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Phenotypic and functional analysis of the mesenchymal stromal cell compartment in the thymus

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On the cover: Immunofluorescence staining of the mouse thymus by Heli Uronen-Hansson ©

“Destitutus ventis, remos adhibe.”

When the winds don’t serve you, take to the oars.

- Latin proverb

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PAPER 1.

Mesenchymal cells regulate retinoic acid receptor-dependent cortical thymic epithelial cell homeostasis.

Katarzyna M. Sitnik, Knut Kotarsky, Andrea J. White, William E. Jenkinson, Graham Anderson, and William W. Agace

Journal of Immunology, 2012 May 15;188(10):4801-9.*

PAPER 2.

Phenotype, ontogeny and functional characteristics of thymic mesenchymal cells

Katarzyna M. Sitnik, Heli Uronen-Hansson, Holger Weishaupt,
Graham Anderson, and William W. Agace

In manuscript

PAPER 3.

Retinoic acid signaling in thymocytes and its impact on T cell development

Katarzyna M. Sitnik, Knut Kotarsky, Andrea J. White, Hanna Johansson,
Rune Blomhoff, Graham Anderson, and William W. Agace

In manuscript

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PAPERS NOT INCLUDED IN THIS THESIS

A novel role for constitutively expressed epithelial-derived chemokines as anti-bacterial peptides in the intestinal mucosa.

Kotarsky K, Sitnik K, Stenstad H, Kotarsky H, Schmidtchen A, Koslowski M, Wehkamp J, Agace WW.

Mucosal Immunology, 2010 Jan; 3(1):40-8

The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut.

Suzuki K, Maruya M, Kawamoto S, Sitnik K, Kitamura H, Agace WW, Fagarasan S.

Immunity, 2010 Jul 23; 33(1):71-833

Chemokine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation.

van de Pavert SA, Olivier BJ, Goverse G, Vondenhoff MF, Greuter M, Beke P, Kusser K, Höpken UE, Lipp M, Niederreither K, Blomhoff R, Sitnik K, Agace WW, Randall TD, de Jonge WJ, Mebius RE.

Nature Immunology, 2009 Nov; 10(11):1193-9

ABBREVIATIONS

FTOC fetal thymic organ culture

FoxN1 forkhead box N1

GFP green fluorescent protein

ICAM-1 intercellular adhesion molecule-1

MAdCAM-1 mucosal addressin cell adhesion molecule-1

MAPK mitogen-activated protein kinase

MHC major histocompatibility complex

PDGFR platelet derived growth factor

RAG recombination activating gene

RANK receptor activator of nuclear factor kappa-B

RORγt retinoic acid receptor-related orphan receptor γt

siRNA small interfering RNA

TNF tumor necrosis factor

VCAM-1 vascular cell adhesion molecule-1

INTRODUCTION

1. Principles of innate and adaptive immunity. Why do we need T cells?

The immune system is composed of specialized tissues, cells and soluble mediators that together serve to protect the host against microbial and parasitic infections. This critical protective function of the immune system relies on its ability to mount an effective but transitory effector response against foreign infectious agents while remaining tolerant towards the body's own and other innocuous components. Acquisition and maintenance of these functional features require the presence of tight regulatory mechanisms, defects in which put the host at risk for developing chronic inflammatory and autoimmune pathologies. In vertebrates, the mechanisms of host defense are distinguished into two branches, innate and adaptive, both required for effectively withstanding pathogen invasion. The principal functional mediators of the innate and adaptive immunity are diverse populations of white blood cells (leukocytes), which are progeny of hematopoietic stem cells located in the bone marrow. The major leukocyte subsets serving adaptive immune functions are B- and T- lymphocytes. Although T lymphocyte progenitors derive from the bone marrow, the main stages of their differentiation into mature T cells occur in the thymus - a primary lymphoid organ whose functionality is the major focus of this thesis work. For better understanding of the general relevance of studies on T cell development, in this section I will describe the basic mechanisms of innate and adaptive immunity putting T cell functionality into the broad context of host immune protection.

In brief, the major differences between the innate and adaptive branches of the immune system concern the specifics of pathogen recognition, the kinetics of effector response development and the ability to generate immunological memory. The innate immune system is uniquely suited to serve on the front line of host defense against newly encountered pathogens, which is due to its ability to rapidly develop an effector response already within hours after microbial invasion. The

innate immune system detects pathogen presence by recognizing groups of conserved microbial molecular structures using a few sensory proteins of limited specificity. Such sensory proteins occur in a secreted form, and as surface and intracellular receptors (so called pattern recognition receptors, PRR) expressed in various combinations by subsets of innate immune cells. PRR triggering is a central event eliciting anti-microbial activity in innate immune cells. Accordingly, PRR activation stimulates macrophages to eliminate bacteria engulfed into phagocytic vesicles while granulocytes, such as neutrophils, are induced through PRR to release anti-microbial factors to the intercellular space. Another consequence of PRR activation is the production of inflammatory mediators (i.e. chemokines and cytokines) that further amplify the innate response by, for example, recruiting circulating granulocytes and macrophage precursors to the site of infection. Last but not least, cytokines generated by the innate immune system promote the initiation and influence the nature of any subsequent adaptive immune response. Contrasting with the above-described features of the innate immune system, the adaptive immune system employs for pathogen recognition a virtually infinite diversity of highly specific T-cell (TCR) and B-cell (BCR) antigen receptors. TCR and BCR recognize antigens in different ways. Whereas BCRs can directly bind to native antigen, TCRs recognize antigenic determinants (generated via breakdown of native antigens inside host's cells) that are presented on the surface of host's cells by members of a group of proteins called MHC molecules (a phenomenon called MHC-restricted antigen recognition). TCR and BCR diversity is generated during lymphocyte development when multiple sets of gene fragments encoded in the germline-inherited TCR and BCR loci, each set corresponding to a portion of the receptor, are randomly recombined to generate complete antigen receptor genes, a process that leads to each individual T- and B-cell expressing a receptor of unique specificity. The resulting repertoire of clonally distributed antigen receptors contains only very low frequency of cells able to recognize a given antigen. Consequently, the adaptive immune system needs a

longer time (usually several days) to develop effector response to a primary infection - a time needed for naive T and B cells to encounter their cognate antigen, expand in number and differentiate into effector cells. These initial stages of the adaptive immune response occur within the lymph nodes (LNs) - organs that serve as platforms where antigens and inflammatory mediators carried by lymph from infected peripheral sites can be efficiently encountered by circulating naive lymphocytes. Effector cells and molecules generated in the LNs during development of the adaptive immune response are ultimately dispersed via circulation to the sites of infection where they execute their functions.

The mechanisms used by effector lymphocytes to fight infections are broadly classified as humoral (i.e. antibody-mediated) and cell-mediated. Most infections evoke both of these forms of adaptive immunity though their relative importance can vary depending on the type of pathogen. Humoral immunity is essentially mediated by B cells, however, as described later in this section, T cells contribute indirectly by providing B cells with important regulatory cues. Following initial recognition of their cognate antigen, B cells proliferate and their progeny differentiates into antibody-secreting plasma cells. Antibodies, which are the soluble form of the BCR, bind to their specific antigens on the surface of pathogens tagging them so they will be more easily recognized and destroyed by the innate immune system, a process that helps to control extracellular pathogen load and prevents/limits spreading of the infection. The other, cell-mediated branch of adaptive immunity relies on the activity of two distinct T cell subsets, cytotoxic and helper, which develop in the thymus and are characterized by specific expression of CD8 and CD4 co-receptors, respectively. $CD8^+$ T cells recognize antigens presented by ubiquitously expressed class I MHC molecules whereas $CD4^+$ cells recognize antigens presented by class II MHC molecules, the steady state expression of which is restricted to a limited number of cell populations, such as dendritic cells (DCs), B cells and thymic epithelial cells. In order to be triggered to proliferate and differentiate into effector cells, naïve $CD8^+$

and $CD4^+$ T cells not only need to recognize their specific antigen-MHC complex but also simultaneously receive additional extrinsic signals. These so called co-stimulatory signals are efficiently provided to both T cell subsets by MHC-I/MHC-II expressing DCs, the major antigen presenting cell (APC) population capable of initiating adaptive immune responses. Differentiated effector $CD8^+$ T cells are cytotoxic and kill host's cells that have been infected with pathogens carrying the specific antigen recognized by the given $CD8^+$ T cell's TCR. In contrast, effector $CD4^+$ T cells function as chief orchestrators of other players of the innate and adaptive immune response, a control they exert via production of cytokines. There are several subsets of effector $CD4^+$ T cells that express distinct cytokine profiles and play discrete functions. For example, interleukin-17 expressing T helper (Th)-17 cells boost neutrophil influx to the site of infection; interferon gamma producing Th-1 cells activate macrophages to become more efficient at killing engulfed pathogens whereas co-stimulatory cues and cytokine cocktails derived from Th-2 and T-follicular helper (Tfh) cells, respectively act on B cells to improve the quality of the antibody response. Acquisition of the various types of effector fate occurs under the influence of extrinsic factors among the progeny of each antigen-activated naive $CD4^+$ cell in the LN. The boost given to the immune response through effector activities of $CD4^+$ and $CD8^+$ T cells is typically needed in order to achieve complete pathogen clearance, a requirement illustrated by severely compromised immunocompetence in subjects suffering from congenital or acquired T cell deficiency. Beside effector T cell populations with pro-inflammatory activity, other T cell subsets exist that display suppressive activity and help keep the immune response in check. These so called regulatory T cells, a subset of which originates directly from the thymus, are of critical importance for prevention of chronic inflammation and autoimmunity.

Finally, the adaptive immune system is uniquely suited to generate immunological memory, which is manifested with heightened (stronger and faster) responsiveness to previously encountered pathogens and relies on generation

during the primary infection of special populations of memory T- and B- cells. Immunological memory is of high relevance to public health as it enables induction of long-term protective immunity in response to vaccination.

2. Mesenchymal cell populations in the secondary lymphoid tissues

Amongst the primary objectives of the studies included in this thesis are the characterization of thus far poorly recognized populations of mesenchymal cells in the thymus, and the comparison of those with currently better-studied mesenchymal stromal subsets in the LNs. To facilitate the understanding and interpretation of these results, in this section I will summarize the current knowledge of the phenotype and functions of the LN stroma.

2.1 Structure and functions of the lymph nodes (LN) - an overview

Lymph nodes (LNs) are organized lympho-stromal structures distributed along the lymph vessel network that play essential functions for the homeostasis and functionality of the adaptive immune system. Amongst the most important roles of the LNs is mediating lymphocyte survival (Link et al., 2007), facilitating lymphocyte encounter of antigens, and organizing immune cell interactions for optimal generation of effector T- and B- cells (for review see (Pereira et al., 2010)). Each LN is exposed to a continuous influx of lymph drained from a certain tissue/organ (see ***Figure 1***). The incoming lymph pours into the sub-capsular sinus, a space underneath the fibrous capsule of the LN that is continuous with the lumen of both afferent and efferent lymphatic vessels. From the sub-capsular sinus, various particular and cellular contents of the lymph, including foreign antigens, can get access to the two underlying anatomical compartments of the LN, the cortex and the medulla. The cortex, which spreads beneath the whole convex surface of the LN, is further compartmentalized into centrally positioned T-cell area and peripherally distributed B cell follicles, each of these regions

supported by the three-dimensional networks of specialized populations of mesenchymal stromal cells. Facing the inner surface of the cortex on one side and the LN concave surface on the other, the LN medulla is composed of macrophage- and plasma cell- filled medullary cords separated by numerous lymphatic sinuses. While both the cortex and the medulla are vascularized, the major ports of entry for circulating naive lymphocytes are the cobblestone-shaped high endothelial venules (HEV) that run through the T-cell zone. Having entered the LN, naive T- and B cells screen the display of peripheral antigens available to them within their respective cortical areas. Lymphocytes that fail to encounter their cognate antigen leave the LN through efferent lymphatics and are returned back to the circulation. In contrast, T- and B-cells that have been activated by their specific antigen are retained in the LN environment where they proliferate and differentiate into effector cells (for review see (Roozendaal and Mebius, 2011)).

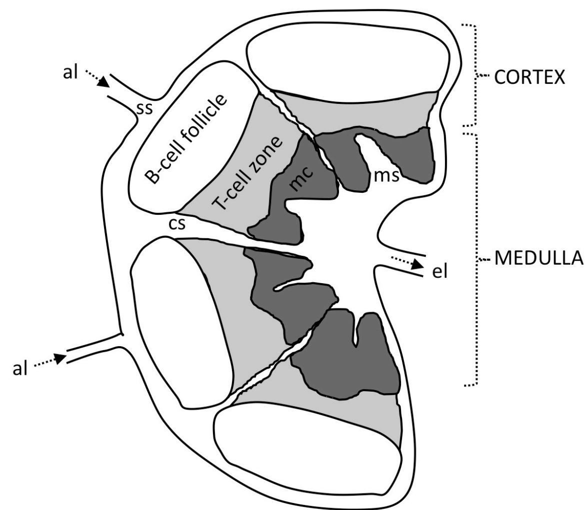

Figure 1 Structure of the lymph node al, afferent lymphatics; el, efferent lymphatics; ss, subcapsular sinus; cs, cortical sinus; ms, medullary sinus; mc, medullary cord; GC, germinal center

2.2 The role of lymphoid tissue organizer (LTo) cells in LN organogenesis

LNs develop at predestined locations throughout the body usually at sites where blood vessels branch. Formation of LN anlagen, which in mice starts from embryonic day 11-12 (E11-E12) after conception, relies on para- and juxtacrine crosstalk between specialized mesenchymal organizer (lymphoid tissue organizer, LTo) and hematopoietic RORγt^{+}IL-7Rα^{+} inducer cell (lymphoid tissue inducer, LTi) populations that seed and remodel the structure of the lymph sacs, the latter being budlike outgrowths from blood vessel wall of newly specified lymphatic endothelium (Coles et al., 2010). Although lymph sacs are the necessary source of lymphatic endothelial cells (LEC) for LN development, they are dispensable for the initial clustering of organizer and inducer cells at the prospective sites of LN formation (Vondenhoff et al., 2009), indicating that LN positioning is determined by other, lymph sac-independent mechanisms. It has been hypothesized that at least at some locations the initial trigger for the start of LN development could be stimulation of putative organizer cell progenitors with RA produced by adjacent neurons (van de Pavert et al., 2009). Accordingly, RA-deficient *Aldh1a2*$^{-/-}$ mice do not develop organizer-inducer cell clusters at most locations, a defect that has been ascribed to the loss at these sites of early mesenchymal cell-derived expression of the LTi chemoattractant, CXCL13 (Honda et al., 2001; Luther et al., 2003; van de Pavert et al., 2009). Whereas signaling pre-requisites determining the positional coordinates for LN anlagen formation remain largely unclear, it is firmly established that once recruited to the LN primordium, lymphotoxin (LT)$\alpha_1\beta_2$-expressing LTi cells drive LTβ receptor-dependent maturation of organizer cells, which is critical to sustain the presence and stimulate further development of the LN anlagen (for review see (Koning and Mebius, 2012)). Accordingly, LTi-deficient ROR$\gamma t^{-/-}$, and LT$\alpha^{-/-}$, LT$\beta^{-/-}$, and LTβR$^{-/-}$ mice lack all peripheral LNs (with exception of LT$\beta^{-/-}$ mice that develop structurally abnormal mesenteric LNs) due to embryonic dissipation of the LN primordia in the absence of mature LTo cells (Benezech et al., 2010; van de Pavert and Mebius, 2010).

Mature LTβR-dependent LTo cells can be identified in the LN anlagen as PDGFRa^{+}gp38^{+}MAdCAM-1^{+}RANKL^{+} population expressing high levels of VCAM-1 and ICAM-1 (Benezech et al., 2010; Cupedo et al., 2004; Okuda et al., 2007). These cells produce highest levels of LTi chemoattractants, CXCL13, CCL19 and CCL21, as well as IL-7 (Benezech et al., 2010; Cupedo et al., 2004). IL-7 has been implicated in fostering LTi cell generation/proliferation/survival (Chappaz et al., 2010) and, together with RANKL, in inducing/maintaining LTi expression of (LT)$\alpha_1\beta_2$ (Cupedo et al., 2004; Yoshida et al., 2002). The important autonomous contributions of IL-7/IL-7R and RANKL/RANK axes to LN organogenesis are illustrated by the absence of several LNs in IL-7$^{-/-}$ (Chappaz et al., 2010) and IL-7R$\alpha^{-/-}$ (Luther et al., 2003) mice, and by complete absence of all peripheral LNs in RANK$^{-/-}$ (Dougall et al., 1999) and RANKL$^{-/-}$ (Kong et al., 1999) mice, respectively. The lineage origin and phenotype of progenitors giving rise to mature organizer cells in the LN anlagen are not yet fully defined and elucidating these questions continues to be a major focus of research in this field. Nevertheless, over the past couple of years several important insights to the ontogeny of LTo cells have been made. Firstly, by combining careful kinetic analyses of mesenchymal phenotype in LN anlagen of wild-type, LTi-deficient and LTβR-deficient mice with *in vitro* LN anlage culture techniques Benezech *et al.* provided evidence to indicate that the precursors of ICAM-1^{hi}VCAM-1^{hi}MAdCAM-1^{+} mature LTo cells are contained within the PDGFRa^{+}gp38^{+}MAdCAM-1^{-}ICAM-1^{int}VCAM-1^{int} population, which expresses basal levels of CXCL13, CCL21, and IL-7 and develops in a LTβR/LTi-independent manner (Benezech et al., 2010). Secondly, a recent publication from the same group (Benezech et al., 2012) showed that the intermediate ICAM-1^{int}VCAM-1^{int} cells found in the inguinal LN anlagen were phenotypically and functionally reminiscent of PDGFRa^{+}gp38^{+} Pref-1^{+} pre-adipocytes present in surrounding fat pads. In a series of elegant grafting experiments these authors showed that cells residing in E18 and in adult inguinal fat pads, as well as

PDGFRa^{+}gp38^{+} cells sorted from adult fat depots, supported LN anlagen growth and contributed to gp38^{+} T-zone FRC and gp38^{+}MAdCAM-1^{+} MRC (see below) stromal cell networks in the LN graft (Benezech et al., 2012). Collectively, these findings demonstrate that fat pad-derived Pref-1^{+}PDGFRa^{+}gp38^{+} pre-adipocytes are a plausible source of LTo progenitors during LN organogenesis and that they can give rise to at least some types of mesenchymal cells found in the LNs of the adult.

2.3 Mesenchymal cell populations in the LNs of the adult

The mesenchymal stromal compartment in adult LNs is phenotypically and functionally heterogeneous. Several specialized subsets of adult LN stroma have been described, the putative developmental link of which to *bona-fide* LTo cells remains unclear.

2.3.1 Follicular dendritic cells (FDC)

B-cell follicles are supported by a population of specialized dendritic-shaped mesenchymal cells called follicular dendritic cells (FDC). Having entered the follicles, B cells migrate in contact with the FDC network (Bajenoff et al., 2006). In addition to supporting B cell migration, FDCs have been implicated in providing several other important functions for B cells. First, FDCs produce soluble factors such as the B-cell activator factor of TNF family (BAFF) and CXCL13, a B-cell survival factor and follicle homing chemokine, respectively (Allen and Cyster, 2008). Further, FDC capture and present free particulate antigens and immune complexes to B cells, facilitating the formation and maturation of the germinal center, a structure in which B cells proliferate and are subject to BCR editing processes necessary to achieve an optimal quality of the humoral response (Allen and Cyster, 2008; Bajenoff and Germain, 2009). *In situ*, FDCs are identified by their expression of complement receptors, CR-1 (CD35)

and CR-2 (CD21), and by staining with antibodies FDC-M1 and FDC-M2. FDC-M1 binds secreted glycoprotein milk fat globule-EGF-factor 8 (MFGE-8), which is expressed by FDCs but is also found on follicle-resident tingible body macrophages, while FDC-M2 recognizes complement component C4 that becomes deposited on FDC surfaces as part of immune complexes (Aguzzi and Krautler, 2010; Allen and Cyster, 2008). Whereas neither of the above markers is entirely FDC-specific, their expression distinguishes FDCs from other mesenchymal populations in the LN. Based on immunohistochemical analyses, FDCs have also been reported to express gp38 (podoplanin), bone marrow stromal cell antigen-1 (recognized by the antibody clone, BP-3) and MAdCAM-1, all of which can be found on other populations of LN stroma (see below) (Katakai et al., 2008; Link et al., 2007). In-depth assessment of FDC phenotype and function, as well as of their putative intra-compartmental heterogeneity, have so far proven challenging due to the paucity of unambiguous FDC-specific markers and lack of reliable methods to isolate these cells. For the same reasons it has been difficult to study the ontogeny of FDCs. Nevertheless, and despite remaining controversies on this subject, accumulating data continue to support the conclusion that FDCs originate from mesenchymal precursors (Allen and Cyster, 2008; El Shikh and Pitzalis, 2012), and a recent study has provided evidence to indicate that FDCs (which are PDGFRβ-negative) develop in the LNs from resident PDGFRβ^+ precursors (Krautler et al., 2012). Although the pathway of FDC differentiation remains obscure, several requirements for FDC development have been established. TNF-TNFR-I and LT$\alpha_1\beta_2$-LTβR signaling axes are each autonomously required for FDC differentiation (TNF and LT) and maintenance (LT) (Allen and Cyster, 2008; Gonzalez et al., 1998; Koni and Flavell, 1999; Liepinsh et al., 2006). B-cell deficient mice lack FDCs (Cerny et al., 1988) and B-cells have been demonstrated to be the major source of TNF required for FDC differentiation in the LNs and the spleen (Endres et al., 1999; Tumanov et al.,

2010), and of $LT\alpha_1\beta_2$ required for splenic but not LN-resident FDC development (Tumanov et al., 2002).

2.3.2 T-zone fibroblastic reticular cells (FRC)

The T-cell area of the LNs contains a specialized population of $gp38^+PDGFR\alpha^+BP\text{-}3^+$ fibroblastic reticular cells (FRCs) that wrap around collagen-rich fibers generating 3D reticular networks and ensheath the blood vessels (Link et al., 2007). FRCs specialize in supporting T cell-DC interactions (Mueller and Germain, 2009), which is thought to aid in inducing adaptive immune responses (Scandella et al., 2008). Accordingly, FRCs produce the chemokines CCL21 and CCL19, which are required for LN homing and specific localization of T cells and DCs in the T-zone (Forster et al., 2008), and the FRC-dependent reticular network acts as the main adhesion substrate on which T cells and DCs migrate (Acton et al., 2012; Bajenoff et al., 2006). In addition to these functions, FRCs have been implicated in maintaining naïve T cell homeostasis via production of the critical T cell survival factor, IL-7 (Link et al., 2007). The cellular and molecular requirements for T-zone FRC development/maintenance remain poorly understood. Studies addressing the development of T-zone stroma in the splenic white pulp and in tertiary lymphoid structures have highlighted the importance of $LT\alpha_1\beta_2$-$LT\beta R$ signaling and suggested a role for LTi cells in FRC differentiation (Glanville et al., 2009; Link et al., 2011; Withers et al., 2007). However, it is important to note that these findings may not necessarily be translatable to FRCs in the LNs. Thus, T- and B-cell deficient $Rag^{-/-}$ mice are completely devoid of $CCL21^+gp38^+$ T-zone stroma in the spleen (Withers et al., 2007) while they still maintain an apparently normal population of $CCL19^+gp38^+$ FRCs in the LNs (Chappaz and Finke, 2010; Fletcher et al., 2011a; Katakai et al., 2008), revealing differential dependence on peripheral lymphocytes of LN- and splenic FRCs.

2.3.3 Other LN mesenchymal populations

The area directly underneath the subcapsular sinus of the LN harbors a population of $gp38^{+}PDGFR\alpha^{+}BP\text{-}3^{+}$ stromal cells that uniquely express RANKL (Fletcher et al., 2011a; Katakai et al., 2008). Similar to FDCs, these so-called marginal reticular cells (MRCs) express MAdCAM-1 and high levels of CXCL13 protein, but are negative for other functional FDC markers, such as CD21/35, FDC-M1 and FDC-M2 (Katakai et al., 2008). So far there has been no clear identified function for MRCs, although it has been suggested that they could be the adult counterparts of LTo cells with which they share overlapping phenotypic traits (Katakai et al., 2008). LNs have also been reported to contain a subset of BP-3-negative $gp38^{+}PDGFR\alpha^{+}$ mesenchymal cells located in the LN capsule and around blood vessels in the LN medulla (Link et al., 2007) and recently, to harbor a distinct $gp38^{-}PDGFRa^{-}$ integrin alpha 7 $(ITGA7)^{+}$ mesenchymal subset with properties of contractile pericytes (Malhotra et al., 2012).

Despite the above-described findings, LN stromal cells remain only partly defined with regard to their population composition, developmental origin(s) and specific functions. We addressed some of these queries in **paper 2** of this thesis in which we used genome-wide expression profiling to compare the phenotype and functions of mesenchymal cell populations from the LNs and the thymus.

3. Biology of the thymus

The thymus is the major site of T cell development. At least four different types of T cells are generated in the thymus from a common progenitor: two major populations of $TCR\alpha\beta$-bearing $CD4^{+}$ helper and $CD8^{+}$ cytotoxic T cells; and two minor subsets of $TCR\gamma\delta^{+}$ T cells and natural killer (NK) T cells, the latter subset characterized by constrained repertoire of $TCR\alpha\beta$ receptors and expression of NK

cell receptors. In the following sections, I will primarily focus on the developmental pathway of CD4$^+$ helper and CD8$^+$ cytotoxic T cells.

3.1 Anatomical structure and stromal cell compartments of the thymus

T-cells develop in the thymus as an outcome of a multi-step differentiation process supported by thymic stromal cells (TSCs). The TSC network, which consists of epithelial and mesenchymal elements, forms the organ capsule and beneath it two anatomically and functionally distinct compartments, the outer cortex and the inner medulla, the latter of which may be discontinuous and split into a number of separate medullary regions. Two phenotypically and functionally specialized populations of cortical (cTECs) and medullary (mTECs) thymic epithelial cells (TEC) form 3D networks in the cortex and the medulla, respectively (see ***Figure 2***). In contrast, thymic mesenchymal cells (TMCs) form the capsule as well as generate a distinct compartment of perivascular cells, traversing both the cortex and the medulla in association with endothelium of the blood vessels. Available imaging data suggest that TMCs only rarely, if at all, intermingle with TECs. Instead, the two cell types appear to form largely non-overlapping compartments, which are in many areas physically separated from each other by a basement membrane. Based on this, terms such as thymic parenchyma and perivascular space (PVS) have been introduced that specifically refer to the basement membrane-separated epithelial and mesenchymal compartments, respectively. In young adult mice, the presence of PVS is best recognized around larger venules located at the cortico-medullary junction (CMJ) and in the medulla. Finally, although not part of the TSC system, another cell type that populates the thymus and plays accessory functions in thymopoiesis are hematopoietic-derived DCs, which densely populate the medulla while showing only sparse distribution in the cortex (Anderson and Takahama, 2012; Kato, 1997; Mori et al., 2007; Odaka, 2009).

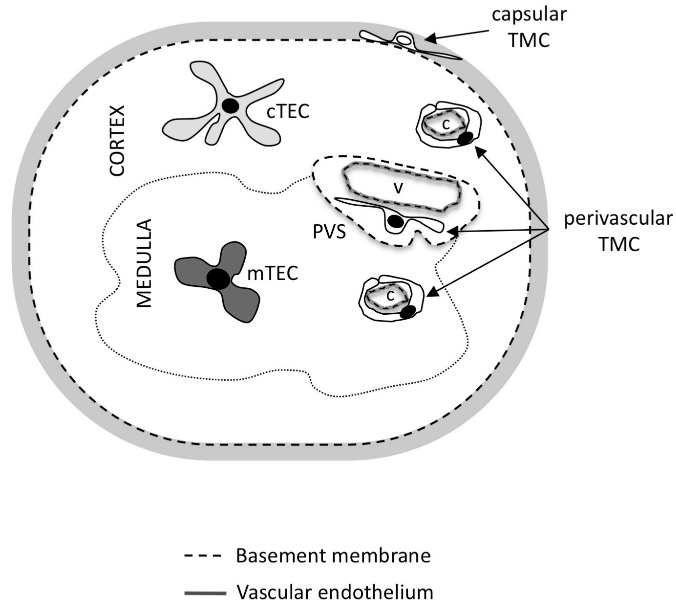

Figure 2 Schematic view of stromal cell compartments in the thymus
cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; TMC, thymic mesenchymal cell; PVS, perivascular space; c, capillary; v, venule

3.2 T-cell differentiation pathway - an overview

T cell development is characterized by a sequential progression of thymocytes through multiple developmental stages with distinct surface phenotype (see ***Figure 3***). Having entered the thymus through PVS-containing venules at the CMJ, immediate descendants of immigrated bone marrow-derived progenitors appear as CD4$^-$CD8$^-$ double-negative (DN) thymocytes and develop progressively through CD44$^+$CD25$^-$ (DN1), CD44$^+$CD25$^+$ (DN2), CD44$^-$CD25$^+$ (DN3) and CD44$^-$CD25$^-$ (DN4) stages within the cortical environment. The DN1 compartment contains a subset of c-kit^{hi} early thymic progenitors (ETP), which represent the *bona-fide* T-cell precursors (Benz et al., 2008; Porritt et al., 2004). ETP and DN2 cells undergo multiple rounds of cell division and progressively loose non-T cell potentials until they finally commit to the T-cell lineage at their

transition to the DN3 stage (Bell and Bhandoola, 2008; Luc et al., 2012; Wada et al., 2008; Yui et al., 2010). DN2 and DN3 cells rearrange their TCRβ chain genes that if successful are assembled into the pre-TCR by pairing with the invariant α chain. Signaling through pre-TCR allows DN3 cells to expand and further progress to $CD4^{+}CD8^{+}$ double positive (DP) stage, a phenomenon called β-selection. Following TCRα chain gene rearrangement, DP cells display low surface levels of mature TCRαβ pairs and are tested for their ability to recognize MHC-self peptide complexes on the surface of cTECs. DP cells that express TCR specificities with low/intermediate avidity to any of the presented peptide-MHC complexes, and thus bear potentially useful TCR specificities, are signaled by the TCR to undergo positive selection (marked by up-regulation of CD69 on their surface) and to become intermediate $CD69^{+}$ $CD4^{+}CD8^{lo}$ cells, which then differentiate into either MHCII-restricted $CD4^{+}$ single positive (SP) or MHCI-restricted $CD8^{+}$ SP cells. In contrast, DP cells with TCRs that cannot signal die of neglect while those that recognize intra-thymically displayed peptides with strong avidity (i.e. potentially auto-reactive cells) undergo apoptosis in a process called negative selection (for review see (Singer et al., 2008); see section **3.6**). The commitment to CD4 and CD8 lineage occurs at the $CD4^{+}CD8^{lo}$ stage as a result of a complex interplay between cell intrinsic and environmental factors (Park et al., 2010)(for review see (Rothenberg, 2009); see section **3.5**). In contrast to DN and DP cells, which develop within the cortical region, newly generated $CD4^{+}$ and $CD8^{+}$ SP cells home to the medulla where they remain for additional 4-5 days before they exit to the circulation via similar type of vessels as the immigrating precursors (McCaughtry et al., 2007; Zachariah and Cyster, 2010). During their medullary residency, SP cells are additionally screened for auto-reactivity on a distinct library of self-peptides presented by mTECs and DCs, as well as progressively acquire functional and phenotypic maturity (for review see (McCaughtry and Hogquist, 2008; Weinreich and Hogquist, 2008); see sections **3.6** and **3.7**).

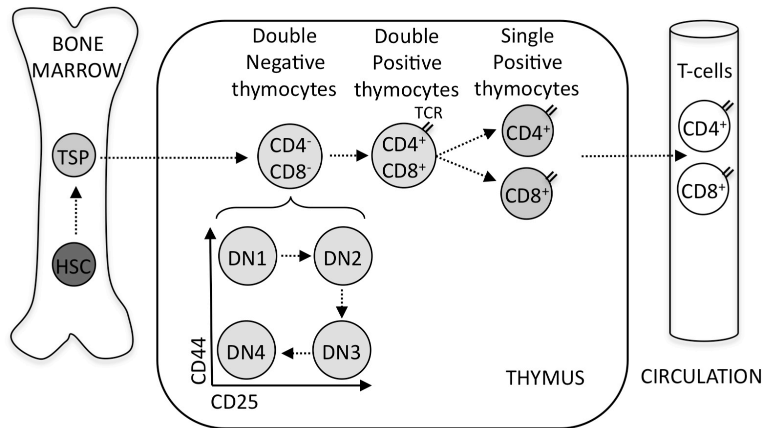

Figure 3 Schematic view of T-cell development TSP, thymus settling progenitor; HSC, hematopoietic stem cell; TCR, T-cell receptor

3.3 Seeding of the thymus by BM-derived progenitors

The thymus does not sustain hematopoietic stem cells (HSC) (Luc et al., 2012). Instead, T cell development is maintained through regular import from the bone marrow of non-self renewing hematopoietic progenitors that are then signaled by the thymic environment to adopt the T cell fate (Bell and Bhandoola, 2008; Luc et al., 2012; Wada et al., 2008; Yui et al., 2010). The precise identity (i.e. the phenotype and commitment stage) of such thymus-settling progenitors (TSPs) has yet not been definitively established. Due to the predictably very low numbers of progenitors homing to the thymus in real-time and the suggested periodic receptivity of the thymus to progenitor settling (Foss et al., 2001, 2002), direct examinations of thymic seeding in steady state have been technically challenging. Thus far, combined evidence from indirect approaches toward the identification of TSPs, focusing on the characterization of the lineage potential of ETPs and the identification of the key homing mediators implicated in thymic settling, have been consistent with candidate TSPs being derived from BM-

resident lymphoid-primed multi-potent progenitors (LMPPs) and/or common lymphoid progenitors (CLPs) ((Luc et al., 2012); for review see (Love and Bhandoola, 2011; Zlotoff and Bhandoola, 2011)). Accordingly, ETPs are highly similar at the molecular level to LMPPs and CLPs, and contain cells with combined granulocyte-monocyte, T- and B-lymphocyte but not megakaryocyte-erythroid lineage potential, which matches the potentials described for LMPPs and a subset of CLPs (Luc et al., 2012). Further, amongst all known BM-progenitors that possess the T cell potential only LMPPs and CLPs rapidly generate thymocytes after intravenous transfer (Schwarz et al., 2007), indicating that thymus settling is selective and suggesting that LMPPs and CLPs may possess the ability to directly colonize the thymus. Indeed, fractions of LMPPs and CLPs have been found to express CCR9, CCR7, and CXCR4, chemokine receptors implicated in mediating the entry of T-cell progenitors into the thymus (Calderon and Boehm, 2011; Krueger et al., 2010; Zlotoff et al., 2010). Future studies are needed to definitively pinpoint the precise characteristics of the TSPs that feed into the thymopoiesis under physiological conditions. It should also be noted that the multi-potent ETP stage may not be obligatory and current evidence does not exclude that this step could be by-passed via thymic homing of more restricted progenitors. Such a possibility has been previously suggested based on the transient appearance of only T cell-restricted progenitors with a DN3-like phenotype in the periphery following irradiation and stem cell transplantation (Lancrin et al., 2002). However, the *in vivo* relevance of these cells toward generating T cells in the thymus after irradiation remains unclear (for review see (Zlotoff and Bhandoola, 2011)).

Irrespective of the nature of TSPs, the process of T-cell progenitor egress from the blood into the thymus is generally assumed to involve similar mechanisms to those by which leukocytes enter the lymph nodes and non-lymphoid tissues (Scimone et al., 2006). The multistep adhesion cascade of leukocyte extravasation involves selectin-dependent rolling on the vessel wall,

followed by chemokine-mediated activation of integrin receptors enabling leukocyte firm adhesion and diapedesis (von Andrian and Mackay, 2000). Consistent with a possibility that T-cell progenitor entry obeys a similar program, efficient thymic settling has been shown to depend on the interaction between P-selectin glycoprotein ligand (PSGL-1) expressed by T-cell progenitors and P-selectin, which is expressed by thymic endothelium (Rossi et al., 2005). Also, and as mentioned above, thymic entry of T-cell progenitors has been shown to depend on their ability to receive signals through the receptors CCR9, CCR7 and CXCR4 (Calderon and Boehm, 2011; Krueger et al., 2010; Zlotoff et al., 2010). Interestingly, thymic expression of P-selectin and the CCR9 ligand, CCL25 appears to rise and fall in periodic cycles (Gossens et al., 2009). This phenomenon has been proposed to regulate progenitor access to the thymus and to explain the earlier observations that the mouse thymus is only periodically receptive to T-cell progenitor homing (Foss et al., 2001, 2002).

3.4 The role of thymic cortex in supporting T cell differentiation

As mentioned above (**3.2**), DN and DP thymocytes develop within the cortical environment (Petrie and Zuniga-Pflucker, 2007). cTECs are thought to critically foster these stages of T-cell development by providing essential cues for DN cell expansion and differentiation, and by mediating positive selection of DP cells. Regarding the former, cTECs are the main source of thymic Delta-like 4 (Dll4) (Koch et al., 2008), which is the principal physiological ligand for Notch-1 expressed by T-cell progenitors. Cell intrinsic Notch-1 signaling is critically required for T-cell lineage commitment and differentiation of DN cells (Benz et al., 2008; Radtke et al., 1999; Wilson et al., 2001). Accordingly, TEC-specific ablation of Dll4 leads to complete abrogation of thymic T-cell development (Koch et al., 2008). Further, cTECs express IL-7 (Alves et al., 2010; Alves et al., 2009; Gray et al., 2007b), an essential regulator of DN cell proliferation and/or survival. Consistently, DN2 and DN3 cells express mediators of IL-7 signaling (Bhandoola

et al., 2007) and mice deficient for IL-7 or IL-7Rα display severe thymic hypoplasia with an incomplete block at the transition to the DN3 stage (Maki et al., 1996; Moore et al., 1996; Prockop and Petrie, 2004). A recent study reported that acute DTR-mediated cTEC ablation *in vivo* resulted in a rapid loss of DN2/DN3 cells followed by disappearance of the DP compartment (Rode and Boehm, 2012), further highlighting the importance of cTECs in supporting DN cell development towards the DP stage.

The importance of cTEC in mediating positive selection has been recognized for many years. Early studies using reaggregate thymic organ cultures (RTOC) and BM chimeras showed that positive selection was critically dependent on the presence of TECs and on the ability of TECs to express MHC molecules (Anderson et al., 1994; van Meerwijk et al., 1997). The autonomous ability of cTECs to present peptides for induction of positive selection was further highlighted by the observation that the transgenic restoration of MHCII expression solely in cTECs in MHCII-deficient mice was sufficient to mediate generation of $CD4^+$ and $CD8^+$ T cells (Laufer et al., 1996). Notably, positive selection is sustained in the absence of DCs and of their MHCII expression, as well as in the context of genetic deficiencies resulting in the lack of mTEC development, indicating that the putative contributions of DCs and mTECs toward positive selection are not essential (Akiyama et al., 2012; Liston et al., 2008; Ohnmacht et al., 2009) One hypothesis that has been put forward to explain the apparent fitness of cTECs to drive positive selection is by displaying an array of self-peptides with unique qualities (Takahama et al., 2012). This hypothesis has gained strength recently with the discovery that proteasome complexes, which generate peptides for presentation by MHC class I, in cTECs uniquely include the enzymatic subunit, β_5t (Murata et al., 2007). Incorporation of β_5t into proteasomes in place of its equivalents found in DCs and mTECs, β_5 and β_5i, selectively reduces proteasome-dependent chymotrypsin-like activity in mouse embryonic fibroblasts (Murata et al., 2007). As the chymotrypsin-like activity is considered important for

the production of peptides with high affinities for MHC-I clefts, cTECs have been proposed to specialize in displaying low-affinity peptides. Although the precise impact of β_5t-dependent peptide processing on the quality of the peptide array presented by cTECs *in vivo* still awaits a formal investigation, β_5t-deficient mice display defective development of $CD8^+$ T cells resulting in the generation of a lymphopenic and abnormally composed $CD8^+$ TCR pool, highlighting the importance of β_5t-dependent peptide production in positive selection (Nitta et al., 2010). Regarding the positive selection of $CD4^+$ T cells, the lysosomal proteases Prss16 (also called thymus specific serine protease (TSSP)), and cathepsin L are highly, but not exclusively, expressed in cTECs, and analyses of mice deficient in Prss16 or cathepsin L have indicated that these enzymes are necessary for optimal positive selection of $CD4^+$ T cells (Bowlus et al., 1999; Gommeaux et al., 2009; Honey et al., 2002; Nakagawa et al., 1998; Viret et al., 2010).

Finally, the cTEC compartment of the postnatal thymus contains so called thymic nurse cells (TNCs), which are characterized as large cells that completely envelop many viable thymocytes within intracellular vesicles. A recent study implicated TNCs in providing microenvironment for DP cells that undergo TCR editing by secondary rearrangements of their TCRα chain genes (Nakagawa et al., 2012). The physiological significance of this process in TCR repertoire generation is not clear.

3.5 CD4/CD8 lineage commitment

Having received a positively selecting TCR signal, DP cells up-regulate CD69 and terminate *Cd8* gene transcription (Brugnera et al., 2000). The latter event leads to the acquisition of a $CD4^+CD8^{lo}$ surface phenotype, which marks a transitional stage during which thymocytes commit to either the helper $CD4^+CD8^-$ or cytotoxic $CD8^+CD4^-$ T-cell fate depending on whether their TCRs bind antigen in the context of MHCII or MHCI, respectively. The molecular underpinnings of the matching of lineage differentiation to MHC-restriction of the TCR remain not

understood. In this regard, it has been proposed that differences in the duration of MHCII-restricted (CD4-dependent) versus MHCI-restricted (CD8-dependent) TCR signaling, which are thought to occur as a result of the asymmetry in co-receptor expression at the $CD4^{+}CD8^{lo}$ stage, initiate distinct signals that direct cells to either the $CD4^{+}$ or $CD8^{+}$ T-cell fate (for review see (Singer et al., 2008)). In contrast to the still enigmatic nature of the initial instructive signal(s), understanding of the intrinsic mechanisms regulating co-receptor expression and functional lineage determination in MHCII- and MHCI-selected thymocytes is steadily increasing. In essence, commitment to the $CD4^{+}$ and $CD8^{+}$ T cell fate is known to be essentially dependent on the activity of the transcriptional factors ThPOK and Runx-1/-3, respectively (for review see (Collins et al., 2009; Rothenberg, 2009; Xiong and Bosselut, 2012)). Accordingly, the zinc-finger transcription factor, ThPOK (also called c-Krox) is induced in MHCII-selected $CD4^{+}CD8^{lo}$ cells reaching maximal level of expression in mature $CD4^{+}$ SP cells, but is essentially absent from MHCI-selected thymocytes (Egawa and Littman, 2008). Helper-deficient mice, which have a spontaneous mutation in the gene encoding ThPOK (*Zbtb7b*, hereafter called *ThPOK*), and mice with targeted deletion of *ThPOK*, both lack $CD4^{+}$ helper T cells as a result of an aberrant redirection of MHC class II-restricted thymocytes to the cytotoxic $CD8^{+}$ T cell lineage (He et al., 2005; Wang et al., 2008). The Runt domain transcription factors, Runx1 and Runx3 have initially been characterized as transcriptional repressors binding to the *Cd4* silencer and negatively regulating CD4 expression in DN and $CD8^{+}$ SP cells, respectively (Taniuchi et al., 2002). Conditional deletion of both Runx1 and Runx3, but not either factor alone, at the DP stage leads to a complete loss of $CD8^{+}$ SP cells and re-direction of MHCI–specific thymocytes to the $CD4^{+}$ T cell lineage (Egawa et al., 2007; Setoguchi et al., 2008), implicating functional redundancy between Runx-1 and Runx-3 in promoting $CD8^{+}$ lineage commitment. Based on the fact that Runx-3, but not Runx-1, is selectively expressed in $CD8^{+}$ lineage cells and the observation that Runx-1 is up-

regulated in $CD8^+$ SP cells in the absence of Runx-3 (Egawa and Littman, 2008), it is presumed that Runx-3 is the primary physiological regulator of the $CD8^+$ lineage commitment.

Positively selected thymocytes are known to relocate from the cortex to the medulla. Consistently, SP cells are selectively accumulated in the medullary region (for review see (Dzhagalov and Phee, 2012)). However, the exact localization of $CD69^+$ $CD4^+CD8^{lo}$ cells during the duration of the lineage commitment process, which has been reported to take approximately 1 and 3 days for MHCII- and MHCI-restricted cells, respectively (Saini et al., 2010), has thus far not been clarified. In this regard, cortical regions are essentially devoid of $CD69^+$ cells that instead are found adjacent to the CMJ and inside the medulla (Choi et al., 2008), suggesting that lineage commitment might at least partially occur in proximity to or even inside the medulla and thus be sensitive to putative extrinsic cues present not only in the cortex but also in the medulla.

3.6 Central tolerance

In addition to maximizing the spectrum of TCRs that are able to recognize foreign antigens, another conceivable consequence of the stochastic assembly of TCR genes during T cell development is generation of TCRs that can recognize the body's own components and potentially attack host tissues. Indeed, it is now firmly established that the intrathymically generated nascent TCR pool contains self-reactive affinities that, in order to prevent autoimmunity, need to be controlled by the mechanisms of central (thymus-dependent) and peripheral (thymus-independent) tolerance. The mechanisms of central tolerance are broadly distinguished into recessive and dominant. Recessive tolerance depends on the process of negative selection that acts to minimize the release from the thymus of functionally competent auto-reactive T cells. In contrast, dominant tolerance involves generation of forkhead box P3 (FoxP3)-expressing T regulatory (T_{reg}) cells with immunosuppressive potential, which serve to dampen the pro-

inflammatory activity of self-reactive T cells that have avoided negative selection and escaped to the periphery (for review see (Fletcher et al., 2011b; McCaughtry and Hogquist, 2008))(Lee et al., 2011)).

3.6.1 Self-reactive T cell removal via negative selection

Negative selection is a collective term used to describe different fates that await a thymocyte whose TCR has encountered its cognate/high affinity peptide ligand in the thymus. The processes triggered by high affinity and/or avidity interaction between the TCR and peptide/MHC complex in the thymus have been described to include receptor editing (Mayerova and Hogquist, 2004), lineage deviation (Pobezinsky et al., 2012) and clonal deletion via induction of apoptosis (Palmer, 2003), the latter of which is thought to be the main physiological mechanism of negative selection. Exactly how the different affinity or avidity of peptide/MHC interaction can be translated by the TCR to induce the qualitatively distinct outcomes of positive and negative selection has not yet been clarified. With regard to the proximal TCR signaling events, it has been proposed that, at least in the case of MHC-I-restricted TCRs, the cellular sensing of positively and negatively selecting signals may be linked to a differential intracellular compartmentalization of members of MAPK pathway, extracellular signal regulated kinase (ERK) and amino-terminal kinase (JNK) (Daniels et al., 2006). Ultimately, signals inducing clonal deletion lead to activation of the apoptotic pathway, which is essentially mediated by the cooperative function of pro-apoptotic BH3-only proteins, Bim and Puma (Gray et al., 2012).

In general, self-antigens utilized to mediate negative selection in the thymus are thought be supplied from the pool of proteins expressed by thymus resident cells and via import of proteins of extra-thymic origin from the blood. Regarding putative thymic populations that could autonomously mediate negative selection by presenting these self-antigens as peptide-MHC complexes, studies have focused on three cell subsets, cortical and medullary TECs, and thymic DCs (for

review see (Derbinski and Kyewski, 2010; McCaughtry and Hogquist, 2008)). Of these, evidence for an autonomous and non-redundant role as negatively selecting APCs *in vivo* has been obtained in mouse models in the case of DCs and mTECs. Thus, compromising APC potential specifically in DCs or mTECs by genetic ablation (DCs) or siRNA-mediated reduction (mTECs) of MHCII expression has been shown to rescue a significant number of $CD4^+$ SP cells from clonal deletion *in vivo* (Hinterberger et al., 2010; Liston et al., 2008). Despite this, and although these questions have been actively studied for several decades, the precise *in vivo* contribution(s) of the APC potential of mTECs versus DCs to negative selection are still being uncovered. Part of the difficulty in studying this topic stems from the fact that at least some antigens can be efficiently transferred in their native and/or processed MHC-bound form from mTECs to DCs (Koble and Kyewski, 2009), which implies that mTECs do not necessarily have to play a critical and/or non-redundant function in the presentation for negative selection of antigens they express. Indeed, evidence for both DC-dependent and DC-independent negative selection to neo-self-antigens whose expression has been selectively targeted to mTECs has been obtained in transgenic mouse models (Hubert et al., 2011; Klein et al., 2001), and a recent report using MHC-tetramer-based identification of Ag-specific T cells indicated that DC were necessary to mediate clonal deletion to a mTEC-expressed endogenous self-antigen (Taniguchi et al., 2012). Interestingly, evidence for similar antigen shuttling from DCs to mTECs has so far not been obtained. Regarding cTECs and negative selection, although data from certain transgenic models have implicated these cells as APCs that could drive negative selection by receptor editing (Mayerova and Hogquist, 2004) or developmental diversion (Pobezinsky et al., 2012), in general there is little evidence to support an autonomous ability of cTEC to mediate clonal deletion *in vivo* (McCaughtry et al., 2008; McCaughtry and Hogquist, 2008). Further, antigen presentation by cTECs alone is not sufficient to establish central tolerance (Laufer et al., 1996).

Despite the unclear role of cTECs in mediating clonal deletion, the ability of the cortical environment *per se* to autonomously drive clonal deletion of thymocytes in response to an ubiquitously expressed endogenous self-antigen has been documented in a TCR transgenic model, and this study implicated cortical DCs as APCs mediating this process (McCaughtry et al., 2008). Thus, it is currently believed that thymocytes can undergo clonal deletion in response to self-antigens encountered in the cortex, however the extent and physiological significance of this process as well as the source(s) of negatively selecting self-ligands in the cortex are less clear. In contrast, it is well recognized that the thymic medullary environment plays an essential role in mediating negative selection for establishment of central tolerance (for review see (Anderson and Takahama, 2012)). Accordingly, genetic deficiencies affecting the size and/or architecture of the medulla (for review see (Akiyama et al., 2012)) as well as blockade of the entry of positively selected thymocytes to the medulla in mice deficient in CCR7 signaling pathway (Nitta et al., 2009) have been shown to result in development of autoimmunity. The apparent link between the optimal establishment of central tolerance and thymocyte exposure to the correctly developed medullary compartment is known to at least in part rely on the unique capacity of mTECs to promiscuously express a broad array of peripheral tissue-restricted self-antigens (TRA) (Takahama et al., 2008), many of which are critically used to purge the T cell repertoire of auto-reactive T cells (DeVoss et al., 2006; DeVoss et al., 2010; Fan et al., 2009; Gavanescu et al., 2007; Shum et al., 2009; Su et al., 2012). Based on microarray analyses it has been estimated that approximately 5-10% of all known mouse genes are turned on in mTECs, in addition to their cell-specific expression fingerprint (Derbinski et al., 2005). Interestingly, evaluations of TRA expression on a single cell level have indicated that at a given time-point each individual TRA is expressed on average by only about 1-3% of total mTECs (Derbinski et al., 2008; Derbinski et al., 2001), raising questions as to how antigens present in such rare cells can efficiently remove their cognate specificities

from the T-cell repertoire. Notably, it appears that negative selection of TRA-reactive thymocytes operates with little buffering capacity with respect to the level of self-antigen expression. Thus, human allelic variants associated with moderate reductions in the level of the intra-thymic expression of certain TRAs, such as insulin and acetylcholine receptor alpha, have been correlated with susceptibility to autoimmune type I diabetes and myasthenia gravis, respectively (Chentoufi and Polychronakos, 2002; Giraud et al., 2007; Pugliese et al., 1997; Taubert et al., 2007).

The only factor identified to date driving the expression of a large group of TRAs in mTECs is a transcriptional regulator, autoimmune regulator (Aire) (Anderson et al., 2002; Derbinski et al., 2005), which is expressed by a subset of post-mitotic MHC2^{hi}CD80^{+} mTECs (Gray et al., 2007a) (also see section **3.9**). Homozygous loss-of-function mutations in AIRE in humans cause autoimmune disease referred to as autoimmune polyglandular syndrome type I (APS-1), or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Peterson and Peltonen, 2005) that is associated with circulating tissue-specific auto-antibodies and in adulthood, development of organ-specific autoimmune diseases, such as gonadal atrophy, type I diabetes, hypothyroidism, and hepatitis (Ahonen et al., 1990). In mice, targeted ablation of the Aire gene results in development of T cell-driven autoimmunity that is causatively linked to the loss of Aire expression in thymic stroma, and with the scope of the disease showing strong dependence on the genetic background (Anderson et al., 2005; Guerau-de-Arellano et al., 2009; Hubert et al., 2009; Venanzi et al., 2008). Several organ-specific autoimmune diseases in the Aire$^{-/-}$ model have been linked to a failure in the appropriate thymic expression of a given TRA under the control of Aire, highlighting the importance of Aire-regulated TRA expression for central tolerance induction (DeVoss et al., 2006; DeVoss et al., 2010; Fan et al., 2009; Gavanescu et al., 2007; Shum et al., 2009; Su et al., 2012). The precise mechanism(s) by which Aire recognizes and activates its target genes in mTECs

have not been fully elucidated. Nevertheless, over the last decade important insights have been made that reveal some critical aspects of Aire's mode of action and of the regulation of TRA expression in general. Firstly, Aire-dependent gene regulation shows unusually high variability amongst individuals, both in terms of the expression level of each particular TRA and the spectrum of up-regulated genes (Taubert et al., 2007; Venanzi et al., 2008). Using mouse models, it has been shown that this variability is fed not only by genetic differences between individuals but also by an intrinsically stochastic activity of Aire itself (Venanzi et al., 2008). In addition, Aire exhibits a probabilistic mode of action on a single cell level i.e. it triggers random patterns of TRA expression in individual mTECs (Derbinski et al., 2008; Villasenor et al., 2008). The former feature is not specific to Aire, but appears a general characteristic of the ectopic gene expression in mTECs (Derbinski et al., 2008; Tykocinski et al., 2010; Villasenor et al., 2008). How does Aire recognize and up-regulate its target genes? One way through which Aire can bind chromatin is via a specific interaction between its plant homeodomain (PHD) -1 and unmethylated histone H3 lysine 4 (H3K4), an epigenetic mark associated with transcriptional silencing (Koh et al., 2008). Notably, although the ability of Aire to bind unmethylated H3K4 is necessary for optimal Aire-dependent TRA expression and prevention of autoimmunity *in vivo* (Koh et al., 2010), the sole presence of this mark in a gene promoter is not sufficient for it to be activated by Aire (Koh et al., 2008). Based on the broad diversity of Aire's binding partners, it has been proposed that on a molecular level Aire could contribute to multiple steps of the transcriptional process (Mathis and Benoist, 2009). Interestingly, it has recently been suggested that Aire may function as a regulator of transcriptional elongation by stimulating the release of stalled RNA polymerase at the promoters of its target genes (Giraud et al., 2012). In addition to regulating the elimination of auto-reactive T cells through transcriptional control of TRAs, other mechanisms of Aire-dependent tolerance have also been suggested. Consistent with such possibility, Aire$^{-/-}$ mice have been

shown to display defects in negative selection against endogenous self-antigens (Kuroda et al., 2005) and transgenic neo-self-antigens (Hubert et al., 2011) whose intra-thymic expression is unaltered in the absence of Aire. The mechanism(s) underlying this phenomenon are not clear. However, interestingly, Aire$^{-/-}$ mice have been reported to display alterations in mTEC and DC compartments, such as altered positioning (mTECs and DCs) (Lei et al., 2011; Yano et al., 2008) and population composition (mTECs) (Dooley et al., 2008; Gray et al., 2007a; Hubert et al., 2009), as well as to display a moderate reduction in the number of thymic T_{reg} cells (Lei et al., 2011).

In summary, the emerging picture is that the thymic medulla to some extent mirrors the self-peptide display encountered by mature T cells in the periphery by exploiting the unique capacity of mTECs to promiscuously transcribe an array of TRAs and common pathways of antigen sampling and processing similar to peripheral APCs. On a global scale, optimal TCR repertoire purging by negative selection seems to be accomplished via complementing contributions to self-antigen supply and to its presentation of mTECs and thymic DCs (Derbinski and Kyewski, 2010; Taniguchi et al., 2012).

3.6.2 FoxP3^{+} T regulatory cell development

As mentioned above (**3.6**), generation of FoxP3^{+} T_{reg} cells represents another mechanism by which the thymus contributes to the induction of tolerance. Based on mouse studies, it is now well established that FoxP3^{+} T cells are required to prevent fatal autoimmunity throughout the lifespan of the host via a mechanism that depends on the immunosuppressive potential of these cells (Fontenot et al., 2005a; Kim et al., 2007; Lahl et al., 2007). Accordingly, genetic deficiency in the X-chromosome encoded transcription factor FoxP3, which itself is required for both the development and function of FoxP3^{+} T_{reg} cells, and acute diphtheria toxin-mediated ablation of FoxP3-expressing cells in mice all result in a lethal lympho-proliferative autoimmune disease characterized by widespread

lymphocyte infiltration and pathology of multiple organs (Fontenot et al., 2003; Fontenot et al., 2005a; Kim et al., 2007; Lahl et al., 2007). Mutations of the human gene FOXP3 cause a fatal autoimmune condition known as immune dysregulation, polyendocrinopathy, enteropathy and X-linked (IPEX) syndrome that shares many phenotypic features with the disease developed by FoxP3$^{-/-}$ mice, consistent with a similar role for FoxP3^{+} T cells in humans (Bennett et al., 2001)(for review see (Sakaguchi et al., 2008)). In addition to FoxP3^{+} T_{reg} cells that develop intra-thymically, FoxP3^{+} immunosuppressive cells can also be induced as one of the effector fates acquired by antigen-activated naive T cells in the periphery. Regarding physiological importance of the thymus-dependent (natural, nT_{reg}) versus peripherally induced (induced, iT_{reg}) FoxP3^{+} T_{reg} cell pools, nT_{reg} cells have long been believed to be essential to prevent the development of wasting systemic autoimmunity. This view is primarily based on the observation that thymectomy of 3 day old mice, a time-point preceding the first wave of nT_{reg} but not conventional CD4^{+} T cell export from the thymus, causes widespread systemic autoimmunity (Sakaguchi and Sakaguchi, 1990). Only recently, the discovery of conserved non-coding DNA sequence (CNS) elements that are differentially required for the induction of FoxP3 expression by nT_{reg} and iT_{reg} cells, and the subsequent generation of mice carrying targeted disruption of these elements (Zheng et al., 2010) have provided tools that allow dissection of the specific functions of nT_{reg} and iT_{reg} under physiological conditions *in vivo*. Studies using CNS-deficient mice have so far indicated that nT_{reg} cells are sufficient to prevent wasting systemic autoimmunity (Josefowicz et al., 2012) and that iT_{regs} function in restraint of allergic Th2-type inflammation at mucosal surfaces in the gut and the lungs (Josefowicz et al., 2012), and in maternal tolerance of the fetus (Samstein et al., 2012).

The nT_{reg} cell compartment generated in the thymus is composed of a major, MHCII-restricted FoxP3^{+} CD4^{+}CD8^{-} population, and a minor (3-4% of total thymic FoxP3^{+} cells) subset of MHCI-restricted FoxP3^{+} CD4^{-}CD8^{+} cells (Fontenot

et al., 2005b; Lee and Hsieh, 2009). Due to their overwhelming abundance, studies have primarily focused on the developmental pathway of $CD4^+$ nT_{reg} cells. Although FoxP3 expression can be induced in DP thymocytes (Fontenot et al., 2005b; Liston et al., 2008) (with $FoxP3^+$ DPs constituting up to 4% of the total thymic $FoxP3^+$ cell pool (Lee and Hsieh, 2009)), nT_{reg} cell generation does not obligatorily involve a $FoxP3^+$ DP intermediate (Wirnsberger et al., 2009). Based on this and the fact that FoxP3-expressing DP and SP cells have been reported to arise concomitantly in kinetic analyses (Fontenot et al., 2005b; Lee and Hsieh, 2009), it is generally believed that the nascent $CD4^+$ nT_{reg} pool is enriched in cells that have up-regulated FoxP3 following their lineage commitment and migration into the medulla. Consistently, $FoxP3^+$ cells are predominantly localized with the bulk of SP cells in the medulla, with remaining cells found in the deep cortex adjacent to the CMJ (Fontenot et al., 2005b; Lahl et al., 2007). Finally, nT_{reg} cell differentiation appears to be intrinsically set to occur within a certain time frame along the $CD4^+$ T cell developmental pathway. Thus, the propensity to develop into an nT_{reg} cell decreases with the medullary dwell time of $CD4^+$ SP cells and is virtually extinguished in mature T cells that have left the thymus (Wirnsberger et al., 2009).

Deciphering of the extrinsic mechanisms regulating nT_{reg} cell differentiation remains a matter of ongoing research in the field. In this regard, much attention has been given to determining whether TCR-signaling plays an instructive role in nT_{reg} fate decision-making process. Despite remaining controversies (Pacholczyk et al., 2007; van Santen et al., 2004), accumulating data from transgenic models support the conclusion that acquisition of nT_{reg} fate by a $FoxP3^-$ thymocyte is promoted by the engagement of its TCR by an agonist self-peptide/MHC complex (Apostolou et al., 2002; Aschenbrenner et al., 2007; D'Cruz and Klein, 2005; Jordan et al., 2001)(for review see (Klein and Jovanovic, 2011; Lee et al., 2011)). Interestingly, it appears that nT_{reg} cells can be induced over a surprisingly wide, 1,000-fold range of peptide-MHC/TCR reactivity, which at least in its higher

range overlaps with conditions sufficient to induce negative selection (Lee et al., 2012). By examining the response of TCR-transgenic thymocytes to titrated amounts of their cognate peptide it has been observed that the numerical output of Ag specific T_{reg} cells is initially positively correlated but finally declines in response to increasing avidity of peptide-MHC/TCR interaction, the decline likely explained by T_{reg} cells eventually succumbing to negative selection (Atibalentja et al., 2009; Feuerer et al., 2007). Consistent with these observations, decreasing the avidity of TCR/peptide-MHC interaction by reducing the levels of MHCII expressed by mTECs in a TCR transgenic model of mTEC-mediated nT_{reg} cell development results in an increased numerical output of Ag-specific nT_{reg} cells (Hinterberger et al., 2010). Regarding other extrinsic cues that have been implicated in nT_{reg} cell generation, $FoxP3^+$ cells are virtually absent in the thymus of mice deficient for common gamma chain ($c\gamma_c$), a component of multiple cytokine receptors including those for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Bayer et al., 2008; Burchill et al., 2007; Fontenot et al., 2005a; Lin et al., 1995). Based on phenotypic assessments of mice bearing single and combined deficiency in the distinct $c\gamma_c$-dependent receptors, it appears that generation of optimal nT_{reg} numbers is dependent on partially redundant activities of IL-2, IL-15, and IL-7 (Bayer et al., 2008; Burchill et al., 2007). Further, nT_{reg} cell development depends on the ability of thymocytes to receive co-stimulation through CD28-B7, and to a lesser extent, CD40-CD40L signaling pathways (Burchill et al., 2008; Proietto et al., 2008b; Spence and Green, 2008; Vang et al., 2010). It has been suggested that one mechanism by which CD28 signaling could impact on nT_{reg} development is by activating c-Rel, a nuclear factor kappa B (NFκB) family member critically required for nT_{reg} cell differentiation and implicated in FoxP3 gene induction (Vang et al., 2010; Zheng et al., 2010). Finally, available data suggest that nT_{reg} cell development does not require a dedicated thymic APC subset. Thus, antigen presentation by both mTECs and DCs supported nT_{reg} cell development *in vitro*,

although distinct optimal peptide doses were required to achieve maximal efficiency of this process (Wirnsberger et al., 2009).

3.7 SP cell maturation and egress from the thymus

Using RAG-GFP transgenic mice, in which the 'age' of SP cells can be assessed based on gradual loss of GFP fluorescence after termination of GFP transgene expression at DP stage, SP cells have been estimated to spend up to 4-5 days in the medulla before being exported to the circulation (McCaughtry et al., 2007). During this time, SP cells undergo a process of phenotypic and functional maturation. Accordingly, newly generated SP thymocytes are characterized by a $CD69^{+}CD24^{hi}CD62L^{lo}Qa2^{-}$ phenotype and are sensitive to negative selection by clonal deletion. In contrast, mature SP cells are $CD69^{-}CD24^{lo}CD62L^{hi}Qa2^{hi}$ and react to agonistic TCR stimuli with proliferation and cytokine production (for review see (Weinreich and Hogquist, 2008)). In addition, an ordered maturation program characterized by phenotypic progression between SP1 ($CD69^{+}6C10^{+}$), SP2 ($CD69^{+}6C10^{-}$), SP3 ($CD69^{-}Qa2^{-}$) and SP4 ($CD69^{-}Qa2^{+}$) stages has been established in the case of $CD4^{+}$ SP thymocytes (Jin et al., 2008).

In order to leave the thymus, SP cells need to up-regulate sphingosine-1-phosphate (S1P) receptor-1 (S1P1), which allows them to be attracted to S1P expressed by TMCs in the PVS (Allende et al., 2004; Matloubian et al., 2004; Zachariah and Cyster, 2010). TMC-derived S1P appears both critical and sufficient to drive optimal SP cell exit from the thymus, although its function can be partially substituted by S1P present in the blood (Pappu et al., 2007; Zachariah and Cyster, 2010). The induction of S1P1 expression in SP cells depends on the function of the transcription factor, Kruppel-like factor (KLF)-2 (Carlson et al., 2006). Both KLF-2 and S1P1 are gradually induced during SP cell maturation reaching optimal levels in mature $CD62L^{hi}$ SP cells after approximately 4 days post positive selection (Matloubian et al., 2004; McCaughtry et al., 2007). The molecular mechanism(s) underlying KLF-2 up-regulation by SP cells, and whether this

process is sensitive to extrinsic regulation, remain poorly understood. To date, activation of forkhead box O (Foxo) transcription factors and release from active inhibition imposed by phosphoinositide-3-kinase (PI3K)-dependent signaling pathway have been implicated in this process (Barbee and Alberola-Ila, 2005; Kerdiles et al., 2010; Sinclair et al., 2008). Also, it appears that the acquisition of egress competence does not require SP cell entry to the medulla (Kurobe et al., 2006).

3.8 Ontogeny and functions of thymic dendritic cell subsets

For better understanding of subset composition and origin of thymic DCs, I will initially provide some background on the classification and ontogeny of DCs in general. Murine DCs are classically divided into $CD11c^{+}MHC2^{+}CD45R(B220)^{-}$ conventional (cDC) and $CD11c^{int}B220^{+}$ plasmacytoid (pDC) populations. In general, cDCs can be distinguished in two main populations, $CD11b^{-}Sirp\alpha^{-}CD8\alpha^{+}$ ($CD8\alpha^{+}$) and $CD11b^{+}Sirp\alpha^{+}CD8\alpha^{-}$ ($CD8\alpha^{-}$), a major fraction of the latter subset expressing CD4 in the spleen. $CD8\alpha^{+}$ and $CD8\alpha^{-}$ cDCs show differential transcription factor requirements for their development/maintenance. Accordingly, $CD8\alpha^{+}$ but not $CD8\alpha^{-}$ cDCs are severely reduced in mice deficient for basic leucine zipper transcription factor, ATF-like 3 (Batf3) and interferon regulatory factor (IRF)-8 while $CD8\alpha^{-}$ cDCs are selectively dependent on IRF-4 and the NFκB family member, RelB (for review see (Satpathy et al., 2011)). The DC compartment of the mouse thymus has been reported to comprise pDCs, IRF-8/Batf3-dependent $CD8\alpha^{+}$ cDCs (Atibalentja et al., 2011; Luche et al., 2011), and phenotypic equivalents of $CD8\alpha^{-}$ cDCs (whose IRF-4/RelB dependence is yet to be definitively shown) (Li et al., 2009; Proietto et al., 2008a; Suzuki et al., 2004; Wu et al., 1998). In contrast to splenic $CD8\alpha^{-}$ cDCs, thymic $CD8\alpha^{-}$ cDCs do not express CD4 mRNA (Vremec et al., 2000) and constitute a minor (ca 20%) fraction amongst the bulk of cDCs (Proietto et al., 2008a). Despite previous observations that cDC subsets could be generated in the spleen and the thymus

from both lymphoid- and myeloid-committed progenitors in irradiated recipients (Manz et al., 2001; Wu et al., 2001), recent lineage tracing data has indicated that the major physiological pathway leading to the generation of cDCs involves myeloid-committed progenitors (Schlenner et al., 2010). The myeloid pathway of DC differentiation from common myeloid progenitors (CMP) has been extensively studied and described to involve a purely DC-restricted BM progenitor called common DC progenitor (CDP). CDPs give rise to pre-pDCs and pre-cDCs, which are restricted to their respective lineages. In contrast to pre-pDCs, which are thought to finalize their development in the BM followed by pDC dissemination to target tissues, pre-cDCs seed lymphoid and non-lymphoid tissues where they complete their differentiation into $CD8\alpha^-$ or $CD8\alpha^+$ cDC subsets under putative influence of yet unknown lineage-determining extrinsic signals (for review see (Satpathy et al., 2011)). Regarding thymic DCs, there is strong evidence from studies using parabiotic mice or transplantation of BM progenitors into irradiated and non-irradiated recipients (Donskoy and Goldschneider, 2003; Li et al., 2009) that $CD8\alpha^+$ cDC subset is generated intrathymically from BM-derived precursors whose development/import is closely linked to that of T-cell progenitors. These results have long been interpreted as supporting a lymphoid origin of thymic $CD8\alpha^+$ cDCs, a view further supported by the fact that ETPs and DN2 cells can be induced to develop into cDCs *in vitro* (Yui et al., 2010). In the light of the aforementioned lineage tracing study of Schlenner *et al.*, which argues against cDCs being generated intrathymically from the bulk of ETPs (Schlenner et al., 2010), and a recent description of an intrathymic subset with phenotypic features reminiscent of pre-cDCs (Luche et al., 2011) one possible scenario reconciling the above observations could be that thymus-homing pre-cDCs are subject to similar gated importation and/or compete for the same intra-thymic environmental niche as T-cell progenitors. In contrast to their $CD8\alpha^+$ counterparts, thymic $CD8\alpha^-$ cDC compartment has been found to be continuously replenished from the blood by what appears to be committed but perhaps semi-mature $CD8\alpha^-$ cDCs (Li et al.,

2009; Proietto et al., 2008b). These studies have also revealed that the thymic pDC compartment is maintained through continuous homing of blood-borne pDCs (Li et al., 2009; Proietto et al., 2008b). Questions remain as to the reasons for the seeming absence/poor efficiency of the developmental branch leading to CD8α^- cDC generation in the thymus *in situ*. Addressing these questions might offer important insights to the so far poorly understood mechanism(s) of pre-cDC commitment.

The migratory nature of thymic CD8α^- cDCs and pDCs has led to a hypothesis that these populations could specifically engage in shuttling of peripheral tissue-derived and/or blood-borne antigens for induction of central tolerance towards these antigens in the thymus. Consistent with this notion, endogenous pDCs and CD11c$^+$MHC2$^+$ cells have been shown to carry peripherally acquired substances or antigens to the thymus (Bonasio et al., 2006; Hadeiba et al., 2012). The putative physiological significance of this route of antigen supply for central tolerance remains to be definitively demonstrated. In this regard, CCR2-deficient mice, which show a specific reduction in the number of CD8α^- cDCs in the thymus, display increased T cell reactivity against serum self-antigens (Baba et al., 2009), suggesting a role for CD8α^- cDCs in the development of central tolerance towards blood-borne proteins. Further, CD8α^- and CD8α^+ cDCs have been reported by one group to be differentially localized within the thymus (Baba et al., 2012; Baba et al., 2009). Accordingly, Sirpα^-CD11c$^+$ cells (corresponding to CD8α^+ cDCs) clustered in the medulla whereas Sirpα^+CD11c$^+$ cells (corresponding to CD8α^- cDCs) were enriched in the PVS at CMJ and inside the cortical parenchyma in close proximity to capillaries, but were essentially absent from deeper medullary regions (Baba et al., 2012; Baba et al., 2009). Mice lacking chemokine XCL1, whose receptor XCR1 is selectively expressed by CD8α^+ but not CD8α^- cDCs (Bachem et al., 2012), display impaired accumulation of CD11c$^+$ cells in the inner medulla (Lei et al., 2011) arguing for a critical role for XCL1-

XCR1 axis in the intrathymic positioning of CD8α^+ cDCs. Notably, XCL1$^{-/-}$ mice display reduced number of thymic T_{reg} cells and defects in central tolerance (Lei et al., 2011), suggesting a potential non-redundant role for CD8α^+ cDCs in nT_{reg} cell development and/or central tolerance maintenance.

3.9 Development of cortical and medullary thymic epithelial cells

3.9.1 Thymus organogenesis - an overview

cTECs and mTECs arise following the formation of the thymic anlagen from the endodermal epithelium of the third pharyngeal pouch (Gordon et al., 2004). Around E10 in the mouse, an invagination of the epithelial monolayer of the third pouch gives rise to the common anlage of the thymus and the parathyroid (see ***Figure 4***). From E11, epithelial cells within the thymus domain of the shared anlage begin to express high levels of FoxN1 (Gordon et al., 2001), a transcription factor that in a cell intrinsic manner critically controls early stages of cTEC/mTEC precursor development and is required for the formation of differentiated cTEC and mTEC compartments, a process instrumental to the generation of a functional thymus. Accordingly, *nu*/*nu* (nude) mice, which carry a homozygous loss of function mutation in the gene *FoxN1*, and mice with targeted deletion of *FoxN1*, develop severely hypoplastic, avascular and alymphoid thymus that is deprived of cTEC/mTEC lineage specific signatures and is not permissive for thymopoiesis (Blackburn et al., 1996; Itoi et al., 2001; Mori et al., 2009; Nehls et al., 1996; Nehls et al., 1994). Between E10 and E12, the thymus-parathyroid anlage becomes enclosed within a mesenchymal capsule (formed by cells that later will give rise to the TMC compartment (see section **3.10**)), and the thymic primordium is colonized by a first wave of lymphoid progenitors. By E12, the paired primordia detach from the pouch via apoptosis and start to migrate caudally towards the chest cavity. Soon after separation from the pharynx, typically by E13, the physical association of the thymus with the parathyroid is lost. By E16-17, the two

thymic lobes, each arising from one of the symmetrically distributed pouch structures on the right and left side of the pharynx, meet together at the final location in anterior mediastinum, above the heart (for review see (Gordon and Manley, 2011)).

3.9.2 Phenotype of cortical and medullary thymic epithelial cells

TECs are generally defined as CD45-negative cells that express the surface marker epithelial cell adhesion molecule, EpCAM, and whose cytoskeleton is composed of intermediate filaments built of cytokeratins. In the adult mouse thymus, cTECs and mTECs can be distinguished based on differential expression of several phenotypic features. *In situ*, cTECs stain positively for cytokeratin -8 and -18, β5t, Enpep glutamyl aminopeptidase (known as Ly51 or BP-1), and C-type lectin receptor, DEC-205 (also known as CD205 or Ly75). In contrast, markers that on thymic sections specifically identify mTECs, or subsets thereof, include cytokeratin -5 and -14, antigens recognized by the antibody clones MTS-10 and ERTR-5, and the lectin UEA-1 (Anderson and Takahama, 2012). By flow cytometry, cTECs and mTECs are typically identified as $CD45^{-}EpCAM^{+}Ly51^{hi}CD205^{+}$ and $CD45^{-}EpCAM^{+}Ly51^{-/lo}CD205^{-}$ cells, respectively. The mTEC population can be further divided based on levels of CD80 and MHCII expression into two main subsets, $CD80^{-}MHC\text{-}II^{low}$ ($mTEC^{low}$) and $CD80^{+}MHC\text{-}II^{hi}$ ($mTEC^{hi}$) (Gray et al., 2006). The $mTEC^{hi}$ population has been further described to be composed of a minor fraction of $Aire^{-}$ transit-amplifying cells and a major population of short-lived post-mitotic $Aire^{+}$ mTECs (Gray et al., 2007a). Comparative assessments of $mTEC^{low}$ and $mTEC^{hi}$ populations have revealed that the $mTEC^{low}$ subset is characterized by limited TRA expression and weak antigen-presenting potential, as measured by the ability to present cognate peptide to naïve CD8 T cells *in vitro*, while $mTEC^{hi}$ cells display a diverse array of TRA and in a similar assay are more efficient APCs (Derbinski et al., 2005; Gray et al., 2006).

3.9.3 Ontogeny of cortical and medullary thymic epithelial cells

cTEC and mTEC lineages are known to arise during thymic development from common $FoxN1^{+}$ progenitor cells (Bleul et al., 2006; Corbeaux et al., 2010; Rossi et al., 2006). Consistently, the existence of a bi-potent cTEC/mTEC progenitor has been demonstrated at a clonal level in E12 thymus (Rossi et al., 2006), and an *in vivo* genetic labeling approach has indicated that bi-potent progenitors are maintained in the post-natal thymus (Bleul et al., 2006). Despite these findings, the "stemness" and phenotypic identity of the common cTEC/mTEC precursor and of its immediate lineage-committed progeny, as well as the extent to which these cells contribute to the maintenance of the post-natal cTEC and mTEC compartments remain elusive (Baik et al., 2013; Bleul et al., 2006; Rossi et al., 2007a) (for review see (Anderson and Takahama, 2012)).

The common cTEC/mTEC progenitor is thought to give rise to lineage-committed cTEC and mTEC progenitors. Initial evidence for the existence of progenitors committed to the mTEC lineage was provided by Rodewald *et al.*, who reported that the thymic medulla is composed of clonally derived 'islets' or clusters of mTECs (Rodewald et al., 2001). In the context of the developmental capacity of mTEC progenitors, much attention has been given to dissecting the differentiation pathway of $Aire^{+}$ mTECs. In this regard, initial precursor-product analyses using cells obtained from cultured embryonic thymus demonstrated that $Aire^{+}$ $mTEC^{hi}$ cells can be generated from $CD40^{-}Aire^{-}MHCII^{-}CD80^{-}CD205^{-}$ precursors via an intermediate, $CD40^{+}Aire^{-}CD80^{-}CD205^{-}$ step that seems phenotypically reminiscent of the $mTEC^{lo}$ population in the adult (Desanti et al., 2012; Rossi et al., 2007b). More recently, it has been shown that $Aire^{+}$ mTECs can also be generated from precursors contained within the bulk of embryonic TECs that have acquired the cTEC-specific marker, CD205, and that $DEC205^{+}$ progenitors likely directly feed into the intermediate $CD40^{+}Aire^{-}CD80^{-}DEC205^{-}$ subset (Baik et al., 2013). The developmental interdependence and relative contribution to the generation of $Aire^{+}$ mTECs *in vivo* of $CD205^{-}$ and $CD205^{+}$ embryonic progenitors

remains to be determined. There is also evidence to suggest that the developmental progression of $Aire^-$ mTEC progenitors toward the $Aire^+$ lineage occurs continuously in the post-natal thymus. Accordingly, assessments of precursor-product relationship in RTOC have indicated that the $mTEC^{low}$ population in the adult contains progenitors of $Aire^+$ $mTEC^{hi}$ cells (Gray et al., 2007a), and analyses of 5-bromo-2-deoxyuridine (BrdU) incorporation and decay have revealed that the adult $Aire^+$ mTEC compartment undergoes a rapid steady-state turnover with a replacement rate of 10-14 days (Gray et al., 2007a). Despite these findings, it is important to note that the frequency of mTEC progenitors within the post-natal $mTEC^{low}$ subset is currently unknown. From available data it also remains unclear whether additional mTEC committed progenitors with the potential to develop into other putative mature mTEC subsets also exist, or whether the same mTEC progenitors can give rise to all mature mTECs. In contrast to mTECs, relatively few studies have examined the differentiation pathway of cTECs. cTEC development in the embryonic thymus has been described to involve progressive acquisition of CD40 and MHCII by immature $DEC205^+$ precursors (Shakib et al., 2009). However, further studies are required to gain a better understanding of the generation of a mature cTEC compartment from bi-potent progenitors, especially in the light of the above-mentioned mixed precursor status of $CD40^-DEC205^+$ TECs at the embryonic stage (Baik et al., 2013) (for review see (Anderson and Takahama, 2012)).

3.9.4 Regulation of thymic epithelial cell development

Development of TECs towards functionally competent cTEC and mTEC compartments appears to be regulated by cell-intrinsic factors as well as extrinsic signals they receive from their local environment. Regarding the former, studies in embryo fusion chimeras have indicated that TEC progenitors are pre-programmed in terms of their maximal division capacity (Jenkinson et al., 2008). Regarding the extrinsic regulation, thymocyte-TEC crosstalk involving DN and DP cells is

implicated in later stages of cTEC development, with DN cells promoting acquisition of the 3D organization, and of CD40 and high levels of MHCII expression by cTECs (Hollander et al., 1995; Shakib et al., 2009), and DP cells influencing cTEC expression of Dll4 (Fiorini et al., 2008). In contrast, mTEC development is known to be influenced by positively selected thymocytes as well as TCRγδ^{+} cells and LTi cells (Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008; Roberts et al., 2012).

While the identity of the cross-talk signals involved in cTEC development remains obscure, the environmental cues underpinning generation of an intact mTEC compartment are known to include stimulation through members of the TNF receptor family, such as RANK, CD40 and LTβR. Signaling through the receptor RANK is cell-intrinsically required for the development of a numerically and functionally intact mTEC compartment (for review see (Ohigashi et al., 2011)). Accordingly, mice deficient in RANK-RANKL axis develop a hypoplastic but architecturally normal medulla with severely reduced number of mTEC^{lo} and, in particular, Aire^{+} mTEC^{hi} subsets (Hikosaka et al., 2008). While the requirement for RANK signaling in the differentiation of Aire^{+} mTECs appears absolute at earlier embryonic stages, CD40-CD40L signaling underpins the development of the residual Aire^{+} compartment in post-natal RANK$^{-/-}$ thymus, revealing a role for both RANK and CD40 in this process (Akiyama et al., 2008). However, despite the ability of the CD40-CD40L pathway to autonomously promote Aire^{+} mTEC generation *in vivo* and *in vitro*, this pathway seems redundant in the presence of an intact RANK-RANKL signal (Akiyama et al., 2008), consistent with the failure of RANK$^{-/-}$, but not CD40$^{-/-}$ thymic stroma to establish central tolerance *in vivo* (Akiyama et al., 2008; Rossi et al., 2007b). The provision of RANKL required for the development of mTECs in the embryonic thymus has been attributed to LTi and TCRγδ^{+} cells, while SP cells appear the main physiological source of RANKL and CD40L regulating the post-natal mTEC compartment (Akiyama et al., 2008; Desanti et al., 2012; Roberts et al., 2012; Rossi et al., 2007b).

The function of LTβR, which is expressed by both epithelial and mesenchymal stromal cells in the thymus (Seach et al., 2008), appears largely distinct from that of RANK and CD40. LTβR-deficient mice display abnormal organization and phenotype of epithelial and mesenchymal elements in the thymic medulla (Martins et al., 2008; Odaka, 2009; Seach et al., 2008; Venanzi et al., 2007), and develop autoimmunity, which has been causatively linked to the disruption of LTβR-signaling in the thymic stroma (Martins et al., 2008). The disrupted medullary architecture observed in the absence of LTβR-dependent signals is characterized by predominant positioning of mTECs in the medullary outskirts and formation of mTEC-free areas filled with mesenchymal cells in the center of the medulla (Martins et al., 2008; Odaka, 2009; Venanzi et al., 2007). LTβR-deficiency has also been reported to result in a 2-fold reduction in the number of mTECs with no apparent impact on $Aire^{+}$ cell frequency (Venanzi et al., 2007; White et al., 2010), and in aberrant gene expression, including loss of the CCR7-ligand, CCL19 and of some Aire-independent TRAs, by the $mTEC^{lo}$ population (Seach et al., 2008). Finally, the thymus of LTβR-deficient mice is completely devoid of $Aire^{-}$ $involucrin^{+}$ Hassall's corpuscles, structures of unclear function expressing markers of mature skin epithelium and thought to be derived from $Aire^{+}$ mTECs (Wang et al., 2012; White et al., 2010), implicating a role for LTβR-signaling in mTEC development beyond the $Aire^{+}$ stage (White et al., 2010). Future studies using conditional knockout mice will be required to dissect the direct and indirect functions of LTβR-signaling in the generation of the functionally competent mTEC compartment.

In addition to hematopoietic cell-derived signals, mesenchymal cells have also been implicated in promoting TEC proliferation resulting in thymus growth (see section **3.10**).

3.10 Ontogeny and functions of thymic mesenchymal cells (TMC)

While the ontogeny, phenotype and functionality of TECs has been extensively studied, far less is known regarding the TMC compartment.

The majority of mesenchymal cells in the thymus are thought to originate from neural crest cells (NCC) (Foster et al., 2008; Muller et al., 2008), a multipotent embryonic population that arises via epithelial-to-mesenchymal transition in the dorsal region of the neural tube (Smith and Tallquist). By E10 in the mouse, NCC populate the 3rd pharyngeal pouch where they accumulate around the newly forming thymic epithelial anlagen and generate the organ capsule as the anlagen detaches from the pharyngeal endoderm at E12 (Gordon et al.). By E13, mesenchymal cells migrate into the thymic epithelial core where they associate with developing blood vessels (Mori et al.). After E15, at least some of these cells express mural cell markers, such as alpha smooth muscle actin (SMA), desmin and NG2 and thus acquire phenotypical characteristics of vascular smooth muscle cells (VSMC) and pericytes. The perivascular and capsular localization of thymic mesenchymal cells (TMC) is maintained throughout all subsequent stages of thymus ontogeny (Foster et al., 2008; Muller et al., 2008).

Experimental ablation (Bockman and Kirby, 1984) or genetic manipulation (Chen et al., 2010; Foster et al., 2010; Griffith et al., 2009) of NCC has demonstrated a role for these cells in the initial determination of thymus size, its separation from the pharynx and its migration to the anterior mediastinum. During later embryonic stages, TMC have been implicated as positive regulators of TEC turnover (Jenkinson et al., 2003; Jenkinson et al., 2007) through their production of fibroblast growth factor (FGF)-7 and -10 (Celli et al., 1998; Revest et al., 2001) and insulin growth factor (IGF)-1 and -2 (Jenkinson et al., 2007), and expression of these factors is maintained in postnatal TMCs (Gray et al., 2007b). In **paper 1** of this thesis, we further identify a role for TMCs as negative regulators of TEC growth in the embryonic thymus through their production of retinoic acid (see section **4**). Regarding direct influence of TMCs on T cell development, TMC-

derived S1P has been shown to regulate thymocyte egress (Zachariah and Cyster, 2010) (see also **3.7**). In addition, TMCs have been reported to express FMS-like tyrosine kinase 3 (Flt-3) ligand (Kenins et al., 2008) and stem cell factor (SCF) (Gray et al., 2007b), factors essentially required for thymocyte progenitor expansion and/or survival *in vivo* (Kenins et al., 2010; Rodewald et al., 1995), suggesting a role for TMCs in the maintenance of thymic progenitor pool.

Several phenotypic markers have been utilized to identify and/or isolate TMC, including platelet derived growth factor receptors α (PDGFRα) and β (PDGFRβ) (Gray et al., 2007b; Jenkinson et al., 2007; Odaka, 2009), Ly51 (Muller et al., 2008) and the glycolipid Forssman antigen recognized by the MTS-15 antibody (Gray et al., 2007b). While these findings suggest phenotypic heterogeneity within the postnatal TMC compartment, in general the phenotypic data have remained scattered, and the overlap between the various phenotypic markers utilized to define TMC as well as the developmental relationship between these putative subsets have remained obscure. We addressed the above issues in **paper 2** of this thesis, in which we show that NC-derived TMCs differentiate into two phenotypically discrete, gp38^{+}PDGFRα^{+} (gp38^{+} TMC) and gp38^{-}PDGFRα^{-} (gp38^{-} TMC) subsets with specialized functions, the former subset primed for production of regulators implicated in TEC homeostasis whereas the latter exhibiting properties of classical pericytes.

4. Biology of retinoic acid (RA)

The vitamin A (retinol) metabolite, retinoic acid (RA) acts as a ligand for nuclear RA receptor (RAR)/retinoid X receptor (RXR) heterodimers, that function as ligand-activated transcriptional regulators. RA-dependent RAR-mediated events (also called RA signaling) have been widely implicated in regulating embryonic patterning and organ development as well as epithelial tissue homeostasis, by impacting on cell proliferation, differentiation and apoptosis (for

review see (Niederreither and Dolle, 2008)). Notably, vitamin A is required for normal immune functions, due at least in part to RA's direct role in regulating the peripheral differentiation and/or activity of effector T cells (Hall et al., 2011; Pino-Lagos et al., 2011). Regarding the thymus, several earlier reports demonstrated that RA could directly affect thymocyte development through effects on DP cell survival. However, these findings are almost entirely based on addition of exogenous RAR agonists *in vitro* or *in vivo*. Notably, despite the evidence of ongoing RA synthesis in the thymus (Kiss et al., 2008), the cellular sources of thymic RA production, as well as the putative function(s) of endogenous RA-signaling in the development of thymocytes or TEC compartments *in vivo* have remained very poorly studied. We addressed these queries in **paper 1** and **3** of this thesis. In these studies, we identify TMCs and the $mTEC^{hi}$ subset as the major cell populations capable of RA production in the thymus. In **paper 1**, in functional experiments involving FTOC, we show that mesenchymal cell-derived RA plays an important role in regulating TEC numbers predominantly within the cTEC compartment. In **paper 3**, we demonstrate that endogenous RAR activity is present in positively selected thymocytes during and after their commitment to the $CD4^+$ and $CD8^+$ lineage, and further provide initial evidence suggesting a key and direct role for RA in thymocyte development *in vivo*.

For better understanding of these results, below I will give a short background on RA biology, focusing on the pathway of RA synthesis and the molecular mechanisms of its function.

4.1 The mechanisms controlling physiological availability of RA

In mammals, the RA precursor retinol is obtained from the diet and transported via chylomicron remnants for storage in the form of retinyl esters in liver stellate cells, the major retinol storage cells of the body. From its liver stores retinol is distributed to peripheral tissues, or delivered via maternal placental transfer to a developing embryo, as a circulating complex with retinol binding

protein (RBP)-4. Regarding the mechanism of retinol uptake by target tissues, the RA responsive gene *Stra6* was identified as a membrane receptor for the retinol-RBP-4 complex (Kawaguchi et al., 2007), indicating that tissue resident STRA6 expressing cells may play an important role in the uptake of circulating retinol although other mechanisms may exist. The physiological availability of RA appears dependent on the net activity of specific RA-synthesizing and RA-metabolizing enzyme pathways. Regarding the former, RA is generated from retinol in a two-step oxidative process via retinal. Two enzyme families, the cytosolic alcohol dehydrogenases (ADHs) and the microsomal retinol dehydrogenases (RDHs), have been implicated in the conversion of retinol to retinal (for review see (Pares et al., 2008)), of which RDH10 has been shown to play a non-redundant role in priming of RA synthesis during embryonic development (Sandell et al., 2007). The oxidation of retinal to RA is carried out by three retinaldehyde dehydrogenases (RALDH1, RALDH2 and RALDH3; encoded by the genes *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*, respectively), of which RALDH2 and RALDH3 are known to play non-redundant developmental functions, as demonstrated by early embryonic and neonatal lethality of mice bearing germ-line mutations in *Aldh1a2* and *Aldh1a3*, respectively. With regard to RA-metabolizing enzymes, members of the cytochrome P450 superfamily, CYP26 -A1, -B1, and -C1 are known to transform RA into more polar derivatives (4-OH or 4-oxo RA). Although it has been debated whether CYP26 products may influence RAR activity, the *in vivo* data indicate a role in the removal of bioactive RA, as demonstrated by development of abnormalities reminiscent of teratogenic effects of excess RA in *Cyp26*-deficient mice (see ***Figure 4***) (for review see (Niederreither and Dolle, 2008)).

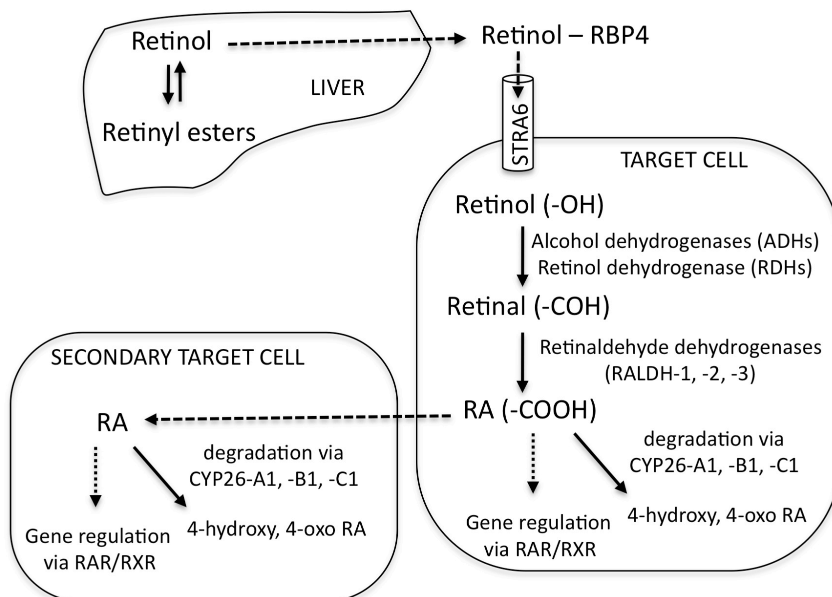

Figure 4 Vitamin A (retinol) metabolism Retinol is obtained from the diet and stored in the form of retinyl esters in liver stellate cells. From its liver stores retinol is distributed to peripheral tissues as a circulating complex with retinol binding protein (RBP)-4. Binding of retinol-RBP4 to its membrane receptor, Stra6 facilitates retinol uptake into target cells where retinol is converted into RA in a two-step oxidation reaction via retinal. RA exerts its biological activity through binding to and modulating the activity of RA receptor (RAR)-retinoid X receptor (RXR) complexes, which function as ligand-activated transcriptional modulators in the nucleus. From its production site, RA can be distributed at paracrine distances to affect the biology of neighboring cells. RA availability is negatively regulated through a degradation pathway mediated by members of the CYP26 enzyme family.

4.2 The mechanism of RAR-mediated gene regulation

The RAR gene family consists of three members, RAR -α, -β and -γ, each giving rise to two main N-terminal isoforms through differential promoter usage and alternative splicing, that act in heterodimeric combinations with any of the three RXR subtypes, RXR -α, -β and -γ. The basic mechanism for transcriptional regulation by RAR/RXR complexes relies on DNA binding to specific RA response elements (RARE) in the regulatory regions of target genes and on ligand-induced conformational changes that direct the dissociation/association of several regulator complexes. The classical RARE is a 5bp spaced direct repeat of a core hexameric motif, PuG(G/T)TCA, referred to as DR5. However, the RAR-RXR heterodimers also bind to direct repeats separated by 1bp (DR1) or 2bp (DR2). According to the classical model of RAR-dependent transcriptional regulation, unliganded DNA-bound RAR-RXR complexes can mediate gene silencing through their association with various co-repressor proteins, such as the nuclear receptor corepressor (NCoR/NCoR1) and silencing mediator for retinoid and thyroid hormone receptors (SMRT/NCoR2), which reside in or recruit high molecular weight complexes endowed with histone deacetylase activity and thereby promote a repressive chromatin structure over the target promoter. Upon RA binding, a resulting conformational change of the ligand-binding domain of the receptor leads to exposure of binding sites for co-activators and dissociation of any pre-bound co-repressors. The co-activators, which essentially include members of the p160 subfamily of steroid receptor co-activators (SRC), promote target gene activation by serving as adaptors recruiting complexes with histone acetyltransferase or methyltransferase, and of nucleosome remodeling activity, that together promote chromatin configuration permissive for active gene transcription. It has also been proposed that activated RARs may influence target gene activation through recruitment of or influence on elements of the basic transcriptional machinery. RA signaling has also been shown to induce dissociation from RAR target genes of other types of repressors, such as Polycomb

group proteins (PcG) that act in large multimeric complexes mediating gene silencing. Which of the above set of molecular players is employed by RARs for gene regulation *in vivo* is thought to ultimately depend on the target gene promoter and cellular contexts.

In addition to the canonical genomic effects, RA signaling has also been implicated in non-genomic biological activities such as the activation of translation and of kinase cascades. Regarding the former, it has been demonstrated that RA activates synthesis of a set of neuronal proteins, including the glutamate receptor GluR1, through a mechanism that involves a RA-dependent release from a block in translation imposed by mRNA-associated RARα (Chen et al., 2008). Regarding direct influence of RA signaling on signal transduction pathways, RARα was recently shown to reside in membrane lipid rafts and activate G-protein-mediated kinase cascades in response to RA (Piskunov and Rochette-Egly, 2012) (for review see (Rochette-Egly and Germain, 2009)).

SUMMARY OF THE PAPERS

Paper 1

***Mesenchymal cells regulate retinoic acid receptor-dependent cortical thymic epithelial cell homeostasis*.**

In this paper, to investigate whether RA signaling played any role in regulating TEC biology, we assessed the impact of pan-RAR antagonist-induced blockade of RA signaling on TEC development in FTOC. Using this model, we demonstrate that endogenous RA signaling promotes TEC cell-cycle exit and restricts TEC cellularity in the fetal thymus. In day 7 FTOC, blockade of RA signaling appeared not to affect CD80^{+}UEA-1^{hi} mTEC numbers but significantly enhanced the number and proportion of CD80^{-} cells expressing high levels of the cTEC marker, CD205. Further, CD80^{-} TECs, which contain both CD80^{-} mTEC and cTEC, showed increased levels of cTEC and decreased levels of mTEC specific gene transcripts when obtained from RAR antagonist treated FTOC. Together, the above results indicate that RA preferentially regulates the size of the cTEC compartment. Using gene expression, biochemical and functional analyses we further show that TMCs are the major source of RA in the embryonic thymus. Consistently, in E15 thymus TMCs but not TECs or thymocytes expressed *Aldh1a2*, showed high-level ALDH activity and induced RAR-dependent β-Gal expression in the RA reporter cell line. In re-aggregate culture experiments, thymic mesenchyme was required for RAR-dependent restriction of CD80^{-} DEC205^{+} TEC numbers, highlighting the importance of mesenchyme-derived RA in modulating TEC turnover. Finally, we show that the RA-generating potential, as demonstrated by RALDH enzyme expression, ALDH activity, and ability to induce RAR-dependent reporter gene activity *in vitro*, is selectively displayed by a discrete, and previously unrecognized, Ly51^{int}gp38^{+} population amongst post-natal TMCs (referred to as gp38^{+} TMC and further characterized in **paper 2** of this

thesis), indicating that mesenchymal cell-derived RA may help in regulating TEC homeostasis in the adult. Collectively, these findings identify RA signaling as a novel mechanism by which thymic mesenchyme influences the development of TEC.

Paper 2

Phenotype, ontogeny and functional characteristics of thymic mesenchymal cells

The idea for **paper 2** was initiated as a result of the phenotypic identification in **paper 1** of what appeared to be multiple mesenchymal subsets in the thymus. Here, using comprehensive phenotypic and genetic lineage tracing analyses, we show that the postnatal PDGFRβ^+ TMC compartment is composed of two distinct neural crest (NC)-derived, gp38$^-$PDGFRα^- (gp38$^-$ TMC) and gp38$^+$PDGFRα^+ (gp38$^+$ TMC) populations, the former further distinguished by smaller cell size, uniform positivity for MTS-15 labeling and high Ly51 expression whereas the latter expressing low to intermediate levels of Ly51 and comprising a major MTS-15$^+$ and a minor MTS-15$^-$ component. Further characterization of mesenchymal populations using microarray analysis, as well as *in situ* localization and kinetic developmental studies revealed a notable functional specialization between gp38$^-$ and gp38$^+$ TMC. Thus, gp38$^-$TMC, in contrast to their gp38$^+$ counterparts, expressed high levels of genes associated with pericytes and vascular smooth muscle contractility and a subset of these cells expressed high levels of smooth muscle actin. We also demonstrated that gp38$^-$TMC were intimately associated with the vascular endothelium (VE) throughout the thymus cortex, CMJ and medulla. Together, these results strongly suggest that gp38$^-$, but not gp38$^+$ cells, are mural cells with classical pericyte and vascular smooth muscle cell functions that support thymic VE. In contrast, gp38$^+$ but not gp38$^-$ TMC appeared the major source of TMC-derived regulatory mediators implicated in TEC homeostasis, such

as IGFs, FGFs and Wnt effectors, indicating that they are likely aiding in regulating TEC compartments. Consistent with this possibility, we demonstrate that gp38^{+} TMC are located laterally in the PVS in close proximity to TEC. Regarding direct contribution of the TMC populations to T-cell development, our immunohistochemical analysis demonstrated that both gp38^{-} and gp38^{+} TMC are resident within the PVS of CMJ vessels, sites of T-cell progenitor immigration and mature T-cell exit, and our microarray analysis showed that both gp38^{+} and gp38^{-} TMC expressed sphingosine kinase-2, while only gp38^{+} TMC expressed high levels of sphingosine kinase-1, key enzymes redundantly involved in sphingosine-1-phosphate (S1P) synthesis. Given the critical function of TMC-derived S1P in thymocyte egress, our data suggest that both TMC subsets are capable of contributing to SP cell exit. Our microarray analysis also showed that gp38^{-} TMC expressed highest levels of SCF and gp38^{+} TMC were the primary source of Flt-3L, both factors implicated in the proliferation and maintenance of thymocyte progenitors, indicating that both TMC subsets may play complementary and potentially non-redundant roles in maintaining thymic progenitor homeostasis.

Microarray-based comparison of gp38^{-} and gp38^{+} TMC to gp38^{-}PDGFRa^{-}, and BP-3^{-} and BP-3^{+} gp38^{+}PDGFRa^{+} lymph node mesenchymal cell (LNMC) subsets demonstrated that despite their different NC origin, gp38^{-} and gp38^{+} TMC (all BP-3^{-} in the thymus) most closely resembled mesoderm-derived gp38^{-} LNMC and BP-3^{-}gp38^{+} LNMC, respectively. BP-3^{+}gp38^{+} LNMC were more similar to BP-3^{-} gp38^{+} LNMC and TMC than to gp38^{-} LNMC and TMC. This data suggest that corresponding TMC and LNMC subsets may develop under the influence of similar extrinsic cues. However, despite their overall transcriptome similarity we observed several notable differences between TMC and LNMC subsets, suggesting some role of the local tissue environment or distinct mesenchymal origin in imprinting on TMC functionality. For example, one noticeable difference between LNMC and TMC was in their chemokine expression profile. Thus, gp38^{+} LNMC expressed high levels of a broad array of CXCL and CCL chemokines

including the chemokines CCL21, CCL19 and CXCL14, compared to either TMC subsets. A marked exception to this was the chemokine CCL25 that was selectively expressed by both TMC subsets.
Finally, kinetic examination of TMC phenotype during thymic development revealed that at E13 the thymus contained a homogenous population of $gp38^{+}PDGFR\alpha^{+}MTS\text{-}15^{-}$ TMC, suggesting that both mature $gp38^{+}$ and $gp38^{-}$ TMC are derived from this population.
Together, these results bring novel insights to the understanding of the phenotypic and functional heterogeneity and ontogeny of TMCs, and provide a panel of markers that should help facilitate future studies on these cells.

<u>*Paper 3*</u>

Retinoic acid signaling in thymocytes and its impact on T cell development

In this manuscript, we investigate the relevance of RA signaling in thymocytes during T-cell development *in vivo*. Initially, using a RA sensitive reporter mouse model, we demonstrate that RAR transcriptional activity is induced in the steady state in $CD69^{+}CD4^{+}CD8^{+}$ and $CD69^{+}CD4^{+}CD8^{lo}$ thymocytes undergoing positive selection and lineage commitment, and continues to be present in both $CD4^{+}$ and $CD8^{+}$ SP cells, with RA sensing further enhanced in recently generated $CD69^{+}$ $CD4^{+}$ SP cells. To address the potential biological significance of RA signaling in $CD69^{+}$DP and SP thymocytes we evaluated T-cell development in *dnRARα-CD4Cre* mice, which express a dominant-negative form of RARα from the DP stage. In a preliminary analysis, *dnRARα-CD4Cre* mice displayed marked alterations in thymocyte subset composition and number. This was most evident by decreased numbers of all thymocyte subsets, with the exception of $CD8^{+}$ SP cells, including ETP and DN2-4 subsets, suggesting that blocking of RAR activity in DP cells and their progeny may indirectly impact on thymocyte precursor entry

and/or survival. *DnRARα-CD4Cre* mice also showed a 4-fold reduction in $CD4^+/CD8^+$ SP cell ratio that was mainly due to disproportional accumulation of mature $CD8^+$ SP cells, consistent with a putative direct role of RA responses in regulating $CD8^+$ SP cell homeostasis through an impact on thymic retention and/or the rate of post-selection expansion of these cells. Notably, mature $CD8^+$ SP cell accumulation did not account entirely for the observed skewing in $CD4^+/CD8^+$ SP cell ratio in *dnRARα-CD4Cre* mice as DP and immature $CD4^+$ SP cells were decreased 1.6-fold in these mice while immature $CD8^+$ SP cells were found in similar numbers to that of control animals. This together with our finding of constitutive RA signaling in $CD69^+$ $CD4^+CD8^{lo}$ cells indicates that RA may impact on the efficiency of MHCI-restricted thymocyte positive selection and/or $CD4^+/CD8^+$ lineage commitment. Together, these preliminary results suggest a key and direct role for RA in thymocyte development. We also investigated the putative intra-thymic source(s) of RA that generated RAR responses in positively selected thymocytes. Total thymocytes and sorted SP cells lacked expression of the critical RA-synthesizing enzymes, indicating that RAR signaling in these cells is due to exogenously derived RA. Gene expression and biochemical analyses indicated that, in addition to $gp38^+$ TMCs, $CD80^+MHC2^{hi}$ mTECs were capable of generating RA, implicating mTEC-derived RA as a likely inducer of RAR transcriptional activity in positively selected thymocytes in the medulla.

DISCUSSION

The studies included in this thesis broaden the current understanding of several aspects of thymus biology. For a detailed discussion of the results the reader is referred to the discussion parts of papers 1, 2, and 3. In this section, I will primarily focus on summarizing the novel insights and future possibilities that our findings bring to the thymic field.

Notably, our data provide a first demonstration of the functional involvement of endogenous RA signaling pathway in thymocyte and TEC development. Regarding the former, while previous studies proposed a role for RA signaling in negative selection (Szegezdi et al., 2003; Szondy et al., 1998), we show that RAR transcriptional activity is constitutively present in positively selected thymocytes *in vivo* albeit at different levels and that inhibition of RA signaling in DP thymocytes and their progeny broadly impacts on thymocyte numbers, including that of ETP and DN2-4 cells, and alters $CD4^+$/$CD8^+$ SP cell ratio *in vivo*, the latter due to preferential accumulation of immature and more significantly mature $CD8^+$ SP cells. These results are consistent with a putative direct and likely pleiotropic role of RAR responses in regulating $CD8^+$ SP cell homeostasis through possible impacts on the initial generation of $CD8^+$ lineage cells as well as the rate of $CD8^+$ SP cell post-selection expansion and/or thymic retention of these cells. Future studies using mixed *dnRARα-CD4Cre*/wild-type bone-marrow chimeras should help dissect the direct and indirect functions of RA at different stages of thymocyte development. Regarding the latter, our results identify RA as a novel negative regulator of TEC proliferation in FTOC that acts preferentially to limit the size of the cTEC compartment. Our preliminary assessment of TEC compartments in 2w old *dnRARα-FoxN1Cre* mice, in which TECs are specifically rendered refractory to RAR activity, showed a selective accumulation of cTEC numbers, confirming that RAR responses in TECs play a direct role in regulating cTEC homeostasis *in vivo* (***Figure 6***).

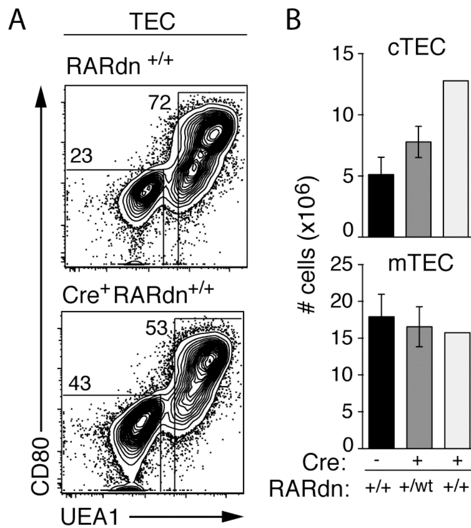

***Figure 6*. RAR responses in TECs play a direct role in regulating cTEC homeostasis *in vivo*.** (A) Representative flow cytometry analysis of TECs and (B) total cTEC and mTEC numbers in 2 week old *dnRARα-FoxN1Cre* and Cre^{-} control mice.

Future studies using the *dnRARα-FoxN1Cre* mouse model will aim to more precisely delineate RA's influence on the cTEC developmental pathway and to address other potential role(s) of RA in TEC homeostasis *in vivo*, and the relevance of this in the regulation of T-cell development. Notably, our demonstration of endogenous RAR transcriptional activity in the mTEC^{hi} subset in the post-natal thymus suggests that in addition to its role in regulating cTEC expansion, RA may potentially act by impacting on development and/or functionality of mature mTEC, which are the primary source of TRAs relevant for central tolerance induction. In summary, our results add RA to the list of thymic pathways that directly and potentially indirectly through regulation of TEC compartments function in T-cell development, contributing to better the

understanding of the impact on immune system's performance associated with vitamin A-deficiency and retinoid-based treatments of cancer and skin diseases.

Our studies provide thus far first assessment of the major thymic populations, including TEC, TMC, DC and thymocyte subsets, with regard to their capacity to synthesize RA. We identify gp38^{+}TMCs and the mTEC^{hi} subset as the major sources of thymic RA in the adult. This, together with our finding that gp38^{+}TMCs are located primarily in the PVS at CMJ and in the medulla while being only rarely associated with the cortical vasculature, suggest that levels of bio-available RA in the thymus may form a gradient with highest levels in the medulla and lowest in the outer cortex. Consistent with this possibility, we detected increased RA sensing in positively selected DP and SP cells that migrate towards or reside in the medulla, whereas RAR activity was undetectable in pre-selection DP cells that engage in random walk within the cortex. We think it likely that mTECs, as opposed to PVS contained TMCs, may be the main providers of RA to positively selected thymocytes in the medulla and that this paracrine crosstalk contributes to CD8^{+} SP cell homeostasis.

Epithelial-mesenchymal crosstalk is known to be important for optimal TEC expansion at the embryonic stages of thymus growth; a process thought to be mediated through TMC-dependent production of TEC mitogens such as FGF-7 and -10 as well as IGF-1 and -2. Our identification of TMCs as the main producers of RA in the embryonic thymus together with our demonstration that TMCs essentially mediate the RAR-dependent restriction of TEC numbers in RTOC and recently, that RA regulates TEC growth in a direct manner in *dnRARα-FoxN1Cre* mice, identify TMC-derived RA as a novel mediator of the epithelial-mesenchymal crosstalk regulating TEC expansion and highlight a role for TMCs as both positive and negative regulators of TEC growth. Interestingly, comparative analysis of the ALDH^{+} and ALDH^{-} fractions within the gp38^{+} TMC population in the adult, which represent cells that possess or lack the RA-generating capacity, respectively demonstrated that both subsets expressed similar levels of FGF-10

and IGF-1 while the former subset expressed significantly higher levels of FGF-7 and IGF-2. Thus, it appears that the same subset of mesenchymal cells can produce both positive and negative regulators of TEC proliferation. The interplay between these pathways for the maintenance of TEC homeostasis in the adult thymus at steady state and in the contexts of thymus regeneration and age-related atrophy remains to be determined.

While TMCs have been implicated in a variety of thymic functions, little is known regarding the phenotypic and functional heterogeneity of TMC subsets. In **paper 2** we demonstrate that postnatal TMCs comprise two phenotypically distinct and functionally specialized NC-derived gp38^{+} and gp38^{-} TMC subsets. We characterize gp38^{-} TMC as mural cells with classical pericyte and vascular smooth muscle cell functions that intimately associate with and support thymic vascular endothelium. In contrast, gp38^{+} TMC were central producers of FGF-7, -10 and IGF-1 and -2 as well as RA, and were located laterally in the PVS in close proximity to TEC, implicating their specialized role in regulating TEC homeostasis. Our data also provide an initial description of the NCC maturation pathway towards mature gp38^{+} and gp38^{-} TMC during thymic ontogeny. Accordingly, we provide preliminary evidence that mature gp38^{+} and gp38^{-} TMC develop from a common gp38^{+}PDGFRα^{+}MTS-15^{-} precursor present in the thymus at E13. Interestingly, a minor population of TMC with this phenotype was observed throughout thymic ontogeny raising the possibility that the thymus maintains a population of TMC progenitors. Notably, E12.5-E15.5 TMC have been shown to contain a multipotent NC-derived progenitor population capable of giving rise to neuronal/glial cells and melanocytes (Yamazaki et al., 2005) and neonatal human and adult mouse thymus have been reported to contain mesenchymal progenitors with the potential to differentiate into osteocyte, chondrocyte, adipocyte and potentially neuronal lineages (da Silva Meirelles et al., 2006; Krampera et al., 2007; Mouiseddine et al., 2008; Siepe et al., 2009). Future studies will aim to determine whether the adult thymus maintains any multipotent

progenitors amongst gp38^{+}PDGFRα^{+}MTS-15^{-} TMCs, as well as address the developmental pathways leading to the differentiation of gp38^{+} and gp38^{-} TMC and the extrinsic cues responsible for their organ-specific molecular fingerprint. Collectively, our results significantly broaden the current understanding of TMC heterogeneity, ontogeny and function.

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