Toward Understanding How the Immune System Establishes a Diverse Yet Self-Tolerant T-Cell Repertoire: Stepwise Roles of Thymic Microenvironments

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Introduction

The thymus is an organ that supports the development and repertoire formation of T lymphocytes (1). Thymic parenchyma consists of leukocytic cells called thymocytes, the majority of which belong to the T-lymphoid lineage, and various stromal cells including thymic epithelial cells (TEC) (2). Thymic stromal cells provide multiple signals to support manifold processes of thymocyte development that are essential for the supply of circulating T lymphocytes (3). In response to these signals, developing thymocytes undergo proliferation, differentiation, and relocation to generate mature T lymphocytes that carry a diverse yet self-tolerant repertoire of T-cell antigen receptors (TCR) (4). These steps of T-lymphocyte development take place in anatomically discrete regions of the thymus where a variety of specialized stromal cells are localized (5).

T lymphocytes arise from hematopoietic stem cell-derived T-lymphoid progenitor cells that migrate to the thymus (6). Most immature hematopoietic cells that have just entered the thymus lack the expression of CD4 and CD8 and therefore belong to CD4/CD8 double-negative (DN) thymocytes (7, 8). The development of DN thymocytes is associated with the dynamic relocation of the cells in thymic parenchyma; T-lymphoid progenitor cells in adult mouse thymus are mostly localized in the corticomedullary junction, the area between deep cortex and medulla (9), whereas thymocytes migrate toward the capsular region of the thymus during differentiation and develop into CD4/CD8 double-positive (DP) thymocytes (10). DP thymocytes expressing TCR on the cell surface are localized in the cortex. DP thymocytes move actively within the cortical microenvironment (11, 12), probably seeking TCR interaction with major histocompatibility complex (MHC)-encoded molecules that are associated with self-peptides. Cortical DP thymocytes that interact via their TCR with the self-peptide–MHC complex are selected for survival or death depending on the avidity of the interaction (13, 14). DP thymocytes that receive TCR signals with ligand interactions of weak avidity and nonextensive aggregation are induced to survive

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and differentiate into mature thymocytes, the process referred to as positive selection (15, 16). By contrast, DP thymocytes that receive TCR signals with ligand interactions of strong avidity and extensive aggregation are destined to die (17, 18), a process referred to as negative selection. During positive selection, the differential kinetics of TCR–ligand interactions determines cell lineage to become either $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single-positive (SP) thymocytes (19). Positively selected thymocytes relocate to thymic medulla, where they further interact with self-peptides displayed in the medullary microenvironment (20). Medullary TEC (mTEC) express a diverse set of genes representing peripheral tissues (21), thereby contributing to the establishment of self-tolerance in thymic medulla. A nuclear factor called autoimmune regulator (AIRE) participates in this promiscuous gene expression in mTEC (22). Consequently, a diverse yet self-tolerant TCR repertoire is formed in the thymus, and mature T lymphocytes with such a TCR repertoire are released to the circulation. Thus, T-cell repertoire formation consists of stepwise fate determinations of thymocyte development in different thymic microenvironments. The dynamic relocation of developing thymocytes within thymic microenvironments is crucial for T-cell repertoire selection.

The aforementioned control of T-lymphocyte development in the thymus is supported in multiple thymic microenvironments that are formed by different sets of thymic stromal cells. Thymic stromal cells are composed of TEC and other stromal cells, including mesenchymal cells, endothelial cells, and hematopoietic cells, such as dendritic cells (DC) and macrophages (23). TEC consist of at least two major populations, cortical TEC (cTEC) and mTEC, which play crucial roles in the development and repertoire formation of T lymphocytes. cTEC and mTEC are derived from common progenitor cells that are generated from the endoderm of the third pharyngeal pouch (24–26). The development of TEC is dependent on many transcription factors, including Tbx1, Hoxa3, Pax1, and Foxn1 (27–30). Thymic mesenchymal cells are also crucial for supporting thymus generation and T-lymphocyte development (31, 32). It is also fascinating to note that thymic stromal cells that support T-lymphocyte development are supported, in turn, by developing thymocytes. The bidirectional signal exchanges between thymocytes and thymic stromal cells are appreciated as “thymic crosstalk” (33–35).

To understand how T lymphocytes are selected to establish a diverse yet self-tolerant repertoire in the thymus, we performed genome-wide screening for genes that are expressed in the thymus and that affect thymus development. We find that β5t-containing thymoproteasomes specifically expressed by cTEC play a pivotal role in the positive selection of CD8 SP thymocytes. We also find that positively selected thymocytes begin expressing CCR7, a chemokine receptor, and thereby relocate to thymic medulla where mTEC produce CCR7 ligands. Furthermore, we find that tumor necrosis factor (TNF) superfamily (TNFSF) ligands, including RANKL and CD40L, are produced by positively selected thymocytes and pivotally regulate mTEC development and thymic medulla formation. Thus, our study shows that sequential encounter of developing thymocytes with a unique set of self-molecules in the cortical microenvironment and another set of self-molecules in the medullary microenvironment is essential for the generation of a diverse yet self-tolerant repertoire of T lymphocytes.

Positive Selection in Thymic Cortex and β5t-Containing Thymoproteasomes

A number of studies support the notion that low-affinity self-peptides present in thymic cortex are responsible for the positive selection of developing thymocytes. However, the nature of positively selecting peptides and positively selecting antigen-presenting cells remains elusive (36). A single MHC–peptide complex expressed by cTEC can produce a diverse repertoire of T lymphocytes (37), suggesting that any peptide that causes low-avidity TCR engagement might be capable of triggering positive selection of thymocytes. It was also suggested that, rather than initial positive selection, subsequent negative selection establishes repertoire formation of T lymphocytes (38). Furthermore, it was shown that the experimental capability of inducing positive selection is not limited to cTEC but can be detected in fibroblasts and DC (39–41). It was also shown that developing thymocytes and thymus-reentering T lymphocytes can induce positive selection of thymocytes (42–45). Thus, the possibility that cTEC, or any other cells in the thymic cortex, have any specialized capability to induce positive selection seemed unlikely until recently.

Our recent discovery of a novel subunit of the 20S proteasome, β5t, has revealed a unique capability of cTEC to support the positive selection of thymocytes (46). Proteasomes are multicatalytic protease complexes that are responsible for regulated proteolysis in eukaryotic cells and for the generation of antigenic peptides presented by class I MHC molecules (47, 48). The 20S proteasome is responsible for the proteolytic activity of the proteasome and is composed of 28 subunits (two α-rings with α1 to α7 subunits and two β-rings with β1 to β7 subunits). Among these subunits, β1, β2, and β5 are responsible for caspase-like, trypsin-like, and chymotrypsin-like catalytic activities, respectively (49, 50). Interferon-γ induces the production of a new set of catalytic subunits, β1i, β2i, and β5i, to replace their constitutive counterparts, β1, β2, and β5, thereby forming immunoproteasomes, a proteasome complex that possesses increased chymotrypsin-like activity and participates in efficient antigen presentation and immune responses (51). On the other hand, the newly identified catalytic subunit β5t is incorporated into the 20S proteasome instead of β5 or β5i, together with β1i and β2i (46). Because this novel proteasome containing β5t is specifically expressed in the thymus and exclusively in cTEC, it is termed a thymoproteasome.

In comparison to β5-containing standard proteasomes and β5i-containing immunoproteasomes, β5t-containing thymoproteasomes exhibit reduced chymotrypsin-like activity but normal caspase-like activity and normal trypsin-like activity (46). Proteasomes are responsible for the production of MHC class I-binding peptides and are the sole enzymes that determine the C-termini of the peptides (52, 53). Hydrophobic C-terminal anchor residues of the peptides are essential for high-affinity peptide binding into the clefts of MHC class I complexes (54). Chymotrypsin-like activity carried by β5 and β5i is important for the production of high-affinity MHC class I ligands (55). Thus, it is possible that cTEC generate a unique set of MHC class I-associated peptides that are different from those present in any other cells.

The generation of $CD4^-$ $CD8^+$ (CD8SP) thymocytes that express high levels of TCR is severely reduced in β5t-deficient mice. The selective reduction of CD8SP

T lymphocytes is also observed in the spleen of these mice. On the other hand, DP and CD4SP thymocytes as well as peripheral CD4 T lymphocytes are unaffected. The absence of β5t does not affect cortical or medullary architecture or overall thymus size, indicating that β5t is essential for neither the development of cTEC nor the generation of normal thymic architecture. These results demonstrate that β5t is required for the development of CD8SP T lymphocytes in the thymus and suggest the possibility that β5t is associated with positive selection of CD8SP T lymphocytes (46).

It is possible that thymoproteasomes in cTEC may be somehow involved in providing costimulatory signals that are specifically required for the generation of CD8SP T lymphocytes rather than CD4SP T lymphocytes. However, since the generation of CD8SP T lymphocytes is specifically affected in β5t-deficient mice, and as the surface expression of MHC class I molecules on cTEC of β5t-deficient mice is comparable to that of normal mice, β5t-containing thymoproteasomes in cTEC are likely involved in producing class I MHC-loaded peptides that provide TCR signals required for positive selection of class I MHC-restricted CD8SP T lymphocytes (46). β5t specifically limits chymotryptic activity that cleaves peptide bonds after hydrophobic amino acid residues in cTEC, and therefore, thymoproteasomes predominantly produce low-affinity class I MHC ligands specifically in cTEC. These low-affinity class I MHC ligands may limit the duration and/or avidity of interaction with TCR and contribute to inducing positive selection of the majority of CD8SP T lymphocytes (46, 56, 57).

The results from β5t-deficient mice reveal that cTEC possess unique protein degradation activity that might lead to the production of a unique set of class I MHC-associated peptides necessary for the generation of CD8 T lymphocytes. This unique protein degradation activity of cTEC might not be limited to class I MHC-associated peptides but might also occur in class II MHC-associated peptides, because cathepsin L, a lysosomal protease that is highly expressed by cTEC and not mTEC (58), is required for the optimal generation of CD4SP T lymphocytes (59, 60). A unique lysosomal degradation activity might be functional in cTEC, which is mediated by cathepsin L in a manner analogous to thymoproteasomes. Thus, the unique character of cTEC protein degradation and self-peptide presentation may be pivotal for the positive selection of thymocytes in both CD4SP and CD8SP lineages (56, 57). These findings of cTEC would not only highlight the significant roles of thymic cortex in T-lymphocyte development but also further our understanding of the molecular mechanisms of T-lymphocyte repertoire selection.

CCR7 Regulates Relocation of Positively Selected Thymocytes to Medulla

Newly generated immature DP thymocytes move randomly in the thymic cortex, as shown by in vitro real-time imaging of intact thymus lobes (11, 61). This “random walk motility” is also observed by noninvasive intravital imaging of thymocytes in the fish thymus (12). On the other hand, immature thymocytes at early ontogeny

before CD4 and CD8 expression appear dormant in the thymus (12). Thus, the differentiation into DP thymocytes possibly coincides with the acquisition of cellular motility, which represents the activity of cells that dynamically seek TCR interaction with the MHC–peptide complex in the cortical microenvironment. DP thymocytes that actively move within the cortex interact with cTEC and pause their motility upon TCR interaction with MHC–peptide ligands (62).

Positively selected thymocytes are induced to survive and to relocate from the cortex to the medulla. Positive selection coincides with the appearance of a thymocyte population that displays rapid and directed migration toward the medulla (11). Upon positive selection, DP thymocytes elevate CCR7 expression on the cell surface (63–65). CCR7 ligands, CCL19 and CCL21, in the thymus are predominantly expressed by mTEC and mostly localized in the medulla (65). Consequently, positively selected thymocytes are attracted to the medulla through CCR7-mediated chemotaxis. We found that positively selected thymocytes in mice deficient for CCR7 or CCR7 ligands are defective in accumulation in the medulla and are localized in the cortex (65, 66). Mice deficient for CCR7 signals exhibit autoimmunity to peripheral tissues (66–68). Thymocytes generated without CCR7 ligands are potent in inducing autoimmune exocrinopathy in mice, and thus are defective in establishing central tolerance (66). Therefore, the migration of positively selected thymocytes to the medulla is essential for the establishment of central tolerance, and the proper relocation of developing thymocytes within multiple thymic microenvironments is necessary for repertoire formation of T lymphocytes (20, 66).

TNFSF Regulation of Medulla Formation

The microenvironment of thymic medulla is mainly composed of mTEC and hematopoietic cells, including mature thymocytes and DC. Similar to cTEC, mTEC are derived from endodermal precursor cells that are generated at the third pharyngeal pouch (25, 26, 69). Thymic medulla is a specialized microenvironment where developing thymocytes establish tolerance to systemic self-antigens, including peripheral tissue-specific antigens. Indeed, T-lymphocyte development within the thymus that is defective in thymic medulla formation leads to failure in establishing self-tolerance, resulting in autoimmune disorders (70–72). In the thymic medulla, mTEC express a variety of "tissue-specific" genes, for example, the gene encoding insulin whose expression is restricted to β-islet cells in the pancreas (73–76). This "ectopic" expression of tissue-specific genes by mTEC is called "promiscuous gene expression" and is responsible for the establishment of self-tolerance through the presentation of tissue-specific antigens to developing thymocytes (21, 77, 78). AIRE, a nuclear protein that is predominantly expressed by mTEC, is associated with the promiscuous gene expression of mTEC (22, 79–82).

The formation of thymic medulla is associated with the development of mature thymocytes in the thymus. An early study showed that medulla formation is defective in *scid* mice, in which thymocyte development is arrested at the DN stage and is restored by reconstitution with wild-type hematopoietic cells (33). Studies of mice

deficient for positive selection, including TCRα-deficient mice, ZAP70-deficient mice, and MHC class I and class II double-deficient mice, confirmed that the formation of thymic medulla is dependent on the generation of positively selected mature thymocytes (83–86). In mice deficient for CCR7 or CCR7 ligands, medulla formation is mildly defective (65, 66), suggesting that optimum development of thymic medulla requires the relocation of positively selected thymocytes. Thus, signals produced by positively selected thymocytes crucially regulate mTEC development and medulla formation.

We recently identified that positive selection promotes mTEC proliferation and thereby nurtures the formation of thymic medulla (87). We found that positively selected thymocytes express RANKL, a TNFSF member ligand (87). The number of mTEC is reduced in mice deficient for RANKL, and the enforced expression of RANKL in mice deficient for positive selection restores mTEC cellularity and medulla formation, indicating that RANKL mediates thymic crosstalk signals for the optimal formation of thymic medulla (87). Osteoprotegerin, a naturally occurring soluble RANKL inhibitor that binds to RANKL and inhibits RANKL binding to its signaling receptor RANK, is expressed by mTEC and regulates mTEC development and medulla formation, because mice deficient for osteoprotegerin exhibit hypercellularity of mTEC and an enlarged thymic medulla (87). The RANKL receptor, RANK, is more strongly expressed in mTEC than in cTEC and is required for the development of mTEC expressing AIRE (87, 88). RANKL is also expressed by $CD4^+$ $CD3^-$ lymphoid tissue inducer (LTi) cells and is involved in the differentiation of mTEC during embryogenesis (88–90).

Positively selected thymocytes also produce CD40L, another TNFSF member ligand (87, 91). In contrast to RANKL, CD40L is not expressed in thymic LTi cells (87). The enforced expression of CD40L in vivo produces an enlarged thymic medulla, suggesting that CD40L signals can promote thymic medulla formation (92, 93). Analysis using fetal thymus organ culture shows that CD40L as well as RANKL facilitates mTEC development through classical and nonclassical NF-κB pathways (94). Mice deficient for both CD40 and RANKL exhibit severe defect in mTEC development and thymic medulla formation, whereas the single deficiency of CD40 causes only a mild defect in thymic medulla (94). Thus, it is likely that RANKL and CD40 cooperate with each other to promote mTEC development. It is also possible that RANKL and CD40 may sequentially regulate mTEC development; RANKL produced by LTi cells plays a role in mTEC development during embryogenesis, whereas RANKL and CD40L produced by positively selected mature thymocytes essentially promote postnatal increase of mTEC cellularity (87, 94).

Concluding Remarks

The fact that the thymus contains at least two distinct microenvironments, the cortex and the medulla, has been known for more than 100 years (95–98). Studies conducted over a span of 40 years since the discovery of the immunological function of the

thymus have remarkably advanced our understanding of lymphocyte biology in relationship to T-lymphocyte development and selection in the thymus. However, the developmental and molecular biology of thymic stromal cells has remained quite vague until recently. Recent achievements have enabled us to study the biology of thymic microenvironments using several molecules that represent key functions of thymic stromal cells. Those molecules include Foxn1, Delta-like ligands, AIRE, and β5t. Several molecules that mediate the development of thymic stromal cells, such as RANKL and CD40L, have also been identified. These outcomes should provide a solid foundation for further studies of thymic microenvironments to better understand the molecular mechanism of the development and repertoire formation of T cells and the therapeutic reconstitution of functional thymus for various clinical situations (99, 100). Unveiling of the molecular identities of thymic microenvironments has just begun.

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