Thymus-specific deletion of insulin induces autoimmune diabetes

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Insulin expression in the thymus has been implicated in regulating the negative selection of autoreactive T cells and in mediating the central immune tolerance towards pancreatic β -cells. To further explore the function of this ectopic insulin expression, we knocked out the mouse *Ins2* gene specifically in the *Aire*-expressing medullary thymic epithelial cells (mTECs), without affecting its expression in the β -cells. When further crossed to the *Ins1* knockout background, both male and female pups (designated as ID-TEC mice for insulin-deleted mTEC) developed diabetes spontaneously around 3 weeks after birth. β -cell-specific autoimmune destruction was observed, as well as islet-specific T cell infiltration. The presence of insulin-specific effector T cells was shown using ELISPOT assays and adoptive T cell transfer experiments. Results from thymus transplantation experiments proved further that depletion of *Ins2* expression in mTECs was sufficient to break central tolerance and induce anti-insulin autoimmunity. Our observations may explain the rare cases of type 1 diabetes onset in very young children carrying diabetes-resistant HLA class II alleles. ID-TEC mice could serve as a new model for studying this pathology.

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Introduction

As a multi-factorial, multigenic metabolic disorder, type 1 diabetes (T1D) is promoted by chronic autoimmune destruction of the insulin-producing pancreatic β -cells, whereas other endocrine cells within the islets and the surrounding exocrine cells are largely spared (Tisch and McDevitt, 1996). Such cellular specificity strongly implicates the fundamental role of β -cell-specific self-antigens in the initiation and progression of the disease. It is well established that specific

major histocompatibility complex (MHC) alleles are major contributors of genetic susceptibility to T1D (Todd *et al*, 1987; Morel *et al*, 1988). Autoreactive T cells are released into the circulation because of the faulty presentation of self-antigens by disease-susceptible MHC molecules, which hinder the negative selection process in the thymus (Trucco, 1992; McDevitt, 2001). To date, only two antigens solely expressed in β -cells have been identified: insulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (Lieberman *et al*, 2003; Gianani and Eisenbarth, 2005). Cytotoxic T cells specific for either antigen were isolated from naïve NOD mice, and were able to transfer T1D to NOD-*scid* mice. However, insulin may have a primary function, because the immune response against IGRP could be prevented by inducing tolerance to (pro)insulin in NOD mice (Krishnamurthy *et al*, 2006). Indeed, in pre-diabetic patients, insulin-specific autoantibodies are detected frequently, long before the onset of clinical symptoms, and their affinities correlate with the progression of the autoimmune attack (Achenbach *et al*, 2004). Similarly, insulin-specific T cells are predominant components of the islet-infiltrating T cells at the pre-diabetic stage in NOD mice. Multiple T cell clones, targeting different insulin epitopes, have been isolated, including both CD4⁺ and CD8⁺ T lymphocyte sub-types (Wegmann *et al*, 1994). Unlike other known autoantigens identified in T1D, such as glutamic acid decarboxylase 65 (GAD 65), tyrosine phosphatase-like protein, IA-2 and IGRP, (pro)insulin is the only autoantigen for which transgenic overexpression in antigen presenting cells (APCs) affects the disease progression in NOD mice (Gianani and Eisenbarth, 2005). Moreover, when a metabolically active, mutant form of insulin (which has alanine in place of tyrosine at the 16th amino-acid position of the B-chain) was transgenically expressed in insulin knockout NOD mice, insulinitis and the development of autoimmune diabetes were totally abolished (Nakayama *et al*, 2005). The altered sequence presumably changes the antigenicity of the dominant insulin B9–23 peptide. These findings further implicate the function of insulin in the autoimmune etiopathogenesis of T1D.

The broad range of tissue-specific genes that are ectopically expressed in the thymus, and the seminal studies of *Aire* knockouts highlight the importance of central selection mechanisms in establishing and maintaining a T cell repertoire that is tolerant towards peripheral tissues (Anderson *et al*, 2002; Kyewski and Klein, 2006). As a putative transcription factor, *Aire* was shown to promote the thymic expression of a number of peripheral tissue antigens (PTAs), such as *Mucin-6* (stomach specific) and interphotoreceptor retinoid-binding protein (*Irbp*, eye specific), which facilitate self/non-self distinction (DeVoss *et al*, 2006; Gavanescu *et al*, 2007). The presentation of PTAs in the thymus enabled the deletion of high-affinity, potentially autoreactive T cells through negative selection. In the *Aire* knockouts, massive T cell infiltration in multiple solid organs was observed following downregulation of PTA expression in the thymus (Anderson *et al*, 2005).

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Indeed, when the thymus of an *Irbp*-knockout mouse was transplanted under the kidney capsule of the thymus atrophic nude mouse, T cell infiltration was detected in the eyes after 10 weeks, suggesting that failure of thymic expression of peripheral antigens is sufficient to cause organ-specific autoimmunity (DeVoss *et al*, 2006).

Similar to other *Aire*-regulated PTAs, insulin expression has been found in the thymus (Jolicœur *et al*, 1994). Variations of this expression may be especially relevant to diabetes. The human IDDM2 locus was mapped to a variable number tandem repeat (VNTR) region 5' proximal to the insulin gene (Pugliese *et al*, 1997; Vafiadis *et al*, 1997). Different VNTR alleles correlate with different thymic insulin expression levels; alleles with shorter repeats result in a reduction of expression and predisposition to T1D. Such a correlation between levels of thymic insulin production and anti-insulin autoimmunity was also suggested by animal studies (Chentoufi and Polychronakos, 2002).

It remains controversial which cells in the thymus are expressing insulin. APCs of bone marrow origin, including thymic dendritic cells and macrophages were reported to express (pro)insulin transcripts (Pugliese *et al*, 2001). In contrast, studies using purified thymic cell populations showed that (pro)insulin was expressed only in thymic epithelial cells (TECs) of the endoderm origin (Palumbo *et al*, 2006). In an attempt to resolve this controversy, Faideau *et al* (2006) created bone marrow chimeras in *Ins2* knockout mice and showed that tolerance to (pro)insulin-2 was due to radio-resistant cells in the thymus, presumably epithelial cells.

To address the importance of thymic insulin expression in mediating immune tolerance towards pancreatic β -cells even in the context of MHC allelic resistance (Pietropaolo *et al*, 2002), we first examined the *Ins2* ectopic expression in specific thymic cell types, and identified endoderm-derived medullary TECs (mTECs) as the major *Ins2*-expressing cells in the thymus, consistent with previous publications (Anderson *et al*, 2002; Palumbo *et al*, 2006). To elucidate the essential role of this mTEC-insulin expression, we developed an animal model in which the mouse *Ins2* gene was specifically deleted in mTECs, whereas its production in the pancreas remained intact. When these mTEC-*Ins2*-deleted animals also lacked (pro)insulin 1, the other isoform of the mouse insulin gene, spontaneous T1D developed at around 3 weeks after birth.

Our study showed that disruption of thymic expression of a single tissue-specific self-molecule is sufficient to trigger autoimmunity towards the relevant tissue and results in pathologic damage even in the presence of disease-resistant alleles of MHC molecules.

Results

Ins2 expression in medullary epithelial cells of the thymus

We chose the *Ins2* gene as the target in our study because it is the predominant isoform of mouse insulin expressed in the thymus (Chentoufi and Polychronakos, 2002), and because the *Ins2* gene alone is sufficient to maintain glucose homeostasis in animals with the *Ins1* gene knocked out (Duvill *et al*, 1997). To examine *Ins2* expression in specific cell types in the thymus of *Ins1* knockout mice, we used a magnetic bead-based isolation method in conjunction with flow cytometry to separate thymic stromal cells into four groups, based on their characteristic surface antigens and molecular signatures. Specifically, hematopoietic lineage-derived thymic APCs were separated as CD45⁺, MHC class II⁺, CD11c⁺ thymic dendritic cells (R1) and CD45⁺, MHC class II⁺, CD11c[−] macrophages and B-cells (R2); whereas TECs were collected as CD45[−], EpCAM⁺, Ly51^{low}, UEA^{high} medullary TECs (R3) and CD45[−], EpCAM⁺, Ly51^{high}, UEA^{low} cortical TECs (R4) (Figure 1A). The purity of the separation was validated by RT-PCR analysis of the expression of cell type-specific genes: CD45 transcripts were only detectable in bone marrow-derived cells (R1 and R2), whereas cytokeratin 2/8 (K-2/8) transcripts were predominantly present in epithelial cells (R3 and R4). As shown in Figure 1B, and consistent with previous publications, mTECs are responsible for nearly all the ectopically expressed *Ins2* in the thymus (Anderson *et al*, 2002; Faideau *et al*, 2006; Palumbo *et al*, 2006).

Generation of an animal model to knockout *Ins2* in *Aire*-expressing mTECs

To elucidate the physiological role of mTEC-specific *Ins2* expression, we chose to use the Cre-Lox system to specifically deplete insulin expression in these particular thymic cells, without affecting its production in pancreatic β -cells (see Supplementary Figure S1 for our strategy). As the first step, two *loxP* sites were genetically engineered to flank the

Figure 1 Analysis of *Ins2* expression in specific thymic stromal cells of *Ins1* knockout mice. (A) Isolation of thymic stromal cells. Left panel, fluorescence-activated cell sorting of bone marrow-derived antigen presenting cells. CD45⁺, MHC II⁺ cells were further gated for CD4[−], CD11c⁺ thymic dendritic cells (R1), and CD4[−], CD11c[−] macrophages and B cells (R2). Right panel, CD45[−], EpCAM⁺ stromal cells were sorted for medullary epithelial cells (R3: Ly51^{low}, UEA^{high}) and cortical epithelial cells (R4: Ly51^{high}, UEA^{low}). The same R1–R4 designation has been used throughout the paper unless otherwise specified. (B) RT-PCR analysis of the mouse *Ins2* gene expression in specific thymic antigen presenting cells (R1–R4). The house keeping *Hprt* gene expression was used as control. K-2/8, cytokeratin 2/8.

mouse *Ins2* gene (designated as *Ins2^l*) (Figure 2). Once homozygous *Ins2^{l/l}* mice were obtained, they were crossed to *Ins1* knockout mice (*Ins1^{-/-}*) to generate double homozygous mice (*Ins1^{-/-}:Ins2^{l/l}*). These mice are viable and euglycemic throughout their lifespan, indicating that the inserted *loxP* sites did not affect insulin production and carbohydrate metabolism. Efficient deletion of the *Ins2* gene was validated by Southern blot analysis of DNA samples isolated from the offspring of *Ins2^{l/l}* mice crossed to germline-expressing, EIIa-Cre mice (data not shown). Furthermore, when these germline-*Ins2*-deleted offspring were further crossed to *Ins1^{-/-}* mice, double homozygous mice (*Ins1^{-/-}:Ins2^{-/-}*) died within 2–3 days postnatally, due to the absence of insulin in pancreatic β -cells (Supplementary Figure S2), in accordance with previous reports (Duvill *et al*, 1997).

It has been shown that the *Aire* gene is expressed predominantly in cells of the thymic medullary epithelium (Figure 1B) and is able to regulate the ectopic expression of the *Ins2* gene in the thymus (Anderson *et al*, 2002). To target

Figure 2 Genetic modification of the mouse *Ins2* gene for thymus-specific deletion. (A) A targeting construct with two *loxP* sites flanking the mouse *Ins2* gene and an *frt*-tagged *Neo* cassette for selection was used to modify the *Ins2* gene through homologous recombination (black dash line). *Neo* cassette within the *loxP*-tagged *Ins2* allele was deleted from the genome by FLP recombinase. A *AseI*, B: *BamHI*, E: *EcoRI*, red bar: 5' external probe. (B) Southern blot analysis of *Ins2*-targeted embryonic stem cell clones. Genomic DNA was isolated, digested using *AseI* and probed with the 5' external probe. * indicates a *loxP*-tagged *Ins2* embryonic stem cell clone. As the result of the genetic modifications, an *AseI* site was introduced to the *loxP*-tagged *Ins2* allele (*Ins2^l*), which appears as the extra 10-kb band on the blot, in addition to the 20-kb band from the wild-type allele (*Ins2⁺*). (C) Agarose gel showing PCR-based genotyping of mice. +, wild type; l, *loxP*-tagged *Ins2* (*Ins2^l*).

mTECs specifically, the putative transcription regulatory element, cloned from the murine *Aire* gene, was used to create an *Aire-Cre* transgene (Figure 3A). Two transgenic lines carrying multiple copies were established and one was selected for further study, as both showed similar expression patterns.

To examine the expression pattern of the *Aire-Cre* transgene, we carried out RT-PCR to analyse the presence of *Cre* transcripts in a number of tissues, including the thymus (Figure 3A). As shown, the *Aire-Cre* transgene was predominantly expressed in the thymus, albeit very weak *Cre* signals were detected in other organs, such as the spleen, the kidney and the pancreas. To further characterize the *Aire-Cre* transgene expression within the thymus, thymic stromal cells were collected from *Aire-Cre* mice and were separated into the four groups (R1–R4) as described above (Figure 1A) and analysed by RT-PCR. As shown in Figure 3B, *Cre* transcripts were present only in the mTECs (R3), but not in the cortical TECs (R4), nor in cells of the hematopoietic lineages (R1 and R2), which is consistent with the expression pattern of the endogenous *Aire* gene. We also examined the presence of *Cre* transcripts in isolated pancreatic islets (IS), to rule out the possibility that the faint *Cre* band observed in the whole pancreas tissue was from islet β -cells. In the purified β -cells, no *Cre* transcripts were observed (Figure 3B).

The effectiveness of the *Aire-Cre* transgene to delete *loxP*-tagged targeted genes was tested by crossing the *Cre* transgenic line to *Rosa26R-lacZ* reporter mice (Soriano 1999). In these animals, *Cre*-mediated recombination generates a functional *LacZ* gene, which is transcribed by means of the universally active *Rosa26* promoter. The *LacZ* signal was only observed in the CD45⁺ cells of the thymus of *Aire-Cre:Rosa26R-lacZ* mice, indicating the epithelial nature of the *Cre* expressing cells (Figure 3C). To rule out any *Cre* recombinase activity in the pancreatic β -cells in the transgenic lines, immunohistochemical analysis of the pancreata was carried out. Pancreatic *LacZ* expression was only detectable in control *Rip-Cre:Rosa26R-lacZ* mice, in which the expression of *Cre* transcripts was driven by the rat insulin promoter (Figure 3D). Thus, the use of the *Aire-Cre* transgene enabled us to target the floxed genes in specific cell types in the thymus.

To prove directly that the *Aire-Cre* transgene can delete the floxed *Ins2* gene in mTECs efficiently, we collected thymus from 6–8-week-old *Aire-Cre:Ins1^{-/+}:Ins2^{l/l}* mice. Furthermore, thymic stromal cells were separated into the four groups (R1–R4). Genomic DNA isolated from each group was analysed by PCR using the primer pairs shown in Figure 2A. As the complementary sequence of the reverse primer was absent when the floxed *Ins2* gene was deleted from the genome, only the intact *Ins2* floxed allele could be amplified. As shown in Figure 3E, the PCR signal of the floxed *Ins2* gene is significantly lower in mTEC samples (R3) than those of other thymic stromal cells, indicating efficient deletion of the floxed *Ins2* gene in mTECs. To ensure that the *Aire-Cre* transgene would not cause deletion of the floxed *Ins2* gene in the pancreatic β -cells, islets were also isolated from these mice, and the genomic DNA was examined. The PCR signal of the floxed *Ins2* alleles was unaffected, consistent with results from the *Rosa26R-lacZ* reporter mice experiments (Figure 3E).

Figure 3 Characterization of *Aire-Cre* transgene expression. (A) Upper panel, schematic drawing of the *Aire-Cre* construct. A 23-kb and an 11-kb genomic fragment were subcloned from the 5' and 3' of mouse *Aire* gene, respectively, and engineered to regulate the Cre gene expression. Lower panel, RT-PCR analysis of *Aire-Cre* transgene expression. (B) RT-PCR analysis of Cre expression in specific thymic stromal cells (R1-R4) isolated from the *Aire-Cre* transgenic mice. (C) *Aire-Cre*:*Rosa26R-lacZ* thymus stained with anti-LacZ antibody (green) and CD45 antibody (red). LacZ⁺ and CD45⁺ cells (yellow). Arrows indicate LacZ⁺, CD45⁺ thymic epithelial cells. (D) Absence of Cre recombinase expression in pancreatic β -cells of *Aire-Cre* mice. Pancreas samples from six-week-old *Aire-Cre*:*Rosa26R-lacZ*, *Rosa26R-lacZ* (negative control), and *Rip-Cre*:*Rosa26R-lacZ* (rat insulin promoter, positive control) mice stained with anti-insulin (red) and anti-LacZ (green) antibodies. (E) Efficient deletion of the floxed *Ins2* allele in mTEC cells collected from *Aire-Cre*:*Ins1*^{-/-}:*Ins2*^{+/l} mice. Genomic DNA was isolated from different thymic stromal cells (R1-R4) and pancreatic islets and the presence of the floxed *Ins2* allele was analysed using PCR, with the same primer pairs depicted in Figure 2B. PCR amplification of the mouse *Ica1* gene was used as control of the genomic DNA input. BM, bone marrow; H, heart; IS, islets; Kd, kidney; Lu, lung; Lv, liver; Pan, pancreas; Sp, spleen; Th, thymus.

Figure 4 Thymic insulin expression in ID-TEC mice. (A) RT-PCR analysis of thymic insulin expression in ID-TEC mice. (B) Real-time PCR quantification of thymic insulin expression in ID-TEC and control mice. (Error bar: s.e.m.) Mk, molecular size marker.

Ecotopic thymic insulin expression in ID-TEC mice

On the basis of the above results, we proceeded to breed the *Aire-Cre* transgenic line with *Ins1*^{-/-}:*Ins2*^{+/l} mice to homozygosity, generating the ID-TEC mice (insulin-deleted thymic epithelial cell; genotype: *Aire-Cre*:*Ins1*^{-/-}:*Ins2*^{+/l}), to knock

out insulin expression specifically in the endoderm-derived thymic epithelial cells. As expected, according to Mendel's law, about a quarter of the newborns from the *Ins1*^{-/-}:*Ins2*^{+/l} and *Aire-Cre*:*Ins1*^{-/-}:*Ins2*^{+/l} mating were ID-TEC pups. Littermates with *Ins1*^{-/-}:*Ins2*^{+/l}, *Ins1*^{-/-}:*Ins2*^{+/l}, or *Aire-Cre*:*Ins1*^{-/-}:*Ins2*^{+/l} genotypes were used as controls unless otherwise specified. The ID-TEC pups were born with normal size and body weight, indicating that the genetic manipulation did not affect their prenatal growth.

As predicted, the levels of thymic *Ins2* expression in ID-TEC mice were drastically lower than those of controls (*Ins1*^{-/-}:*Ins2*^{+/l} mice) (Figure 4A). Real-time PCR analysis showed that thymic *Ins2* mRNA expression is knocked down to less than 10% of the controls (Figure 4B). The observed, very low residual insulin mRNA expression could come from the small population of bone marrow-derived, CD45⁺ thymic APCs that may express low levels of insulin mRNA, or could be due to the reported abnormal upregulation of (pro)insulin expression in bone marrow-derived APCs under various experimental and/or hyperglycemic situations (Kojima *et al*, 2004; Carlsen and Cilio, 2008).

ID-TEC mice develop spontaneous diabetes within 3 weeks after birth

In early postnatal life, ID-TEC pups have normal size and weight compared with their control littermates and have normal blood sugar levels. When pancreata collected from newborn pups (day 1 postnatal) were examined by immunohistochemistry, islet structures were observed to be normal with abundant insulin-producing β -cells (Figure 5A). These data further confirmed the absence of *Aire-Cre* transgene expression in the islet β -cells of ID-TEC mice.

At postnatal day 10, ID-TEC mice remained normoglycemic with similar plasma insulin levels and pancreatic insulin contents compared with controls (Figure 5B and C). However, within the following week, ID-TEC pups started to develop elevated blood sugar levels (Figure 5D). By 3 weeks, in striking contrast to control mice, severe hyperglycemia developed in both male and female ID-TEC mice (Figure 5D). Consistently, circulating insulin levels were significantly lower than those of controls, indicating insufficient β -cell function (Figure 5E). Immunohistochemical examination of pancreata, collected from diabetic ID-TEC mice (blood glucose ~ 500 – 600 mg/100 ml), showed that only a small number of insulin-positive β -cells were still present at 4 weeks after birth, whereas glucagon-secreting α -cells remained largely intact (Figures 5F). In some islet-like areas, granulocytic invasion was prominent, indicating the active scavenging of islet tissues after acute damage (Supplementary Figure S3). Taken together, these data clearly showed that a drastic loss of insulin-producing β -cells occurred in ID-TEC mice during the postnatal time window of 2–3 weeks.

Insulin-specific, T cell mediated islet β -cell destruction in ID-TEC mice

The specific loss and damage of β -cells in ID-TEC mice lead us to investigate the autoimmune nature of the disease. As expected, pancreata collected from non-diabetic littermate controls were largely free of infiltrating lymphocytes (Supplementary Figure S4), whereas numerous CD4⁺ and CD8⁺ T cells (i.e. insulinitis) were found in diabetic ID-TEC pancreata when examined by immunohistochemistry at 3 weeks (Figure 6A–D). In addition, B-cells and macrophages are also observed in the islets, reflecting an ongoing inflammatory response (Figure 6E and F). Moreover, islet infiltrations of different severities were observed in pancreata of pre-diabetic 14-day-old ID-TEC pups (Supplementary Figure S5). To further characterize the anti-islet autoimmunity, we analysed the presence of anti-insulin autoantibody (IAA) in the sera of ID-TEC mice with an ELISA-based colorimetric assay. As shown in Figure 6G, the light absorption signals from ID-TEC sera were well above those from littermate controls, showing the existence of IAA in the circulation of ID-TEC mice (Figure 6H). Consistently, sera collected from 6–8-week-old diabetic ID-TEC mice were able to recognize islet cells in sections prepared from C57BL/6 *Rag1*^{−/−} (*Rag1*) mice.

Interestingly, these clear signs of autoimmunity were found in the ID-TEC mice that, in fact, were derived from three mouse lines with either C57BL/6 (the *Aire-Cre* and *Ins1*^{−/−} lines) or 129:C57BL/6 mixed (the *Ins2*^{+/+} line) backgrounds, all of which carried the H-2^b MHC haplotype, which would normally convey resistance to islet autoimmunity.

To examine the antigen-specificity of the T cells present in the ID-TEC mice, ELISPOT assays were carried out. On stimulation with insulin, IFN- γ -secreting T cells were found

Figure 5 ID-TEC mice develop spontaneous diabetes within 3 weeks after birth. (A) Normal islet development of ID-TEC mice at birth. Pancreata from control and ID-TEC mice were collected at postnatal day 1, and stained using anti-insulin (green) and glucagon (red) antibodies. (B) Plasma insulin levels of 10-day-old ID-TEC pups. (C) Pancreatic insulin contents of 10-day-old ID-TEC pups. Pancreata were collected from three litters and insulin content in each pancreas was measured and plotted.—mean insulin levels; Δ , control littermates; filled red circle, ID-TEC pups. (D) Blood glucose levels of ID-TEC (red line, $n = 7$) and control mice (blue line, $n = 11$). (E) Plasma insulin levels of diabetic, 4-week-old ID-TEC mice. (F) Pancreatic sections stained with anti-insulin (green) and anti-glucagon (red) antibodies.

Figure 6 Diabetes developed in ID-TEC mice is autoimmune in nature. (A–F) Pancreata from ID-TEC mice were collected at postnatal day 21. Pancreatic sections were stained with anti-CD4 (red) (A–C), anti-CD8 (D), anti-B220 (E) and anti-F4/80 (F) antibodies (red). Higher magnification ($\times 400$) of a damaged islet with infiltrating T cells is shown in (B). Sections (A), (B), (D) and (F) were counter stained with anti-glucagon antibody (green). Nuclei were stained with Hoechst 33342 in all the sections (blue). Damaged islets are outlined by dashes. (G) Sera collected from 3–8-week-old diabetic ID-TEC mice and age-matched controls (1:50 dilution) were subjected to insulin autoantibody (IAA) assay. Data are presented as absorbance at 450 nm (OD₄₅₀). —, mean value. $**P < 0.01$. (H) Sera collected from 6–8-week-old diabetic ID-TEC mice or control mice were used to stain islet sections from C57BL/6 *Rag1*^{−/−} mice (1:50 dilution). Bound mouse antibody was revealed with goat anti-mouse IgG. (I) Presence of autoreactive T cells responding specifically to insulin in ID-TEC mice. Suspensions of splenocytes were pooled from three animals, and cultured overnight with whole insulin protein, whole GAD65 protein, insulin B-chain peptide 9–23 (Ins B9–23), or GAD65 peptide 206–220 (GAD 206–220). The frequency of IFN- γ -producing cells was measured by ELISPOT assays in triplicate. The upper panel shows one representative result from three independent assays. Filled bar, ID-TEC mice; open bar, controls. Representative microscopic pictures of ELISPOT assay using insulin as antigen are shown in the lower panel: ID-TEC splenocytes (left), controls (right).

in ID-TEC mice. Almost 50% of the spots were distinctly larger, indicating a high level of IFN- γ production by these T cells (Figure 6I). We also examined the insulin peptide B9–23, an immunodominant peptide found in both NOD mice and human T1D patients (Abiru *et al*, 2001; Alleva *et al*, 2001), and observed similar levels of response as those of insulin. In contrast, neither whole GAD65 protein nor its immunodominant peptide in NOD mice, peptide 206–220 (GAD 206–220), was able to stimulate a response at a level higher than littermate controls. The limited T cell response to the whole GAD protein and GAD 206–220 also suggests that epitope spreading had not yet occurred in ID-TEC mice. We were unable to detect any IL-4-secreting T cells using the same set of antigens, suggesting a primary TH1 response to insulin in ID-TEC mice (data not shown).

ID-TEC thymus is sufficient to induce spontaneous islet autoimmunity

To further show that the lack of insulin expression in thymic epithelial cells is sufficient to induce anti-insulin autoimmunity, we collected thymi from 2-week-old ID-TEC mice and transplanted them under the kidney capsules of athymic nude mice. At 16 weeks after thymus transplantation, the recipients were sacrificed and pancreata collected for histological evaluation. Flow cytometry analysis of lymph nodes and spleens obtained from thymus-transplanted recipients showed the success of T cell reconstitution (Figure 7A). Although no T cell infiltrates were found in pancreata from recipients of control thymi (Figure 7B), insulinitis was readily observed in all pancreata collected from nude mice transplanted with the ID-TEC thymi (Figure 7C–E). These

Figure 7 ID-TEC thymus is sufficient to transfer islet autoimmunity. (A) Representative FACS results showing the successful T cell reconstitution in nude mice with thymus transplantation underneath the kidney capsule. (B–E) At 16 weeks after thymus transplantation, pancreata were collected and stained with anti-CD4 (red) and anti-insulin (green) antibodies. (B) A representative pancreas section from nude mice transplanted with thymi from littermate controls, in which *Ins2* was intact, showing the absence of T cell infiltration. (C–E) Representative pancreata collected from nude mice transplanted with ID-TEC thymi.

experiments further showed the presence of thymus-derived, insulin-specific autoreactive T cell clones in the ID-TEC mice.

Islet autoimmunity can be transferred by both CD4⁺ and CD8⁺ T cells of ID-TEC mice

The autoreactivity of ID-TEC T cells towards islet β -cells was further shown by adoptive transfer experiments. Although limited by the number of cells that could be obtained from the extremely young mice, twenty million splenocytes pooled from a number of diabetic ID-TEC mice were infused into immune-deficient *Rag1* mice, which have the same histocompatibility background. At 1–2 weeks after the adoptive transfer, blood glucose levels of the recipients were elevated, compared with animals that received splenocytes from controls (205 ± 18 versus 127 ± 12 mg/100 ml, $n = 5$, $P < 0.01$, Student's *t*-test). Moreover, autoreactive T cells targeting insulin were found among the splenocytes collected from the adoptively transferred *Rag1* mice, as shown by ELISPOT assays for IFN- γ -producing cells (Figure 8A). The recipients remained modestly hyperglycemic for 2 months after the adoptive transfer and the presence of T cells in the proximity of the islets was readily detectable (Figure 8B). Taken together, these results showed that the T cell repertoire of diabetic ID-TEC mice includes cells that specifically recognized islet autoantigens, among them insulin or its peptides, although they were not able to develop a strong insulinitis.

To further understand the functions that different groups of T cells had in mediating islet infiltration and destruction,

T cells were isolated from the spleen and the lymph nodes of ID-TEC mice as effector CD4⁺CD25⁻ T cells and CD8⁺ cytotoxic T cells, and transferred to *Rag1* mice either alone or together. At 4 weeks after the adoptive transfer, pancreata of *Rag1* mice were collected and analysed by immunohistochemistry. Insulinitis and islet β -cell destruction were observed in *Rag1* mice adoptively transferred with either CD4⁺CD25⁻ or CD8⁺ T cells, indicating that both cell types were capable of reacting to islet β -cells and eliciting an autoimmune response (Figure 8C and D). These results indicated that insulin-reactive T cells were present in both CD4⁺ and CD8⁺ T cell compartments in ID-TEC mice, and both types of T cells could home to the pancreas and cause damage to the islet β -cells.

ID-TEC mice displayed unimpaired immune tolerance towards autoantigens other than insulin

To exclude the possibility that our genetic manipulations cause generalized defects in central negative selection, we examined immune tolerance to male-specific HY autoantigens in ID-TEC mice. It is well established that thymocytes specific for HY minor antigens are negatively selected in the male thymus, whereas they can survive the selection process in the female thymus due to the absence of male antigen expression and presentation within the female thymic medulla (Simpson *et al*, 2002). Consequently, HY autoantigen-specific T cells were absent in the periphery of the male mouse, but can readily be detected in females on HY autoantigen challenge.

Figure 8 Both CD4⁺ and CD8⁺ T cells can transfer islet autoimmunity in immune-compromised *Rag1*^{-/-} mice. (A) Anti-insulin autoimmunity can be transferred to C57BL/6 *Rag1*^{-/-} hosts, which carry the same MHC haplotype (H2^b) as ID-TEC mice, by adoptive transfer of ID-TEC splenocytes. At 3 weeks after reconstitution, splenocytes were pooled from two animals, cultured overnight with either whole insulin or GAD 206–220 peptide as negative control. One representative result from three independent ELISPOT assays is shown. C, controls; I, ID-TEC (B) Detection of T cell infiltration in *Rag1*^{-/-} pancreata after adoptive transfer of ID-TEC splenocytes. At 4 weeks after the transfer, pancreata were collected and stained with anti-CD4 (red) and anti-insulin antibody (green). (C, D) Pancreata were collected from *Rag1*^{-/-} mice adoptively transferred with either CD4⁺CD25⁻ T cells (C) or CD8⁺ T cells (D), and stained with anti-CD4 (C) or anti-CD8 (D) antibody (red). Insulin staining is shown in green.

Thus, examination of the presence of T cells specific to the HY autoantigens enabled us to evaluate the overall efficacy of thymic negative selection in ID-TEC mice.

We first examined the thymic expression of male-specific HY autoantigen expression in ID-TEC mice. As shown in Figure 9A, expression of genes encoding H2^b-restricted HY autoantigens (*Uty*, *Smcy* and *Dby*) was unaffected in the male ID-TEC thymus. Tetramer analysis of CD8⁺ T cells specific to *Uty* (Millrain *et al*, 2001), one of the immunodominant HY autoantigens, showed that *Uty*-specific CD8⁺ T cells were essentially absent in the spleens of male mice, but existed in large numbers in female spleens of both ID-TEC and control mice (Figure 9B). We also examined the presence of male autoantigen-specific CD4⁺ cells in the spleens of both male and female ID-TEC mice through ELISPOT assays. The male-specific peptide, *Dby*, presented by MHC class II molecule (I-A^b), can stimulate IFN- γ secretion in numerous CD4⁺ T cell clones within female spleens, whereas these clones were essentially absent in male spleens (Figure 9C). These data showed unimpaired negative selection of both CD4⁺ and CD8⁺ thymocytes specific to HY autoantigens in the thymus of male ID-TEC mouse, indicating that mTEC-specific insulin

deletion does not generally compromise central selection, but is responsible exclusively for insulin-specific autoimmunity.

As impaired peripheral tolerance could also contribute to islet-specific autoimmunity, we also examined the presence of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (T_{reg}) in ID-TEC mice. Pancreatic lymph nodes, mesenteric lymph nodes and spleens from ID-TEC mice were collected and analysed for the percentage of T_{reg} cells within the T cell population. Similar levels of T_{reg} cells were found in the CD4⁺ T cell population in ID-TEC mice as in littermate controls, suggesting that the observed β -cell-specific immune destruction was not due to an overall quantitative deficiency in the T_{reg} compartment (Supplementary Figure S6).

To examine the functional capability of T_{reg} cells in suppressing the pathologic actions of autoreactive T cells in ID-TEC mice, we adoptively transferred CD4⁺CD25⁻ (T_{eff}) and CD8⁺ (T_{cyto}) T cells into immune-deficient *Rag1* mice, with or without CD4⁺CD25⁺ (T_{reg}) cells. At 4 weeks after the transfer, pancreata of recipient *Rag1* mice were collected and analysed by immunohistochemistry. In striking contrast to the severe insulinitis and islet β -cell destruction observed in *Rag1* mice adoptively transferred with T_{eff} and T_{cyto} cells

Figure 9 Unimpaired negative selection of HY autoantigens and normal T_{reg} cell development in male ID-TEC mice. **(A)** Thymi collected from male ID-TEC mice were separated as in Figure 1 (R1, thymic dendritic cells; R2, macrophage per B-cell; R3, mTEC). Isolated cells were subjected to RT-PCR analysis of expression of the genes encoding the immunodominant male-specific autoantigens (*Uty*, *Smcy* and *Dby*). **(B)** FACS analysis of *Uty*-specific CD8⁺ T cells in the spleens of male and female ID-TEC mice. CD8⁺ T cells were isolated from the spleens using magnetic beads and stained with anti-CD3 and anti-CD8 antibodies, together with D^b/Uty pentamers (ProImmune). Representative FACS data are shown, which were gated on the CD3⁺CD8⁺ population. **(C)** ELISPOT analysis of IFN- γ -secreting CD4⁺ T cell clones stimulated with the immunodominant male-specific HY *Dby* peptide. Data are presented as mean $\pm$ s.e.m. Lower panel, representative microscopic pictures of the ELISPOT assay with male (left) or female (right) ID-TEC splenocytes are shown. **(D, E)** Pancreata collected from *Rag1*^{-/-} mice adoptively transferred with CD4⁺CD25⁻ effector T cells and CD8⁺ T cells, with (D, E) or without (F, G) an equal number of CD4⁺CD25⁺ T_{reg} cells, were stained with either anti-CD4 (D, F) or anti-CD8 (E, G) antibodies (red), counter-stained with anti-insulin antibody (green) and with Hoechst 33342 for nuclei (blue).

(Figure 9D and E), neither peri-insulinitis nor insulinitis was found when T_{reg} cells were co-transferred (Figure 9F and G), showing the suppressive capability of ID-TEC T_{reg} cells in inhibiting the autoimmune destruction of islet β -cells in adoptive transferred *Rag1* mice *in vivo*.

Discussion

Our results on ID-TEC mice established a direct link between thymic ectopic expression of a single tissue-specific gene (i.e., insulin) and the development of a self-tolerant T cell repertoire towards specific peripheral tissues. Such a correlation was shown in immunologically naïve animals, without any manipulation of the natural developmental process of the immune system, or disturbance to the overall thymic negative selection of autoreactive T cells specific to other autoantigens. Moreover, full-blown, spontaneous, autoimmune diabetes develops in ID-TEC mice around 3 weeks after birth. This is in striking contrast to the previously described *Ins2* total knockout mice, in which mild anti-insulin autoimmunity

occurred only after immunization with the (pro)insulin-2 molecule, with neither pathological destruction of β -cells nor T cell infiltration into the pancreas (Faideau *et al*, 2006). Although thymic expression of (pro)insulin-2 molecules was reduced in both mouse models, the presence of functional alleles of the *Ins1* gene, even with its limited expression in the thymus, might explain the only partial breakdown of insulin tolerance observed in the *Ins2* total knockouts. In agreement with Faideau *et al* (2006), ID-TEC control littermates with even one functional *Ins1* allele (genotype *Aire-Cre:Ins1*^{+/-}:*Ins2*^{+/+}) remained euglycemic and showed no sign of insulin autoimmunity through life (our unpublished observation). The *Ins1* knockout in the genetic background of our ID-TEC mice avoids these pitfalls. Our data clearly show that in the absence of *Ins1*, depletion of *Ins2* expression in the thymus is sufficient to impair negative selection in ID-TEC mice, resulting in the migration of *Ins2*-specific, autoreactive T cells out of the thymus. Under these circumstances, autoantigen(s) derived from (pro)insulin-2 molecules was sufficient to drive the autoimmune response

of Ins2-specific T cells in the periphery, causing the progressive destruction of pancreatic β -cells, even in the absence of any evident environmental trigger or manipulation.

In theory, there are a number of ways that will allow insulin epitopes to be presented to T cells in the thymus to mediate central tolerance: bone-marrow-derived thymic APCs and macrophages can take up and transport circulating insulin molecules to the thymus (Li *et al*, 2009); APCs and macrophages could express (pro)insulin ectopically in the thymus (Pugliese *et al*, 2001); endoderm-derived TECs, especially those within the medullary region, express and present to the T cell directly (Faideau *et al*, 2006); or peptides derived from the insulin molecules, produced from mTEC, could be cross-presented by bone marrow-derived APCs to mediate the deletion of the autoreactive T cells within the thymus (Surh and Sprent, 1994). Our data clearly showed that circulating insulin was not effective in mediating negative selection of Ins2-specific T cells in our young animals, as no statistical difference of either pancreatic insulin contents or circulating insulin levels was detected between ID-TEC pups and control littermates at birth and by postnatal day 10. Furthermore, it was previously shown that the efficacy of antigen presentation by dendritic cells increased, at least, 100–1000-fold when an anti-DEC-205 antibody-based strategy was used to facilitate the antigen internalization into the target cell as compared with natural uptake from the circulation (Hawiger *et al*, 2001; Dudziak *et al*, 2007; Mukhopadhyaya *et al*, 2008). Thus, it is conceivable that expression in mTECs could be more efficient, or even indispensable, for effective autoantigen presentation to generate a self-tolerant T cell repertoire. Although we did not observe the presence of *Ins2* transcripts in bone marrow-derived thymic APCs under our experimental conditions (Figure 1B), the residual (<10%) insulin transcripts observed in the ID-TEC thymus might come from these bone marrow-derived cells under the hyperglycemic conditions of the diabetic ID-TEC mice. Indeed, there are a number of reports showing elevation of ectopic insulin expression in bone marrow-derived cells under these abnormal conditions (Kojima *et al*, 2004; Carlsen and Cilio, 2008). Furthermore, overexpression of autoantigens, including insulin, in bone marrow-derived APCs has been shown to attenuate or prevent autoimmune diabetes progression in NOD mice. Whether this regained insulin ectopic production in the immune cells could re-establish central and/or peripheral tolerance remains to be investigated, and the ID-TEC mice would serve as an ideal experimental model.

Ins2 transcripts were also drastically downregulated in the thymus of *Aire* total knockout mice, but only a portion of animals showed anti-islet autoimmunity, with no report of pathologic destruction of pancreatic β -cells (Anderson *et al*, 2002). Our Cre–Lox approach is likely to be more effective at eliminating *Ins2* expression in specific *Aire*-expressing thymic cells. Furthermore, thymic *Ins1* expression in the *Aire* knockout mice might help to eliminate insulin-specific autoreactive T cells, as it is not affected by *Aire* expression. In addition, differences between *Aire* total knockouts and our ID-TEC mice could also result from other important aspects of *Aire* function, such as control of the interactions between T lymphocytes and TECs, or the regulation of TEC differentiation (Cheng *et al*, 2007; Gillard *et al*, 2007). Nevertheless, our data show that the negative selection checkpoint is facilitated by insulin produced by *Aire*-expressing,

endoderm-derived mTECs, although bone marrow-derived cells in the ID-TEC mice may also have a function by cross-presenting insulin peptides expressed in thymic epithelium (Surh and Sprent, 1994; Gray *et al*, 2007), or picked up from circulation (Li *et al*, 2009).

Recently, it was found that *Aire* is also expressed in a subset of CD45⁺, MHC II⁺, EpCAM⁺ stromal cells (defined as extrathymic *Aire*-expressing cells, eTACs) in the peripheral lymphoid organs (Gardner *et al*, 2008). Autoantigen expression in eTACs could enable the peripheral deletion of autoreactive T cells. This interesting new aspect of the *Aire* gene places it in a pivotal position in regulating the gene expression network essential for both central and peripheral tolerance. However, there was little overlap observed between the set of *Aire*-regulated genes in medullary TECs and eTACs, and insulin was not among the genes found to be influenced by *Aire* in eTACs in the periphery. Similar to their *Aire*-driven *Adig* transgene, our *Aire*–Cre transgene is also expressed at very low levels in spleen. It remains to be determined whether *Aire*–Cre is also expressed in eTACs to mediate *Ins2* deletion in peripheral tissues and its potential immunologic significance. Regardless, our thymus transplantation data clearly demonstrated the dominant role of central insulin expression in regulating islet autoimmunity, as ID-TEC thymus alone is sufficient to generate insulin-reactive, islet-infiltrating T cells, even though neither *Ins1* nor *Ins2* gene was affected in peripheral lymphoid tissues.

Similar to NOD mice, immune cells of both adoptive and innate immunity were found infiltrating the pancreatic islets. However, we did not observe the signature massive T lymphocyte infiltration (i.e. peri-insulitis and insulitis) in our adoptive transfer experiments, like that classically seen in pre-diabetic NOD mice and in humans. One distinct difference is the early, rapid onset of the islet destruction in our ID-TEC animals. The chronic disease progression in NOD mice provides an opportunity for the amplification of the inflammatory process by epitope spreading and bystander effects. However, it has been suggested that a small number of active effector cells may be sufficient to destroy the β -cells of an islet (von Herrath and Homann, 2004). Thus, the classic image of insulitis may reflect an expanded, less specific process occurring after the initial islet β -cell damage; a later phase that we simply did not observe, perhaps also due to the autoimmune resistant H-2^b MHC of the ID-TEC animals in comparison with the autoimmune prone H-2^d allele of the NOD mice (von Herrath and Nepom, 2009). The large spot sizes observed in ELISPOT assays of IFN- γ production indicate the strength of the TH1-type response developed once the insulin-reactive T cells are stimulated (Hesse *et al*, 2001). In the ID-TEC mice, this strong response mediated by a small number of cells may cause pathological damage to islet β -cells at an earlier age. Consistently, islets with severe damage could be found in *Rag1* mice transplanted with, as little as, 5×10^5 CD8⁺ T cells from ID-TEC mice. Islet destruction accompanied with massive T cell infiltration was also detected in CD4⁺CD25⁺ effector T cell-transplanted *Rag1* mice. Thus, as the consequence of abolished thymic insulin expression, insulin-reactive T cells are present in both CD4⁺ and CD8⁺ compartments in ID-TEC mice.

Neither our thymus transplantation nor T cell adoptive transfer experiment recapitulated the full-blown clinical

diabetes seen in ID-TEC pups, although islet-specific autoimmunity was evident. In both cases, the T cells transferred or migrating from the transplanted ID-TEC thymus would expand in the lymphopenic environment of an immune-compromised adult mouse, a situation clearly different from that of a newborn pup (Garcia *et al*, 2000). In addition, studies of neonatal mice showed that the development of effector T cells precede that of T_{reg} cells (Monteiro *et al*, 2008). Within the neonatal environment, establishment of proper T_{eff} : T_{reg} ratio is crucial to prevent autoimmune disease later in adult life. Indeed, delay of T_{reg} migration from the thymus through thymectomy at day 3, has been associated with organ-specific autoimmune disease (Sakaguchi 2004). Without negative selection of insulin-specific T cells, the initial wave of effector T cells seeding the periphery of ID-TEC pups would contain a substantially higher number of insulin-specific T cells, which could disturb the proper ratio of T_{eff} : T_{reg} cells. This unchecked expansion of insulin-reactive effector T cells could inflict irreversible damage to the β -cells before the expansion of T_{reg} cells to establish tolerance. We are currently generating insulin-specific T cell clones from ID-TEC mice to test this possibility. Another unique feature in neonatal pups is the wave of excessive β -cell apoptosis during the 2nd and the 3rd week of postnatal life (Scaglia *et al*, 1997; Trudeau *et al*, 2000). The shedding of islet antigens through the apoptotic process was able to activate APCs and initiated insulinitis in pre-diabetic NOD mice. As a high-level expression of the *Aire-Cre* transgene was observed in the newborn ID-TEC thymus (Y Fan *et al*, our unpublished observations), negative selection of insulin-specific thymocytes could be compromised at birth due to the Cre-mediated abrogation of insulin expression. Although neither insulinitis nor loss of insulin content was observed in ID-TEC pancreas at day 10, elevated blood sugar levels were observed in the 3rd post-natal week, when signs of islet inflammation are present. Thus, β -cell apoptosis might have an indispensable function in initiating and amplifying the islet-specific autoimmune response in ID-TEC mice, which was absent in transplantation models using adult immune-compromised mice as recipients. Alternatively, but not mutual exclusively of the above mechanisms, other peripheral self-antigen tolerance mechanisms could limit the progression of islet autoimmune destruction.

In the ID-TEC mouse model, the ability to negatively select insulin-specific autoreactive T cells within the thymus is undermined by the total absence of the self-antigen expression, even in the presence of disease-resistant H-2^b MHC molecules. Our data support the conclusion that (pro)insulin is a major autoantigen for the initiation and/or progression of T1D, as previously hypothesized from studies of clinical cases, suggesting the association of diabetes susceptibility with genetic differences that affect insulin expression (Walter *et al*, 2003). Our data can also explain the rare cases of T1D with rather early onset in children expressing protective HLA alleles. The ID-TEC mice could serve as a new model to study this particular type of T1D, as both male and female ID-TEC mice develop spontaneous diabetes around 3 weeks after birth with 100% penetration, in striking contrast to the NOD animal model in which 80–90% females develop the disease over a period of 20 weeks (from 12 weeks to 40 weeks), a gender preference not observed in human T1D cases. Our study also suggests that the functionality of transcription factors able to regulate thymic self-antigen expression should

be examined in young patients to help diagnosis. Disease prevention strategies may need to address the antigenic role of insulin in the development of T1D.

Materials and methods

Mice

Loxp-tagged *Ins2* mice were generated through standard gene targeting methods as described by Fan *et al* (1999). *Aire-Cre* transgenic mice were obtained through pronuclear injection of the linearized Cre construct into fertilized eggs collected from C57BL/6 mice. Transgenic lines were subjected to multiple rounds of breeding with *Ins1*^{−/−}:*Ins2*^{+/+} mice to obtain ID-TEC mice. Thus, all the animals in the study are on the 129:B6 mixed genetic background, with the MHC H-2^b haplotype shared by both the 129 and C57BL/6 strains.

Heterozygous *loxp*-tagged *Ins2* mice were first crossed to Flp recombinase-expressing mice (129S4/SvJaeSor-*Gt(ROSA)26Sor*^{tm1(FLP1)Dym}/J, Jackson Laboratory, Bar Harbor, ME) to delete the *Neo* cassette, and then interbred to obtain homozygous *Ins2*^{+/+} mice. Subsequently, *Ins2*^{+/+} mice were crossed to *Ins1* knockout mice (a gift from Dr Jacques Jami) to obtain *Ins2*^{+/+}:*Ins1*^{−/−} mice.

Immune deficient C657BL/6 *Rag1*^{−/−} mice (B6.129S7-*Rag1*^{tm1Mom}/J), Rip-Cre mice (B6.Cg-Tg(*Ins2-Cre*)25Mgn/J) and Rosa26R-lacZ (B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J) mice were all purchased from the Jackson Laboratory, Bar Harbor, ME. All mice were housed in a specific pathogen-free animal facility at Rangos Research Center, Pittsburgh. All experiments were carried out in accordance with institutional guidelines.

Constructs

Detailed procedures to obtain the constructs used in the study (the *Ins2* targeting construct and the *Aire-Cre* construct) are available on request. Briefly, to constitute the *Aire-Cre* plasmid, DNA segments flanking the *Aire*-coding region were subcloned from a BAC plasmid containing the mouse *Aire* gene. The *Aire-Cre* construct was assembled from a 23-kb DNA fragment containing the sequence 5' of the mouse *Aire* gene and including its first exon, a PCR product of the Cre recombinase cDNA, and an 11-kb fragment 3' of the last exon of the *Aire* gene.

Blood glucose and insulin levels

Blood glucose levels were monitored using the Ascensia Contour blood glucose monitoring system (Bayer HealthCare LLC, Mishawaka, IN). Blood insulin levels were measured using the Mercodia Ultrasensitive Mouse Insulin Elisa Kit (Mercodia AB, Sweden), following the manufacturer's protocol.

RNA analysis

The total RNA of individual thymi or pooled thymi was isolated using an RNA minikit, according to the manufacturer's protocol (Qiagen). Real-time PCR was carried out on cDNA prepared from DNase I-treated RNA (Superscript III cDNA kit, Invitrogen) using the LightCycler FastStart DNA Master SYBR Green I kit, and analysed using the LightCycler 2 system (Roche Applied Science).

Primer pairs used in the study

Hprt:	F 5'-GGATACAGGCCAGACTTTGTTGGA-3' R 5'-CAACAGGACTCCTCGTATTTCAG-3'
Aire:	F 5'-AATCTCCGCTGCAAATCCTGCTCT-3' R 5'-ACTGCAGGATGCCGTCAAATGAGT-3'
CD45:	F 5'-AGGACCTCAAACAGAAGCTTCCCA-3' R 5'-TTGCAGAGAGGCTGGAGATCTTT-3'
Cytokeratin 2/8:	F 5'-AGGAGCTCATTCGGTAGCTG-3' R 5'-TCTGGGATGCAGAACATGAG-3'
Cre:	F 5'-TCGCTGGCGTTTCTATGACGAGAA-3' R 5'-ATCACTCGTTGCATCGACCGTAA-3'
Insulin:	F 5'-CACCCAGGCTTTTGTCAG-3' R 5'-AGATGCTGGTGCAGCACT-3'
β -actin:	F 5'-TGGAAATCCCTGTGGCATCCATGAAA-3' R 5'-TAAACGCAGCTCAGTAACAGTCCG-3'

Histology

Pancreata and thymi were collected, fixed in 4% paraformaldehyde for 3 h at 4°C, and placed in 30% sucrose overnight. Cryosections,

5 μ m thick, were cut and stained using primary antibodies. Antibodies used in the study: CD3, CD45, CD4 and CD8 (BD Biosciences, Franklin Lakes, NJ); insulin (Santa Cruz Biotechnology, Santa Cruz, CA); glucagon (Zymed, San Francisco, CA); β -galactosidase (Chemicon, Temecula, CA).

Adoptive transfer experiments

Spleen, mesenteric and pancreatic lymph nodes were collected from ID-TEC mice. Single cells were prepared and subjected to magnetic bead isolation first for CD4⁺ T lymphocytes following manufacturer's protocols (Miltenyi Biotec, CD4⁺ T cell isolation kit), followed by FACS sorting of CD25⁻ and CD25⁺ cells. Purities above 95% were achieved routinely. The CD8⁺ T cell isolation kit was used for CD8⁺ T cell isolation (Miltenyi Biotec). Equal numbers (5×10^5) of T cells of the different subsets were mixed and injected into *Rag1*^{-/-} mice through the tail vein (i.v.).

ELISPOT assay

ELISPOT assays were carried out using the BD mouse IFN- γ ELISPOT set, according to the manufacturer's specifications (BD Biosciences). Splenocytes (3×10^6) from 2–3 mice were pooled and cultured overnight with 0.1 mg/ml insulin (Sigma-Aldrich), whole GAD65 protein (Abnova), insulin B chain peptide 9–23 (SHLVEA-LYLVCGERG, AnaSpec), GAD65 peptide 206–220 (TYEIAPVFVL-LEYVT, Sigma-Aldrich) or medium alone. Assays were repeated and carried out in triplicate and averaged. To evaluate immune response to male-specific HY autoantigen, 2×10^5 splenocytes were incubated with 10 μ g/ml *Dby* peptide (NAGFNSNRANSSRSS, AnaSpec) overnight.

Thymus transplantation

Thymic lobes were isolated from 2-week-old ID-TEC pups or littermate controls, and cultured in transwells at 37°C in the presence of 1.35 mM deoxyguanosine (Sigma-Aldrich) for 10 days to eliminate hematopoietic cells. Two lobes were grafted beneath the kidney capsule of each 6–8-week-old nude mouse. At the end of the study (16 weeks post thymus transplantation), pancreata were collected for histological analysis.

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Tetramer analysis of CD8⁺ T cells specific to HY autoantigen Uty

Animals were injected with 5×10^5 male splenocytes collected from littermate controls of ID-TEC mice intraperitoneally (IP). At 2 weeks after the initial priming, splenocytes were separated using CD8⁺ T cell isolation kit (Miltenyi Biotec) and stained with CD3, CD8 and Pro5 MHC class I D^b/Uty pentamer, following manufacturer's protocols (ProImmune).

Insulin autoantibody (IAA) assay

A 96-well EIA plate was coated with 2 μ g of heat-inactivated (95°C, 30 min) human insulin in 100 μ l of PBS overnight. Wells were blocked with PBS with 10% BSA for 2 h at room temperature, and probed with mouse sera collected from 3–8-week-old ID-TEC mice and age-matched controls (1:50 dilution, 100 μ l in each well) for another 2 h. Next, biotin-conjugated rat anti-mouse IgM (BD Biosciences) was added to the well (1:5000 dilution) and incubated for 30 min, followed by incubation with horseradish peroxidase-conjugated streptavidin (1:4000) for 15 min. Lastly, ACE substrate (BD Biosciences) was added to each well and the absorbance was measured at 450 nm using Bio-Rad Microplate Reader.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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