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Understanding the Thymic Microenvironment: the Cellular and Molecular Basis of T Cell Development

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Objectives: The thymus is a primary lymphoid organ that provides specialized microenvironment for T cell development. A variety of thymic stromal cells form the thymic tissue architecture and critically regulate the development and repertoire selection of T cells. **Methods:** We reviewed historical and recent studies on thymic stromal cells, especially focusing on the well-characterized functions of thymic epithelial cells (TECs) and the significance of as yet less characterized non-TEC thymic stromal cells and hematopoietic antigen-presenting cells in the regulation of T cell development. **Results:** Cortical TECs (cTECs) induce positive selection of diverse and functional T cells, while medullary TECs (mTECs) establish T cell tolerance via the negative selection of auto-reactive T cells and their conversion into regulatory T cells. These modes of T cell tolerance induction are also mediated by hematopoietic antigen-presenting cells such as dendritic cells and thymic B cells. Thymic mesenchymal cells, a prominent component of non-TEC thymic stromal cells, support the development and maintenance of TECs and thereby T cell production. **Conclusion:** Understanding the cellular and molecular basis for thymic stromal subsets will provide invaluable information toward in vivo reconstitution of the thymic microenvironment for future therapeutic applications.

Keywords: Thymus Gland, Thymocytes, Central Tolerance, T Cell Antigen Receptor, Cell Microenvironment

Introduction

T lymphocytes (T cells) are central players in the adaptive immune system. Specific recognition of peptide antigens displayed with major histocompatibility complex (MHC) proteins by diverse T cell antigen receptors (TCRs) and the coreceptor CD4 or CD8 play a major role in immune responses against foreign antigens in humans and mice. The specificity of antigen recognition by TCR is stringently maintained so that T cells are reactive to foreign antigens but tolerant to self-antigens [1, 2]. The development of T cells and the formation of their TCR repertoire occur primarily in the thymus [3]. This article reviews the cellular and molecular mechanisms by which these processes are carried out.

The thymus is an organ exquisitely specialized for supporting T cell development. The thymic microenvironment is composed of a variety of stromal cells, including thymic epithelial cells

(TECs), endothelial cells and fibroblasts [4]. These thymic stromal cells form a three-dimensional meshwork architecture that hosts hematopoietic stem cell-derived immature T cells (thymocytes) and critically supports their development. Certain hematopoietic antigen-presenting cells, such as dendritic cells (DCs) and thymic B cells, also participate in controlling the formation of T cell repertoire. The thymus is subdivided into two discrete regions, the cortex and medulla. The cortex is the outer region of the thymus, where a stromal meshwork houses densely packed, immature thymocytes, while the medulla is the inner region, packed with enriched stromal cells and less densely localized mature thymocytes. The most characteristic stromal components that distinguish the cortex and medulla are two different TEC subsets: cortical TECs (cTECs) and medullary TECs (mTECs). The cortical stromal architecture is mainly composed of cTECs, whereas the medullary microenvironment is heterogeneous, being composed of mTECs, mesenchymal fibroblastic reticular cells (FRCs), DCs and B cells. The thymus also contains blood vessels that are composed of endothelial cells and mesenchymal cells and are enriched in the cortico-medullary junction [5-7]. These thymic stromal cells provide multiple signals to guide the differentiation, migration, proliferation, survival and death of developing thymocytes, thus playing essential roles in supporting the adaptive immune system [8, 9].

This article provides an overview of the stepwise process of T cell development in the thymus, then reviews our current understanding of the development and function of thymic stromal cells, particularly focusing on the two well-characterized thymic stromal cell subsets, cTECs and mTECs. In addition, recent advances on less well characterized thymic stromal cells and hematopoietic antigen presenting cells, such as DCs and B cells, are also discussed.

1. T cell development in the thymus

The most immature thymocytes, which are called early T cell progenitors (ETPs), are derived from hematopoietic stem cells in the fetal liver or adult bone marrow [10]. These ETPs belong to CD4/CD8 double negative (DN) thymocytes and undergo developmental programs that go through DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$) stages. During the DN2 and DN3 stages, TCRβ-VDJ rearrangement occurs. Successful rearrangement of the TCR β-chain leads to further differentiation into the DN4 stage. This process, called 'β selection', ensures commitment to the T cell lineage. DN4 thymocytes proliferate and express CD4 and CD8 co-receptors, giving rise to CD4/CD8 double positive (DP) thymocytes. These differentiation processes are associated with a relocation of thymocytes: in the adult thymus, ETPs first enter the thymus via vessels at the cortico-medullary junction, developing DN2 and DN3 thymocytes migrate across the cortex toward the subcapsular region, and the generation of DP thymocytes occurs in the outer cortex.

In the cortex, DP thymocytes undergo TCRα-VJ rearrangement, as a result expressing the αβTCR on the cell surface. DP thymocytes move randomly in the cortex, probably seeking pMHC ligands for their newly generated TCR in the cortical microenvironment. Interaction of the αβTCR with peptide-MHC (pMHC) complexes presented in the cortical microenvironment leads to the decision of the fate of DP thymocytes. DP thymocytes that receive a low avidity TCR interaction with self pMHC survive and differentiate into mature thymocytes. Cells expressing MHC class II-reactive TCR are fated to the CD4 single positive (SP) lineage, while those with MHC class I-reactive TCR are fated to the CD8SP lineage, the process referred to as "positive selection". In contrast, DP thymocytes expressing TCR strongly reactive to self pMHC (self-reactive cells) die by apoptosis, a process referred to as "negative selection". DP thymocytes that fail to express pMHC-reactive TCR are also destined to die, a process referred to "null-selection" or "death-by-neglect". Positively selected CD4SP or CD8SP thymocytes relocate to the medulla by chemotactic migration. In the medulla, mTECs express a variety of peripheral tissue-restricted antigens (TRAs), at least partly by virtue of autoimmune regulator (Aire), a nuclear factor expressed in mTECs. TRAs are presented autonomously by mTECs or cross-presented by DCs, such that SP thymocytes reactive to TRAs are deleted by negative selection or induced to differentiate into Foxp3-expressing regulatory T (Tregs) cells. Consequently, mature SP thymocytes that have completed the cortical and medullary selection processes, which thereby express diverse yet self-tolerant TCRs, are released into the circulation as naïve T cells.

In addition to classical T cell development, the thymus also supports the development of unconventional (non-classical) T cells, such as γδT cells, invariant natural killer T (iNKT) cells, and natural T helper 17 (nTh17) cells. γδT cells belong to a distinct T cell lineage that expresses TCRγ and TCRδ chains and recognizes

native non-peptide and peptide antigens, such as stress-induced proteins. αβT and γδT cell development diverges at the DN2 and DN3 stages. iNKT cells represent an unconventional αβT cell subset expressing invariant Vα14-Jα18 TCR that recognizes glycolipid antigens presented by MHC-like CD1d molecules, playing a role in the regulation of the innate and adaptive immune responses [11]. These iNKT cells are positively selected by CD1d/glycolipid complexes expressed on the surface of DP thymocytes [12]. nTh17 cells comprise an unconventional $CD4^+$ αβT cell subset that potentially contributes to both protective and pathological inflammatory responses. The development of nTh17 cells requires the expression of MHC class II and self-antigens in mTECs and the cytokines IL-6 and transforming growth factor (TGF) β [13-15].

2. cTEC development

cTECs are the predominant cells that form the stromal architecture in the thymic cortex. Marker proteins that identify cTECs in the adult thymus include cell-surface proteins such as CDR1, CD205 and CD249 (Ly51), as well as intracellular proteins such as keratin-8, keratin 18 and β5t. Both cTECs and mTECs are derived from common endodermal progenitor cells that reside in the third pharyngeal pouch [16-18]. The common TEC progenitors progress to the transitional progenitor stage, a process dependent on the transcription factor FOXN1 [19, 20]. Such transitional TEC progenitors express cTEC-associated genes, such as CD205, β5t, CCRL1, and IL-7, and give rise to both cTECs and mTECs [20-22]. The generation of mature, functional cTECs from transitional TEC progenitors requires signals delivered by developing DN thymocytes, most likely through intercellular signals that have yet to be identified [19, 23].

2.1 cTECs support the thymic architecture and early T cell development

Mutant mice lacking normal cTEC development, either intrinsically or inducibly, exhibit disorganized cortical architecture and a massive loss of thymic cellularity, indicating that cTECs are required for the formation and maintenance of the thymic architecture and for optimizing T cell development [24-28]. cTECs provide a variety of the molecules required for the survival, proliferation, differentiation and migration of immature thymocytes. Dll4, a Notch ligand expressed by cTECs, is both necessary and sufficient for T-lineage determination of early lymphoid progenitors in the thymus [29-31]. IL-7 is also predominantly produced by cTECs [32] and promotes the survival, proliferation, and differentiation of thymocytes [33-35]. Outward migration of DN thymocytes from the cortico-medullary junction to the subcapsular region is mediated by the chemokines Ccl25 and Cxcl12, which are produced by cTECs, and their respective receptors CCR9 and CXCR4, which are expressed in DN thymocytes [36-39]. CCRL1 is highly expressed in cTECs and is an atypical non-signaling chemokine receptor that binds CCL25, as well as CCL19 and CCL21 [27]. It promotes the outward migration of DN thymocytes via still-unknown mechanisms [40, 41]. Vascular-cell adhesion molecule-1 (VCAM-1) is expressed by cTECs and its receptor integrins α4β1 and α4β7 are expressed by DN thymocytes. They are also important for intimate stromal interactions and the outward migration of DN thymocytes [42]. DN thymocytes turn back inward and differentiate into DP thymocytes in the subcapsular region.

2.2 cTECs control positive selection

The most widely recognized function of cTECs is the induction of T cell positive selection. The thymus from cTEC-deficient mice exhibits impaired positive selection and an altered TCR repertoire [28]. As described above, a low affinity TCR engagement by pMHC complexes induces positive selection of functional T cells, whereas a high affinity TCR-pMHC interaction leads to negative selection of self-reactive (potentially harmful) T cells. Recent studies support the idea that cTECs have unique proteolytic and antigen processing capabilities for producing the MHC-bound peptides that are required for positive selection.

For the MHC class I system, cTECs are equipped with a unique type of proteasome. Proteasomes are huge protease complexes that are responsible for producing MHC class I-bound peptides as well as handling the turnover of intracellular proteins [43]. Peptides with C-terminal hydrophobic anchor residues are produced by the chymotrypsin-like activity of the proteasomes, an effect which is mediated by β5 catalytic subunits. Unlike most somatic cells that express 'standard proteasomes' possessing the standard β5 subunit, or immune cells and interferon (IFN) γ-stimulated cells that express β5i subunit-containing 'immunoproteasomes' [44, 45], cTECs express a specialized type of proteasome, called a 'thymoproteasome', that contains the β5t subunit [46, 47]. β5t is exclusively expressed by cTECs

throughout the lifespan of mice [48], thus constituting a specific marker of cTECs. In mice deficient for β5t, cTECs express β5- and β5i-containing proteasomes and display a spectrum of MHC class I-bound peptides that are different from those in β5t-sufficient cTECs [49, 50]. In these mice, positive selection of MHC class I-restricted thymocytes is substantially reduced, leading to a marked reduction (only 20% of the wild-type) and altered repertoire of CD8 T cells, indicating that an optimal positive selection of CD8 T cells requires the β5t-dependent peptide repertoire in cTECs. The β5t-dependent peptides also determine the antigen responsiveness of positively selected CD8 T cells [51]. A recent study identified unique cleavage motifs in β5t-dependent MHC class I-bound peptides that confer a low affinity TCR interaction and have the capacity to induce positive selection [52]. This uniqueness of the peptide motifs might be attributed to the peptide cleavage preference difference that exists between β5t and the other subunits, β5 and β5i [46, 52]. Collectively, these various characteristics show that cTECs regulate positive selection of CD8 T cells by producing a unique set of MHC class I-bound peptides with a low affinity for the TCR.

Furthermore, in the case of the MHC class II system, cTECs have unique protein degradation and antigen processing mechanisms for inducing the positive selection of CD4 T cells. Lysosomal proteases, such as cathepsin L and thymus-specific serine protease (TSSP), are highly expressed in mature cTECs. Mice deficient in cathepsin L exhibit a reduced positive selection of polyclonal CD4 T cells [53, 54]. TSSP-deficient mice exhibit a defective positive selection of CD4 T cells having certain TCR specificities [55-57]. It was also shown that cTECs exhibit high levels of constitutive autophagy [58], an auto-degradation process that facilitates the loading of endogenous peptides onto MHC class II. Mice with defective autophagy induction, specifically in TECs, display altered repertoire selection in certain CD4 T cells. The nature of the MHC class II-bound, positive selection-inducing peptides produced by cTECs remains to be elucidated.

The thymic cortex is also the site at which thymocytes reactive to self-antigens are deleted by negative selection. It is estimated that nearly six times as many thymocytes undergo negative selection compared with positive selection and 75% of this negative selection occurs in the cortex [59], most frequently the inner cortical region [60]. However, negative selection, in any experimental model yet tested, was observed to be normal in β5t-deficient mice [49], TSSP-deficient mice [55] and cTEC-deficient mice [28], indicating that cTEC-specific peptides are not required for cortical negative selection. Rather, it is the cortex-resident DCs that appear to be responsible for the negative selection that occurs in the cortex [60].

2.3 cTECs form 'thymic nurse cells'

Another noteworthy feature of cTECs is the unusually intimate interactions they have with developing thymocytes. A study published in 1980 demonstrated unique multicellular complexes comprised of huge epithelial cells that engulf many living thymic lymphocytes within their intracellular vesicles, a discovery made in cell suspensions prepared by enzymatically dissociating thymus tissues [61]. This multicellular complex (or the epithelial cell that forms this complex) was called 'thymic nurse cell (TNC)'. This study, as well as many later studies (reviewed in [62]), hypothesized that TNCs provide microenvironment for positive and negative selection, although the precise cell lineage and function of the TNC-forming thymic epithelium remained elusive at that time. A recent report found that a majority of cTECs, but not mTECs, tightly interact with thymocytes and that approximately 10% of the cTECs in the adult mouse thymus form thymocyte-wrapping complexes that are identical to the previously described TNCs [63]. TNC formation is less detected in the case of strong positive selection, but is readily detectable in the null-selection case. TNC-enveloped lymphocytes are enriched in long-lived, unselected DP thymocytes undergoing secondary TCRα-VJ rearrangement. Thus, TNCs are formed upon persistent cTEC-DP thymocyte interaction and facilitate secondary TCRα rearrangement. Given that the efficiency of secondary TCRα rearrangement is controlled by DP thymocyte survival [64], the microenvironment within intra-TNC vesicles may ensure the survival of enclosed DP thymocytes. Secondary TCRα rearrangement is required for increasing the opportunity for positive selection, thereby maximizing developmental efficiency of functional T cells, including allo-reactive T cells [65]. The mechanisms by which unselected thymocytes are enclosed into and positively selected thymocytes are released from the TNC complexes, and by which the intra-TNC microenvironment promotes survival and/or continued TCR rearrangement in DP thymocytes, remain to be determined.

3. mTEC development

mTECs are distinguished by the expression of cell-surface proteins such as claudin-3, claudin-4 and CD80, as well as intracellular proteins such as keratin-5, keratin-14 and Aire. The fucose-binding lectin Ulex europaeus agglutinin 1 (UEA1) is also a widely used marker of mTECs. As described above, mTECs emerge from TEC progenitors that express cTEC-associated genes. Immature mTECs expressing low levels of MHC class II and CD80 ($mTEC^{lo}$) differentiate into MHC class II^{hi} $CD80^{hi}$ mature mTECs ($mTEC^{hi}$) expressing Aire [66, 67, 68]. In the adult thymus, $mTEC^{lo}$ is a heterogeneous cell population that includes both developing immature mTECs and developed mature mTECs at the 'post-Aire' stage [69, 70]. The 'post-Aire' $mTEC^{lo}$ cells constitute a distinct mTEC subpopulation expressing chemokines such as CCL21 [71]. These cells further undergo terminal differentiation that is characterized by the expression of involucrin and the stratified squamous epithelia that resemble Hassall's corpuscles observed in the human thymus [72, 73].

3.1 Developing T cells promote mTEC development and thymic medulla formation

The formation of the thymic medulla is defective in mice deficient for early T cell development [74]. In particular, mice deficient for positive selection show a marked reduction of the thymic medullary regions and mTEC cellularity, without any effect on the thymus size or cortical architecture [75-78], indicating that the positively selected SP thymocytes induce the development of mTECs, which, in turn, provide the microenvironment required for the selection and maturation of SP thymocytes.

The mechanism of thymocyte-dependent mTEC development can be accounted for the ligand-receptor interactions that activate the transcription factor nuclear factor-κB (NF-κB) in mTECs. Mice deficient in NF-κB signaling molecules such as TNF receptor-associated factor 6 (TRAF6) [79], NF-κB-inducing kinase (NIK) [80] or IκB-kinase α (IKK α) [81], or the NF-κB subunits, Bcl-3 [82], NF-κB2 (p52) [83, 84] or RelB [85, 86], exhibit defective $Aire^+$ mTECs, mTEC development and thymic medulla formation. These NF-κB pathways for thymic medulla formation are activated by the TNFR superfamily receptors RANK, CD40 and LTβR that are expressed in mTECs, and their TNF superfamily ligands, RANKL, CD40L and lymphotoxins (LTs), respectively, that are expressed by lymphoid cells, mostly SP thymocytes [87-89].

RANKL, a major mediator of mTEC development, is produced by lymphoid tissue inducer (LTi) cells and γδT cells in the embryonic thymus and SP thymocytes and iNKT cells in the postnatal thymus [88-92]. CD40L and LTs are expressed predominantly by SP thymocytes [87-90]. These TNFSF ligands have cooperative as well as distinct, non-redundant functions in mTEC development. During embryogenesis, RANKL and LTs trigger the differentiation of $Aire^+$ mTECs from $Aire^-$ progenitors [93], while in the adult thymus RANKL and CD40L promote the proliferation of $Aire^+$ mTECs [89, 94]. LTs also regulate the development of a distinct subset of mTECs expressing CCL21 [71, 95, 96] and the terminal differentiation of mTECs [73].

RANKL signaling in mTECs up-regulates the transcription factor Spi-B, which in turn induces the expression of some TRAs, co-stimulatory molecules and osteoprotegerin (OPG) [97]. OPG is an inhibitory decoy receptor for RANKL and represses RANKL-mediated mTEC development and expansion [89, 97, 98], implying a fine-tuning mechanism of mTEC development and function.

3.2 mTECs express TRAs and establish T cell tolerance

In the medulla, a diverse array of TRAs, the expression and function of which are primarily restricted to peripheral tissues, are transcribed in mTECs (reviewed in [9]). SP thymocytes reactive to these TRAs are deleted from the conventional T cell pool through deletion by negative selection or differentiation into $Foxp3^+$ Tregs. As shown by many studies, T cells that are produced in mice lacking normal mTEC development cause autoimmune disorders, indicating that mTECs are essential for establishing central tolerance [69, 79-88, 94]. TRA expression displays a mosaic pattern, as each TRA protein is expressed in only 1-3% of mTECs so that the number and epitope density of TRAs can be optimized for presentation to SP thymocytes [9], likely through epigenetic mechanisms in which a single mTEC coexpress a set of TRA genes which are clustered in chromosomes and colocalized to nuclear subdomains [99].

A substantial fraction of TRAs is controlled by Aire [100], a nuclear protein predominantly expressed in mTECs. Aire-driven TRA expression is crucial for the negative selection of TRA-reactive SP thymocytes [101-103] and generation of $Foxp3^+$ Tregs [104-106] in the medulla. Complete deficiency of Aire gene results in autoimmune polyendocrinopathy syndrome type 1 (APS1) or autoimmune polyendocrinopathy-candidiasis-

ectodermal dystrophy (APECED) in humans [107, 108], and similar organ-specific autoimmune disorders in mice [100, 109, 110], indicating that Aire is essential for the establishment of self-tolerance. Recently, it has been demonstrated that mutations in the AIRE gene, including mutations with partial or dominant effects, are more frequently found than previously appreciated and may cause a variety of autoimmune manifestations in human populations [111]. The expression of Aire in mTECs is controlled by a cis-regulatory element that contains NF-kB-binding sites upstream of the Aire coding sequence [112, 113]. Aire expression is also related to sex, as males exhibit higher Aire expression in mTECs compared with females in humans and mice, and because androgen and estrogen exert promoting or inhibitory effects, respectively, on Aire expression in cultured mTECs [114, 115]. Such sex hormone regulation of Aire expression may contribute to the higher female susceptibility to autoimmune diseases that is widely reported.

The function of Aire in mTECs remains to be determined and in fact is controversial. A series of studies showed that Aire directly promotes the transcription of target TRA genes via transcriptional elongation and pre-mRNA processing (reviewed in [116]), while others have shown that Aire controls the differentiation program of mTECs that includes TRA expression. The latter model is supported by the evidence that Aire-deficient mice exhibit abnormal thymic medulla formation and mTEC development [68, 70, 72, 117-119]. They also display defective T cell tolerance against Aire-independent TRAs [103, 110], and Aire-driven genes include non-TRA proteins, such as cytokines, chemokines, MHC class II peptide-loading factors and proteases [103, 120-123]. Furthermore, it has been reported that more than half of the total TRAs are expressed by mTECs in an Aire-independent manner [100, 123-125].

A recent report identified the transcription factor Fezf2 as a regulator of TRA expression in mTECs [126]. Fezf2 is predominantly expressed by mTECs in the thymus and controls the expression of a substantial number of TRA genes, mostly Aire-independent ones, via direct binding to the promoters of target TRA genes. Mice deficient for Fezf2 in TECs exhibit autoimmune disorders in multiple peripheral organs, and the spectrum of autoimmunity in Fezf2-deficient mice is different from that in Aire-deficient mice. These data indicate that Fezf2 and Aire play non-redundant and mutually complementary roles in regulating TRA expression so as to ensure T cell tolerance. Fezf2-deficient mice exhibit a disorganized thymic medulla and reduced mTEC numbers, demonstrating the role of Fezf2 in regulating mTEC development. It is still unknown whether and how genetic variants or mutations of Fezf2 are associated with human autoimmune diseases, although Fezf2 is required for the development of neurons [127] and its mutations have been associated with autism [128, 129].

3.3 mTECs regulate Treg development

The process by which tolerance is induced by mTECs also involves the development of Foxp3$^+$ Treg cells, which are essential for protection from autoimmunity [130]. In mice deficient in mTEC development or mTEC expression of MHC class II, the thymic development of Foxp3$^+$ Treg cells is impaired [79, 80, 82, 131]. Studies using neo-self antigen transgenic mice showed the generation of Foxp3$^+$ Treg cells specific for the self-antigen expressed by Aire+ mTECs [104, 132]. Recently accumulating data have provided a link between mTEC TRA expression and the development of TRA-specific Tregs. Foxp3$^+$ Treg cells reactive to endogenous self antigens are generated in an Aire-dependent manner [105]. T cells infiltrating self-tissues in Aire-deficient mice expressed TCRs that were preferentially expressed by Foxp3$^+$ Treg cells in Aire-sufficient mice, suggesting that Aire directs self-reactive T cells into the Treg lineage [133]. The number of Foxp3+ Treg cells was shown to be significantly reduced in Fezf2-deficient mice, suggesting that Fezf2-dependent TRAs also contribute to Treg cell development [126].

Terminally differentiated mTECs form unique swirled epithelial structures, called 'Hassall's corpuscles', which may provide a microenvironment for the generation of Treg cells. Hassall's corpuscles produce thymic stromal lymphopoietin (TSLP), which activates thymic DCs in order to promote the differentiation of CD4SP thymocytes into Foxp3$^+$ Tregs [134].

mTECs may also provide intrathymic niches for Tregs. The number of thymic Foxp3+ Tregs is likely controlled by the mTEC cellularity and size of the medulla, as the thymus from OPG-deficient mice contains an increased number of Foxp3$^+$ Tregs [97]. The increased Foxp3$^+$ Tregs in the OPG-deficient thymus include a substantial number of recirculating Tregs that re-enter the thymus from the periphery [135].

3.4 mTECs regulate thymocyte migration

DP thymocytes that receive positive selection signals differentiate

into CD4SP or CD8SP thymocytes and express the chemokine receptor CCR7 on the cell surface [136]. The CCR7 ligand chemokines CCL19 and CCL21 are produced by mTECs [136] and medullary fibroblasts [137], and attract CCR7-expressing SP thymocytes from the cortex to the medulla [136, 138, 139]. During residence in the medulla, SP thymocytes are exposed to antigens presented by mTECs and DCs. CCR7-mediated medulla migration is required to ensure negative selection of self-reactive SP thymocytes [95, 140]. Indeed, mice deficient in CCR7 or CCR7 ligand chemokines exhibit organ-specific autoimmunity [139, 141, 142]. mTECs produce another chemokine, Xcl1, which mediates medullary accumulation of thymus-resident DCs that contribute to Treg development [122].

SP thymocytes that have completed developmental programs and repertoire selection are exported from the thymus into the circulation. This export is controlled by chemotactic signaling via sphingosine-1 phosphate (S1P) and its receptor S1P1. Mature SP thymocytes express a high level of S1P1 and then migrate toward a gradient of S1P [139, 143, 144] that is provided by neural crest-derived perivascular cells (pericytes) in the cortico-medullary junctions [145] and circulating blood [146].

4. Thymic DCs

The negative selection of self-reactive SP thymocytes and generation of $Foxp3^+$ Treg cells in the thymus require not only mTECs, but also thymic DCs. Thymic DCs are derived from hematopoietic precursor cells and predominantly localized in the medulla, with a small fraction sparsely localized in the cortex. Through direct presentation of endogenously expressed antigens and indirect 'cross-presentation' of antigens expressed by other cells, thymic DCs contribute to the induction of T cell tolerance against self-antigens, including Mtv-encoded superantigens, blood-borne antigens, and TRAs expressed by mTECs [147-151].

One report estimated that a substantial portion (approximately half) of Aire-dependent negative selection and Treg development is mediated by the cross-presentation of TRAs by thymic DCs [152]. This cooperation between mTECs and DCs might be mediated by unidirectional, intercellular transfer of mTEC-derived proteins to DCs [153]. These interactions among mTECs, DCs and CD4SP thymocytes require medullary accumulation of thymic DCs for optimal Treg cell induction which depends on the chemokine Xcl1 [122] as well as CCR7-mediated medulla migration of CD4SP thymocytes [154].

5. Thymic B cells

Recent studies have highlighted thymic B cells and their impact on T cell selection. It has been reported that the thymus in both humans and mice [155, 156] contains a small population of B cells (0.2-0.5% of the thymic cellularity) predominantly in the medulla [157]. Thymic B cells are derived from both intrathymic B lymphopoiesis [157, 158] and the immigration of peripheral B cells [159, 160], and phenotypically are classified as the B2-type 'mainstream' B cells. MHC class II and the co-stimulatory molecules CD80 and CD86 are highly expressed in thymic B cells [159, 160, 161], suggesting that these cells are capable of antigen presentation to developing SP thymocytes. Surprisingly, unlike peripheral B cells, thymic B cells express the Aire protein and a set of Aire-dependent TRAs, albeit at a low level [160]. As in the case of the development of $Aire^+$ mTECs, $CD80^+Aire^+$ thymic B cells are differentiated from $CD80^-Aire^-$ B cells, and this process requires CD40L-CD40 signaling mediated by cell-cell interaction with self-reactive CD4SP thymocytes. Thymic B cells can induce negative selection of CD4SP thymocytes reactive to cognate antigens under a variety of experimental conditions [159, 160, 162]. It is also suggested that thymic B cells contribute to the generation of thymic Tregs [163, 164]. Thus, thymic B cells might play a role in establishing central tolerance, such that CD4 helper T cells are made tolerant to B cell antigens presented in secondary or tertiary lymphoid tissues.

6. Thymic fibroblasts

In addition to TECs, neural crest-derived mesenchymal cells are a prominent component of non-hematopoietic stromal cells in the thymus. During thymic organogenesis, mesenchymal cells contribute to the development of the thymic rudiment [165, 166] and TEC proliferation [167], and enter the thymic rudiment to form the blood vessel architecture [168, 169]. In the adult thymus, mesenchymal cells are predominantly localized to the capsule and medulla. Medullary mesenchymal cells, characterized by specific markers such as ER-TR7, MTS15 and platelet-derived growth factor receptor (PDGFR) [137, 170], form a conduit-like structure that resembles the FRC network in secondary and tertiary lymphoid organs, so these cells are known as thymic FRCs [154]. Thymic FRCs highly express LTβR, IL-7, and chemokines such as CCL19 and CCL21 [96, 137], suggesting a role in regulating T cell development. Thymic FRCs also produce podoplanin (also called gp38), a mucin-like

membrane protein that forms an immobilized CCL21 gradient, which is likely important for the targeting of newly differentiated Treg cells to the medulla [154].

A recent study reported that fibroblast-specific protein 1 (FSP1, also known as S100a4) is a good marker of thymic fibroblasts [171]. Deletion of FSP1-expressing cells in FSP1-thymidine kinase transgenic mice resulted in a prominent reduction in mature mTECs, thymus atrophy and a marked delay of thymus regeneration after chemical-induced injury. These results suggest that FSP1-expressing thymic fibroblasts are critical for the maintenance and regeneration of mTECs. However, FSP1-driven cell depletion might be inappropriate for studying the specific roles of medullary FRCs, because FSP1 expression is detectable not only in medullary FRCs, but also in thymic capsular fibroblasts and mTECs. A characterization of these cell subsets based on intrathymic location and functional molecules will be required to better understand the significance of thymic fibroblasts. Whether and how thymic fibroblasts contribute to the medulla formation and T cell development are thus still open questions.

Conclusion

This report has reviewed the historical and more recent studies on thymic stromal cell subsets, including both the well-characterized TECs and as yet less well characterized non-TEC stromal cells, and also the most recently highlighted thymic antigen-presenting cells such as DCs and B cells. Accumulating evidence continues to reveal the cellular and molecular basis of thymic stromal cells, leading to advances in our understanding of how the thymic microenvironment supports T cell immunity. Based on such advances, several recent studies reported the in vivo reconstitution of the thymic microenvironment for producing functional and self-tolerant T cells [31, 172-174]. Such current and still emerging findings on the development, function, and reconstitution of thymic microenvironment will provide invaluable information toward insight for future therapeutic applications.

Conflict of Interest

The authors state no conflict of interest.

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