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A Mimic of Viral Double-Stranded RNA Triggers Fulminant Type 1 Diabetes-like Syndrome in Regulatory T Cell-Deficient Autoimmune Diabetic Mouse

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Human fulminant type 1 diabetes (FT1D) is an extremely aggressive disease. The delay of proper diagnosis results in high mortality. However, the pathophysiology of this disease remains unclear. We took advantage of CD28-deficient NOD (CD28−/− NOD) mice, which have limited numbers of regulatory T cells and develop aggressive autoimmune diabetes, to create a FT1D model that mimicked the disease in humans. Young CD28−/− NOD mice were injected with polyinosinic-polycytidylic acid to activate innate immunity in an effort to induce diabetes onset. In this model, innate immune cell activation precedes the onset of diabetes similar to ~70% of FT1D patients. Eighty-three percent of CD28−/− NOD mice developed diabetes within 1–6 d after injection of polyinosinic-polycytidylic acid. Moreover, T cells infiltrated the pancreatic exocrine tissue and destroyed α cells, an observation characteristic of human FT1D. We conclude that an FT1D-like phenotype can be induced in the background of autoimmune diabetes by a mimic of viral dsRNA, and this model is useful for understanding human FT1D. The Journal of Immunology, 2011, 187: 4947–4953.

Because of its obscure etiology, several models of FT1D, based on distinct mechanisms but having an abrupt onset of diabetes, have been proposed. For instance, the insulin receptor substrate 2-deficient C57BL/6 mouse that develops diabetes has been proposed as a pure nonautoimmune model of FT1D (13). Alternatively, the encephalomyocarditis virus-induced diabetic mouse has been proposed as a model of disease caused by viral infection (14). Finally, the adoptive transfer of BDC2.5 TCR-transgenic T cells, derived from an insulin granule-reactive T cell clone (15), into NOD neonates provoked aggressive autoimmune diabetes and widespread destruction of pancreatic cells, both α and β cells as well as exocrine tissue, suggesting that FT1D may be caused by an autoimmune mechanism in a situation of immature regulatory T cell (Treg) function. However, none of these models fully mimics the multiple aspects of the human disease including autoimmune responses, induction of disease in the majority of patients following infection, and T cell reactivity.

Therefore, we took advantage of CD28-deficient NOD (CD28−/− NOD) mice as a possible model of human FT1D. Previous findings in this model have shown that this mouse develops profound T1D as well as autoimmune exocrine disease due to limited Treg numbers (16, 17). Given the more rapid course of FT1D in humans and the proposed role of viral infection in disease pathogenesis, we examined the effect of administration of a mimic of dsRNA, polyinosinic-polycytidylic acid [poly(I:C)], which has been suggested to increase pathogenic T cells and Tregs in conventional NOD mice (18), to CD28−/− NOD mice. Moreover, poly(I:C) mimics one of the candidate viruses that might be related to FT1D, an RNA enterovirus (12), which triggers the same TLRs. Thus, we examined whether poly(I:C) treatment of CD28−/− NOD mice alters the disease phenotype to more closely mimic FT1D. In this study, we report that injection of the dsRNA mimic [poly(I:C)] into CD28−/− NOD mice at 8 wk of age led to rapid development of diabetes within 1–6 d after administration and showed an FT1D-like phenotype. This rapid disease development was due to the initiation and/or acceleration of the innate arm of immunity.
Materials and Methods

Mice

Male and female CD28 heterozygous knockout (KO) NOD mice were generated as previously described (19). A CD28 heterozygous KO male and CD28 heterozygous KO NOD female were intercrossed, and the offspring were screened for homozygous (CD28−/− NOD mice), heterozygous, and wild-type genotypes and used for the experiments. All animals were maintained under specific pathogen-free conditions in the Laboratory Animal Section of Keio University School of Medicine (Tokyo, Japan) according to the guidelines for animal welfare.

To induce diabetes, 20–50% of the area of each islet. As control, CD28−/− NOD mice were tested for glucosuria every day from 56–62 d of age, and twice a week thereafter (9–20 wk of age). After two consecutive positive readings of glucosuria based on a Tes-Tape (Shionogi), a blood glucose measurement using Glutest-AceR (Sanwakagakufu Airway) was performed. Mice exhibiting a blood glucose concentration of ≥250 mg/dl were considered diabetic.

Histological analysis

The pancreas harvested from each mouse was fixed in 3.7% paraformaldehyde and embedded in paraffin. Thin sections, 10 μm apart, were stained with H&E for grading of insulitis and exocrine infiltration. For glucagon staining, tissues were deparaffinized and stained with anti-glucagon Abs (Funakoshi). To quantify a cell area, glucagon-positive area was measured using Adobe Photoshop CS4 Extended (Adobe Systems) and NanoZoomer 2.0-HT (Hamatsu Photonics) and divided by whole islet area determined by H&E staining. To identify the infiltrating cell types, each pancreas was inflated with OCT compound and snap-frozen in liquid nitrogen, and thin sections were fixed in 4% paraformaldehyde/PBS and stained with a HistoMouse Max Kit (Invitrogen) using the following mAbs: anti-mouse CD4 (H129.19; BD Biosciences), anti-mouse CD8α (53-6.7), anti-mouse CD11b (M1/70), anti-mouse CD19 (H129.19), FITC rat anti-mouse CD8a (53-6.7), anti-mouse CD11b (M1/70), anti-mouse CD19 (ID3), anti-mouse CD11c, and NKG2D (191004; R&D Systems). The stained tissues were examined by fluorescence microscopy.

Insulitis was graded according to the following criteria: grade 0: no insulitis; grade 1: peri-insulitis with mononuclear cell infiltration in <20% of the area of each islet; grade 2: moderate insulitis with mononuclear cell infiltration in 20–50% of the area of each islet; and grade 3: severe insulitis with mononuclear cell infiltration in >50% of the area of each islet.

Grading of mononuclear cell infiltration of the exocrine area. Grading was performed as follows: grade 0: no infiltration of periductal region; grade 1: infiltration of several mononuclear cells in periductal region; grade 2: mononuclear cell clustering in periductal region but not limiting plate; grade 3: focal acinar infiltration; and grade 4: widespread periductal and acinar infiltration or regeneration of ducts.

Poly(I:C) administration

Eight-week-old CD28−/− NOD or wild-type NOD mice were injected with poly(I:C), 200 μg/mouse (Sigma-Aldrich), i.p. for 7 d or until the onset of diabetes. As control, CD28−/− NOD mice were injected with PBS for 7 d.

Flow cytometry to evaluate population of mononuclear cells in pancreas

The spleens from mice were aseptically removed and cell subsets examined by flow cytometric analysis. After lysing RBCs, the cells were washed three times with PBS. Splenocytes were suspended at an appropriate concentration and stained with the following mAbs: PE rat anti-mouse CD4 (H129.19), FITC rat anti-mouse CD8α (53-6.7), rat IgG2a anti-mouse CD19 (ID3; in this case, FITC anti-IgG2a [RG711.30] was used as the second Ab), PE rat anti-mouse CD11b (M1/70), PE rat anti-mouse CD49b (DX5), or PE rat anti-mouse CD11c. To perform flow cytometric analysis of the pancreas, collagenase IV (0.2%; Worthington) was injected into the common bile duct, and the distended pancreas was removed and incubated at 37°C for 17 min with shaking. After washing with PBS, nonenzymatic solution (Sigma-Aldrich) was added to the pancreas, followed by incubation for another 5–10 min. Then the pancreas was dissected with a cell strainer and stained with the same mAbs as for the splenic cell preparations. The stained cells were analyzed with an Epics Altra (Beckman). The spleens from mice were aseptically removed and cell subsets examined by flow cytometric analysis. After lysing RBCs, the cells were washed three times with PBS.

Evaluation of mRNA expression

Total RNA was extracted from the pancreas and spleen using an RNaseasy Mini kit (Qiagen). During the procedure, DNase treatment was performed according to the manufacturer’s protocol. The extracted RNA was reverse transcribed using Not I-d(T)18 primer and a First-Strand cDNA synthesis kit (Amersham Biosciences) according to the manufacturer’s instructions. Semiquantitative RT-PCR was conducted for IL-2, IFN-γ, IL-4, IL-10, TGF-β, Foxp3, granzyme B, IL-18, IL-12p40, and GAPDH (internal control) in an ABI Prism 7700 sequence detector (Applied Biosystems). The primer and probe sequences were used as previously described (20). All reactions were performed using TaqMan Universal MasterMix (Applied Biosystems). The levels of mRNA expression were normalized to that of the GAPDH PCR product amplified from the same sample ([sample PCR product/GAPDH PCR product] × constant).

Statistical analysis

All statistical analyses were performed using StatView (SAS Institute). Results are presented as mean ± SEM. Fisher’s exact tests were used to compare the incidence of diabetes and histological grades. Other mean values among the groups were compared by Mann–Whitney U test or, in some instances, by ANOVA.

Results

Exocrine tissue infiltration and reduced a cell area in CD28−/− NOD mice

First, we evaluated the incidence of diabetes in CD28−/− NOD mice and confirmed that the incidence in our colony was comparable with that in the previous report (19). Approximately 75–80% of both male and female CD28−/− NOD mice became overtly diabetic between 8 and 12 wk of age, which was a slightly higher incidence as compared with the prior report (19). The pancreatic tissue was next examined histologically, especially focusing on exocrine tissue infiltration, which was scored as shown in Fig. 1, and a cell area, which was quantified as described in the Materials and Methods, because pancreatic biopsies and autopsies in FTID patients showed mononuclear cell infiltration into exocrine tissue and a reduction of a cell area (2, 21, 22). CD28−/− NOD mice (10–13-wk-old female, n = 3), at 1 wk after the onset of diabetes, showed more severe exocrine tissue mononuclear cell infiltration of grade 3 or higher in 4.4% (22 out of 498 total ducts counted) of ducts, as compared with wild-type female NOD mice examined 1 wk after becoming diabetic; exocrine tissue mononuclear cell infiltration score in this cohort of diabetic NOD mice between 17 and 22 wk of age (n = 3) was grade 3 or higher in 0.2% (1 out of 430 total ducts counted) of ducts (p < 0.0001). Insulitis score in islets of grade 3 or higher was not statistically significantly different between the groups (CD28−/− NOD 63.6%, wild-type NOD 61.5%). A reduction in...
the α cell area, the glucagon-positive area, was observed in diabetic CD28−/− NOD mice as compared with diabetic wild-type NOD mice (Fig. 2, α cell area out of islet area; CD28−/− NOD [total 28 islets observed] 9.2 ± 2.1%, wild-type NOD [total 10 islets observed] 30.2 ± 11.0%; p < 0.01). Thus, the pancreas of CD28−/− NOD mice showed histological characteristics comparable to those in humans with FT1D.

Rapid induction of diabetes in 8-wk-old CD28−/− NOD mice treated with poly(I:C)

Given the rapid course of FT1D in humans and the link to viral infections, we examined whether diabetes and the histological characteristics of the pancreas in FT1D in CD28−/− NOD mice could be accelerated to more closely mimic human FT1D. In a pilot study, we could not induce diabetes within a week when we administered poly(I:C) to 4-wk-old CD28−/− NOD mice (n = 5), a stage showing no insulitis, whereas we could efficiently induce diabetes when we administered poly(I:C) at 8 wk of age (n = 3), a stage of intraislet insulitis. Therefore, 8-wk-old CD28−/− NOD mice were injected with poly(I:C) and followed for disease incidence. Eighty-three percent (15 out of 18) of the mice developed overt diabetes within 7 d, with some as early as 1 d after administration (Table I). In contrast, only 27% (4 out of 15) of control, PBS-injected 8-wk-old CD28−/− NOD mice developed overt diabetes within the same period of time (p = 0.0016). None (0 out of 10) of the poly(I:C)-injected, 8-wk-old, wild-type NOD mice developed diabetes within 7 d postinjection as compared with PBS-injected, age-matched, wild-type NOD mice (0%, 0 out of 9), although this was not a statistically significant difference. Thus, poly(I:C) administration clearly accelerated overt diabetes in 8-wk-old CD28−/− NOD mice. Importantly, poly(I:C)-injected CD28−/− NOD mice presented with marked mononuclear cell infiltration in the exocrine tissue as well as a reduction of α cell area.

Increased NK and/or cytotoxic T cell function in poly(I:C)-injected CD28−/− NOD mice

In human FT1D patients, the majority of the pancreatic infiltrate consists of macrophages and T cells (1, 21). To determine the similarities between the mouse model and human disease, we histologically examined the pancreatic tissue of poly(I:C)-injected CD28−/− NOD mice at 1 wk postinjection. There were CD11b cells, presumably macrophages, and T cells in the lesion as predicted from the human data. However, NK cells were also observed in both the exocrine area and islets (Fig. 3). CD11c-positive cells, dendritic cells (DCs), were observed in the islets, but the numbers seemed fewer overall (Supplemental Fig. 2). There was no significant difference in the CD4-, CD8-, CD19-, and DX5-positive cells, dendritic cells (DCs), were observed in the islets, but the numbers seemed fewer overall (Supplemental Fig. 2). There was no significant difference in the CD4-, CD8-, CD19-, CD11b-, and CD11c-positive cell populations between poly(I:C)-injected CD28−/− NOD mice and PBS-injected CD28−/− NOD mice at 1 wk postinjection (CD4: 17.3 ± 4.0%, 26.8 ± 3.8%; CD8: 7.4 ± 1.5%, 11.7 ± 1.8%; CD19: 17.3 ± 4.1%, 13.7 ± 3.9%; CD11b: 6.0 ± 1.6%, 2.4 ± 0.5%; and CD11c: 1.0 ± 0.4%, 0.5 ± 0.1%, respectively) when the pancreas was disrupted and examined by flow cytometry. In contrast, the percentage of DX5-positive NK cells was significantly higher in the poly(I:C)-treated group (3.0 ± 0.5%) as compared with the PBS-treated group (1.5 ± 0.5%, p < 0.05; Figs. 4A, 5), suggesting that NK cells seemed to be involved in the pathogenesis. There was no significant difference between the two groups regarding the populations of these mononuclear cells in the spleen (CD4: 34.4 ± 2.5%, 39.7 ± 2.6%; CD8: 14.6 ± 1.6%, 14.2 ± 1.4%; CD19: 23.6 ± 5.5%, 25.2 ± 7.1%; CD11b: 7.9 ± 1.5%, 5.3 ± 0.5%; and CD11c: 3.3 ± 0.7%, 3.3 ± 0.0%, respectively; Fig. 4B).

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** α cell area was reduced in CD28−/− NOD mice. A. The pancreas harvested from CD28−/− NOD mice or wild-type (WT) NOD mice at 1 wk after the onset of diabetes was fixed in 3.7% paraformaldehyde and embedded in paraffin. For glucagon staining, tissue was deparaffinized and stained with anti-glucagon Abs. B. A reduction of the α cell area, the glucagon-positive area, was observed in diabetic CD28−/− NOD mice (n = 28) as compared with diabetic WT NOD mice (n = 10). x-axis indicates the proportion of α cell area in the islet area (%). **p < 0.01 versus WT.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Histological appearance of pancreas from poly(I:C)- (A) or PBS-injected (B) CD28−/− NOD mice at 1 wk after injection. Original magnification ×100.

| Table I. Diabetes incidence of poly(I:C)-injected CD28−/− NOD mice |
|-----------------|-----------------|-----------------|
|                  | PBS             | Poly(I:C) KO     | Poly(I:C) Wild   |
| Diabetes incidence | 4/15 (27%)      | 15/18 (83%)*    | 0/10 (0%)       |

*p = 0.0016 versus PBS group, p < 0.0001 versus poly(I:C) wild group.

PBS, PBS-injected 8 wk-old CD28−/− NOD mice; poly(I:C) KO, poly(I:C)-injected 8-wk-old CD28−/− NOD mice; poly(I:C) Wild, poly(I:C)-injected 8-wk-old WT NOD mice.
There was lower Foxp3 mRNA expression in the pancreas from poly(I:C)-injected CD28−/− NOD mice as compared with that in PBS-injected CD28−/− NOD mice, whereas higher IL-10 expression was observed in the pancreas from poly(I:C)-injected CD28−/− NOD mice as compared with that from PBS-injected CD28−/− NOD mice (p < 0.05, respectively; Fig. 6A). These differences were not observed in the spleen (Fig. 6B). There was lower IL-2 and IFN-γ mRNA expression in the pancreas from poly(I:C)-injected CD28−/− NOD mice as compared with those in PBS-injected CD28−/− NOD mice, although the differences did not reach statistical significance. There was no significant difference in TGF-β and IL-4 expression between the two groups. The level of IL-18, which is considered to be produced by activated macrophages, was significantly elevated in both the pancreas (p < 0.05; Fig. 6A) and spleen (p < 0.01; Fig. 6B) in the poly(I:C) group as compared with the PBS group. Moreover, granzyme B level, which is expressed in NK and/or cytotoxic T cells, was significantly higher in both the pancreas (p < 0.05; Fig. 6A) and spleen (p < 0.01; Fig. 6B) in the poly(I:C) group as compared with the PBS group (23). IL-12p40 level showed no significant difference between the poly(I:C) and PBS groups. Together, these results suggest that poly(I:C) may stimulate CD11b cells, which stimulate NK cells (24) and/or cytotoxic T cells. The activated NK cells and/or cytotoxic T cells might not be regulated by Tregs in the local lesion, because there are no Tregs around to control this effector cell tissue destruction, as Tregs have been shown to block NK function (25) and/or cytotoxic T cells, leading to a vicious cycle.

**Discussion**

Although human FT1D is an aggressive disease, its etiology remains unknown because of a lack of a suitable animal model. In this study, we found that poly(I:C)-injected CD28−/− NOD mice developed an FT1D-like phenotype. The characteristics of FT1D in humans are as follows: first, ~70% of cases have a history of flu-like symptoms before onset of the disease (9); second, the development of diabetes is very rapid and not only β cells but also α cells are destroyed (12); and third, lymphocytic infiltration is observed in both islets and exocrine tissue (21).

First, we used poly(I:C) dsRNA to mimic an acute RNA virus infection, similar to enterovirus infection that has been previously linked to FT1D. Injection of poly(I:C) accelerated diabetes onset in 8-wk-old Treg-deficient CD28−/− NOD mice. It has been reported that poly(I:C) injection to wild-type NOD mice results in an increase of both pathogenic cells and Tregs (18). Therefore, we speculate that administration of poly(I:C) to CD28−/− NOD mice resulted in an increase of pathogenic cells while having a minimal effect on Tregs due to the deficiency of costimulation. Previous results have shown that Tregs are especially sensitive to CD28 costimulation blockade, leading to reduced Treg development and survival in the periphery (26, 27). In this regard, it is important to point out that injection of poly(I:C) and subsequent IL-2 production did not result in major recovery of Tregs. In fact, we observed a relative decrease in Foxp3 mRNA expression after poly(I:C) administration, consistent with the hypothesis that Treg deficiency cannot be overcome by cytokines and may in fact be further disabled during a virus-like inflammatory response. Given the similarities of these results and the human disease, it can be speculated that humans prone to FT1D may have a defect in Tregs such that upon dsRNA exposure during infection, the relative effect of a TLR agonist results in preferential effector T cell activation. It is interesting to note that there was indeed higher expression of IL-10 in the pancreas from poly(I:C)-injected CD28−/− NOD mice. IL-10 is classically described as a suppressive cytokine and thus would not be expected to increase in a pathogenic setting. However, several studies have shown that IL-10 can promote T cell proliferation in the local lesion (28, 29). A further point regarding poly(I:C) treatment is that injection of poly(I:C) to 4-wk-old CD28−/− NOD mice did not result in induction of diabetes, as described. This result suggests that pre-existence of intraislets may be necessary to accelerate diabetes in CD28−/− NOD mice following poly(I:C) injection. These results fit with

**FIGURE 4.** Flow cytometric analysis of mononuclear cells in pancreas (A) and spleen (B) from poly(I:C)- (n = 10) or PBS-injected (n = 8) CD28−/− NOD mice at 1 wk after injection. y-axis indicates the population of mononuclear cells (%). To perform flow cytometric analysis of the pancreas, 0.2% collagenase IV was injected into the common bile duct, and the distended pancreas was removed and incubated at 37°C for 17 min with shaking. After washing with PBS, nonenzymatic solution was added to the pancreas followed by incubation for a further 5–10 min. Then the pancreas was dissected with a cell strainer and stained with mAbs. The stained cells were analyzed with an Epics Altra (Corixa). *p < 0.05 versus PBS.

**FIGURE 5.** Representative figure of flow cytometric analysis of NK cells (DX5-positive cells) in pancreas from poly(I:C)- or PBS-injected CD28−/− NOD mice at 1 wk after injection.
fact that FT1D in humans is rare in childhood but has a higher incidence in adults.

Second, not only $\beta$ cells but also $\alpha$ cells were destroyed in this model, similar to the observation in human FT1D (2). It has been reported that alpha cell mass does not change in conventional NOD mice even after cyclophosphamide treatment (30). One of the possible explanations for this may be a difference in cell weakness between $\alpha$ and $\beta$ cells; $\beta$ cells may be weaker under stress circumstances such as inflammation (31). Therefore, $\beta$ cells may be destroyed more rapidly than other islet cells such as $\alpha$ cells in usual T1D. In FT1D, however, the speed of destruction and aggressiveness of inflammation may be stronger and include more antigenic specificity than in typical T1D, leading to a reduction in $\alpha$ cell mass as well as $\beta$ mass cell mass.

Third, it has been reported that CD28$^{-/-}$ NOD mice exhibit exocrine tissue inflammation. In fact, under some circumstances, even old NOD mice, protected from $\beta$ cell destruction and diabetes, can exhibit some degree of exocrine tissue infiltration (16). In this setting, amylase was observed to be a major target Ag (16, 32). The difference in severity of exocrine disease in different models may suggest either regulatory or specificity differences. It is interesting to note that AIRE$^{-/-}$ NOD mice, which exhibit altered central tolerance, also exhibit exocrine tissue destruction (33), supporting some Ag spreading in these settings. It is also possible that the deficiency in peripheral tolerance (i.e., reduced Tregs) in the CD28$^{-/-}$ NOD environment leads to enhanced inflammation in exocrine tissue. In this regard, it is important to note that poly(I:C) injection to CD28$^{-/-}$ NOD mice resulted in complete loss of expression of the Treg gene, Foxp3, in the pancreas (2), even though CD28$^{-/-}$ NOD mice exhibit exocrine tissue destruction (33), supporting some Ag spreading in these settings. It is also possible that the deficiency in peripheral tolerance (i.e., reduced Tregs) in the CD28$^{-/-}$ NOD environment leads to enhanced inflammation in exocrine tissue. In this regard, it is important to note that poly(I:C) injection to CD28$^{-/-}$ NOD mice resulted in complete loss of expression of the Treg gene, Foxp3, in the pancreas (2), even though CD28$^{-/-}$ NOD mice already had very few Tregs. This loss of any residual Treg expression may tip the balance to more severe exocrine tissue destruction in this setting. Although we observed a higher amylase level in serum from CD28$^{-/-}$ NOD mice as compared with wild-type NOD, the difference was not statistically significant (data not shown). One of the reasons might be that the wild-type NOD mouse is predisposed to the development of autoimmune pancreatitis, although wild-type NOD mice do not display overt exocrine pancreas autoimmunity; NOD mice treated with certain cytokines can mount an immune response against the exocrine pancreas (16). Thinking about the human situation, however, not all but approximately half of FT1D patients show high amylase levels, so an increase of amylase is not a definitive feature. Moreover, even though amylase is a target autoantigen, it is not necessarily increased in serum; although insulin is a target Ag in T1D as well, an increased level of insulin in serum is not observed. Regarding poly(I:C)-treated CD28$^{-/-}$ NOD mice, we could not obtain sufficient serum samples to measure amylase, probably because of the dehydration caused by severe hyperglycemia; therefore, we measured another pancreatic exocrine enzyme, lipase. Again, we did not observe a significant difference between poly(I:C)-treated CD28$^{-/-}$ NOD versus controls (data not shown), although the poly(I:C) group showed a slightly higher level. We speculate that we injected poly(I:C) until diabetes development or just 7 d, but in the human situation, virus can expand for a longer period of time; thus, the stimulation in our system may not have been enough to cause massive exocrine damage, and this is a limitation of our study.

Regarding the issue of the mechanism of spontaneous diabetes in CD28$^{-/-}$ NOD mice versus that of accelerated diabetes in poly(I:C)-treated CD28$^{-/-}$ NOD mice, we think they are different. When we compared CD28$^{-/-}$ NOD mice with age-matched wild-type NOD, higher IFN-$\gamma$ and lower IL-10 and TGF-$\beta$ expression were observed (Supplemental Fig. 1); thus, spontaneous diabetes in CD28$^{-/-}$ NOD is considered to develop in a condition of a Th1-dominant state and reduction of Treg function. In contrast, poly(I:C) seems to activate macrophages based on the significantly higher expression of IL-18 in both the pancreas and spleen from poly(I:C)-treated CD28$^{-/-}$ NOD mice as compared with PBS-treated CD28$^{-/-}$ NOD (Fig. 6), and the IL-18 production will expand NK and cytotoxic T cells based on the high granzyme B level in poly(I:C)-treated mice as compared with PBS-treated mice (Fig. 6). Therefore, we think that the mechanisms of pathogenesis of diabetes development, an antiendocrine response, in spontaneous CD28$^{-/-}$ NOD and poly(I:C)-induced CD28$^{-/-}$ NOD are different. Regarding the antiexocrine response, however, we did not observe a significant difference in lipase level between poly(I:C)-treated CD28$^{-/-}$ NOD versus PBS-treated CD28$^{-/-}$ NOD (data not shown) and therefore speculate that the essential difference between the two is in the antiendocrine response. Thinking about the human situation, 70% of FT1D patients have a history of
a viral infection episode right before the onset, but the rest of the patients have no history of viral infection; thus, the pathophysiology of FT1D seems not to be homogenous and to be caused by different mechanisms. Therefore, we propose that both spontaneous and accelerated models can be used as models of human FT1D.

When we consider the involvement of IL-18, one might argue about the involvement of IL-12 as well. However, there was no significant difference in IL-12p40 level between the poly(I:C) and PBS groups; IL-12p40 seems not to be involved in the specific pathogenesis of this poly(I:C)-induced CD28−/− NOD model. This result fits with the observation in human FT1D (34) that IL-18 is promptly secreted from virus-activated [in our case, poly(I:C)-activated] macrophages and acts independently of IL-12 (35).

From this point of view also, we think that this system is different from previous observation in standard NOD mice that artificial induction of IL-18 induced IL-12p40 in the periphery, leading to generation of a Th1-type response and acceleration of diabetes onset (36).

In human FT1D, macrophages and T cells (1) expressing CD8 (21) were observed in both islet lesions and exocrine area. In this model, we found not only macrophages and CD8 cells but also CD4 in both islet lesions and exocrine area. The lack of detection of CD4 T cells in human FT1D studies to date may have been due to the timing of histological examination. In fact, in recent studies of a human FT1D case by our group, we observed CD4-positive T cells in the islets (Y. Okawa, A. Hashiguchi, and A. Shimada, manuscript in preparation). Thus, not only CD8 cells but also CD4 cells may be important during the disease process in human FT1D.

Regarding DCs, we observed CD11c-positive cells in immunohistochemical (Supplemental Fig. 2) and flow cytometric studies, but there was no evident difference between the poly(I:C) and PBS groups, suggesting that DCs seem not to be mainly involved in the pathophysiology of this system.

Perhaps the most interesting observation in the mouse studies was the increase of NK cells in the pancreas (Figs. 3, 4A, 5). Although this cell subset has not been observed to date in the human disease setting, it is intriguing to speculate that there is a direct connection between the potential role of viral infection and innate immune activation in this disease. In an animal model, it has been suggested that NK cells exist in the local lesion for only a short period of time (37). Therefore, we can still discuss the involvement of NK cells in the human situation as well. Recent studies have implicated NK cells in NOD diabetes (38, 39), supporting the link. Although an impaired NK cell response has been reported that NOD-scid mice expressing IFN-γ in NOD diabetes under normal circumstances. In fact, some studies have suggested that IFN-γ can protect NOD mice from developing diabetes (41). Although we think that NK cells have important roles in the accelerated diabetes phenomenon, NK cells may not be enough to cause this phenotype because it has been reported that NOD-scid mice expressing IFN-γ/β in islets did not become diabetic (42). We think that poly(I:C) administration activates macrophages, resulting in activation of NK and/or cytotoxic T cells in a Treg-deficient condition and causing aggressive diabetes.

In conclusion, we propose that the poly(I:C)-injected CD28−/− NOD mouse is a suitable model of human FT1D, and this model clearly indicates that an FT1D-like phenotype can be induced by autoimmunity. In this regard, we speculate that human FT1D may develop in high-risk subjects for T1D with limited numbers of Tregs or Treg dysfunction combined with an encounter with a viral infection such as enterovirus. Further detailed evaluation is necessary to examine this concept in a clinical setting.

Disclosures
A.T. was supported by Baxter. The other authors have no financial conflicts of interest.

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Suppl Figure 1
mRNA expression levels in pancreas from poly (I:C) injected CD28-/- NOD mice at 1 week post treatment, PBS injected CD28-/- NOD mice at 1 week post treatment, or age matched wild type NOD mice.
*P<0.05 vs. PBS group. Y axis indicates arbitrary units.
Suppl Figure 2

CD11c positive cells observed in both poly (I:C) injected CD28−/− NOD (left) and PBS injected CD28−/− NOD (right) mice.