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IN VIVO DIABETOGENIC ACTION OF CD4+ T LYMPHOCYTES REQUIRES FAS EXPRESSION AND IS INDEPENDENT OF IL-1 AND IL-18

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Abstract

CD4+ T lymphocytes are required to induce spontaneous autoimmune diabetes in the NOD (Non Obese Diabetic) mouse. Since pancreatic β cells upregulate Fas expression upon exposure to pro-inflammatory cytokines, we studied whether the diabetogenic action of CD4+ T lymphocytes depends on Fas expression on target cells. We assayed the diabetogenic capacity of NOD spleen CD4+ T lymphocytes when adoptively transferred into a NOD mouse model combining: a) Fas-deficiency, b) FasL-deficiency, and c) the SCID mutation. We found that CD4+ T lymphocytes require Fas expression in the recipients' target cells to induce diabetes.

IL-1β has been described as a key cytokine involved in Fas up-regulation on mouse β cells. We addressed whether CD4+ T cells require IL-1β to induce diabetes. We also studied spontaneous diabetes onset in NOD/ICE (Interleukin-1 Converting Enzyme) deficient mice, in NOD/IL-1β deficient mice, and CD4+ T cell-adoptively transferred diabetes into NOD/SCID IL-1β-deficient mice. Neither IL-1β nor IL-18 are required for either spontaneous or CD4+ T-cell adoptively transferred diabetes.

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We conclude that $CD4^+$ T cell-mediated β cell damage in autoimmune diabetes depends on Fas expression, but not on IL-1β, unveiling the existing redundancy regarding the cytokines involved in Fas upregulation on NOD β cells *in vivo*.

Keywords

Autoimmune diabetes; β cell apoptosis; Fas; $CD4^+$ T cells

Introduction

Autoimmune diabetes (Type 1 Diabetes mellitus or T1D) is a T-cell mediated condition characterized by the selective destruction of insulin-producing β cells [1]. Three major effector pathways for β cell destruction have been proposed for T1D: the Fas/FasL [2] and perforin [3] pro-apoptotic pathways, and cytokine-induced β cell death via iNOS [4]. The most extensively pursued mechanism has been the Fas(CD95)/FasL(CD95L) pathway, which seems to be one of the main pathways involved in cytokine-induced β cell death [5,6]. Fas death receptor belongs to the TNFR family, and trimerizes once engaged by its trimeric ligand, 3FasL, a member of the TNF family. Fas trimerization triggers the death cascade by inducing extrinsic apoptosis. Fas expression on β cells is upregulated by IL-1β in conjunction with IFN-γ in mice [6-8]. Moreover, chemical depletion of macrophages, the main producers of IL-1β upon activation, abrogates diabetes onset [9] in NOD mice, one of the most studied animal models for T1D [1]. In addition, IL-1β is involved in NO-mediated β cell death by necrosis [10, 11]. However, apoptosis and not necrosis has been reported to be the main mechanism responsible for spontaneous diabetes onset in T1D [10, 12]. IL-1β can induce β cell death through Fas up-regulation and NO generation via NFκB signaling [5, 11]. Moreover, lymphocytes up-regulate Fas and FasL on their cell surface upon activation, becoming an important source of FasL, and therefore, cell death inducers for nearby cell types expressing Fas, including β cells [13].

NOD mice deficient in either Fas (NOD/lpr) or FasL (NOD/gld) do not develop spontaneous diabetes and NOD/lpr mice are resistant to adoptively transferred diabetes [14, 15]. Interestingly, β cell specific Fas deficiency impairs spontaneous diabetes onset [16, 17]. Moreover, transgenic expression of FasL on β cells exacerbates the diabetic phenotype in NOD mice [14, 18] suggesting that there may be a gradual up-regulation of Fas on β cells during the course of islet infiltration prior to diabetes onset, and the early presence of FasL on neighboring β cells might accelerate fratricidal β cell death. $CD4^+$ T cells are required to promote insulitis and diabetes in NOD mice [19]. All of the above mentioned suggest a scenario in which the reciprocal activation of macrophages and $CD4^+$ T cells, upon receipt of an inflammatory signal in the local pancreatic environment, triggers IL-1β and IFN-γ production by macrophages and Th1 $CD4^+$ T cells respectively. Both cytokines, in turn, up-regulate Fas on β cells causing their death as soon as the Fas receptor is engaged by its ligand, FasL. Nonetheless, several reports have questioned the relevance of Fas-induced β cell death in T1D [20-23]. Several of these studies rely on a single $CD4^+$T cell specificity, which could be masking the overall *in vivo* scenario, composed of several $CD4^+$T cell clones and/or effector mechanisms.

The overall aim of our study was to understand the role of Fas and $CD4^+$ T lymphocytes in the induction of β cell death, and hence, autoimmune diabetes. In the current report, we show that the diabetogenic activity of $CD4^+$ T lymphocytes is Fas-dependent, and, moreover, despite the fact that IL-1β can mediate upregulation of Fas on islets, IL-1β is not required to promote diabetes in NOD mice.

Results

Purified primed spleen CD4$^+$ T cells require Fas expression to trigger β cell death

Fas expression on β cells has been reported to promote β cell apoptosis for the development of diabetes [14, 16, and 17]. We aimed to establish the role of Fas and FasL on CD4$^+$ T cell-mediated β cell apoptosis in autoimmune diabetes. For that purpose it was necessary to avoid the pleiotropic effects of Fas deficiency in NOD lpr/lpr mice [24], which affects the T and B cell repertoire [25]. To this end we purified splenic CD4$^+$ T cells from 8 to 20 week old pre-diabetic (not exhibiting glycosuria) female NOD mice (at this age islet-specific CD4$^+$ T cells should be primed since insulitis is already observed in 8 week old females [1 and 26]), and adoptively transferred 15 million of these CD4$^+$ T cells into NOD/SCID female recipients (deficient in both, T and B cells) combining Fas-deficiency and FasL-deficiency. In this series of experiments, the 2 different types of NOD/SCID recipient females were deficient in FasL (gld/gld) [27], and either Fas-deficient (lpr/lpr) or Fas sufficient (lpr/+) (Table 1). Fas deficiency in the NOD/SCID recipients addressed the requirement of Fas expression by CD4$^+$ T cells alone to cause diabetes, Fas deficiency on APCs (Antigen Presenting Cells) should not interfere with antigen presentation.. FasL deficiency (gld) in the NOD/SCID recipients ensures that the only source of FasL are the transferred activated CD4$^+$ T cells.

Mice sufficient for Fas were significantly more susceptible to diabetes development upon CD4$^+$ T cell transfer than Fas-deficient recipients (47% and 6% respectively, $p<10^{-3}$ log-rank test) (Figure 1). Our experiments demonstrate that primed CD4$^+$ T cells require the Fas-death receptor pathway on recipients, presumably in the pancreatic β cell compartment, to mediate their diabetogenic action (Figure 1).

We tested if transgenically expressed FasL on beta cells accelerated the Fas mediated beta cell death by CD4+ T cells. Two types of splenic CD4$^+$ T cells were used for these experiments, either from diabetic (detectable glycosuria and glycemia above 200mg/dL) or non-diabetic (not exhibiting glycosuria) NOD female donors: and 12.5 million of CD4$^+$ T cells were transferred per recipient. The recipient mice were FasL-sufficient NOD/SCID females and either transgene positive or negative for the RIP-FasL transgene (Figure 2) (Table 1). Interestingly, mice expressing the FasL transgene on β cells that received CD4$^+$ T cells from a diabetic donor exhibit a certain trend, although not significant ($p = 0.059$ log-rank test), to develop delayed diabetes compared to transgene negative littermates (at day 107 post-transfer 57% (4/7) of transgene positive recipients developed diabetes compared to 100% (5/5) of transgene negative littermates) (Figure 2A).

In contrast, when spleen CD4$^+$ T cells from a non-diabetic donor female were transferred, no difference in either cumulative incidence or kinetics of disease was found between transgene negative or positive recipients ($p>0.9$, log-rank test) (Figure 2B) (Table 1). The difference between these two results (Figure 2A and Figure 2B) may be due to the fact that fully activated islet-specific CD4$^+$ T cells from a diabetic donor are more susceptible to Fas-induced apoptosis upon engagement with FasL [28]. This tendency to develop a higher incidence of diabetes that was detected in recipient mice which do not overexpress FasL on β cells could suggest a state of immune privilege towards immune attack by activated islet-antigen-specific CD4+ T cells as is suggested in Figure 2B.

NOD mice do not require IL-1β either to develop spontaneous or adoptively transferred diabetes mellitus

IL-1β is one of the key pro-inflammatory cytokines believed to upregulate Fas in the course of T1D development. Caspase 1, also known as ICE (Interleukin-1 Converting Enzyme), is responsible for processing the immature pro-cytokines IL-1 and IL-18 into their

corresponding mature cytokine forms [29]. NOD mice deficient for Caspase 1 develop autoimmune diabetes normally ($p>0.9$, log-rank test) (Figure 3), which has also been described in another report [30].

However, since Caspase 1 deficiency affects both IL-1 and IL-18 processing, we studied IL-1β KO mice. Spontaneous diabetes in NOD/IL-1β KO mice is indistinguishable to that of WT and heterozygous littermates ($p>0.6$, log-rank test) (Figure 4). Additionally, IL-1β deficient NOD/SCID recipient mice are equally susceptible to autoimmune diabetes than IL-1β sufficient NOD/SCID recipient mice when adoptively transferred with either total NOD spleen cells ($p>0.4$, log-rank test) (Figure 5) or purified $CD4^+$ T cells ($p>0.5$, log-rank test) (Figure 6). We conclude from these results that, contrary to our expectations, IL-1β is neither essential for spontaneous nor transferred diabetes.

Discussion

Fas expression on target cells is required for $CD4^+$ T cell induced diabetes

Here we show that Fas expression is required for the adoptive transfer of diabetes by $CD4^+$ T cells. $CD4^+$ T cells are essential effectors in the induction of islet infiltration and β cell death [19], but so far no clear link has been delineated between $CD4^+$ T cells and the molecular pathway triggered to cause the destruction of β cells. We have observed that primed $CD4^+$ T cells require the presence of Fas on NOD/SCID recipients to cause T1D. The expression of Fas within islets has mostly been associated with intra-islet macrophages, dendritic cells and to a lesser extent to infiltrating lymphocytes [31]. Fas expression is, however, up-regulated on islet cells upon exposure to cytokines [6-8]. Fas has been detected by cytometric analysis of β cells in in vivo models of accelerated, but not spontaneous, diabetes [32]. Two recent reports have revealed that Fas is actually necessary to induce β cell apoptosis in NOD mice [16, 17]. Although in pancreatic islets from Fas-deficient NOD/SCID lpr/lpr mice there are other cell types in addition to pancreatic β cells, which are also deprived of Fas expression, mostly dendritic cells and macrophages (31). These mice, when adoptively transferred with spleen cells from either prediabetic or diabetic NOD donor do not develop diabetes (2). In this experimental approach, donor splenocytes included Fas-sufficient macrophages, dendritic cells and other hematopoietic subpopulations that could replace the Fas-deficient recipient cell types. Nonetheless total spleen cells from a Fas-sufficient donor is not able to transfer diabetes to Fas-deficient NOD/SCID recipients, which clearly suggests that Fas deficiency on β cells is responsible for the absence of diabetes onset. Moreover, in our experimental setting, the adoptively transferred $CD4^+$ T cells are already primed, and therefore only require proper antigen presentation by local antigen presenting cells (dendritic cells and macrophages) to activate their effector functions.

Our results are consistent with a scenario in which Fas-deficiency on target pancreatic β cells, and not on other cell types (macrophages and dendritic cells), is responsible for the impaired diabetes induction. Our results are supported by those from Nakayama et al. [33], who showed that blockade of Fas/FasL interaction early in life prevents insulitis and diabetes.

Previous reports [20-23] questioning the role of Fas in $CD4^+$ T cell-induced autoimmune diabetes studies rely on a single $CD4^+$T cell specificity, using a TCR transgenic model. We propose that these monoclonal cells probably overrepresent one effector mechanism rather than the panoply of mechanisms involved in the overall in vivo scenario when a polyclonal population of effector cells, composed of several $CD4^+$T cell clones, mediate diabetes. Therefore, our study suggests that the diabetogenic action of NOD $CD4^+$ T lymphocytes is very probably dependent on Fas expression on target cells.

Effect of FasL expression on β cells

Our results indicate that diabetogenic $CD4^+$ T cells may have an impaired ability to transfer diabetes into NOD/SCID recipients which over-express FasL on β cells compared to transgene-negative recipients. This could indicate immune privilege acquired by β when they encounter activated, diabetogenic $CD4^+$ T cells. These data seem to be in apparent contradiction to that reported previously [14], in which overexpression of FasL in wild type NOD mice accelerates diabetes onset. This paradox of FasL expression on β cells could imply that expression of FasL on β cells favors an autoaggressive repertoire while the immune repertoire is maturing. In NOD/SCID mice, however, T and B cell subsets are missing, which might otherwise contribute to that final configuration of the immune repertoire in the islet. Last but not least, β cell - specific transferred T cells are mostly activated, and hence, expressing Fas on their surface. Nevertheless further work should be done to resolve this paradox.

Redundancy of IL-1β in autoimmune diabetes

Here we report that IL-1β does not play an essential role in spontaneous autoimmune diabetes although progression to diabetes is slower in NOD/IL-1R KO mice [34], the overall impact on the disease is not remarkable. Thus, caution should be exercised when translating *in vitro* studies in which islets or β cell lines are exposed to IL-1β since the results may not necessarily correspond to what is actually taking place *in vivo* during disease progression.

Although IL-1β seems to play a crucial role in β cell destruction in islet transplantation models [35-38], it does not do so in the NOD model of spontaneous diabetes. This may be explained by the fact that during transplantation, the immune system is activated because of a strong inflammatory environment developing in and around the entire graft. However in spontaneous T1D the immune response is cell-targeted and the pro-inflammatory environment is mostly limited to the islet. Therefore IL-1β may help to exacerbate the spontaneous β cell attack, but in its absence, other mechanisms may replace it (e.g. IFN-γ and /or TNF-α). Therefore, diabetogenic $CD4^+$ T cells do not require Il-1β to mediate Fas-dependent β cell death.

In conclusion, given the key role of Fas in $CD4^+$ T cell induced β cell death, it is now necessary to determine how the timing of its expression on β cells is critical to trigger T1D. Such studies will provide new insight into preventive and or therapeutic approaches for T1D.

Materials and methods

Mice

Mice were kept in SPF conditions (Specific Pathogen Free), in a dark-light 12h dark/light cycle and fed *ad libitum* using standard rodent diet chow (Panlab, Cornellà, Spain). All animal experimentation procedures performed in this work have been overseen and approved by the Institutional Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA), and the Institutional Animal Care and Use Committee (IACUC) at Yale University, in accordance with the European and U.S. Regulations on Animal Experimentation respectively. Mice carrying the SCID mutation were kept on Gobens-trim antibiotic mixture (Sulfametoxazol 1.2 g/l and trimetoprim 0.24g/l) 3 days a week (Normon, Madrid; Spain). Idd susceptibility loci [19] were checked in all backcrossing procedures into the NOD genetic background.

Generation of NOD/lpr and NOD/gld mice

Mice homozygous for either the lpr mutation (Fas deficiency) [24] or the gld mutation (FasL mutation) [27] respectively, were purchased from the Jackson Lab on the C57BL/6 genetic background (The Jackson laboratories, Bar Harbor, ME, USA). After intensive backcrossing onto the NOD genetic background, we reached the 9th generation (N10) for both the *lpr* mutation and the *gld* mutation. The *lpr* and gld mutations respectively were genotyped by PCR according to the protocols provided by The Jackson Laboratories (The Jackson Laboratories, Bar Harbor, ME; USA).

Generation of NOD/Interleukin One Converting Enzyme deficient (NOD/Caspase 1 KO) mice

Caspase 1 KO mice obtained initially on the 129Sv-C56BL/6 mixed background [29] and backcrossed onto the NOD background. We reached the N14 generation (13th backcross), and used it for our studies. Mice were genotyped as described [29].

Generation of NOD/Interleukin 1 beta deficient (IL-1β) mice

Mice deficient in IL-1β have been previously described elsewhere [39]. We have backcrossed mice carrying this mutation originally in the B10.RIII (H2<r>(71NS)/Sn) genetic background into the NOD background. We have intercrossed mice in the N9 (8th backcross) generation. Mice were genotyped as described [39].

Genotyping of the SCID mutation in the different NOD strains

NOD/SCID mice [40] were purchased from The Jackson Lab (The Jackson laboratories, Bar Harbor, ME, USA). The *scid* mutation was genotyped by using the PCR protocol recommended by the Jackson laboratory.

NOD/SCID RIPFasL transgenic mice

NOD/RIPFasL *line 24* transgenic mice (NOD mice overexpressing FasL on pancreatic β cells)[14] , were outcrossed onto NOD/SCID mice several times, in order obtain NOD/SCID mice overexpressing FasL on pancreatic β cells (NOD/SCID RIPFasL transgenic mice). The RIP FasL transgene was genotyped as previously published [14].

Assessment of diabetes

Female mice from each strain were monitored weekly for the development of glycosuria with Medi-Test Glucose 3 (Macherey-Nagel, Düren, Germany) starting at 3 weeks of age in case of natural history. In case of adoptive transfer, recipient female mice were monitored twice a week for glycosuria after adoptive transfer was performed.

Diabetes was confirmed by measuring glycemia with the Accu-Check test strips (Accutrend, Roche Diagnostics GmbH, Mannheim, Germany) with values over 200 mg/dl.

Once diabetes was diagnosed, diabetic individuals were sacrificed, and autoimmunity was confirmed by histological examination of pancreata, which were fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin-eosin (Sigma, St Louis, MO, USA) to assess the presence of mononuclear infiltrates in the pancreatic islets (insulitis).

Adoptive transfer experiments

$CD4^+$ T cells from total splenocytes pooled from multiple donors were purified by negative selection (Miltenyi Biotec, Bergisch Gladbach, Germany). The prediabetic or diabetic status of the donors was assessed by measuring urine and blood glucose levels, and glycemia

levels above 200mg/dL were considered to be indicative of diabetes onset in the donor. Depending on the experiment from 12.5 to 15 million of cells were transferred intravenously in physiological saline. Purity of isolated CD4+ T cells (≥95%) was checked by Flow Cytometry (BD FACSCalibur, Becton Dickinson, New Jersey, USA). All donors and recipients were female mice.

Statistical Analysis

Survival curves were analyzed using the log-rank test.

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Abbreviations

IFNγ	gamma Interferon
IL-1β	Interleukin 1 beta
T1D	Type 1 Diabetes Mellitus
TNFR	Tumor Necrosis Factor Receptor
SPF	Specific Pathogen Free

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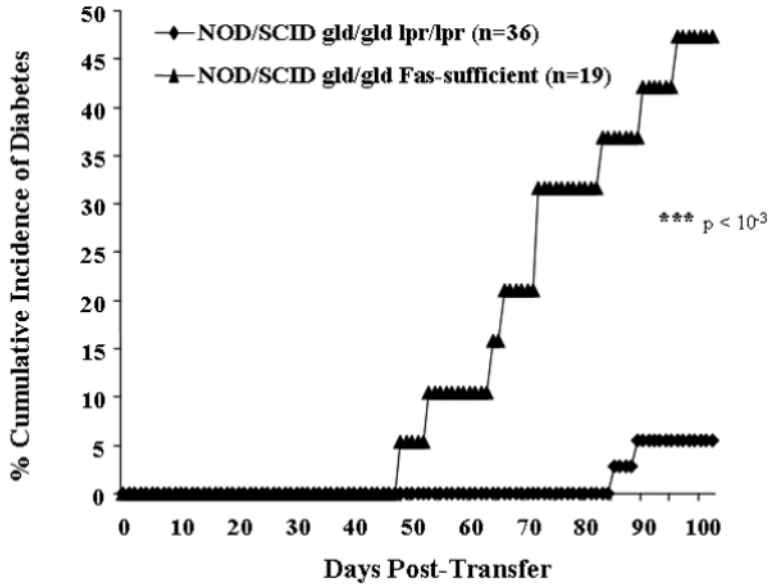

Figure 1. Primed splenic CD4$^+$ T cells require the presence of Fas on recipient cells to induce adoptively transferred diabetes

15 million splenic CD4$^+$ T cells from non-diabetic female NOD donors (ranging from 8 to 15 weeks of age) were adoptively transferred into NOD/SCID/gld/gld (FasL-deficient) female mice, which were either Fas sufficient (lpr/+) (closed triangles) or Fas deficient (lpr) (closed diamonds). Recipient mice were then monitored for diabetes onset as readout of β cell destruction. n=16 independent experiments. $p<10^{-3}$ log-rank test.

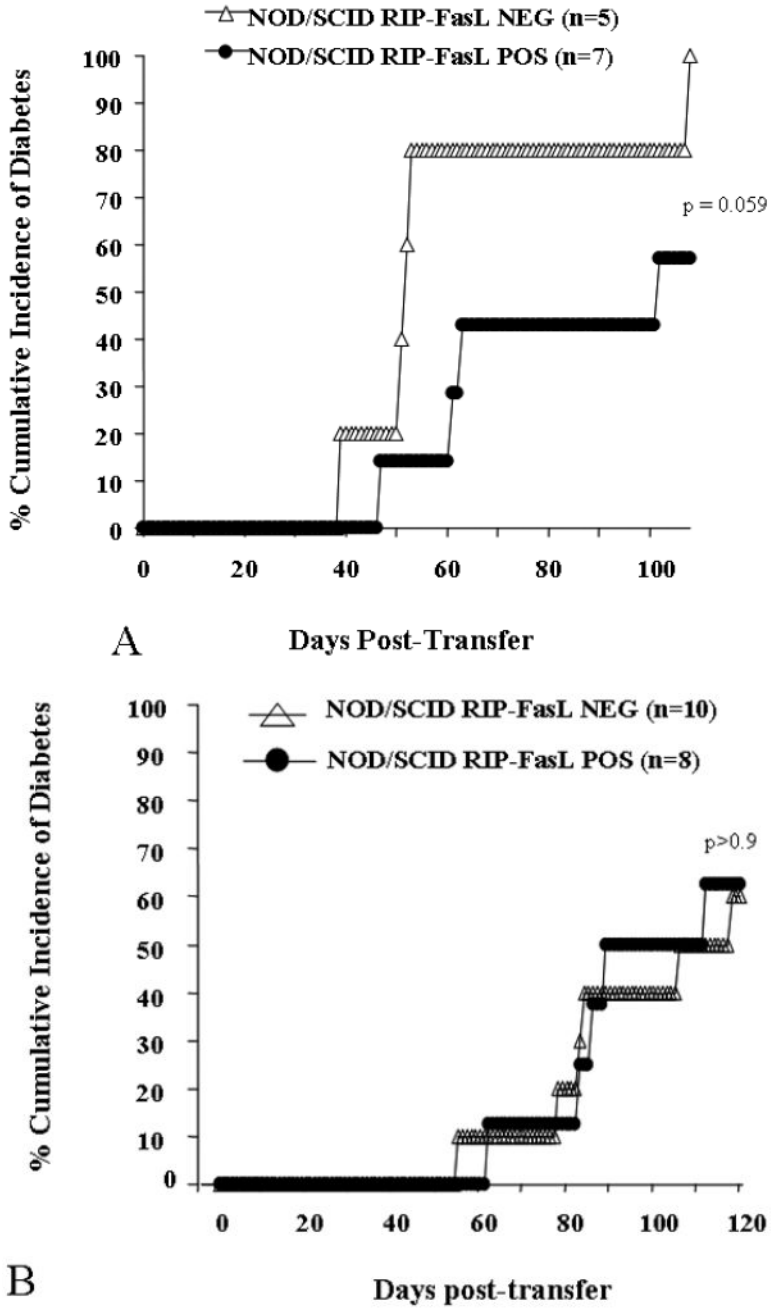

Figure 2. Overexpression of FasL on β cells protects them from splenic CD4$^+$ T cells from diabetic donors, but not splenic CD4$^+$ T cells from non diabetic donors

NOD/SCID RIP-FasL transgene-positive (closed circles) and -negative (open triangles) mice were transferred with 12.5 million splenic CD4$^+$ T cells from diabetic (A) or 15 million splenic CD4$^+$ T cells from non diabetic (B) female NOD mice. Recipient mice were then monitored on a weekly basis for diabetes onset as readout of β cell destruction. (A) n=4 independent experiments, p = 0.059 log-rank test. (B) n=6 independent experiments, p>0.9, log-rank test.

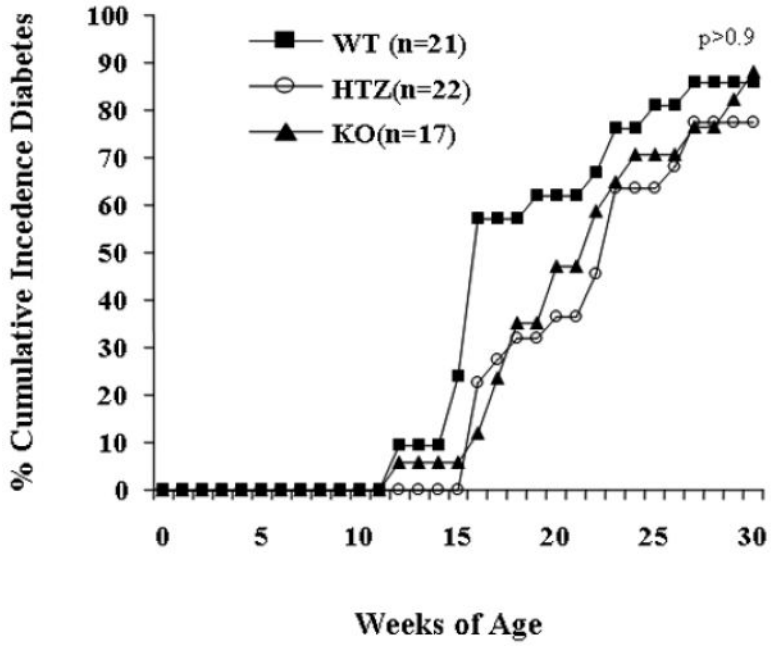

Figure 3. Spontaneous diabetes incidence in NOD/ Caspase 1-deficient mice

Caspase 1 deficient (KO) (closed triangles), heterozygous (HTZ) (open circles) and wild type (WT) (closed squares) NOD female mice were monitored for diabetes onset on a weekly basis as readout of β cell destruction. p>0.9, log-rank test.

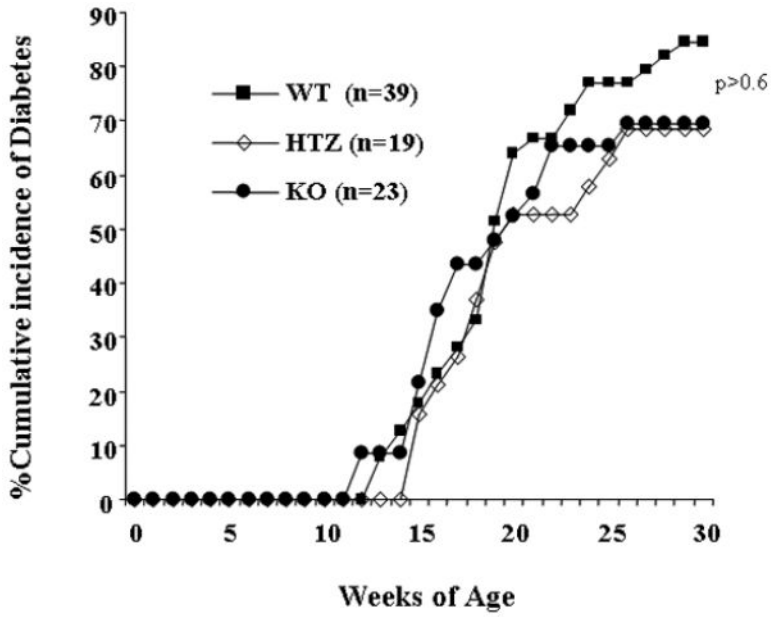

Figure 4. IL-1β-deficiency does not impair spontaneous diabetes development in NOD mice

IL-1β-deficient (KO) (closed circles), heterozygous (HTZ) (open diamonds) and wild type (WT) (closed squares) NOD female mice were monitored for diabetes onset as readout of β cell destruction. p>0.6, log-rank test.

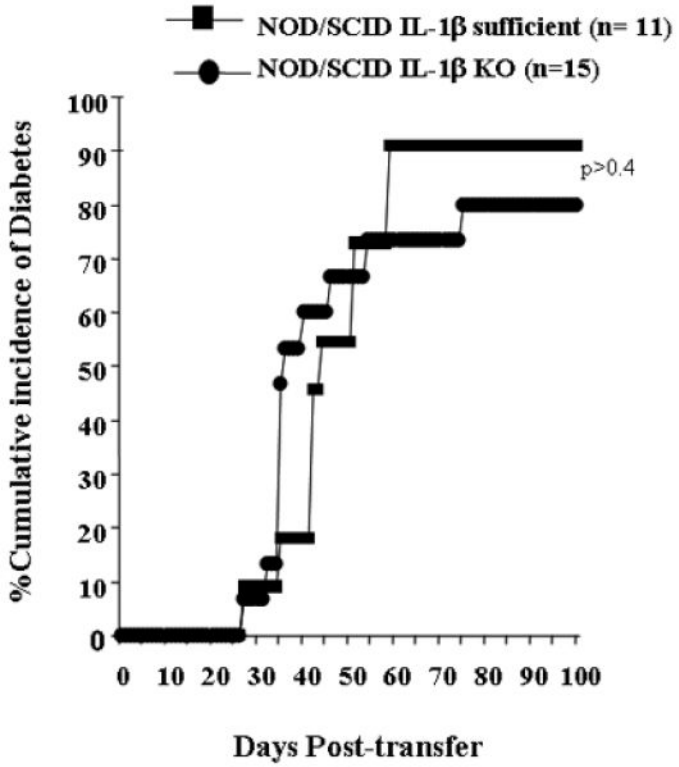

Figure 5. IL-1β-deficient NOD/SCID mice are equally susceptible to adoptively transferred diabetes by total spleen cells as IL-1β-sufficient NOD/SCID mice

NOD/SCID female mice either sufficient (closed square) or deficient (KO) (closed circle) for IL-1β were adoptively transferred with 15 million spleen cells from non diabetic NOD donors and monitored for diabetes onset as readout of β cell destruction (n=5 independent experiments). p>0.4, log-rank test.

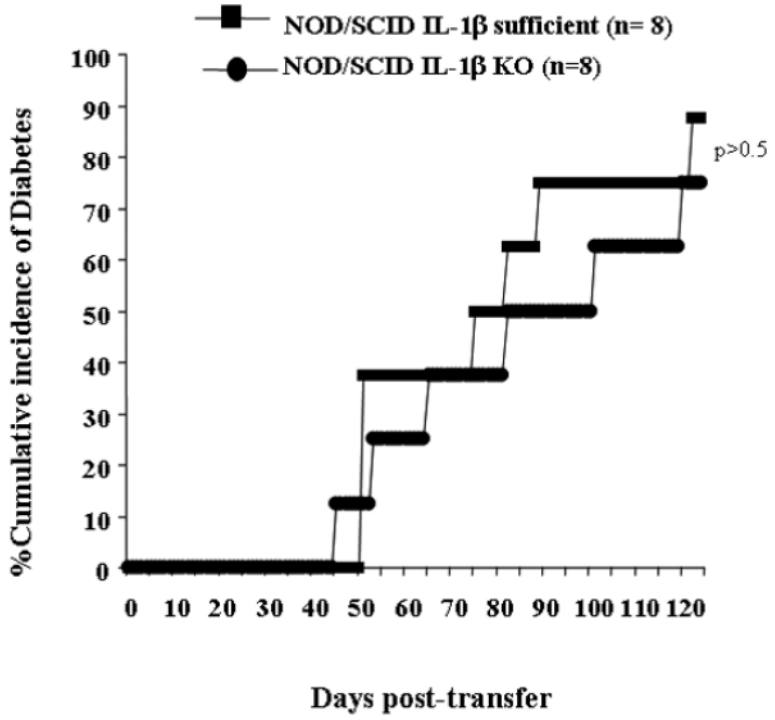

Figure 6. IL-1β-deficient and IL1β-sufficient NOD/SCID mice are equally susceptible to adoptively transferred diabetes by purified spleen CD4$^+$ T cells

NOD/SCID female mice either sufficient (closed squares) or deficient (KO) (closed circles) for IL-1β were adoptively transferred with 15 million CD4$^+$ T cells from non diabetic NOD donors and monitored for diabetes onset as readout of β cell destruction (n= 2 independent experiments). p>0.5, log-rank test.

Table 1

Genetically modified mouse strains used in Figures 1 and 2.

Recipient mouse strain	**Deficiency in**	**Molecules expressed**	**Expression on β cells**	**Donor mouse strain**	**Cells transferred**	**Expected expression of Fas on cells transferred**	**Used in Figure/s**
NOD/SCID/RIP-FasL^{+}	B, T lymphocytes (SCID)	**a) FasL transgene b) Fas (endogenous)**	**a) Yes b) Yes**	NOD female	CD4+ T cells (from either diabetic or not diabetic donor	- Yes (diabetic donor) - No (pre-diabetic donor)	Fig. 2A Fig. 2B
NOD/SCID/RIP-FasL^{-}	B, T lymphocytes (SCID)	**a) FasL transgene b) Fas (endogenous)**	**a) No b) Yes**	NOD female	CD4+ T cells (from either diabetic or not diabetic donor	- Yes (diabetic donor) - No (pre-diabetic donor)	Fig. 2A Fig. 2B
NOD/SCID/ gld/gld/ lpr/lpr	a) B, T lymphocytes (SCID) b) FasL (all cell types affected) (gld) c) Fas (all cell types affected) (lpr)			NOD female	CD4+ T cells from pre-diabetic donor	- No (pre-diabetic donor)	Fig. 1
NOD/SCID/ gld/gld/ lpr/+	a) B, T lymphocytes (SCID) b) FasL (all cell types affected) (gld)	**a) Fas (endogenous)**	**a)Yes**	NOD female	CD4+ T cells from pre-diabetic donor	No (pre-diabetic donor)	Fig. 1