Autoreactive T cells induce beta cell necrosis and not apoptosis or RIPK3-dependent necroptosis in a type 1 diabetes model

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ABSTRACT

Type 1 diabetes (T1D) results from T cell-mediated destruction of pancreatic beta cells. The mechanisms of beta cell killing *in vivo*, however, remain unclear. Here we altered expression levels of critical cell death proteins in mouse islets and tested their ability to survive T cell-mediated attack using an *in vivo* graft model. Loss of the BH3-only proteins BIM, PUMA or BID did not protect beta cells from this death. Overexpression of the anti-apoptotic protein BCL-2 or combined deficiency of the pro-apoptotic multi-BH domain proteins BAX and BAK also failed to prevent beta cell destruction. Furthermore, loss of function of the death receptor FAS or its essential downstream signaling molecule FADD in islets was also not protective. Using electron microscopy we observed that dying beta cells showed features of necrosis. However, islets deficient in RIPK3, a critical initiator of necroptosis, were still normally susceptible to T cell-mediated destruction. Remarkably, simultaneous inhibition of apoptosis and necroptosis by combining loss of RIPK3 and overexpression of BCL-2 in islets did not protect them against immune attack either. Collectively, our data indicate that beta cells die by necrosis in T1D, and that apoptosis and necroptosis are both dispensable for this process.

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by beta cell destruction in pancreatic islets of Langerhans, resulting in insulin deficiency and hyperglycemia. Studies in human patients as well as rodent models have revealed that beta cells in T1D are mainly destroyed by diabetogenic T cells that have been activated by beta cell-specific antigens (1; 2). While much progress has been made in understanding the development and activation of such auto-aggressive T cells, the mechanisms of beta cell killing in T1D remain poorly understood.

The main pathways of cell death include apoptosis, necrosis and necroptosis, which result in distinct morphological features that can be discriminated by transmission electron microscopy. Apoptosis can be activated through two distinct but ultimately converging pathways: the death receptor (extrinsic) pathway and the BCL-2 protein family regulated (intrinsic) pathway. Caspases are the executioners in both pathways that lead to distinct apoptotic morphology, including nuclear fragmentation and chromatin condensation (3). The death receptor pathway is initiated by ligation of death receptors, such as FAS or TNFR1 that have an intra-cellular death domain. Caspase-8 is then activated by the adaptor FAS-associated death domain (FADD) upon recruitment of both of these proteins to the death receptor associated DISC (death inducing signaling complex). This results in the activation of the executioner caspases (particularly caspase-3 and caspase-7), which unleash the demolition of the doomed cell.

The BCL-2-regulated apoptotic pathway is activated by developmental cues or cellular stresses, such as cytokine deprivation or ER stress. It is regulated by the balance between pro- and anti-apoptotic members of the BCL-2 protein family. The pro-apoptotic BH3-only proteins (e.g. BIM, PUMA, BID) inhibit the pro-survival BCL-2-like proteins, and some of them can also directly activate the pro-apoptotic multi-BH-domain proteins BAX and BAK. They cause mitochondrial outer membrane permeabilisation (MOMP), which initiates release of cytochrome c, apoptosome activation and caspase activation. Previous studies have shown that FAS-induced killing of beta cells requires amplification of the caspase cascade by engagement of the BCL-2-regulated apoptotic pathway through BID, which is directly activated by caspase-8 mediated proteolysis (4). Other BH3-only proteins, including BIM and PUMA, have also been shown to play a role in the death of beta cells in culture in response to pro-inflammatory cytokines or high glucose concentrations (5-7).

Necroptosis has recently emerged as an important genetically programmed mechanism of cell death (8). In particular, ligation of death receptors can trigger necroptosis through the receptor interacting serine/threonine protein kinases RIPK1 and RIPK3 to activate the pseudokinase Mixed Lineage Kinase domain-like (MLKL1) (9; 10). Hypoxia, deregulated calcium flux and free radicals, have been shown to induce cell death with features reminiscent of necroptosis, but killing in response to these insults does not require RIPK1, RIPK3 or MLKL (10). A role for necroptosis or other forms of necrotic cell death in the killing of beta cells in T1D has not yet been demonstrated.

The non-obese diabetic (NOD) mouse is a model of T1D with similar pathological features to T1D in humans. While beta cell destruction clearly occurs as a result of autoreactive T cell-mediated attack, it has been challenging to determine the mechanisms that kill beta cell death in T1D. This is due to the protracted time that it takes for the disappearance of beta cells during disease development, the quick clearance of dying cells, as well as lack of islet specific gene knock-out strains on the NOD background. Histological examination of sections from pancreata of diabetic patients and pre-diabetic NOD mice have revealed TUNEL+ beta cells, implicating apoptosis as the mechanism for their killing (11-15). However, TUNEL staining detects DNA breaks that can occur in both apoptosis and necrosis; so it is not specific enough to define the apoptotic death pathway (16). In fact, both apoptosis and necrosis have been reported in islets treated *in vitro* with inflammatory stimuli, such as cytokines and death receptor ligands (e.g. FasL, TNF) (5; 17-19). While *in vitro* studies shed light on mechanisms of beta cell killing in T1D, they may not fully reflect the *in vivo* environment. It is therefore necessary to use more defined approaches *in vivo* to resolve the question of how beta cells are killed in T1D.

Here we used an adoptive transfer model initially developed by Katz and co-workers to examine which death pathways are essential for T cell-mediated beta cell destruction (20). We have tested the role of regulators of both apoptosis and necroptosis proteins in diabetogenic T cell-mediated beta cell destruction. To our surprise, loss of proteins that are critical for BCL-2-regulated and death receptor apoptotic pathways did not protect beta cells against T cell-mediated destruction. Using transmission electron microscopy, we observed widespread necrotic but not apoptotic beta cells in islets under T cell attack. However, loss of RIPK3, a kinase

essential for death receptor-mediated necroptosis, did not prevent T cell-mediated beta cell destruction. Our data suggest that in T1D, beta cells die by necrosis that is either uncontrolled or follows some presently non-defined controlled program.

RESEARCH DESIGN AND METHODS

Mice

Mice were bred and maintained at the St. Vincent's Institute, and all experiments were approved by the Institutional Animal Ethics Committee. *NODBDC2.5*, *NODlpr*, *NODFADDdn*, *NOD.Bid*$^{-/-}$ as well as *RipBcl-2*, *Bim*$^{-/-}$, *Puma*$^{-/-}$ and *Bak*$^{-/-}$*Bax*$^{fx/fx}$ (all on a C57BL/6 background) mice have been described before (21-28). *Mip-luc-VU* mice (FVB/N background) were kindly provided by Dr Alvin Powers (Vanderbilt University, USA) (29). *Caspase8*$^{fx/fx}$ (C57BL/6 background) mice were kindly provided by Dr Stephen Hedrick (UCSD, USA) (30). *Rosa26.Cre-ER* (C57BL/6 background) mice were obtained from the Jackson Laboratory (31). *RIPK3*$^{-/-}$ (C57BL/6 background) mice were obtained from Genentech (32).

Tamoxifen treatment

Tamoxifen (Sigma-Aldrich) was dissolved in corn oil at a concentration of 40 mg/mL and injected intra-peritoneally (i.p.) into mice (200 mg/kg body weight) twice a week for 3 weeks.

Islet isolation and culture

Islets of Langerhans were isolated using collagenase P (Roche, Basel, Switzerland) and histopaque-1077 density gradients (Sigma-Aldrich) as previously described (33). For grafting, islets were handpicked and cultured as previously described (33).

Streptozotocin injection and islet grafting

Streptozotocin (Sigma-Aldrich) was dissolved in ice-cold PBS prior to i.p. injection into 8-week old *NODscid* mice (0.25 mg/g body weight). Blood glucose levels were measured at 72 h after injection. Mice with a blood glucose reading of 15 mM or higher were considered diabetic and used as recipients. Recipient mice were grafted under the kidney capsule with 400 islets isolated from donor mice (33) and their blood glucose was monitored on every second day. Mice were kept for 10 days after normo-glycemia was restored before adoptive transfer of BDC2.5 T cells.

Adoptive T cell transfer

Spleens and pancreatic lymph nodes from *NODBDC2.5* mice were dispersed through a 70-μm strainer and red blood cells (RBCs) were lysed with RBC lysis buffer (155 mM NH_4Cl, 10 mM Tris.HCl, pH 7.5). Dissociated cells were stained with antibodies to CD4, CD25 and the clonotypic BDC2.5 TCR (34). Diabetogenic BDC2.5 T cells were collected by sorting $CD4^+BDC2.5^{high}CD25^-$ cells on a FACS Aria (BD Bioscience) and injected intra-venously (i.v.) into recipient mice ($1x10^6$ T cells/mouse).

Immunohistochemistry

Islet grafts were snap frozen in OCT (Sakura Finetek, Torrance, CA). Five-micrometer sections were cut and stained with antibodies to insulin followed by secondary antibodies against guinea-pig IgG coupled to horseradish peroxidase (Dako Corp., Carpinteria, CA, USA). Staining was developed with diamino-benzidine

(Sigma-Aldrich) and sections were counter stained with haematoxylin (Sigma-Aldrich).

Electron Microscopy

For transmission electron microscopy, islet grafts were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.08M Sorenson's phosphate buffer at room temperature for 4 h. Sections were then prepared by the electron microscopy facility at the Peter MacCallum Cancer Center. Images were taken on the JEOL JEM-1011 transmission electron microscope.

IVIS

In vivo imaging of bioluminescent signal was performed using Xenogen IVIS-200 (PerkinElmer, Waltham, MA). Mice were kept under isoflurane (1.5%) anesthesia and given an i.p. injection of D-luciferin (Thermo Fisher Scientific Australia Pty Ltd, 150 mg/kg body weight). Images were then taken continuously with 1-min exposure and 2-min interval for 20 min.

RESULTS

Islet destruction by diabetogenic T cells

To examine the mechanisms of islet destruction by T cells, *NODscid* mice were depleted of endogenous beta cells and made diabetic by a single high dose injection of streptozotocin. These mice were then grafted under the kidney capsule with 400 wild-type NOD islets, after which the blood glucose of the recipients dropped to the

normo-gylcaemic range (5 to 10 mM; Figure 1A). Mice were then adoptively transferred with $1x10^6$ diabetogenic BDC2.5 $CD4^+$ T cells to induce T cell-mediated destruction of the grafted islets. Because *NODscid* mice lack T and B cells, they do not develop allo-rejection of islets from other strains, such as those on a C57BL/6 background. In addition, BDC2.5 T cells only recognize antigen presented by $I\text{-}A^{g7}$ on *NODscid* antigen presenting cells, so they are able to kill islets from any mouse strain (20). After transfer of BDC2.5 T cells, the recipient's blood glucose rose above 15 mM on day 8, suggesting the grafted islets were destroyed by the transferred T cells (Figure 1A). Histological analysis of graft sections confirmed infiltration and accumulation of leukocytes and disappearance of beta cells (Figure 1B).

We also monitored the loss of grafted islets after T cell transfer by monitoring the loss of beta cell-specific transgenic luciferase expression using the In Vivo Imaging System (IVIS). Islets expressing luciferase from MIP-Luc-VU mice were grafted into diabetic *NODscid* mice, which then received PBS or $1x10^6$ BDC2.5 T cells. The bioluminescence signal from islets, measured by IVIS after i.p. injection of luciferin, weakened substantially over time after adoptive transfer of T cells (Figure 1C&D). By the time when the blood glucose had risen to diabetic levels, the luciferase signal was hardly detectable, indicating dramatic loss of beta cells (Figure 1D). For the islet recipient mice that had not received BDC2.5 T cells, the bioluminescence signal from the transplanted islets was stable, indicating they remained intact (Figure 1C). Overall, these data validate that this experimental setup reflects conditions in T1D, in which beta cells are destroyed in a T cell-dependent manner.

Role of the death receptor apoptosis pathway in T cell mediated beta cell death destruction.

Activated T cells have been shown to express FASL, which triggers FAS mediated apoptosis in target cells. To test whether FAS mediated apoptosis plays a role in T cell-mediated beta cell destruction, islets from *NODlpr* mice that carry a loss of function mutation in the *Fas* gene were grafted into diabetic *NODscid* mice, and BDC2.5 T cells were transferred into such recipients after normoglycaemia had been restored. Upon T cell transfer, recipients of wild-type (*NOD*) or *NODlpr* islets became diabetic at the same rate, suggesting that FASL and FAS do not play major roles in T cell mediated killing of islet beta cells (Figure 2A).

While FASL and FAS are dispensable for T cell-mediated beta cell killing, other death ligands (e.g. TNF, TRAIL) may initiate the death receptor apoptotic pathway to kill beta cells. Therefore we investigated the impact of T cells on beta cells lacking functional FADD (an adaptor that is essential for all death receptor mediated apoptosis) (35) or BID (a BH3-only protein that is critical for death receptor induced apoptosis in some but not all cell types) (4). Notably, islets from $BID^{-/-}$ mice, or those expressing a dominant negative form of FADD (dnFADD) were not protected from T cell-mediated beta cell destruction (Figure 2B). We also bred Caspase-8 floxed mice with Rosa26-Cre-ERT2 mice (expressing in all cells a latent tamoxifen inducible Cre recombinase). Administration of tamoxifen significantly reduced the Caspase-8 expression in islets from *Caspase* $8^{fx/fx}$*creER*^{Tm} mice (Figure 2C). However, this loss of Caspase-8 did not prevent islet cell death after adoptive transfer of BDC2.5 T cells

(Figure 2D). Collectively, these results show that the death receptor apoptosis pathway is not essential for diabetogenic T cell mediated killing of beta cells in T1D.

Role of the BCL-2-regulated apoptosis pathway in T cell-mediated beta cell destruction.

In vitro studies have shown that the BCL-2-regulated apoptotic pathway plays a major role in the killing of beta cells in response to diverse cytotoxic insults. In particular, BIM and PUMA are critical for glucotoxicity and ER stress induced killing of beta cells (5-7). To determine whether these BH3-only proteins play a role in T cell-mediated beta cell killing, we grafted islets from mice lacking key pro-apoptotic BCL-2 family members into diabetic *NODscid* mice and examined occurrence of diabetes after adoptive transfer of BDC2.5 T cells. Loss of the BH3-only proteins BIM and PUMA did not protect islets from T cell-mediated killing (Figure 3A).

While this result suggests that on their own BIM and PUMA are dispensable for T cell mediated beta cell killing, multiple BH3-only proteins might cooperate in this process in a highly redundant manner. To inhibit the BCL-2 regulated apoptotic pathway completely, we used islets from transgenic mice expressing the anti-apoptotic protein BCL-2 under the control of rat insulin promoter (*Rip-Bcl-2* mice). Islets from these mice are protected against apoptosis induced by inflammatory cytokine treatment, ER stress and staurosporine *in vitro* (6; 7; 22). However, *Rip-Bcl-2* islets were not protected from BDC2.5 T cell mediated destruction (Figure 3B).

Since combined loss of the pro-apoptotic multi BH domain proteins BAX and BAK completely block the BCL-2-regulated apoptotic pathway in diverse cell types (36), we sought to generate islets deficient in both BAX and BAK to confirm the lack of a role for this pathway in T cell-mediated beta cell killing. Mice with whole body knockout of *Bak* and a conditionally targeted allele of *Bax* (*Bak*$^{-/-}$*Bax*$^{fx/fx}$) were crossed with *Rosa26-Cre-ERT2* mice (see above). When *Bak*$^{-/-}$*Bax*$^{fx/fx}$*Cre-ER*^{TM} mice were treated with tamoxifen, islet expression of BAX protein was substantially reduced (Figure 3C). However, these islets were not resistant to T cell-mediated destruction *in vivo*, as their recipients became diabetic in the same time frame as the recipients of wild-type islets (Figure 3D). Collectively, these results show that the BCL-2 regulated apoptotic pathway is not essential for T cell-mediated destruction of islet beta cells, and is consistent with lack of protection from autoimmune diabetes in *Rip-Bcl-2* mice on a NOD background (22).

Role of the necroptotic pathway in T cell mediated beta cell destruction.

Necroptosis is a genetically programmed process for cell killing induced by ligation of death receptors (37). We sought to determine whether necropotosis plays a role in T cell mediated destruction of beta cells. To do this, we obtained islets from mice deficient in RIPK3, a kinase that is essential for death ligand induced necroptosis (38-40). When islets from wild-type or *RIPK3*$^{-/-}$ mice were transplanted into diabetic *NODscid* mice followed by adoptive transfer of BDC2.5 T cells, no difference in diabetes onset was observed (Figure 4A). This indicates that death ligand induced necroptosis does not play a major role in T cell-mediated beta cell destruction.

Impact of combined inhibition of both apoptosis and necroptosis on T cell mediated destruction of islet beta cells.

It is possible that apoptosis and necroptosis act in a highly redundant manner in T cell mediated destruction of beta cells. To examine this hypothesis we generated *Rip-Bcl-2;RIPK3*$^{-/-}$ mice in which apoptosis is inhibited due to over-expression of BCL-2 and necroptosis is blocked due to loss of RIPK3. However, when these islets were challenged *in vivo* by diabetogenic T cells, no protection against destruction was observed (Figure 4B). This indicates that apoptosis and necroptosis are both dispensable for T cell mediated destruction of beta cells in T1D.

Necrotic morphology of dying beta cells under attack of diabetogenic T cells identified by electron microscopy.

The fact that blocking both apoptosis and necroptosis could not protect beta cells from T cell mediated killing suggested other death mechanisms may be responsible. Transmission electron microscopy (TEM) is the definitive method to identify the form of cell death because necrosis, apoptosis and autophagy all have distinct morphologies. The adoptive T cell transfer model we used has the advantage that the grafted islets are concentrated in a defined area under the kidney capsule, and the islet cell death occurs over a short period of time. The grafts were harvested for TEM on day 8 after BDC2.5 T cell transfer, when a large number of beta cells are in the process of dying and the recipients are about to become diabetic (Figure 1A). Beta cells were readily identified by their electron-dense insulin granules. Intact beta cells showed electron-dense mitochondria with clear cristae structure, electron-light nuclei,

and few empty vesicles without insulin (Figure 5A). Dying beta cells, on the contrary, displayed swollen mitochondria, accumulation of empty vesicles, rupture of the plasma membrane, and leaking of intracellular contents, all features of necrosis (Figure 5B-E). We did not find any chromatin condensation and nuclear fragmentation in dying beta cells that would have been consistent with apoptotic beta cell death. Moreover, no accumulation of double membrane vesicles were observed, indicating autophagy does not play an important role in beta cell death. We also observed the same necrotic features in dying *RIPK3*$^{-/-}$ beta cells that are protected from death ligand induced necroptosis (Figure 5F). These data suggest that diabetogenic T cells kill beta cells through induction of necrosis.

DISCUSSION

Type 1 diabetes results from the killing of pancreatic beta cells by autoreactive T cells. Understanding the mechanism of beta cell death, therefore, is critical for the development of targeted therapies to preserve beta cell mass. Apoptosis has long been thought to be the major mechanism of T cell-mediated beta cell death. Evidence supporting this conclusion includes the detection of TUNEL positive beta cells in prediabetic mice and *in vitro* studies using beta cells cultured with a variety of cytotoxic stimuli (5-7; 11-15). However, few *in vivo* studies have been conducted so far to confirm that apoptotic signaling pathways mediate beta cell death. In the 15 years since the description of beta cell apoptosis in mouse models of T1D there have been enormous advances in understanding the pathways of cell death in great detail at a molecular level. We took advantage of this knowledge and re-tested the hypothesis that T cell-mediated beta cell killing in T1D occurs by apoptosis using an established

transfer model. Our results show that blocking the death receptor and BCL-2-regulated apoptosis pathways was unable to prevent beta cell death induced by diabetogenic BDC2.5 T cells. Using electron microscopy we observed beta cells with necrotic, but not apoptotic, morphology, suggesting that T cells induce necrotic cell death of beta cells.

While originally considered as an unregulated type of cell death, in recent years at least one pathway to necrosis that is induced by death ligands, now called necroptosis, has been recognized as a programmed process for killing cells. Necroptosis has been postulated to play a role in a variety of disease conditions, such as ischemic stroke and cardiac infarction(8). We examined the role of the death receptor-activated necroptosis pathway in T1D by using islets from *RIPK3*$^{-/-}$ mice but did not observe protection from T cell-mediated beta cell killing. This suggests that T cells may kill beta cells through induction of necrosis that may not be programmed. Although less is known about this type of cell death, it can be activated by oxidative stress or calcium overload, and occur independent of the RIPK1, RIPK3 and MLKL (9). It is currently impossible to test the role of this pathway genetically because no essential components of this pathway are known; indeed, this process may not even be programmed.

In our study, beta cell destruction was induced by BDC2.5 T cells, which are beta cell specific $CD4^+$ T cells, potent in mediating islet inflammation and beta cell death (25). Diabetogenic $CD4^+$ T cells are not only required for the development of $CD8^+$ T cell mediated autoimmunity, but also able to induce beta cell death without cytotoxic $CD8^+$ T cells (41; 42). During the development of diabetes, both $CD4^+$ and $CD8^+$ T

cells are present in inflamed islets (43). While $CD8^+$ T cells play a dominant role in beta cell killing (using perforin and granzymes for target cell destruction), $CD4^+$ T cells are equally capable of inducing beta cell death because when the effector function of $CD8^+$ T cells is inhibited, $CD4^+$ T cells continue to kill beta cells and diabetes can still develop (44; 45). Our findings about the pathways by which BDC2.5 T cells kill beta cells are therefore pathologically relevant and reflect what occurs *in vivo* in beta cells under attack by autoreactive T cells. A similar experimental setup may be used to study $CD8^+$ T cell-mediated beta cell killing. However, the availability of donor islets is more limited because the islets have to come from strains expressing H-$2K^d$ to allow direct interaction between NOD $CD8^+$ T cells and beta cells to take place (46).

It is not clear what effector mechanism induces the necrosis of beta cells. BDC2.5 T cells do not interact directly with beta cells but most likely activate myeloid cells to kill beta cells (47). Macrophages are known to release cytokines and ROS to kill target cells. While cytokines, such as IFNγ and TNFα, can induce beta cell killing by either apoptosis or necrosis *in vitro*, our previous results have shown that inhibition of cytokine signaling in beta cells did not prevent their destruction *in vivo* (48). Beta cells are highly susceptible to ROS as they express lower level of antioxidants (49; 50). Oxidative stress has been shown to induce necrosis (9); targeting ROS may therefore provide insight into the mechanisms of beta cell death in T1D. Collectively, our data suggest that beta cells die by necrosis in T1D. Since necrosis is known to induce inflammation, it is possible that necrosis of beta cells helps to further stimulate the activity of immune cells in islets, thus contributing to the overall pathology in the islets.

AUTHOR CONTRIBUTIONS

YZ, SF, LE and HET performed experiments and analyzed data. YZ and HET designed the study and wrote the manuscript. WWW, KDM, JA, AS, DCH, TWHK contributed reagents, provided intellectual input and edited the manuscript. HET is guarantor of this manuscript.

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Figure Legends

Figure 1. BDC2.5 T cells destroy islet grafts in the adoptive transfer model. **A)** 400 NOD islets were grafted under the kidney capsule of a *NODscid* mouse previously made diabetic by injection of streptozotocin, and blood glucose levels were monitored thereafter. BDC2.5 T cells were transferred on day 11 after islet grafting. **B)** Frozen sections of islet grafts from *NODscid* recipients with or without BDC2.5 T cell-induced diabetes were stained with an antibody to insulin. Magnification 200X. **C, D)** Islets expressing luciferase were grafted under the kidney capsule of diabetic *NODscid* recipients that later received PBS (C) or BDC2.5 T cells (D). Blood glucose levels and bioluminescence signals from the grafted islets were measured. Representative data from three experiments are shown.

Figure 2. The death receptor apoptosis pathway is not required for BDC2.5 T cell-induced beta cell killing. **A, B, D)** 400 islets of the indicated genotypes were grafted under the kidney capsule of diabetic *NODscid* mice. After blood glucose levels had returned to normal, BDC2.5 T cells were transferred into the grafted recipients. Blood

glucose levels were monitored and the incidence of diabetes is shown. **C)** Western blot analysis of Caspase-8 levels using islet lysates from *Caspase 8*$^{fx/fx}$*Cre-ER*^{TM} mice that had been treated with tamoxifen or vehicle.

Figure 3. The BCL-2-regulated apoptosis pathway is not required for BDC2.5 T cell-induced beta cell killing. **A, B, D)** 400 islets of the indicated genotypes were grafted under the kidney caspsule of diabetic NOD*scid* mice. After blood glucose levels had returned to normal, BDC2.5 T cells were transferred into the grafted recipients. Blood glucose levels were monitored and the incidence of diabetes is shown. **C)** Western blot analysis of BAX levels in islet lysates from *Bak*$^{-/-}$*Bax*$^{fx/fx}$*Cre-ER*^{TM} mice that had been treated with tamoxifen or vehicle.

Figure 4. Combined inhibition of necroptosis and apoptosis does not protect beta cells from BDC2.5 T cell-mediated killing. **A and B)** 400 islets of the indicated genotypes were grafted under the kidney capsule of diabetic *NODscid* mice. After blood glucose levels had returned to normal, BDC2.5 T cells were transferred into the grafted recipients. Blood glucose levels were monitored and the incidence of diabetes is shown.

Figure 5. Dying beta cells under attack by BDC2.5 T cells show features of necrosis. Representative TEM of **A)** Intact beta cells in the graft; **B, C, D)** Dying beta cells in the graft; **E)** higher magnification of the square in D; and **F)** dying *RIPK3*$^{-/-}$ beta cells in the graft. White arrow head: intact mitochondria; Black arrow head: swollen mitochondria; White arrow: empty vesicles; Black arrow: ruptured membrane and

leaking of intracellular contents. White scale bar: 5 μm; Black scale bar: 10 μm. Results are representative of three experiments.

Figure 1.

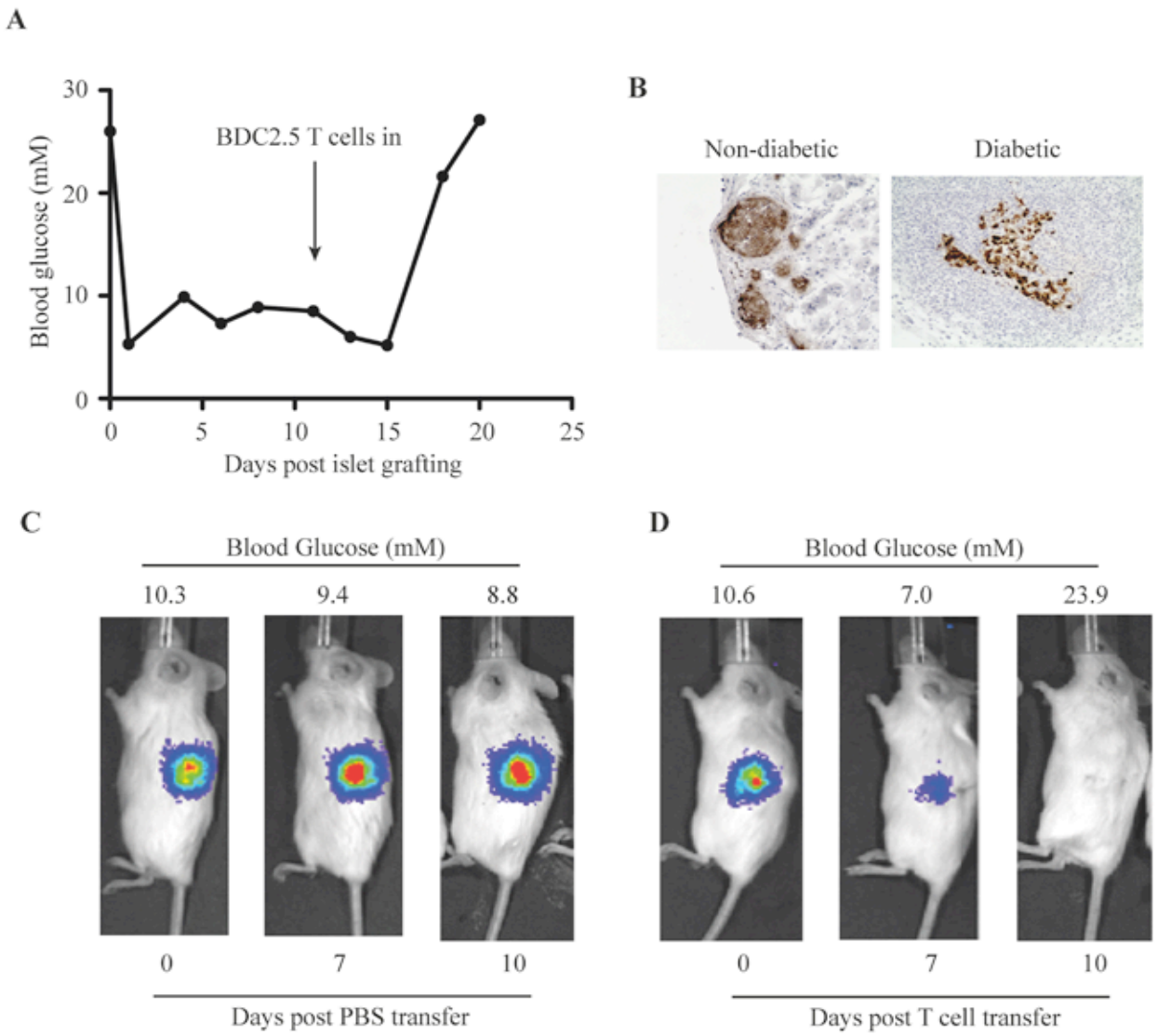
A
Blood glucose (mM)
30
20
10
0
BDC2.5 T cells in
0
5
10
15
20
25
Days post islet grafting
B
Non-diabetic
Diabetic
C
Blood Glucose (mM)
10.3
9.4
8.8
0
7
10
Days post PBS transfer
D
Blood Glucose (mM)
10.6
7.0
23.9
0
7
10
Days post T cell transfer

Figure 2.

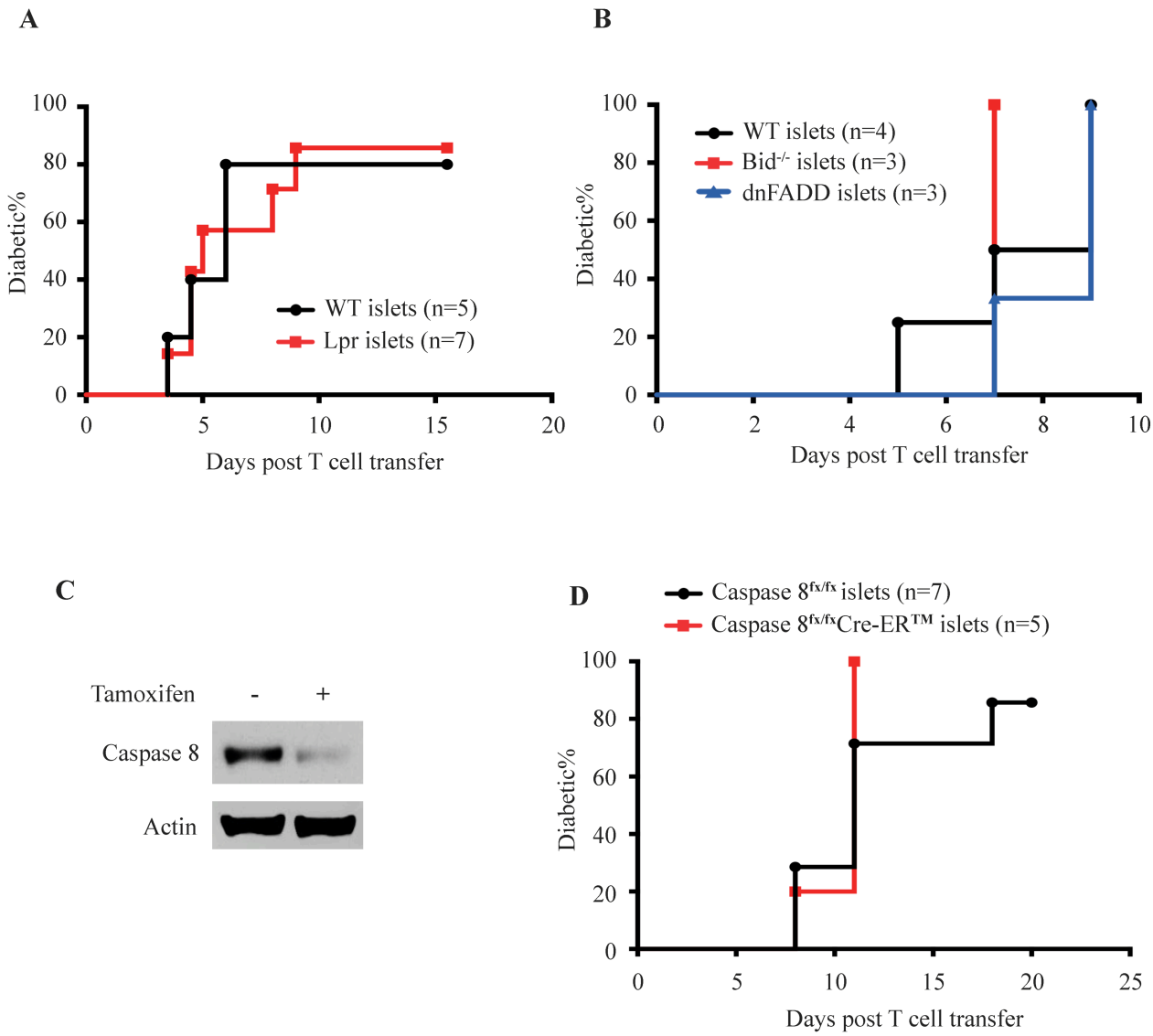
A
100
80
60
40
20
0
Diabetic%
0
5
10
15
20
Days post T cell transfer
WT islets (n=5)
Lpr islets (n=7)
B
100
80
60
40
20
0
Diabetic%
0
2
4
6
8
10
Days post T cell transfer
WT islets (n=4)
Bid-/- islets (n=3)
dnFADD islets (n=3)
C
Tamoxifen
-
+
Caspase 8
Actin
D
Caspase 8fx/fx islets (n=7)
Caspase 8fx/fxCre-ER™ islets (n=5)
100
80
60
40
20
0
Diabetic%
0
5
10
15
20
25
Days post T cell transfer

Figure 3.

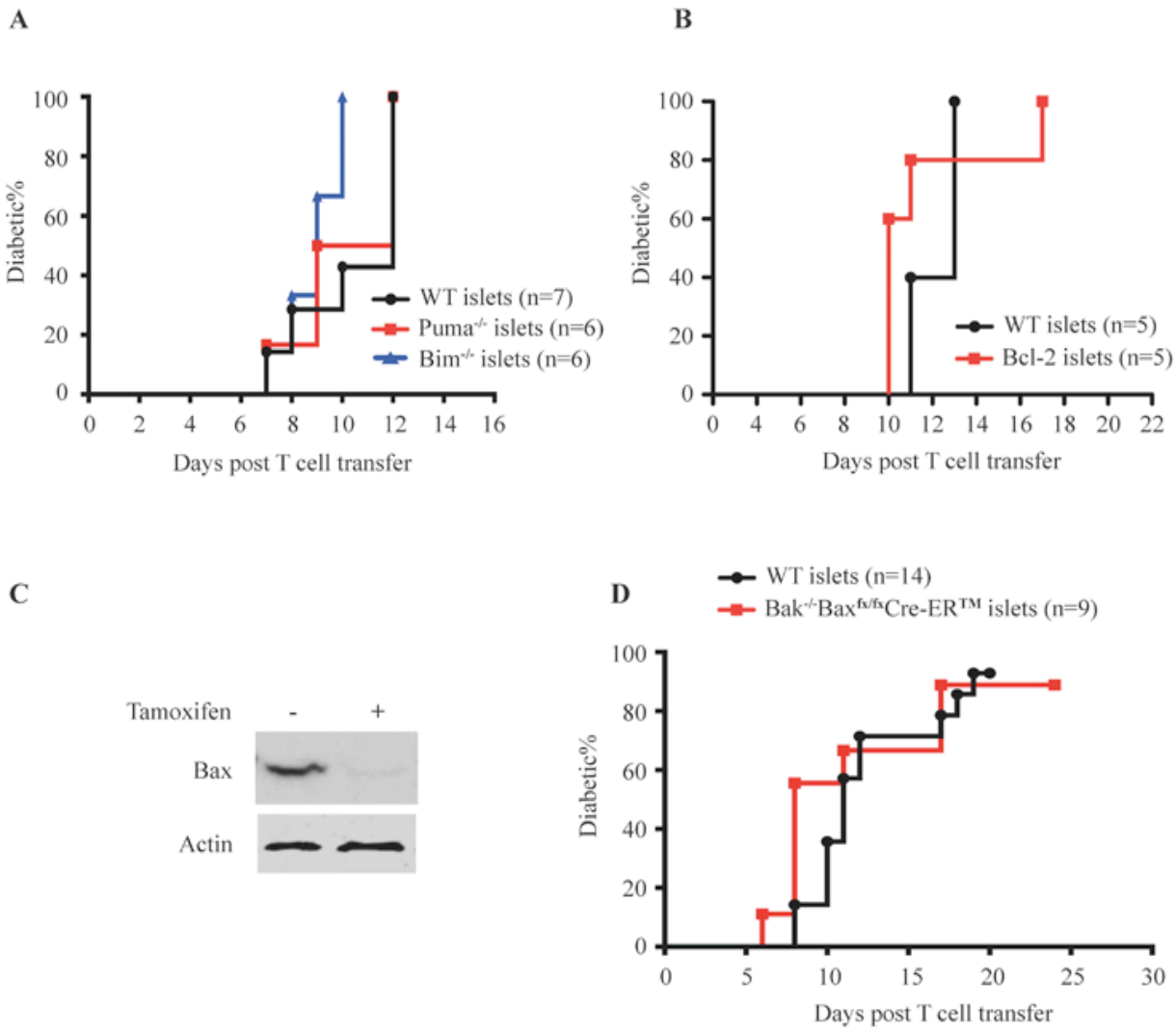
A
Diabetic%
Days post T cell transfer
WT islets (n=7)
Puma-/- islets (n=6)
Bim-/- islets (n=6)
B
Diabetic%
Days post T cell transfer
WT islets (n=5)
Bcl-2 islets (n=5)
C
Tamoxifen
-
+
Bax
Actin
D
WT islets (n=14)
Bak-/-Baxfx/fxCre-ER™ islets (n=9)
Diabetic%
Days post T cell transfer

Figure 4.

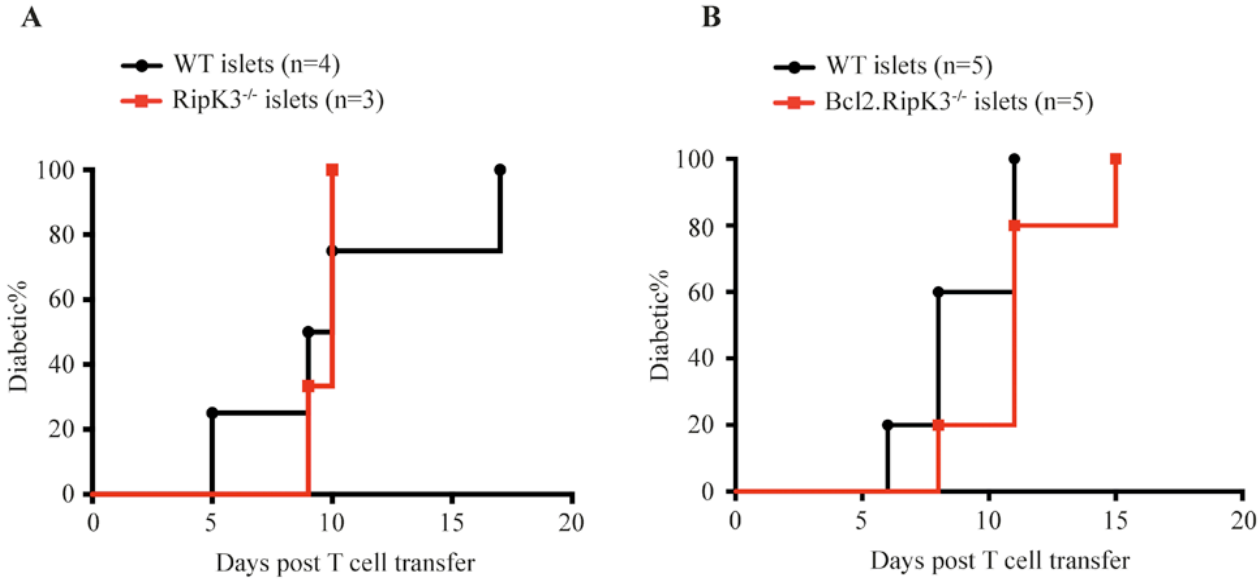
A
WT islets (n=4)
RipK3-/- islets (n=3)
Diabetic%
100
80
60
40
20
0
0
5
10
15
20
Days post T cell transfer
B
WT islets (n=5)
Bcl2.RipK3-/- islets (n=5)
Diabetic%
100
80
60
40
20
0
0
5
10
15
20
Days post T cell transfer

Figure 5.