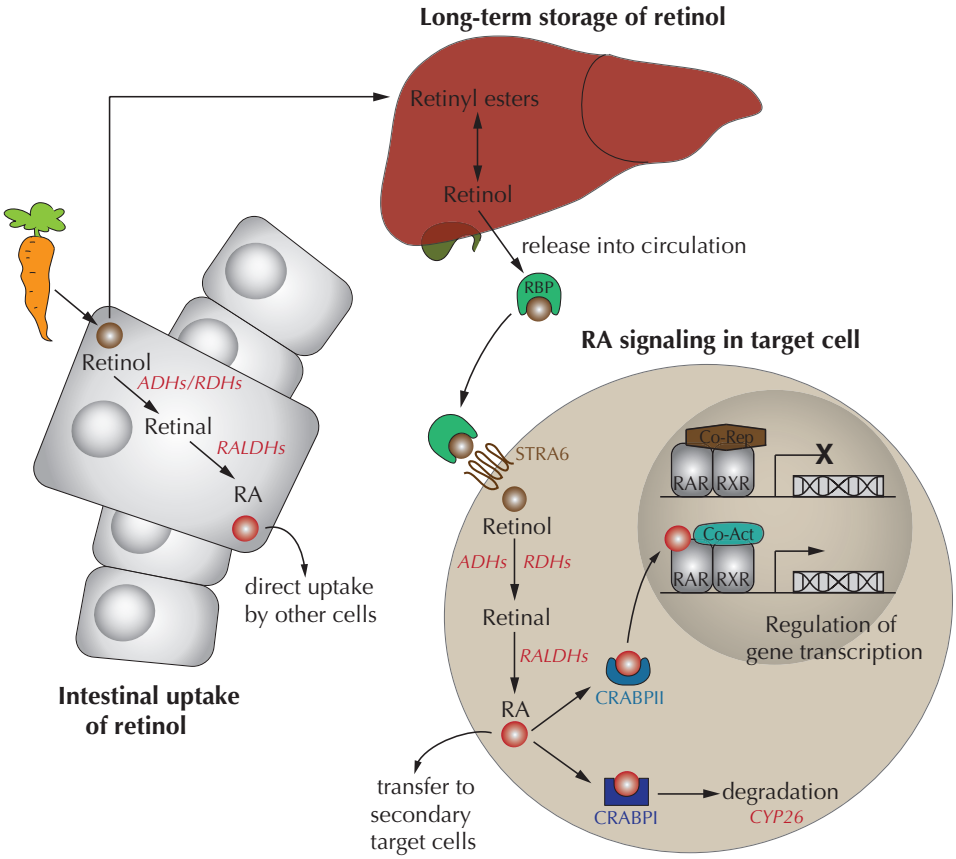

Figure 8 - Metabolism and function of RA. Retinol is taken up by small intestinal epithelial cells and either metabolized into RA directly and secreted into the intestinal milieu or transferred to the liver for long-term storage in the form of retinyl esters. RBP-bound retinol released from the liver into the circulation translocates into the cytosol of target cells via STRA6 and other surface receptors. The two step oxidative conversion of retinol to RA via retinal is controlled by ADH/RDH and RALDH enzyme families. Cytosolic RA bound to CRABPI is marked for degradation by CYP26 enzymes while CRABPII-bound RA will be transported into the nucleus where it exerts its function as a ligand of RAR-RXR receptor complexes to initiate target gene transcription.

Three enzyme families are involved in RA synthesis: Ubiquitously expressed alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs) can mediate the reversible reaction of retinol to retinal[331], while the final oxidation of retinal to RA is controlled by retinaldehyde dehydrogenases (RALDHs) of which the three isoforms RALDH1, RALDH2 and RALDH3 (encoded by the genes Aldh1a1, Aldh1a2 and Aldh1a3) are highly relevant in terms of regulating levels of available RA[331, 332].

The cell-specific expression of RALDHs means that RA production is limited to certain tissues and it is generally assumed that RA-responding cells either express RALDHs themselves or are closely associated with cells that do. However, RA can influence more remote cells via transport in extracellular vesicles or bound to serum proteins, although the exact mechanisms of this mode of RA transfer are not completely understood[333].

Within the target cell, amounts of cytosolic RA are further regulated by RA-metabolizing CYP26 enzymes and binding to the cellular retinoic acid-binding protein 1 (CRABPI) targets RA for this degradation pathway[334]. In contrast, binding to CRABPII mediates transport of RA to the nucleus where it acts as a ligand for heterodimeric RA receptor (RAR)-retinoid X receptor (RXR) complexes[335] (**Figure 8**). Of note, *all-trans* RA, the main RA isoform occurring *in vivo*, displays a 50-fold stronger activation of RARs than RXRs[336], indicating that ligand-dependent activation of the RAR-RXR complex is predominantly induced through binding of RA to RAR. In contrast, the main purpose of the RXR domain seems to be to enhance the binding of the receptor dimer to the DNA and to increase the transcriptional activity of RARs[337]. However, apart from RAR, RXRs also form complexes with other nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), and as part of these receptor dimers RXRs can be activated by fatty acids to control metabolic processes and lipid biosynthesis[338].

RA ligation of RAR-RXR dimers is the prerequisite for RA-mediated gene regulation, the details and variations of which will be discussed in the following section.

Mechanisms of RA-regulated gene expression

As mentioned above, the nuclear receptors through which RA is signaling belong to the RAR and RXR families, both of which containing three subtypes (RARα, -β, -γ and RXRα, -β, -γ) that can pair in any form of heterodimeric combination. RAR-RXR receptor dimers bind to specific RA responsive elements (RARE) in the regulatory regions of RA target genes, with the classical RARE being a direct repeat (DR) of a core motif, PuGGTCA, separated by a 5 bp spacer (DR5), although additional 2 bp- and 1 bp-spaced (DR2/DR1) RAREs have been described as well [339].

The main mechanism of RA-mediated gene regulation is the direct transcriptional activation of RAR-RXR complexes that subsequently act to induce gene expression. In the absence of RA as ligand, RAR-RXR dimers are associated with various corepressors such as nuclear receptor corepressor (NCoR) 1 and NCoR2 that further recruit histone deacetylases (HDACs) to mediate gene silencing via induction of repressive chromatin structures over the promoter of the target gene. Conversely, RA binding of RAR induces a conformational change of the receptors that causes the release of bound corepressors and instead leads to the association of coactivators belonging to the p160 steroid receptor coactivator family. Subsequent recruitment of histone acetyltransferases/methyltransferases and nucleosome remodeling complexes induces a permissive chromatin landscape that enables target gene transcription[340] (**Figure 8**).

Apart from directly inducing gene transcription through RAR activity on RARE sites, RA signaling has also been implicated in several non-canonical ways of transcriptional regulation. Firstly, RA-ligated RAR-RXR dimers can influence the activity of transcription factors that are not direct RA target genes in the classical sense, such as the dimeric transcription factor complex activator protein-1 (AP-1), by blocking its ability to bind DNA and disrupting its dimerization[341]. Moreover, RA-RAR interactions have been shown to induce the expression of microRNAs and non-coding RNAs, both of which are able to degrade mRNA of other transcriptional regulators and/or act as adaptors for chromatin remodeling complexes, notably during differentiation and developmental processes[342, 343, 344].

Interestingly, RA-mediated gene regulation does not only occur on the transcription level but also directly affects translation events in the cytoplasm. Here, unligated RARα functions as an mRNA-binding protein that effectively inhibits translation of several neural proteins including glutamate receptor 1 (GluR1). Binding of cytoplasmic RA then induces the release of mRNA-associated RARα and enables translation, thus demonstrating an important mechanisms of rapid and transient regulation of protein expression by RA and RARs that occurs independent of their nuclear functions[345, 346].

RA signaling in the immune system

Models for studying RA signaling in vivo

Most of the early work regarding the role of RA signaling in immunity has been performed in vitamin A-deficient (VAD) animals that lack systemic RA signaling due to absence of retinol in the diet. Importantly, the generation of VAD mice (or any other species) has to be initiated on the level of the previous generation by placing pregnant females on VAD diet from E11 onwards and the experimental window in which VAD mice can be used is rather short, as systemic absence of vitamin A strongly affects general physiology and is eventually lethal[347]. More recently, various transgenic models have been developed that allow for a continuous ablation of RA signaling, for example by using conditional knockouts of any of the RAR isoforms[348, 349, 350] or by cell type-specific expression of a dominant-negative RARα transgene (dnRAR), a truncated version of RARα that cannot induce RA-mediated gene transcription due to lack of its signaling domain[351] (**Figure 9**). The dnRAR model has been used for the work in Paper I and II and its advantages and limitations will be discussed in the synopses of these papers.

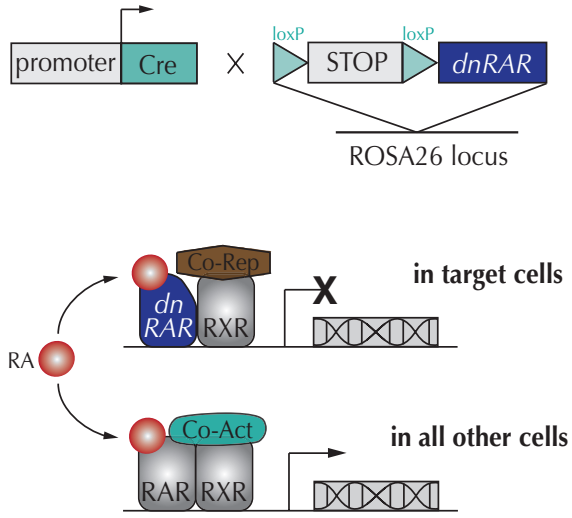

Figure 9 - Expression of a dominant-negative RARα construct (dnRAR) blocks RA signaling. Mice bearing the dnRAR construct behind a loxP-flanked STOP cassette in their ROSA26 locus are crossed to mice expressing the Cre recombinase under the control of a cell-specific promoter (such as Foxn1 or Cd4). The offspring of such cross breeding will express the dnRAR in the cells of interests due to Cre-mediated removal of the STOP cassette which abrogates RA signaling in these cells. In contrast, RA signaling will function normally in all other cells.

RA signaling in immune system development

In line with its role in organ development and differentiation during embryogenesis, RA is required for the development of SLOs and the availability of maternal RA during fetal development determines the functionality of the adult immune system. Consequently, offspring of mice treated with RA inhibitors during their pregnancy display reduced numbers and decreased size of LNs and small intestinal Peyer's patches, due to the impaired differentiation and functionality of LTi cells, a subset of type 3 innate lymphoid cells (ILC3s)[352]. More precisely, RA signaling controls LTi differentiation through direct regulation of RAR-related orphan receptor γt (RORγt) gene expression, a key transcription factor for ILC3 development and maturation[352]. Importantly, absence of RA signaling in LTi cells during early life only (up to 2 weeks of age) led to impaired $CD8^+$ T cell effector responses to viral infections during adulthood[352], underscoring the importance of RA signaling *in utero* and during early life for the establishment of a functional immune system.

In primary lymphoid organs such as the thymus, RA signaling has been implicated in controlling apoptosis of DP thymocytes, with opposing effects depending on the involved RAR isoform. Thus, while RARγ agonists lead to massively reduced DP numbers and thymic involution[353], RARα agonists and RA at physiological concentrations have been shown to prevent TCR signaling-induced deletion of self-reactive thymocytes[354, 355]. This RARα-mediated protection of apoptosis in response to strong activation signals is thought to occur via inhibition of Nur77 activity and Bim synthesis[355]. Of note, these studies either investigated the effects of RA signaling in *in vitro* systems such as fetal thymic organ cultures (FTOC) or made use of synthetic RAR agonists that were injected into mice at high concentrations. Thus, the physiological role of RA signaling in developing thymocytes *in vivo* remains incompletely understood. In terms of the epithelial compartment of the thymus RA signaling has been suggested as a potential mechanisms of epithelial-mesenchymal crosstalk that regulates TEC homeostasis, as TMC have been identified as producers of RA *in vivo* and treatment of FTOC with RA-inhibitors resulted in TEC expansion[290].

Regulation of T cell effector functions by RA signaling

During postnatal life, RA is commonly associated with maintaining the balance between protective immune responses and peripheral tolerance, with several additional factors controlling the variable effects of RA signaling in different contexts. These factors include the amount of available RA, the cell type-specific expression of RAR isoforms and the local cytokine milieu. In its role as a potent regulator of gene expression RA has been shown to affect the differentiation, maturation and functionality of most cells of the innate and adaptive immune system[356]. However, considering that the work presented in this thesis focuses on T cell development and peripheral T cell effector functions, the following section will be limited to the role of RA signaling in T cell responses.

The regulation of inflammatory and tolerogenic immune responses is of particular importance in the intestine, where the local immune system encounters various pathogen-derived antigens while being constantly exposed to commensal bacteria and innocuous food antigens that need to be tolerated. In this regard it is worthwhile noting the elevated levels of RA in the small intestinal microenvironment, owing mainly to the retinol metabolizing activity of the local epithelium and SI $CD103^+$ DCs[333]. While SI epithelial cells constitutively express Aldh1a1[357], SI $CD103^+$ DCs upregulate the expression of the RA target gene Aldh1a2 upon RA imprinting, in response to RA signals derived from intestinal epithelial cells, stromal cells and the bile[328, 358, 359]. RA-imprinted $CD103^+$ DCs migrate to the draining mLNs where they present intestinal-derived antigen to naive T cells while providing RA signals that induce the expression of gut-homing molecules CCR9 and $\alpha4\beta7$ during priming[360, 361]. Of note, *in vivo* induction of gut-homing properties is dependent on mLN stromal cells that, in contrast to peripheral LN stromal cells, express high levels of Aldh1a1 and Aldh1a3[362, 363]. Thus, the local stromal network further increases the availability of RA (and possibly other signals) in the mLN that confer a permissive environment for the induction of gut-homing lymphocytes.

In terms of T cell effector functions RA signaling has been studied extensively in the context of $CD4^+$ T cell differentiation and lineage stability, where RA seems to elicit distinct effector responses depending on the prevailing homeostatic or inflammatory conditions. During steady state, RA promotes the TGF-β-dependent generation of pTregs by inducing FoxP3 gene expressing while inhibiting Th17 differentiation via blocking of IL-6R and IL-23R signaling[364, 365, 366]. Additionally, RA counteracts cytokines that inhibit pTreg conversion and promote effector differentiation such as

IL-4, IL-21 and IFN-γ produced by $CD4^+$ $CD44^{high}$ effector T cells[367]. This immunosuppressive role of RA dominates during homeostatic conditions and is important for maintaining peripheral tolerance, however, RA signaling can have opposing functions during settings of infection and inflammation where it is required for the generation of functional Th1[368, 369, 370], Th2[371, 372], Th17 responses[368, 370, 373] and for lineage stability of Th1 cells[374]. In general, the contrasting role of RA signaling in T helper cell differentiation and pTreg induction is determined by a varying spectrum of synergizing cytokines and other environmental factors, as well as the concentration of RA itself. Thus, it has been shown that low levels of RA promote Th17 differentiation *in vitro*, while high levels suppress Th17 differentiation in favor of Treg generation[375].

Compared to $CD4^+$ effector responses, less is known regarding the role of RA signaling in $CD8^+$ T cell functions, although RA is thought to enhance anti-tumor[376] and anti-viral immunity[377]. Indeed, production of RA within the tumor microenvironment of melanoma-bearing mice increases the accumulation of tumor-specific $CD8^+$ T effector cells and RA is required for $CD8^+$ T cells to mediate antitumor immunity and limit tumor growth[378]. In terms of viral infections, RA signaling-impaired $CD8^+$ T cells were shown to predominantly adopt an MPEC phenotype during the effector phase after vaccinia virus infection that was characterized by impaired effector functions, although this did not seem to affect viral clearance[379]. It is noteworthy however that the role of RA in naive $CD8^+$ T cells prior to infection has not been addressed in the study by Allie at al. and it remains to be seen if and how abrogated RA signaling affects the naive $CD8^+$ T cell compartment.

Present investigation

Aims of the thesis

The overall aim of this thesis work was to study the impact of retinoic acid signaling on thymic epithelial stroma and T cell functionality, as well as the characterization of mesenchymal stromal cell compartments in lymphoid organs.

Specifically, the aims of the included studies were:

I. To assess the role of RA signaling in TEC homeostasis and functionality with regards to their ability to support T cell development

II. To determine the impact of RA signaling in developing thymocytes and $CD8^+$ T cells for their development and generation of effector functions

III. To characterize TMC heterogeneity and investigate the ontogeny and developmental relationship of mesenchymal cells in lymphoid organs

The following section provides a summary of the main findings of the work included in this thesis, as well as a brief discussion of open questions and limitations regarding the individual studies.

For a complete overview and detailed discussion of the results, the reader is referred to the original publications (for Paper I and III) and the manuscript (for Paper II) in the back of this book.

Summary and Discussion of the Papers

Paper I

Retinoic Acid Signaling in Thymic Epithelial Cells Regulates Thymopoiesis

TECs are essential for the generation of functional naive T cells by mediating survival, differentiation and selection of developing thymocytes. Despite their non-redundant role in T cell development, knowledge of the signals controlling TEC homeostasis and differentiation is limited. Previous work by our group identified thymic mesenchymal cells as producers of the vitamin A metabolite RA and demonstrated increased TEC proliferation *in vitro* in FTOCs upon addition of a RAR-antagonist [290]. To better understand the significance of RA signaling in TECs *in vivo* we generated *Foxn1Cre.dnRAR*$^{lsl/lsl}$ mice, whose TEC compartment is unresponsive to RA, and used these mice to analyze TEC homeostasis, gene expression and functionality in the absence of RA signaling.

Key findings

- Adult *Foxn1Cre.dnRAR*$^{lsl/lsl}$ mice display increased numbers of cTEC and enhanced cTEC proliferation
- RA controls gene expression of distinct biological pathways in cTEC and mTEC^{lo}, but not mTEC^{hi}
- Absence of RA signaling leads to an accumulation of putative bipotent progenitors within the cTEC compartment and reduced mTEC numbers during early postnatal periods
- Enhanced TEC regeneration after glucocorticoid-induced thymic injury in the absence of RA signaling
- Reduced generation of CD4SP and CD8SP thymocytes in *Foxn1Cre.dnRAR*$^{lsl/lsl}$ mice
- Impaired RA signaling in TEC leads to altered response of naive CD8^{+} T cells to *in vitro* TCR stimulation

Discussion

This study was initiated based on previous *in vitro* findings regarding the potential role for RA signaling in TEC homeostasis and the identification of RA-producing TMCs in the adult thymus[290]. Generation of *Foxn1Cre.dnRAR*$^{lsl/lsl}$ mice allowed us to perform detailed *in vivo* analysis of the impact of RA signaling in TECs, thereby confirming and considerably extending the prior results.

The expansion of the cTEC compartment observed in the absence of RA signaling was mostly due to an increase in the Sca-1^{high} subset, a subpopulation of Ly51^{+} cTECs that has been described to contain immature bipotent TEPCs[164, 165]. An accumulation of immature progenitors at the expense of functionally mature TECs might explain why RA signaling-deficient TECs displayed suboptimal functionality in terms of supporting thymopoiesis. While we did not observe alterations in the expression of genes associated with promoting thymocyte development or survival in cTECs at 8w of age, gene expression analysis of younger mice could provide additional insight into the mechanisms of RA-mediated gene regulation in early life, as this is the phase when thymic output and TEC function peak. Additionally, it would be of interest to characterize the accumulating Sca-1^{high} cTEC subset further, ideally on a single cell basis, to determine if the increase predominantly affects cells that harbor true progenitor potential.

In terms of identifying direct RA target genes in TECs, additional chromatin immunoprecipitation (ChIP)-sequencing experiments should aid in determining direct binding sites of RARs. However, it is important to bear in mind that RAR-binding RAREs can be found in various places of RA target genes, not necessarily only in the promoter. Additionally, identification of specific histone modifications such as activating and silencing marks and marks of ongoing transcription are warranted to clearly identify induction or repression of gene expression in response to RA signaling. Finally, RA and RARs can function as regulators of gene transcription and translation in ways that extend beyond the classical mechanism of RAR binding to the DNA, the effect of which would be not detectable by RAR ChIP-sequencing. In this regard, it would also be important to determine the targets of other transcription factors controlled by RA, as it is likely that many effects of RA signaling are mediated indirectly, through activation of additional transcriptional regulators that induce or repress gene expression by itself.

The transgenic mouse model used in this study makes use of the expression of the transcription factor Foxn1 in TECs to drive cell-specific Cre-mediated removal of the STOP cassette in front of the dnRAR construct. As with any model using the Cre-loxP system there is the risk of off-target effects; however, we did not observe any alterations in TEC and thymocyte phenotype of pure *Foxn1Cre* mice compared to their wild type littermates. Of note, Foxn1 is also expressed in the skin, most notably in hair follicles (hence the hairless phenotype of the *nude* Foxn1-deficient mouse mutant) and in keratinocytes that form the stratified skin epithelium[380]. Thus, it is fair to assume that RA signaling would also be impaired in epithelial cells in the skin in the *Foxn1Cre.dnRAR*$^{lsl/lsl}$ model. Despite previous reports suggesting a role for RA signaling in skin development and homeostasis[381, 382, 383], we did not observe any aberrant skin phenotype in these mice. To the best of our knowledge, *Foxn1Cre.dnRAR*$^{lsl/lsl}$ mice do not display evident signs of spontaneous skin inflammation nor were they more (or less) susceptible to imiquimod-induced psoriasis compared to wild type littermates (V. Bekiaris, personal communication).

The abrogation of RA signaling in TECs in *Foxn1Cre.dnRAR*$^{lsl/lsl}$ mice is a result of the expression of the dnRAR construct, which encodes for a dominant-negative RARα isoform that lacks the final 59 amino acids in the carboxy-terminal RA binding domain, thereby impairing downstream signaling ability and possibly affecting receptor-ligand interactions[351]. Importantly, the dnRAR construct has been introduced into the Rosa26 locus and thus does not replace the normal RARα gene. Nonetheless, dnRAR overexpression was shown to inhibit the function of naturally expressed RARα and even that of other RAR isoforms, suggesting that it can be used to effectively block RA signaling[351]. While many studies have made use of the dnRAR construct to ablate RA signaling in various cell types, the full extend of its biological activity and possible side effects remains unclear. Considering that RARs usually form heterodimers with RXRs it is possible that overexpressed dnRAR might sequester RXRs in inactive complexes and thus prevents them from associating with other nuclear receptors such as PPARs, COUP-TFII, thyroid hormone receptor and vitamin D_3 receptor[356]. In that case, dnRAR expression would not only interrupt RA signaling but also impair other biological pathways controlled by RXR and its alternative binding partners. While it is currently difficult to control for any indirect effects of dnRAR activity, it is important to bear possible off target effects in mind when using the dnRAR model.

Paper II

The Role of Retinoic Acid Signaling in CD8$^+$ T Cell Development and Function

RA signaling is known to play an important role in shaping peripheral T cell responses, most notably through the induction of gut-homing receptors, generation of peripheral Tregs and control of Th differentiation and lineage stability. Compared to the extensive body of knowledge concerning RA signaling in CD4$^+$ T cells, much less is known on how RA influences CD8$^+$ T cells. In this study, using *CD4cre. dnRAR$^{lsl/lsl}$* mice with abrogated RA signaling in developing thymocytes and peripheral T cells, we aimed to investigate how RA signaling affects CD8$^+$ T cell development and the functionality of naive and effector CD8$^+$ T cells.

Key findings

- Block of RA signaling in developing thymocytes perturbs T cell development and leads to the accumulation of mature CD8SP thymocytes
- CD8SP thymocytes developing in the absence of RA signaling display reduced levels of TCR expression and a skewed TCR repertoire
- Abrogated RA signaling skews the naive CD8$^+$ T cell compartment towards a CD44^{hi} memory-like phenotype in the absence of antigen encounter
- The common B cell marker B220 is upregulated on a subset of mature CD8SP thymocytes and naive CD44^{hi} peripheral CD8$^+$ T cells in *CD4Cre.dnRAR$^{lsl/lsl}$* mice
- Naive CD8$^+$ T cells from *CD4Cre.dnRAR$^{lsl/lsl}$* mice display enhanced survival and expansion upon TCR stimulation
- RA signaling regulates effector gene expression in a similar manner in splenic and mLN-primed CD8$^+$ T cells
- RA signaling-impaired CD8$^+$ T cells display defective CTL activity

Discussion

Our investigations of T cell development and $CD8^+$ T cell functionality in *CD4Cre.dnRAR*$^{lsl/lsl}$ mice have revealed an important role for RA signaling during thymopoiesis, maintenance of naive $CD8^+$ T cells and the generation of cytotoxic effector responses.

These results contribute to a better understanding of the significance of RA signaling for T cell development in particular, as analysis of the thymic phenotype of *CD4Cre.dnRAR*$^{lsl/lsl}$ mice has been neglected in previous studies that focused entirely on peripheral effector responses[378, 379, 384]. However, when addressing the functional competence of peripheral T cells in *CD4Cre.dnRAR*$^{lsl/lsl}$ mice, it is important to bear the thymic development of these cells in mind, as any functional impairment might be connected to aberrant development in the absence of RA signaling. In this regard, we plan to further investigate the functionality of the accumulating phenotypically mature CD8SP thymocyte population, addressing their egress competence and proliferative capacity upon TCR stimulation. Furthermore, the expression of B220 on these cells remains puzzling and warrants additional experiments in order to determine its significance. Currently, attempts to stain for B220-expressing CD8SP thymocytes in thymic sections are on the way to determine the anatomical location of these cells. Additionally, we plan to test the survival and viability of $B220^+$ CD8SP thymocytes to determine if the upregulation of B220 is related to impaired apoptosis and/or marks cells that were supposed to die. Interestingly, after sorting CD8SP thymocytes from Cre^- and Cre^+ mice for subsequent RNA-seq experiments, we observed a high degree of sample degradation selectively in the $B220^+$ CD8SP subset of Cre^+ mice, possibly indicating increased cell death in these cells. However, seeing as this was a stand-alone observation that can have multiple reasons, we aim to investigate this further by using *in vitro* cultures with varying conditions.

In contrast to the previously undocumented observations of altered T cell development in the thymus of *CD4Cre.dnRAR*$^{lsl/lsl}$ mice, an impaired CTL response in the absence of RA signaling has been reported previously in the setting of a vaccinia virus infection using the same mouse model[379]. Importantly, depending on the viral dose and route of infection, the primary $CD8^+$ T cell response during vaccinia virus infection has been shown to rely on $CD4^+$ T cell help[210, 385]. As the dnRAR construct is also expressed in $CD4^+$ T cells in *CD4Cre.dnRAR*$^{lsl/lsl}$ mice, it is possible that the impaired generation of a $CD8^+$ effector response in this setting might be due to insufficient help from $CD4^+$ T cells lacking RA signaling. However, it seems unlikely

that impaired $CD4^+$ T cell help alone is responsible for suboptimal CTL response, as we could show similarly reduced effector response and killing efficiency in *CD4Cre.dnRAR*$^{lsl/lsl}$ mice on an OT-I background that were immunized with the model antigen ovalbumin. In fact, we further showed that the defects we observed in CTL activity were cell-intrinsic to the $CD8^+$ T cell compartment. However, if these findings were to be followed up by additional experiments to test the ability of RA signaling-impaired $CD8^+$ T cells to clear actual pathogens *in vivo*, it would be advisable to choose an infection model that does not involve $CD4^+$ T cell help to exclude any confounding effects from that aspect.

Paper III

Context-Dependent Development of Lymphoid Stroma from Adult $CD34^+$ Adventitial Progenitors

While mesenchymal stromal cells are critically important for the function of lymphoid organs, their functional specialization and ontogeny remains poorly understood, especially in the thymus. Expression of the surface markers PDPN, PDGFRα and PDGFRβ is used to identify TMC and LNMC populations, with $PDPN^+$ LNMC further distinguished into distinct subsets of FRCs, FDCs and MRCs. A similar functional distinction of TMCs has not been described and it is unclear how TMC and LNMC subsets relate to each other developmentally. In this study, we performed comparative analysis of the TMC and LNMC compartments and subsequently identified a conserved population of $CD34^+$ multipotent precursors whose *in vivo* developmental potential we addressed using reaggregate organ grafts.

Key findings

- TMC and LNMC can be divided into $PDPN^-$ and $PDPN^+$ subsets, with $PDPN^-$ and $PDPN^+$ TMC displaying a higher population similarity with their respective LNMC counterparts than between each other
- $PDPN^+$ LNMC, but not TMC, contain a major fraction of $BP\text{-}3^+$ cells that comprises FRCs, FDCs and MRCs, while $PDPN^-$ TMC and LMNC mainly represent contractile and non-contractile pericytes

- CD34$^+$ PDPN$^+$ mesenchymal cells are localized within the adventitial vascular niche of postnatal lymphoid and non-lymphoid organs and can differentiate into BP-3$^+$ PDPN$^+$ FRCs, MRCs and FDCs as well as PDPN$^-$ pericytes *in vivo*, depending on the environmental context
- In the thymus, CD34$^+$ cells and PDPN$^-$ pericytes arise from a common anlage-seeding mesenchymal precursor population and the maturation and/or maintenance of the CD34$^+$ TMC compartment requires LTβR signaling

Discussion

Prior to this study the knowledge regarding the functional heterogeneity and developmental origin of mesenchymal stromal cells in lymphoid organs remained limited, despite their essential role in maintaining functional thymic and LN environments. By combining detailed flow cytometry analysis with gene expression profiling and *in situ* immunofluorescence analysis we were able to identify and define the functional niches of distinct mesenchymal stromal cell subsets in primary (thymus) and secondary (LN) lymphoid organs. In particular, we identified two mesenchymal subsets, PDPN$^-$ pericytes and PDPN$^+$ CD34$^+$ adventitial cells, that were shared between thymic and LN environments and represent specialized, non-redundant components of the vascular niche. Importantly, the multi lineage potential of the CD34$^+$ adventitial cell population was demonstrated *in vivo* using lymphoid organ reaggregates where CD34$^+$ cells were able to give rise to various mesenchymal stroma-like subsets, including BP3$^+$ FRCs, FDCs and MRCs as well as integrin α7$^+$ pericytes, depending on the environmental context.

Two forms of reaggregate organ cultures were used in this study, reaggregate thymic organ cultures (RTOC) and reaggregate lymph node organ cultures (RLOC), to specifically address the role of the surrounding lymphoid environment in the development of CD34$^+$ adventitial cells. The use of reaggregate organ cultures has the advantage that the development of a selected cell population can be traced in a defined context, by isolating these cells from reporter mice and introducing them to cell suspensions obtained from embryonic thymus or lymph node structures. However, reaggregate organ cultures have their limits as models for lineage tracing, mostly due to low and variable recovery of the progeny of the potential precursor population and the technical challenges involved in the generation and

transplantation of the reaggregates. In this regard, while the absence of a particular cell population in a recovered graft might be solely due to the developmental potential of the input population, one cannot exclude the possibility that the *ex vivo* generation phase of the reaggregate and its grafting to an ectopic site (under the kidney capsule) might impact more strongly on certain cell subsets and possibly impair their development. Additionally, using the RTOC/RLOC setups described above, we were only able to investigate the multi-lineage potential of the CD34$^+$ adventitial cell subset on a population level, with the exact progenitor properties of individual CD34$^+$ cells remaining to be determined.

Irrespective of the question regarding individual precursor potential, our identification of a distinct CD34$^+$ mesenchymal stromal cell population in lymphoid organs was confirmed in a recent study investigating the transcriptional profiles of LN stromal cells on a single cell level[386]. This detailed RNA-sequencing based analysis revealed nine LN stromal clusters, one of them being CD34$^+$ adventitial cells. Furthermore, this study confirmed our findings regarding the localization of these cells in association with the vasculature and a potential function as regulators of endothelial cell homeostasis[386]. In addition, another study has found CD34$^+$ cells to be a part of a novel subset of so-called medullary FRCs (MedRCs) that are important in plasma cell homeostasis by providing specialized survival niches through the production of the plasma cell survival factors IL-6, CXCL12 and BAFF, as well as ECM proteins[387]. To what extend CD34$^+$ adventitial cells contribute to the function and/or development of the MedRC population remains to be seen, although the fact that CD34$^+$ cells produce high levels of CXCL12[386] indicates that these cells might play an additional role in the homeostasis of hematopoietic cells.

Lastly, we demonstrated that not any particular cell intrinsic properties but rather the tissue-specific environment is determining the development of CD34$^+$ cells into pericytes and BP-3$^+$ subsets, with the latter only being generated in the setting of RLOC grafts. However, the exact nature of the tissue-specific signals responsible for the differences in TMC and LNMC composition in general and the LN-specific generation of BP-3$^+$ PDPN$^+$ mesenchymal calls in particular remain unclear. In line with a previous report[388], our study showed that lack of T and B cells in Rag-deficient mice leads to a reduction, but not complete absence of BP-3$^+$ cells in LNs, indicating that additional signals govern the development of this population. In fact, it is possible that instead of an inductive signal in the LNs a repressive signal in the thymus is controlling the fate of mesenchymal cell progenitors. Seeing as epithelial

cells are the major stromal cell component of the thymus but are absent from LNs, TECs are a likely candidate for the provision of said signal(s). In support of this notion it has been shown that progressive reduction in TECs establishes a LN-like environment in the thymus, including the development of FDC and FRC-like cells[389]. Furthermore, a similar phenotype has been observed in ephrin B-deficient mice that display large epithelial-free areas in the thymus that are instead occupied by ERTR7^{+} fibroblasts and ECM structures reminiscent of FRC networks in LNs[390].

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