

How does thymus infection by coxsackievirus contribute to the pathogenesis of type 1 diabetes?

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Through synthesis and presentation of neuroendocrine self-antigens by major histocompatibility complex proteins, thymic epithelial cells (TECs) play a crucial role in programming central immune self-tolerance to neuroendocrine functions. Insulin-like growth factor-2 (IGF-2) is the dominant gene/polypeptide of the insulin family that is expressed in TECs from different animal species and humans. *Igf2* transcription is defective in the thymus of diabetes-prone bio-breeding rats, and tolerance to insulin is severely decreased in *Igf2*^{-/-} mice. For more than 15 years now, our group is investigating the hypothesis that, besides a pancreotropic action, infection by coxsackievirus B4 (CV-B4) could implicate the thymus as well, and interfere with the intrathymic programming of central tolerance to the insulin family and secondarily to insulin-secreting islet β cells. In this perspective, we have demonstrated that a productive infection of the thymus occurs after oral CV-B4 inoculation of mice. Moreover, our most recent data have demonstrated that CV-B4 infection of a murine medullary (m) TEC line induces a significant decrease in *Igf2* expression and IGF-2 production. In these conditions, *Igf1* expression was much less affected by CV-B4 infection, while *Ins2* transcription was not detected in this cell line. Through the inhibition of *Igf2* expression in TECs, CV-B4 infection could lead to a breakdown of central immune tolerance to the insulin family and promote an autoimmune response against insulin-secreting islet β cells. Our major research objective now is to understand the molecular mechanisms by which CV-B4 infection of TECs leads to a major decrease in *Igf2* expression in these cells.

Keywords: enterovirus, coxsackievirus, thymus, self-tolerance, type 1 diabetes, insulin family, insulin-like growth factor 2

Introduction

The major genetic determinants of type 1 diabetes (T1D) are the class II major histocompatibility complex (MHC) on chromosome 6 – which accounts for almost 50% of the genetic susceptibility – as well as a number of non-MHC genes, including the variable number of tandem repeat (VNTR) alleles upstream of the *INS/IGF2 (IDDM2)* locus, *PTPN22*, *CCR5*, *IL2RA*, *IL10*, and *CTLA4*. However, only 10% of the individuals bearing a genetic predisposition will develop T1D, and more than 50% of

monozygotic twins are discordant for the disease, which illustrates the implication of environmental influences in T1D pathogenesis (1) as for all autoimmune diseases.

Type 1 diabetes occurrence has been related to a number of viruses but epidemiological studies have provided the strongest evidence that enteroviral infections, in particular, by coxsackievirus B (CV-B), are frequent events preceding T1D onset (2–7). Human enteroviruses include human pathogens, such as poliovirus, CV-B, rhinovirus, and echovirus. Using RT-PCR detection, CV-B genome was detected in 5 out of 12 (42%) newly diagnosed T1D patients and in 1 of 12 (8%) patients during the course of T1D. None of T2D patients and none of 15 healthy controls had enterovirus sequences in their blood (8). CV-B4 E2 can persistently infect human β cells (9) and a CV-B4 variant infects β cells leading to a disturbance of proinsulin synthesis and insulin secretion (10). The mechanism most accredited to explain the link between CV-B infection and T1D is a specific tropism of the virus for insulin-secreting islet β cells (11) – that is, mediated by their expression of the specific virus receptor – and a bystander activation of autoreactive T cells by antigens released by β cells after their damage caused by CV-B infection (12). Another crucial study has shown that CV-B4 is able to infect β cells in patients with T1D and that such infection is associated with both inflammation and severe β -cell functional disturbance (13). The persistent aspect of enterovirus infection is also an important factor to take into account [for a complete review, see Ref. (14)]. Very recently, this scenario received a strong support through the Diabetes Virus Detection (DiViD) study that detected a low-grade enteroviral infection in the islets of Langerhans collected from living patients newly diagnosed with T1D (15). This study does not prove a causal relationship between enterovirus infection and T1D, but is the first to detect enterovirus in pancreatic islets from patients close to the time of their diagnosis of T1D. The association between T1D and viral infections has also been previously reinforced by a genetic linkage between T1D susceptibility and host determinants of the antiviral response, such as the antiviral oligoadenylate synthase (OAS1) and the interferon-induced helicase (IFIH1), which intervene in innate immunity by recognition of RNA genome of picornaviruses, such as enteroviruses (16, 17). Besides this pancreotropism of CV-B, we have been exploring for a long time another mechanism that could play an essential and complimentary role in the development of the diabetogenic autoimmune response, namely, thymus infection.

Thymus-Dependent Central Self-Tolerance to Islet β Cells

As previously demonstrated that the thymus epithelium plays a unique role in programming central self-tolerance to neuroendocrine functions [complete reviews in Ref. (18–20)], as well as to many tissue-related antigens (21). Following gene transcription in the thymus, neuroendocrine precursors are processed not according to the classical model of neurosecretion but for presentation by, or in association with, the thymic MHC machinery. In the thymus, MHC presentation of neuroendocrine self-peptides promotes two intimately associated but

paradoxical events: (1) negative selection and deletion of self-reactive T cell clones and (2) Generation of self-specific regulatory T (tTreg) cells that are able to inhibit in the periphery those “forbidden” self-reactive T cells that escaped thymic clonal deletion. The AutoImmune REgulator (AIRE) protein controls intrathymic transcription of neuroendocrine genes, including all the members of the insulin gene family (22) that are transcribed in the murine thymus according to the following hierarchy: *Igf2* > *Igf1* > *Ins2* > *Ins1*. Thymic self-antigen expression and AIRE function are also regulated by epigenetic and post-translational mechanisms (23).

There is now mounting evidence that a defect in intrathymic negative selection is implicated in the development of autoimmune endocrine diseases, such as T1D (24–27), although this is still discussed for the non-obese diabetic (NOD) thymus (28, 29). Contrary to *Igf1* and *Ins2*, *Igf2* transcription is defective in the thymus of diabetes-prone of bio-breeding (BB) rats (30), one of the two animal models of T1D with the NOD mouse. In humans, *INS* transcripts are measured at a lower level in the thymus from fetuses with short class I VNTR alleles, the second genetic trait (*IDDM2*) of T1D susceptibility (31, 32). Both VNTR alleles and AIRE determine the concentration of *INS* transcripts in the human thymus (33). In the mouse, *Ins2* is predominantly transcribed in the thymus, while *Ins1* expression is dominant in islet β cells, which leads to a higher immunological tolerance to *Ins2*. This explains why the breeding of *Ins2*^{-/-} mice onto the NOD background accelerates insulinitis and diabetes onset (34), whereas insulinitis and diabetes are markedly inhibited in *Ins1*^{-/-} congenic NOD mice (35). There is now firm evidence that *Ins1* codes for the primary insulin-derived autoantigenic epitopes tackled by the autoimmune diabetogenic process (36, 37). In addition, there is a very rapid onset of autoimmune diabetes after a thymus-specific *Ins1* and *Ins2* deletion resulting from the crossing of *Ins1*^{-/-} mice with mice presenting a specific *Ins2* deletion in Aire-expressing medullary thymic epithelial cells (TECs) (38). The insulin transactivator *Mafa* also regulates *Ins2* transcription in the thymus and targeted *Mafa* disruption induces appearance of anti-islet antibodies (39).

Tolerogenic Properties of IGF-2: Multiple Facets

Given the direct relationship between the expression level of a protein/peptide in the thymus and the immunological tolerance to this protein/peptide (40), the hierarchical profile of the intrathymic expression of insulin-related peptides (*IGF-2* > *IGF-1* > insulin) suggests that tolerance to insulin-like growth factor-2 (*IGF-2*) is high and that tolerance to insulin is low. This is indirectly supported by the fact that insulin is the primary autoantigen of T1D (36, 37) while no autoimmune response against *IGF-2* has ever been reported. Conversely, the highly immunogenic properties of insulin might actually be related to its very low expression in rare medullary (m) TEC subsets. Recently, the alternate variant *INS-IGF-2* has been identified as a novel autoantigen in T1D (41), but there is still no data about the expression of this hybrid protein in thymic epithelium. Spontaneous autoimmune diabetes does not develop in *Igf2*^{-/-} mice although these mice display

a marked lower tolerance to insulin, which evidences that *Igf2* expression mediates cross-tolerance to insulin and is required for the programming of a complete immunological tolerance to this protein (42). The homologous sequences Ins B9-23 and IGF-2 B11-25 compete for binding to the MHC-II DQ8 allele, and their presentation to PBMCs isolated from DQ8⁺ T1D adolescents induce distinct cytokine profiles with a regulatory profile for IGF-2 B11-25 that is not observed for Ins B9-23 (43). Two recent studies have further evidenced the tolerogenic properties of IGF-2 by enhancement of Treg cell functions in an experimental model of food allergy (44), as well as promotion of antigen-specific Breg cell properties (45).

Our studies have also shown that the blockage of IGF-mediated signaling in the thymus severely interferes with T-cell growth and differentiation blocks T-cell differentiation (46), which was further confirmed by the demonstration that an antibody to CD222 (the IGF-2 receptor, an endosomal transporter that regulates protein trafficking) plays a central function in the initiation of T-cell signal transduction (47).

Therefore, the predominant expression of IGF-2 in the thymus is not only associated with a higher immunological tolerance to this protein but also seems to confer significant tolerogenic properties to IGF-2- and IGF-2-derived antigen sequences. On these experimental bases, we have proposed the novel concept of “negative self-vaccination” that is under current development through DNA vaccine methodology (48).

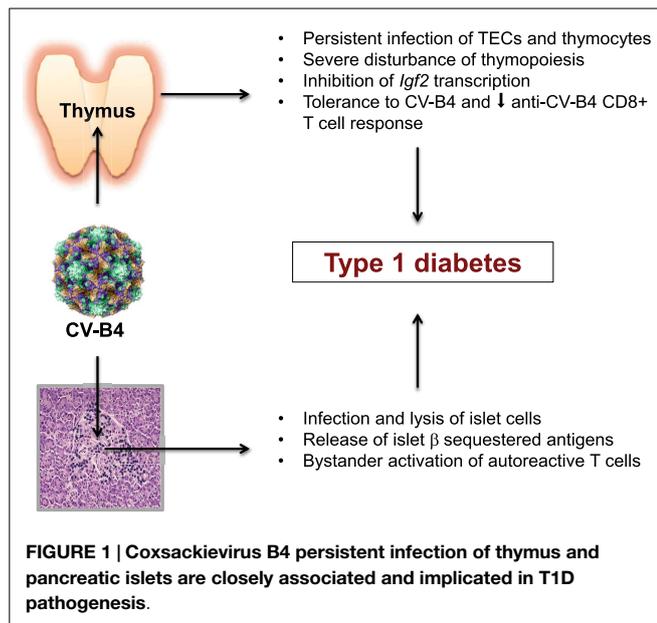
Thymus Infection by Enteroviruses

Given the programming of self-tolerance to islet β cells in the thymus and its defect in the development of the autoimmune diabetogenic response, we investigated the question of a putative role played by an enteroviral infection in an acquired dysfunction of the three major properties of this primary lymphoid organ: thymopoiesis, establishment of central self-tolerance, and generation of self-antigen-specific tTreg cells. A persistent replication of CV-B4 E2 (a “diabetogenic” CV-B strain) and JBV (a prototype CV-B strain) in primary cultures of human TECs was demonstrated by detection of positive- and negative-strand viral RNA in extracts from cell cultures, by immunofluorescence staining of the VP1 capsid protein, and by release of infectious particles up to 30 days after culture inoculation without any apparent cytolytic effect. The persistence of CV-B4 infection was associated with an increased rate of TEC proliferation and with an increase in the secretion of the cytokines IL-6, LIF, and GM-CSF in the supernatants. CV-B4 replication was not restricted to the CV-B4 E2 strain and did not depend on the genetic background of the host. However, cytokine secretion in human TEC cultures infected with CV-B4 E2 was higher than in cultures infected with CV-B4 JBV (49). Therefore, although they are considered as cytolytic viruses, enteroviruses can infect persistently some tissues, such as thymus and pancreas.

Coxsackievirus B4 E2 is also able to infect human fetal thymic organ cultures (FTOC). Viral RNA was detected by quantitative RT-PCR in CV-B4 E2-infected human FTOC, which supported high yields of virus production, as well as in flow-sorted thymic T cell populations for 7 days after infection. In FTOC,

double positive CD4⁺CD8⁺ thymocytes were the principal target cells of infection and were progressively and severely depleted with no sign of apoptosis. Of note, massive thymic depletion of developing T cells and the subsequent CD4⁺CD25⁺ tTreg cells was shown previously to result in systemic autoimmunity (50). CV-B4 E2 replication caused a major up-regulation of MHC class I expression on thymic T cells and TECs. This MHC class I up-regulation was correlated with markers of CV-B4 infection (viral RNA quantification, release of infectious particles), and this was the result of a direct infection rather than caused by production of soluble factors, such as interferon- α (51). Interestingly, Krogvold et al. also reported an overexpression of MHC class I in the islets of all the patients included in their recent study (15). CV-B4 E2 was similarly shown to disturb T-cell differentiation in infected murine FTOC (52). In concordance with previous observations (53), CV-B4 oral inoculation of outbred mice results in a systemic spreading of viral RNA and a detection of viral RNA in thymus, spleen and blood up to 70 days after inoculation (54). Finally, CV-B4 infection of a murine mTEC line induces a dramatic decrease in *Igf2* transcription and IGF-2 production in long-term cultures of this cell line, while *Igf1* transcripts were much less affected and *Ins2* transcripts were not detected in these experimental conditions (55). Inoculation of the mTEC line with CV-B3, CV-B4 JVB, or echovirus 1 also induced a decrease in IGF-2 production, while herpes simplex virus 1 stimulated IGF-2 production. As already cited, a defect of *Igf2* expression in the thymus was suggested to play a role in the development of autoimmune diabetes in the diabetes-prone BB rat (30). Although these effects need to be reproduced *in vivo*, they strongly support our hypothesis that CV-B4 infection of the thymus could disrupt central self-tolerance to the insulin family, and could also enhance CV-B4 virulence through induction of central immunological tolerance to this virus. We are currently investigating the molecular mechanisms responsible for the CV-B-induced decrease of thymic IGF-2 expression in this mTEC line and *in vivo* after oral inoculation of CD1 mice. Since the CV-B-mediated effects in mTEC line are more pronounced on IGF-2 protein than on *Igf2* transcription, we concluded that post-transcriptional and/or post-translational mechanisms could be both involved.

As previously discussed by Zinkernagel (56), fetal exposure to maternal enterovirus infections should also be taken into account. One study has shown that enterovirus infection during the first trimester of pregnancy is not associated with a higher risk for T1D in the childhood (57), but another one has evidenced that such maternal enterovirus infection was a risk factor in offspring diagnosed with T1D between 15 and 30 years of age (58). More recently, a study has investigated that the effects of CV-B4 E2 oral inoculation of CD1 mice at days 4, 10, or 17 of gestation. Severe inflammation of the pancreas and higher glucose blood levels were observed only when dams were previously infected and, in particular, at day 17, thus, in the late phase of pregnancy (59). CV-B4 E2 oral inoculation of pregnant mice is also associated with fetal thymus infection and disturbance of T-cell differentiation (Jaidane, personal communication). Obviously, the question of maternal-fetal transmission of enterovirus infection highly deserves to be further investigated.



Conclusion: A Model Associating CV-B-Induced Dysfunction of Central Tolerance and Peripheral Bystander Activation

In addition to the necessity of standardization for the serological and RT-PCR detection of CV-B infection as recommended by Gale and Atkinson (60), there is also an urgent need for a thorough investigation of the relationships between CV-B and the host immune system (Figure 1). What is our current knowledge about this point? CV-B4 is able to persistently infect α and β cells in human pancreatic islets, and to cause functional impairment and β -cell death characterized by nuclear pyknosis. The CV-B4-induced damage to the islet cells causes release and presentation of sequestered islet antigens. Through bystander activation, autoreactive T cells initiate the diabetogenic autoimmune process. Now, with regard to the origin of these autoreactive T cells,

more and more experimental evidence points to the generation in the thymus of “forbidden” T cell clones due to a failure of the central tolerogenic mechanisms. This thymus defect results in a progressive enrichment of the peripheral T cell repertoire with self-reactive T cells and a decreased generation of self-antigen tTreg cells. From our collaborative work, it appears that CV-B4 is also able to persistently infect the epithelial and lymphoid compartments of the thymus. CV-B4 infection of the thymus leads to increased secretion of diverse cytokines synthesized in TECs, to a severe depletion of double positive CD4⁺CD8⁺ thymocytes, and to marked up-regulation of MHC class I molecules expressed by TECs and double positive thymic T cells. Moreover, CV-B4 infection of a murine mTEC line induces a marked decrease in *Igf2* transcription and IGF-2 production. Therefore, a CV-B4 persistent infection of the thymus may lead to significant thymus and immune dysregulation that associates:

- A significant impairment of thymus-dependent self-tolerance issued from the decrease in the presentation of insulin family related self-antigens, and putatively a direct viral interference with self-antigen presentation (61).
- An induction of central tolerance to CV-B4 and a secondary decrease of anti-CV-B4 CD8⁺ T-cell mediated response, so that further exposure to the virus could promote more severe damage to the peripheral target tissues.

If further research confirmed such rational assumption based on our new knowledge of thymus functions, then an anti-CV-B4 vaccination could be considered as a strategy for T1D prevention in regions with a high incidence of this disease such as in Scandinavian countries (62).

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