For Debate

# Programing of the Autoimmune Diabetogenic Response in the Thymus during Fetal and Perinatal Life

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## Abstract

*The presentation of self-peptides in the thymus is responsible both for negative selection of self-reactive T cells emerging during stochastic TCR recombination in fetal life, as well as positive selection of self-specific regulatory thymic T (tTreg) cells during and after perinatal life. The combination of these two sequential processes programs central self-tolerance, a fundamental property of the adaptive immune system. A defect in intrathymic self-presentation, either genetic or acquired, is the earliest event in the pathogenesis of autoimmunity already during fetal development. This defect is necessary but not sufficient for the appearance of a classical autoimmune disease like type 1 diabetes (T1D). Environmental factors are required for activation of the diabetogenic autoimmune response that targets insulin-secreting β cells in pancreatic Langerhans' islets. Based on epidemiological studies, viral infections have been suspected for a long time to be one of those environmental factors. In this Debate article, we present a series of experimental data that support the hypothesis that, following vertical transplacental transfer, viruses might infect the fetal thymus and disturb already in utero central self-tolerance orchestrated by this organ.*

*"It is with logic that we prove but it is with intuition that we discover."*
*Henri Poincaré (1854-1912)*

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## Central Self-Tolerance of Neuroendocrine Functions

Following his formulation of the clonal selection theory, Frank Macfarlane Burnet already hypothesized that the thymus could be the site for the deletion of T-cell clones with reactivity to self and that such 'forbidden' T lymphocytes could play an essential role in autoimmunity (1). At the end of 80s, several groups independently demonstrated clonal deletion

of self-reactive T cells in the thymus (2-5), and the true biochemical nature of self-antigens encountered in this organ was then immediately questioned. In 1986, the hypothalamic neuropeptide Oxytocin (OT) was shown to be synthetized in thymic epithelial cells (TECs), including thymic 'nurse' cells (TNCs), from different species (6-8). Although functional specific neurohypophysial receptors are expressed by distinct thymic T-cell subsets (9,10), it is noteworthy that thymic OT is not secreted by TECs but that OT precursor is processed for antigen presentation by TEC major histocompatibility complex (MHC) proteins. This is also the case for the neurotensin (NT) precursor (11,12). Quite economically, TECs synthetize one dominant member per neuroendocrine family: OT for neurohypophysial peptides, NT for neuromedins, cortistatin for somatostatins, and insulin-like growth factor 2 (IGF-2) for the insulin family. Thus, the biochemical identity of neuroendocrine self can be defined as follows (see 13 for complete review):

- A hierarchy characterizes the profile of expression of neuroendocrine-related peptides in thymus epithelium: OT > Vasopressin (VP), IGF-2 > IGF-1 >> Insulin. This hierarchy is very important since tolerance to a peptide is directly proportional to its concentration in the thymus (14).
- The dominant neuroendocrine self-peptide precursor includes sequences that are highly conserved during evolution of its family.
- A neuroendocrine self-peptide is important for individual (IGF-2) and species (OT) preservation.
- Some specific epigenetic regulation of their expression in the thymus may exist (for example, absence of parental imprinting of Igf2 in the thymus).
- And most importantly, neuroendocrine self-peptide precursors are not processed for classic secretion but for antigen presentation by thymic MHC proteins.

## Thymus and Autoimmunity

In the early 2000s, the Heidelberg group of the late Bruno Kyewski reported the promiscuous gene expression (PGE) of tissue-restricted antigens (TRAs) in medullary (m) TECs and this mechanism was proposed to be a crucial key for our common knowledge of central immune self-tolerance and autoimmunity (15,16). However, contrary to neuroendocrine self-peptides, thymic TRAs do not behave as accessory signals binding to cognate receptors during T-cell differentiation, and PGE is a unique property of mTECs while neuroendocrine self-peptides are synthesized in cortical (c) and mTECs. The elegant studies performed by the Kyewski laboratory played an important role for the recognition by immunologists of the importance of thymus-dependent central self-tolerance (17-19). Moreover, an increasing number of studies pointed out a defect in the establishment of central self-tolerance as the earliest event in the programing of organ-specific autoimmune diseases (20,21). For example, Igf2 transcription is defective in the thymus of diabetes-prone BioBreeding rat, an animal model of human T1D (22).

This novel view of the thymus as the primary rampart against autoimmunity culminated with the identification by positional cloning of the AutoImmune REgulator (AIRE) gene (23). Autoimmune polyendocrinopathy candidiasis and ectodermal dystrophy (APECED) or Autoimmune Polyglandular Syndrome-I (APS-I) is an autosomal recessive monogenic autoimmune disease affecting children already in very early life. The three major symptoms are hypoparathyroidism, adrenal deficiency and chronic muco-cutaneous candidiasis. Other endocrine autoimmune diseases may be associated such as type 1 diabetes, vitiligo, autoimmune thyroiditis, premature gonadal failure, alopecia, pernicious anemia. A defect in negative selection of autoreactive T cells was demonstrated in $Aire^{-/-}$ mice (24). These mice have a phenotype much less severe than APECED children but they also develop autoantibodies and lymphocytes infiltrates in peripheral organs (25). Most importantly, this autoimmune phenotype was linked to a marked decrease in intrathymic transcription of several TRAs and neuroendocrine self-peptides such as OT, IGF-2 and neuropeptide Y (25). The molecular actions of AIRE have been investigated in a huge number of following studies but our current knowledge in this field is out of the scope of this debate article. It is nevertheless important to mention that another factor, the fasciculation and elongation protein zeta family zinc finger-2 protein, FEZF2, also intervenes as another transcriptional modulator of the intrathymic expression of different neuroendocrine self-peptides and TRAs (26,27).

These studies have also important implications from the evolutionary point of view. Since their appearance in early forms of life, the neuroendocrine and innate immune systems coevolve without any sign of neuroendocrine autotoxicity/autoimmunity. Some 500 millions years ago, the emergence in sharks and rays of two transposon-like recombination-activating genes was responsible for the development of the adaptive immune system and its progressive inherent risk of autoimmunity. The appearance of the adaptive immune system exerted an evolutionary pressure so potent that, according to Paul Ehrlich's prediction of 'horror autotoxicus', a unique thymus also appeared in sharks and rays with the specific role of orchestrating immune self-tolerance. Through transcription of dominant neuroendocrine representative precursors in TECs, the thymus so ensured a harmonious and peaceful coevolution of the neuroendocrine system and adaptive immunity. Far from being only the lymphoid organ responsible for T-cell development as long thought, the thymus now appears first as a cemetery for developing T cells; it has been estimated

that, from 100 hundred T-cell progenitors migrating through the thymus, only 3% are leaving this organ (28) either as self-tolerant and competent T cells against foreign non self-antigens or, mainly after birth, as self-reactive Treg cells that inactivate peripheral forbidden self-reactive T-cell clones having fortuitously escaped negative selection in the thymus. This novel knowledge on the unique and essential role of thymus-dependent central self-tolerance is currently paving the way for the development of innovative treatments against autoimmune diseases such as the inverse self-vaccination against T1D which aims at restoring T-cell tolerance to islet ß cells using T1D-related self-antigens (29,30).

## Viral Etiology of T1D

Intrathymic programing of an autoimmune response is necessary but not sufficient for the appearance of a clinical autoimmune disease and several strong arguments favor the intervention of environmental factors for the initiation of this type of chronic disease. The concordance index of T1D in monozygotic twins is less than 50%, and there is an impressive North-South gradient in T1D incidence with a maximum in Scandinavian countries. The environmental agents that are suspected include viral infections, anti-tumor immunotherapy, endocrine disruptors and sex steroids, nutrition and vitamin D deficiency, gut microbiota, and even stressful events (in particular for autoimmune thyroiditis).

The viral etiology of T1D has been nicely summarized in a recent review (31) and the question of an in-utero viral infection in T1D pathogenesis has already been proposed (32). Since more than 20 years, we have been investigating the hypothesis that an infection of the thymus by the enterovirus coxsackie B4 (CV-B4) could interfere with the establishment of central self-tolerance to islet ß cells.

There are various mechanisms by which CV-B4 can contribute to T1D development through interplay between the virus, and innate and adaptive immunity (33-35). CV-B4 can infect pancreatic cells, especially islet ß cells and ductal cells and CV-B4 infection persists in these cells as reported by our team (35-37). CV-B4 can infect monocytes and macrophages, resulting in the production of interferon alpha (IFN-α) and inflammatory cytokines that play a role in activating an autoimmune response against ß cells (38-42). The infection of monocytes with CV-B4 relies on enhancing antibodies, which increase the viral load in the host and the pathogenic effect of the virus (43-47).

## Thymus Dysfunction Induced by CV-B4

As reminded above, CV-B4 targets ß cells, and this is most probably involved in T1D pathogenesis. However, the development of this autoimmune disease needs the activation of preexisting T lymphocytes directed towards ß cell antigens. Therefore, it was hypothesized that CV-B4 could disturb the tolerance to ß cells at a central level.

It was decided to investigate first whether CV-B4 is able to infect human TECs. It was observed that CV-B4 persisted in primary cultures of human TECs, which resulted in an increase in the secretion of the cytokines IL-6, LIF, and GM-CSF in the supernatants (48). In a second step, CV-B4 E2 was able to infect human fetal thymic organ cultures (FTOC). Double positive CD4+CD8+ thymocytes were the principal target cells of infection and were progressively depleted. MHC class I expression by thymocytes was also markedly upregulated by CV-B4 infection (49) but this was not dependent on IFNα, which is known to be a major player in T1D pathogenesis (50).

Then, the infection of thymus with CV-B4 in an in-vivo model was developed. Outbred mice (Swiss mice) were inoculated with CV-B4 p.o. Viral RNA was detected in thymus, spleen and blood up to 70 days after inoculation (51). These observations prompted us to study further the impact of CV-B4 on mouse thymus. It was shown that the diabetogenic CV-B4 E2 strain disturbs T-cell differentiation in murine FTOC (52). The next purpose was to investigate the result of a persistent infection of TEC with CV-B4 on genes of the insulin family using a murine TEC line. This cell line expressed IGF2 and IGF1 but not insulin. A persistent infection with CV-B4 of this TEC line resulted in a dramatic decrease in Igf2 transcription and IGF-2 production in long-term cultures, while Igf1 transcripts were much less affected (53). This is noteworthy since IGF-2 is the self-peptide of the insulin family involved in central tolerance of the insulin family (see above). Thus an impaired expression of thymic Igf2 might result in a defect of negative selection of self-reactive thymocytes as well as a decrease in the generation of self-reactive Treg cells. The combination of these two mechanisms so would promote an autoimmune attack against ß cells (54). Molecular pathways responsible for the CV-B4-induced decrease of thymic Igf2 expression in a murine TEC line are under current investigation (55).

Since the thymus negative selection already operates during fetal life, we investigated whether CV-B4 E2 can reach the fetal thymus during the course of an in-utero infection. Pregnant Swiss albino mice were inoculated with the diabetogenic CV-B4 E2 strain. In-utero CV-B4 E2 infection of offspring thymus was shown by detection of viral RNA and infectious virus in the organ (56). Moreover, CV-B4 infection of the fetal thymus induced significant changes in the percentage of thymic T-cell subsets as analyzed by flow cytometry. Infection at 10 days of gestation blocked the double negative (DN) to double positive (DP) transition and then the DP to single positive (SP) differentiation, in particular to SP8 (56). In this murine model, it thus appears that CV-B4 infection during pregnancy can promote a

disturbance of T-cell differentiation. Whether CV-B4 infection on fetal thymus may impact the central development of autoimmunity is currently investigated in our laboratories. Another consequence of the thymus infection by CV-B4 might be the progressive induction of immune tolerance towards this virus, which will render CV-B4 more virulent and cytotoxic towards islet ß cells.

## Conclusions (table 1)

An increasing number of studies support the idea that a dysfunction of the thymus programs the development of different organ-specific autoimmune responses such as the childhood autoimmune T1D. Such dysfunction is either genetic (AIRE and FEZF2 mutations) or acquired following thymus viral infection. Besides T1D, it may now be assumed that the majority of organ-specific autoimmune diseases are determined during fetal and perinatal period although the intervention of environmental factors is required later in life for the clinical appearance of such diseases.

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## Disclosure

The authors Vincent Geenen and Didier Hober have nothing to disclose.

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*Table 1. Thymus dysfunction in the development of organ-specific autoimmune response*

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| **Thymus physiology** |
| • Transcription of neuroendocrine self-peptides and tissue antigens |
| • Negative selection of self-reactive T cells (already in fetal life) |
| • Positive selection of self-reactive tTreg cells (starting from perinatal period). |
| **Defect of thymus-dependent central tolerance during fetal and perinatal life** |
| **Genetic** |
| Aire and Fezf2 mutations |
| **Acquired** |
| Viral infection of the thymus |
| • Release of 'forbidden' self-reactive T-cell clones (already in fetal life) |
| • Decrease in positive selection of self-reactive tTreg cells (starting from perinatal period) |
| **Bridge between self-reactive T cells and peripheral target antigens** |
| Role of environmental factors (viruses, anti-tumor immunotherapy, endocrine disruptors and sex steroids, gut microbiota, diet, vitamin D deficiency, stress…) |

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