

Critical Roles of Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand in Type 1 Diabetes

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis of tumor cells but not most normal cells. Its roles in normal nontransformed tissues are not clear. To explore the potential roles of TRAIL in type 1 diabetes, we examined the consequences of TRAIL blockade or TRAIL deficiency in two animal models of autoimmune diabetes. In the first model, NOD mice received an injection of a soluble TRAIL receptor to block TRAIL function. This significantly accelerated the diabetes and increased the degree of autoimmune inflammation in both pancreatic islets and salivary glands. The GAD65-specific immune responses were also significantly enhanced in animals that received the soluble TRAIL receptor. In the second model, we treated normal and TRAIL-deficient C57BL/6 mice with multiple low-dose streptozotocin to induce diabetes. We found that both the incidence and the degree of islet inflammation were significantly enhanced in TRAIL-deficient animals. On the basis of these observations, we conclude that TRAIL deficiency accelerates autoimmune diabetes and enhances autoimmune responses. *Diabetes* 52:2274–2278, 2003

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a type 2 membrane protein of the TNF superfamily. It preferentially induces apoptosis of tumor cells but not most normal cells (1), with the exception of hepatocytes (2), neural cells (3), and thymocytes (4,5), which are sensitive to TRAIL-induced apoptosis. In humans, TRAIL interacts with at least four membrane receptors that all belong to the TNF receptor superfamily. TRAIL receptor 1 (TRAIL-R1 or death receptor 4) (6) and TRAIL receptor 2 (TRAIL-R2, death receptor 5, TRICK2, or KILLER) (7,8) have cytoplasmic death domains, and can activate both caspases and nuclear factor (NF)- κ B pathways. The other two receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), have

truncated death domains. They are not capable of activating caspase cascade but may activate NF- κ B and block apoptosis (9). In addition, osteoprotegerin may be a soluble receptor for TRAIL, which can inhibit osteoclastogenesis and increase bone density (10). In contrast to these receptors in humans, only one membrane TRAIL receptor has been identified in mice, which shares the highest homology with human death receptor (DR) 5 (8). The mouse decoy receptors share low homology with those of humans and do not have intracellular domains (11). Although TRAIL and its receptors are expressed in a variety of mouse and human tissues, their roles in health and diseases are not clear.

Type 1 diabetes is an autoimmune inflammatory disease of the pancreatic islets. In both human type 1 diabetes and its rodent models, pancreatic β -cells that produce insulin are selectively destroyed by infiltrating inflammatory cells (12,13). The cyclophosphamide (CY)-accelerated diabetes and the low-dose streptozotocin (STZ)-induced diabetes are putative mouse models of human type 1 diabetes (14). The disease can be induced by a single injection of CY in NOD mice or by multiple injections of low-dose STZ in susceptible strains of animals (14,15). Both models share many clinical and histologic features with human type 1 diabetes and require the participation of T-cells and macrophages (16,17). To explore the roles of TRAIL in diabetes, we studied these two disease models in animals that were either deficient in TRAIL or treated with a blocking TRAIL receptor. We found that TRAIL played a critical role in the pathogenesis of diabetes.

RESEARCH DESIGN AND METHODS

Mice. NOD and normal C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice deficient in TRAIL (TRAIL^{-/-}) were generated by homologous recombination as described (18). All TRAIL^{-/-} mice used in this study were backcrossed for more than 10 generations to C57BL/6 mice. Mice were housed in the University of Pennsylvania animal care facilities under pathogen-free conditions. All procedures used were pre-approved by the Institutional Animal Care and Use Committee.

Reagents. GAD65 (524–543) peptide was synthesized by Research Genetics-Invitrogen (Huntsville, AL) using Fmoc (fluorenylmethoxycarbonyl) method and purified through high-performance liquid chromatography. Peptide purity was determined by capillary electrophoresis, and the composition was verified by mass spectrometry. STZ and CY were purchased from Sigma. Rat anti-mouse interleukin (IL)-2, IL-10, and γ -interferon (IFN- γ) antibodies were purchased from Pharmingen (San Diego, CA). Enzyme-linked immunosorbent assay (ELISA) for IL-2, IL-10, and IFN- γ was performed using paired monoclonal antibodies specific for corresponding cytokines per the manufacturer's recommendations.

Production of a soluble TRAIL receptor. The recombinant human soluble (s)DR5 was produced using the *Pichia pastoris* system as we described (19). The purified soluble DR5 (sDR5) contains 1–2 ng of lipopolysaccharide (LPS) per mg of protein, as determined by Limulus amoebocyte lysate assay. This is comparable to human serum albumin (HSA) purchased from Sigma, which contains 1–4 ng of LPS per mg of protein. Before this study, we had

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CY, cyclophosphamide; DR, death receptor; ELISA, enzyme-linked immunosorbent assay; HAS, human serum albumin; IFN- γ , γ -interferon; IL, interleukin; i.p., intraperitoneally; LPS, lipopolysaccharide; NF, nuclear factor; sDR5, soluble DR5; STZ, streptozotocin; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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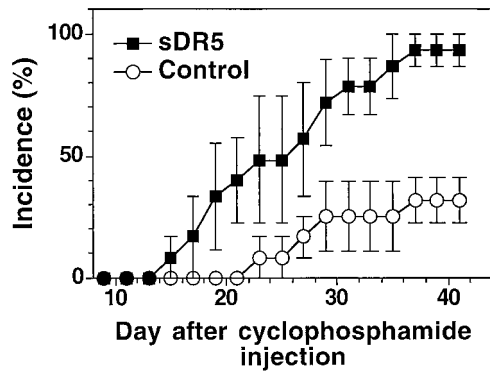


FIG. 1. TRAIL blockade exacerbates diabetes in NOD mice. Seven- to eight-week-old female NOD mice, 14 mice per group, received an i.p. injection of CY to precipitate diabetes as described in RESEARCH DESIGN AND METHODS. sDR5 or HSA, 200 μ g/mouse, was injected i.p. once every other day starting 2 days before the CY injection. The differences between the two groups are statistically significant as determined by Mann-Whitney *U* test ($P < 0.001$). Data are pooled from three independent experiments.

determined that this level of LPS had no effect on the development of diabetes using LPS-free PBS as a control.

Induction and clinical evaluation of diabetes. For NOD mice, pre-diabetic (7- to 8-week-old) female mice were challenged intraperitoneally (i.p.) with 200 mg/kg CY to precipitate the disease. For C57BL/6 (B6) mice, diabetes was induced by multiple low-dose injections of STZ. Briefly, male B6 mice, 7–9 weeks old, were injected i.p. once a day for 5 consecutive days with 40 mg/kg body wt STZ dissolved in citrate buffer (pH 4.5). Mice were tested once every other day for urinary glucose levels using the keto-Diastix kit (Bayer, Elkhart, IN). Mice were considered diabetic when the urinary glucose levels were ≥ 300 mg/dl on two consecutive tests.

Histochemistry and quantification of inflammatory lesions. Pancreas and submandibular salivary glands were fixed in 10% formalin, sectioned, and stained with hematoxylin/eosin. The degree of islet inflammation was scored as follows: 0, normal; 1, peri-insulinitis with mononuclear cell infiltration affecting $\leq 25\%$ of the circumference; 2, peri-insulinitis with mononuclear cell infiltration affecting $>25\%$ of the circumference; 3, mild to moderate insulinitis with intra-islet mononuclear cell infiltration but good preservation of islet architecture; and 4, severe insulinitis with numerous intra-islet inflammatory cells and loss of normal islet architecture. The degree of salivary gland inflammation was measured on the basis of the number of inflammatory lesions detected in each tissue sections. An inflammatory lesion is defined as an infiltrate inside the parenchyma of the gland consisting of a minimum of 10 mononuclear cells (20).

Cell culture. For cytokine assays, splenocytes were cultured at 1.5×10^6 cells/well in 0.2 ml of DMEM (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum and various amounts of GAD65 peptide. Culture supernatants were collected 40 h later, and cytokine concentrations were determined by ELISA. For proliferation assays, 0.5×10^6 cells/well were used. [3 H]thymidine was added to the culture at 48 h, and cells were harvested 16 h later. Radioactivity was determined using a flatbed β -counter (Wallac, Gaithersburg, MD).

Statistical analysis. The significance of differences in disease severity, day of onset, and cytokine concentrations was determined by ANOVA.

RESULTS

TRAIL blockade exacerbates CY-induced autoimmune diabetes. To examine the consequences of TRAIL blockade on type 1 diabetes, sDR5 or a control protein was injected i.p. into prediabetic female NOD mice for a total of 38 days. The sDR5 is a truncated human DR5 that effectively blocks TRAIL function in both mouse and human systems as we reported (19). Diabetes was induced by a single CY injection 2 days after the first sDR5 treatment. As

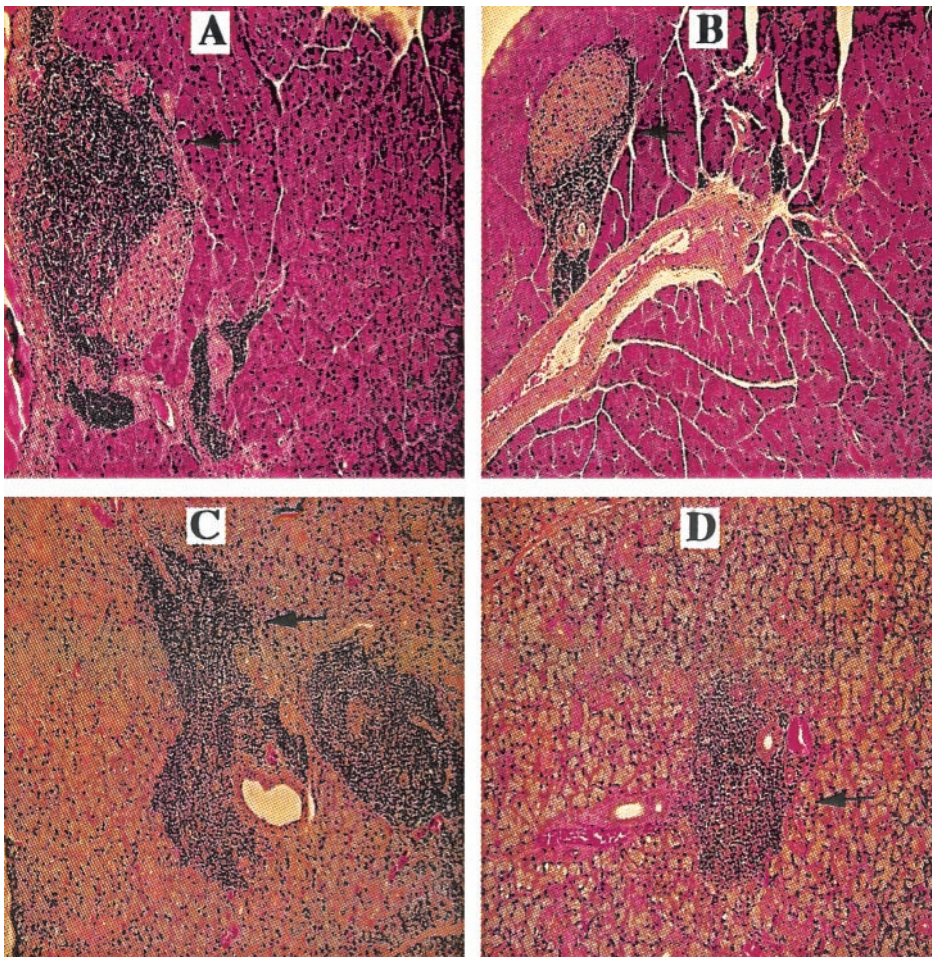


FIG. 2. Histologic examination of pancreatic islets and salivary glands. NOD mice were treated as described in Fig. 1 and killed 42 days after CY injection. Pancreatic (A and B) and submandibular salivary gland (C and D) sections were stained with HE (hematoxylin/eosin). A and B: Pancreatic sections of sDR5- and HAS-treated mice, respectively. C and D: Salivary gland sections of sDR5- and HAS-treated mice, respectively. Magnification 100 \times . Arrows indicate inflammatory lesions.

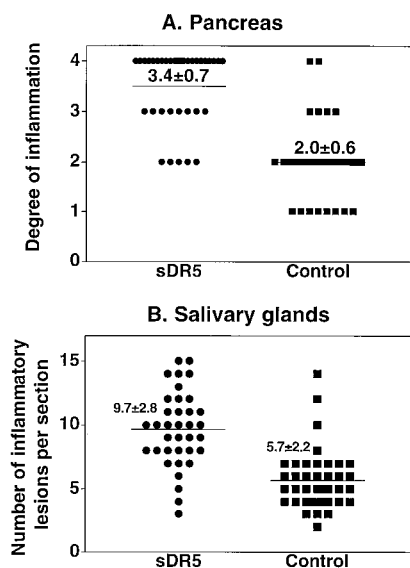


FIG. 3. Quantitative analyses of inflammatory lesions in the pancreas and salivary glands. Mice were treated as described in Fig. 1. The degree of inflammation in the pancreas (*A*) and salivary glands (*B*) was graded as described in RESEARCH DESIGN AND METHODS. A minimum of three cross sections per organ were examined. Results shown are from a total of 28 mice. Each data point represents a cross section. Numbers in the graphs represent means \pm SD of each group. The differences between the two groups in both *A* and *B* are statistically significant ($P < 0.0001$).

shown in Fig. 1, control NOD mice developed diabetes 23 days after the CY injection, reaching a maximal incidence of 31.6% on day 37. By contrast, the disease was significantly accelerated in mice that were treated with sDR5. Diabetes was first detected on day 15, and by day 37, $>90\%$ of mice were diabetic. Of the 14 mice, 2 died of diabetes in the sDR5-treated group, whereas none died in the control group. Consistent with these results, Delovitch and Mi recently showed that sDR5 accelerated diabetes in NOD mice that received transfer of diabetogenic T-cells (unpublished data). Thus, chronic blockade of TRAIL by sDR5 significantly exacerbated diabetes in NOD mice.

TRAIL blockade enhances the degree of inflammation in the target organs. To investigate the effect of TRAIL blockade on the formation of inflammatory lesions in target organs, we performed histochemical studies of pancreas and submandibular salivary glands. As shown in Fig. 2, multiple inflammatory lesions in pancreas (Fig. 2*A* and *B*) and submandibular salivary gland (Fig. 2*C* and *D*) were detected in sDR5-treated mice. The inflammatory lesions consisted mostly of lymphocytes and macrophages and, in the case of the pancreas, centered around the islets. By contrast, in control animals, much reduced degrees of inflammation were observed. Figure 3 summarizes the results of these analyses. It is evident that TRAIL blockade significantly increased the degree of inflammation in both pancreas and submandibular salivary gland.

TRAIL blockade enhances anti-GAD65 immune responses. In NOD mice, activation of autoreactive lymphocytes is essential for the development of diabetes. To determine whether TRAIL blockade affected the functions of diabetogenic T-cells, we studied anti-GAD65 T-cell responses in mice after TRAIL blockade. Mice were treated with either sDR5 or a control protein as described in Fig. 1, and anti-GAD65 T-cell responses were determined ex

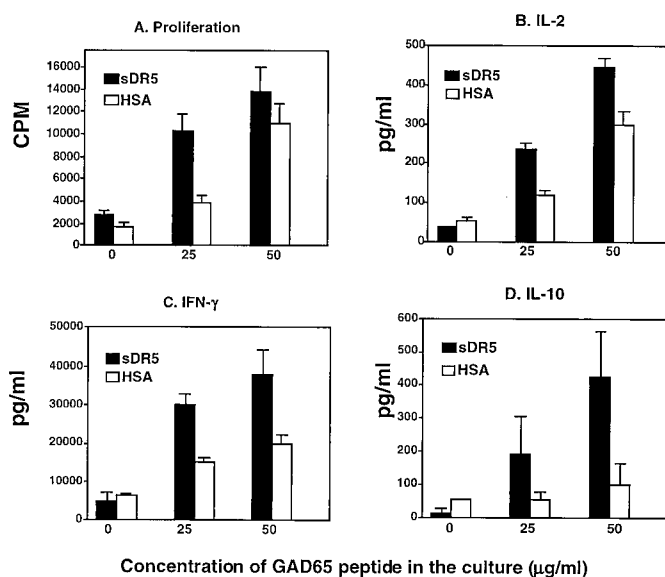


FIG. 4. GAD65-specific proliferative and cytokine responses. Female NOD mice, five mice per group, were treated with CY, sDR5, or HSA as described in Fig. 1. Mice were killed 38 days after CY injection, and splenocytes were tested for anti-GAD65 immune responses as described in RESEARCH DESIGN AND METHODS. Data presented are representative of two experiments. The differences between sDR5 and HSA groups are statistically significant ($P < 0.01$) for all of the cultures with GAD peptide.

vivo 5 weeks after the first injection of the sDR5. As shown in Fig. 4, in response to GAD65 peptide, splenocytes of control mice proliferated and produced both T helper type 1 (IL-2 and IFN- γ) and type 2 (IL-10) cytokines. These responses were significantly increased in mice that were treated with sDR5. To determine whether anti-GAD65 β -cell responses were also affected by sDR5 blockade, we tested serum anti-GAD65 antibodies by GAD65-specific ELISA. Anti-GAD65 IgG1 was significantly increased in mice that were treated with sDR5 as compared with the controls (Fig. 5), whereas anti-GAD65 IgG2a was not detected in either of the groups (data not shown). These results indicate that TRAIL blockade in NOD mice enhanced both cellular and humoral immune responses to GAD65.

TRAIL knockout mice are more susceptible to STZ-induced diabetes. For elucidating the roles of TRAIL in

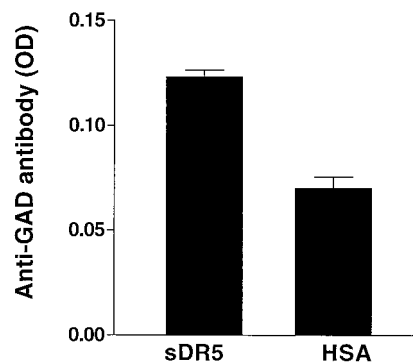


FIG. 5. GAD65-specific antibody responses. Female NOD mice, five mice per group, were treated with CY, sDR5, or HSA as described in Fig. 1. Mice were bled retro-orbitally 38 days after the first CY injection, and anti-GAD65 IgG1 antibodies were determined by ELISA using recombinant murine GAD65 as antigen. Each data point represents mean \pm SD of OD450 from five mice. The experiments were repeated twice with similar results. The difference between sDR5 and HSA groups is statistically significant ($P < 0.01$).

TABLE 1
Exacerbation of STZ-induced diabetes in TRAIL^{-/-} mice*

Mice	Incidence (%)†	Day of onset (mean ± SEM)‡	Range of urine glucose (mg/dl)§	Degree of insulinitis (mean ± SEM)
TRAIL ^{+/+}	7/13 (53.8)	20.3 ± 7.4	500–1,000	0.9 ± 0.15
TRAIL ^{-/-}	12/13 (92.3)	10 ± 4.3	1,000–2,000	3.2 ± 0.18

*Data are pooled from three independent experiments. †The cumulative disease incidence. The difference between the two groups is statistically significant ($P < 0.001$). ‡The difference in day of onset between the two groups is statistically significant ($P < 0.05$). §Range of urine glucose levels of diabetic mice 50 days after the first STZ injection. (The urine glucose levels of nondiabetic mice were < 100 mg/dl.) The difference between the two groups is statistically significant ($P < 0.0001$). ||Mean insulinitis grades of diabetic mice. A total of 50 islets were scored for each group. The difference between the two groups is statistically significant ($P < 0.0001$).

vivo, TRAIL-deficient mice have been recently generated by gene targeting (18). TRAIL-deficient mice develop normally and acquire a structurally normal immune system. To examine the consequences of TRAIL deficiency on type 1 diabetes, we injected low-dose STZ (40 mg STZ/kg body wt) into normal and TRAIL-deficient C57BL/6 mice for 5 consecutive days (days 0–4). Diabetes was monitored by both urine glucose levels and histochemistry of pancreatic islets. We found that the onset of the diabetes was significantly accelerated and the incidence increased in the TRAIL^{-/-} mice. Specifically, as shown in Table 1, the incidence of the diabetes was increased from 54% in TRAIL^{+/+} group to 92% in TRAIL^{-/-} group. The majority of pancreatic islets in TRAIL^{+/+} mice seemed normal or mildly infiltrated by lymphocytes and myeloid cells. By contrast, most pancreatic islets in TRAIL^{-/-} mice were either destroyed or heavily infiltrated by inflammatory cells 10 days after STZ injection. Figure 6 summarizes the results of our histochemical analysis. It is apparent that TRAIL deficiency significantly increased the severity of the diabetic inflammation.

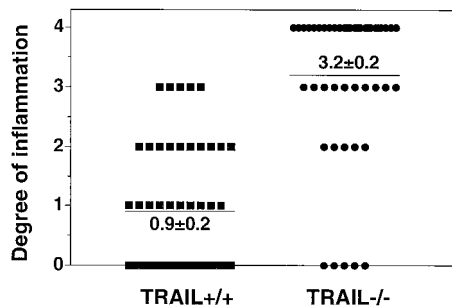


FIG. 6. TRAIL-deficient mice are more susceptible to STZ-induced diabetes. Normal (TRAIL^{+/+}) and TRAIL-deficient (TRAIL^{-/-}) B6 mice received injections of multiple low doses of STZ as described in RESEARCH DESIGN AND METHODS. Diabetes was monitored and recorded as in Table 1. Fifty-five days after the first STZ injection, mice were killed and their pancreata were examined by histochemistry. The degree of islet inflammation was scored as described in RESEARCH DESIGN AND METHODS. Results shown are from a total of 10 mice. Each data point represents a tissue section. Numbers in the graphs represent means ± SD of each group. The differences between the two groups are statistically significant ($P < 0.0001$).

DISCUSSION

The TNF family consists of at least 13 homologous proteins, which play crucial roles in a wide range of biological processes, including apoptosis, immunity, inflammation, and development. Like many members of this family, TRAIL is expressed by a variety of cell types, including T-cells (21), natural killer cells (22,23), monocytes (24), and β -cells (25). In addition to inducing apoptosis of tumor cells, TRAIL seems to have very limited cytotoxicity to normal cells, with the exception of activated T-cells (26), human hepatocytes (2), human brain cells (3), and thymocytes (4,5). The goal of this investigation was to determine the potential roles of TRAIL in autoimmune diabetes. Results presented here strongly suggest that one of the functions of TRAIL in vivo is to inhibit autoimmune inflammation in the islets of Langerhans. Blocking endogenous TRAIL with sDR5 eliminates this inhibition and exacerbates autoimmune diabetes. Thus, unlike TNF- α , which may promote the development of type 1 diabetes (27), TRAIL inhibits insulinitis and suppresses autoimmune diabetes.

The multiple low-dose STZ-induced diabetes is an inflammatory disease of the pancreatic islets with clinical and histochemical features similar to those of human type 1 diabetes (14,15). Both T-cells and macrophages are involved in mediating β -cell injury in this disease (17). TRAIL and its receptors are constitutively expressed by these two cell types and therefore may regulate diabetes through acting on one or both of these cells. Our finding that autoreactive T-cell function was enhanced in NOD mice that were treated with sDR5 suggests that TRAIL may inhibit diabetes by suppressing the functions of autoreactive T-cells. This is consistent with our recent observations in other models of autoimmunity, i.e., collagen-induced arthritis in DBA/1 mice (19) and experimental autoimmune encephalomyelitis in C57BL/6 mice induced by myelin oligodendrocyte glycoprotein (28), in that TRAIL acts as an inhibitor of autoreactive T-cell activation. However, it should be pointed out that TRAIL may not only inhibit the functions of autoreactive lymphocytes; in vitro, TRAIL also blocks DNA synthesis and cell cycle progression of T-cells activated by anti-CD3 antibody (19), indicating that the TRAIL effect is not restricted to a particular T-cell type. In addition to regulating lymphocyte functions in the periphery, TRAIL mediates negative selection of thymocytes (4). However, whether TRAIL is involved in thymic selection of autoreactive T-cells recognizing islet antigens remains to be established.

In summary, by studying NOD mice that were treated with sDR5 and C57BL/6 mice deficient in TRAIL, we have established that TRAIL plays a crucial role in inhibiting diabetic inflammation and autoreactive T-cell activation. These findings may have significant implications for our understanding of the pathogenesis of autoimmune diabetes and for developing novel strategies for the treatment and prevention of the disease.

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