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# Effective Destruction of Fas-deficient Insulin-producing β Cells in Type 1 Diabetes

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## Abstract

In type 1 diabetes, autoimmune T cells cause destruction of pancreatic β cells by largely unknown mechanism. Previous analyses have shown that β cell destruction is delayed but can occur in perforin-deficient nonobese diabetic (NOD) mice and that Fas-deficient NOD mice do not develop diabetes. However, because of possible pleiotropic functions of Fas, it was not clear whether the Fas receptor was an essential mediator of β cell death in type 1 diabetes. To directly test this hypothesis, we have generated a β cell–specific knockout of the *Fas* gene in a transgenic model of type 1 autoimmune diabetes in which $CD4^+$ T cells with a transgenic TCR specific for influenza hemagglutinin (HA) are causing diabetes in mice that express HA under control of the rat insulin promoter. Here we show that the Fas-deficient mice develop autoimmune diabetes with slightly accelerated kinetics indicating that Fas-dependent apoptosis of β cells is a dispensable mode of cell death in this disease.

Key words: Fas receptor • diabetes • β cell death • autoimmunity • conditional knockout

## Introduction

Nonobese diabetic (NOD) mice are thought to represent a suitable animal model of human type 1 diabetes (1, 2). In this model, the disease depends on the activation of autoimmune T cells by peptides presented by the particular NOD class II (g7) MHC allele that controls disease. In addition, several T cell receptor transgenic models of type 1 diabetes have been developed in which particularly high frequencies of CD4 or CD8 T cells specific for antigens expressed in β cells cause pancreatic islet cell infiltration with subsequent destruction of β cells (3, 4). In the NOD and in some transgenic models (3, 5, 6), the disease can be precipitated when CD4 T cells from diabetic mice are transferred into immunodeficient recipients where diabetes develops within 1–2 wk. Nevertheless, the mechanism of β cell destruction has remained obscure. It became clear that perforin-deficient NOD mice exhibited a delayed onset of disease (7, 8), but it was not obvious whether the late phase of β cell destruction was affected. On the other hand, it was shown that Fas-deficient lpr/lpr mice on the NOD background did not develop diabetes (9). Additional analyses had suggested that Fas was present on β cells (9) and that expression increased during development of the disease. Other studies, however, also revealed that in Fas-deficient NOD mice there was no infiltration of pancreatic islets by mononuclear cells (10), thus leaving open the question of whether Fas was directly involved in β cell death. More recent studies using a dominant negative *Fas* mutation (11) or in vivo anti-Fas L antibody treatment (12) were interpreted to indicate that Fas may have a role in promoting predominantly early stages of the disease. To address a potential role of Fas in β cell destruction conclusively, we have produced a conditional *Fas* allele ($fas^{fl}$) in which the death domain-encoding exon 9 is flanked by loxP sites, allowing for cell type–specific Fas inactivation (unpublished data). To analyze whether Fas inactivation in pancreatic β cells resulted in resistance to the development of diabetes, the $fas^{fl}$ allele and a rat insulin promoter (RIP)–controlled Cre transgene (13) were introduced into a transgenic model of type 1 diabetes in which a transgenic TCR recognizes peptide 111–119 of influenza hemagglutinin (HA) expressed under control of the RIP and presented by class II $E^d$ MHC molecules (TCR-HA, Ins-HA) (5). The onset and development of diabetes in I-$E^d$ homozygous mice carrying the five transgenes (TCR-$HA^{+/-}$, Ins-$HA^{+/-}$, $Fas^{fl/fl}$, RIP-$Cre^{+/-}$ mice) was compared with

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*Abbreviations used in this paper:* HA, hemagglutinin; NOD, nonobese diabetic; RIP, rat insulin promoter.

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that in mice with only four transgenes lacking the RIP-cre transgene (TCR-HA$^{+/-}$, Ins-HA$^{+/-}$, Fas$^{fl/fl}$, RIP-Cre$^{-/-}$ mice) and thereby expressing Fas in β cells.

## Materials and Methods

*Mice and Genotyping.* TCR-HA mice and Ins-HA mice were bred as heterozygous transgenic mice and are on the BALB/c background. Fas$^{fl}$ mice derived from C57Bl/6 were created by homologous recombination using a conditional *Fas* allele in which the exon 9 has been flanked by loxP sites. RIP-Cre mice were on a mixed 129sv, C57Bl/6, and DBA-2 background (13). All animals were maintained in a pathogen-free facility in accordance with the guidelines of the Committee on Animals of Harvard Medical School. Genotyping of transgenes was determined by PCR on tail DNA. Primer sequences were 5′-GGCTACCATGCGAACAATTCACCCG-3′ and 5′-CTCCGTCAGCCATAGCAAATTTCTG-3′ for the *HA* transgene, 5′-ACAAGGTGGCAGTAACAGGA-3′ and 5′-ACAGTCAGTCTGGTTCCTGA-3′ for the *TCR-HA* transgene, 5′-CGATGCAACGAGTGATGAGG-3′ and 5′-CATCGCTCGACCAGTTTAGT-3′ for the *Cre* transgene, and 5′-TGCAGTTGCTGAGATGAACCATTTTCTCTGTCT-3′ (P1) and 5′-GGCTTTGGAAAGGAATTTCCTCCTAAGAGG-3′ (P2) for WT and floxed *Fas* alleles. A 430-bp amplified product indicated the presence of a WT *Fas* allele, whereas a 470-bp amplified product indicated the presence of a floxed *Fas* allele, since the sense primer was located in exon 9 and the antisense one 3′ to the loxP site downstream of exon 9 (Fig. 1 A). Homozygosity for I-E$^{d}$ was determined by staining peripheral blood leukocytes.

*RIP-Cre–mediated Fas Recombination in Islet DNA.* DNA was prepared from the thymus, liver, heart, and islets isolated at the Harvard Medical School Islet Core Rodent Isolation. RIP-Cre–mediated *Fas* recombination was assessed using a sense primer with the sequence 5′-GTCCTCTATTATCCTCATCATGAG-3′ (P3) located upstream of the 5′ loxP site and the antisense primer located downstream of the 3′ loxP site (P2). A 1.7-kb amplified product indicated the presence of intact exon 9, whereas a 260-bp amplified product indicated the presence of a deleted exon 9.

*Diabetes Monitoring and Cyclophosphamide Administration.* Development of spontaneous diabetes was assessed by measuring blood glucose twice a week with an automatic glucometer (Accu-chek Advantage; Roche). Cyclophosphamide (Sigma-Aldrich) in PBS was injected intraperitoneally at a dose of 200 mg/kg. Blood glucose levels of cyclophosphamide-treated mice were monitored daily.

*Adoptive Transfer of Diabetes.* CD4$^{+}$ TCR-HA–expressing cells were purified by sorting lymphocytes from spleen and LN of RAG-2$^{-/-}$, TCR-HA$^{+/-}$ mice stained with the 6.5 (anti–TCR-HA) and anti-CD4 (GK1.5; BD Biosciences) mAbs. $10^5$ sorted 6.5$^{+}$CD4$^{+}$ cells were i.v. injected into either RAG-2$^{-/-}$, Fas$^{fl/fl}$, Rip-cre$^{+/-}$, Ins-HA$^{+/-}$ or RAG-2$^{-/-}$, Fas$^{fl/fl}$, Rip-cre$^{-/-}$, Ins-HA$^{+/-}$ mice. Blood glucose levels of the recipients were tested daily.

## Results and Discussion

The efficacy and tissue specificity of Cre-mediated deletion of *Fas*–exon 9 was assessed by PCR on DNA prepared from purified islets and various tissues from Cre-expressing and -nonexpressing Fas$^{fl}$ homozygous mice. Fig. 1 A depicts a schematic representation of mutated and WT *Fas* alleles and the position of the primers used to assess littermate genotype and β cell-specific loss of functional *Fas* allele. Fig. 1 B shows that, regardless of Cre expression, DNA of non-pancreatic origin yielded a single 1.7-kb P2/P3 PCR product indicative of the sole presence of *Fas*–exon 9 (Fig. 1 A). Even traces of recombined *Fas*–exon 9 would have been detected by the presence of a 260-bp fragment, which because of its short size would be favored over the 1.7-kb fragment. Evidence for islet-specific Cre-mediated deletion of exon 9 came from the single 260-bp P2/P3 PCR product that was obtained from islets of Cre-expressing mice, whereas a single 1.7-kb band was detected from the islets of Cre-nonexpressing mice. PCR analysis of the same samples using the P1/P2 primers that hybridize to WT nonrecombined but not to recombined *Fas* alleles (Fig. 1 A) yielded a single 470-bp band in nonpancreatic tissues of mice irrespective of RIP-Cre expression and in islets of mice lacking RIP-Cre (Fig. 1 B). A much fainter 470-bp band was seen

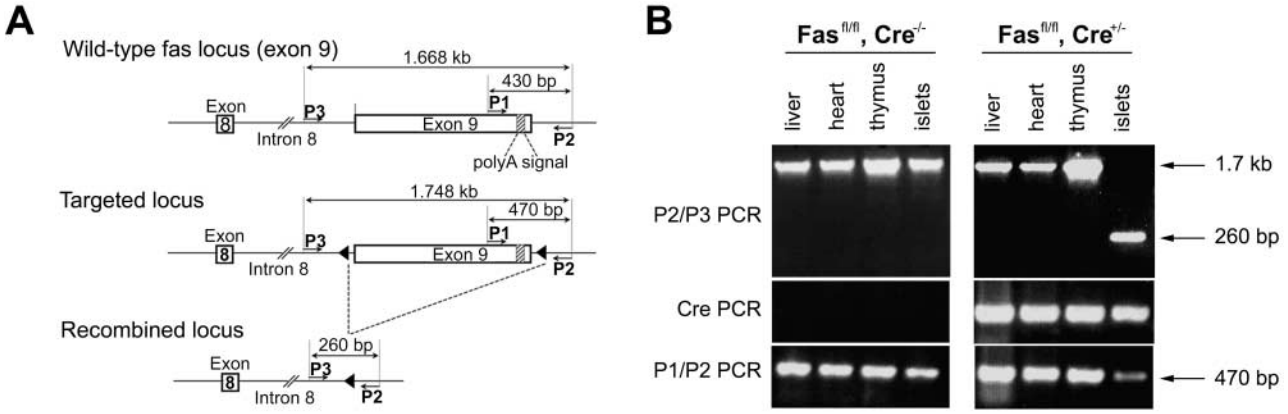


**Figure 1.** Successful elimination of the floxed *Fas*–exon 9 by homologous recombination in pancreatic β cells. (A) Representation of WT *Fas*, floxed *Fas*, and recombined floxed *Fas* alleles, and location of the primers used in PCR analyses. Cre-mediated recombination of the floxed *Fas* allele results in the deletion of exon 9. (B) PCR analysis on DNA from liver, heart, thymus, and purified islets of one Fas$^{fl}$ homozygous and one Fas$^{fl}$ homozygous, RIP-Cre heterozygous mouse.

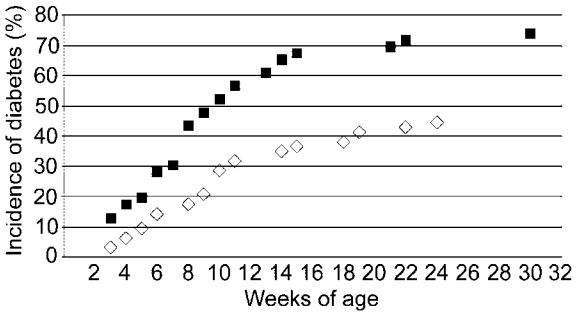


**Figure 2.** Effective destruction of β-islet cells from Fas-deficient and Fas-competent TCR-HA, Ins-HA transgenic mice. Blood glucose levels of $Fas^{fl/fl}$, $Rip\text{-}Cre^{+/-}$, $TCR\text{-}HA^{+/-}$, $Ins\text{-}HA^{+/-}$ (■, $n = 46$) and $Fas^{fl/fl}$, $Rip\text{-}Cre^{-/-}$, $TCR\text{-}HA^{+/-}$, $Ins\text{-}HA^{+/-}$ (◇, $n = 63$) mice were assessed over a period of 32 wk. Mice were considered diabetic after two successive blood glucose measurements ≥300 mg/dl.

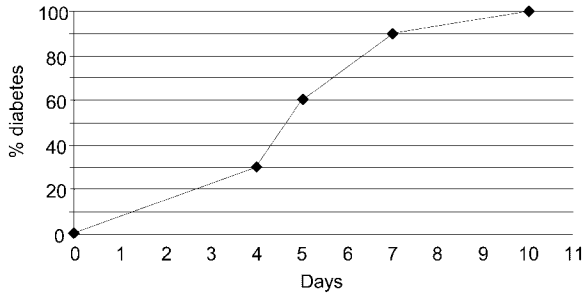


**Figure 3.** Cyclophosphamide-induced diabetes in $TCR\text{-}HA^{+/-}$, $Ins\text{-}HA^{+/-}$, $Fas^{fl/fl}$, $RIP\text{-}Cre^{+/-}$ mice. Mice ($n = 10$) were injected with 200 mg/kg of cyclophosphamide on day 0 and monitored for their blood glucose levels every day.

in islets of Cre-expressing mice (Fig. 1 B). This faint band is likely to reflect the fact that whole islets such as those used in the present study contain cells other than β cells (~30%), e.g., non–β endocrine cells, macrophages, endothelial cells, etc., lacking Cre expression. Thus, the absence of amplification of a 260-bp P2/P3 PCR product (which will be favored over the 1.7-kb P2/P3 PCR product) in nonpancreatic tissues and its detection only in Cre-expressing islets and the diminution of the 470-bp fragment intensity in Cre-expressing islets strongly suggest that the RIP-Cre–mediated recombination resulted in tissue-specific deletion of functional Fas in the vast majority of β cells. Since the development of diabetes with blood sugar levels exceeding 200 mg/dl requires destruction of well over 50% of all β cells, the conditional knockout was suitable to reveal a possible essential role of Fas in the development of diabetes, since any Fas-dependent destruction of β cells could not exceed 50% in these particular mice, and thus be insufficient to result in diabetes. Previous work using a RIP-Cre transgenic mouse line identical to that of the present study could not find evidence for a toxic effect of the transgene to the β cells, their function, or the glucose tolerance of RIP-Cre transgenic mice (13, 14). Furthermore, the $Fas^{fl}$ heterozygous, TCR-HA, Ins-HA, RIP-Cre transgenic breeders used in this study to generate the Fas-competent and -deficient TCR-HA, Ins-HA mice behaved identically to TCR-HA, Ins-HA double transgenic mice with respect to diabetes development, fertility, and frequency of the expected genotypes among their litter. Thus, we could exclude a role for the RIP-Cre transgene in potential phenotypical differences between RIP-Cre–expressing and –nonexpressing mice. To assess the impact of Fas expression by β cells in their T cell–mediated destruction, we compared the development of spontaneous autoimmune diabetes in the TCR-HA, Ins-HA transgenic model in which the floxed *Fas* alleles are recombined by the Cre recombinase ($TCR\text{-}HA^{+/-}$, $Ins\text{-}HA^{+/-}$, $Fas^{fl/fl}$, $RIP\text{-}Cre^{+/-}$ mice) to that of transgenic littermates lacking Cre expression ($TCR\text{-}HA^{+/-}$, $Ins\text{-}HA^{+/-}$, $Fas^{fl/fl}$, $RIP\text{-}Cre^{-/-}$ mice) (Fig. 2). The incidence and onset of diabetes in the latter quadruple transgenic littermates was not different from that in TCR-HA, Ins-HA double transgenic mice, indicating that neither the introduced floxed *Fas* alleles nor the mixed genetic background brought by the Fas floxed and RIP-Cre transgenic mice did perturb autoimmunity. The elimination of *Fas* by Cre recombinase did not produce a delay in the onset but some acceleration of the onset of diabetes (Fig. 2). A similar conclusion, namely that Fas deficiency did not delay the onset of diabetes, was reached when diabetes was analyzed in TCR-HA, Ins-HA mice transgenic mice with a β cell–specific deletion of *Fas* that were treated with 200 mg/kg cyclosphosphamide. This treatment is known to precipitate diabetes in NOD and TCR-HA, Ins-HA mice with similar kinetics, perhaps by interfering with regulatory (suppressive) T cells that control the development of disease. The RIP-Cre–expressing mice came down with diabetes 4–5 d after treatment with the drug, indicating that also under these conditions Fas did not play a discernible role in β cell death caused by autoimmune T cells (Fig. 3). Finally, it was tested

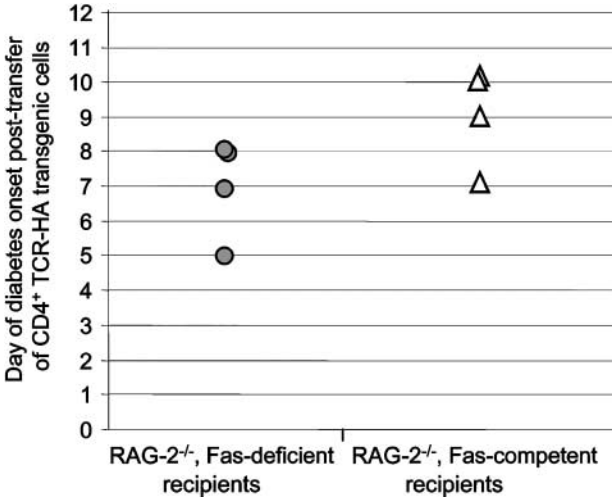


**Figure 4.** Development of diabetes in Fas-deficient and Fas-competent $RAG\text{-}2^{-/-}$, $Ins\text{-}HA^{+/-}$ recipients injected with $CD4^+$ TCR-HA transgenic cells. $6.5^+CD4^+$ cells from $RAG\text{-}2^{-/-}$, $TCR\text{-}HA^{+/-}$ mice were sorted, and $10^5$ cells were transferred into $RAG\text{-}2^{-/-}$, $Fas^{fl/fl}$, $Rip\text{-}cre^{+/-}$, $Ins\text{-}HA^{+/-}$ (●, $n = 4$) and $RAG\text{-}2^{-/-}$, $Fas^{fl/fl}$, $Rip\text{-}cre^{-/-}$, $Ins\text{-}HA^{+/-}$ (△, $n = 4$) on day zero. Blood glucose levels were monitored daily.

whether purified $CD4^+$ TCR-HA–expressing T cells from TCR-HA transgenic RAG-$2^{-/-}$ mice could cause β cell destruction in the absence of $CD8^+$ T cells by transferring them into Ins-HA transgenic mice on the RAG-$2^{-/-}$ background lacking or expressing fas on β cells. As shown in Fig. 4, diabetes developed in both types of recipients within a short time period after injection of $10^5$ $CD4^+$ T cells.

In both the NOD model (2) and in several TCR transgenic models (3, 5, 6), the autoimmune disease can be transferred with purified CD4 T cells into immunodeficient mice that themselves do not have any T cells. Since only a few activated $CD4^+$ T cells express perforin but most express Fas ligand, it was conceivable that such T cells destroyed β cells by a Fas L–Fas interaction that required direct cell contact. Our findings refute this hypothesis and are consistent with the notion that β cells from Fas-deficient mice that were transplanted into Fas-competent mice are effectively destroyed by diabetogenic T cells (14–16) even though it is not clear in the latter scenario whether destruction of the transplanted β cells occurs by the same mechanism as that of β cells in situ. Our results are also consistent with the finding that a dominant negative *Fas* transgene in NOD mice had little effect on the development of diabetes when splenocytes from diabetic animals were transferred into x-irradiated transgenic nondiabetic recipients (11).

By eliminating the Fas L–Fas apoptotic system as the essential mediator of β cell death in type 1 diabetes, our results should shift the focus to other mediators of apoptotic cell death that do not require direct contact between T cells and β cells. With regard to this notion, we may point out that there is apparently no significant expression of class II MHC on β islet cells (1) and perhaps even more conclusively that antigen-specific contact between T cells and β cells is not required for the T cell–mediated destruction of β cells (17). Thus, the search for mediators of β cell destruction needs to focus on molecules that play an essential role in β cell destruction in type 1 autoimmune diabetes and differ from Fas. The identification of such essential mediators of β cell death would perhaps permit interference with the disease once the process of destruction has begun and first symptoms are detectable.

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## References

1. Bach, J.F., and D. Mathis. 1997. The NOD mouse. *Res. Immunol.* 148:285–286.
2. Tisch, R., and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. *Cell.* 85:291–297.
3. Degermann, S., C. Reilly, B. Scott, L. Ogata, H. von Boehmer, and D. Lo. 1994. On the various manifestations of spontaneous autoimmune diabetes in rodent models. *Eur. J. Immunol.* 24:3155–3160.
4. Scott, B., R. Liblau, S. Degermann, L.A. Marconi, L. Ogata, A.J. Caton, H.O. McDevitt, and D. Lo. 1994. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. *Immunity.* 1:73–83.
5. Sarukhan, A., A. Lanoue, A. Franzke, N. Brousse, J. Buer, and H. von Boehmer. 1998. Changes in function of antigen-specific lymphocytes correlating with progression towards diabetes in a transgenic model. *EMBO J.* 17:71–80.
6. Katz, J.D., C. Benoist, and D. Mathis. 1995. T helper cell subsets in insulin-dependent diabetes. *Science.* 268:1185–1188.
7. Kagi, D., B. Odermatt, P. Seiler, R.M. Zinkernagel, T.W. Mak, and H. Hengartner. 1997. Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. *J. Exp. Med.* 186:989–997.
8. Amrani, A., J. Verdaguer, B. Anderson, T. Utsugi, S. Bou, and P. Santamaria. 1999. Perforin-independent beta-cell destruction by diabetogenic CD8(+) T lymphocytes in transgenic nonobese diabetic mice. *J. Clin. Invest.* 103:1201–1209.
9. Chervonsky, A.V., Y. Wang, F.S. Wong, I. Visintin, R.A. Flavell, C.A. Janeway, Jr., and L.A. Matis. 1997. The role of Fas in autoimmune diabetes. *Cell.* 89:17–24.
10. Itoh, N., A. Imagawa, T. Hanafusa, M. Waguri, K. Yamamoto, H. Iwahashi, M. Moriwaki, H. Nakajima, J. Miyagawa, M. Namba, et al. 1997. Requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice. *J. Exp. Med.* 186:613–618.
11. Savinov, A.Y., A. Tcherepanov, E.A. Green, R.A. Flavell, and A.V. Chervonsky. 2003. Contribution of Fas to diabetes development. *Proc. Natl. Acad. Sci. USA.* 100:628–632.
12. Nakayama, M., M. Nagata, H. Yasuda, K. Arisawa, R. Kotani, K. Yamada, S.A. Chowdhury, S. Chakrabarty, Z.Z. Jin, H. Yagita, et al. 2002. Fas/Fas ligand interactions play an essential role in the initiation of murine autoimmune diabetes. *Diabetes.* 51:1391–1397.
13. Kulkarni, R.N., J.C. Bruning, J.N. Winnay, C. Postic, M.A. Magnuson, and C.R. Kahn. 1999. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell.* 96: 329–339.
14. Allison, J., and A. Strasser. 1998. Mechanisms of beta cell death in diabetes: a minor role for CD95. *Proc. Natl. Acad. Sci. USA.* 95:13818–13822.
15. Kim, Y.H., S. Kim, K.A. Kim, H. Yagita, N. Kayagaki, K.W. Kim, and M.S. Lee. 1999. Apoptosis of pancreatic beta-cells detected in accelerated diabetes of NOD mice: no role of Fas-Fas ligand interaction in autoimmune diabetes. *Eur. J. Immunol.* 29:455–465.
16. Pakala, S.V., M. Chivetta, C.B. Kelly, and J.D. Katz. 1999. In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor alpha. *J. Exp. Med.* 189:1053–1062.
17. Sarukhan, A., O. Lechner, and H. von Boehmer. 1999. Autoimmune insulitis and diabetes in the absence of antigen-specific contact between T cells and islet beta-cells. *Eur. J. Immunol.* 29:3410–3416.