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Structure and function of the thymic microenvironment

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1. ABSTRACT

Organs are more than the sum of their component parts – functional competence requires that these parts not only be present in the appropriate proportions, but also be arranged and function together in specific ways. The thymus is an excellent example of the connection between cellular organization and organ function. Unlike more familiar organs, such as lung or kidney, the thymus is not organized into easily identifiable structures such as tubes and ordered cell layers, but instead is a complex meshwork of microenvironments through which T cell progenitors migrate, receiving signals that instruct them to differentiate, proliferate, or die. Proper thymic organization is essential to the optimal production of a functional T cell repertoire. During aging, the thymus undergoes involution, largely due to degradation of the TEC microenvironmental compartment, which then fails to support optimal thymocyte development resulting in reduced output of naïve T cells. This review will summarize the current state of understanding of the composition and organization of thymic microenvironments and the mechanisms that promote their proper development and function.

2. INTRODUCTION: THYMUS STRUCTURE AND FUNCTION THROUGH THE LIFESPAN

2.1. What is the thymic microenvironment?

The thymus is the primary immunological organ that is responsible for the production of self-restricted, self-tolerant T cells. It consists of developing T cells, or thymocytes, supported by a complex cellular network containing a variety of resident cell types, including thymic epithelial cells (TEC), dendritic cells, vasculature, and mesenchymal cells. These cell types comprise multiple functional microenvironments that restrict immigrating lymphoid progenitor cells (LPCs) to the T cell fate, then direct and support these thymocytes to develop from immature progenitors into mature cells, shaping this emerging repertoire such that it is both self-tolerant (will not attack the body's own cells) and self-restricted (recognizes peptide antigens in the context of 'self' MHC). The thymus is built during embryonic stages and maintained postnatally through both thymic epithelial cell-autonomous mechanisms and complex intercellular crosstalk interactions. During development and in the steady-state thymus, thymic epithelial cells (TECs) produce growth, differentiation and survival factors required for

thymocyte maturation and present self-peptide/MHC complexes that mediate positive or negative selection. Thus, T cell development in the thymus is not a cell autonomous process, but requires interactions with the thymic microenvironments that provide signals for their survival, proliferation, and differentiation. A steady supply of naïve T cells is essential for optimal function of the peripheral adaptive immune system and is directly correlated with thymus organ size, and with the structure and function of the thymic microenvironment. Failure of these events results in immunodeficiency or autoimmunity.

In the thymus, lymphoid progenitor cells undergo a series of progressive differentiation steps via interactions with the non-lymphoid stromal cell microenvironments that they encounter during a stereotypical migration path through the thymus organ. Unlike the bone marrow, self-renewing hematopoietic stem cells (HSCs) do not reside in the thymus, and so there is no HSC microenvironmental stem cell "niche". However, there is evidence for the existence of one or more types of thymic epithelial progenitor or stem cells in the postnatal thymus, that, if they exist, presumably reside in specific microenvironments of their own. Thus, there is not really a single thymic microenvironment, but multiple ones, each of which promotes specific events in T cell differentiation, or supports stem or progenitor cells that maintain the thymic epithelial component of the stromal environment. The question of the nature of the thymic microenvironment is thus a complex one that remains poorly understood. In this chapter, we will review aspects of the current literature addressing the fetal development, postnatal function, and aging-related decline of the thymic cellular microenvironment(s). We will discuss the microenvironmental components with an emphasis on TEC subsets required for thymocytes to differentiate into functional T cells, and address the question of whether thymic epithelial stem or progenitor cells maintain these microenvironments.

2.2. Overview of thymus development and decline

The thymus originates from endodermal cells from the ventral third pharyngeal pouches during mid-gestation in mouse embryos (1). Once the endodermal cells are specified to become thymic epithelial cells (TECs), a complex set of cellular interactions takes place between TECs, surrounding mesenchyme, and immigrating lymphoid and endothelial progenitor cells. By late gestation, the resulting fetal thymus has a well-developed mesenchymal capsule, contains numerous differentiating thymocytes, has initiated cortical and medullary TEC differentiation programs, and is connected to the blood stream via a network of blood vessels. After birth, the thymus continues to develop and organize its compartmental structure, expanding in size and increasing output of naïve T cells to the peripheral environment (2-4). The thymus then reaches a period of relative homeostasis, in which the complex thymic microenvironments and TECs are maintained in a steady state with turnover of TECs but no net expansion or loss. At some point (although the exact timing of onset is controversial), the thymus enters a period of decline, resulting in thymic atrophy, or involution (5, 6). This process can be thought of as a failure of homeostasis, in that the cellular components characteristic of the homeostatic thymic stroma are no longer maintained, but instead undergo a gradual disorganization of thymic compartments and functional decline. While the mechanisms underlying these processes are as yet poorly characterized and highly controversial, the final product is a clearly deteriorated thymus, with severely reduced output of naïve T cells.

T cell development in the thymus is not a cell autonomous process, but rather requires interactions with TECs that provide signals for T lineage specification/lineage commitment, and thymocyte proliferation, differentiation, survival, and repertoire selection (7, 8). The thymus is organized into regions that contain different populations of TECs and developing thymocytes. The outer compartment is the cortex, the inner region is termed the medulla, and the zone where they meet is the corticomedullary junction (CMJ). Within the mature postnatal thymus, developing thymocytes undergo a stereotypical migration through complex microenvironments, in which they interact with different epithelial and other cells to promote their differentiation and survival (Figure 1) (9, 10). Thymocyte-derived signals are in turn indispensable for development of the unique three-dimensional TEC meshwork and for proper compartment formation and organization. This well-established mutually inductive process is termed "cross-talk" (8, 11, 12), and contributes to the regulation of thymus organogenesis, homeostasis and involution, although the molecular basis for these interactions is poorly understood. Since mechanisms operating in the fetal thymus are required for initial TEC differentiation and compartment organization, they are also obvious candidates for mechanisms that could fail in involution. Thus, to understand thymic degeneration during aging, or devise therapeutic strategies for rebound, it is important to understand the normal ontogeny of the postnatal steady-state thymus including the molecular and cellular mechanisms that contribute to its initial development.

2.3. Cellular components of the thymic microenvironment

More than 99% of the cells in the young adult thymus are developing thymocytes. The remaining cells constitute the stromal cells that comprise the different regional microenvironments needed for proper thymocyte differentiation (Figure 1). In addition to TECs, the thymic stroma includes mesenchymal cells, primarily of neural crest (NC) origin and endothelial cells that form the vasculature. The NC and endothelial cells enter the fetal thymus, and together form the thymic vasculature. The thymus also contains other non-T cell lineage hematopoietic-derived cells, including dendritic cells, macrophages, and B cells. The thymus contains several subsets of dendritic cells, which either migrate into the thymus from the periphery or differentiate directly within the thymus. B cells can also either migrate in or develop *in situ*, and have different phenotypes depending on their origin. Although these cells are not strictly considered stromal cells, they can play specific roles in the

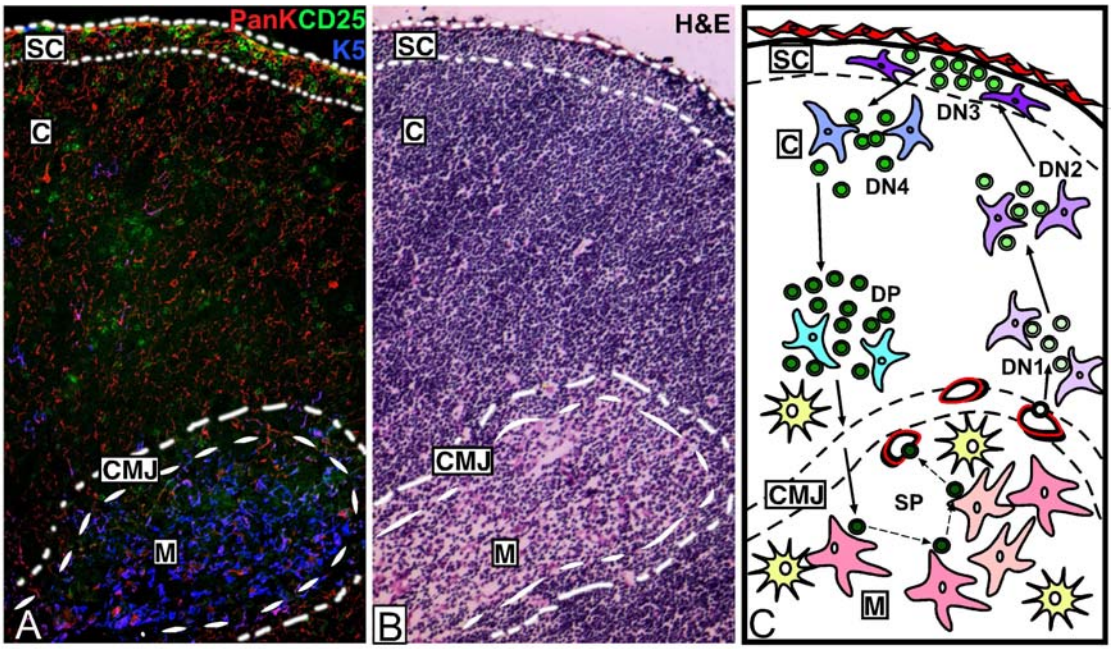

Figure 1. Structure of the postnatal thymus. Major thymus regions in all panels are indicated as subcapsule (SC), cortex (C), corticomedullary junction (CMJ) and medulla (M). Left and middle panels: transverse sections of a 1 month-old mouse thymus stained with CD25, pan-keratin, and keratin 5 (green, red, blue; left) or hematoxylin and eosin (H&E, middle). Right panel: cartoon of the major events in thymocyte differentiation. Small circles are thymocytes shown as progressively darker green at each differentiation stage. Amorphic cells are TECs, colors indicate regional differences in TEC-based microenvironments (purples and blues are cTECs, pinks are mTECs). Dentritic cells are shown as yellow 'starburst' cells, and are primarily in the medulla. Neural crest-derived mesenchymal cells in the outer capsule and surrounding the large vessels at the CMJ are in red. Other cell types and structures, including macrophages, B cells, and capillaries, are not shown for simplicity. Progenitors enter through large blood vessels at the CMJ. Double negative (DN) thymocytes migrate through the stroma, proliferating and differentiating in response to different microenvironments in the cortex. Double positive (DP) thymocytes then undergo positive selection. In the medulla, individual single positive (SP) T cells interact with multiple mTECs for negative selection before exiting the thymus, again at the CMJ.

microenvironment. Dendritic cells are primarily localized to the medulla and play an important role in negative selection by presenting tissue-restricted self-antigens to eliminate self-reactive thymocytes or drive them into the T regulatory cell lineage. Dendritic cells are efficient antigen presenting cells, but do not themselves produce tissue-restricted antigens. Rather, mTECs supply self-antigens to dendritic cells for efficient cross-presentation to thymocytes (13, 14). Macrophages presumably play a scavenger role, disposing of the many thymocytes that undergo apoptosis due to a failure of positive selection or as a consequence of negative selection (15). The role of B cells, if any, is more obscure. However, their production in the thymus can be used as a readout of specific aspects of microenvironmental function, such as induction of Notch-mediated signaling that establishes T as opposed to B lineage commitment (16-18). These various stromal cell types thus provide added complexity to the composition of the thymic milieu, but the major functions of the thymus primarily depend on TECs.

2.4. Phenotypic hallmarks of the thymic microenvironment during involution

There are several hallmarks of aging–associated involution that are easily assayed by histological approaches and more quantitative flow cytometric techniques. Key classically defined morphological changes are cortical thinning, disintegration of the CMJ, and significant reduction in size. These characteristics are all easily identified using hematoxylin and eosin stained paraffin sections (Figure 2A) or staining of frozen sections with compartment-specific markers (Figure 2B, C). Histological (A-C) and thymocyte cellularity (D) analysis throughout postnatal life in the mouse indicates that there is a close correlation between thymocyte numbers and stromal organization. After a very rapid logarithmic increase in thymus size and cellularity in the first postnatal week, thymus size and cellularity level off at around 3 weeks postnatal, with maximum size and cortical thickness at about 4 weeks. We observe the first clear drop in T cell numbers and thymus size between 6 and 7 weeks, cortical thinning is observed at 2 months, and by 3 months we observe the first signs of disorganization at the CMJ (regarded as an early hallmark of the onset of involution). After 3 months, a significant decrease occurs in the frequency of the subset of medullary TECs defined by binding high levels of the lectin, *Ulex europaeus* agglutinin-1 (UEA-1). By 6 months the thymus is already dramatically smaller, and CMJ degeneration is clearly progressing. In addition to depletion of the TEC compartment, accumulation of adipocytes in the perivascular space is another characteristic feature of the

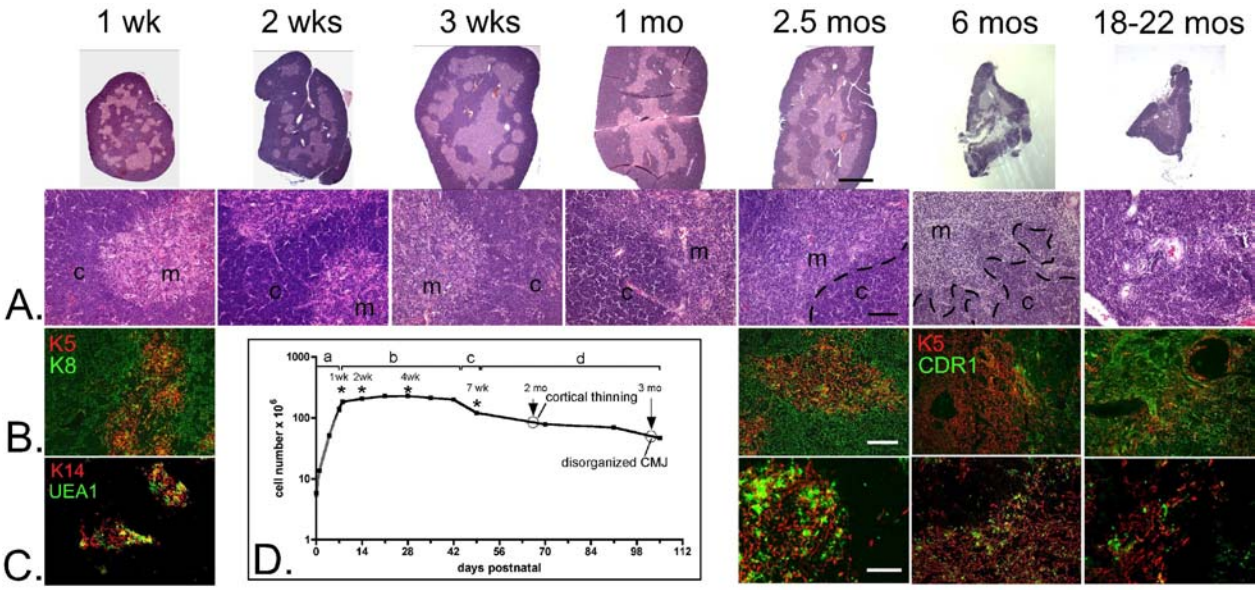

Figure 2. Lifespan analysis of thymus structure in mice. A. H&E stained sections of mouse thymus from 1 week postnatal through 18-22 months of age. The panels in the 2nd row of the figure are higher magnification images of those shown in the first row, to highlight the structure of the CMJ. Note changes in the size and cortico-medullary structure of the thymus. Cortical thinning is visible at 2.5 months (10 weeks), although the CMJ is well-organized (dotted line). By 6 months the thymus is dramatically smaller and CMJ degeneration is well underway, with a convoluted cortical-medullary interface (dotted line). At 18 months the thymus is largely disorganized at the cellular level. B. Marker analysis using major cortical (green, K8 or CDR1) and medullary (red, K5) markers. C. Visualizing medullary integrity with two medullary markers K14 and UEA-1. The medulla is tightly organized at 1 week through ~ 3 months, when UEA-1^{hi} cells decrease and the medullary boundary begins to become disorganized. D. Total thymocyte numbers through the first 3 months of life, showing the initial increase in size (a), period of relative homeostasis (b), initial decline in numbers (c) and beginning of the long gradual decline associated with involution (d).

aging thymus (19-21). Although the precise origin of intrathymic adipocytes remains unknown, recent evidence suggests that adipocytes may arise via epithelial to mesenchymal transition (22). Involution becomes progressively more severe at subsequent ages, ultimately resulting in a near total breakdown of compartmental organization.

Alterations in composition of the thymus stromal compartment have been quantified by flow cytometric analysis of stromal cell subsets during thymus involution. The results have shown changes in the frequency of TECs relative to mesenchymal cells, a reduced ratio of mTECs to cTECs, reduced numbers of both major TEC subsets, specific reductions in the levels of MHC Class II expression and decline in the numbers of UEA-1+ cells. However, it is important to recognize that morphological changes in TECs with aging can result in changes in the ease of isolation of specific subsets. Therefore, specific changes in the relative frequencies of different TEC subsets must also be validated by independent methods, such as immunostaining in tissue sections. Technical difficulties in isolating thymus stromal cells and their cellular morphology make it difficult to accurately quantify changes in specific subsets during involution. New approaches to evaluating changes in the thymic stromal composition and organization are needed to move the field forward.

3. FORMATION OF THE MICROENVIRONMENT DEPENDS ON CROSSTALK BETWEEN TECS AND MULTIPLE CELL TYPES

A critical and pervasive characteristic of the mechanisms involved in developing and maintaining the thymic microenvironment is that of crosstalk. In this context, crosstalk refers to signaling interactions between different cell types that are required for specific stages of differentiation and/or to maintain the structure and function of the mature thymus. Although first identified as occurring between developing TECs and thymocytes, crosstalk interactions are being described between multiple cell types in the thymus, and are more likely best thought of as a network of interactions between multiple cell types.

3.1. Thymocyte and TEC differentiation are interdependent

Early stages of thymocyte differentiation occur in the cortex where thymocytes interact with cortical TECs (cTECs) that provide essential signaling molecules for thymocyte differentiation, proliferation, and survival (9, 10). The stereotypical migration of these cells within the thymus suggests that TEC subsets in specific locations throughout the thymus supply different signaling molecules that promote thymocyte maturation. TECs play an essential role in shaping the T cell repertoire by presenting self-peptide/MHC complexes that positively or negatively select thymocytes, resulting in T cells that are self-restricted, i.e.

respond to foreign peptides in the context of self-MHC molecules on antigen presenting cells (APCs), and self-tolerant, i.e. fail to mount an immune response to self-peptide/MHC complexes on APCs . Positive selection occurs in the cortex where $CD4^+CD8^+$ thymocytes bearing TCRs with moderate affinity for self-peptide/MHC complexes presented by cTECs are rescued from programmed cell death, terminate either CD8 or CD4 expression and migrate into the medulla (reviewed in (23)). In the medulla, self-peptide/MHC complexes on mTECs and DCs signal thymocytes with high affinity TCRs to undergo apoptosis. This negative selection process purges exported T cells of many self-reactive clones that are capable of causing autoimmunity (24). It is now well established that mTECs play an indispensable role in establishing central tolerance and preventing autoimmunity due to their unique ability to express tissue restricted antigens (TRAs) (25). In addition, mTECs transfer self-epitopes to dendritic cells which are highly efficient in inducing central tolerance (13, 14, 26). Expression of a wide array (but not all) TRAs by mTECs is a regulated by the *Aire* (a*utoimmune regulator*) gene (27). Expression of Aire and its target TRAs provide an essential deterrent to autoimmunity since patients or mice deficient in Aire develop multiorgan autoimmune disease. In addition, TRAs presented by mTECs promote the development of CD4+CD25+Foxp3+ T regulatory cells and NKT cells both of which actively repress self-reactive peripheral T cells (28-30).

Just as TECs are indispensable for thymocyte development, thymocyte-derived signals are required for the generation of functional cortical and medullary thymic epithelial compartments (reviewed in (31, 32). Mice in which thymocyte development is blocked at or prior to the $CD4^-CD8^-CD44^+CD25^+$ (DN2) stage have severely hypoplastic thymi with a highly disorganized epithelial compartment that is arrested at an immature developmental stage characterized by co-expression of keratin 8 (K8) and K5 and lack of a three-dimensional meshwork (33, 34). In contrast, mice in which thymocyte development is blocked at the later $CD4^-CD8^-CD44^-CD25^+$ (DN3) stage have a well-organized cortical epithelial compartment that contains both K8+K5- and K8+K5+ TEC subsets (33, 35, 36) although it is not completely mature (37). Thymocyte-derived signals are also required for mTEC formation. Development of the medullary epithelial compartment is severely impaired when thymocyte development is blocked at or prior to the CD4+ CD8+ double positive (DP) maturation stage (38, 39). This suggests that signals from positively selected thymocytes play a role in development and/or expansion of mTECs in the adult thymus. In the fetal thymus, signals from lymphoid tissue inducer (LTi) cells are required for initial differentiation of Aire expressing mTECs (40). Both mature SP thymocytes and LTi cells express ligands that activate members of the tumor necrosis factor (TNF) receptor superfamily including receptor activator of NFkB (RANK), CD40 and lymphotoxin-β receptor (LTβR) which are expressed on TECs (41-44). The absence of these receptors, their ligands or components of the downstream signaling pathways impairs mTEC development and organization resulting in defective central tolerance and the appearance of autoimmune disease.

3.2. Epithelial-mesenchymal interactions and the role of NCCs in thymus development

Epithelial-mesenchymal interactions are a common scenario during organogenesis. Mutually inductive interactions between the endoderm and neural crest (NC)-derived mesenchyme are essential during thymus development (reviewed in (7, 31). Using tissue recombination experiments, Auerbach originally demonstrated the importance of NC-derived mesenchyme in development of epithelial thymus rudiment explants (45). This concept was supported by neural crest ablation experiments in chicks that resulted in variable defects in thymus development (46). However, heterotopic transplant experiments using chick:quail chimeras indicated that NC-derived signals do not induce initial organ formation, since endodermal explants taken prior to neural crest cell (NCC) migration were capable of forming a functional thymus in an ectopic location (47). Similar studies in mice using lineage tracing and transplantation experiments also support an entirely endodermal origin for TECs (1). Nevertheless, NC-derived signals are essential for thymus development. The specific role played by NCCs varies throughout ontogeny and in the postnatal thymus. At the outset of thymus organogenesis NCCs are involved in patterning third pharyngeal pouch endoderm by setting the border between thymus and parathyroid fated domains resulting in appropriate allocation of endodermal progenitors to each domain (48). Signals from NC-derived mesenchyme promote separation of the thymus rudiment from pharyngeal endoderm as well as detachment of the developing thymus from the parathyroid (48). Subsequently, NC-derived cells play a role in migration of fetal thymus lobes into the thoracic cavity. Specifically, epithelial-mesenchymal interactions involving BMP signaling are required for thymic capsule formation, thymus-parathyroid separation and organ migration (49). A recent report demonstrated that EphB-ephrinB2 interactions regulate NCC mobility, and that deletion of ephrinB2 from NCCs results in failure of thymus organ migration and ectopic positioning of thymic lobes (50).

NCCs are essential for TEC proliferation and outgrowth of the thymus rudiment, primarily via fibroblast growth factor (FGF) signaling. Reciprocal FGF signaling between third pouch epithelium-derived FGF8 and NCCs expressing FGF10 has been implicated in 3rd pouch formation and initial outgrowth of the organ primordium. In the developing limb bud epithelial cells produce FGF8 that regulates expression of FGF10 in the underlying mesenchyme (51, 52). Similarly, *Fgf10* expression in the perithymic mesenchyme is dependent on *Fgf8* expression in the pouch endoderm and/or ectoderm (53). After pouch formation, FGF7 and FGF10 produced by perithymic NC-derived mesenchyme activate the corresponding receptor (FgfR2IIIb) on fetal TECs to promote their proliferation (54-56). *FGFR2-IIIb* mutants develop severe thymus hypoplasia after E12.5, and $Fgf10^{-/-}$ mutants display reduced TEC proliferation, indicating that NCC-derived FGF7 and -10 signals are required for thymic epithelial cell

(TEC) proliferation (56). Removing the mesenchymal capsule from E12 fetal thymi prior to transplantation inhibits thymus growth *in vitro* and results in hypoplastic thymi after transplantation under the kidney capsule (55, 57). However, in the absence of NCCs, the transplanted thymuses developed TEC subsets that support thymocyte maturation indicating that NCC-derived signals are required for TEC proliferation but not differentiation. These results also suggest that expansion of the TEC compartment is necessary to provide sufficient intrathymic niches to support thymocyte progenitors.

NC cells regulate thymus size and morphogenesis in part via secretion of BMP4 and WNT family proteins (49, 58, 59). A recent study reported reduced expression of Bmp4 and Wnt3 in mesenchymal cells from MafB deficient embryos (60). The MafB transcription factor is predominantly expressed by NC-derived mesenchyme, and its absence indirectly affects epithelial function. Epithelial cells in the MafB-deficient thymus rudiment express low levels of CCL21 and CCL25, chemokines known to attract thymocyte progenitors to the fetal thymus (57, 61). As result the number of hematopoietic cells in the MafB-deficient fetal thymus is reduced (60).

Thymic mesenchyme has also been proposed to directly participate in thymocyte development, although this role is less well supported and molecular mechanisms have not been identified (62). Although the question of whether NC-derived mesenchymal cells directly or indirectly regulate thymocyte development is unresolved, the overall question of NCC function in the thymus is an important issue that will affect strategies designed to restore thymus function and T cell output after age or disease associated involution. The proportion of mesenchymal cells increases as TEC numbers decrease during aging-related thymic involution (63). As a result, their contribution to the microenvironment increases, and could contribute to changes in the function of the microenvironment with age.

3.3. Thymus compartment formation and elaboration of the vascular network

A crucial but understudied component of the thymic architecture is the network of capillaries and blood vessels sometimes referred to as the thymic blood vessel tree. A capillary network throughout the cortex provides for oxygen delivery, as in other tissues. However, blood vessels in the thymus provide an additional critical function. Although the initial immigration of LPCs into the thymus occurs by directly traversing the epithelium in response to chemokines (64), in the postnatal thymus LPCs enter and leave the thymus via blood vessels located at the cortico-medullary junction, or CMJ (65). In spite of the crucial role blood vessels play in thymus function, almost nothing is known about the development of blood vessels in the thymus during ontogeny. CD31+ endothelial precursors first enter the thymic primordium at E12.5 (66), and the intrathymic vasculature is functionally connected to the vasculature outside the thymus by E14.5 (64) (JL Bryson *et al*, unpublished data). The architecture of the thymic vasculature relative to thymic compartments was first described by a study using 3D reconstructions of vessels versus medullary regions (67). This study concluded that different regions of the thymus were associated with specific types of vessels: capillaries in the cortex, medium sized vessels associated with medullary regions, and larger vessels without a consistent localization. The association of vasculature with medullary condensations in both wild-type and Rag mutant thymi suggested that interactions between vasculature and mTECs are responsible for organizing the medullary compartment, although the directionality of signaling was not determined. A more recent study also concluded that Fgf7 originating from blood vessels promotes mTEC expansion, although a direct role in mTEC differentiation is less clear (54).

As in all tissues, the vasculature is composed of more than one cell type, with endothelial cells forming vessels that are enclosed by tightly associated mesenchymal cells. In both fetal and adult thymic vasculature, NCC-derived mesenchyme surrounds the endothelial cells (68, 69). Thus, NCC-endothelial progenitor interactions are likely necessary for correct formation of the vasculature. NCC-derived pericytes also participate directly in vascular function in the postnatal thymus, as those at the CMJ have been shown to promote thymocyte egress via expression of S1P (70). TECs are also closely associated with fetal and postnatal thymic vasculature, and proper TEC differentiation is required for both initial (66) and later development and maturation of the fetal thymic vasculature (JL Bryson, *et al.* unpublished data). However, the signals mediating this crosstalk have not been definitively identified. One obvious candidate is vascular endothelial growth factor, or VEGF. TECs, thymic mesenchyme, and a subset of immature thymocytes (CD25+ DN cells) have all been implicated as sources of VEGF in the fetal thymus (66, 69, 71), and may direct remodeling of the thymic vasculature during perinatal medullary expansion (71). Current evidence suggests that TEC-derived VEGF may be important for formation of the capillary bed in the thymic cortex (69, 71), while mesenchyme-derived VEGF may support the development of larger vessels (69). The functional significance of apparent VEGF expression on immature thymocytes is less clear. Furthermore, VEGF is unlikely to be the only signaling pathway involved in the complex process of thymic vascularization. Thus, multiple crosstalk signals between TECs, NC mesenchyme, and endothelial cells (and possibly thymocytes) are likely required for proper patterning and maturation of the thymic vasculature.

4. EVIDENCE FOR THYMIC EPITHELIAL STEM/PROGENITOR CELLS

4.1. Defining stem/progenitor cells

Identification of the cellular mechanisms underlying development and maintenance of the postnatal thymus are critical to understanding all stages of the thymus life cycle, from organogenesis to involution. All tissues and organs in the body must be actively maintained by replacing cells lost due to normal turnover, injury, or disease; this process is generally referred to as homeostasis and involves generation of new differentiated cells. This

can be achieved at the cellular level by different mechanisms, essentially stem cell-based or stem cell-independent. Stem cells are defined operationally as cells that have the capacity to generate one or more other cell types by differentiation, and also have the capacity to self-renew - in other words to divide such that each daughter cell is a perfect copy of the parent cell. Stem cells are often regarded as cells that can contribute to maintenance of a given tissue or organ throughout the lifespan of the organism. The primary distinction between 'stem cells' and other cells that can differentiate to generate other cell types is the property of self-renewal; the term 'progenitor cell' is generally used to describe cells that can proliferate to some extent without overtly differentiating, but which do not self-renew (72). In tissues in which cell replenishment occurs via a stem cell-based mechanism, new differentiated cells are generated as a result of stem cell division and differentiation. The stem cells may divide asymmetrically, to generate one new stem cell and one more differentiated daughter, or symmetrically to generate either two new stem cells, at least one of which subsequently differentiates, or two new more differentiated cells. The resulting mature, terminally differentiated progeny may be generated directly, or via intermediate progenitor cells that may be restricted to a specific sub-lineage. Examples of this mode of tissue maintenance occur in the blood, skin and the intestinal crypts (73-76). 'Tissue stem cells' in these and other organs may be targets for therapeutic treatment of aging, injury, or disease. However, it appears that not all tissues use a stem cell-based mechanism for cell replacement during homeostasis; some maintain themselves by a simpler mechanism, by proliferation of differentiated cells themselves with apparently no *de novo* differentiation at the adult stage. This mechanism has been described principally in the liver (77) and may operate in the pancreas (78) as well as in other organs.

It is worth noting that both of these mechanisms pertain to adult organs; furthermore, it is not necessarily the case that the molecular and cellular mechanisms operating at fetal stages during organogenesis are the same as those operating in the adult during homeostasis and tissue repair and regeneration. It is an open question for most organ systems when and how the stem cell types present in the adult arise during development. In some systems at least, the mechanisms that drive organ generation during embryonic and fetal development are not present in the adult organ under normal homeostatic conditions. However, the notion that molecular programs used in organogenesis may be re-deployed during tissue repair is currently the subject of investigation, and has some experimental support. For example, a recent report established that fetal mechanisms normally absent from the postnatal pancreas are induced in an acute injury model, where they play an essential role in the facultative stem cells that regulate organ regeneration in that model (79). Collectively, effective therapeutic intervention is significantly improved by knowledge of the specific cellular mechanisms operating in the target tissue.

4.2. Identification of fetal thymic epithelial stem/progenitor cells

The debate regarding the existence and potential identity of thymic epithelial stem or progenitor cells (TESC, TEPC) has been complicated to some degree by a largely semantic confusion arising from assumptions that fetal and postnatal progenitors should have the same properties. Therefore, we will first describe current understanding of progenitor cells in the fetal thymus, and then separately discuss evidence for a postnatal TESC.

During fetal development, the thymus arises from the endoderm of the third pharyngeal pouches (1). These bilateral structures form at around embryonic day 9.0 (E9.0) in the mouse, and it has been unequivocally demonstrated from E9.0, the third pharyngeal pouches contain some cells specified to the TE lineage (1). In terminology often applied to developing organs, these E9.0 third pharyngeal pouch cells can therefore be described as 'founder cells' for the thymic epithelial lineage – in other words, the earliest cell type which will adopt thymic epithelial but not other fates (80). While it has not been formally proven that these cells are irreversibly committed to TE fate, this is the earliest stage at which a 'TE committed' cell type has been demonstrated.

The first genetic evidence for a TEPC phenotype was provided by a study addressing the nature of the defect in *nude* mice, that suggested that in the absence of *Foxn1*, TEC lineage cells undergo maturational arrest and persist as progenitors, marked by the two antibodies MTS20 and MTS24 (81). Ontogenic analysis demonstrated that the proportion of $MTS20^{+}24^{+}$ epithelial cells is highest in the early thymus primordium, decreasing to less than 1% in the postnatal thymus (82), consistent with the expression profile expected of markers of fetal tissue progenitor cells. The protein bound by MTS20 and MTS24 was subsequently identified as Plet-1 (83), a membrane-associated protein uniformly expressed in the third pharyngeal pouch endoderm from its formation at E9 until primordium formation at E11.5 (82-84). The functional capacity of isolated $MTS20^{+}24^{+}$ cells and $MTS20^{-}24^{-}$ cells in the fetal thymus was assessed using ectopic transplantation. These studies showed that until at least E15.5, transplantation of limiting numbers of $MTS20^{+}24^{+}$ cells under the kidney capsule was sufficient to establish a completely functional thymus, while the $MTS20^{-}24^{-}$ population was unable to do so (82, 85), demonstrating that a potent TEPC activity resided in the $MTS20^{+}24^{+}$ population. This conclusion was subsequently challenged by a study showing that at E15.5, both the $MTS24^{+}$ and $MTS24^{-}$ TEC compartments could form a functional thymus upon transplantation. However, this study both used large cell numbers for transplantation, and lacked analysis of the input population and therefore could not determine precursor:progeny relationships (86). As none of these experiments contained clonal analyses, they were unable to determine whether the $MT20^{+}24^{+}$ population is heterogeneous with respect to progenitor function. Therefore, while the $Plet1^{+}$ population itself may be functionally heterogeneous, and it is probable that some $Plet1^{-}$ intermediate progenitor cell types may exist in the fetal thymus at later stages, the earliest currently identified founder cells for the TEC lineage are $Plet\text{-}1^{+}$ third pharyngeal pouch cells. Our unpublished observations further establish that diminishing Plet1 expression

correlates with acquisition of differentiation markers during thymus ontogeny (Nowell and Blackburn, unpublished).

Two studies subsequently addressed the potency of TEPC at a clonal level. The hypothesis that TEPC are maintained in a state of maturational arrest in the absence of Foxn1 was tested and confirmed by an elegant study in which postnatal clonal reactivation of a conditional null allele of Foxn1 was shown to result in generation of functional thymus tissue containing organized cortical and medullary regions (87). This established unequivocally that in the absence of *Foxn1*, some persisting TECs have bi-potent progenitor activity. However, since the block in TE development in Foxn1$^{-/-}$ thymi occurs in fetal development shortly after formation of the thymus primordium, these data demonstrated the existence of fetal TEPC, but did not address TEPC potential in the postnatal thymus. A second study used injection of single E12.5 TEC into fetal thymic lobes to demonstrate the existence of a bipotent TEPC that could contribute to both cortical and medullary TEC compartments (88).

Current evidence supports the existence of cortical and medullary sub-lineage specific progenitor cells from relatively early in organogenesis. Analysis of allophenic chimeras indicated the presence of medullary TEPC, as in this study no direct lineal relationship could be found between individual medullary islets and the surrounding cortical areas at least in early development (89). In this study, the mTEPC activity was shown to persist until at least E15.5, but the immunophenotype of the mTEPC was not determined. This issue was addressed in a later study, which identified the Cldn3,4^{hi}, UEA1^{hi} subpopulation of fetal TEC as progenitors for the Aire^{+} subpopulation of mTEC (90). Additionally, evidence suggests the existence in fetal thymus development of a cortical sub-lineage specific progenitor, characterized by expression of CD205 (91). A remaining question is the timing of emergence of the sub-lineage progenitors during organogenesis, and the extent to which they persist in the late fetal and postnatal organ.

4.3. Evidence for TESC/TEPC in the postnatal thymus

The first evidence for a common stem/progenitor cell activity in the postnatal thymus was provided by analysis of a subset of human thymic epithelial tumors that were found to contain cells that could generate both cortical and medullary sub-populations, suggesting that these arose from epithelial stem cells (92). These data, and subsequent data based mainly on shared marker expression in cultured cells have been extensively reviewed elsewhere (93) and will not be revisited here.

Further data regarding the phenotype of thymic epithelial progenitors came from analysis of mice with a secondary block in thymus development resulting from a primary T cell differentiation defect, which suggested that epithelial cells that co-express K5 and K8 have cTEC progenitor activity (33). This conclusion was based on the observation that in mice with complete, early block in thymocyte differentiation, the postnatal thymus is characterized by the predominance of K8^{+}K5^{+} TECs. Normal cTEC development and architecture developed upon restoration of T cell differentiation, which lead to the conclusion that a precursor:progeny relationship existed between K8^{+}K5^{+} TECs and K8^{+}K5^{-} cTECs. It is unclear whether the TEC phenotype observed in these mouse mutants corresponds to an authentic differentiation arrest of a normally occurring TEC population, or reflects an abnormal state induced by the thymocyte differentiation block; also, the differentiation of cTEC from a minor subpopulation of undetermined phenotype in the mutant thymi cannot be excluded. Nevertheless, these data in conjunction with data indicating that all TEC in the developing thymic primordium at E11.5 share expression of these markers are consistent with the proposal that the K8^{+}K5^{+} population of the adult thymus contains a TEPC activity. As K8 and K5 are co-expressed by only a population of cells at the cortico-medullary junction and scattered in the cortex in the postnatal thymus, their distribution pattern is also consistent with this hypothesis. Furthermore, a population of Plet1^{+} TEC also exists in the postnatal thymus as a minor subpopulation of mTEC, and overlaps partially with the medullary population of K8^{+}K5^{+} TECs. Based on extrapolation from the characteristics of fetal Plet1^{+} TEC, it is tempting to speculate that these Plet1^{+}K8^{+}K5^{+} cells may be postnatal TEPC/TESC, however at present no data directly address this possibility.

The notion that the postnatal thymus is maintained principally by a common TEPC/TESC that generates both cortical and medullary TEC must be treated with caution, however, since the chimera study discussed above revealed no evidence for this type of activity (89). Furthermore, a lineage analysis based on low frequency epithelial-specific Cre recombination in the postnatal thymus demonstrated the existence of apparently clonal proliferative units that were limited to either medullary or cortical TEC, or spanned both cortical, cortico-medullary junction and medullary regions (87). These studies are often taken to provide support for sub-lineage restricted stem/progenitor cells in the postnatal thymus. However, neither demonstrates this definitively, as in both cases the data would also be consistent with proliferation of terminally differentiated epithelial cells - or a mixture of these activities. Also, the relatively early time of recombination (2 weeks postnatal) in the latter study leaves open the possibility that some or all of the activities detected are from residual fetal cells that are not maintained in the adult steady-state thymus. Similarly, while some recent data could be interpreted to support the existence of TE stem cells in the postnatal thymus, in each case the data are also consistent with proliferation of committed progenitor or terminally differentiated epithelial cells. For example, the changes seen in thymi upon castration demonstrate that regenerative capacity persists in the involuted thymus. While this would be consistent with persisting stem/progenitor cell activity, some mature cTEC and mTEC cells are still present in the involuted thymus, leaving open the possibility that regeneration could be due to their proliferation. In this regard, evidence that Fgf7 is mitogenic for all adult TECs provides a possible mechanism for proliferation-based expansion of differentiated cells during rebound (94). It is also possible

that a facultative stem cell activity could be induced under regenerating conditions, similar to the oval cell activity induced by certain types of liver damage and the $Ngn3^+$ ductal cell activity induced by partial duct ligation in the pancreas (95). Thus, at the present time, the question of whether the postnatal thymus is maintained during homeostasis by a stem cell or progenitor cell based mechanism, or by proliferation of terminally differentiated cells, remains unresolved.

Several recent publications have begun to shed light on the molecular requirements for postnatal TEC maintenance and the potency of postnatal TEC. Analysis of the p63 knock out phenotype revealed the requirement for this protein, a critical regulator of the stratified epithelial cell program, in postnatal TEC. *p63*$^{-/-}$ mice develop thymus hypoplasia that was suggested to result from loss of a TESC compartment (96). This study suggested an important connection between the mechanisms underpinning maintenance of the thymus and those that maintain other stratified epithelial cells. Recently, this issue has been explored further through functional experiments that demonstrated that postnatal rat TEC can be cultured clonally and indefinitely under the same culture conditions as skin/hair follicle keratinocytes (97). These cultured TEC, which as a population express Plet1 and a variety of stem cell-associated markers including p63, were shown to contribute to the thymic microenvironment in the presence of carrier fetal thymic epithelial cells. This assay was short-term, and therefore did not address whether the cultured cells had TESC activity. Furthermore, the contribution to these thymus reaggregates was largely limited to mTEC. However, the cultured rat TECs were able to contribute to both epidermal and hair follicle lineages in a skin morphogenetic assay, and could maintain this contribution for the long term. These cells therefore functioned as classical skin stem cells once reprogrammed to adopt the skin/hair follicle molecular program – notably, this reprogramming was induced by their microenvironment rather than by genetic intervention. While it is unclear at present how this study relates to the presence of epithelial stem cells in the postnatal thymus, the findings are of great interest and merit further investigation. In addition to these studies, a recent study has addressed whether a Foxn1-negative cell type may exist at the base of the postnatal TEC hierarchy (98), and concludes that postnatal TEPC express Foxn1, based on use of three different approaches to ablating Foxn1-expressing postnatal TEC. However, although technically elegant, these approaches do not exclude all scenarios for Foxn1-negative progenitors, including the possibility that Foxn1-positive niches are required to indirectly to maintain Foxn1-negative TESC.

5. REGULATION OF THE THYMIC MICROENVIRONMENT: PROLIFERATION, DIFFERENTIATION, OR BOTH?

5.1. Foxn1 is a key regulator of TEC differentiation and proliferation throughout the thymus lifespan

Perhaps the best-known mouse mutants affecting thymic epithelial cells carry mutations at the nude locus, which encodes Foxn1 (99-101). Foxn1 is a forkhead transcription factor required for all TEC differentiation (81, 98). The requirement for Foxn1 for multiple stages of fetal TEC differentiation has been summarized many times, including in a recent review (102). Here, we will focus on the most recent studies regarding the role of Foxn1 in the postnatal thymus.

The early studies of Foxn1 gene expression showed widespread expression in postnatal cTEC and mTEC (101). In contrast, an analysis of Foxn1 protein distribution concluded that Foxn1 protein was not detected in most TECs in the postnatal thymus, and that its presence did not correlate with the expression of the known functional markers Dll4 and CCL25. Based on these data, this study predicted that Foxn1 did not play a significant role in postnatal thymus function, and further suggested that Foxn1-negative TECs may play an important role in postnatal thymus function (103). More recent studies from the Manley lab (104) and others (103) (Nowell and Blackburn, unpublished) have shown that Foxn1 expression is dynamically modulated in different TEC populations, and suggests that that this quantitative requirement for specific levels of Foxn1 may be essential for specific TEC subpopulations to develop or be maintained (104)(Nowell and Blackburn, unpublished). Furthermore, analysis of Foxn1 gene expression using a lacZ allele suggested that most TEC retain Foxn1 gene expression through 12 months of age (104), suggesting that the method of detecting Foxn1 expression may be critical to identifying TECs expressing lower Foxn1 levels. A recent report used a diptheria toxin-based approach to eliminate Foxn1-positive TECs during fetal or postnatal development to conclude that all functional TECs develop from Foxn1-positive cells, and that only Foxn1-lineage TEC can function to support thymocyte development (98). These studies are not completely definitive with respect to the expression of Foxn1 in TESC/TEPC, as mentioned above (section 4.3), but do strongly support a substantial and ongoing requirement for Foxn1 in the postnatal thymus. Other studies using an allele of Foxn1 expressing Cre recombinase to activate marker genes or delete other genes in TECs also clearly show that Foxn1 is expressed in the vast majority, if not all, TECs in both the fetal and adult thymus at some point in their development (16, 105, 106), even if they reside in cysts or have subsequently down regulated *Foxn1* expression (98, 107). Partial deletion of the Foxn1 gene using a conditional allele also supported a requirement for Foxn1 in the postnatal thymus (108, 109). Thus, the balance of data clearly supports a model in which Foxn1 is expressed in most TECs at some level throughout life, and that Foxn1 remains a critical regulator of both the fetal and postnatal thymus.

Genetic data also show that proper modulation of Foxn1 levels is required to generate and maintain the correct composition, structure, and function of the postnatal thymus. Reduced *Foxn1* expression has been identified as an early event in thymic involution (110). The consequences of this level of down regulation were identified by analysis of an allele of Foxn1 in which premature postnatal down regulation of Foxn1 expression

to ~30% of young adult levels occurs (104). This reduced level of Foxn1 induces a premature involution phenotype associated with specific changes in the proliferation and differentiation of specific TEC subsets. These data also suggest that specific TEC subsets require different levels of Foxn1 for their development and/or maintenance, and that modulating Foxn1 levels functions to balance or coordinate TEC differentiation and proliferation during formation and maintenance of the thymic microenvironment. Reduced Foxn1 expression with age is likely to be a major factor contributing to disintegration of the TEC network and inability to support T cell development during involution, and is therefore a strong candidate for a target for inducing thymic rebound. Thus, the balance of available studies clearly supports the conclusion that Foxn1 plays a critical role in most if not all TEC differentiation and is a major regulator of the thymic microenvironment throughout the lifespan.

5.2. The role of cell cycle regulation in maintaining the size of the thymic microenvironment

A variety of genetic studies have provided evidence demonstrating that regulating TEC proliferation is a key component of the cellular mechanisms that maintain the postnatal thymus. The first such study was the analysis of a transgenic mouse line expressing Cyclin D1 from a Keratin 5 promoter (K5.CyclinD1) (111). These mice showed dramatic and continuous thymus growth, resulting in an outsized thymus that did not undergo aging-associated involution, and eventually caused the death of the animal. Analysis of TEC subsets using subset-specific markers showed that this hyperplastic thymus had a relatively normal organization, and displayed no characteristics of thymoma or thymic lymphoma. Analysis of thymocyte differentiation, including assaying selection using TCR transgenic models, showed no defects in T cell development, only increased overall numbers. Interestingly, no thymus phenotypes were observed in mice with constitutive activation of CDK4 (112, 113), which could be explained by low levels of CDK4 in TECs and/or by CDK4-independent functions of Cyclin D1 in these cells. These data indicate that over-expressing Cyclin D1 in TECs results in increased capacity for normal thymocyte differentiation, presumably through an increased availability of microenvironmental niches.

A number of transgenic and knockout strains of mice with alterations in the cell cycle regulatory machinery have further been shown to have thymic phenotypes. For instance, transgenic mice overexpressing E2F2 (but not E2F1) in the thymus led to thymic carcinomas (114, 115), suggesting a role for E2F2 in the expansion of thymic epithelial cells (TECs) (114), while a mild decrease in thymus size was observed in *E2f1;E2f2* double knockout animals (116). TEC hyperplastic phenotypes have been observed in transgenic mice expressing viral oncoproteins such as E7 and SV40 LT that can inactivate members of the retinoblastoma (RB) family (117-120), consistent with a role for RB family members in the thymic epithelium. Finally, the analysis of mice with mutations in p16 family members and p21 family members, the two families of small cell cycle inhibitors controlling the kinase activity of Cyclin/CDK complexes suggest specific roles for $p18^{Ink4c}$ and $p27^{Kip1}$ in suppressing the expansion of TECs (121) (122). p27 in particular is expressed at higher levels in the developing thymus (123) and low levels of p27 correlate with poor prognosis in patients with thymoma (124, 125). Loss of p27 function in mice increases thymocyte cellularity as a direct consequence of an expanded TEC compartment that maintains proper organization and function (122). This phenotype is similar to that of over-expression of CyclinD1 in TECs, although perhaps not as severe.

While collectively these data strongly support a role for the extended RB pathway in the expansion of the thymic epithelium, major issues need to be addressed before the role of cell cycle regulation in TECs is understood. Many of these experiments did not specifically manipulate these genes in TECs, and non-cell autonomous effects due to crosstalk cannot be ruled out. In addition, none of these studies explored a role for this pathway at different stages over the entire lifespan. Thus, the stage-specific roles of the RB pathway in the formation, growth, maintenance, and/or involution of the thymus remain to be determined, in particular whether they play a role in aging-associated involution. Furthermore, the extra- and intra-cellular signals converging on members of the RB pathway to normally regulate TEC proliferation and differentiation are unknown. Finally, the molecular and cellular mechanisms by which the RB pathway may regulate TEC expansion and function, in particular the balance between regulating proliferation and differentiation, and their relationship to other known regulators of TEC differentiation such as Foxn1 and the NFKB family, represent intriguing future areas of investigation.

6. CLINICAL AND TRANSLATIONAL RELEVANCE OF UNDERSTANDING THE THYMIC MICROENVIRONMENT

Thymic output is quantitatively and qualitatively correlated with peripheral immune function. Loss of thymic output occurs during aging, as well as due to a wide variety of conditions including genetic disorders, AIDS, and cancer therapies such as irradiation and chemotherapy. The reduced output of naïve T cells after age-related thymus involution in humans severely impairs the ability to respond to newly encountered antigens. Older people are less likely to develop protective immunity after vaccination and therefore are more susceptible to new infectious diseases such as West Nile virus (126). Diminished thymus function is a particular problem for patients who have undergone therapeutic bone marrow transplantation (BMT) (127, 128). The cytoablative treatment used to prepare patients for BMT damages the thymus microenvironment. This poses a serious risk even to young patients, who would otherwise sustain robust T cell output from a functional thymus. T cell deficiency resulting from treatment-induced thymus damage renders these patients susceptible to infection for months after transplantation. Restoration of thymus function would thus be extremely efficacious in reducing post-transplant morbidity and mortality. Patients with T cell-based immunodeficiency

from AIDS are also vulnerable. Overall, the identification of mechanisms to prevent or revert thymus involution would benefit a large patient population.

Important proof of concept studies in infants presenting with full DiGeorge Syndrome have demonstrated that transplantation of neonatal human thymus tissue leads to generation of a functional adaptive immune system (129-131). In these studies, *in vitro* cultured neonatal thymus fragments were transplanted into the quadriceps muscle of full DiGeorge syndrome patients presenting with no circulating T cells, aged between 51 and 127 days on the date of grafting. In the initial study, five children were transplanted. Three died of complications arising from DiGeorge Syndrome but unrelated to the thymus transplant. However, the two surviving patients developed host T cells which exhibited a normal TCRbV repertoire, showed robust responses to mitogen and developed appropriate B cell responses to tetanus and pneumococcus vaccinations. Interestingly, one of these grafts was completely unmatched at HLA. TRECS were detected in both patients after but not before grafting (129). This study has now been enlarged and followed up for more than 12 years (130-137), and recently the same approach has been successfully extended to treating human *nude* patients (138). These studies provide proof of principle that cell replacement approaches in general can work for increasing thymus function, and that thymus grafting into an ectopic location, at least of neonatal tissue, can be successful in reconstituting a functional T cell population.

Aging-associated immunodeficiency represents a key component of the pathophysiological effects of aging and has multiple well-documented effects on the quality of life and health. Hallmarks of aging with respect to immune function include enhanced susceptibility to infection, poor responses to vaccination, and increased autoimmunity, all of which are factors that increase morbidity and mortality in the elderly. Even in middle-aged people, reduced thymic function is a contributing factor in the ability to combat infectious diseases such as influenza. Immune deficiency is exacerbated by diseases including cancer and AIDS, and is a major side effect of chemotherapy, radiation, and adult bone marrow transplantation, all of which are compounded by the effects of aging-related immunosenescence. A major component of immunosenescence is the loss of T cell production from the thymus as a result of aging-associated involution. Age-related T cell abnormalities that contribute to reduced immune system function include a decline in the frequency and function of naïve peripheral T cells as well as a decline in the expansion of memory T cells leading to a restricted T cell repertoire. The diminished capacity of peripheral T cells to proliferate and produce cytokines in older individuals is thought to result from defects accumulated after prolonged T cell residence in the periphery (139). As the reduced number (and repertoire) of naïve T cells in the peripheral pool is due to diminished output from the aged thymus (140), many of the age-associated changes in peripheral T cells are directly or indirectly related to reduced thymic output. The incidence of autoimmune diseases also increases with age and may be mechanistically linked to thymic involution. Regenerating thymic output of naïve T cells thus has the potential to substantially improve immune status for a wide variety of patients and the elderly, while prevention or amelioration of thymic involution could reduce the incidence of autoimmunity. Due to the high demand and clinical need for therapies to ameliorate immunodeficiency caused by aging, disease, or disease treatment, some preclinical and clinical studies to enhance thymus function are already in progress or on the horizon (reviewed in (141, 142)). A better understanding of the biology underlying involution should lead to the development of more specific and effective strategies for reversing or even preventing age-related involution, thus improving overall immune function in the aged and aging population.

Understanding the development and function of the thymic microenvironment is critical for any efforts to reverse or prevent its degeneration from aging or disease. Knowledge of the mechanisms by which cells in the thymus differentially translate extracellular signals to achieve homeostasis could lead to the development of strategies that manipulate thymic output in patients and normal individuals during aging.

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Abbreviations: EC: thymic epithelial cell, NCC: neural crest cell

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