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REVIEW

Thymoproteasome: Role in Thymic Selection and Clinical Significance as a Diagnostic Marker for Thymic Epithelial Tumors

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Abstract The thymoproteasome is a specialized type of proteasomes expressed exclusively in the thymic cortex. It has a unique catalytic subunit β5t with unusual enzymatic activity. The thymoproteasome exhibits lower chymotrypsin-like activity than other forms of proteasomes such as constitutive proteasomes and immunoproteasomes. Its cleavage specificity appears uniquely suited for the production of peptides that mediate positive selection of $CD8^+$ T cells. Similar to major histocompatibility complex molecules and T/B-cell receptors, the thymoproteasome occurs only in jawed vertebrates, suggesting that it evolved concomitant with the cardinal elements of adaptive immunity. β5t can be used as a marker in the differential diagnosis of thymic tumors. It is expressed in most type B and some type AB thymomas, but not in type A thymoma, thymic carcinoma, or tumors of non-thymic epithelial origin.

Keywords Thymoproteasomes · β5t · Thymus · Positive selection · Thymoma · Thymic carcinoma

Abbreviations

cTEC	Cortical thymic epithelial cell
DC	Dendritic cell
IFN-γ	Interferon-γ
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cell
TAP	Transporter associated with antigen processing
TCR	T-cell receptor

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Introduction

The proteasome constitutes the central proteolytic component of the ubiquitin–proteasome system required for the maintenance and regulation of various cellular processes (Coux et al. 1996; Tanaka et al. 2012). In the immune system, proteasomes are responsible for the generation of peptides presented by major histocompatibility complex (MHC) class I molecules (Kloetzel 2001; Rock and Goldberg 1999; Tanaka and Kasahara 1998). To date, three types of proteasomes have been identified in jawed vertebrates; the constitutive or housekeeping proteasome highly conserved from yeast to man; the immunoproteasome, which is induced by stimulation with interferon (IFN)-γ in most tissues, but is constitutively expressed in immune tissues such as spleen and thymus (Tanaka and Kasahara 1998); and the thymoproteasome expressed exclusively in the thymic cortex (Murata et al. 2007; Tomaru et al. 2009). Accumulated evidence indicates that the latter two forms of proteasomes play important roles in adaptive immunity. The immunoproteasome efficiently produces antigenic peptides capable of binding to MHC class I molecules, thus facilitating elimination of virus-infected and tumor cells by cytotoxic T cells (Tanaka and Kasahara 1998). On the other hand, the thymoproteasome plays a crucial role in positive selection of $CD8^+$ T cells (Murata et al. 2008; Nitta et al. 2010; Takahama et al. 2010, 2012).

In the thymus, positive and negative selection regulates the development of T cells; both processes involve the recognition of self-peptides bound to self-MHC molecules (Klein et al. 2009). Positive selection is the process by which developing T cells expressing T-cell receptors (TCRs) that bind to self-MHC molecules are rescued from apoptosis; this process is mediated mainly by MHC molecules expressed on cortical thymic epithelial cells

(cTECs). Negative selection, the process by which developing T cells expressing self-reactive TCRs undergo apoptosis, is mediated mainly by MHC molecules expressed on medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) that present self-peptides possibly encountered by T cells in the periphery. mTECs express a diverse set of peripheral tissue antigens; this phenomenon known as promiscuous gene expression is partly controlled by the autoimmune regulator gene (Kyewski and Klein 2006). Notably, the majority of proteasomes expressed in mTECs and medullary DCs are immunoproteasomes (Nil et al. 2004), whereas the majority of proteasomes expressed in cTECs are thymoproteasomes (Murata et al. 2008; Nitta et al. 2010; Takahama et al. 2010, 2012). Therefore, the differential distribution of the two forms of proteasomes may lead to the production of differential sets of MHC class I-binding peptides in cortex and medulla, thereby influencing positive and negative selection of $CD8^+$ T cells.

This review will provide a brief overview of proteasomal antigen presentation, focusing on the role of the thymoproteasome in thymic selection. We also discuss the diagnostic significance of a thymoproteasome-specific subunit as a marker for thymic tumors.

Proteasome and Antigen Processing

The recognition of non-self antigens by T cells is a crucial process in launching adaptive immune responses. Cell surface-expressed MHC class I molecules present antigenic peptides that can be specifically recognized by cytotoxic T cells (Rock and Goldberg 1999). Recognition fully depends on the binding of antigenic peptides into the groove of MHC class I molecules (Falk et al. 1991; Rammensee et al. 1995). The proteasome has an essential role in the generation of peptides presented by MHC class I molecules (Kloetzel 2001; Tanaka and Kasahara 1998). Indeed, inhibition of proteasome activity abolishes MHC class I antigen presentation almost completely (Rock et al. 1994; Schwarz et al. 2000). Peptides generated by proteasomes are transported into the lumen of the endoplasmic reticulum by the transporter associated with antigen processing (TAP), where they dock to the peptide-binding groove of the MHC class I molecule (Kloetzel 2001; Momburg and Hämmerling 1998; Reits et al. 2000; Rock et al. 1994) (Fig. 1).

The 26S proteasome, the active form of the proteasome, is composed of the 20S core complex, also known as the 20S proteasome, and two 19S regulator complexes that are responsible for the activation of the 20S core complex as well as for the binding and unfolding of ubiquitinated substrates (Glickman et al. 1998; Voges et al. 1999). Proteolysis is conducted in the 20S core complex composed of 14 non-identical subunits that form four stacked rings of seven subunits each, $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ (Baumeister et al. 1998; Tanaka 2009). The proteolytic activity is exerted by three of the β subunits, β1, β2, and β5, which have caspase-like (cleavage after acidic residues), trypsin-like (cleavage after basic residues), and chymotrypsin-like (cleavage after hydrophobic residues) activities, respectively (Fig. 2).

There are at least two possible reasons why proteasomes emerged as the main protease generating MHC class I-binding peptides. First, the carboxyl-terminal hydrophobic residues are, in most cases, the most important anchor residues for binding to MHC class I molecules, and the chymotrypsin-like activity of the β5 subunit is responsible for the production of peptides that carry carboxyl-terminal hydrophobic residues (Rock and Goldberg 1999). Second,

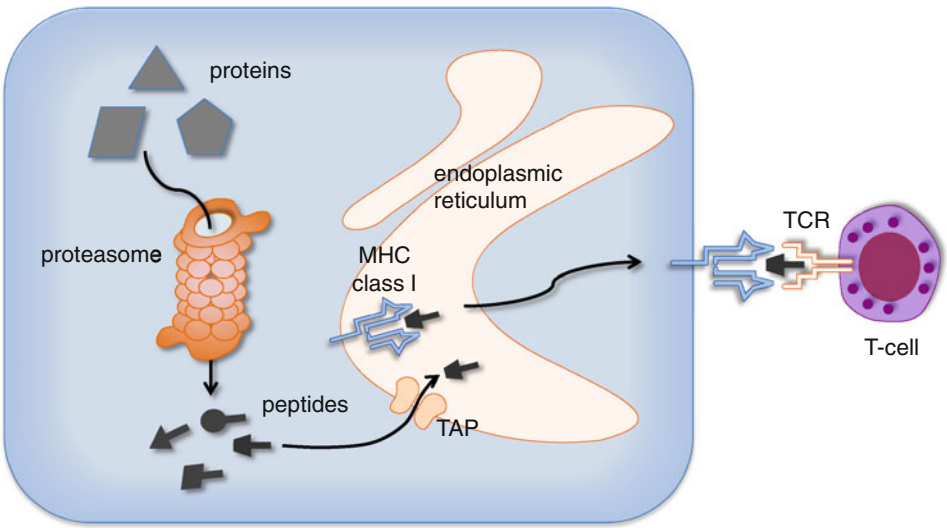

Fig. 1 Overview of antigen presentation. Proteasomes degrade self- and non-self-proteins into peptides. Peptides thus generated are transported into the lumen of the endoplasmic reticulum by TAP. They are then loaded onto MHC class I molecules, and the peptide-loaded MHC class I molecules are transported to the cell surface. $CD8^+$ T cells recognize peptide/MHC class I complexes by their TCRs

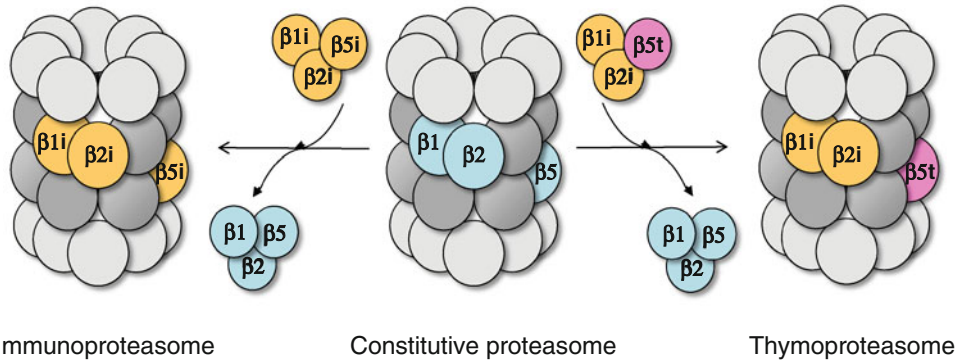

Fig. 2 Three types of 20S proteasomes. The constitutive proteasome conserved from yeast to human contains three catalytically active β subunits: β1, β2, and β5. IFN-γ stimulation induces the production of three subunits, β1i, β2i, and β5i, which are preferentially incorporated into 20S proteasomes, producing the immunoproteasome. In thymic cortex, a unique catalytic subunit β5t is expressed; this subunit is incorporated into 20S proteasomes in place of β5 or β5i, forming the thymoproteasome composed of three catalytically active subunits: β1i, β2i, and β5t

proteasomes generate peptides of 8–10 amino acids in length and thus of a size suited for transport by TAP and binding to MHC class I molecules (Kisselev et al. 1999).

Upon IFN-γ stimulation, a specialized type of 20S proteasomes is induced, in which β1, β2, and β5 subunits are replaced by three catalytically active, IFN-γ-inducible β subunits β1i, β2i, and β5i, respectively (Kloetzel 2004; Tanaka and Kasahara 1998) (Fig. 2). Immunoproteasomes have strong chymotrypsin-like activity required for MHC class I ligand production. In addition to the chymotrypsin-like activity, trypsin-like activity also plays a role in MHC class I ligand generation, because some basic residues can also serve as anchor residues for binding to MHC class I molecules (Kloetzel 2004). The immunoproteasome exhibits increased chymotrypsin-like as well as trypsin-like activities. This property of the immunoproteasome favors the production of MHC class I-binding peptides, thereby increasing the efficiency of antigen presentation in cells harboring this type of proteasome. Phylogenetically, immunoproteasomes occur only in jawed vertebrates, indicating that they originated from more ancient constitutive proteasomes concomitant with the emergence of MHC- and T/B-cell-receptor-based adaptive immunity (Kandil et al. 1996). It is thought that, along with many other genes involved in this form of adaptive immunity, the genes coding for β1i, β2i, and β5i subunits were formed through two rounds of whole-genome duplication (Flajnik and Kasahara 2010; Kasahara et al. 1996).

Structure, Expression, and Evolution of Thymoproteasomes

While searching for proteasome-related genes in genome databases, a gene, the product of which is closely related to β5 and β5i subunits, was identified in mice and humans (Murata et al. 2007). The gene coding for this subunit has a single exon in both species, and is expressed exclusively in the thymus (Murata et al. 2007). This subunit, named β5t, is incorporated into 20S proteasomes together with β1i and β2i (Fig. 2). Sequence alignment of β5t with β5 and β5i suggested that β5t has a unique peptidase activity different from β5 and β5i (Murata et al. 2007). The peptidase specificity of proteasomes is determined by the structure of the S1 pocket (Groll et al. 1997; Unno et al. 2002); the S1 pockets of β5 and β5i are mostly composed of hydrophobic amino acids, whereas that of β5t is mostly composed of hydrophilic amino acids. Indeed, β5t-containing thymoproteasomes were shown to display only weak chymotrypsin-like activity but comparable caspase-like and trypsin-like activities, in comparison to constitutive proteasomes and immunoproteasomes (Murata et al. 2007). When the β5t subunit was discovered, it was unclear whether it could actively participate in protein degradation to produce unique peptides, or whether it could act as a catalytically inactive bystander. However, assays using activity-based proteasome probes demonstrated that β5t is a catalytically active subunit. The substrate preference of β5t is biased towards peptides with hydrophilic side chains, which is distinct from that of β5 or β5i (Florea et al. 2010). It is, therefore, reasonable to assume that a repertoire of peptides generated by thymoproteasomes will be different from that generated by constitutive proteasomes or immunoproteasomes.

The thymoproteasome is characterized by its pattern of expression; in mice, β5t is expressed exclusively in cTECs responsible for positive selection of developing T cells (Murata et al. 2007) (Fig. 3). The majority of proteasomes present in mouse cTECs is the thymoproteasome, with constitutive proteasomes and immunoproteasomes occupying only a small fraction. β5t expression is strictly restricted to cTECs both in the postnatal and embryonic

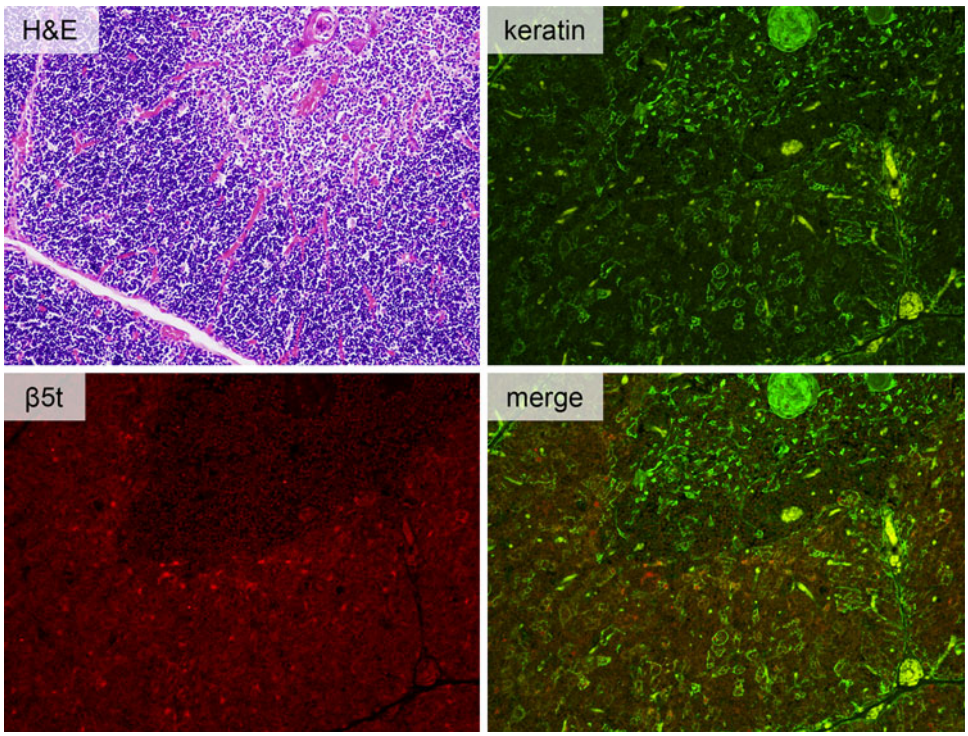

Fig. 3 Expression of the β5t subunit in the mouse thymus. The *left upper panel* shows H&E staining of neonatal thymic tissues. Immunofluorescence staining of thymic tissues demonstrates that β5t-expressing cells are located only in the cortex (*left lower panel*), and that most of the β5t-expressing cells are cytokeratin-positive cTECs

periods, suggesting the involvement of thymoproteasomes in the development of cTECs. By contrast, the autoimmune regulator gene, which is strongly expressed in mTECs but not in cTECs (Anderson et al. 2002), is expressed at lower levels in non-thymic immune tissues such as lymph nodes, fetal liver, and spleen as well as in embryonic stem cells (Nishikawa et al. 2010). The forkhead transcription factor Foxn1 responsible for the nude mutation (Nehls et al. 1994) is a pivotal regulator of thymic epithelium lineage development. The expression of β5t in cTECs is dependent on Foxn1 (Ripen et al. 2011), suggesting that Foxn1 contributes to the regulation of β5t transcription either directly or indirectly via regulation of cTEC development. On the contrary, β5t expression in cTECs is independent from the RelB-regulated formation of thymic medulla or from the development of thymocytes (Ripen et al. 2011). Like its mouse counterpart, human β5t is expressed exclusively in thymic cortex and is incorporated into proteasomes with β1i and β2i, forming 20S proteasomes with subunit compositions characteristic of thymoproteasomes (Tomaru et al. 2009). Notably, human β5t appears to be expressed also in some cortical DCs (Tomaru et al. 2009).

Similar to immunoproteasomes, thymoproteasomes are unique to jawed vertebrates (Boehm 2009; Flajnik and Kasahara 2010; Kasahara et al. 2008; Takahama et al. 2010). However, unlike immunoproteasome-specific subunit genes that most likely arose by whole-genome duplication, the gene coding for β5t appears to have emerged by tandem duplication from the β5-encoding gene (Sutoh et al. 2012).

Roles of Thymoproteasomes in T-Cell Selection

Analysis of β5t-deficient mice showed that the generation of CD8 single-positive thymocytes was severely impaired, suggesting that the thymoproteasome is involved in the development of $CD8^+$ T cells in the thymus (Murata et al. 2007). The number of $CD4^+CD8^+$ and $CD4^+CD8^-$ T cells as well as other lineages of immune cells, including $\gamma\delta^+$ T cells, $CD8\alpha\alpha^+$ intraepithelial lymphocytes, natural killer cells, natural killer T cells, B cells, DCs, and macrophages, was not altered in β5t-deficient mice (Murata et al. 2007; Nitta et al. 2010). Neither the cellularity of cTECs nor the corticomedullary architecture of the thymus was affected, indicating that impaired generation of CD8 single-positive thymocytes was not due to defective cTEC development. In female transgenic mice carrying an MHC class I-restricted male-antigen-specific TCR gene, the generation of CD8 single-positive thymocytes was severely impaired in the absence of β5t (Nitta et al. 2010). Furthermore, although surface expression levels of MHC class I molecules were not affected, cTECs displayed self-peptides not typically presented by MHC class I molecules in wild-type mice (Murata et al. 2007; Nitta et al. 2010). These observations strongly suggest that the thymoproteasome produces a set of MHC class I-binding peptides required for positive

selection of $CD8^+$ T cells. Interestingly, the number of $CD8^+$ T cells in β5t-deficient mice was decreased by 70–80 % as compared to that in normal mice, but $CD8^+$ T cells were not completely depleted (Murata et al. 2007; Nitta et al. 2010). Thus, the thymoproteasome is required for positive selection of the majority, but not all, of the $CD8^+$ T cell repertoire.

In thymic selection, T-cell progenitors are positively selected upon interaction with cTECs in the cortex, and after migration into the medulla, they encounter β5i-expressing mTECs and DCs, both potent inducers of negative selection (Fig. 4). Self-peptides displayed on cTEC-expressed MHC class I molecules are derived mainly from the cleavage products of thymoproteasomes and partly from those of constitutive proteasomes (Murata et al. 2007; Nil et al. 2004), whereas those displayed by mTEC and DCs are mainly produced by immunoproteasomes. Thus, self-peptides presented to the thymocytes presumably differ significantly between cortex and medulla. By contrast, the majority of proteasomes in cTECs are immunoproteasomes in β5t-deficient mice (Nitta et al. 2010). Thus, in these mice, self-peptides presented to the thymocytes are presumably similar between cortex and medulla (Hogquist and Xing 2010).

A decrease in $CD8^+$ T cells may occur in β5t-deficient mice because efficient thymic selection requires the presentation of distinct sets of peptides between cortex and medulla (Hogquist and Xing 2010; Klein et al. 2009). However, mice forced to express a single peptide/MHC class I complex on all cells in the body show a decreased number of $CD8^+$ T cells, but $CD8^+$ T cells in such mice express a diverse TCR repertoire. Thus, the differential display of peptides in cortex and medulla is not an absolute requirement for thymic selection (Wang et al. 2009). Besides contributing to the differential display of peptides in cortex and medulla, β5t may facilitate the display of unique peptide/MHC class I complexes on cTECs, such that in their absence, positive selection itself is impaired (Hogquist and Xing 2010; Murata et al. 2008; Takahama et al. 2008, 2010, 2012). According to the classic affinity/avidity model of thymic selection, peptides causing low-avidity TCR engagement are thought to trigger positive selection of thymocytes (Fukui et al. 1997; Ignatowicz et al. 1996). The thymoproteasome with reduced chymotrypsin-like activity may generate low-affinity MHC class I-binding peptides that provide low-avidity interactions between self-peptide/MHC class I complexes and TCRs (Murata et al. 2007, 2008; Takahama et al. 2008, 2010, 2012).

A Mouse Model with Aberrant Expression of β5t

Since physiological expression of β5t is localized to the thymic cortex, we hypothesized that aberrant expression of β5t in vivo might cause the development of immunologic abnormalities. To test this hypothesis, we have generated a transgenic mouse model with ubiquitous expression of β5t

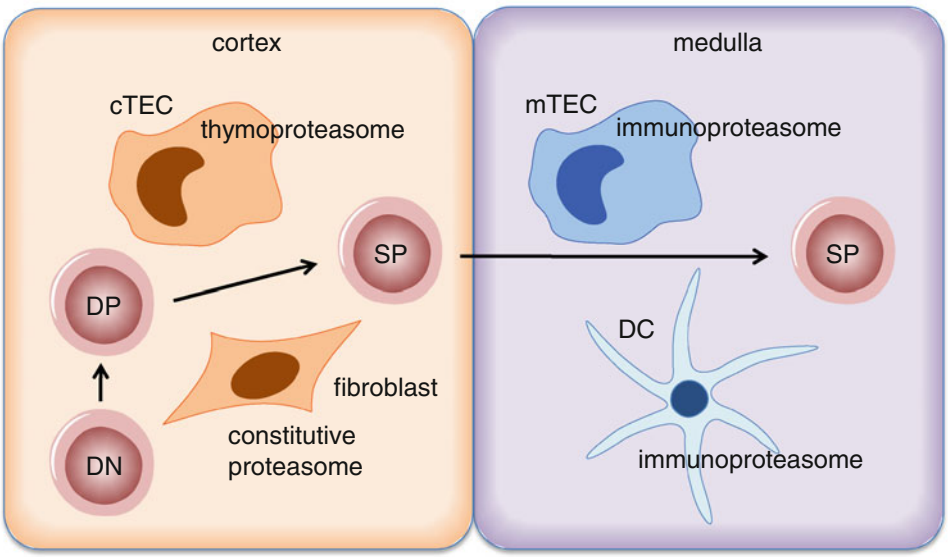

Fig. 4 Roles of proteasomes in thymic selection. Positive selection is mediated predominantly by cTECs expressing thymoproteasomes. According to the classic affinity/avidity model of thymic selection, low-affinity interactions between TCR and self-peptide/MHC class I complexes in thymic cortex are thought to induce the survival of double-positive (DP) cells and their differentiation to single-positive (SP) cells. Negative selection is mediated by mTECs and DCs, both expressing immunoproteasomes. High-affinity TCR interactions with self-peptide/MHC class I complexes induce deletion of self-reactive thymocytes. *DN* double-negative thymocytes

(Tomaru et al. 2012a). In transgenic mice, the CD4/8 ratio was increased in peripheral blood mononuclear cells and thymocytes. However, these mice developed neither overt autoimmunity nor immunodeficiency (unpublished data). Intriguingly, the transgenic mice exhibited a shortened lifespan and age-related phenotypes. Forced ubiquitous expression of β5t decreased a proportion of β5-containing constitutive proteasomes and β5i-containing immunoproteasomes in most tissues, leading to a systemic decrease in proteasomal chymotrypsin-like activity. Therefore, the aforementioned phenotypes of the transgenic mice were thought to result from the altered protein degradation caused by the decreased chymotrypsin-like activity. Proteasomal activity is decreased with age (Gaczynska et al. 2001; Tonoki et al. 2009). Because the proteasome plays an essential role in cellular processes, an age-associated decline in proteasome function has been widely assumed to contribute to the development of age-related metabolic disorders and to the aging process itself (Dahlmann 2007). Consistent with this assumption, when the transgenic mice were fed a high-fat diet, they were more prone to develop age-related metabolic disorders such as obesity and hepatic steatosis as compared to wild-type mice.

β5t as a Diagnostic Marker for Thymic Epithelial Tumors

Tumors of the thymus account for less than 1 % of all neoplasms; however, they encompass entities with a broad spectrum of different morphologic and clinical features (Rosai 1999). Thymomas are neoplasms arising from or exhibiting differentiation towards TECs, with a variable number of non-neoplastic lymphocytes. Thymic carcinomas are malignant epithelial tumors with overt cytological atypia, almost invariable invasiveness, and lack of organotypic features; they show a worse outcome with poor survival and increased recurrence in comparison with thymomas (Müller-Hermelink et al. 2004). In 1999, the World Health Organization published a classification of thymic epithelial tumors, which divided the tumors into types A, AB, B1, B2, B3, and C, on the basis of the histological assessment of the morphology, the degree of cytological atypia of neoplastic epithelial cells, and the relative amount of a non-neoplastic lymphocytic component (Rosai 1999). Subsequently, the term "type C thymoma" was replaced by "thymic carcinoma" in the revised World Health Organization classification (Müller-Hermelink et al. 2004).

Thymoma and thymic carcinoma often cause a diagnostic problem due to their rarity and their great variability in the cytological and structural patterns. Much effort has been made to search for markers specific for cTECs or mTECs. However, until recently, there were no reliable markers. Also, no thymus-specific marker was available that could help distinguish thymic carcinoma from type B3 thymoma. Previously, CD5, c-kit, and glucose transporter 1 were reported as useful markers for thymic carcinoma (Hishima et al. 1994; Kojika et al. 2009; Nakagawa et al. 2005; Pan et al. 2004). However, these markers are not thymus-specific, and are expressed in various normal and neoplastic cells. Foxn1 and CD205 are thymus-expressed molecules important for thymic organogenesis and positive selection of thymocytes, respectively (Small and Kraal 2003; Su et al. 2003). However, they are expressed in some malignant tumors of non-thymic origin including lung carcinomas and can be expressed in both thymoma and thymic carcinoma (Nonaka et al. 2007). Our recent work showed that β5t is expressed in most cases of type B thymoma and less frequently in type AB thymomas (thymoma characterized by a variable mixture of type A and type B thymoma components), but not in type A thymomas or thymic carcinomas (Yamada et al. 2011), demonstrating that immunostaining for β5t is useful for the differential diagnosis of various types of thymomas and thymic carcinomas (Fig. 5). Importantly, expression of β5t was not observed in tumors arising from non-thymic tissues, indicating that β5t is a specific marker for thymoma (Yamada et al. 2011).

Thus far, morphologic classifications of thymic epithelial tumors have been approached from a variety of viewpoints. For example, thymic epithelial tumors were classified based on the histogenesis of neoplastic epithelial cells into medullary thymoma, mixed thymoma, predominantly cortical thymoma, cortical thymoma, well-differentiated thymic carcinoma, and high-grade thymic carcinoma (Kirchner et al. 1992; Marino and Müller-Hermelink 1985). These subtypes of tumors are thought to correspond to type A thymoma, type AB thymoma, type B1 thymoma, type B2 thymoma, type B3 thymoma, and thymic carcinoma, respectively (Müller-Hermelink et al. 2004). Our observation that β5t is expressed in most type B and some type AB thymomas, but not in type A thymoma (Yamada et al. 2011) supports this interpretation and indicates that β5t is a reliable marker for detecting neoplastic epithelial cells of type B component differentiating towards cTECs.

Although relatively rare, thymomas can arise in non-thymic tissues such as neck, chest wall, pleura, lung, and heart (Yan et al. 2010). These ectopic thymomas are thought to arise from the remnants of thymic tissues that failed to properly descend into the anterior mediastinum or migrated aberrantly during embryonic development (Chan and Rosai 1991). Our recent work showed that β5t is also a useful marker for the diagnosis of cervical ectopic thymoma (Tomaru et al. 2012b).

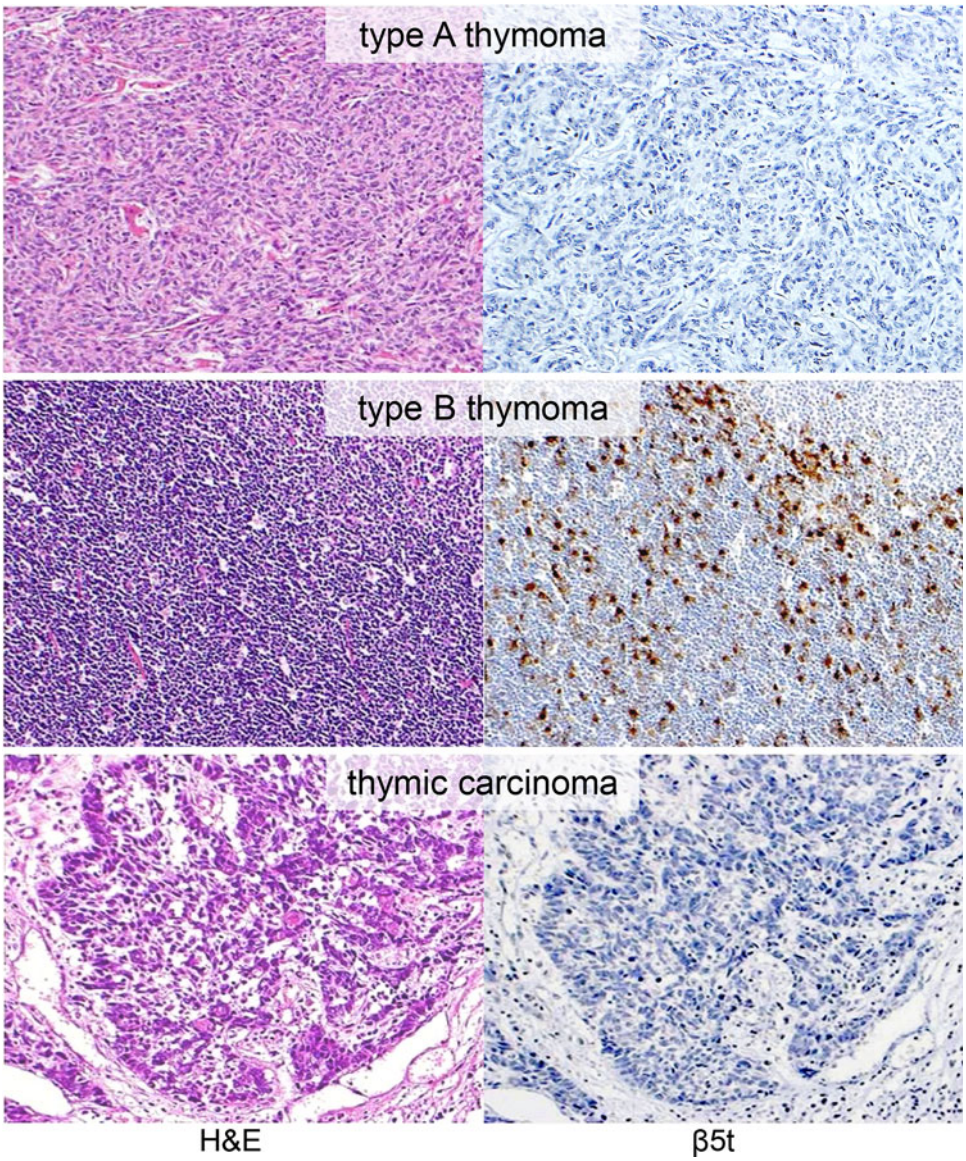

Fig. 5 Expression of β5t in thymic epithelial tumors. β5t is detected in most cases of type B thymoma, but not in type A thymoma or thymic carcinoma. β5t is expressed in neoplastic epithelial cells differentiating towards cTECs

Thymomas are frequently associated with autoimmune diseases including myasthenia gravis. Conversely, approximately 30 % of patients with myasthenia gravis are associated with thymomas, in particular type B thymoma (Nakagawa et al. 2003; Okumura et al. 2002). It has been suggested that thymoma-associated T cells are involved in the onset of paraneoplastic myasthenia gravis (Fujii 2012; Marx et al. 2010; Okumura et al. 2008). In thymomas, the epithelial and lymphoid components closely mimic the cell organization in normal thymus; thus, thymomas or the "sick thymus" may generate T cells that are self-reactive due to impaired maturation (Shelly et al. 2011). Indeed, thymocytes derived from cortical areas with increased proliferation rates are known to show self-reactivity (Theofilopoulos et al. 2001). Future study should address whether dysregulated β5t expression in neoplastic epithelial cells is involved in aberrant T-cell development leading to autoimmunity.

Conclusions

Niedermann and colleagues suggested that the differential expression of constitutive and immunoproteasomes in cTECs and mTECs might influence self-peptide repertoires presented by MHC class I molecules during thymic selection (Nil et al. 2004). Subsequently, cytotoxic T lymphocytes in wild-type and β5i-deficient mice were found to use distinct TCR Vβ genes for an ovalbumin-derived epitope (Osterloh et al. 2006), suggesting the involvement of the β5i subunit in the formation of T-cell repertoires. With the discovery of thymoproteasomes, the

investigation addressing the role of proteasomes in thymic selection has clearly entered a new era. To understand the molecular mechanism by which the thymoproteasome regulates thymic selection, it is important to define the cleavage specificity of thymoproteasomes more precisely, elucidate the characteristics of peptides generated by thymoproteasomes, and eventually identify naturally processed peptides displayed by cTEC-expressed MHC class I molecules. Apart from its role in thymic selection, accumulating evidence indicates that β5t can be used as a marker in the pathologic diagnosis of thymic epithelial tumors. Further analysis of β5t expression, in particular in relation to the biological behaviors, such as the degree of malignancy or the association with paraneoplastic immune dysfunction, may help formulate a biologically more meaningful classification of thymic epithelial tumors.

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Conflict of interest The authors have no competing financial interests.

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