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The Thymic Insulin-like Growth Factor Axis: Involvement in Physiology and Disease

Abstract

A repertoire of neuroendocrine-related genes is transcribed in the non-lymphoid compartment of the thymus, transposing the dual physiological role of this organ at the molecular level in T-cell development towards the establishment of central T-cell self-tolerance. The "neuroendocrine self" has been defined as a series of antigen sequences processed from precursors predominantly expressed in the thymus and first encountered by differentiating T-lymphocytes in their early life. All the members of the insulin gene family are expressed in the thymus according to a precise hierarchy and cellular topography, whereby IGF-II (epithelium of the subcapsular cortex and medulla) exceeds IGF-I (macrophages), which in turn far exceeds INS (rare subsets of medullary epithelial cells). This hierarchy in the degree of their respective thymic expression explains why IGF-II is more tolerated than IGF-I, and much more so than insulin. Evidence has been found for significant regulatory/tolerogenic properties in the IGF-II B:11 – 25 sequence after analysis of the cytokine secretion profile in peripheral blood mononuclear cells isolated from ten DQ8+ type 1 diabetic adolescents. In the thymus, IGF ligands and receptors also intervene in the control of T-cell proliferation and differentiation. Here, we also discuss how a disturbance in the intrathymic IGF-mediated signaling could contribute to the pathogenesis of T-cell leukemia.

Key words
Thymus epithelium · Self-tolerance · Autoimmunity · Type 1 diabetes · T-cell leukemia · Tolerogenic vaccine

Abbreviations

AIRE/AIRE:	autoimmune regulator gene/protein
CD:	cluster differentiation
DC:	dendritic cell
FTOC:	fetal thymic organ culture
IGF:	insulin-like growth factor
IGFBP	IGF binding protein
INS:	human insulin gene
Ins:	mouse insulin gene
IR:	immunoreactive
MHC:	major histocompatibility complex
NKA:	neurokinin A
NOD:	non-obese diabetic
OT:	oxytocin
PBMCs:	peripheral blood mononuclear cells
TEC:	thymic epithelial cell
TNC:	thymic nurse cell
TCR:	T-cell receptor for the antigen
T1D:	type 1 diabetes
VNTR:	variable number of tandem repeats.

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Physiology of the Thymus: a Broad Overview

Together with diversity and memory, self-tolerance is a fundamental property of immune response. The thymus is a unique lymphoid structure specialized in both generation of T-cell receptor (TCR) diversity to antigens and establishment of central self-tolerance. The latter results from the clonal deletion of self-reactive T-cells emerging during random intrathymic recombination of variable TCR segment genes [1], as well as the generation of self-antigen-specific regulatory T-cells (T_R) [2,3]. Even though some degree of tolerance induction occurs in primary hematopoietic sites (fetal liver and bone marrow), antigen-dependent B cell tolerance is primarily due to absence of thymus-derived T-cell help [4].

The dual physiological role of the thymus in T-cell development (thymopoiesis) and self-tolerance induction is ensured by the cellular components of the thymic stroma (Fig. **1**). Thymic epithelial cells (TECs) represent around 80% of the thymic stroma and are distributed in three regions – subcapsular (or outer) cortex, inner cortex, and medulla. During embryonic development, TECs derive from epithelial stem cells identified in the primitive endoderm [5,6]. In the outer cortex, thymic "nurse cells" (TNCs) are large TECs that engulf up to 50 thymocytes (immature T-cells) inside leaflets in the TNC plasma membrane. TNCs contain the subcellular equipment and enzymatic machinery necessary for antigen processing and presentation [7]. Issued from bone marrow, thymic dendritic cells (DCs) are located at the corticomedullary junction, while macrophages are distributed throughout the thymic stroma without any precise topography. From primitive hematopoietic sites, T-cell progenitors migrate into the thymus, proliferate into the outer cortex, and pursue their differentiation program from cortex to medulla on contact with thymic stromal cells. Presentation of self-antigens to randomly rearranged TCR constitutes one major component of the multiple signaling pathways between thymic stromal cells and pre-T-cells. This process is responsible for the deletion of self-reactive ■T-cell T-cells■, and is very powerful since only 1 – 2% of pre-T-cells will leave the thymus in a state of competence against non-self and tolerance to self.

To explain this dual role of the thymus, the first model attributed different properties to TECs/TNCs, DCs and macrophages. Since DCs and macrophages are dedicated antigen-presenting cells, they were attributed negative selection of self-reactive T-cell, whereas TECs/TNCs would be in charge of T-cell proliferation and differentiation. This model was abandoned when the ability of TECs, in particular medullary TECs, to present self-antigens and to induce T-cell negative selection was demonstrated [8]. According to the more recent "affinity-avidity model" [9], clonal deletion is the fate of T-cells bearing a TCR with high affinity for self-antigens presented at high density by thymic major histocompatibility complex (MHC) molecules. T-cells with a low-affinity TCR or confronted with self-antigens at too low density will die of "neglect", while those with intermediate affinity/avidity will be selected for further development. It is, however, important to note that the affinity of a given TCR for its specific antigen is rather low (K_D around 10^{-7} M), so the biological meaning of lower affinities may be questioned.

A significant advance in our understanding of thymic physiology was gained with the demonstration that a repertoire of neuroendocrine-related as well as peripheral antigen-encoding genes are transcribed thymic stromal cells [10 – 15]. Based on the intrathymic transcription of neurohypophysial and tachykinin genes, our group proposed another model to explain at the molecular level the paradox of thymus physiology. Oxytocin (OT) and neurokinin A (NKA) are expressed in TECs/TNCs as the dominant members of the neurohypophysial and tachykinin families, respectively [16,17]. Thymic OT and NKA precursors engage two distinct types of interactions with developing T-cells. On the one hand, these precursors are the source of ligands that bind with high affinity to neuroendocrine receptors expressed by pre-T-cells. In this type of cryptocrine signaling [18,19], those ligands are not secreted but targeted to the outer surface of thymic stromal cell plasma membrane. On the other hand, the same precursors un-

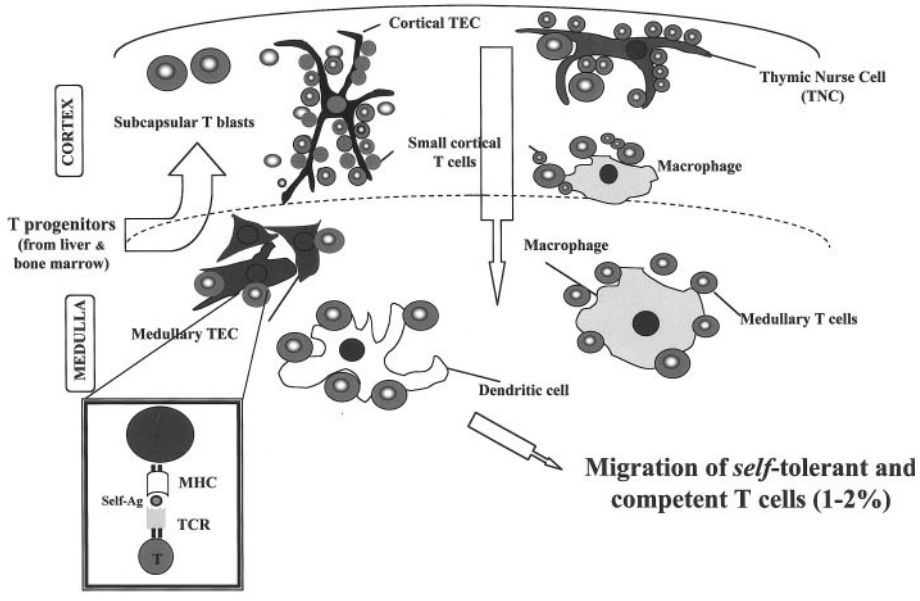

Fig. **1** T-cell differentiation in the thymus microenvironment. From primitive hematopoietic sites (fetal liver, then bone marrow), possibly under the influence of still undefined chemokines, T-cell progenitors enter the thymus at the corticomedullary junction and proliferate in the subcapsular cortex. From outer cortex to medulla, T-cell differentiation is promoted through different signaling pathways activated from their contact with thymic stromal cells (TNCs, cortical and medullary TEC, dendritic cells and macrophages). Engagement of TCR signaling driven by presentation of self-antigens (Ag) by thymic MHC molecules plays a pivotal role in T-cell negative and positive selection. At the end of their intrathymic journey, only 1 – 2% of immature T-cells leave the organ in a state of self-tolerance and self MHC-restricted competence. *Reprinted and adapted from reference 22, Copyright 2003, with permission from Elsevier.*

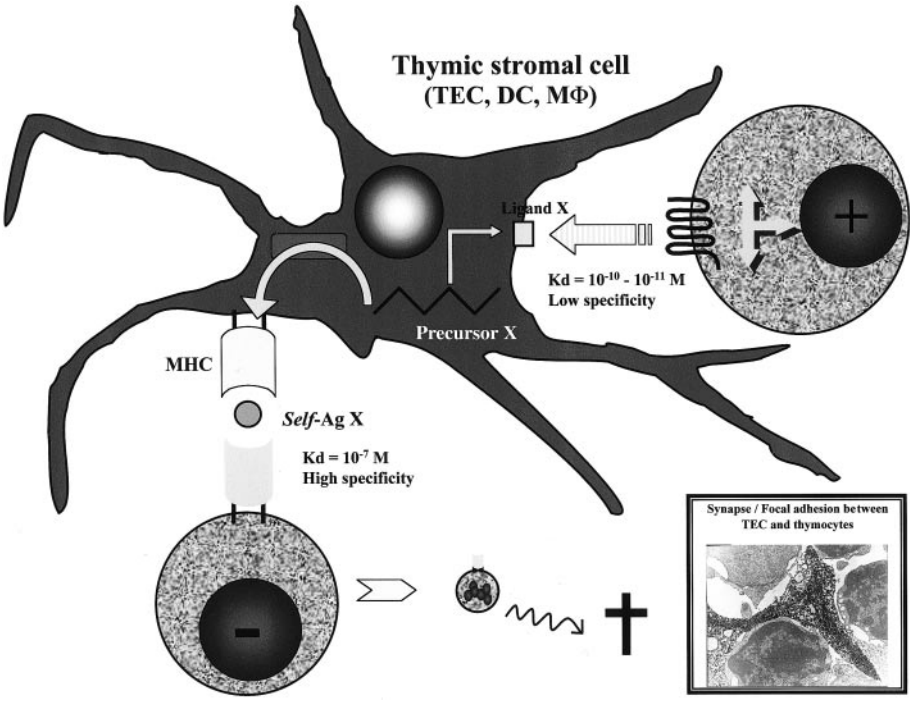

Fig. **2** The dual role of thymic neuroendocrine precursors in T-cell differentiation. Precursors encoded by neuroendocrine-related genes in thymic stromal cells (TEC, DC and macrophages [M4>]) are the source of two types of interactions with thymocytes (pre-T-cells). ■ 1) They deliver ligands that are not secreted but targeted to the outer surface of thymic stromal cell plasma membrane. These ligands bind with high affinity to neuroendocrine-type receptors expressed by thymocytes. This cryptocrine signaling in the "immunological synapse" between thymic stromal cells and pre-T lymphocytes can activate various intracellular pathways (such as phosphoinositide turnover, phosphorylation of focal adhesion-related kinases…). ■ 2) Through MHC pathways, the antigenic processing of thymic neuroendocrine precursors leads to the presentation of self-antigens by thymic MHC proteins. Deletion of T-cell clones bearing a rearranged TCR specific for MHC/neuroendocrine self-antigen complexes has been proposed to be responsible for the establishment of central immunological self-tolerance of neuroendocrine families. *Reprinted and adapted from reference 22, Copyright 2003, with permission from Elsevier.*

dergo another type of processing and deliver self-antigens that are presented by the thymic MHC machinery [20, 21]. A negative signal could result from this low-affinity, but specific binding of the complex MHC/self-antigen to its cognate TCR. According to this model, presentation of neuroendocrine self in the thymus network is responsible for the establishment of the central T-cell self-tolerance of neuroendocrine principles (Fig. **2**) [22].

The Thymic Insulin-like Growth Factor Axis

During further experimentation with this working model, we used in-depth immunohistochemistry (IHC) some time ago with the objective of identifying the dominant member of the insulin family expressed in the thymic environment. Using a battery of specific polyclonal and monoclonal antibodies, a strong immunoreactivity for insulin-like growth factor (IGF) II was detected in the epithelial compartment of human and rat thymus glands [23]. Although the protein was detected in cell bodies in primary human TEC cultures, no IGF-II could be detected in the incubation media. In addition, evidence was found for immunoreactive (IR) IGF-II at the outer surface of cultured human TEC plasma membrane using confocal microscopy (Achour et al., PhD Thesis, University of Liege, Faculty of Science). IR IGF-I was also identified in thymic stromal cells with a thymic macrophage-like morphology and topography. With two monoclonal antibodies directed against distinct epitopes of insulin, we did not find any significant immunoreactivity in human thymic lobules. Nevertheless, when reanalyzing our data in the light of recent reports (see ■below■), some faint insulin immunoreactivity could be considered in some subsets of medullary TEC. From those studies, we concluded that IGF-II is by far the dominant insulin-related protein first encountered by immature T-cells during their differentiation process in the thymus [23].

Van Buul-Offers et al. [24] generated transgenic mice carrying one of three human IGF-II minigenes containing different non-coding exons preceding the coding exons 7, 8 and 9, spaced by truncated introns. Those constructs were placed under transcriptional control of the MHC H-2K^b promoter-enhancer and contained the SV40 small-t intron and early polyadenylation signal. Overexpression of IGF-II did not affect overall body growth in these transgenic normal and dwarf mice, but provoked a marked thymic hyperplasia suggesting a role for IGF-II in thymic development by paracrine/autocrine action. By *in situ* hybridization, transcripts of the IGF-II transgene were found at high density in the thymic non-lymphoid medulla and in scattered positive cells in the thymic cortex. Intense IR IGF-II staining was observed by

IHC with the same distribution of IGF-II mRNA [25]. Moreover, IGF-II overexpression in these transgenic mice increases thymic cellularity and stimulates the production of normal mature T-cells with a slight polarization towards the CD4+ phenotype [26].

Components of the IGF axis have been further investigated in the normal human thymus. Promoters P3 and P4 are active in the control of IGF-II transcription by human TECs. Transcripts of type I and II IGF receptor genes were detected in human lymphoid Jurkat T-cells but not in cultured human TECs. Using Northern blot analysis, genes encoding IGF-binding proteins (IGFBP)-2 to 6 (but not IGFBP-1) were found to be expressed in TECs with a dominance of IGFBP-4. Lymphoid Jurkat T-cells only express IGFBP-2, but at quite high levels [27].

The functional relevance of the thymic insulin-like growth factor axis has been investigated using murine fetal thymic organ cultures (FTOCs). Neither growth hormone nor IGF-I influenced thymopoiesis in this experimental model [26]. In murine fetal thymic lobes, IGF-II and IGF-I transcripts were detected in TECs and macrophages, respectively [28]. Treatment of FTOCs with an anti-IGF-I antibody did not affect thymopoiesis. However, T-cell differentiation at early stages (CD4 – 8-, double negative) was severely inhibited when FTOCs were treated with antibodies against IGF-II, IGF type I receptor, and even IGF type II receptor [29]. In addition, no significant effect on thymopoiesis or T-cell differentiation was observed after TOC treatment with a specific antibody directed against (pro)insulin. These findings and the thymopoietic effects of IGF-II overexpression in a transgenic model strongly suggest that IGF-II, rather than IGF-I or insulin, is an important tissue factor for thymopoiesis.

Central Self-tolerance of the Insulin Family

While there is ample evidence that the thymic IGF axis is implicated in regulation of T-cell development, the important question arises as to its involvement in the establishment of central T-cell self-tolerance. The members of the insulin gene family are all transcribed in the thymic stromal cells with a precise topography. As discussed above, IGF-II is transcribed by TECs in the whole cortex and in medulla, while IGF-I is expressed by thymic macrophages. INS is transcribed by some subsets of medullary TECs [30], and these cells are now identified as a unique cell type that can express "promiscuously" and randomly a large number of tissue-specific genes with the potential of inducing central self-tolerance in peripheral tissue antigens [15]. Thus, immune self-tolerance of a family that is crucial for vital aspects such as fetal development, postnatal growth, and glucose metabolism is established at the central level through the intrathymic expression of INS, IGF-I and IGF-II.

Experimental evidence also exists that the degree of tolerance to a given protein is closely correlated with its intrathymic concentration [31,32]. From several studies, a hierarchy appears in the expression pattern of insulin-related genes/proteins. IGF-II, IGF-I, and proinsulin concentrations are 96.7 ± 10.6, 42.9 ± 5.0 and 1 – 10 ng/g wet weight, respectively in the human thymus [33,34]. In parallel with this hierarchy, immune tolerance to IGF-II is higher than it is to IGF-I, and much higher than to insulin. This is indirectly reflected by the frequency and titer of antibodies obtained after active immunization with the three peptides [35]. Using the same line of reasoning, the high occurrence of anti-insulin autoantibodies in the normal population [36] could be linked to the low level of INS expression within the human thymus. The IGF-II protein contains peptide sequences that have been highly conserved throughout evolution of the insulin family. Because of this close homology, thymic IGF-II would be a good candidate for inducing central immune self-tolerance of the whole insulin family although the tolerance to insulin *per se* would be weaker. This again might explain why B and T-cell autoreactivity to insulin has been equally observed in diabetic and related non-diabetic individuals [36].

In order to gain further insight into the immune tolerance mediated by IGF-II, we have immunized wild type and IGF-II$^{-/-}$ mice (heterozygote couples kindly provided by A. Efstratiadis and C. Graham) with the whole IGF-II protein. All mice developed a primary humoral response (immunoglobulin [Ig] M directed to IGF-II) but, as expected, only IGF-II$^{-/-}$ mice developed IgG to IGF-II (at high titer). This result indicates the presence of IGF-II specific CD4+ T-cells since help provided by these latter is a prerequisite for the Ig isotypic switch. Preliminary attempts to clone those "forbidden" IGF-II specific T-cells have, however, failed, and endogenous IGF-II invalidation seems to interfere with T-cell proliferation even in presence of the IGF-II protein in fetal calf serum-supplemented culture medium (Hansenne et al., manuscript in preparation).

Intrathymic Development of the Diabetogenic Autoimmune Response

Until recently, the question of a defect in the thymic establishment of self-tolerance has not been intensively investigated as a factor involved in the development of the diabetogenic autoimmune response specifically directed against insulin-secreting islet β-cells. However, data from several studies add to the confirmation of this hypothesis. As early as in 1982, neonatal thymectomy had been shown to prevent the emergence of diabetes in an animal model of type 1 diabetes (T1D), the Bio-Breeding (BB) rat [37]. The therapeutic benefit of thymectomy might actually result from the removal of a defective thymic censorship responsible for continuous release and enrichment of the peripheral T-cell pool with self-reactive T-cell clones. In contrast, the occurrence of diabetes is prevented by the transplantation of thymus from diabetes-resistant (BBDR) to diabetes-prone (BBDP) BB rats [38]. Thymus transplantation from NOD mice to diabetes-resistant mouse strains was also shown to induce autoimmune diabetes in the recipients [39]. Bone marrow transplantation was ineffective in preventing autoimmune phenomena in MRL$^{/+}$ mice, whereas thymus transplantation proved to work [40]. Grafts of pure thymic epithelium from NOD mouse embryos to newborn C57BL/6 athymic mice induced CD4 and CD8 T-cell-mediated insulitis and sialitis [41]. At the histological level, a defect in thymus tolerogenic function could result from disorganization in the tissue environment such as the presence of giant perivascular spaces as observed in NOD mouse thymus [42]. Epithelial defects have also been characterized in the thymus of BB rats [43].

The development of the diabetogenic autoimmune process may result from a defect in the establishment of thymic central self-tolerance through abnormalities of transcription or processing of β-cell-specific autoantigen-encoding genes. IGF-II transcripts could not be found in the thymus of more than 80% BBDP rats in close agreement with the incidence of diabetes in this BB rat strain (86%) [44]. This gene defect was specific of the thymus since IGF-II mRNA was shown in the brain and liver of BBDR rats. Two independent groups have shown that the levels of INS transcripts were low in the thymus from deceased fetuses with genetic susceptibility to T1D (presence of VNTR class I alleles), while they were higher in thymus from fetuses bearing protective alleles (VNTR class III alleles) [33,34]. Another study has also reported low expression of insulin within the thymus of NOD mice [45], while mice with thymus-restricted insulin defect developed a strong proinsulin-specific T-cell reactivity [46]. Also, an acceleration of autoimmune diabetes is observed in NOD mice with drastically reduced Ins2 expression [47]. Thus, thymic insulin also contributes to central self-tolerance despite its low expression in the thymus. It still remains to be determined whether this contribution could be mediated through the generation of insulin-specific Tr cells. With regard to other β-cell autoantigens, it is interesting to note that GAD67 is the dominant GAD isoform expressed in the thymus whereas GAD65 is the autoantigen implicated in the peripheral diabetogenic autoimmunity against β-cells [48]. An alternative splicing of IA2 occurs in the thymus, which leads to the intrathymic presentation of IA-2 antigens different from those involved in the peripheral autoimmune reaction directed to islet β-cells [49]. The AIRE (AutoImmune Regulator) protein is a transcription factor involved in the control of intrathymic expression of "promiscuous" genes encoding peripheral autoantigens [50–52]. Several mutations of the AIRE gene are responsible for the development of autoimmune polyglandular syndrome type 1 (APS-1) or APECED syndrome (Autoimmune PolyEndocrinopathy, Candidiasis and Ectodermal Dystrophy). AIRE expression is maximal in the thymus [53,54], and thymus transplantation from Aire$^{-/-}$ mice to normal mice is followed by the appearance of several autoimmune lesions in grafted mice [55]. The profile of gene expression was studied by microarrays in the thymus of Aire$^{-/-}$ mice and thymic levels of transcripts from several genes (including IGF-II, Ins2, Ot and neuropeptide Y) were severely decreased in these thymus samples compared to normal ones [55]. AIRE was further confirmed to control negative selection of pancreatic-specific T-cells [56].

Towards a Thymus-based Tolerogenic Approach for T1D Prevention and Cure

Thus, in parallel with a physiological role of this organ in the establishment of central immune self-tolerance, a thymus defective for this censorship of self-reactivity increasingly appears to exert a crucial influence in the development of organ-specific autoimmunity. As early as in 1973, Sir Frank Macfarlane Burnet hypothesized and provided some preliminary data supporting this novel concept according which the origin of autoimmunity resides in a defect in self-tolerance setting (programming) during the process of T-cell differentiation within the thymus environment [57]. Consequently, an efficient and secure prevention and/or cure of devastating autoimmune diseases such as T1D could be based upon knowledge of the powerful tolerizing mechanisms in the thymus. This strategy may first rely on the dominant IGF-II derived thymic self-antigen(s) of the insulin family. As previously stated, insulin is poorly expressed in the thymus, and this fact may explain why insulin – or insulin-derived epitopes – appears so immunogenic in some experimental models [58,59]. Likewise, insulin administered either orally or subcutaneously does not exert any significant tolerogenic effect that could protect residual β-cell mass from the destructive autoimmune process [60–62]. On the basis of the hierarchy in the intrathymic expression of insulin-related genes, we have explored the hypothesis that IGF-II would be a more appropriate choice for designing an antigen-driven tolerogenic approach in T1D prevention. Preliminary analyses revealed that the major autoantigenic epitope of insulin (sequence B: 9–23) and the homologous sequence of IGF-II (B: 11–25) share the same affinity and equally compete for binding to the MHC class II allele DQ8 conferring major susceptibility to TID (Wücherpfennig, personal communication). In a preclinical study, we investigated the cytokine profile elicited by the DQ8 presentation of these sequences to PBMCs isolated from ten T1D DQ8+ adolescents. In accordance with a previous study [63], insulin B: 9–23 elicited a predominant immunogenic profile (high IFN-γ and IL-4, low IL-10), whereas IGF-II B: 11–25 treatment was associated with a regulatory/tolerogenic profile (high IL-10 and IL-1 (VIFN-γ ratio) ([64]; Geenen et al., manuscript in preparation). From these data, it appears that IGF-II B: 11–25 may regulate T-cell activity either by acting at the same CD4 TCR as a natural "altered peptide ligand" of insulin B: 9–23, or by stimulating CD4+ T_R previously selected in the thymus. These preliminary results support the idea that IGF-II derived self-antigen(s) might constitute the base for a theoretical anti-T1D efficient tolerogenic (or "negative") vaccine [65]. It may be expected that a complete tolerogenic vaccination procedure could include self-antigen sequences derived from GAD67 and protein derived from alternatively spliced IA-2 that are dominantly expressed in the thymus for presentation to pre-T-cells.

Involvement of the Thymus in the Pathogenesis of Lymphoid Leukemia

Besides induction of central immune self-tolerance, the other physiological property of the thymus – T-cell generation or thymopoiesis – should not be neglected. Contrary to common belief, thymopoiesis is maintained until late in life [66], and the persistence of a functional thymus plays an important role in immune recovery following chemotherapy and highly active antiretroviral therapy [67–69]. In 1944, Jacob Furth and colleagues showed that thymectomy prevents the development of lymphoid leukemia in mice of the AKR strain [70]. Still more recently, this report was selected by Donald Metcalf as one of the outstanding papers in biology [71]. The crucial role exerted by the thymus in the development of lymphoid leukemia in AKR mice was confirmed in other experimental model, as shown in particular in the studies performed by Henry Kaplan and colleagues. Thymic function evaluated by quantification of recent thymic emigrants is severely compromised in childhood T-cell hematopoietic malignancies [72]. So, if the development of autoimmune disease can now be considered as resulting from a defect in thymic central negative

selection of "forbidden" self-reactive clones, the emergence of lymphoid leukemia may be regarded as the result of abnormal thymic T-cell development. Amongst specific T-cell differentiation products, pre-TCR mediates survival and proliferation of late CD4 – 8- (double negative) T-cells [73], while mature TCRocP regulates further development to the CD4+ and CD8+ single positive T-cells [74]. The essential role of pToc and Notch-mediated signaling on T-cell tumor genesis and development of T-cellacute lymphoblastic T-cell leukemia was recently demonstrated in very elegant studies [75, 76]. However, as outlined above, many other signaling molecules present in the thymic environment [77], including components of the thymic IGF axis, are implicated in tight control of T-cell development.

With regard to AKR mouse, several studies have shown that this mouse strain develops hypoglycemia and thymic hyperplasia in association with a very high rate of spontaneous lymphoid leukemia. Pansky et al. [78] reported for the first time the existence of a biologically and IR insulin-like factor in the AKR thymus. Some difference with native insulin appeared in this paper, however, since thymic IR insulin was assayed at 150 µIU/g in acetone extracts but could not be detected in acid-alcohol extracts (contrary to pancreatic IR insulin). Interestingly, the authors discussed the concordance of their data with other studies of hypoglycemia associated with large tumors of epithelial and mesodermal origin [79]. Though Ins2 is expressed in the mouse thymus, it seems very unlikely that the very low concentration of thymic insulin (see above) is responsible for this insulin-like immunoreactivity and biological activity. Most probably, this factor corresponds to IGF-II. The close homology between IGF-II and insulin may explain significant cross-reactivity with the poorly specific anti-insulin antibodies used at that time. In addition, the biological effects of IGF-II are mediated by the insulin and IGF type I receptors. Moreover, the hypoglycemic effects of IGF-II [80] as well as the significant binding affinity of IGF-II to insulin receptors may explain the biological activity of thymic extracts on glucose metabolism. Therefore, although this remains to be demonstrated, given the impact of the IGF axis revealed in FTOC (see above), the syndrome of hyperglycemia and lymphoid leukemia in AKR female mice might in fact result from IGF-II overexpression by a hyperplasic thymic epithelium with subsequent secretion of IGF-II in the bloodstream as well as abnormal thymic T-cell proliferation and generation. Since thymic T-cell subsets express different types of IGF [81] and neurohypophysial [82] receptors, it may be expected that such neuroendocrine receptors can be identified as new cluster differentiation (CD) markers. It is worth noting that IGF type II receptors have already been identified as CD222 [83]. Based on the mitogenic and survival-promoting activities of thymic IGFs, the use of selective IGF receptor antagonists might well be considered in the future as a potential adjuvant therapy of T-cell leukemia.

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