Cleavage of Protein Kinase C δ by Caspase-3 Mediates Pro-inflammatory Cytokine-Induced Apoptosis in the Pancreatic Islet

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Summary

In type 1 diabetes (T1D), autoreactive immune cells infiltrate the pancreas and secrete pro-inflammatory cytokines that initiate cell death in insulin producing islet β-cells. Protein kinase C δ (PKCδ) plays a role in mediating cytokine-induced β-cell death; however, the exact mechanisms are not well understood. Here we explored the role of PKCδ in mediating pro-inflammatory cytokine-induced apoptosis in both mouse and human islets. Our results support a role for PKCδ activity in mediating cytokine-induced apoptosis and inhibiting PKCδ with a cell permeable inhibitor, δV1-1, also protected against cytokine-induced apoptosis in mouse and human islets. Pro-inflammatory cytokines increased PKCδ activity and nuclear translocation, and caspase-3 cleavage of PKCδ was required for cytokine-induced apoptosis. Our results support a role for PKCδ in regulation of pro-apoptotic signaling in pancreatic islets and suggest PKCδ may play a role in mediating cytokine-induced apoptosis in pancreatic β-cells in T1D.

Keywords: Protein Kinase C δ, pancreatic islet, β-cell, apoptosis, pro-inflammatory cytokines, caspase-3, type 1 diabetes

Introduction

Type 1 diabetes (T1D) is characterized by the selective immune-mediated destruction of insulin producing β-cells in pancreatic islets (Lightfoot, Chen and Mathews, 2012; Pugliese, 2017). Clinical onset of T1D results in life-long dependence on exogenous insulin and is associated with long-term complications including, ophthalmic, kidney, cardiovascular, and neurological disorders (Nathan, 1993). While current therapeutic strategies aim to control glucose levels, they do not stop disease progression nor prevent disease complications. Furthermore, current clinical trials to prevent or reverse disease outcomes although have had limited success, including disease remission and incomplete recovery of normoglycemia (Ovalle *et al.*, 2018; Herold *et al.*, 2019). The exact mechanisms regulating β-cell death during T1D pathogenesis are not fully understood, making it difficult to develop effective therapies to preserve endogenous β-cells.

In T1D, pro-inflammatory cytokines secreted from autoreactive immune cells play a crucial role in β-cell apoptosis (Lu *et al.*, 2020). Studies have shown that the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin 1-beta (IL-1β), and interferon-γ (IFN-γ) work synergistically to induce islet dysfunction and β-cell death *in vitro* (Wachlin *et al.*, 2003; Farnsworth *et al.*, 2016; Demine *et al.*, 2020) . In the β-cell, cytokine-mediated apoptosis has been shown to occur through either activation of mitogen-activated protein kinase (MAPK), activation of nuclear factor kappa B or activation of signal transducer and activator of transcription 1 (STAT1), all of which lead to activation of caspase-3 and apoptosis (Tomita, 2017; Lu *et al.*, 2020). Our lab has previously shown that pro-inflammatory cytokines disrupt gap junction coupling, calcium (Ca^{2+}) signaling and impairs insulin secretion in both mouse and human islets via upregulation of protein kinase C delta (PKCδ) activity (Farnsworth *et al.*, 2016).

PKCδ regulates many cellular functions including cell proliferation, differentiation, and apoptosis (Reyland, 2009). In response to apoptotic stimuli, such as DNA damaging H_2O_2 and IR radiation (Yuan *et al.*, 1998; Reyland *et al.*, 1999; Konishi *et al.*, 2001) the serine or tyrosine sites on PKCδ are phosphorylated leading to a conformational change in PKCδ into its active state where it can then relay intercellular signaling messages through phosphorylation and initiate apoptosis (Kikkawa, Matsuzaki and Yamamoto, 2002). PKCδ has been shown to mediate fatty acid–induced apoptosis in human β-cells (Eitel *et al.*, 2003) and studies utilizing a mouse model with overexpression of a kinase-negative PKCδ protected against high fat diet mediated apoptosis (Hennige *et al.*, 2010). Similarly, a whole-body knockout of PKCδ protected against β-cell death in streptozotocin (STZ) induced diabetes in mice *in vivo* and cytokine treated β-cells *in vitro* (Cantley *et al.*, 2011). However, PKCδ is ubiquitously expressed throughout the body and a global knockout can lead to spontaneous B-cell proliferation resulting in increased autoimmunity (Kuehn *et al.*, 2013).

While a role for PKCδ in mediating β-cell apoptosis has previously been identified in mouse islets, the mechanism by which PKCδ regulates apoptosis has not been well defined in the β-cell. In normal epithelial cells and some cancer cells, PKCδ regulates etoposide mediated apoptosis, where activated PKCδ is cleaved by caspase-3 into a 40kDa catalytically active fragment (Reyland *et al.*, 1999). Caspase cleavage exposes a nuclear localization signal and induces nuclear localization of PKCδ to initiate the apoptotic signaling cascade (DeVries-Seimon *et al.*, 2007). The goal of this study is to understand the role of PKCδ in mediating cytokine-induced β-cell death. As caspase-3 activation plays a role in cytokine-mediated β-cell death, we hypothesized that cytokine-mediated activation of PKCδ leads to cleavage by caspase-3 and nuclear translocation, resulting in an increase in pro-apoptotic signaling. We utilized isolated islets from mice with a β-cell-specific knockout of PKCδ or human islets treated with a cell permeable PKCδ specific inhibitor, δV1-1. Improved understanding of the cytokine-mediated apoptotic pathway in human β-cells will further elucidate key mechanisms in the pathogenesis of T1D and will provide new avenues towards developing targeted therapies to preserve β-cell mass in T1D.

Results

Characterization of the PKCδ$^{fl/fl}$ x MIP-Cre^{ER} mouse

To assess the role of PKCδ in mediating cytokine-induced β-cell death, we developed a β-cell specific knockout of PKCδ (PKCδ-βKO). β-cell specificity was achieved using mouse insulin promoter (MIP) Cre-ER on the C57Bl/6 mouse background, which is expected to produce mosaic expression in β-cells based on previous results with the inducible Cre-ER system (Guo, Yang, and Lobe 2002). PKCδ-βKO islets had 58% less PKCδ protein compared to control (PKCd$^{fl/fl}$) as determined by western blot ($p=0.015$, Figure 1A and B). No significant alterations in glucose tolerance levels were observed between the PKCδ-KO and control mice using an intraperitoneal glucose tolerance test (Figure 1C).

PKCδ Mediates Cytokine-Induced Apoptosis in Mouse and Human Islets

To determine if PKCδ is required for cytokine-induced apoptosis in the β-cell, isolated PKCδ-βKO and control islets were treated with or without a cocktail of proinflammatory cytokines TNF-α (10ng/ml), IL-1β (5 ng/ml), and IFN-γ (100ng/ml) for 24hr. A significant increase in apoptotic cells was observed in cytokine treated control islets compared to untreated control islets ($p<0.001$, Figure 2A). The percentage of apoptotic cells was significantly reduced in the cytokine treated PKCδ-βKO islets compared to control controls ($p=0.003$, Figure 2A). Mouse C57Bl/6 and human islets were also treated with or without cytokines and with or without the PKCδ inhibitor δV1-1. Both mouse ($p<0.001$) and human ($p=0.02$) islets treated with cytokines displayed a decrease in the percentage of apoptotic cells with δV1-1 treatment compared to cytokine only control (Figure 2B and C). This data supports a role for PKCδ in mediating cytokine-induced apoptosis in pancreatic β-cells.

Cytokine Treatment Induces Nuclear Translocation and Activity of PKCδ in Islets

Nuclear translocation of PKCδ is an indication of activation and is associated with the pro-apoptotic functions of PKCδ. To investigate whether cytokines induce nuclear translocation of PKCδ in pancreatic islet β-cells, PKCδ-βKO islets were pre-treated with a virus to overexpress GFP fused to the c-terminus of PKCδ (GFP-PKCδ, Supplemental Figure 1) overnight and cultured with or without cytokines for 24 hours. GFP-PKCδ was expressed in PKCδ-βKO islets to reduce interference from endogenous PKCδ and to avoid cell death due to supraphysiological levels of PKCδ. Prior to imaging, islets were stained with NucRed live to determine nuclear versus cytosolic PKCδ localization. In untreated islets we observed diffuse staining for PKCδ throughout the cytosol and the nucleus, with nuclear GFP intensity generally lower than cytosolic GFP (Figure 3A). We observed an increase in GFP fluorescence in the cell nuclei with cytokine treatment compared to untreated controls (Figure 3A, red arrows). Furthermore, at both 3 ($p=0.034$) and 24hr ($p<0.001$) timepoints, the fluorescence intensity in the nucleus normalized to the cytosol significantly increased in cytokine treated islets compared to untreated controls (Figure 3B). We also translocation of GFP-only using an adenoviral vector to confirm that GFP was not influencing the observed nuclear translocation with cytokine treatment. No significant change in the fluorescence intensity in the nucleus normalized to the cytosol was observed in PKCδ-βKO islets treated with GFP-only virus with cytokine treatment compared to untreated controls (Figure 3C). Finally, we tested changes in nuclear activity of PKCδ with cytokine treatment using a nuclear targeted PKCδ specific FRET-based activity sensor (δCKAR) in MIN6 cells. Expression of δCKAR was primarily localized to the nucleus (Figure 3D) and fluorescence lifetime imaging (FLIM) was performed using a threshold on photon counts to calculate the average lifetime in the nuclear region of the cells (Figure 3E) to determine changes in FRET with cytokine treatment. We found that PKCδ activity as measured by FLIM increases in the nuclei of cytokine treated cells compared to untreated controls after 1.5 hours of treatment ($p=0.039$, Figure 3D and E).

Cytokine-Induced Cleavage of PKCδ by Caspase-3

In several cell types, cleavage of PKCδ yields a constitutively active fragment that localizes to the nucleus to promote apoptosis. Therefore, subcellular fractionation and western blot were used to observe changes in both intact (75kDa) and cleaved (45kDa) fragments of PKCδ in both the cytosolic and nuclear fractionations of human islet lysates (Figure 4A). Staining of histone cluster 1 H3D in the nuclear fraction and not in the cytosolic fraction confirmed separation of the cytosolic and nuclear lysate (Figure 4A). In contrast to the results from Figure 3A and B, quantification of the blot stained for PKCδ and β-actin revealed no significant changes in intact PKCδ (78kDa) in the cytosolic fraction compared to the nuclear fraction when cytokine treated samples were normalized to untreated samples (Figure 4B). This suggests that PKCδ translocation measured in Figure 3A and B was mainly the PKCδ fragment, as GFP is fused to the c-terminus (Supplemental Figure 1) and will identify both intact and cleaved PKCδ. A significant increase in the PKCδ fragment (45kDa) was observed in the nuclear fraction compared to the cytosol ($p=0.047$, Figure 4B). Overall, these results support cytokine-mediated cleavage of PKCδ.

To determine if caspase-3 cleaves PKCδ during cytokine-induced apoptosis, and if caspase cleavage is necessary for cytokine-induced apoptosis, PKCδ-βKO islets were transduced with a virus encoding a GFP-PKCδ peptide, where the sequence that can be cleaved by caspase-3 has been mutated to prevent PKCδ cleavage (CM-GFP-PKCδ). Islets treated with viruses coding for GFP-only, GFP-PKCδ, or CM-GFP-PKCδ were cultured for 24h with or without cytokines (Supplemental Figure 1). Cytokines caused significant increases in apoptosis under all conditions tested (Figure 4C). No significant differences were observed in cytokine-induced apoptosis levels between the PKCδ-βKO alone and GFP-only treatment (Figure 4C), indicating that GFP does not contribute to islet apoptosis. There was a significant increase in cytokine-induced apoptosis in islets expressing GFP-PKCδ compared to islets with no virus pre-treatment ($p<0.001$, Figure 4C), indicating normal function of the GFP-PKCδ peptide in the islet. The cytokine-treated CM-GFP-PKCδ islets showed a significant reduction in the percentage of dead cells compared to cytokine treated GFP-PKCδ islets ($p<0.001$, Figure 4C) and similar levels to GFP-only treated cells indicating the inhibition of caspase-3 cleavage can mitigate PKCδ mediated apoptosis. Translocation of the CM-GFP-PKCδ to the nucleus was also measured as previously described and an increase in the nuclear to cytosolic ratio of the GFP intensities was observed with cytokine treatment ($p=0.009$, Figure 4D), indicating increased nuclear translocation of the intact PKCδ determined as described above. These results suggest that PKCδ is cleaved by capase-3; however, cleavage is not required for nuclear translocation with cytokine treatment.

To further verify the role of caspase-3 in cytokine-mediated apoptosis and regulation of PKCδ, mouse and human islets were treated for 24hr with the caspase-3 inhibitor ZEVD-FMK with or without cytokines (Figure 5). Inhibiting caspase-3 protected against cytokine-induced apoptosis in both mouse ($p<0.001$) and human islets (Figure 5A and B). To determine if inhibiting caspase-3 altered cleavage of PKCδ with and without cytokine treatment, western blot analysis of PKCδ and β-actin was performed on control and PKCδ-βKO mouse islet lysates treated for 24 hours with or without ZEVD-FMK and with or without cytokines (Figure 5C). While inhibiting caspase-3 had no significant impact on the level of full length PKCδ normalized to β-actin (Figure 5D), we observed a decrease in the level of the PKCδ fragment (45kDa) normalized to β-actin (Figure 5E) or normalized to intact PKCδ (Figure 5F) in ZEVD-FMK treated islets compared to untreated controls. A similar decrease in levels of the PKCδ fragment was observed in islets treated with cytokines and ZEVD-FMK compared to cytokines alone ($p=0.05$, Figure 5E). Overall, these results support the conclusion that caspase-3 cleavage of PKCδ is required for PKCδ mediation of cytokine-induced β-cell apoptosis.

Cytokine-Activated PKCδ Mediates Pro-Apoptotic Signaling

Finally, we investigated PKCδ-mediated changes in pro-apoptotic signaling, including Bax and C-Jun N-terminal kinases (JNKs). Control and PKCδ-βKO islets were either untreated or treated with cytokines for 24 hours and levels of Bax, phospho-JNK (p-JNK), JNK, and β-actin were analyzed via western blot (Figure 6). We found that Bax levels were similar in both control and PKCδ-βKO islets either untreated or treated with cytokines for 24hr (Figure 6A and B). JNK1 (46kDa) and JNK2 (54kDa) were both observed with JNK staining of the western blot (Figure 6C); however, only the quantification of JNK1 is shown as JNK2 levels did not significantly change in control versus PKCδ-βKO islets either in untreated or cytokine treated conditions (data not shown). An increase in p-JNK/JNK1 was observed with cytokine treatment in both the control and PKCδ-βKO islets (p=0.016, Figure 6D). Overall, this data supports a role for PKCδ in regulating pro-apoptotic JNK signaling in the islet.

Discussion

The goal of this study was to characterize the intracellular signaling mechanisms underlying PKCδ regulation of cytokine-mediated β-cell death in pancreatic islets. Utilizing an inducible β-cell specific PKCδ KO mouse as well as a small peptide specific inhibitor of PKCδ we determined that PKCδ activity mediates cytokine-induced islet apoptosis in both mouse and human islets. We also determined that cytokines induced nuclear translocation and activity of PKCδ and that caspase-3 cleavage of PKCδ is required for cytokine-mediated islet apoptosis. Ultimately, activated PKCδ was shown to regulate pro-apoptotic JNK activity, suggesting a downstream mechanism of PKCδ-mediated apoptosis as shown in Figure 7. Elucidating novel roles of PKCδ in mediating β-cell death has provided further insight into apoptotic signaling mechanisms that may occur in T1D. Combined with the protective effects of PKCδ inhibition with δV1-1, the results of this study have the potential to aid in the development of novel β-cell targeted therapies to prevent or delay β-cell death and preserve β-cell function in T1D.

Inhibition of PKCδ Protects Against Cytokine-Induced Death

Pro-inflammatory cytokines play a critical role in mediating islet death and dysfunction. Several studies have identified a potential role for PKCδ in mediating cytokine-induced β-cell death. For example, a knockout of PKCδ or expression of a kinase negative PKCδ protected against cytokine-induced islet death and improved glucose homeostasis and β-cell function in high-fat diet mice *in vitro* (Frangioudakis *et al.*, 2009; Hennige *et al.*, 2010; Cantley *et al.*, 2011). While these studies strongly support a role for PKCδ in mediating cytokine-induced islet death, the results are confounded by the effects from a whole body PKCδ KO as PKCδ is ubiquitously expressed (Kuehn *et al.*, 2013). To determine the role of PKCδ specifically in mediating β-cell death, we generated a mouse line with an inducible β-cell specific KO of PKCδ (PKCδ-βKO). Our results indicate that KO of PKCδ specifically in the β-cell protects against cytokine-induced apoptosis. Contrary to previously published results indicating that loss of PKCδ impairs glucose tolerance, our PKCδ-βKO mice were normoglycemic compared to control controls (Uchida *et al.*, 2007; Schmitz-Peiffer and Biden, 2010). While this may be due to loss of only ~50% of PKCδ protein in the islets of our transgenic mouse, this may also be indicative of confounding effects of a whole-body KO from loss of PKCδ in the liver, where previous studies have indicated that global PKCδ KO impairs insulin sensitivity (Bezy *et al.*, 2011; Khromov *et al.*, 2014). It has been reported that PKCδ is present in α-cells which comprise ~20% of a pancreatic islet (Peterson *et al.*, 2020). This supports a higher β-cell specific knockout since PKCδ reduction in the whole islet was measured in this study.

To confirm our results with a genetic KO of PKCδ, we also utilized a cell permeable specific inhibitor of PKCδ, δV1-1, an FDA approved peptide that specifically inhibits PKCδ activation. We found inhibiting PKCδ activation with δV1-1 protects against cytokine-induced islet apoptosis in both mouse and human islets. Collectively, this strongly supports a role for PKCδ in mediating cytokine-induced apoptosis specifically in the β-cell and our results in human islets strongly

support a role for PKCδ in human diabetes pathology. While no direct evidence for activation of PKCδ in islet β-cells has been shown in T1D or T2D, our results suggest that cytokine-induced activity of PKCδ may promote β-cell death under inflammatory conditions associated with diabetes. Future studies are warranted to investigate the role of PKCδ in β-cell death during diabetes pathogenesis and to determine therapeutic efficacy of δV1-1 in protecting against β-cell death.

Cytokine-Activated PKCδ Translocates to the Nucleus

Intact PKCδ is an isoenzyme of ~78kDa and is comprised of a regulatory and catalytic domain separated by a hinge region (Basu, 2003; Malavez, Gonzalez-mejia and Doseff, 2008). In the presence of apoptotic stimuli, such as ionizing radiation and etoposide, PKCδ has been shown to be proteolytically cleaved into a constitutively active catalytic fragment (~40kDa) which can undergo translocation from the cytosol to the nucleus (Emoto *et al.*, 1995; DeVries, Neville and Reyland, 2002; Steinberg, 2004). In this study nuclear translocation and accumulation of PKCδ was observed with cytokine-treated islets. Furthermore, PKCδ was cleaved into a smaller 45kDa fragment in the presence of pro-inflammatory cytokines. In this study, we were not able to investigate the difference in activity between intact PKCδ and the 45kDa fragment. However, the observed increase in PKCδ activity in the nucleus suggests that PKCδ may mediate cytokine-induced apoptosis through phosphorylation and subsequent activation of downstream mediators of apoptosis. Previous studies in keratinocytes have shown that PKCδ mediates activation of the STAT3 transcription factor, which is regulated by cytokine signaling (Gartsbein *et al.*, 2006). It has also been suggested PKCδ may also regulate transcription factor p53 (Reyland, 2009). Other potential substrates include other kinases and structural proteins (Reyland, 2009). In the β-cell, PKCδ has also been shown to activate the FOXO1 transcription factor in response to free fatty acids, leading to β-cell apoptosis (Hennige *et al.*, 2010). These studies support that nuclear translocation and activity of PKCδ may regulate cytokine-induced apoptosis via activation of nuclear transcription factors and support future studies to understand which nuclear signaling pathways PKCδ regulates. Additionally, some studies have shown that the active fragment of PKCδ inhibits activity of DNA-dependent protein kinase, shifting cellular response towards DNA damage-induced apoptosis instead of DNA damage repair (Kikkawa, Matsuzaki and Yamamoto, 2002). Activation of caspase-3 is a hallmark of both DNA damage-induced and cytokine-induced apoptosis, motivating our investigation into the role of caspase-3 in PKCδ regulation of apoptosis (Bacqueville and Mavon, 2008).

Cleavage of PKCδ by Caspase-3 Mediates Cytokine-Induced β-cell Apoptosis

Active caspase-3 targets a myriad of intracellular proteins, ultimately resulting in cell death (Collier *et al.*, 2011). Our results indicate that caspase-3 cleaves cytokine-activated PKCδ into a 45kDa fragment and that cleavage is required for PKCδ mediated cytokine-induced apoptosis in the islet. While we did not investigate if PKCδ is cleaved before or after nuclear translocation, DeVries-Seimon et al. provide evidence that supports intact PKCδ translocates to the nucleus before being cleaved by caspase-3 in etoposide treated parC5 cells (DeVries-Seimon *et al.*, 2007). This is further supported by our results where expression of a mutant PKCδ that cannot be cleaved by caspase-3 is still translocated to the nucleus in the presence of cytokines. Caspase-3 has also been shown to undergo nuclear translocation in α-Fas or etoposide treated HepG2 cells only after activation, further suggesting that cleavage of PKCδ occurs in the nucleus (Kamada *et al.*, 2004).

While caspase activation alone can initiate apoptotic signaling that leads to cell death, our results indicate that inhibition of PKCδ is sufficient abolish cytokine-induced apoptosis. This suggests that cytokine-activated PKCδ may activate caspase-3 prior to cleavage by activated caspase-3. This is supported by previous studies in neuroblastoma cells, where etoposide activation of PKCδ initiates a positive feedback loop that activates caspase-3 prior to cleavage of

PKCδ into a 40kDa fragment (Day, Wu and Safa, 2009). Overall, our results support a novel mechanism of cytokine-induced apoptosis involving PKCδ and caspase-3 that may present a unique pathway to prevent inflammation-induced β-cell death in diabetes.

Cytokine-Activated PKCδ Regulates Pro-apoptotic Signaling

As our results indicate that PKCδ mediates cytokine-induced apoptosis via nuclear translocation and activity, the final goal of this study was to determine if PKCδ regulates pro-apoptotic signaling in the β-cell. PKCδ regulation of pro-apoptotic signaling has been characterized in several cell types. For example, in previous studies in lung cancer cells exposed to ionized radiation, activation of PKCδ leads to conformational changes and activation of pro-apoptotic Bax and Bak, ultimately resulting in apoptosis (Choi *et al.*, 2006). The results from our study showed no change in Bax levels in islets treated with cytokines or between the control and PKCδ-βKO islets. This suggests Bax may not be regulated by PKCδ and that Bax may not be involved in cytokine-mediated apoptosis under the cytokine cocktail and timescales in this study. The role of Bax in cytokine-mediated β-cell death varies in the literature substantially. While some studies have demonstrated increased levels of Bax with cytokine treatment that correlated with increased cell death, another study has shown that silencing Bax in INS-1 derived cells did not protect cells treated with pro-inflammatory cytokines IL-1β and IFN-γ (Collier *et al.*, 2006). While Bax may play a role in mediating β-cell death, our results support that Bax does not mediate β-cell death by pro-inflammatory cytokines and is not regulated by cytokine-activated PKCδ. Although not investigated in this study, it is possible there could have been alterations in gene expression.

In addition to Bax we also analyzed PKCδ-mediated changes in JNK activity. JNKs are a protein kinase family that are involved in various cellular functions including regulating cell death (Bonny *et al.*, 2001). Environmental stress factors including cytokine induced inflammation can stimulate JNK activation, specifically JNK1 (Bonny *et al.*, 2001; Liu, Minemoto and Lin, 2004). Our results show a significant increase in activated JNK levels compared to total JNK1 (46kDa) but not JNK2 (54kDa) with cytokine treatment. This is consistent with studies in mouse fibroblasts, where JNK1 but not JNK2 was required for TNF-α induced apoptosis (Liu, Minemoto and Lin, 2004). PKCδ-βKO islets also showed a decrease in JNK levels compared to the control islets suggesting that JNK activity is mediated by PKCδ with cytokine treatment. Inhibiting PKCδ has also been shown to downregulate JNK activity in parotid gland cells as well as protected against irradiation-induced apoptosis *in vivo*, supporting these results (Humphries *et al.*, 2006). Overall, PKCδ regulation of JNK activity suggests that PKCδ regulates cytokine-induced apoptosis via activation of nuclear transcription factors either directly or via JNK translocation to the nucleus (Bonny *et al.*, 2001). Furthermore, as PKCδ did not mediate Bax levels, this suggests that PKCδ does not mediate the mitochondrial apoptosis signaling pathway. Further characterization of the downstream signaling pathways mediated by PKCδ will provide novel insight into cellular targets to protect pancreatic islets from cytokine induced death.

Conclusion

To summarize, we identified a role for PKCδ in mediating cytokine-induced death and have shown that inhibiting PKCδ protects pancreatic β-cells from cytokine-induced apoptosis in both mouse and human islets. Under inflammatory cytokine treatment PKCδ translocates from the cytosol to the nucleus and is cleaved by caspase-3. Cleavage by caspase-3 was also found to be required for PKCδ mediated cytokine-induced cell death. Furthermore, our data suggests that PKCδ mediates the activity of the proapoptotic JNK. Overall, the results from this study provide novel insight into the role of PKCδ in mediating cytokine-induced apoptosis in pancreatic islets and supports a role for PKCδ in mediating cytokine-induced apoptosis in T1D in humans. Furthermore, the results of this study have clinical implications as targeted inhibition of this novel PKCδ mediated apoptotic pathway may preserve β-cell mass in T1D.

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Author Contributions

J.C., N.F., M.E.R., and R.B. contributed to experimental design, J.C., N.F., and R.P., contributed to data collection, J.C., N.F., M.E.R., and R.B. contributed to data analysis, J.C. and N.F. wrote the manuscript, and J.C., R.P., M.E. R., R.B. and N.F. contributed to editing and approving the final manuscript.

Declaration of Interests

The authors declare no competing interests.

Figure Legends

Figure 1: Development of a β-cell specific inducible KO of PKCδ in C57Bl/6 mice. (A) Representative western blot of PKCδ and β-actin controls from islets isolated from two control and two age and gender matched PKCδ-βKO mice 2 weeks after tamoxifen injection. (B) Quantification of western blots from panel A of PKCδ protein normalized to β-actin in islet isolated from control and PKCδ-βKO mice two weeks after tamoxifen injection (n=3). $P<0.05$ is statistically significant based on a paired-sample t-test. (C) Blood glucose levels in 8-15 week old control and age and gender matched PKCδ-βKO mice 2 weeks after tamoxifen injection following a glucose tolerance test (n=3).

Figure 2: PKCδ Mediates Cytokine-Induced Islet Apoptosis. (A) Percent apoptotic cells in isolated control or PKCδ-βKO mouse islets treated for 24h with a cytokine cocktail compared to untreated islets in 4-5 islets per experiment (n=16). (B) Percent apoptotic cells in C57Bl/6 mouse islets treated for 24h with a cytokine cocktail and the PKCδ inhibitor δV1-1 in 4-5 islets per experiment as indicated (n=7). (C) Percent apoptotic cells in human islets from four donors treated for 24h with a cytokine cocktail and the PKCδ inhibitor δV1-1 as indicated (n=4). * Indicates significant difference from control for control and PKCδ-βKO islets respectively ($p<0.01$). $p<0.05$ indicates statistical significance based on ANOVA with Tukey's post-hoc analysis.

Figure 3: Cytokines Induce Nuclear Translocation and Activity of PKCδ. (A) Representative images of GFP-PKCδ (green) in PKCδ-βKO islets co-stained with NucRedLive (red) and either untreated or treated with cytokines for 24 h. Red arrows point to nuclei with increased fluorescence intensity compared to cytoplasm in cytokine treated samples. (B) Quantification of GFP intensity in the nucleus normalized to intensity in the cytoplasm of each cell in 4-5 islets per experiment (3h n=4, 24h n=10). (C) Quantification of GFP intensity in PKCδ-βKO islets co-stained with NucRedLive (red) and transduced with GFP only and treated with cytokines for 24 hours

(n=4). (D) Representative images YFP and CFP emission with CFP excitation using the FRET sensor for PKCδ (δCKAR) targeted to the nucleus. (E) Representative images of fluorescence lifetime imaging in MIN6 cells transfected with the FRET-based PKCδ activity sensor in E. Images on the top represent the number of photon counts and images on the bottom represent the calculated fluorescence lifetime in ns for untreated (left panel) and cytokine treated (right panel) MIN6 cells after 1.5h culture. (F) Quantification of PKCδ activity by fluorescence lifetime imaging of the FRET sensor for PKCδ activity in the nucleus in MIN6 cells either untreated or treated with cytokines for 1.5 hours (n=3). In panels B and C, $p<0.05$ indicates a significant difference as determined by ANOVA with Tukey's post-hoc analysis. In panel E, $p<0.05$ indicates a significant difference as determined by students T-test.

Figure 4: Cleavage of PKCδ is Required for Cytokine-Mediated Islet Apoptosis. (A) Representative western blots of PKCδ, β-actin, and nuclear marker histone cluster 1 H3D in lysates from human islets treated for 24 hours with or without cytokines and fractionated into cytosolic and nuclear fractions. Two bands were identified for PKCδ staining, one at 75kDa representing intact PKCδ and one at 45kDa representing a cleaved version of PKCδ. (B) Quantification of the blots in A of either intact PKCδ (75kDa) or the 45kDa PKCδ fragment represented as cytokine treated samples normalized to untreated control samples. (C) Percent apoptotic cells in mouse PKCδ-βKO islets treated for 24h with a cytokine cocktail and either GFP-only, GFP-PKCδ, or CM-GFP-PKCδ virus compared to untreated islets in 4-5 islets per experiment (n=4). * Indicates a significant difference from untreated islets ($p<0.01$). (D) Quantification of GFP fluorescence intensity in the nucleus normalized to intensity in the cytosol in PKCδ-βKO mouse islets transfected with the CM-GFP-PKCδ virus as in C and either untreated or treated with cytokines for 24h in 4-5 islets per experiment (n=3). $P<0.05$ indicates a significant difference based on ANOVA.

Figure 5: Inhibiting Caspase-3 Protects Against Cytokine Induced Apoptosis and Prevents PKCδ Cleavage. (A) Percent apoptotic cells in mouse C57Bl/6 islets treated for 24h with a cytokine cocktail and the caspase-3 inhibitor Z-DEVD-FMK as indicated compared to untreated islets (n=4). (B) Percent apoptotic cells in human islets treated for 24h with a cytokine cocktail and the caspase-3 inhibitor Z-DEVD-FMK as indicated compared to untreated islets (n=3). * Indicates a significant difference from untreated islets ($p<0.01$). (C) Representative western blots of PKCδ and β-actin in lysates from C57Bl/6 mouse islets treated with or without cytokines and with or without the caspase-3 inhibitor Z-DEVD-FMK for 24 hours as in A and B. (D) Quantification of the blots in C of intact (75kDa) PKCδ normalized to β-actin, where data was normalized to untreated controls for each experiment (n=5). (E) Quantification of the blots in C of the PKCδ fragment (45kDa) normalized to β-actin, where data was normalized to untreated controls for each experiment (n=5). (F) Quantification of the blots in C of the PKCδ fragment (45kDa) normalized to intact PKCδ (75kDa), where data was normalized to untreated controls for each experiment (n=5). In D-F, * indicates a significant difference from untreated controls as determine by 95% CI. $p<0.05$ in all panels indicates a significant difference based on ANOVA.

Figure 6: PKCδ mediates pro-apoptotic signaling. (A) Representative western blots of Bax and β-actin in lysates from control and PKCδ-βKO mouse islets treated with or without cytokines for 24 hours. (B) Quantification of the blots in A, where Bax levels were normalized to β-actin for each condition (n=9-12). (C) Representative western blot of JNK, p-JNK, and β-actin in lysates from control and PKCδ-βKO mouse islets treated with or without cytokines for 24 hours. (D) Quantification of the blots in C, where p-JNK was normalized to JNK1 (46kDa) levels (n=5-7). In panels B and D $p<0.05$ indicates a significant difference based on ANOVA with Tukey's post-hoc analysis.

Figure 7: Proposed Mechanism for PKCδ Regulation of Cytokine-Induced β-cell Death. Cytokines activate PKCδ, which in turn promotes caspase-3 activation by cleavage of pro-caspase-3. Activated PKCδ also promotes activation of JNK by phosphorylation, which translocates to the nucleus and promotes apoptosis through regulation of nuclear transcription factors. Activated PKCδ is translocated to the nucleus and cleaved by caspase-3. We propose that nuclear activity of cleaved fragment of PKCδ may also regulate nuclear transcription factors that promote apoptosis in the β-cell.

STAR Methods

Resource Availability

Lead Contact: For further information or requests for resources or reagents please contact the lead contact, Nikki Farnsworth (nfarnsworth@mines.edu).

Materials Availability: All materials, reagents, or mouse lines generated in this study will be made available upon request without restriction.

Data and Code Availability: All data or code generated in this study will be made available upon request without restriction.

Experimental Model and Subject Details

Mice: All experiments with mice were approved by the University of Colorado Denver Institutional Animal Care and Use Committee (Protocols 000929 and 00024). Animals were housed in a temperature-controlled facility with access to food and water ad libitum and were on a 12h light/dark cycle. PKCδ$^{LoxP/LoxP}$ (PKCδ$^{fl/fl}$) (Bezy *et al.*, 2011). animals were a generous gift from Dr. Ronald Kahn and mouse insulin promoter (MIP) Cre^{Er} (Wicksteed *et al.*, 2010) and C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbour, ME). To generate a β-cell specific knockout of PKCδ, PKCδ$^{fl/fl}$ and MIP-Cre^{Er} mice were bred, which we will refer to as control (PKCδ$^{fl/fl}$) and PKCδ-βKO (PKCδ$^{fl/fl}$MIPCre^{Er}). control and PKCδ-βKO were bred in house and genotyping was performed by Transnetyx (Cordova, TN) using real-time PCR with the following primer sets: Cre (Forward: TTAATCCATATTGGCAGAACGAAAAC, Reverse: CAGGCTAAGTGCCTTCTCTACA), PKCδ$^{fl/fl}$ (Forward: CTCCCCACCGATTAGTGTTGAAAA, Reverse: GGACGTAAACTCCTCTTCAGACCTA), PKCδ^{WT} (Forward: GGCAGTTATCTGACTCTTGCAGCT, Reverse: CCAAATAGGAACAACAGGTGCCTCT).

Human Islets: Human islets were obtained from the Integrated Islet Distribution Program (IIDP) from the following donors: SAMN17928660, SAMN18196260, SAMN18091343, and SAMN23079315 with an average 95 ± 3% viability and purity upon receipt.

Cell Lines: MIN6 cells were cultured in DMEM with 10% FBS and 5% penicillin/streptomycin at 37°C and were passaged at ~80% confluency using 0.25% trypsin. MIN6 cells used in this study ranged from passage 23-30 as previous studies have shown loss of glucose responsiveness after passage 35 in culture (Cheng *et al.* 2012).

Method Details

Materials: Recombinant mouse and human pro-inflammatory cytokines (TNF-α (410-MT, 210-TA), IL-1β (401-ML, 201-LB), and IFN-γ (485-MI, 285-IF) were purchased from R&D systems

(Minneapolis, MN). RPMI 1640, DMEM, Hanks Balanced Salt Solution (HBSS), fetal bovine serum (FBS), penicillin, streptomycin, YO-PRO1 (Y3603), NucBlue Live ReadyProbes Reagant (R37605), fluorescein diacetate, lipofectamine™ 3000 transfection reagent (L3000015), Halt™ Protease & Phosphatase Inhibitor Cocktail (1861281) and caspase inhibitor Z-DEVD-FMK (FMK004) were purchased from Fisher Scientific (Pittsburgh, PA). Collagenase (C9263), tamoxifen, corn oil, D-glucose, ethylenediaminetetraacetic acid (EDTA, EDS), ethylene glycol-bis(2-amino-ethylether)-N,N,N'N'-tetraacetic acid (EGTA), HEPES, Magnesium chloride hexahydrate ($H_{12}Cl_2MgO_6$), propidium iodide, were purchased from Millipore-Sigma (Saint Louis, MO). Potassium chloride was purchased from Avantor Performance Materials (Center Valley, PA). PKCδ inhibitor, δV1-1 was purchased from AnaSpec Inc. (AS-65111, Fremont, CA). Western blot buffers and reagents were purchased from Azure biosystems (Dublin, CA). Bacterial vectors encoding FRET sensors for PKCδ activity targeted to the nucleus (δCKAR) were a kind gift from Dr. Alexandra Newton (Kajimoto *et al.*, 2010).

Fasting Glucose Tolerance Test (GTT): Mice were fasted for 16hr with access to water ad libitum and fasting glucose measurements were taken from the tail vein prior to intraperitoneal injection of 200mg/kg glucose. Blood glucose was monitored over 2hr post glucose bolus via the tail vein.

Islet Isolation and Culture: Control and PKCδ-βKO mice were injected with 50mg/kg tamoxifen in corn oil once daily for five consecutive days to induce recombination. Islets were isolated from 8–12-week-old C57Bl/6 or 8–12-week-old age and sex matched control and PKCδ-βKO mice two weeks after the first tamoxifen injection. For islet isolation, animals were injected with 100mg/kg ketamine and 8mg/kg xylazine and euthanized via exsanguination. Islets were isolated by injecting the pancreas with 12.5mg/mL collagenase, pancreas removal, and subsequent enzymatic digestion at 37˚C. Islets were handpicked into 1640 RPMI Medium (Millipore-Sigma) with 10% FBS, 10,000 U/mL Penicillin and 10,000µg/mL Streptomycin and incubated overnight at 37ºC and 5% CO_2. Both mouse and human islets were cultured overnight at 37ºC and 5% CO_2 prior to use in experiments.

Adenoviral Vectors: Adenoviral vectors for expression of GFP-only, GFP fused to the c-terminus of PKCδ (GFP-PKCδ), and PKCδ with a D→A327 point mutation that can't be cleaved by caspase-3 with GFP fused to the c-terminus (CM-GFP-PKCδ) were generated by Dr. Mary Reyland as previously described (DeVries, Neville and Reyland, 2002; Humphries et al., 2008) and the expressed proteins are shown in Supplemental Figure 1. Briefly, mouse PKCδ was cloned into the pEGFP-N1 adenoviral vector and the point mutation in PKCδ in the CM-GFP-PKCδ vector was generated by PCR using the primers and site mutagenesis kit descried by DeVries, Neville, and Reyland 2002.

Islet Treatments and Viability Measurements: PKCδ-KO and control islets were cultured for 24hr either untreated or treated with a cytokine cocktail at 1X relative cytokine concentration (1 RCC, 10ng/ml TNF-α, 5ng/ml IL-1β, 100ng/ml IFN-γ). Human and C57Bl/6 mouse islets were treated with a cytokine cocktail containing human or mouse recombinant cytokines respectively and 1µM of a PKCδ inhibitor δV1-1 for 24hr. Islets were stained with either propidium iodide or an apoptotic specific stain YO-PRO1 and NucBlue or fluorescein diacetate (FDA). Imaging was performed either on a Leica STELLARIS 5 LIAchroic laser supply unit, with a 40X water immersion objective. 405nm, 488nm, and 514nm solid state lasers were used for excitation and emission was collected with HyD spectral detectors or on a Zeiss LSM 780 with 488nm or 514nm excitation laser. All islets were imaged as a Z-stack consisting of 3 images 8-10µm apart and live/dead cells were counted manually in ImageJ (NIH). To determine if caspase-3 mediates cytokine-induced apoptosis, islets from female C57Bl/6 mice or isolated human islets were cultured for 24hr with or without cytokines and with or without the caspase-3 inhibitor Z-DEVD-FMK (10µl/ml). Islets were

co-stained with YO-PRO1 (1hr) and NucBlue as described above and imaged with a Leica Stellaris confocal microscope with a 40X water immersion objective. Live and dead nuclei were manually counted in ImageJ in 5-10 islets per treatment per experiment. To determine if cleavage of PKCδ by caspase-3 mediates cytokine-induced β-cell death, PKCδ-KO islets were either untreated or treated with a GFP-only expression virus (GFP), GFP-PKCδ, or a GFP-tagged cleavage mutant of PKCδ (CM-GFP-PKCδ, 1:2000) which cannot be cleaved by caspase-3, all for 24hr with or without cytokines. Islets were co-stained with YO-PRO1 and NucBlue as described above and imaged with a Leica Stellaris confocal microscope. Live and dead nuclei were manually counted in ImageJ in 5-10 islets per treatment per experiment.

PKCδ Translocation to the Nucleus: PKCδ localization and translocation was determined with a GFP adenoviral vector, a GFP-tagged PKCδ adenoviral vector (GFP-PKCδ) or a GFP-tagged cleavage mutant of PKCδ which cannot be cleaved by caspase-3 (CM-GFP-PKCδ) for 24h. Viral vectors were generously provided by Dr. Mary Reyland and were constructed as described above. Mouse PKCδ-KO islets were used to reduce potential deleterious contributions from endogenous PKCδ. Isolated islets were cultured with GFP-PKCδ for 24 hours prior to culture for 3 or 24hr with or without cytokines. The GFP-PKCδ virus was also cultured simultaneously with cytokine treatment. Islets were co-stained with NucRed or NucBlue prior to imaging. Islets were imaged either on a Leica Stellaris confocal or a Zeiss LSM 800 confocal with 40X water immersion objective and a 405nm/488nm/514nm excitation laser for each respective dye. Nuclear translocation was determined in ImageJ by normalizing the GFP pixel intensity within each nucleus as determined by colocalization with NucRed or NucBlue, against its respective cytosolic GFP pixel intensity. The ratio of nuclear to cytosolic fluorescence intensity for all GFP-expressing cells was averaged for each islet, in 3-5 islets per experiment.

Determining Nuclear PKCδ Activity: PKCδ activity in the nucleus was determined with a PKCδ specific Förster resonance energy transfer (FRET)-based kinase activity sensor (δCKAR) containing a nuclear localization sequence, which was generously given by Dr. Alexandra Newtown and previously described (Kajimoto *et al.*, 2010). The δCKAR construct was transfected into MIN6 cells using lipofectamine™ 3000 transfection reagent per the manufacturer's instructions. Transfected islets were treated for 1.5hr with or without cytokines and changes in FRET were measured with time-resolved fluorescence lifetime imaging (FLIM) on a Zeiss LSM 780 confocal with a tunable infrared Coherent Chameleon Ultra II laser (Zeiss, Oberkochen, Germany. FLIM imaging was done in 2-photon mode. Fluorescence was excited at 720nm with fs-pulses generated by a Coherent Chameleon laser. The microscope is equipped with an ISS FastFLIM acquisition unit and a Becker and Hickl SPC-150E TCSPC acquisition card which allow for fluorescence lifetime imaging in frequency domain and time correlated single photon counting mode respectively in 2 photon imaging. FLIM images were acquired for MIN6 cells over 3 independent experiments. The average FRET-sensor lifetime in 3-10 MIN6 cells was analyzed in ISS VistaVision software with a threshold of 100-200 photon counts applied prior to lifetime calculations with a logarithmic fit and bin size of 1, and the average lifetime was calculated in a manually drawn region of interest for each cell.

Subcellular Fractionation: Subcellular fractionation was conducted as previously described on humans islets (Farnsworth *et al.*, 2016). Briefly, human islets were washed with 1X TBS and placed in cold fractionation buffer containing 20mM HEPES, 10mM KCl, 2mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM DTT and 1mM phosphatase and protease inhibitor cocktail. Samples were incubated on ice for 15min before being passed through a 25G needle 20 times, incubated on ice for 20min and centrifuged at 3,000 rpm for 5min. The supernatant, which contained the cytosolic content was transferred to a separate tube and the nuclear pellet was resuspended with fractionation buffer, passed through a 25G needle, and centrifuged at 3,000 rpm for 10min. The

supernatant was discarded, and the pellet was resuspended with TBS/0.1wt% SDS and sonicated.

Western Blotting: Mouse and human islets were cultured for 24hr with treatments as indicated. Mouse islets were washed once with PBS and lysed by sonication for 30s in lysis buffer containing 100mM NaCl, 50 mM TrisHCl, 10 mM MgCl2, 1 mM dithiothreitol, and with a protease & phosphatase inhibitor cocktail (5ul/ml). Human islets were fractionated into cytosolic and nuclear components as described above. Protein concentration was measured with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) per manufacturer's instructions. Samples were run on 4–15% mini-PROTEAN® TGX protein gels (Bio-Rad) and transferred to either a PVDF (AC2105; Azure Biosystems) or nitrocellulose (AC2107; Azure Biosystems) membrane. PVDF membranes were blocked in chemi-blot blocking buffer (AC2148; Azure Biosystems) for 2hr and probed with the following antibodies for >2hr at 4°C, all at a 1:1,000 dilution: anti-Bax (ab32503; Abcam, Cambridge, MA), anti-PKCδ (ab182126; Abcam); anti-β-actin (NC9426659; Fisher Scientific); anti-p-JNK (sc-6254, Santa Cruz, CA), and anti-JNK (sc-7345; Santa Cruz). Secondary anti-rabbit (102649-670; VWR) or anti-mouse (626520; Fisher Scientific) horseradish peroxidase-conjugated antibodies diluted 1:5000-1:10,000 for 2hr at room temperature. All membranes were imaged using an Azure c600 imaging system (AC6001; Azure Biosystems) and protein quantification was performed in ImageJ using densitometric analysis. Membranes containing fractionated samples were were stripped, and re-stained with nuclear marker histone cluster 1 H3D (sc-134355; Santa Cruz) at a 1:100 dilution and anti-mouse horseradish peroxidase-conjugated secondary diluted 1:10,000. Blot were imaged and quantified as described above.

Quantification and Statistical Analysis: Statistics were performed using Origin software (OriginLabs, Northampton, MA). Two sample t-test and one-way ANOVA with Tukey's post hoc analysis were performed as indicated. A p-value of <0.05 was considered statistically significant.

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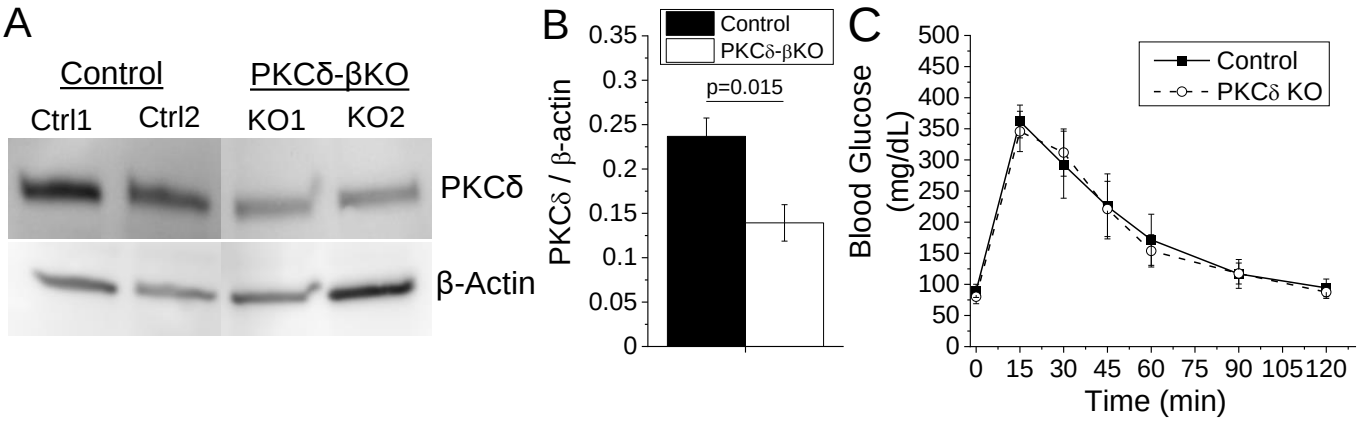
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PKCδ-βKO
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Ctrl2
KO1
KO2
PKCδ
β-Actin
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PKCδ / β-actin
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
C
Control
PKCδ KO
Blood Glucose (mg/dL)
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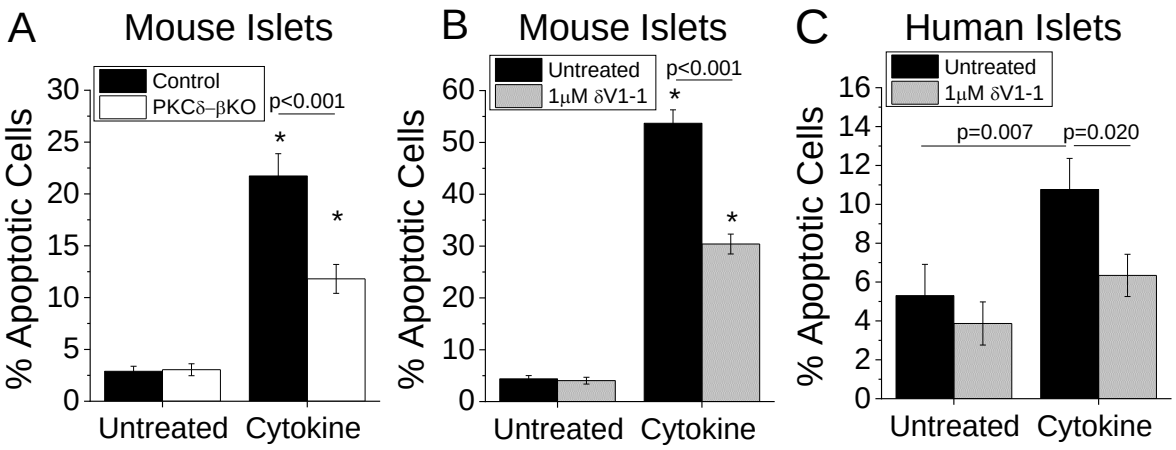
400
350
300
250
200
150
100
50
0
0 15 30 45 60 75 90 105 120
Time (min)

Figure 1


A
Mouse Islets
Control
PKCδ−βKO
p<0.001
% Apoptotic Cells
Untreated
Cytokine
B
Mouse Islets
Untreated
1μM δV1-1
p<0.001
% Apoptotic Cells
Untreated
Cytokine
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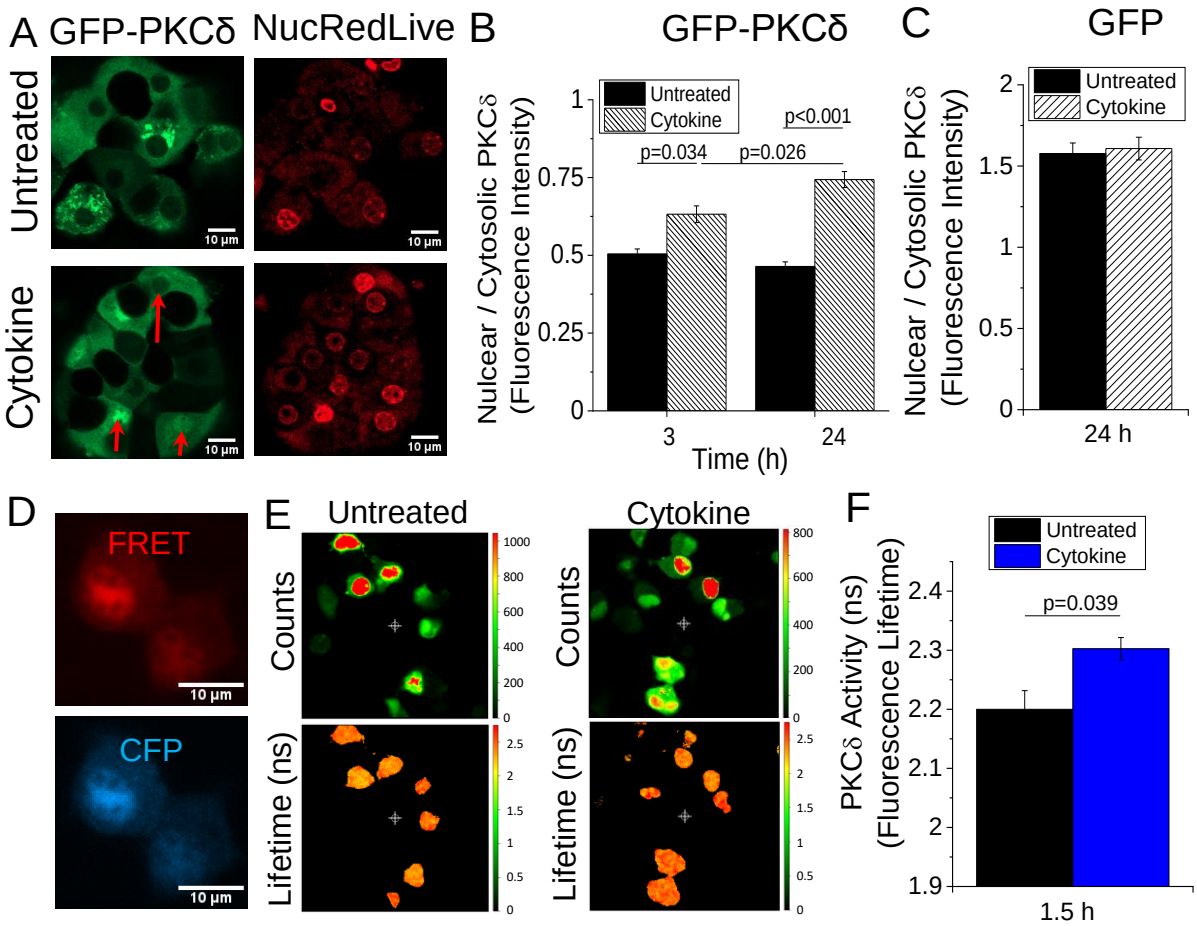
p=0.020
% Apoptotic Cells
Untreated
Cytokine

Figure 2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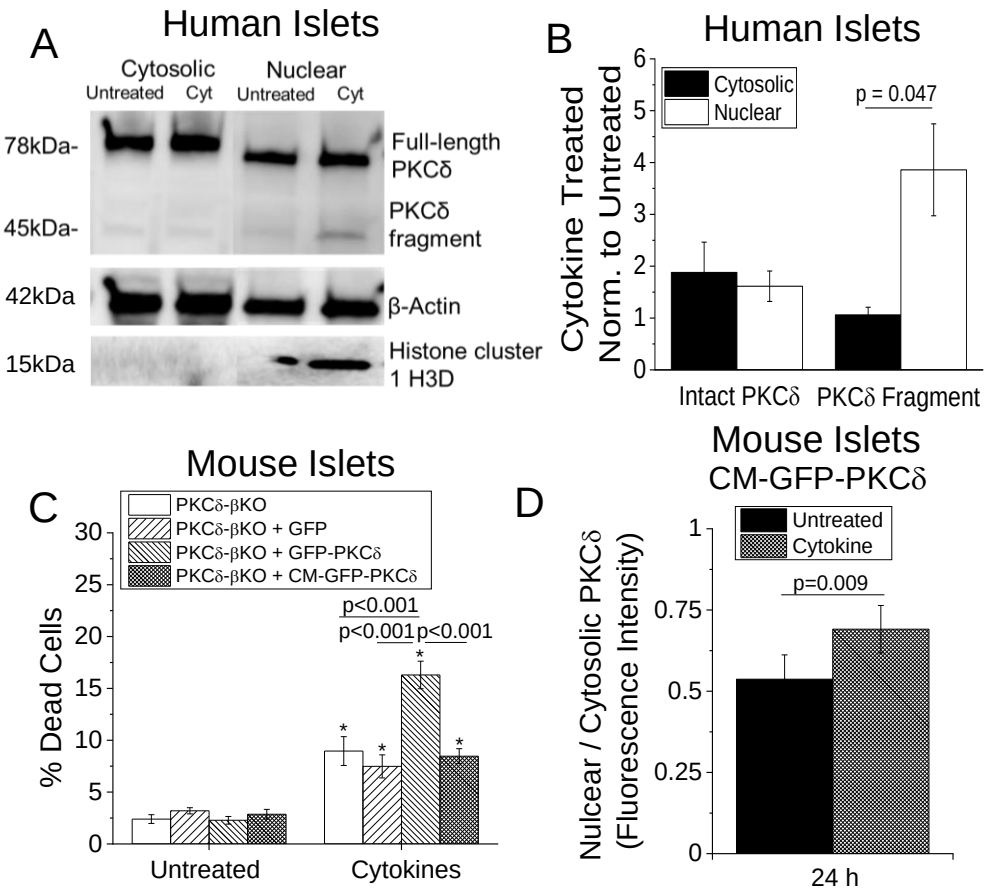
GFP-PKCδ
NucRedLive
Untreated
Cytokine
10 µm
B
GFP-PKCδ
Untreated
Cytokine
p<0.001
p=0.034
p=0.026
Nulcear / Cytosolic PKCδ (Fluorescence Intensity)
Time (h)
3
24
C
GFP
Untreated
Cytokine
Nulcear / Cytosolic PKCδ (Fluorescence Intensity)
24 h
D
FRET
CFP
E
Untreated
Cytokine
Counts
Lifetime (ns)
F
Untreated
Cytokine
p=0.039
PKCδ Activity (ns) (Fluorescence Lifetime)
1.5 h

Figure 3


A
Human Islets
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Cyt
Untreated
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78kDa-
45kDa-
42kDa
15kDa
Full-length PKCδ
PKCδ fragment
β-Actin
Histone cluster 1 H3D
B
Human Islets
Cytosolic
Nuclear
p = 0.047
Cytokine Treated Norm. to Untreated
Intact PKCδ
PKCδ Fragment
C
Mouse Islets
PKCδ-βKO
PKCδ-βKO + GFP
PKCδ-βKO + GFP-PKCδ
PKCδ-βKO + CM-GFP-PKCδ
p<0.001
p<0.001
p<0.001
% Dead Cells
Untreated
Cytokines
D
Mouse Islets
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Untreated
Cytokine
p=0.009
Nulcear / Cytosolic PKCδ (Fluorescence Intensity)
24 h

Figure 4

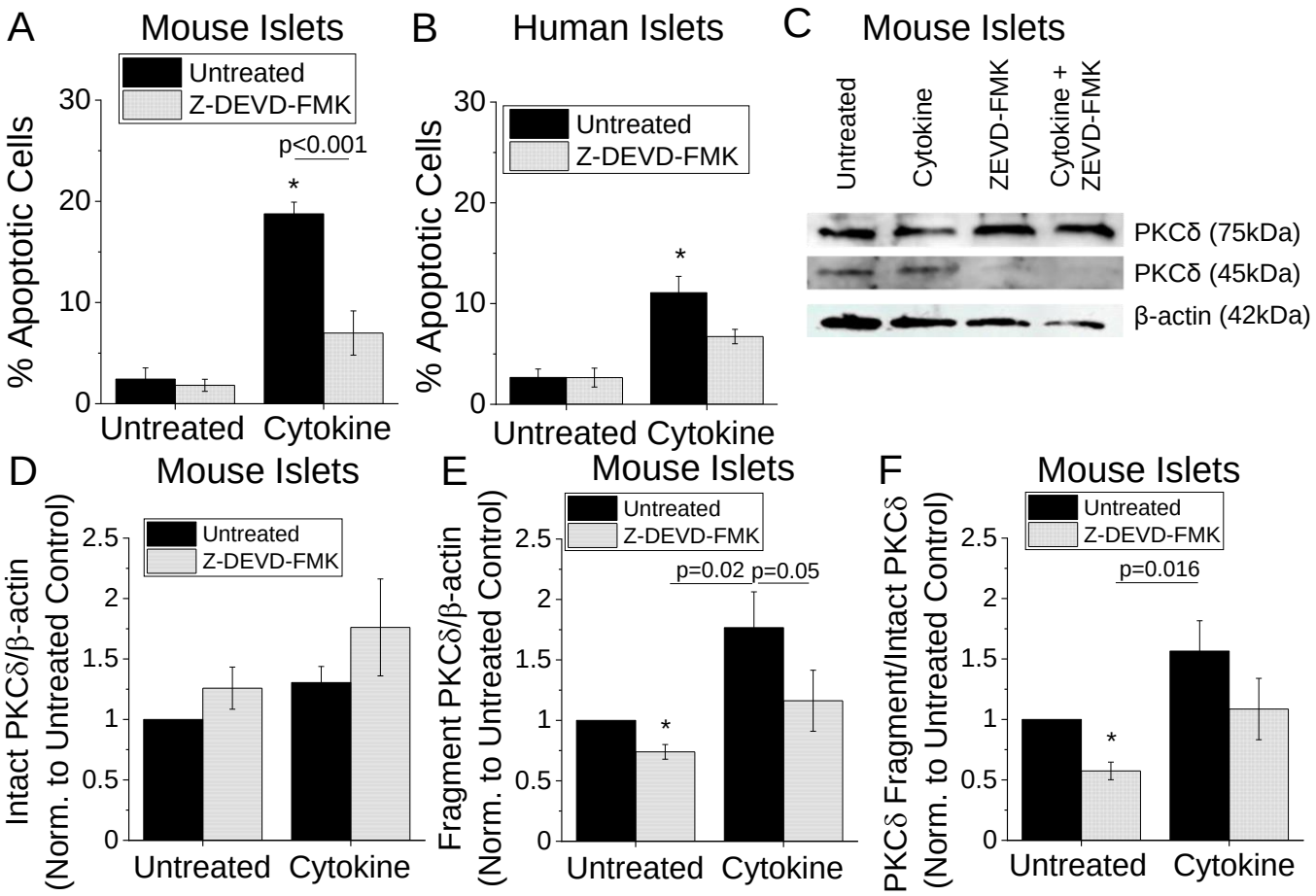

A
Mouse Islets
Untreated
Z-DEVD-FMK
% Apoptotic Cells
p<0.001
*
Untreated
Cytokine
B
Human Islets
Untreated
Z-DEVD-FMK
% Apoptotic Cells
*
Untreated
Cytokine
C
Mouse Islets
Untreated
Cytokine
ZEVD-FMK
Cytokine + ZEVD-FMK
PKCδ (75kDa)
PKCδ (45kDa)
β-actin (42kDa)
D
Mouse Islets
Untreated
Z-DEVD-FMK
Intact PKCδ/β-actin (Norm. to Untreated Control)
Untreated
Cytokine
E
Mouse Islets
Untreated
Z-DEVD-FMK
Fragment PKCδ/β-actin (Norm. to Untreated Control)
p=0.02
p=0.05
*
Untreated
Cytokine
F
Mouse Islets
Untreated
Z-DEVD-FMK
PKCδ Fragment/Intact PKCδ (Norm. to Untreated Control)
p=0.016
*
Untreated
Cytokine

Figure 5

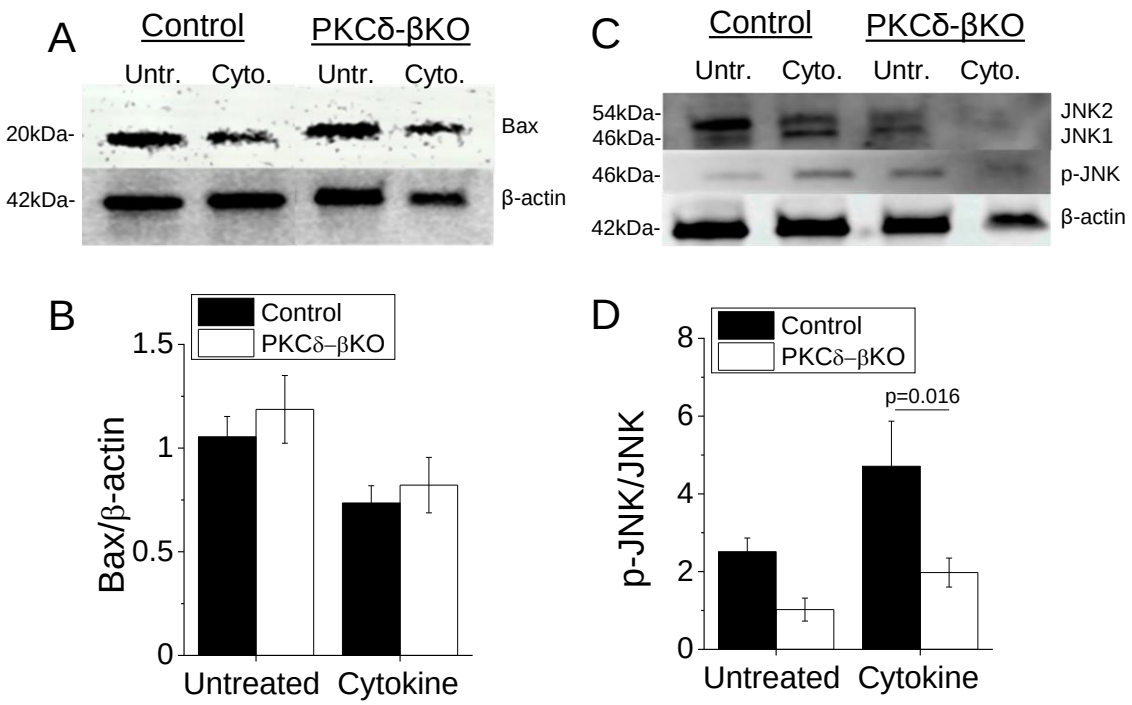

A
Control
PKCδ-βKO
Untr.
Cyto.
Untr.
Cyto.
20kDa-
42kDa-
Bax
β-actin
C
Control
PKCδ-βKO
Untr.
Cyto.
Untr.
Cyto.
54kDa-
46kDa-
46kDa-
42kDa-
JNK2
JNK1
p-JNK
β-actin
B
Control
PKCδ–βKO
Bax/β-actin
1.5
1
0.5
0
Untreated
Cytokine
D
Control
PKCδ–βKO
p-JNK/JNK
8
6
4
2
0
p=0.016
Untreated
Cytokine

Figure 6

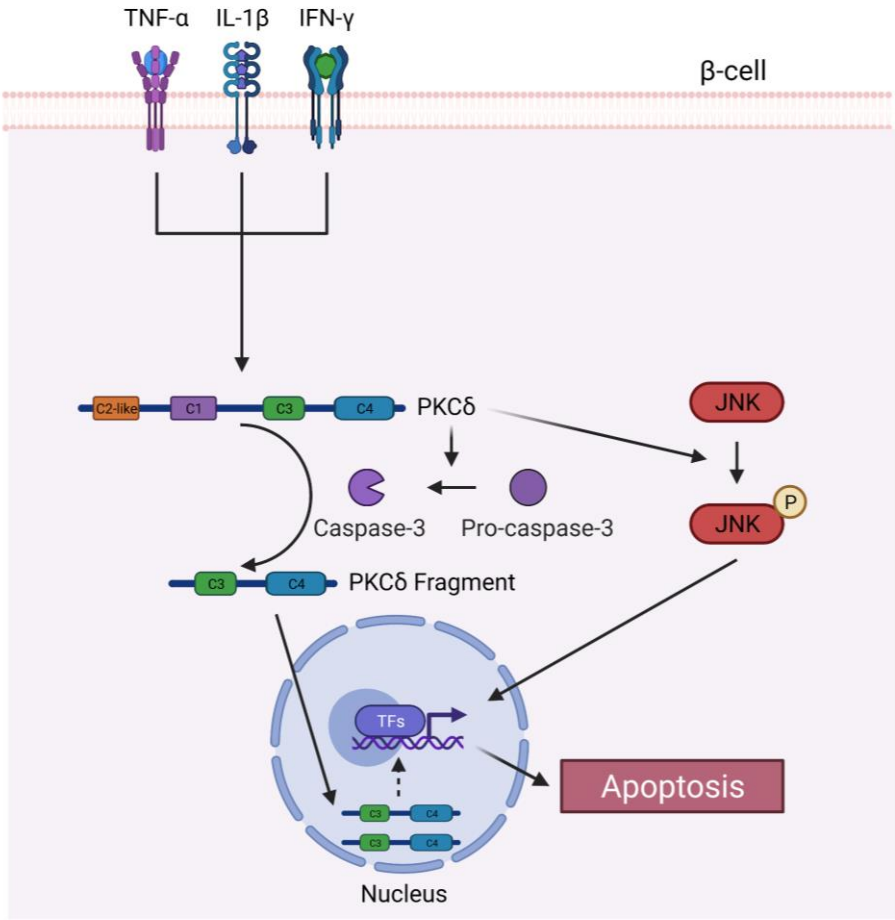
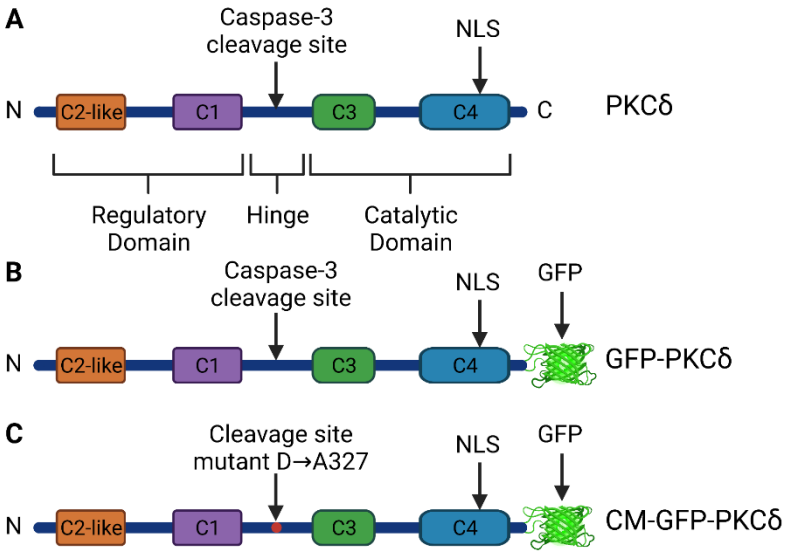

TNF-α
IL-1β
IFN-γ
β-cell
C2-like
C1
C3
C4
PKCδ
JNK
P
JNK
Caspase-3
Pro-caspase-3
C3
C4
PKCδ Fragment
TFs
Apoptosis
Nucleus

Figure 7



Supplemental Figure 1: Schematic diagrams of native PKCδ and Modified PKCδ Expressed Through Viral Vectors. (A) Native PKCδ contains regulatory and catalytic domains connected by a hinge domain. The nuclear localization sequence is located in the C4 region of the catalytic domain near the C-terminus and the site where PKCδ can be cleaved by caspase-3 is located in the hinge region. (B) The GFP-PKCδ viral construct expresses PKCδ with EGFP fused to the C-terminus of the protein. (C) The CM-GFP-PKCδ viral construct expresses PKCδ with D327 in the caspase-3 cleavage site mutated to A327 and EGFP fused to the C-terminus.