Multi-parameter single-cell kinetic analysis reveals multiple modes of cell death in primary pancreatic β-cells

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Summary

Programmed β-cell death plays an important role in both type 1 and type 2 diabetes. Most of what is known about the mechanisms of β-cell death comes from single time-point, single parameter measurements of bulk populations of mixed cells. Such approaches are inadequate for determining the true extent of the heterogeneity in death mechanisms. Here, we characterized the timing and order of molecular events associated with cell death in single β-cells under multiple diabetic stress conditions, including hyperglycemia, cytokine exposure, nutrient deprivation and endoplasmic reticulum (ER) stress. We simultaneously measured the kinetics of six distinct cell death mechanisms by using a caspase-3 sensor and three vital dyes, together with brightfield imaging. We identified several cell death modes where the order of events that usually define apoptosis were not observed. This we termed ‘partial apoptosis’. Remarkably, complete classical apoptosis, defined as cells with plasma membrane blebbing, caspase-3 activity, nuclear condensation and membrane annexin V labeling prior to loss of plasma membrane integrity, was found in only half of the cytokine-treated primary β-cells and never in cells stressed by serum removal. By contrast, in the MIN6 cell line, death occurred almost exclusively through complete classical apoptosis. Ambient glucose modulated the cell death mode and kinetics in primary β-cells. Taken together, our data define the kinetic progression of β-cell death mechanisms under different conditions and illustrate the heterogeneity and plasticity of cell death modes in β-cells. We conclude that apoptosis is not the primary mode of adult primary β-cell death.

Key words: Islet β-cells, Apoptosis, Necrosis, Live-cell imaging, Fluorescence resonance energy transfer, FRET

Introduction

Functional β-cell mass is maintained by a balance between β-cell growth, survival, proliferation, differentiation and programmed cell death (Bonner-Weir, 2000; Dor et al., 2004). Indeed, proper maintenance of β-cell mass requires low levels (∼0.1%) of β-cell apoptosis (Butler et al., 2003; Finegood et al., 1995; Scaglia et al., 1997). Increased β-cell death is present in both type 1 and type 2 diabetes. Type 1 diabetes is characterized by the specific autoimmune destruction of pancreatic β-cells by pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ) released from infiltrating T-cells (Cnop et al., 2005; Mathis et al., 2001), which leads to an almost complete ablation of β-cell mass (Meier et al., 2005; Nakanishi and Watanabe, 2008). The residual β-cell mass in patients suffering from type 1 diabetes might represent a potential source of β-cells that can be expanded by preventing β-cell death due to the ongoing autoimmune attack and by promoting β-cell proliferation (Meier et al., 2005). Additionally, because the onset of type 1 diabetes can be triggered by the initial apoptosis of β-cells, understanding the mechanisms of β-cell death might eventually allow us to target this destructive pathway to prevent or delay the onset of this disease (Mathis et al., 2001). The progression of type 2 diabetes is most commonly characterized by initial β-cell compensation, followed by a decrease in insulin secretion due, in part, to increased β-cell death (Cnop et al., 2005). As a consequence of both forms of diabetes, chronic hyperglycemia persists, and this has been demonstrated to induce further β-cell apoptosis (Federici et al., 2001; Maedler et al., 2002; Maedler et al., 2001).

Islet transplantation is a promising therapy for type 1 diabetes (Robertson, 2004). However, the high level of β-cell death during the isolation, pre-transplant culturing, transplantation procedure and post-transplant engraftment stages often dictates the success of the transplant and the requirement of islets from multiple donors (Robertson, 2004). Additionally, transplanted islets are susceptible to the recurrent autoimmune attack, and caspase-3-dependent apoptosis in the transplanted islets can be induced by the same immunosuppressants that are required to prevent graft rejection for successful islet transplantation (Johnson et al., 2009; Robertson, 2004; Shapiro et al., 2000). The rate of human β-cell death in culture can range from 2 to >20%, even in the highest quality preparations, while proliferation remains virtually absent (Alejandro and Johnson, 2008; Dror et al., 2007; Kulkarni et al., 2012). Thus, enhancing β-cell survival is critical for therapeutic interventions for both type 1 and type 2 diabetes.

Mechanistic studies on the initiation and progression of β-cell death might make significant contributions to the prevention and treatment of type 1 diabetes and type 2 diabetes, in addition to improving the success of islet transplantations. Numerous intrinsic and extrinsic signals are required for the maintenance of functional β-cell mass by providing pro-survival and pro-death...
signals. There are three commonly characterized forms of cell death distinguished by morphological features: apoptosis, necrosis and autophagic cell death (Galluzzi et al., 2009; Kroemer et al., 2009). Understanding the redundancy and exclusivity of different mechanisms of cell death has important implications for the detection and therapeutic manipulation of cell death. The assessment of the different modes of β-cell death is often misleading because it usually relies on the use of single-parameter end-point measurements in bulk cell populations.

Guidelines provided for the interpretation for different modes of cell death (e.g. apoptosis, autophagy and necrosis) require multiple readouts, but these guidelines are often overlooked and are rarely examined simultaneously in living cells (Galluzzi et al., 2009; Klionsky et al., 2008; Kroemer et al., 2009).

Here, we report a novel six-parameter live-cell imaging approach that enables single-cell analysis of multiple cell death mechanisms and the application of this approach to the study of primary and transformed pancreatic β-cells exposed to multiple...
distinct stresses. Our results illustrate the heterogeneity in cell death kinetics and demonstrate that the majority of primary β-cells die through mechanisms that are distinct from those strictly defined as apoptosis. These observations have implications for therapeutic efforts to block β-cell death.

**Results**

**Detection of apoptotic and non-apoptotic forms of β-cell death**

The potential for multiple modes of cell death within the β-cell population makes the development of therapeutics complicated. Our attempt to determine the predominant modes of cell death began with the development of a multiple-parameter live-cell assay for the detection of different phases of cell death. With the use of vital dyes and a caspase-3 activity reporter expressed under the control of the **Ins1** promoter (Fig. 1A,B), we were able to track the onset of specific events during the progression of cell death and classify the mode of cell death for individual β-cells. Culturing cells in a low concentration of Hoechst 33342 allowed us to detect nuclear condensation. Importantly, we employed a concentration of Hoechst 33342 that does not have significant effects on cell survival when compared with that of cells that were not exposed to the nuclear dye (Fig. 1C). AlexaFluor647-conjugated annexin V allowed for the detection of phosphotidylserine translocation from the inner leaflet to the outer leaflet of the plasma membrane, an early event in the classical apoptotic cascade (Koopman et al., 1994; Reutelingsperger and van Heerde, 1997). The dilution of AlexaFluor647-conjugated annexin V employed also did not induce significant levels of cell death (Fig. 1D). The detection of late-phase cell death was monitored by propidium iodide (PI) incorporation, which marks the irreversible compromise of the plasma membrane. Cells were transduced with lentiviral particles carrying the EBFP2–GFP caspase-3 fluorescence resonance energy transfer (FRET) sensor expressed under the control of the **Ins1** promoter, as well as mRFP driven by the **Pdx1** promoter (Fig. 1B). The temporal tracking of caspase-3 activation (decrease in FRET), plasma membrane blebbing (brightfield imaging), nuclear condensation, annexin V incorporation, PI incorporation, and changes in **Ins1** or **Pdx1** promoter activities can be observed in a representative MIN6 cell treated with cytokines (Fig. 1E; supplementary material Movies 1–3). Owing to interference in the red channel and the weak activation of the **Pdx1** promoter in primary cells, we were unable to simultaneously monitor **Pdx1** promoter activity and PI incorporation in primary β-cells. Nevertheless, these results clearly demonstrate that multiple molecular events in programmed cell death can be distinguished at the single-cell level.

We next sought to determine the length of time required for each cell death mechanism and the relative order in which they occur at the single-cell level. The time of the onset of plasma membrane blebbing, caspase-3 activation, PI incorporation and nuclear condensation was assessed relative to that of annexin V incorporation, and 3D dot plots of these results in individual cells were made, allowing for the identification of different modes of cell death (Fig. 2A,B). Cells that underwent classical apoptosis were defined as undergoing plasma membrane blebbing, caspase-3 activation, nuclear condensation and annexin V incorporation prior to PI incorporation. Cells that underwent ‘partial apoptosis’ displayed PI incorporation simultaneously with one or more of the other events. Cells lacking all apoptotic features prior to PI incorporation were considered necrotic. Individual primary mouse β-cells undergoing different types of cell death were categorized (Fig. 3A–D). Of the apoptotic cells analyzed, plasma membrane blebbing always occurred first, followed by nuclear condensation and caspase-3 activation. Annexin V incorporation occurred last before PI incorporation in all apoptotic cells (Fig. 3A). Complete loss of GFP was used as an indication of protein loss, which was often coincident with PI incorporation. Caspase-3 activation is

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**Fig. 2. Different modalities of β-cell death identified through single-cell analysis of the morphological features of cell death.** (A,B) Dispersed mouse islet cells stably expressing the β-cell-specific caspase-3 reporter and stained with Hoechst, PI, and AlexaFluor647-conjugated annexin V were imaged for 60 hours. Cells were treated in 5 or 20 mM glucose RPMI medium with cytokines (25 ng/ml TNF-α, 10 ng/ml IL-1β, and 10 ng/ml IFN-γ), 1 μM thapsigargin or serum-free conditions. β-cells that underwent cell death during the time course were analyzed for the time of onset of plasma membrane blebbing, nuclear condensation, caspase-3 activation, annexin V incorporation and PI incorporation. 3D plots of the time of onset of the indicated cell death events relative to the onset of annexin V incorporation for individual primary mouse β-cells are shown (scales are in hours).
often associated with apoptosis (Kroemer et al., 2009). We found two types of distinct ‘partial apoptotic’ cell death involving caspase-3 activation (Fig. 3B,C). The first form of partial apoptosis involved plasma membrane blebbing and caspase-3 activation with a lack of nuclear condensation (Fig. 3B; supplementary material Movie 4). The second form of partial apoptosis manifested as nuclear condensation, caspase-3 activation and prolonged period of oncosis with a lack of plasma membrane blebbing (Fig. 3C; supplementary material Movie 5). We also identified a small number of cells undergoing necrotic cell death, which lacked any of the apoptotic cell death features including caspase-3 activation (Fig. 3D; supplementary material Movie 6). Collectively, our data suggest that nuclear condensation, caspase-3 activation and plasma membrane blebbing exist in both apoptotic and partial apoptotic cell death (Fig. 4). Annexin V incorporation rarely occurred in cells undergoing partial apoptotic cell death (Fig. 4B). Perhaps, cells that were initially undergoing apoptosis were re-routed to other forms of cell death owing to the cellular state induced by specific stress conditions.

Temporal progression of cell death events in distinct stress conditions
It is not known whether the time courses of specific cell death events or the temporal relationships between cell death events are dependent on the type of stress. We asked this question by exposing primary β-cells to hyperglycemia, cytokines, endoplasmic reticulum (ER) stress and/or nutrient deprivation, and determining the onset of cell death events relative to that of annexin V incorporation (Fig. 5). Detection of plasma membrane blebbing under all conditions was variable. The delay in the initiation of nuclear condensation and caspase-3 activation
following plasma membrane blebbing was dependent on the treatment conditions. Often cells with concurrent annexin V and PI incorporation did not display plasma membrane blebbing, suggesting activation of non-apoptotic pathways. Depending on the treatment conditions, the average length of time between the detection of the first cell death morphological feature and last phase of cell death (loss of membrane integrity) was between 10 and 20 hours. Remarkably, some cells initiated cell death up to 2 days prior to the loss of membrane integrity. Significant differences can also be observed between the absolute time of onset of different events for cells treated under low- and high-glucose conditions (Fig. 6). These data provide evidence of a critical window of time for reversing cell death after it has been initiated.

**Primary β-cells predominantly undergo non-apoptotic cell death**

Apoptosis is the main mode of cell death commonly assessed in the β-cell field (Chu et al., 2010; Cistola and Small, 1991; Cnop et al., 2005; Federici et al., 2001; Maedler et al., 2001; Maedler et al., 2002; Maedler et al., 2001; Mathis et al., 2001), whereas contributions from other modes of cell death are often overlooked. Using our multiple-parameter single-cell analysis, we were able to distinguish between apoptotic, partial-apoptotic and non-apoptotic modes of primary β-cell death, which cannot be disassociated using cell population analyses of cell death (Fig. 7A,B). We found changes in the relative proportion of β-cells undergoing cell death displaying all four to none of the apoptotic features assayed under the different stress conditions (Fig. 7C). Cells were considered apoptotic if they underwent all four apoptotic morphological and/or biochemical changes (plasma membrane blebbing, nuclear condensation, caspase-3 activation, and annexin V incorporation) prior to PI incorporation. Using these stringent criteria, we determined that primary mouse β-cells predominately undergo non-apoptotic cell death in low-glucose conditions (Fig. 7D). Under conditions of ER-stress or serum-withdrawal, no cells were observed to follow the canonical apoptotic pathway. Interestingly, under serum-containing conditions, exposure to high glucose or a cytokine cocktail of TNF-α, IL-1β and IFN-γ increased the proportion of primary β-cells undergoing apoptotic cell death. Similarly, high glucose increased the proportion of apoptosis in cells with thapsigargin-induced ER stress from 0% to 16% (Fig. 7D). As a control to demonstrate that our multi-parameter assay could detect
multiple modes of cell death, including complete classical apoptosis, the same study was conducted using the MIN6 β-cell line, which has been characterized as predominantly undergoing apoptotic cell death. Indeed, under the same stress conditions, MIN6 cells predominantly underwent complete classical apoptosis (Fig. 7E–H). Taken together, these data demonstrate that primary mouse β-cells and the MIN6 cell line do not undergo classically defined apoptosis to the same extent, and glucose controls the mode of programmed cell death exhibited by individual primary β-cells.

**Discussion**

Apoptosis is the most well-studied mode of β-cell death and it is characterized by a stereotypical order of molecular events. Few, if any studies, have examined whether the apoptotic series of events occurs in single cells. In the present study, we clearly demonstrated that most pancreatic β-cells undergo forms of cell death that differ from strictly defined apoptosis. Our studies began with the elucidation of the complex and variable interplay between cell death mechanisms within single β-cells. The progression of β-cell death events was simultaneously assessed using a live-cell assay that detected the temporal induction of plasma membrane blebbing, nuclear condensation, caspase-3 activation, annexin V incorporation, PI incorporation and loss of GFP. We characterized the kinetic interrelationships between cell death events in the context of multiple diabetes-associated conditions that can trigger the onset of β-cell death, including prolonged exposure to hyperglycemia, inflammatory cytokines and ER stress (Chu et al., 2010; Cistola and Small, 1991; Cnop et al., 2005; Federici et al., 2001; Maedler et al., 2001; Mathis et al., 2001). Although some kinetic features were common to all forms of β-cell death, we also found significant differences in event timing and order between distinct stresses.

The molecular mechanisms and progression of programmed cell death through apoptosis have been characterized, mostly on
the basis of bulk cell population biochemical and flow cytometry studies, as a series of events preceding the loss of plasma membrane integrity. The widely accepted temporal and mechanistic model of apoptosis posits that plasma membrane blebbing, chromatin condensation and fragmentation, rounding up of the cell, and cellular and nuclear volume reduction all occur prior to the final loss of membrane integrity, which can be imaged by the incorporation of normally membrane-impermeant dyes, like PI, within the nucleus (Kroemer et al., 2009).

Apoptosis can be triggered by two distinct signaling networks that share many regulatory components. Extrinsic apoptosis proceeds when death receptors at the plasma membrane are activated by extracellular ligands, including TNF-α, Fas ligand and TRAIL (Mathis et al., 2001; Spencer and Sorger, 2011). Following assembly of the death-inducing signaling complexes, initiated at the cytoplasmic tails of the death receptors, initiator procaspases-8 and -10 are activated, which in turn activates effector procaspases-3 and -7 to generate active proteases (Spencer and Sorger, 2011). Intrinsic apoptosis results from exposure to ultraviolet light, cellular stress and toxins. The cascade of signaling events that follows often involves the activation of pro-apoptotic Bcl-2 family members (Bax, Bak, Bad, Bid), triggering mitochondrial outer membrane permeabilization prior to the activation of caspases (Kroemer et al., 2009; Mathis et al., 2001; Spencer and Sorger, 2011). Studies by our group and others have also focused on the role of ER and cytosolic Ca^{2+} dynamics in β-cell survival and function upon the initiation of the intrinsic apoptotic pathway (Dror et al., 2008; Gwiazda et al., 2009; Luciani et al., 2009; Varadi and Rutter, 2002). We have previously noted robust glucose-dependent differences between the mechanisms of β-cell death with respect to the involvement of notch signaling, netrin signaling, carboxypeptidase E, ATP-citrate lyase, Uchl1 and intracellular Ca^{2+} release channels (IP3R, RyR) (Chu et al., 2012; Chu et al., 2010; Dror et al., 2008; Dror et al., 2007; Jeffrey et al.,

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**Fig. 6.** Stress treatments influenced the absolute onset of cell death molecular events in single mouse β-cells. (A,B) Mouse β-cells carrying a caspase-3 sensor were stained with Hoechst, PI and AlexaFluor647-conjugated annexin V. Cells were treated with the indicated treatments under 5 mM (A) and 20 mM glucose (B) and imaged for 60 hours at 37˚C and 5% CO₂. The absolute time for the onset of plasma membrane blebbing, nuclear condensation, caspase-3 activation, annexin V incorporation, PI incorporation and protein loss were determined (n=15–63, mean±s.e.m., * P<0.05 compared with 5 mM glucose).
Consistent with other reports, glucose does indeed modulate β-cell death levels (Federici et al., 2001; Hoorens et al., 1996; Maedler et al., 2001; Van de Casteele et al., 2003). Here, we found differences in the kinetics and mode of the core cell-death events between cells treated in low glucose and cells treated in high glucose. To the best of our knowledge, this is the first comprehensive single-cell analysis of β-cell death modulation by glucose.

The roles of signaling molecules involved in the temporal cascade of events leading to β-cell apoptosis, autophagy and necrosis have not been well characterized at the level of individual cells, resulting in the under-appreciation of non-apoptotic and pseudo-apoptotic forms of cell death. Notwithstanding, β-cell death through necrosis has also been implicated in the pathogenesis of diabetes (Fujimoto et al., 2010; Steer et al., 2006). Necrosis is often defined as cell death lacking the characteristics of apoptosis or autophagy. In addition, key morphological features of necrosis include plasma membrane rupture and swelling of cytoplasmic organelles (Golstein and Kroemer, 2007; Kroemer et al., 2009). Although initially believed to be an uncontrolled form of cell death, there is accumulating evidence supporting the notion that necrotic cell death is regulated by a defined set of signaling events induced by oxidative stress, loss of Ca²⁺ homeostasis and/or ischemia (Golstein and Kroemer, 2007; Kroemer et al., 2009). In the conditions tested, a small fraction of β-cells underwent classical necrosis, characterized by oncosis, plasma membrane rupture and the lack of apoptotic features (including plasma membrane blebbing, nuclear condensation, caspase-3 activation and annexin V incorporation) prior to loss of plasma membrane integrity. However, we were able to identify other forms of partial apoptotic cell death that shared some of the apoptotic characteristics (Golstein and Kroemer, 2007; Kroemer et al., 2009). The term necroptosis has been used to describe similar atypical apoptosis-like forms of programmed cell death (Osborn

Fig. 7. The level of different β-cell death modalities is determined by specific stress treatments. Primary mouse β-cells or MIN6 cells carrying a caspase-3 sensor were stained with Hoechst, PI and AlexaFluor647-conjugated annexin V. Cells were imaged with Molecular Devices ImageXpress Micro at 37°C and 5% CO₂. Cells were treated in 5 or 20 mM glucose RPMI medium with cytokines (25 ng/ml TNF-α, 10 ng/ml IL-1β and 10 ng/ml IFN-γ), 1 μM thapsigargin or serum-free conditions. (A,B) Population analysis of islet cell death represented by the percentage of PI-positive cells (n=8–30, mean±s.e.m.). (C) The proportion of primary β-cells displaying zero to four of the apoptotic features (n=15–63). (D) The percentage of apoptotic and non-apoptotic (includes partial apoptotic and necrotic cells) cell death within the pool of primary β-cells that died during the time course (n=3–4, mean±s.e.m., *P<0.05 compared with serum-containing treatment with the same glucose level). (E,F) Population analysis of MIN6 cell death represented by the percentage of PI-positive cells (n=3–11, mean±s.e.m.). (G) The proportion of MIN6 cells displaying zero to four of the apoptotic features (n=17–30). (H) The percentage apoptotic and non-apoptotic cell death within the pool of MIN6 cells that died during the time course (n=3–4, mean±s.e.m.).
et al., 2010; Vandenabeele et al., 2010). Without characterization of additional mechanisms at the single-cell level, we were not able to further classify these cell death modalities or determine their precise molecular mechanisms. We speculate that apoptosis was initially triggered in some of the cells but, owing to the change in cellular environment (e.g. lack of sufficient ATP, loss of Ca\(^{2+}\) homeostasis, or ischemia), apoptosis could not proceed to completion. Not surprisingly, apoptosis and necrosis sometimes share common signaling pathways involving mitochondrial membrane permeabilization through activation of proapoptotic Bcl-2 family members (Golstein and Kroemer, 2007; Kim et al., 2003). When exposed to stress conditions, direct inhibition of caspase activation or depletion of ATP (required for caspase activation) can inhibit apoptotic signaling and favor necrotic cell death, suggesting that cell death modalities co-exist within the same cell and have the potential to substitute for each other (Eguchi et al., 1997; Kim et al., 2003; Leist et al., 1997). In our studies of individual \(\beta\)-cells, partial apoptotic cell death played a major, if not exclusive, role in \(\beta\)-cell death under all the diabetes-related stress conditions tested. Additionally, we were able to determine the relative onset of each cell death feature and quantify their contribution to cells undergoing ‘partial apoptosis’ by tracing single cells over time. The complex interplay between different modes of cell death makes the development of therapeutics for preventing \(\beta\)-cell death more complicated.

Therapeutic interventions preventing \(\beta\)-cell death have the potential to treat diabetes. When looking at nuclear condensation or caspase-3 activation alone, our data are consistent with others estimating the duration of apoptosis in \(\beta\)-cells to be 2.5 hours under unstressed conditions or 90–110 minutes under stressed conditions (Köhler et al., 2003; Saisho et al., 2009). When we define the duration of \(\beta\)-cell death as the length of time between the detection of the first cell death morphological feature and last phase of cell death (loss of membrane integrity), we determined that the duration of cell death was usually between 10 and 20 hours. In some cells, plasma membrane blebbing was initiated up to 2 days prior to the loss of membrane integrity. Thus, we provided evidence of a critical therapeutic window of time for reversing cell death after it has been initiated. Comprehensive understanding of the different modes of \(\beta\)-cell death and the functional state of \(\beta\)-cells under varying stress conditions will provide mechanistic insight into diabetes initiation and progression.

Materials and Methods

Primary islet isolation and cell culture

Pancreatic islets were isolated from 12- to 20-week-old male and female C57BL/6J mice (Ax, Bar Harbor, MA, USA) using collagenase and filtration. Mice were housed in accordance with the University of British Columbia Animal Care Committee guidelines. The islets were further handpicked using a brightfield microscope. Islets were cultured overnight (37 \(^\circ\)C, 5% CO\(_2\)) in RPMI1640 medium (Invitrogen, Burlington, ON, Canada) with 9.9 mM glucose (Sigma), 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin (Invitrogen) and 10% (v/v) FBS (Invitrogen).

Preparation of lentiviral particles

Viral particles were prepared as described (Curran and Nolan, 2002; Szabat et al., 2009). Briefly, HEK293T cells at 60% confluency were transfected with CPRAEv, pCI-VSFG and FIV-pTiger-triple reporter constructs using FuGENE6 (Roche) under serum-free and antibiotics-free conditions. At 48 and 72 hours following transfection, viral particles were collected from the culture medium and concentrated by centrifugation at 50,000 \(\times\) g. The viral pellet was resuspended in Tris-NaCl-EDTA buffer (50 mM Tris, 100 mM NaCl, 0.5 mM EDTA, pH 7.4) and stored at −80 \(^\circ\)C. Concentrated virus were titrated in MIN6 cells infected with a serial dilution of the virus particles in 96-well plates and imaged for reporter gene expression on ImageXpress\(\textsuperscript{\textregistered}\) (Molecular Devices, Sunnyvale, CA, USA) 48 hours following infection.

Multi-parameter time-lapse cell death assay

For cell death assays, MIN6 and dispersed islet cells were seeded into 96-well plates and infected with lentiviral particles carrying the triple reporter construct at MOI of 5. Cell death experiments were conducted 48 hours following lentiviral infection. Cells were stained with 50 ng/ml Hoechst 33342 (Invitrogen), 0.5 \(\mu\)g/ml propidium iodide (PI, Sigma) and 3:500 dilution of annexin V conjugated to AlexaFluor647 (Invitrogen). Following treatments, cells were imaged with ImageXpress\(\textsuperscript{\textregistered}\) (Molecular Devices) every 5–15 minutes at 37 \(^\circ\)C and 5% CO\(_2\). Data are expressed as mean±s.e.m. unless otherwise indicated. Results were considered statistically significant when \(p<0.05\) using Student’s \(t\)-test or ANOVA, where appropriate (GraphPad Prism; GraphPad, La Jolla, CA).

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Author contributions

Y.H.C.Y. designed research, performed research, analyzed data and wrote the manuscript. J.D.J. designed research, edited the manuscript and takes full responsibility for all results.

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References


Death kinetics in single β-cells
Movie 1. Live cell kinetic analysis of cell death in MIN6 β-cells. MIN6 cells stably expressing RFP under the Pdx1 promoter and caspase-3 eBFP-devd-eGFP FRET sensor under the Ins1 promoter were stained with Hoechst, PI, and annexinV-647. The cells were treated with a cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ) and imaged every 5 min for 49 hours. Representative single cell movies were taken 13 hr 20 min following treatment with cytokine cocktail. The various fluorescent dyes and sensors are spectrally and spatially distinguishable to allow for the detection of different modes of cell death and maturation states. Ratiometric imaging of caspase-3 FRET sensor. The FRET probe is cleaved upon activation of caspase-3 and -7 leading to the loss of FRET between eBFP2 and eGFP. FRET with eGFP was measured and normalized to eBFP2 emission intensity.

Movie 2. Live cell kinetic analysis of cell death in MIN6 β-cells. MIN6 cells stably expressing RFP under the Pdx1 promoter and caspase-3 eBFP-devd-eGFP FRET sensor under the Ins1 promoter were stained with Hoechst, PI, and annexinV-647. The cells were treated with a cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ) and imaged every 5 min for 49 hours. Representative single cell movies were taken 13 hr 20 min following treatment with cytokine cocktail. The various fluorescent dyes and sensors are spectrally and spatially distinguishable to allow for the detection of different modes of cell death and maturation states. Fluorescent imaging of Hoechst (blue), annexinv-647 (green), and PI (red) combined with brightfield imaging.
Movie 3. Live cell kinetic analysis of cell death in MIN6 β-cells. MIN6 cells stably expressing RFP under the Pdx1 promoter and caspase-3 eBFP-devd-eGFP FRET sensor under the Ins1 promoter were stained with Hoechst, PI, and annexinV-647. The cells were treated with a cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ) and imaged every 5 min for 49 hours. Representative single cell movies were taken 13 hr 20 min following treatment with cytokine cocktail. The various fluorescent dyes and sensors are spectrally and spatially distinguishable to allow for the detection of different modes of cell death and maturation states. Fluorescent imaging of Hoechst (blue), Ins1 promoter activity (green), and Pdx1 promoter activity (red) combined with brightfield imaging.

Movie 4. Live cell kinetic analysis of cell death in primary mouse β-cells. Dispersed mouse islet cells stably expressing the β-cell specific caspase-3 reporter and stained with Hoechst, PI and annexinV-647 were treated with a cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ) and imaged every 12 min for 60 hours. Representative single cell movies were taken 7 hr 39 min following treatment with cytokine cocktail. Fluorescent imaging of Hoechst (blue), annexinV-647 (green), PI (red), and GFP (grey). Representative single cell movie of a cell undergoing partial-apoptosis.
Movie 5. Live cell kinetic analysis of cell death in primary mouse β-cells. Dispersed mouse islet cells stably expressing the β-cell specific caspase-3 reporter and stained with Hoechst, PI and annexinV-647 were treated with a cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ) and imaged every 12 min for 60 hours. Representative single cell movies were taken 7 hr 39 min following treatment with cytokine cocktail. Fluorescent imaging of Hoechst (blue), annexinV-647 (green), PI (red), and GFP (grey). Representative single cell movie of a cell undergoing partial-apoptosis.

Movie 6. Live cell kinetic analysis of cell death in primary mouse β-cells. Dispersed mouse islet cells stably expressing the β-cell specific caspase-3 reporter and stained with Hoechst, PI and annexinV-647 were treated with a cytokine cocktail (25 ng/mL TNF-α, 10 ng/mL IL-1β, 10 ng/mL IFN-γ) and imaged every 12 min for 60 hours. Representative single cell movies were taken 7 hr 39 min following treatment with cytokine cocktail. Fluorescent imaging of Hoechst (blue), annexinV-647 (green), PI (red), and GFP (grey). Representative single cell movie of a cell undergoing necrosis.