Thymus-Dependent T Cell Tolerance of Neuroendocrine Functions

Principles, Reflections, and Implications for Tolerogenic/Negative Self-Vaccination

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ABSTRACT: Under the evolutionary pressure exerted by the emergence of adaptive immunity and its inherent risk of *horror autotoxicus*, the thymus appeared some 500 million years ago as a novel lymphoid structure able to prevent autoimmunity and to orchestrate self-tolerance as a cornerstone in the physiology of the immune system. Also, the thymus plays a prominent role in T cell education to neuroendocrine principles. Some self-antigens (oxytocin, neurotensin, insulin-like growth factor 2 [IGF-2]) have been selected to be predominantly expressed in thymic epithelium and to be presented to thymus T cells for educating them to tolerate other antigens related to them. In the insulin family, *IGF2* is dominantly transcribed in cortical (c) and medullary (m) thymic epithelial cells (TECs), whereas the insulin gene (*INS*) is expressed at low level by only a few subsets of mTECs. Intrathymic transcription of both *IGF2* and *INS* is under the control of the autoimmune regulator (*Aire*) gene. The highest concentrations of IGF-2 in the thymus explain why this peptide is much more tolerated than insulin, and why tolerance to IGF-2 is so difficult to break by active immunization. The high level of tolerance to IGF-2 is correlated to the development of a tolerogenic/regulatory profile when the sequence B11-25 of IGF-2 (homologous to the autoantigen insulin B9-23) is presented to DQ8+ type 1 diabetic patients. Since subcutaneous and oral insulin does not exert any tolerogenic properties, IGF-2 and other thymus self-antigens related to type 1 diabetes (T1D) should be preferred to insulin for the design of novel specific antigen-based preventive approaches against T1D.

KEYWORDS: thymus; central tolerance; autoimmunity; self-antigens; AIRE; regulatory T cells (T_R); type 1 diabetes

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INTRODUCTION

Some 500 million years ago, although some rudiment of immune diversity already existed in jawless fishes (e.g., lamprey),[1] novel adaptive immunity emerged in cartilaginous fishes (e.g., shark and ray). Specialized recombination machinery in somatic lymphoid cells is the fundamental property of adaptive immunity and is responsible for the random generation of a huge diversity of immune receptors (BCRs and TCRs) able to recognize non-self antigens. The emergence of this novel form of immune defense exerted such a potent pressure that novel structures and mechanisms appeared along the paths of lymphocyte traffic to impose immunological self-tolerance, that is, the inability of the immune system to attack the host organism. Together with the generation of diversity and memory, self-tolerance is a cornerstone in physiology and homeostasis of the immune system. The progressive rise in the level of immune diversity and complexity also explains why failures of immunological self-tolerance (such as organ-specific autoimmune diseases) are more and more frequently detected in parallel with evolution, the maximum being observed in the human species. The first thymus also appeared in cartilaginous fishes concomitant with the emergence of adaptive immunity. Though some forms of tolerance induction already take place in primary hematopoietic sites (fetal liver and bone marrow), antigen-dependent B cell tolerance is predominantly due to an absence of T cell help. So, among all lymphoid structures, the thymus is the only organ specialized in the establishment of immunological self-tolerance.

The thymus crucially stands at the crossroads between the immune and neuroendocrine systems.[2] Within this organ responsible for thymopoiesis (T cell generation), the neuroendocrine system regulates the process of T cell differentiation from the very early stages. In addition, T lymphocytes undergo in the thymus a complex educative process that establishes central T cell self-tolerance of neuroendocrine principles. The thymus is a very unique place wherein these is a permanent confrontation between ancient, almost constant, neuroendocrine principles and a recent system equipped with a sophisticated machinery promoting stochastic generation of response diversity. Contrary to a previous assumption, the thymus functions throughout life and plays a fundamental role in the recovery of a competent T cell repertoire after intensive chemotherapy or during highly active antiretroviral therapy.[3,4] Finally, the thymus is an important site for the generation of self-antigen specific regulatory T cells (T_R) that suppress in the periphery the activation of self-reactive T cells that have escaped the thymus central censorship.[5,6]

DEVELOPMENTAL BIOLOGY OF THYMIC EPITHELIUM

Epithelial cells of the thymic cortex (cTEC, including thymic "nurse" cells [TNC]) and medulla (mTEC) originate from a common progenitor derived

around embryonic day 11 (E11) from the endoderm of the third pharyngeal pouch.[7,8] Using lineage tracing analysis in whole embryo culture, no evidence was found for a contribution from the ectoderm of the third pharyngeal cleft. Further development of this primitive epithelial rudiment depends upon a contribution from the cephalic neural crest. Some human diseases (and animal models) include a defective thymus development, leading to primary immune deficiencies. DiGeorge's syndrome associates congenital absence (or hypoplasia) of thymus and parathyroids with defects in the heart and truncal vessels. This syndrome partly results from a migration failure of the cephalic neural crest. Mice in whom the homeobox A3 gene (*Hoxa3*) has been disrupted present thymic aplasia, parathyroid hypoplasia, and frequent defects in heart and great vessels.[9] Wild animals with immune deficiencies most closely related to DiGeorge's syndrome are "nude" mice with hairlessness and lack of thymic development resulting from defects in TECs. The "nude" phenotype is caused by mutations in the *nude* gene on murine chromosome 11 that encodes the transcription factor winged-helix nude (*whn*) or forkhead box N1 (*Foxn1*).[10] Wnt glycoproteins and dependent signaling were shown to regulate *Foxn1* expression in TECs.[11] In the absence of functional *Foxn1*, TECs are arrested at an immature progenitor stage (with expression of $MTS20^+$ and $MTS24^+$ determinants) and do not differentiate into epithelial subregions.[12] Different studies indicate that a common progenitor of TECs might exist, with a marker phenotype of $MTS20^+$ $MTS24^+$ cytokeratin 5 ($K5^+$) and $K8^+$. Once further identified, such TEC progenitor lines could be used for restoring thymus function in DiGeorge's syndrome or in immunosenescence, as well as for improving the final outcome after bone marrow and organ transplantations.

Five other transcription factors, paired box gene 1 (*Pax1*), and 9 (*Pax9*), eyes absent 1 homologue (*Eya1*), and sine oculis–related homeobox 1 homologue (*Six1*), also contribute to the ontogeny of thymic epithelium. In mice, these genes are coexpressed only in the pharyngeal endoderm and in the cephalic neural crest–derived mesenchyme (with the exception of *Pax1* and *Pax9*).[7] Important developmental signaling pathways (fibroblast growth factors [FGFs], bone morphogenetic proteins [BMP], and sonic-hedgehog homologue [Shh]) are also implicated in cell–cell interactions between thymic epithelium and mesenchymal cells, as well as the surrounding neural crest–derived mesenchyme (reviewed in Ref. 13).

From fetal liver and then bone marrow, T cell progenitors migrate into the thymus through the boundary between cortex and medulla, undergo around 20 division cycles in the outer cortex, and then differentiate after presentation of peptides by major histocompatibility complex (MHC) proteins expressed by thymic antigen-presenting cells (APCs), that is, cTECs, mTECs, dendritic cells (DCs), macrophages, and rare thymus B cells. The random rearrangement of related β then α loci generates an enormous diversity of TCRs, a great number of which are able to bind peptide/MHC ligands with high affinity and to be negatively selected. Negative selection can occur in both the cortex and

the medulla,[14] though mTECs display the most complete APC competence. At the end of the differentiation process in the thymus, only ± 5% of naïve thymus T cells (thymocytes) will leave the organ in a state of self-tolerance and competence against infectious non-self antigens.

THE NEUROENDOCRINE SELF

From the investigation of the intrathymic expression of neuroendocrine-related self-peptide precursor genes, a series of specificities could be listed to define the nature of "neuroendocrine self." [15] (1) Neuroendocrine self-antigens usually correspond to peptide sequences that have been highly conserved throughout the evolution of one given family. (2) A hierarchy characterizes their expression pattern. In the neurohypophysial family, oxytocin (OT) is the dominant peptide synthesized by TEC/TNCs from different species. The binding of OT to OT receptor (OTR) expressed by pre-T cells induces a very rapid phosphorylation of focal adhesion–related kinases.[16–18] This event could play a major role in the promotion of "immunological synapses" between immature T lymphocytes and thymus APCs. Concerning the tachykinin family, neurokinin A (NKA)—but not substance P (SP)—is the peptide generated from the processing by TECs of the preprotachykinin A (*PPT-A*) gene product.[19] With regard to the insulin gene family, all members are expressed in the thymus network according to a precise hierarchy and topography: *IGF2* (cTEC/TNC and mTEC) > *IGF1* (thymic macrophages) > *INS* (mTEC).[20–23] This hierarchical pattern is significant since the tolerogenic response primarily concerns the dominant epitopes of a protein family. Contrary to (pro)insulin, the blockade of thymic IGF-mediated signaling, at the level of IGF ligands (in particular IGF-2) or IGF receptors, interferes with the early stages of T cell differentiation in fetal thymic organ cultures (FTOCs).[24] (3) Neuroendocrine precursors are not processed according to the classic model of neurosecretion, but they undergo antigenic processing for presentation by—or in association with—MHC proteins. (4) This processing differs between thymic APCs and dedicated peripheral APCs. At least for some neuroendocrine self-antigens (OT and neurotensin), such differences imply that presentation by thymic APCs is not tightly restricted by MHC alleles as much as presentation of infectious non self antigens and autoantigens by peripheral dedicated APCs (macrophages, DCs, and B cells).

During ontogeny of Balb/c mice, *OT* transcripts are detected on E13 both in thymus and brain, whereas vasopressin (*VP*) gene transcription starts on E14 in the brain and is clearly detected in the thymus only on E15.[25] The earlier *OT* expression in the thymus strongly supports the role of thymic OT in the induction of central T cell tolerance of the neurohypophysial peptides before their appearance in the hypothalamic magnocellular neurons. The expression of the neurohypophysial receptor genes (*OTR*, *V1*, *V2,* and *V3*) was investigated on murine $CD4^-$ $CD8^-$ T cell lines, as well as on murine T cell subsets. *OTR*

transcripts are detected in $CD4^{-}CD8^{-}$, $CD4^{+}$ $CD8^{+}$, and $CD8^{+}$ cells, while a very faint *V3* expression is restricted to $CD4^{+}$ $CD8^{+}$ and $CD8^{+}$ cells. *V1* and *V2* expression could not be detected on any T cell subset. In FTOCs, a specific OTR antagonist increases late T cell apoptosis, confirming the involvement of OT/OTR signaling in T cell proliferation/survival.[25,26]

REGULATION OF SELF-EXPRESSION

The progressive accumulation of evidence that genes/proteins considered to be expressed only in the brain, in the neuroendocrine system, and in peripheral organs are also synthesized in the thymus finally brought into question the common view of the establishment of immunological self-tolerance and the development of organ-specific autoimmunity (completely reviewed in Ref. 27). In 1993, the term "promiscuous" was introduced to specify this unique promoter use by TECs compared to other somatic cells.[28] Another advance in the current reappraisal of the crucial role played by the thymus in preventing autoimmunity came from the identification of the gene of which mutations are responsible for the polyendocrine autoimmune disease, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) or autoimmune polyglandular syndrome type 1 (APS-1).[29,30] The gene *Aire* (autoimmune regulator) encodes a protein with structural and functional features suggesting a transcription factor. The generation of $Aire^{-/-}$ mice revealed that Aire primarily functions within TECs where its expression is maximal and where it controls transcription of genes encoding neuroendocrine self-antigens (including *Ot*, *Igf2*, *Ins2,* and *Npy*), as well as a series—but not all—tissue-restricted antigens.[31] Although Aire controls the transcription of these two neuroendocrine-related genes, *Ins2* expression is restricted to mTEC, while *Igf2* transcripts are detected in cTEC/TNCs, as well as in mTECs. The existence of epigenetic mechanisms in the Aire control of self-expression is also strongly supported by the discovery that the set of promiscuous transcripts expressed by human mTECs includes several groups of chromosomally clustered genes.[32] Such epigenetic regulation is further suggested by loss of imprinting and overexpression of *IGF2* in human mTECs.[33] Nevertheless, it remains unclear why TECs are the only somatic cells (with the exception of multipotent stem cells) to transcribe such a diversity of neuroendocrine and tissue-restricted antigens, and why this diversity seems to be correlated with the stage of TEC differentiation.

Aire promotes negative selection of self-reactive thymus T cells and, perhaps more importantly, improves the overall efficiency of antigen presentation by mTECs.[34,35] As previously mentioned, a difference between the central and peripheral mechanisms was again evidenced at this level since antigen presentation by DCs in the periphery is more efficient in the absence of Aire.[36] On the other hand, no any significant defect of the T_R lineage was observed in *Aire*-deficient mice. Very interestingly, thymic Aire expression decreases

and autoimmunity develops in lymphotoxin (LT) α or β receptor–deficient mice.[37] Although it is still not clear whether the LT receptor directly controls Aire expression or indirectly via regulation of mTEC development, this study illustrates the importance of lymphoepithelial crosstalk in thymus physiology. Human thymic stromal lymphopoietin (TSLP) has also been reported to induce *AIRE* expression in human DC.[38] Although helix-loop-helix transcription factors of the forkhead family (Foxn1, Foxp3, Foxo, and Foxj1) are major modulators of the immune development and responses, Aire seems to be until now the unique molecular determinant involved in the control of self-expression inside the thymus.

Altogether, those studies contributed to reevaluate the physiological importance of thymus-dependent central tolerance and "recessive" clonal deletion—as opposed to the "dominant" tolerance by T_R generation—in the prevention of autoimmunity and "horror autotoxicus" of the organism, so to speak in a conceptual view like that proposed by Ehrlich in 1901.[39] Self-tolerance homeostasis and prevention of autoimmunity by central and peripheral tolerogenic mechanisms acting synergically have, however, to be considered together as evidenced by the severe and multiple autoimmune organ deficiencies as observed in *Foxp3*- and *Foxj1*-deficient mice.[40,41]

PRESENTATION OF NEUROENDOCRINE SELF-ANTIGENS

While a vast repertoire of neuroendocrine-related and tissue-restricted self-antigens is expressed by TECs, the coupling of their thymic transcription to the MHC presentation of derived epitopes has not been extensively investigated. This point is, however, fundamental since some authors recently reported that the promiscuous gene expression of an autoantigen gene (H/Ka subunit of the gastric membrane protein H^+/K^+ ATPase) did not result in negative selection of effector self-reactive T cells.[42]

Using an immunoaffinity column prepared with a mAb to the monomorphic part of human MHC-I molecules, we identified in proteins extracted from human TEC plasma membranes a 55-kDa protein that was labeled both by anti-MHC-I and antineurophysin antibodies.[43] This membrane protein may represent a hybrid protein with a neurophysin domain (10 kDa) and a MHC-I heavy chain domain (45 kDa). Formation of such a hybrid protein could reside either at the posttranscriptional level (such as a transsplicing mechanism), or at the posttranslational level (such as the ATP-dependent binding of ubiquitin to proteins in proteolysis). The MHC-I domain would be implicated in the membrane targeting of this 55-kDa protein, while neurophysin would bind OT for presentation to thymus T cells. If this assumption were correct, this would mean that, both in the hypothalamo-neurohypophysial axis and in the thymus, the neurophysin part of the OT precursor fulfills the same function: binding of OT and transport to the surface of magnocellular neurons or TEC/TNCs. If

true, this explanation would imply that the immune system has adopted during evolution a component of the neurohypophysial peptide biosynthesis for the development of tolerance to the self-antigen OT of this family. Interestingly, with regard to this hypothesis, it was recently demonstrated that a component of lipid metabolism, the binding protein apolipoprotein E, is used by the immune system for binding lipid antigens and delivering them into DC endosomal compartments containing CD1.[44] Further studies are needed to verify whether IGF-binding proteins could also be involved in the intrathymic presentation of IGF-2 to thymus T cells. This hypothesis is nevertheless plausible since several genes encoding these important components of the IGF system are transcribed in the thymus network.[22]

Cultured human TECs contain ± 5 ng neurotensin (NT) per 10^6 cells, of which 5% are associated with plasma cell membranes. HPLC analysis of immunoreactive (ir)-NT present in human TECs revealed a major peak of ir-NT corresponding to intact NT1-13. Ir-NT was not detected in the supernatant of human TEC primary cultures. Using an immunoaffinity column with an anti-MHC-I mAb, NT-related peptides were retained on the column and were eluted at basic pH just as antigens bound to MHC-I proteins.[45] The C-terminal sequence of NT includes tyrosine, leucine, and isoleucine, all residues that can be used for anchorage to most of the MHC-I alleles. Thus, NT and NT-derived C-terminal fragments could behave as natural ligands for a majority (if not all) of MHC-I alleles. This hypothesis stands in agreement with the high degree of conservation of NT-related C-terminal region throughout evolution.

DEFECTIVE CENTRAL TOLERANCE AS A PRIMARY EVENT FOR THE DEVELOPMENT OF AUTOIMMUNE ENDOCRINOPATHIES

In 1992, a defect in the process of T cell education to recognize and to tolerate the neurohypophysial self-Ag OT was hypothesized to play a pivotal role in the development of hypothalamus-specific autoimmunity and "idiopathic" diabetes insipidus.[46] As already hypothesized by Burnet in 1973, the pathogenesis of autoimmune diseases could result from the appearance of "forbidden" self-reactive effector T cell clones in the peripheral repertoire.[47] Since the thymus is the primary site for induction of self-tolerance, thorough investigation of a defective thymic censorship should provide the scientific community with important keys to understand the mechanisms underlying the development of autoimmunity. A number of abnormalities of thymic morphology and cytoarchitecture have been described for several autoimmune disorders. Apoptosis of self-reactive T cells is also defective in the thymus of NOD mice.[48] The expression of Aire is maximal in murine mTECs, but is absent in TECs of diabetic NOD mice.[49] The expression of insulin-related genes was analyzed in thymus, liver, and brain of a common animal model of type 1 diabetes (T1D),

the biobreeding (BB) rat. A thymus-specific defect of *Igf2* expression was evidenced in more than 80% of diabetes-prone BB rats (BBDP).[50] This defect could explain both lymphopenia, including a lack of antigen-specific T_R cells that control autoimmune diabetes, as well as absence of central self-tolerance of insulin family in BBDP rats. Further experimental data arguing for a role the promotion of β-cell self-tolerance by thymic insulin-related peptides came from other recent experiments showing that susceptibility to diabetes was correlated with the intrathymic levels of *Ins2* expression.[51,52] As a consequence of the defective thymic censorship, self-reactive T cells bearing TCRs oriented against dominant epitopes of insulin-related peptides could continuously migrate from the thymus and enrich the peripheral pool with self-reactive T cells exhibiting a potential cytotoxic power against islet β cells. Under certain environmental influences, a molecular "bridge" could be installed between the target autoantigenic epitopes, leading to activation of the self-reactive T cell pool and subsequent β-cell destruction.

T1D (juvenile or insulin-dependent diabetes) is a chronic devastating disease resulting from an autoimmune response specifically oriented against pancreatic islet β cells, the only cells secreting insulin according to the endocrine model. In accordance with the above hypothesis, *INS* transcripts were measured at lower levels in the thymus of human fetuses with short class I variable number of tandem repeats (VNTR) alleles, a genetic trait of T1D.[53,54] A very recent study also provided evidence that both *IDDM2* alleles and *AIRE* expression could influence the level of *INS* expression in the human thymus.[55]

THEORETICAL PRINCIPLES OF "NEGATIVE SELF-VACCINATION"

The study of neuroendocrine gene expression and precursor processing in the thymus led to the identification of neuroendocrine self-peptides. With regard to insulin-related gene expression in the thymus, IGF-2 —a prominent fetal growth factor—was identified as the dominant self-peptide precursor of the insulin family expressed in the thymus from different species. This observation is in close accordance with the theory of self-recognition, which, according to F.M. Burnet, is not an inherited property but is gradually acquired in the course of fetal life. Although the tolerogenic properties of neuroendocrine self-peptides remain to be further documented, they are strongly suspected from what is known about the immunological tolerance of classic hormones. The development of specific antibodies by active immunization (i.e., experimental breakdown of self-tolerance) revealed that OT is more tolerated than VP, and that IGF-2 is also more tolerated than IGF-1, and much more than insulin. Some cases of diabetes insipidus result from an autoimmune process against VP-producing hypothalamic neurons (infundibulohypothalamitis).[56–58] Insulin is the primary autoantigen tackled by the autoimmune

response observed in T1D, and the intrinsic immunogenicity of insulin might result from its very low expression in the thymus. On the contrary, autoimmunity has never been observed against OT and IGF-2. The strong tolerance of these peptides, resulting from the high expression of *OT* and *IGF2* in the thymus, may be considered as the consequence of some evolutionary pressure to protect fundamental processes, such as species reproduction and individual ontogeny, respectively.[59–61] The putative pathogenic role of "forbidden" self-reactive T cells against IGF-2 deserves to be further analyzed through immunization of *Igf2*$^{-/-}$ mice.[62] In this latter model, nevertheless, the absence of IGF-2 was clearly shown to decrease immunological tolerance to insulin.

Thus, while VP and insulin behave as the immunogenic autoantigens of their respective families, OT and IGF-2 may be viewed as the tolerogenic self-peptide precursors of the neurohypophysial and insulin families, respectively. In this perspective, recent experiments have shown that, compared to insulin B9-23, the presentation of the homologous peptide sequence IGF-2 B11-25 to PBMC purified from DQ8+ T1D adolescents elicits a tolerogenic/regulatory profile with a higher IL-10/IFN-γ ratio and a lower IL-4 secretion.[63] Perhaps it is now appropriate to distinguish an autoantigen from a self-antigen, in the sense that peripheral autoantigens possess "altered" peptide sequences compared to homologous thymic self-antigens. Though they are highly evolutionarily related, they are not identical and this biochemical difference could drive completely opposite immune responses (i.e., immunogenic vs. tolerogenic responses). Undoubtedly, the reevaluation of thymus-dependent central self-tolerance will lead to the design of novel strategies to cure and prevent severe autoimmune diseases (such as T1D) that constitute the heavy tribute paid by mankind, for the diversity, complexity, and efficiency of human immune defenses.

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