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Tolerance to Proinsulin-2 Is Due to Radioresistant Thymic Cells

Béatrice Faudeau,* Chantal Lotton, * Bruno Lucas, *† Isabelle Tardivel,* John F. Elliott, ‡ Christian Boitard,* and Jean-Claude Carel*§

Proinsulin is a key Ag in type 1 diabetes, but the mechanisms regulating proinsulin immune tolerance are unknown. We have shown that prepriproinsulin-2 gene-deficient mice (proins-2−/−) are tolerant to proinsulin-2. In this study, we analyzed the mechanisms underlying T cell-mediated tolerance to proinsulin-2 in 129/Sv nonautoimmune mice. The expression of one proinsulin-2 allele, whatever its parental origin, was sufficient to maintain tolerance. The site of proinsulin-2 expression relevant to tolerance was evaluated in thymus and bone marrow chimeras. CD4+ T cell reactivity to proinsulin-2 was independent of proinsulin-2 expression in radiation-sensitive bone marrow-derived cells. A wt thymus restored tolerance in proins-2−/− mice. Conversely, the absence of the prepriproinsulin-2 gene in radioresistant thymic cells was sufficient to break tolerance. Although chimeric animals had proinsulin-2-reactive CD4+ T cells in their peripheral repertoire, they displayed no insulin or insulin Abs, suggesting additional protective mechanisms. In a model involving transfer to immunodeficient (CD3ε−/−) mice, naive and proinsulin-2-primed CD4+ T cells were not activated, but could be activated by immunization regardless of whether the recipient mice expressed proinsulin-2. Furthermore, we could not identify a role for putative specific T cells regulating proinsulin-2-reactive CD4+ T in transfer experiments. Thus, proinsulin-2 gene expression by radioresistant thymic epithelial cells is involved in the induction of self-tolerance, and additional factors are required to induce islet abnormalities.

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3 Abbreviations used in this paper: VMTR, variable number of tandem repeats; mTEC, medullary thymic epithelial cell; MBP, myelin basic protein.

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Aire-deficient mice (29). Thymic proinsulin expression is thought to be of prime importance, but proinsulin expression in bone marrow-derived APCs such as dendritic cells and macrophages may also be involved in the regulation of self-tolerance (30). Moreover, bone marrow-derived APCs may acquire islet-derived proinsulin from the periphery and present it to developing thymocytes, resulting in negative selection, as described for myelin basic protein (31). Overexpression of the preproinsulin-2 gene in the thymus (32) or in bone marrow-derived APCs (33) is sufficient to induce tolerance to preproinsulin epitopes and to delay diabetes in NOD mice, but the physiological relevance of these observations remains to be evaluated.

We and others (26, 34) have shown that T cells from proinsulin-2-deficient mice react with proinsulin-2 and that the expression of proinsulin-2 in wt mice induces tolerance. This model can be used to evaluate the site of proinsulin-2 expression relevant to tolerance induction in a nonautoimmune situation. We report here studies based on thymus and bone marrow chimera and adoptive transfer of CD4+ T cells.

Materials and Methods

Mice

We used preproinsulin-2 knockout mice (proins-2−/− mice) (25), in a 129/SvPas/Ico background, bred in specific pathogen-free conditions. Mice were hemizygous or homozygous for the mutated allele as typed by PCR analysis of tail DNA, as described previously (34). Heterozygous proins-2+/− mice with paternal or maternal proins-2 expression were obtained by crossing proins-2−/− mice with (H11001/H9255) mice and (H11002/H9255) mice, all similarly immunized with proinsulin-2 in CFA and tested for proinsulin-2 reactivity as described above. The other recipients were tested similarly, but without proinsulin-2 immunization.

Memory CD4+ T cells.

CD4+ T cells from the draining popliteal lymph nodes of proinsulin-2-primed proins-2−/− mice were purified, as described above. We tested the reactivity to proinsulin-2 of the purified CD4+ T cells in vitro, and then injected them i.v. into proins-2−/−CD3ε−/− mice or proins-2−/−CD3ε−/− mice (5 × 106 cells/mouse). Two months after CD4+ T cell transfer, half the recipients were immunized with proinsulin-2 in CFA and tested for proinsulin-2 reactivity as described above. The other recipients were tested similarly, but without proinsulin-2 immunization.

Immunization with recombinant mouse proinsulin-2

Mouse proinsulin-2 was produced as a fusion protein, with a (His)6 tag immediately upstream of the proinsulin sequence. It was purified and tested for the presence of endotoxin, as described previously (35). Mice were bred as homozygous lines and typed by PCR analysis of tail DNA, as described previously (34). Heterozygous proins-2+/− mice with paternal or maternal proins-2 expression were obtained by crossing proins-2−/− mice with (H11001/H9255) mice and (H11002/H9255) mice, all similarly immunized with proinsulin-2 in CFA and tested for proinsulin-2 reactivity as described above. The other recipients were tested similarly, but without proinsulin-2 immunization.

Thymic and bone marrow chimeras

Bone marrow chimeras. Bone marrow samples were depleted of CD4+ and CD8+ T cells using anti-CD4 (GK1.5) and anti-CD8 (Lyt-2) Ab and magnetic beads coupled to anti-rat Ig.Recipient mice were gamma-irradiated (10 Gy from a 137Cs source), and their bone marrow was reconstituted by injection (5 × 106 cells i.v.). Recipient mice were given neomycin sulfate (4 mg/ml) in drinking water for 2 wk after bone marrow transplantation, and bone marrow reconstitution was allowed to proceed for 8–12 wk before testing for proinsulin-2 reactivity.

Thymic and bone marrow chimeras. Mice underwent thymectomy by aspiration at the age of 4–6 wk. The completeness of thymectomy was confirmed at autopsy. Thymus grafting was performed 1–4 wk later, by placing two to three neonatal thymic lobes under the left kidney capsule or under both kidney capsules when two kinds of thymic lobes were used. One week after thymus grafting, bone marrow transplantation was performed. The mice were tested for proinsulin-2 reactivity after reconstitution.

Adoptive transfer of CD4+ T cells into immunodeficient mice

Naïve CD4+ T cells. Cells from peripheral lymph nodes (inguinal, axillary, maxillary) of unprimed proins-2−/− mice were depleted of macrophages, granulocytes, CD8+ T cells, and B cells, as described above. Purified CD4+ T cells were injected i.v. into proins-2−/−CD3ε−/− mice or proins-2−/−CD3ε−/− mice (5 × 106 cells/mouse). The recipient mice were monitored as described above.

Adoptive transfer of CD4+ T cells into immunocompetent mice

Peripheral lymph node CD4+ T cells from unprimed proins-2−/− mice were prepared as described above and injected i.v. into proins-2−/− 129 wt mice (50 × 106 or 10 × 106 cells/mouse). Two days after CD4+ T cell transfer, the recipients were immunized with proinsulin-2 in CFA and tested for proinsulin-2 reactivity.

Histology

The pancreas was removed from mice at autopsy, and fixed in 10% formaldehde in PBS. Paraffin-embedded sections were cut and stained with H&E.

Results

CD4+ T cells from 129 proins-2−/− mice proliferate and produce IFN-γ in response to proinsulin-2 immunization

We previously reported that preproinsulin-2 expression prevents the CD4+ T cell response to proinsulin-2 in wt 129 mice, whereas proins-2−/− mice display significant IFN-γ and IL-2 responses following immunization against proinsulin-2. We first evaluated side-by-side cellular proliferation by CFSE dilution assay and IFN-γ production by ELISA in CD4+ T cell from three proins-2−/− and three wt mice, all similarly immunized with proinsulin-2 (Fig. 1). In CD4+ T cells from wt mice, in vitro recall with proinsulin-2 for 72 h induced a marginal CFSE dilution (∆CFSE = 0.8 ± 1.3%, mean ± SD) and absent or minimal specific IFN-γ secretion (∆IFN-γ = 25 ± 14 pg/ml). In contrast, CD4+ T cells from 129 proins-2−/− mice clearly proliferated (∆CFSE = 5.6 ± 1.8%) and secreted IFN-γ (∆IFN-γ = 288 ± 107 pg/ml) in response to proinsulin-2 in vitro. Assessment of CFSE dilution or IFN-γ secretion 48 or 96 h after in vitro recall gave similar results. These results indicate that both techniques can clearly distinguish CD4+ T cell reactivity of proins-2−/− and wt mice after immunization with proinsulin-2. Thus, we decided to use specific IFN-γ secretion at 72 h as a marker of CD4+ T cell reactivity to proinsulin-2 in additional experiments.

Effect of a single functional proinsulin-2 allele on proinsulin-2 reactivity

Because the number of copies of the preproinsulin-2 gene controls proinsulin-2 expression in the thymus (26), we investigated whether the CD4+ T cell response to proinsulin-2 depended on the
number of proinsulin-2 gene copies (one or two) and whether the parental origin of the proinsulin-2 allele expressed affected this response. Because the proinsulin-2 gene is subject to genomic imprinting, at least in the yolk sac (37), we generated proins-2mat and proins-2mat/mat mice. No IFN-γ secretion by purified CD4+ T cells was detected in response to proinsulin-2, following immunization in heterozygous animals (Fig. 2), indicating that one or two proinsulin-2 alleles and whether the parental origin had no effect on the T cell response to proinsulin-2.

The proinsulin-2 genotype of bone marrow-derived cells does not influence the induction of CD4+ T cell tolerance

We investigated the effect of bone marrow-derived cell genotype on immune tolerance by constructing bone marrow chimeras, using wt and proins-2−/− mice. Upon immunization with proinsulin-2, CD4+ T cells from proins-2−/− animals transplanted with wt bone marrow produced IFN-γ in response to proinsulin-2, as did CD4+ T cells from proins-2−/− → proins-2−/− control chimeras (Fig. 3). Conversely, CD4+ T cells from wt animals transplanted with proins-2−/− bone marrow from wt → wt controls chimeras did not respond to immunization with proinsulin-2 (Fig. 3). Because congenic markers are not available on the 129 background, we used parallel bone marrow-transplanted mice to evaluate the chimerism. Five control recipient mice were irradiated and transplanted with T cell-depleted bone marrow from B6 CD45.1 congenic H-2 compatible mice. After reconstitution, >99% of the thyocytes, 94.7 ± 2.8% (mean ± SD) of the splenic CD4+ T cells, 99 ± 1% of splenic CD19+ B cells, and 95.5 ± 1.3% of peritoneal macrophages were from donor origin, confirming the validity of our experimental approach. Thus, the proinsulin-2 genotype of radiation-sensitive bone marrow-derived cells does not affect tolerance induction.

The proinsulin-2 genotype of radioresistant cells from the thymic stroma is involved in CD4+ T cell tolerance induction

The proinsulin-2 reactivity of bone marrow chimeras indicated that tolerance occurred when proinsulin-2 was produced in radioresistant cells of non-bone marrow origin. We evaluated the relative contributions of proinsulin-2 expression in thymic and islet cells by generating thymus and bone marrow chimeras. CD4+ T cells from chimeras with a proins-2−/− thymus in a wt environment...
were intolerant to proinsulin-2 because they secreted IFN-γ secretion in vitro after immunization with recombinant proinsulin-2 (Fig. 4A). Therefore, the absence of the preproinsulin-2 gene in thymic radioreistant cells is sufficient to break tolerance in an otherwise proinsulin-2-sufficient mouse. Conversely, CD4+ T cells from chimera with a wt thymus in a proins-2−/− environment were fully tolerant to proinsulin-2, and did not respond to proinsulin-2 immunization (Fig. 4B). Thus, expression of the preproinsulin-2 gene in radioreistant cells from the thymus is sufficient to induce tolerance in an otherwise preproinsulin-2-deficient mouse. Despite the simultaneous presence of proinsulin-2-specific CD4+ T cells in their peripheral repertoire and proinsulin-2 in their islet β cells, wt chimera recipients displayed no islet infiltration (Fig. 4C) or insulin Abs (data not shown) up to 3 mo after bone marrow transplantation.

Islet-derived preproinsulin-2 does not affect memory CD4+ T cell response to proinsulin-2

These chimera experiments suggest that the thymic epithelium plays a key role in regulation of the CD4+ T cell response to proinsulin-2. The presence of proinsulin-2-reactive T cells in the peripheral repertoire was not sufficient to initiate islet abnormalities. We therefore investigated the effects of an activated T cell population not purged by the thymic filter, to establish a model of central tolerance failure. We used a transfer model in which CD4+ T cells from proinsulin-2-primed proins-2−/− mice were injected into immunodeficient recipients with and without proinsulin-2 gene expression. The proinsulin-2 reactivity of the transferred CD4+ T cells was assessed, at the time of transfer, by determining in vitro IFN-γ secretion, as described previously (data not shown). Two months after transfer, proinsulin-2 reactivity was not detected ex vivo, regardless of whether the recipient produced islet-derived proinsulin-2 (Fig. 5A). Therefore, islet-derived proinsulin-2 cannot drive the expansion of a pre-established specific memory CD4+ T cell pool. Reactivation of proinsulin-2-specific CD4+ T cells by immunization of the recipient mice with proinsulin-2 two mo after transfer resulted in the full recovery of ex vivo responsiveness to proinsulin-2, regardless of whether the recipient produced proinsulin-2 (Fig. 5A). No insulin autoantibodies or leukocyte infiltrates were detected in any of the recipient mice (data not shown), suggesting that islet cells are ignored by the specific memory CD4+ T cells present in the injected pool. Therefore, proinsulin-2-specific activated memory T cells and proinsulin-2-producing β cells can coexist without activation, the induction of deletion, or anergy.

Iset-derived preproinsulin-2 does not affect the naive CD4+ T cell response to proinsulin-2

Because memory T cells are generally believed to be less susceptible to tolerance induction than naive T cells, we performed similar adoptive transfer experiments using unprimed CD4+ T cells from proins-2−/− mice. Consistent with the results obtained in the previous experiment, we found no evidence of T cell priming by islet-derived proinsulin-2 two mo after transfer (Fig. 5B). Recipient mice immunized with proinsulin-2 mounted a full T cell response to proinsulin-2. Thus, proinsulin-2-specific naive CD4+ T cells were not deleted or anergized by islet-derived proinsulin-2. No insulin autoantibodies or leukocyte infiltrates were detected up to 2 mo after transfer (data not shown). These results indicate that islet-derived preproinsulin-2 is ignored by proinsulin-2-specific naive CD4+ T cells from proins-2−/− mice.

Do dominant mechanisms play a major role in tolerance to proinsulin-2 in 129 wt mice?

Thymic proinsulin-2 expression in wt mice could result in two nonmutually exclusive outcomes in developing thymocytes: the deletion of autoreactive anti-proinsulin-2 thymocytes and/or the positive selection of proinsulin-2-specific regulatory T cells. To further evaluate these mechanisms, we decided to inject intolerant CD4+ T cells from unprimed proins-2−/− mice into immunocompetent wt mice and evaluate whether putative specific regulatory T cells would affect reactivity to proinsulin-2. Two groups of wt mice received either 50 × 10^6 or 10 × 10^6 CD4+ T cells from unprimed proins-2−/− mice and were immunized with proinsulin-2 two days later. Reactivity to proinsulin-2 was detected among CD4+ T cells from recipient mice in a dose-dependent manner (Fig. 6), indicating that the cellular environment of the wt mice had not affected proinsulin-2-reactive CD4+ T cells from proins-2−/− mice. The results of this experiment rule out a major contribution of dominant mechanisms and proinsulin-2-specific regulatory cells in CD4+ T cell tolerance to proinsulin-2.

To further evaluate this point, we used a second approach generating chimeras with two thymuses originating from both a wt and a proins-2−/− mouse (Fig. 4D). Although we cannot evaluate the respective roles of the two thymuses in the peripheral T cell pool,
immediately reconstituted by i.v. injection of T cell-depleted bone marrow cells from wt mice (5 x 10^6 cells/mouse i.v.). The mice were immunized 10–12 wk later with 50 g of recombinant proinsulin-2 emulsified in CFA. CD4+ T cells purified from the spleen 10 days later were stimulated in vitro alone or in the presence of the immunizing protein (20 g/ml); IFN-γ concentrations were determined as described in Fig. 2. Data are compiled from two independent experiments.

B, Proins-2 recipients: proins-2−/− mice underwent thymectomy at 4–5 wk of age, and three or four thymic lobes from newborn wt or proins-2−/− mice were implanted under the kidney capsules. Recipients were then irradiated (10 Gy) to ablate their immune systems, which were immediately reconstituted by i.v. injection of T cell-depleted bone marrow cells from wt mice (5 x 10^6 cells/mouse i.v.). The mice were immunized 10–12 wk later with 50 μg of recombinant proinsulin-2 emulsified in CFA. CD4+ T cells purified from the spleen 10 days later were stimulated in vitro alone or in the presence of the immunizing protein (20 μg/ml); IFN-γ concentrations were measured as described in Fig. 2. Data are compiled from two independent experiments.

FIGURE 5. Response to proinsulin-2 in CD3e-deficient mice after immune reconstitution with CD4+ T cells from proins-2−/− mice. CD3e-deficient mice were injected i.v. with the following: purified CD4+ T cells from proins-2−/− mice immunized with proinsulin-2 emulsified in CFA 10 days previously (5 x 10^6 cells/mouse) (A); or purified CD4+ T cells from unprimed proins-2−/− mice (5 x 10^6 cells/mouse) (B). Two months after injection, CD4+ T cells were purified from the spleens of recipient mice by negative selection and incubated in vitro with APCs alone or in the presence of the immunizing protein (20 μg/ml). IFN-γ concentrations were determined as described in Fig. 2. Data are compiled from two independent experiments.

A


B


FIGURE 4. Immunization of thymic and bone marrow chimeras with recombinant proinsulin-2. A, wt recipients: wt mice underwent thymectomy at 4–5 wk of age, and two or three thymic lobes from newborn proins-2−/− mice or wt mice were implanted under the kidney capsules. Recipients were then irradiated (10 Gy) to ablate their immune systems, which were immediately reconstituted by i.v. injection of T cell-depleted bone marrow cells from wt mice (5 x 10^6 cells/mouse i.v.). The mice were immunized 10–12 wk later with 50 μg of recombinant proinsulin-2 emulsified in CFA. CD4+ T cells purified from the spleen 10 days later were stimulated in vitro alone or in the presence of the immunizing protein (20 μg/ml); IFN-γ concentrations were measured as described in Fig. 2. Data are compiled from two independent experiments. If A, C, Typical pancreatic histology in wt recipients of proins-2−/− thymus glands and wt bone marrow transplants. D, wt recipients of thymic grafts of dual origin: wt mice underwent thymectomy at 4–5 wk of age and were grafted with two thymic lobes from newborn proins-2−/− mice and two thymic lobes from newborn wt mice under each kidney capsule. Recipients received a T cell-depleted bone marrow cells from wt mice and were tested as described in A.
we checked the functionality of thymus grafts by FACS analysis and expressed the results as ratios of wt to combined thymuses, therefore reflecting the contribution of the wt thymus to the total thymic mass. The wt/wt + proins-2 /-/thymocyte ratio was 55 ± 25% (range, 25–87%). The wt/wt + proins-2 /-thymocyte ratio was 55 ± 28% (range, 21–88%). These results indicate that, on average, both thymuses were equally functional, although experimental variation could shift the balance in favor of the wt or the proins-2 /-thymus. After reconstitution, T cell reactivity to proinsulin-2 could be detected after immunization in four of six mice, indicating that cells originating from the wt thymus had not regulated proinsulin-2-specific CD4+ T cells generated by the proins-2 /-thymus in these animals. In contrast, two of six mice behaved differently in this experiment because no reactivity to proinsulin-2 could be detected. These mice (Fig. 4D) had wt/wt + proins-2 /-thymocyte ratios similar to the other four mice, ruling out a trivial explanation where only the wt thymus has contributed to the peripheral pool. Our interpretation of the results in these two mice is that the reconstitution might have been incomplete. However, we cannot rule out the generation of regulatory cells by the wt thymus as a possible explanation for the results in these two mice. Although the results of these dual thymus transplant experiments are not straightforward, we interpret them as indicative that regulatory mechanisms do not play an essential role.

**Discussion**

We have previously shown that reactivity to proinsulin-2 detected in mice lacking this protein is abolished by proinsulin-2 expression (34). In this study, we evaluated the site of proinsulin-2 gene expression relevant for regulation of the CD4+ T cell response. A single functional proinsulin-2 gene allele was sufficient to induce tolerance, whatever its parental origin. The proinsulin-2 genotype of radioreistant cells of the thymic stroma was sufficient to modulate the induction of T cell tolerance, with bone marrow-derived and islet cells playing no detectable role. In addition, our transfer experiments indicated that islet-autoreactive T cells were insufficient to cause disease. These results demonstrate the role of central tolerance in the regulation of proinsulin-2 reactivity and indicate additional mechanisms to protect the islets exist if central tolerance fails. Our model can be used to evaluate the mechanism of proinsulin-2 tolerance in a physiological situation—in contexts other than transgenic expression of the Ag and/or the TCR and spontaneous autoimmunity.

Several methodological aspects of our study should be discussed. First, we immunized the mice to assess CD4+ T cell responses to proinsulin-2, as in our previous study where we established that islet proinsulin-2-autoreactive CD4+ T cells are functionally eliminated in wt mice (34). In fact, a response to proinsulin-2 has also been detected in unprimed 129 proins-2 /-mice (26), but the response level was too low in our hands to base our mechanistic assessment of T cell tolerance. Second, we focused on CD4+ T cells because islet autoimmunity is known to involve both CD4+ and CD8+ T cells. However, islet-reactive CD4+ T cells, although generally insufficient to induce islet pathology on their own, play an essential role in autoimmune islet responses and therefore warrant the mechanistic analysis that was performed in this study (38–40). Third, because our assessment of tolerance was purely functional, one potential limitation concerns the possibility that immune reconstitution in the mice may have involved at least some peripheral CD4+ T cells originating from the recipient. We were unable to test this because no suitable congenic marker in the 129/Sv background is available. However, in a similar protocol using C57BL/6 mice congenic mice, ~90% of CD4+ T cells were found to be of donor origin, whether the mice were irradiated with 8 Gy or 11 Gy (41). Moreover, the absence of a proinsulin-2 response in proins-2 /- recipients of wt thymus glands is consistent with donor CD4+ T cells making a major contribution in our system.

Our chimera experiments suggest that the thymic epithelium plays a key role in the regulation of CD4+ T cell responses to proinsulin-2. It is now well established that this β cell protein is also expressed in the thymus, both in mice (42, 43) and humans (14, 15). However, it remains unclear which cells in the thymus express the preproinsulin gene. In some studies, this gene was reported to be expressed in bone marrow-derived cells: murine thymic dendritic cells (44), and human dendritic cells and macrophages, in the thymus and peripheral lymphoid organs (30). However, in studies using highly purified thymic cell populations, expression was found to be restricted to mTEC in mice (27) and humans (45). Previous studies exploring the mechanism by which the ectopic thymic expression of β cell-specific Ags leads to T cell tolerance have been conducted in transgenic mice, but conflicting results have been obtained. Mice overexpressing neo-self Ags under control of the rat insulin promoter (40, 42) are generally self-tolerant to these Ags and express them to various extents in rare cells of the thymic medulla (46). The level of thymic expression and the impact on specific T cell tolerance induction differs between transgenic mouse founder lines. Additional studies with transgenic Aire-deficient mice have shown that this transcription factor is involved in establishing central tolerance to neo-self Ag expressed under control of the rat insulin promoter, through the deletion of Ag-specific T cells (22, 29). However, it is unclear whether the results of these studies can be generalized, because of potential artifacts due to the use of transgenic technology. The functional data presented here suggest that promiscuous proinsulin-2 expression by thymic epithelial cells plays a much more important role than proinsulin-2 expression by bone marrow-derived APCs in the induction of CD4+ T cell central tolerance.

Our system allowed us to decipher the mechanism of CD4+ tolerance to proinsulin-2. Within the limits of a polyclonal system where we cannot monitor specific cell populations but can only
measure T cell responses, our results in Figs. 4D and 6 are consistent with a deletional mechanism of central tolerance playing a predominant role in CD4+ T cell tolerance to proinsulin-2. This is consistent with results obtained in Aire-null mice, where thymic expression of proinsulin or proinsulin promoter-driven transgenes is not detectable (22). When backcrossed to the Aire-null background, the RIP-OVA/OT-II (47) and RIP-HEL/J3A9 (29) transgenic systems have been used to evaluate the mechanisms by which thymic expression of self-Ags regulate autoimmunity, and, in both cases, negative selection of effector cells was the predominant mechanism (29, 47). Whether additional specific regulatory cells also play a role in our system cannot be ruled out because our assessment of tolerance is binary (response vs no response), whereas regulatory cells might be expected to finely modulate the amplitude of the response. In the dual-origin transplant experiment (Fig. 4D), the amplitude of the response seems decreased in comparison with the experiment where only proins-2+/− thymuses were implanted. However, the interexperimental quantitative variability precludes any definitive conclusion on this point. Similarly, the lack of detectable response in two of the six mice in the dual transplant experiment of Fig. 4D might reflect the role of regulatory T cells, although other interpretations are possible. Indeed, expression of tissue-specific Ags by thymic epithelial cells has been shown to lead to the selection of Ag-specific regulatory CD4+CD25+ T cells, raising the issue of whether similar mechanisms were operating in our system (48, 49). In addition, in humans, insulin VNTR class III alleles are associated with both high insulin transcription level in the thymus and higher release of the regulatory T cell-associated cytokine IL-10 by memory CD4+ T cells (50). Altogether, our experiments provide indirect evidence to the mechanisms of T cell tolerance and strongly suggest that deletion occurs, without ruling out the generation of regulatory T cells as an additional mechanism.

The relevance of the essential role of thymic epithelial cells to other somatic self-Ags has been evaluated in transgenic and knockout mice. For myelin basic protein (MBP), tolerance involves the thymic deletion of reactive T cells. However, tolerance resulted from the MBP being imported from bone marrow-derived APCs, rather than from the synthesis of MBP in the thymus (31). Expression of the serum amyloid P component in mTEC has been shown to mediate T cell tolerance, but peripheral serum amyloid P production had the same effect, indicating that several mechanisms may operate for a single Ag (27).

We also investigated whether “intermediate” expression levels, such as those observed in heterozygous mice (26), affected central T cell tolerance. This issue is important because, in humans, VNTR class I alleles give thymic insulin levels only one half to one third of those attained with class III alleles, but they predispose the individual to type 1 diabetes (14, 15). The immunization of heterozygous mice functionally confirmed that a single functional allele was sufficient to confer tolerance and that imprinting of the proinsulin-2 gene in the thymus was unlikely. These results functionally confirm the expression data, which directly demonstrated that proinsulin gene expression is not imprinted in murine thymus (15). However, we cannot rule out the possibility of small quantitative differences in the number of T cell precursors not detected by the assay used. The effect of the level of thymic expression on tolerance is unclear, because the results obtained appeared to depend on the model used. In transgenic systems using heterozygous Aire−−/− mice as a means of reducing thymic expression, deletion was complete if the neo-Ag was expressed under control of the thyroglobulin promoter, but incomplete if the rat insulin promoter was used (29). In our model, using physiological proinsulin expression, central tolerance remained a well-buffered process, which is disrupted only if there is a large decrease in thymic expression.

We provide evidence that Ag expression by the thymic epithelium plays a major role in regulation of the CD4+ T cell response to proinsulin-2, but we also investigated the role of peripheral Ag in regulating a self-reactive T cell response resulting from a failure of central tolerance mechanisms. Our data indicate that islet proinsulin-2 is ignored by proinsulin-2-reactive CD4+ T cells. Surprisingly, even proinsulin-2-primed CD4+ T cells appeared to be less abundant after expansion in CD3ε−/− mice, indicating that memory T cell populations are not preferentially expanded, even if their specific Ag is expressed in the periphery. A similar attrition of Ag-specific T cells has been reported for virus-specific CD8+ T cells, using a similar protocol (51). The lack of islet infiltrate in our chimeras and transfer experiments contrasts with our previous findings (34), in which proinsulin-2-producing islets were the target of a mononuclear infiltrate after their transfer into intolerant mice. This difference demonstrates the role of local factors (e.g., a local vascular or inflammatory process in the vicinity of the grafted islets) in the initiation of such a response.

In conclusion, our results demonstrate the functional role of proinsulin-2 expression by radioresistant thymic epithelial cells in the induction of self-tolerance and suggest that additional factors are involved in triggering islet abnormalities. They raise questions as to whether central tolerance to proinsulin-2 is induced directly by mTEC or whether neighboring APCs are also involved (52, 53). Our study system can be used to evaluate factors, such as Toll-like receptors agonists (54), capable of targeting the proinsulin-2 T cell response to islet cells. Finally, our results, like those obtained by overexpressing the proinsulin-2 gene in the thymic epithelium of NOD mice (32), indicate that the manipulation of central tolerance should be further investigated in organ-specific autoimmune diseases, such as diabetes.

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Disclosures
The authors have no financial conflict of interest.

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