UNDERSTANDING AND PREVENTING AUTOIMMUNE BETA CELL DESTRUCTION: PROTECTION PROVIDED BY *mt-ND2*[a]

By

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To my Family

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LIST OF ABBREVIATIONS

$\Delta\Psi m$	Mitochondrial membrane potential
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BB-DP	BioBreeding-diabetes-prone
CAD	Caspase activated deoxyribonuclease
CML	Cell-mediated lymphocytotoxicity
CTL	Cytotoxic T lymphocyte
Cyt c	Cytochrome c
DC	Dendritic cell
DMPK	Dystrophia myotonica-protein kinase
Endo G	Endonuclease G
ER	Endoplasmic reticulum
FADD	Fas-Associated Death Domain
FPIR	First phase insulin release
GAD	Glutamic acid decarboxylase
GLUT	Glucose transporter
GSH	Glutathione
GSIS	Glucose stimulated insulin secretion
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HLA	Human leukocyte antigen
HMGB1	High-mobility group box 1

HSP	Heat shock protein
IAP	Inhibitor of apoptosis
ICA	Islet cell antibody
IFNγ	Interferon gamma
IGRP	Glucose-6-phosphatase catalytic subunit-related protein
IL-1β	Interleukin 1 beta
IL-1R	Interleukin 1 receptor
INS	Insulin
iNOS	Inducible form of nitric oxide synthase
K_{ATP}	ATP-sensitive potassium [channel]
MHC	Major histocompatibility complex
MODY	Maturity-onset diabetes of the young
MOMP	Mitochondrial outer membrane permeabilization
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial ROS
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
NOD	Non-obese diabetic
$^{\cdot}OH$	Hydroxyl radical
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cell
PFA	Paraformaldehyde
PHHI	Persistent hyperinsulinemic hypoglycemia of infancy
PPI	Preproinsulin
PTPRN	Protein tyrosine phosphatase, receptor type, N

RIP	Rat-insulin promoter
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SO_2^-	Superoxide
STZ	Streptozotocin
SUR	Sulfonylurea receptor
T1D	Type 1 diabetes
TCA	Tricarboxylic acid cycle
TCR	T cell receptor
TMRM	Tetramethylrhodamine methyl ester
TNFα	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
VNTR	Variable number of tandem repeats
ZnT8	Solute carrier family 30, member 8
Z-VAD-FMK	Pan-caspase inhibitor

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UNDERSTANDING AND PREVENTING AUTOIMMUNE BETA CELL DESTRUCTION: PROTECTION PROVIDED BY *mt-ND2*^{a}

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Mitochondria are indispensable in the life and death of many types of eukaryotic cells. In pancreatic β cells, mitochondria play an essential role in insulin secretion, a hormone that regulates blood glucose levels. Unregulated blood glucose is a hallmark symptom of diabetes. The onset of type 1 diabetes (T1D) is preceded by autoimmune-mediated destruction of β cells. Predisposition for T1D is in part determined by genetic factors. While many of the proposed causative genes for T1D impact immune function/regulation, the contribution of T1D-susceptibility or resistance genes at the β cell level cannot be excluded at this time. Particularly, the effect of T1D-associated sequence variation in the mitochondrial DNA (mtDNA) and the role of mitochondria in human β cell death have not been assessed. Therefore, the importance of mitochondria in immune destruction of the human β cell line, βLox5, and two derivative cell lines was investigated. Specifically, the contribution of two mitochondrial alleles, *mt-ND2*$^{a/c}$, to the response of these cell lines to pro-death effectors was examined.

Previously, our group defined a mutation in the mouse mitochondrial gene *mt-Nd2* that prevents T1D. A single nucleotide transversion, C4738A (mouse) and C5178A (man), causes an amino acid change that is associated with T1D resistance. Although the mouse *mt-Nd2*^{a} allele has been shown to decrease mitochondrial reactive oxygen species (mtROS) production after damage, no published data exist on the protective mechanism provided by the human ortholog, *mt-ND2*^{a}.

Similar to islet cells, βLox5 cells express common T1D autoantigens, are targeted by autoreactive effectors, and die by mechanisms implicated in disease progression. Thus, this human β cell line is a practical source of human β cells for cytotoxicity assays. Cytoplasmic hybrids (cybrids) encoding either *mt-ND2*^{c} (βLox5-*ND2*^{c}) or *mt-ND2*^{a} (βLox5-*ND2*^{a}) were developed after depleting βLox5 cells of their mtDNA, followed by fusion with donor platelets. Compared to βLox5-*ND2*^{c}, βLox5-*ND2*^{a} was more resistant to cytokines, Fas-induced killing, as well as autoreactive $CD8^{+}$ T cells. Resistance correlated with lower levels of mtROS generation. These results indicate that, like in the mouse, *mt-ND2*^{a} protects human β cells from insults associated with T1D by suppressing mtROS production in response to pro-death signals.

CHAPTER 1
LITERATURE REVIEW

Type 1 Diabetes

Type 1 diabetes (T1D) is a chronic, multifactorial disorder that results from interplay of genetic and environmental factors. T1D accounts for 5-10% of reported cases of diabetes, representing approximately 2 million individuals in the United States. Autoimmune attack and functional inhibition of the insulin-producing β cells in the pancreas leads to the inability of β cells to metabolize glucose, and thus results the hallmark clinical symptom of diabetes: abnormally high blood glucose levels. During the course of the disease, T lymphocytes become reactive to β cell antigens and islet cell antibodies produced by B cells are also detected. High titers of autoantibodies correlate with T1D, making them valuable prognostic markers for disease risk. Subsequent to the measurement of autoantibodies against β cell antigens, a decline in first phase insulin release (FPIR) can be measured in those at risk for developing T1D, allowing further susceptibility to be determined [1]. However, the development of the destructive pathological lesion, known as insulitis, and the steps leading to T1D in humans are not well understood. Identification and study of immune cell infiltration in T1D patients has been problematic [2]. As a result, the majority of our knowledge of the pathology of T1D stems from animal models that develop insulin-requiring diabetes, either spontaneous autoimmune or experimentally-induced, as well as from *in vitro* studies using primary islets and β cell lines, from human and murine sources. *In vitro* experiments are particularly advantageous when assessing the specific contributions of individual effector molecules and molecular pathways to β cell destruction. A breakdown of self-tolerance renders β cells susceptible to an arsenal of immune cells and their killing

mechanisms; each must then be analyzed independently in order to formulate targeted treatment options.

Cellular Effectors in T1D Development

Immunohistological examination of pancreatic tissues from patients with T1D has demonstrated that, in contrast to the animal models of spontaneous T1D, insulitis is a rare event in humans [2]; when present, the following cell types have been identified in the islets: lymphocytes that consisted mostly of $CD8^+$ T cells but include B cells as well as $CD4^+$ T cells, macrophages, and dendritic cells (DCs) [3-6]. Unfortunately, human samples with established T1D do not delineate the successive events that culminate in autoreactive lymphocyte activation and β cell killing, and only recently has information emerged on the nature of insulitis in T1D-free autoantibody positive organ donors [2,6,7].

To understand T1D development, the key mechanisms in destructive insulitis have been studied in great depth using non-obese diabetic (NOD) mice and BioBreeding-diabetes-prone (BB-DP) rats. NOD mice spontaneously develop autoimmune T1D. Female NOD mice develop spontaneous T1D at a high rate (~90%). A similar rate is observed in both sexes of BB-DP rats [8]. Macrophages, DCs, B cells, and T cells of different subtypes are found in NOD islets during the early stages of insulitis. Not surprisingly, splenocytes from diabetic and prediabetic NOD mice transfer T1D to immunodeficient NOD (NOD-*Scid*) mice [9]. Similarly, athymic nude NOD.Cg-*Foxn1*^{nu} (NOD-*Nude*) mice do not develop T1D [10], thereby providing evidence that T cells are required for T1D onset in this mouse model. Adoptive transfer of T1D by injection of splenic cell subsets into immunodeficient NOD.CB17-*Prkdc*^{scid} (NOD-*Scid*) mice

substantiated that $CD8^+$ T cells in the absence of $CD4^+$ T cells cannot cause disease, and that $CD4^+$ T cells are able to transfer T1D without $CD8^+$ T cells only when isolated from donors with active disease [11]. As expected, T cell depletion using a regimen of anti-CD3 or Anti-thymocyte globulin prevents T1D and results in remission of recent onset T1D in NOD mice [12,13]. These studies have established T1D as a T cell-mediated disorder in animal models. Nevertheless, other cell types are also involved in the initiation and maintenance of destructive insulitis. Macrophages contribute to T1D by secreting proinflammatory cytokines and chemokines that help recruit and activate lymphocytes, and DCs participate in antigen-specific autoreactive cell activation [14-16]. B cells, in addition to their antibody-secreting actions, are important antigen-presenting cells (APCs). Mouse and human studies have demonstrated a role of B cells as APCs in T1D [17,18]. In contrast, the presence of autoantibodies, while useful markers for T1D risk [19] as they indicate autoreactive T cell activation, do not appear to be directly pathogenic to β cells [20,21].

Molecular Mechanisms of β Cell Death

Fas/FasL and perforin/granzyme pathways in T1D: Activated $CD8^+$ cytotoxic T lymphocytes (CTLs) are armed with several molecular mechanisms to lyse and kill target cells; current theory portends that the two major pathways are 1) FasL on lymphocytes interacting with Fas on target cells, and 2) cytoplasmic granule release of perforin and granzyme molecules. Individual cell types differ in the mechanism of cell death induced by the interaction of FasL on the CTL with Fas on the target cell [22]. Type I cells activate a cascade of proteases, known as caspases, that cleave apoptotic substrates independent of the mitochondria. Conversely, in type II cells an amplification

of the caspase cascade requires cleavage of the BH3 protein Bid and death occurs intrinsically. Cleaved Bid translocates to the mitochondria, leading to cytochrome c release, which results in the formation of the apoptosome and downstream activation of effector caspases [23]. Bid-deficient C57BL/6 mouse islets are resistant to Fas-induced apoptosis *in vitro* [24]. Therefore, based on the above criteria, β cells were classified as type II cells. However, the signaling decision has now been shown to be dependent on the level of Fas receptor expressed on the cell surface [25]. β cells upregulate Fas expression on the cell surface after cytokine treatment [26-29]; as a result, both type I and II signaling may occur within β cells *in vivo*. I posit that the local inflammatory conditions determine the death signals by impacting both the autoreactive CTLs as well as the β cells.

Upon antigen recognition, CTLs release perforin and granzyme directed at the target cell. Perforin is required for the proapoptotic actions of granzyme, a serine protease that activates effector caspases and promotes the intrinsic pathway of apoptosis. Dispersed mouse and human islets die by caspase-dependent apoptosis when perforin and granzyme are added together, but by osmotic lysis (necrosis) when perforin is added alone at high concentrations [30].

The Fas/FasL and perforin pathways have been implicated in human T1D. Pancreata from T1D patients stain positive for Fas expression on β cells and FasL on infiltrating cells [31]. Further evidence exists for a role of the Fas/FasL pathway in the pathogenesis of T1D based on *in vivo* studies using the NOD mouse model of the disease. Specific β cell disruption of Fas signaling via the expression of a dominant-negative point mutation in a death domain of the Fas receptor (Fas^{cg}) [32] or a

dominant-negative form of Fas-Associated Death Domain (FADD) [33], both resulted in delayed T1D progression and decreased incidence at 210 and 250 days, respectively. Neutralizing the actions of FasL prevented the reoccurrence of autoimmunity that is normally seen after syngeneic islet transplantation into diabetic NOD mice [34]. The importance of perforin for T1D progression has been analyzed in NOD mice as well. Despite the observation that perforin deficient NOD.Pzf$^{-/-}$ mice develop insulitis to a similar extent as littermate controls, T1D incidence is significantly reduced and disease onset is delayed [35]. Nonetheless, the exact contribution of each pathway has been shown to differ depending on the disease model used and whether β cell killing is tested *in vivo* or using primary islets and β cell lines *in vitro*.

In vitro CTL-Mediated Killing of Pancreatic Islets

Little is known about the mechanisms involved in the killing of human islets by autoreactive CTLs. Preproinsulin-specific CTLs required cell-to-cell contact to selectively lyse β cells in dispersed human islet preparations; however, the mechanism of killing was not investigated further [36]. Thus far, mechanistic studies involving CTL killing of human islets have been accomplished using viral-specific CTL clones and human islets pulsed with the appropriate viral peptide [37]. Peptide-specific, human leukocyte antigen (HLA)-restricted killing of human islets was found to be perforin-dependent, with Fas-mediated killing only observed after pretreatment with proinflammatory cytokines. Interestingly, pan-caspase inhibition failed to protect human islets from CTL-mediated killing, indicating that perforin-mediated killing of islets by CTLs occurs through non-apoptotic mechanisms when apoptosis is blocked [37]. Indeed, although Fas/FasL as well as perforin and granzyme B-induced apoptosis in β

cells was found to be dependent on the proapoptotic BH3-only protein Bid [24,30], Bid-deficient NOD mice develop insulitis and progress to T1D at a similar rate as wild-type NOD mice [38].

CTL effector mechanisms important for β cell destruction have been widely studied *in vitro* with the use of diabetes-prone NOD mice that are transgenic for T cell receptors (TCR) of diabetes-causing T cell clones. This technique has allowed researchers to overcome the difficulty of isolating autoreactive T cells from wild-type NOD mice due to their low precursor frequency. Specifically, $CD8^+$ CTLs derived from NOD.Cg-Tg (TcraTcrbNY8.3) [NOD-NY8.3] mice that recognize an epitope of glucose-6-phosphatase catalytic subunit-related protein [*G6PC2* or IGRP], a T1D β cell autoantigen, have been widely used for such studies [39-41]. Initially, NOD-NY8.3 T cells were found to kill islets exclusively via Fas [42], but further studies demonstrated that NOD-NY8.3 T cells effectively destroy islets from Fas-deficient NOD mice [NOD.MRL-*Fas*^{lpr} (NOD-*Fas*^{lpr})]. However, autoreactive T cells from NOD8.3 mice lacking perforin (NOD-NY8.3-*Prf1*$^{-/-}$) were unable to lyse NOD-*Fas*^{lpr} islets [43]. Similar results were observed when a non-β cell target cell line was pulsed with IGRP peptide [43], suggesting that both the perforin/granzyme pathway and the Fas/FasL pathway can be utilized by NOD8.3 CTLs to exert their diabetogenic potential. When these data are considered along with the studies with the viral antigen described above, one could propose that the antigen recognized may dictate the mechanism of killing and not the target cell itself.

The importance of the Fas and perforin pathways has also been investigated *in vitro* with islets expressing ovalbumin (OVA) under the control of the insulin promoter

(RIP-mOVA) as targets and OVA-specific OT-I CTLs as effector cells [44]. Again, CTL-mediated β cell death was predominantly perforin dependent and, in the absence of perforin, the OVA-specific CTLs failed to lyse Fas-deficient islets. Taken together, these results demonstrate that *in vitro* systems, despite providing support for the involvement of specific pathways in CTL-mediated β-cell killing, do not necessarily account for the redundancy in CTL effector mechanisms existing *in vivo*.

β Cell Lines for *in vitro* CTL Studies

Primary islets are composed of a mixture of hormone-producing cells; however, only β cells are selectively destroyed in T1D. Thus, β cell lines represent valuable tools in understanding and preventing autoimmune β cell destruction. Rodent β cell lines have proven most useful in immunological studies due to the inherent phenotypic instability that limits their use in functional studies [45].

With the use of murine cell lines, our group found that the previously described NOD-derived β cell line, NIT-1 [46], is susceptible to the diabetogenic AI4 $CD8^+$ T cell clone, while a similarly derived cell line, NIT-4, only differing in the allotype of *mt-Nd2*^{a}, which is associated with decreased basal mitochondrial ROS production [47,48], is resistant to killing [49]. Because IFNγ priming of NIT-4 cells rendered them susceptible to AI4 CTL killing [49] and IFNγ treatment has been shown to upregulate major histocompatibility complex (MHC) Class I and surface Fas expression [50], it is possible that AI4 CTL-killing is Fas dependent, but we have yet to test the exact mechanism.

$GAD65_{546-554}$-specific CTLs have also been used to lyse NIT-1 cells [51]; however, due to the lack of GAD65 expression by the cell line, NIT-1 cells required transfection with GAD65 prior to killing. The authors concluded that killing was perforin dependent

as the $GAD65_{546-554}$-reactive CTLs expressed perforin but not FasL, and the transfected NIT-1 cells had undetectable levels of Fas. The apparent dominance of perforin over the Fas/FasL pathway in the killing of β cell lines *in vitro* remains to be confirmed with inhibition studies. Additionally, the type of cell death triggered by Fas ligation and cytotoxic granules, whether apoptosis or necrosis, demands attention.

Significantly less work has occurred with human β cell lines. Similar to what is expected with rodent β cell lines, the ideal *in vitro* β cell model should remain stable with passages and maintain insulin secretion in response to glucose stimulation, as well as preserve the expression of other β cell-specific markers and autoantigens [52]. Given the differences between primary rodent and human β cells, several human β cell lines have been established. These cell lines are HP62 [53], CM [54], NES2Y [55], βLox5 [56], NAKT-15 [57], and EndoC-βH1 [58] (Table 1-1).

As discussed below, most of the human β cell lines available do not serve as models of normal β cell function but have proven useful for specific *in vitro* assays (Table 1-2). The recently developed insulin-secreting EndoC-βH1 cell line represents a promising tool for both immunological and functional studies, as these cells maintained glucose stimulated insulin secretion (GSIS) after 75 passages [58]. EndoC-βH1 cells were generated from human fetal pancreas cells that were transduced to express the SV40 T antigen under the control of the rat insulin promoter; as a result, only insulin-producing cells become immortalized. The reversibly immortalized NAKT-15 cell line [57] was initially reported to be a functional human β cell line that displayed molecular characteristics of pancreatic β cells, maintained insulin secretion, and reversed

chemically induced diabetes in mice. Unfortunately, since the initial report, no further studies have been published.

Created two decades ago, the HP62 cell line was obtained from the transfection of human islet cells with a plasmid vector encoding SV40 viral DNA [53]. Early passages of these cells secreted glucagon and somatostatin (passage 3); insulin was later detected, but secretion, or synthesis alone, was short lived (passages 6 and 7, respectively). Interferon gamma (IFNγ) priming of HP62 led to an increase in the expression of HLA Class II molecules [53], suggesting a possible application of this cell line in immunological studies. Consequently, the HP62 cell line has since been used to study the cytotoxicity and modulatory effects of cytokines on the expression of adhesion molecules that facilitate $T_{effector}$-target cell contact [59,60]. Due to its endocrine lineage, HP62 cells have also been useful in confirming the expression and functionality of endotoxin receptors measured in isolated human islets [61]. Previously, sera from T1D patients containing islet cell antibodies (ICA) failed to react with this cell line [62], casting doubt on its antigenicity and β cell likeness. Still, along with the human insulinoma cell line, CM [54], HP62 cells have continued to be tested as β cells in cytotoxicity assays, with responses similar to those of primary islet cells [63-65].

The CM cell line was generated from a patient with a malignant pancreatic insulinoma [54]. Although CM cells lose insulin secretion with long-term passage, the cell line retains many β cell-specific characteristics [66,67]. CM cells grown in medium containing high glucose stimulated proliferative responses of T cells isolated from T1D patients to a greater extent when compared to control subjects; the same pattern was not observed in low glucose conditions [68], but the findings further suggested their

usefulness in cell-mediated lymphocytotoxicity (CML) assays. Indeed, glutamic acid decarboxylase (GAD)-specific $CD8^+$ T cell clones expanded from T1D patients lysed CM and HP62 in ^{51}Cr release assays [65]. The mechanism of CTL killing was not elucidated; however, transfection of the cell lines with B7-H4, an inhibitory co-signal molecule expressed on the cell surface, decreased the percent specific lysis measured, indicating the GAD-reactive T cells killed the β cells by a mechanism requiring direct cell contact. The CM insulinoma cell line has been valuable as a β cell model in a wide range of *in vitro* assays [63-65,68-76], but not without criticism [77,78]. In addition to poor GSIS, several chromosomal abnormalities were noted in CM cells. While genetic defects in the CM cell line are not surprising due to their tumorigenic source [79], these cells are likely most beneficial when analyzing killing mechanisms utilized by autoreactive immune effectors to destroy human β cells, rather than in functional assays.

Another pancreatic cell line, NES2Y, was derived from the islets of a patient with persistent hyperinsulinemic hypoglycemia of infancy (PHHI). Similar to the β cells of PHHI patients, NES2Y cells constitutively secrete insulin due to loss-of-function mutations resulting in defective ATP-sensitive potassium (K_{ATP}) channel activity [55]. NES2Y cells also lack expression of the homeodomain transcription factor, PDX-1 [55]. These cells have been useful in determining the contribution of normal calcium signaling and PDX-1 expression/function within the β cells [80-83]. Transfection studies with genes encoding the K_{ATP} channel subunits [sulfonylurea receptor (SUR) 1 and Kir6.2] and PDX-1 not only rescued GSIS and glucose-regulated insulin promoter activity [84], but also highlighted the requirement of both SUR1 and Kir6.2 for K_{ATP} channel function

[82], as well as the role of PDX-1 in the control of insulin gene transcription upon glucose stimulation [80]. The functional outcomes of mutations in the PDX-1 gene, associated with maturity-onset diabetes of the young (MODY), have also been studied in NES2Y cells transfected to express normal or mutant forms of this gene [85,86]. Despite the aforementioned dysfunctions, NES2Y cells have been utilized as human β cells in cell death assays. Mechanisms of fatty acid-induced toxicity, unrelated to T1D but linked to β cell death in type 2 diabetes, were previously tested in these cells [87,88]. In the context of T1D, NES2Y membranes successfully induced proliferation of autoreactive T cells isolated from new onset T1D patients that were selected for granule membrane reactivity [89], and NES2Y cells, like CM cells, have been used to study regulatory components that protect against apoptotic stimuli, namely tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [75,76]. Efforts are still underway to create a physiologically relevant β cell line from NES2Y cells [90]; nonetheless, the continued expression of β cell antigens [89] by this cell line makes it an attractive potential target of autoreactive CTLs.

Transformation with oncogenes and enhanced telomerase activity (SV40 T antigen, Ras^{val12}, and hTRT) of adult pancreatic islets enriched for β cells led to the development of the βLox5 cell line [56]. βLox5 cells are not responsive to high glucose challenge with increasing passage number; however, the cell line can be manipulated to regain β cell function [91,92]. Interestingly, independent of secretory function, late passage cells express HLA Class I molecules on their cell surface that can be further induced by IFNγ, and maintain the expression of autoantigens recognized by T cells in human T1D, such as insulin (low), IA-2, IGRP, GAD65, GAD67, and ZnT8 (Chapter 4).

Similarly, Fas receptors were measured on the cell surface of the βLox5 cell line, adding to its worth as a CTL target. To mimic Fas-mediated CTL killing of human β cells, βLox5 cells were incubated with IFNγ (to increase surface Fas) and CH-11, a human anti-Fas antibody, and it was found that these cells died by caspase-dependent apoptosis after Fas stimulation [29]. As will be shown later (Chapter 4), βLox5 are also lysed by IGRP-reactive $CD8^+$ T cells. Therefore, direct CTL killing mechanisms can be tested with the βLox5 cell line.

Soluble Mediators in T1D

Reactive Oxygen Species and Proinflammatory Cytokines

Prior to the advent of animal models that develop spontaneous autoimmune diabetes, investigators seeking to destroy β cells and induce insulin-requiring diabetes would do so using alloxan or streptozotocin (STZ). Both alloxan and STZ are free radical generators that are selectively toxic to β cells due to their structural resemblance to glucose. These compounds gain entry into murine β cells via Glucose transporter 2 (GLUT2) (Fig. 1-1: #1) [93]. Once inside the β cell, oxidative reactions occur with thio containing enzymes such as glucokinase (Fig. 1-1: #1 and #2) and aconitase, resulting in impaired glucose sensing, mitochondrial dysfunction, and necrotic cell death (Fig. 1-1: #3) [94-97]. The findings that alloxan-induced β cytotoxicity could be prevented by antioxidants [98-100] and that β cell death consequent to STZ exposure could be partially prevented with superoxide dismutase [101] were instrumental in our understanding of β cell susceptibility to both oxidative and immune-mediated stress. This led to the observation that in comparison to other tissues, β cells had reduced or absent activity of antioxidants [102-107]. The explanation for the low levels of

antioxidants in β cells remains unclear, as upregulation of antioxidants in these cells does not have a significant impact on glucose-stimulated insulin secretion [105,108-110]. However, the reduction of defenses against oxidative stress results in β cells being exquisitely sensitive to ROS damage caused by toxins or inflammation.

There is a preponderance of evidence indicating that β cell dysfunction can result from inflammation-induced oxidative stress. Free radical-mediated β cell dysfunction and death can be due to either murder (exogenous) or suicide (endogenous). The source of exogenous reactive oxygen species (ROS) or reactive nitrogen species (RNS) production is likely activated macrophages, which are present in high numbers in the preclinical insulitic infiltrates [111,112]. The oxidative burst of activated macrophages can destroy co-cultured islet cells [113,114] through the release of highly reactive oxygen species [e.g.; superoxide (SO_2^-), hydroxyl radical (^-OH), nitric oxide (NO)]. The initial cytotoxic effects of NO are thought to be mediated via the destruction of intracellular iron-containing enzymes, including members of the Kreb's cycle and the electron transport chain, resulting in the reduction of energy metabolism [115-118]. The impact of NO in decreasing ATP production blunts insulin secretion and induces necrosis (Fig. 1-1: #4) [119,120]. Potent tissue-damaging oxygen radicals can be derived from free cytosolic Fe^{2+} by the Fenton reaction as well as through arachidonic acid metabolism, which destroy organelle membranes and membrane-associated enzymes via lipid peroxidation [121].

Additional cytotoxic free radicals in the prediabetic state are likely generated within the β cells themselves in response to cytotoxic mixtures of monokines and lymphokines [122-124]. Post-mortem pancreatic tissue samples from patients with T1D demonstrate

increased interferon alpha (IFNα) expression, and interferon gamma (IFNγ)-secreting lymphocytes have been identified in the islets [125]. Tumor Necrosis Factor alpha (TNFα) and Interleukin 1 beta (IL-1β)-producing macrophages and DCs have been detected in patients with recent-onset T1D [126]. In the NOD mouse and the BB-DP rat, the expression of monokines such as IL-1β, IFNα, TNFα, and type 1 cytokines including IFNγ, TNFβ, IL-2, and IL-12 have been reported to associate with destructive insulitis [127]. Islet-infiltrating $CD4^+$ and $CD8^+$ T cells are sources of cytokines, particularly the potent macrophage activator, IFNγ.

IFNγ potentiates the effects of IL-1β and TNFα, which impair the function of rodent and human β cells individually, and are highly cytotoxic when combined [122,128,129]. This toxicity mediated by the combined action of monokines and IFNγ has been attributed to the induction of NO synthase (iNOS) and the subsequent production of NO [130,131]. Cytokines synergize to activate NF-kB, the major transcription factor for iNOS expression (Fig. 1-1: #5) [107]. Use of transgenic mouse models has shown that β cell overexpression of iNOS, and the ensuing elevation of islet NO, kills β cells independent of insulitis [132] (Fig. 1-1: #4). Recent work suggests that the superoxide-producing phagocyte NADPH Oxidase (NOX) is expressed in β cells [133-135]. NOX can be activated in response to proinflammatory cytokines resulting in ROS production and β cell damage and death [133]. Cytokines can also generate potent cytotoxic aldehyde moieties (malondialdehyde, butanal, pentanal, 4-hydroxynonenal, and hexanal) capable of lipid peroxidation [136], perhaps through the activation of NOX or excessive mitochondrial ROS production [137].

In vitro Cytokine-Induced β Cell Killing

As mentioned above, of the cytokines identified *in vivo*, *in vitro* cytokine killing experiments have focused on the individual and combined actions of IFNγ, IL-1β, and TNFα. Of note, when cultured with whole islets, cytokines are not specifically cytostatic or cytotoxic towards β cells [125,127]. Although this might dispute the cytotoxic role of cytokines *in vivo*, it is possible that *in vivo* β cell-specific autoreactive cells at very close proximity to their intended target, the β cells, could produce significant damage by secreting cytokines.

In vitro, cytokine-mediated destruction of mouse islets occurs through NO-dependent necrosis with some contribution from apoptosis (Fig. 1-1: #5, 6) [138]. When compared to untreated whole mouse islets, a six-day incubation period with IFNγ, IL-1β, and TNFα led to about a 13% survival rate in wild-type mouse islets, versus a 98% survival of iNOS knockout ($iNOS^{-/-}$) islets. Only after nine days in culture did cytokines reduce $iNOS^{-/-}$ islet-viability to about 82%; survival in the wild-type islets at day nine was 8% of untreated cells. When $iNOS^{-/-}$ β cells were purified and treated with cytokines, very little, if any, necrosis was measured, suggesting that apoptosis accounted for the small reduction in viability noted in the mouse islets after the nine days in cytokine-containing media [138]. In comparison, IL-1β alone can kill rat islets via NO-dependent necrosis [139]. The inhibitory effect of cytokines on human islets was also shown to correlate with NO production [140,141]. However, preventing iNOS function was insufficient to prevent human β cell death [141]. This is likely a result of higher expression of the stress protein heat shock protein 70 (HSP70) in human islets

compared to rodent islets [142], as heat shock alone can prevent cytokine-induced rat islet inhibition and NO toxicity [143,144].

Collectively, cytokine killing assays with isolated islets suggest that β cells die by necrosis as well as apoptosis. To understand the cytotoxicity of cytokines specifically on β cells, the described studies have also been performed with β cell lines. We have used a human β cell line to study the cell death pathways activated after cytokine (TNFα and IFNγ) incubation. In addition to NO-independent necrotic cell death, caspase-dependent and caspase-independent apoptosis was detected. All forms of cell death appeared to be dependent on functional mitochondria, as mitochondrial DNA (mtDNA) deficient cells were resistant to cytokine killing [29].

The human islet cell line HP62, as well as the human insulinoma cell CM, were treated with the cytokines TNFα or IFNγ, but not in combination. Only TNFα was cytotoxic to HP62 (~15% cell death) but not to CM, and the mechanism of killing remains unresolved [64]. Other groups have reported that treatment of murine β cell lines with cytokines can trigger different apoptotic pathways. For instance, two studies proved that the β cell lines INS-1 and NIT-1 (rat and mouse-derived respectively) died by caspase-dependent apoptosis [145,146]. As well, another mouse β cell line, MIN6, activates caspases in response to cytokine incubation [147]. In addition, NO produced in MIN6 cells with cytokine treatment elicited the unfolded protein response [148], implicating endoplasmic reticulum (ER) stress-mediated apoptosis in cytokine-induced β-cell death. Alternatively, when investigated using cytokine-treated INS-1 cells, induction of ER stress was not observed [149].

Interestingly, in a recent report, several strategies were employed to analyze cytokine-induced cell death of two INS-1-derived cell lines [150]. The authors compared the type of cell death induced by cytokines to the apoptotic cell death known to be prompted by camptothecin and found that cytokine-induced β-cell destruction is not an apoptotic process. Therefore, *in vitro*, cytokines are capable of activating a multitude of cell death pathways that, although not the same in all the models used, ultimately lead to the common outcome of β cell demise. The common denominator in all *in vitro* models outlined, however, is necrotic cell death. Thus necrosis, a potent promoter of inflammation, might be the most relevant cell death pathway triggered by cytokines in the context of T1D.

Immunomodulatory Effects of Cytokines

In addition to being β-cytotoxic [151], cytokines are able to control the type of immune response mounted [152], and prime β cells for heightened immune surveillance and clearance. To date, there are a considerable number of reports that have sought to identify the impact of a single cytokine in T1D.

Overexpression of IFNα in β cells using a transgenic approach with the rat-insulin promoter (*RIP-IFNα)* in the non-autoimmune diabetes prone C57BL6 mouse strain, resulted in β cell dysfunction, death, and onset of diabetes [153]. While non-autoimmune-prone *RIP-IFNβ* transgenic mice exhibit β cell dysfunction but do not develop spontaneous T1D [154], in the NOD background, β cell-specific overexpression of IFNβ precipitates T1D [155]. Similarly, in NOD mice, disruption of the gene encoding IFNγ does not prevent T1D development [156] but β cell specific transgenic expression of IFNγ under the control of the rat insulin promoter (*RIP-IFNγ*) stimulates insulitis and

T1D only in immune competent animals [157]. This supports a role for interferons in promoting β cell recognition and killing by cellular effectors *in vivo*, with IFNα being seemingly more potent in eliciting an immune response in the absence of a predisposing immune system. Indeed, viral-induced IFNα production represents a potential environmental factor linked with T1D [158].

On the other hand, disruption of IL-1β signaling protects *in vitro* against IFNγ and TNFα-induced mouse islet cell death, but does not alter spontaneous T1D incidence [159,160]. TNFα, also implicated in T1D pathogenesis, has been shown to promote different disease outcomes depending on the timing of expression [161]. This effect may now be explained by recent data indicating that TNFα boosts regulatory T cells (Tregs) *in vivo* [162].

Mitochondria and the Mechanisms of Cell Death

Mitochondria are required for proper β cell function. Functionally and structurally sound mitochondria are essential for GSIS [163,164]. However, these organelles are also important as regulators of cell death. Apoptotic stimuli lead to proapoptotic Bcl family member activation, mitochondrial membrane permeabilization, and the release of proapoptotic proteins like cytochrome c, apoptosis-inducing factor (AIF), as well as endonuclease G (Endo G). Proapoptotic Bcl family members include Bax, Bak, Bid, Bim, Bad, and PUMA. Anti-apoptotic members such as Bcl-2, and Bcl-XL hold these proteins in check. Consistent with this, human islets overexpressing Bcl-2 show increased resistance to the combined actions of IFNγ, IL-1β, and TNFα, as measured by decreased DNA fragmentation, cell death, and β cell dysfunction after a 5-day incubation period [165]. Similarly, knockdown of the proapoptotic member Bim with

small interfering RNA (siRNA) technology decreased IFNγ and TNFα-induced cell death of dispersed human islet cells and INS-1E cells [166]. Cytokine-induced INS-1E cell death was reduced by 10% with Bim knockdown. Bim knockdown also rescued viability of primary rat β cells and dispersed human islets incubated with IFNγ and TNFα by 10% & 7%, respectively. Treatment of INS-1E cells with the combination of IFNγ and IL-1β for 24 h led to cell death that was prevented by knocking down the proapoptotic Bcl member PUMA (20% to 15% death) but not Bim [167]. Together, these studies support an important role for the mitochondria in β cell death through the differential activation of pro- and anti-apoptotic Bcl family members by distinct stimuli. The experiments and results described clearly demonstrate that variations in key treatment conditions, such as incubation period or cytokine concentration, occur and may account for some of the discrepancies mentioned previously. Cell death is a dynamic process and "grey zones" are sure to exist connecting the different effectors, as well as the modes of death. Therefore, the time point where death is investigated is likely as important as the mechanism of induction.

Also known as programmed cell death, apoptosis is an energy-requiring process. Consequently, mitochondria, being the powerhouses of the cell, can control the form of cell death through their supply of energy. Endogenous inhibitors of mitochondrial respiration and production of ATP via oxidative phosphorylation, such as NO, lead to modest decreases in cellular ATP concentrations that may result in a switch from apoptosis to necrosis in metabolically suppressed cells (i.e. β cells in pre-T1D) that have already been signaled for apoptotic cell death. Mitochondria are also major sources of cellular ROS, and mitochondrial respiratory chain inhibition induces ROS

production from complexes I and III. Cellular ROS, regardless of where they are produced, can lead to caspase-dependent apoptosis in β cells [137]. However, although not tested in β cells, high levels of hydrogen peroxide (H_2O_2) have been shown to inhibit caspases [168,169] and promote necrosis. Therefore, excessive or continued mitochondrial ROS production represents another mechanism by which mitochondria may determine the fate of the cell and the choice of death mechanism.

Genetics of Autoimmune T1D

The contribution of inheritance in the development of early-onset diabetes was noted decades ago [170]. However, our current classification of T1D as a complex polygenic disease is a result of candidate gene testing and genome-wide association studies that have identified over 40 chromosomal regions linked with T1D susceptibility [171]. The highest genetic risk for T1D is encoded within the HLA Class II region. Class II HLA (DP, DR, and DQ) molecules present antigen to $CD4^+$ T cells, a process not only important for immune activation, but also tolerance induction. The specific amino acid residues in, or impacting the structure of the binding pocket of these molecules, determine their peptide-binding function [172]. For instance, non-aspartic acid residues at β57 of DQB are associated with susceptibility to T1D [173], with the protective β57Asp being linked to increased MHC Class II αβ dimers [174]. Class I HLA alleles, although to a lesser extent, also associate with T1D risk [175-177]. Disease-related variants might be involved in the presentation of self-antigen to autoreactive T cells [178], but their exact role is not well understood.

Candidate gene studies have identified several other non-HLA loci that predispose to, or protect against, T1D progression. These include variants within the insulin gene

(*INS*), single nucleotide polymorphisms (SNPs) in the protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*), gene variants in cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) and interleukin 2 receptor alpha (*IL2RA*) [171], *AMACO* [179], *ICAM1* [180], as well as the mitochondrially encoded gene *mt-ND2* [181].

Variable number of tandem repeats (VNTR) polymorphisms in the 5' region of the *INS* gene determine the risk or protective variants. Class I VNTR alleles (length of 26-63 repeats) are associated with susceptibility, whereas the class III VNTR alleles (140-210 repeats) are linked with protection. Class III VNTR alleles allow for higher *INS* expression in the thymus compared to the class I VNTR alleles [182,183]. Tissue-specific protein expression in the thymus is required for central tolerance, therefore, lower insulin in the thymus leads to improper clearance of autoreactive T cells during development. The *PTPN22*, *CTLA4*, and *IL2RA* genes are involved in T-cell mediated responses. Interestingly, the T1D-associated SNP in the *PTPN22* gene results in gain-of-function inhibition of T cell signaling [184] that disturbs tolerance induction. The *CTLA4* gene product is also a negative regulator of T-cell activation but little is known about the role of the T1D-associated polymorphism. *IL2RA* encodes the α-subunit of the IL-2 receptor through which IL-2 signaling occurs. While IL-2 signaling is important for both effector and regulatory T cell functions, studies in the NOD mouse model of T1D indicate that disease alleles correlate with impaired regulatory T cell function [185].

In addition, an mtDNA-encoded susceptibility SNP in *mt-ND2* and *mt-Nd2*, has been identified both in humans and in animal models, respectively [181,186]. The risk allele (*mt-Nd2*^{c}) leads to higher mitochondrial ROS production in NOD [48]; thus, the protection afforded by the resistant allele (*mt-Nd2*^{a}) was found to be due to decreased

mitochondrial ROS (mtROS) generation the level of the β cell [47,49]. Although the C to A nucleotide substitution that results in a leucine to methionine amino acid change likely has the same effect of reduced mtROS production in humans, the mechanism remains to be tested. Here, the human β cell line βLox5 was employed to investigate the contributions of *mt-ND2*^{a}.

Table 1-1. Human β cell lines

Cell Line	Cell Origin	Insulin	GSIS	Method	Ref.
HP62	Pancreatic Islets	Early	Early	SV40 T-antigen	[53]
CM	Insulinoma	Yes	Early	Malignant Insulinoma Subculture	[54]
NES2Y	PHHI Islets	Yes	No	Continual Islet Cell Subculture	[55]
NISK9	PHHI Islets	Yes	Yes	NES2Y transfected with Kir6.2, SUR1, and PDX-1	[84]
βLox5	Adult β Cells	Yes	Early	Floxed SV40 T-antigen, Ras^{val12}, and hTERT	[56]
NAKT-15	Pancreatic Islets	Yes	Yes	Floxed SV40 T-antigen, hTERT, and EGFP	[57]
EndoC-βH1	Fetal Pancreas	Yes	Yes	RIP-SV40 T-antigen, and hTERT	[58]

PHHI, persistent hyperinsulinemic hypoglycemia of infancy; GSIS, glucose stimulated insulin secretion

Table 1-2. Utility of human β cell lines for *in vitro* cytotoxicity assays

Cell Line	T Cell Assays/ Direct Killing	Killing Mechanism	Cytokine Sensitivity	Killing Mechanism	Ref.
HP62	CD56+ NKT cell target; TRAIL	N/T	IFNγ+TNFα	N/T	[59,63,64]
CM	T1D T cell stimulation; $CD56^+$ NKT cell target; TRAIL	TRAIL death inhibited by Bcl-2 & XIAP	TNFα	ROS	[63,64,68,70,74-76]
NES2Y	Granule-specific T cell stimulation; TRAIL	TRAIL death inhibited by Bcl-2 & XIAP	N/T	N/T	[75,76,89]
NISK9	N/T	N/T	N/T	N/T	
βLox5	Fas-FasL	Caspase-dependent	IFNγ+TNFα	Caspase-dependent/ independent; ROS	[29]
NAKT-15	N/T	N/T	N/T	N/T	
EndoC-βH1	N/T	N/T	N/T	N/T	

TRAIL, Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand; N/T, Not Tested; ROS, Reactive Oxygen Species; XIAP, X-linked Inhibitor of Apoptosis Protein

TRAIL, Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand; N/T, Not Tested; ROS, Reactive Oxygen Species; XIAP, X-linked Inhibitor of Apoptosis Protein

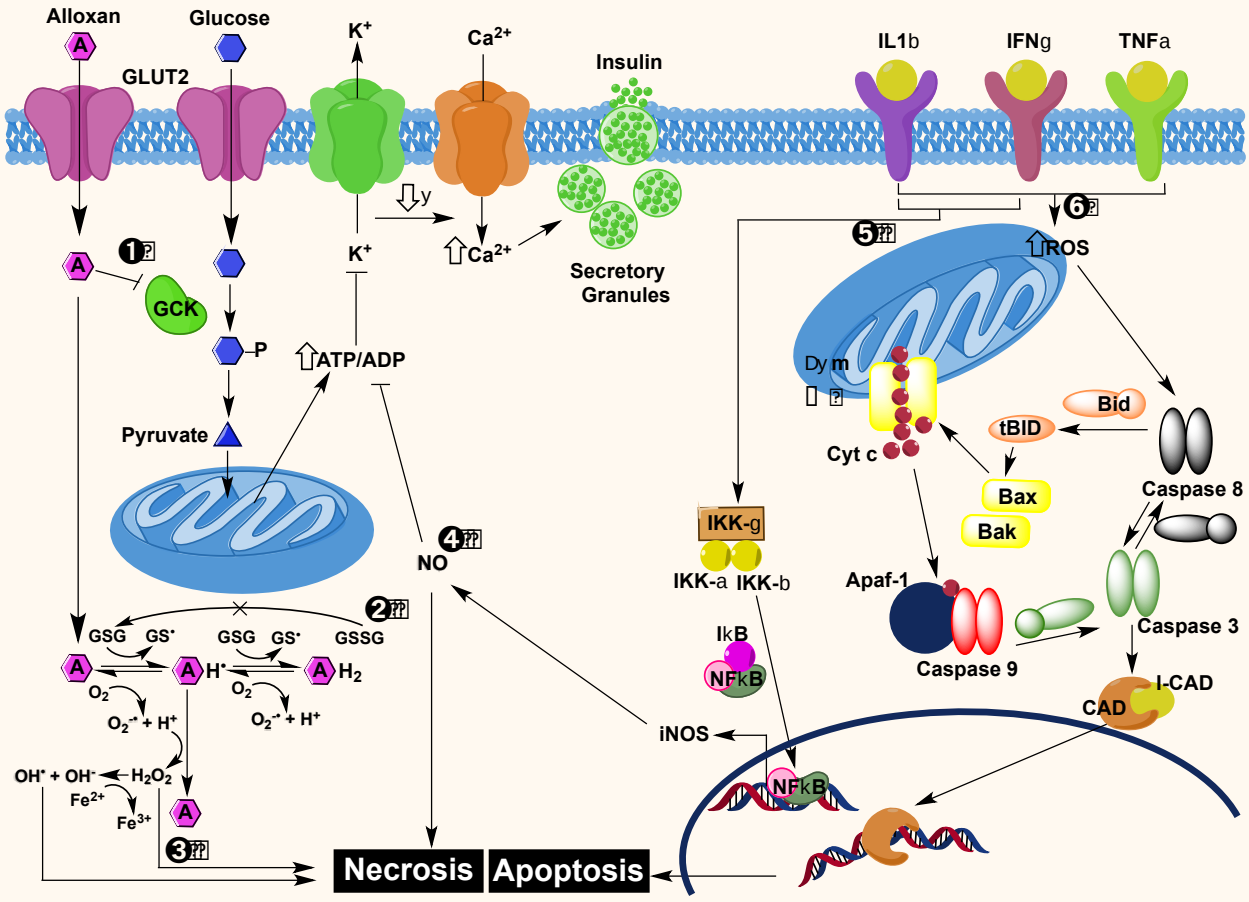

Figure 1-1. Oxidative stress-induced β cell dysfunction and death. 1. Alloxan (A) is a cytotoxic ROS generating glucose analogue that preferentially accumulates in β cells via the GLUT2 glucose transporter. Alloxan prevents glucose-stimulated insulin secretion by inhibiting glucokinase activity, the enzyme responsible for the rate-limiting step of glucose catabolism, as well as enzymes associated with mitochondrial ATP production. ROS are generated in a cyclic reaction between alloxan and its reduced product, dialuric acid (AH_2). Autoxidation of dialuric acid generates superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and, in the presence of a metal catalyst through the Fenton reaction, hydroxyl radicals ($OH^{\cdot}$). 2. Glutathione (GSH) is consumed within the cell for redox cycling, thereby producing oxidized glutathione (GSSG). However, because β cells display low glutathione reductase activity, β cells are unable to maintain redox balance and undergo necrotic cell death. 3. β cells also exhibit low levels of catalase and glutathione peroxidase, the main H_2O_2-inactivating enzymes, which contributes to the high susceptibility of β cells to ROS. 4. Exogenous Nitric Oxide (NO) production promotes β cell dysfunction by preventing increases in ATP/ADP ratios through the inhibition of aconitase, a tricarboxylic acid (TCA) cycle enzyme, and complex IV of the electron transport chain. NO causes necrotic β cell death. 5. The combination of IL1β and IFNγ leads to the induction of NFkB-responsive stress genes such as inducible Nitric Oxide

Synthase (iNOS) by the β cell, further driving dysfunction and necrosis by endogeneous NO production. 6. When added together, IL1β, IFNγ and TNFα result in increased mitochondrial ROS production, thereby activating caspases which cause changes in the mitochondrial membrane potential ($\Delta\Psi m$) and mitochondrial membrane permeabilization (7), this allows the release of proapoptotic proteins such as cytochrome c (Cyt c) and amplification of the caspase cascade via the apoptosome (Cyt c, Apaf-1, and Caspase 9). Ultimately, effector caspases (Caspase 3) activate Caspase Activated Deoxyribonuclease (CAD), a DNA-degrading enzyme. DNA cleavage promotes apoptotic cell death. 6. Mitochondrial ROS production induced by the interaction of TNFα with TNFR1 or FasL with Fas contributes to Caspase 8 activation, potentiating of the caspase cascade.

CHAPTER 2
ROLE OF THE MITOCHONDRIA IN IMMUNE-MEDIATED APOPTOTIC DEATH OF THE HUMAN PANCREATIC β CELL LINE βLox5

Introduction

Insulin-dependent, or type 1 diabetes mellitus (T1D) results as a consequence of the specific autoimmune destruction of the pancreatic islet β cells. While better understood in animal models, the exact progression to T1D in humans remains elusive, in part due to the limited human pancreatic samples available for research and the fact that the islets collected are obtained *postmortem* resulting in variable quality and functional capacity [187]. Consequently, animals that develop diabetes spontaneously and resemble the human form of the disease, like the NOD mouse and the BB-DP rat, as well as β cell lines derived from murine sources, are heavily relied upon for a mechanistic understanding of the pathogenesis of the disorder [8].

Studies performed using animal models of T1D as well as primary human donor islets have proposed several direct and indirect mechanisms of β cell destruction. For instance, in the NOD mouse, insulitis begins with the activation of macrophages and DCs within the pancreatic islets. These resident specialized antigen-presenting cells locally produce chemokines and cytokines that recruit and activate autoreactive T and B lymphocytes [14]. Additionally, soluble mediators, such as cytokines and free radicals, both RNS and ROS produced by the infiltrating immune cells and the β cell themselves, can lead to β cell death. In previous studies, IL-1β, IFNα, TNFα, and type 1 cytokines (IFNγ, TNFβ, IL-2, and IL-12) were found to correlate with destructive insulitis in the T1D prone NOD mouse and the BB rat [127]. Pancreatic samples from patients with T1D

were also shown to contain the cytokines IFNα and IFNγ, TNFα-producing lymphocytes, as well as TNFα and IL-1β-expressing macrophages and DCs [125].

In vitro studies on the cytotoxicity of cytokines to β cells suggest that individual proinflammatory cytokines can either enhance or inhibit insulin secretion depending on dose and length of exposure. However, when added in combinations, IL-1β, IFNγ, and TNFα induce death and dysfunction of both human and rodent islets [188]. The impact of cytokines on mouse and rat islets is mainly through NO-mediated necrosis with minor contributions of apoptosis [138,188-197]. Studies reporting observations after exposing human islets to cytokines have been less clear, likely due to differences in experimental systems [198] as well as the health of the isolated human islets used [199,200]. Taken together, it is rational to propose that when treated with cytokines, human islets die by both necrotic and apoptotic mechanisms.

Furthermore, cytokines can either alone or in combination change the surface of islet cells, thereby enhancing the potential for immune surveillance by cytotoxic T cells (CTLs). Predictably, molecules elevated by cytokines, such as MHC class I and Fas, have been correlated with destructive insulitis in both mice and humans [3]. β cell surface remodeling by cytokines, combined with the fact that T1D is considered to be a T cell dependent disorder, imply that, *in vivo*, cytokines are responsible for providing an inflammatory environment conducive for T cell recognition and destruction of the insulin-producing cells. In this proinflammatory milieu, recognition of autoantigens by CTLs leads to direct β cell lysis. CTL specific killing mechanisms that are thought to be involved in β cell destruction include the Fas/FasL pathway and perforin/granzyme release. In cytotoxic assays using a population of NOD-derived, autoreactive $CD8^+$ T

cells specific for an epitope of IGRP, a known T1D autoantigen [39,41], NOD islets were killed only when at least one of these pathways was left intact [42,43].

Most of these studies have been performed using both β cells and CTLs derived from animal models. Yet, the appreciated genetic and immunopathologic differences between animal models of the disease and humans attest that diabetogenesis in humans could be distinctive and highlights the need for a human β cell line that can be used for the study of death in the context of autoimmune-mediated destruction. In this report, the usefulness of a cell line derived from purified adult β cells, βLox5 [56], was tested in assays of β cell death; the mitochondrial contributions to human β cell killing by immune effectors were investigated as well. βLox5 cells were exposed to direct killing by an activating human Fas antibody, CH-11, in addition to indirect killing by the proinflammatory cytokines IFNγ and TNFα. The data presented show that, similar to primary islets and β cell lines derived from animals, βLox5 cells are killed after ligation of Fas by caspase-dependent apoptosis, whereas these cells die by caspase-dependent and –independent apoptosis together with necrosis after incubation with TNFα and IFNγ. Importantly, βLox5 cells depleted of their mitochondrial DNA (mtDNA) were resistant to proinflammatory cytokine-induced killing, implicating a role for mitochondria-associated cell death mechanisms in the progression to T1D in humans.

Materials and Methods

Cell Line and Reagents

The βLox5 cell line was kindly provided by Dr. Fred Levine (Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA). βLox5 cells were maintained in low glucose (1 mg/mL) DMEM (Cellgro, Manassas, VA),

supplemented with 10% FBS (HyClone, Fisher Scientific, Pittsburgh, PA), 1% MEM non-essential amino acids (Cellgro), 1% penicillin-streptomycin (Gemini Bio-Products, West Sacramento, CA) solution, 0.02% BSA (Sigma, St. Louis, MO) and 15 mM HEPES (Cellgro) (VC-DMEM). Recombinant human IFNγ was obtained from BD Biosciences (San Jose, CA). Recombinant human TNFα and recombinant IL-1β were purchased from R&D Systems (Minneapolis, MN). Fas agonistic antibody (Clone CH-11) was purchased from Millipore (Temecula, CA). A monoclonal antibody to HMGB1 was acquired from Abcam Inc. (Cambridge, MA). An antibody to hCD95 (Fas)-PE-Cy5 and the isotype control were purchased from BD Biosciences. Annexin V-APC and propidium iodide (PI) were purchased from Invitrogen (Carlsbad, CA).

Generation of βLox5 ρ^0 Cells and Cybrid βLox5 Cells

βLox5 ρ^0 cells were cultured in high glucose (4.5 mg/mL) DMEM (Cellgro) supplemented with 10% FBS (HyClone, Fisher Scientific), 1 mM sodium pyruvate (Sigma), 50 mg/L Uridine (Sigma), and 1% penicillin-streptomycin (Gemini Bio-Products). mtDNA was depleted by culturing cells in the above medium supplemented with 100 ng/mL Ethidium Bromide (EtBr) for 6 months. Depletion of mtDNA was confirmed by: 1) PCR; 2) confocal microscopy imaging; 3) failure of βLox5 ρ^0 cells to survive in pyruvate- and uridine-free medium.

Cybrid βLox5 cells were generated as described before [201]. Briefly, cybrid cells were made by fusing βLox5 ρ^0 cells with mtDNA donor platelets from a healthy individual under the presence of 50% (W/V) polyethylene glycol 1500 (Roche). Cells were cultured in the medium for βLox5 ρ^0 cells during the first 3 days after fusion and then in selective medium (uridine and pyruvate-free DMEM supplemented with 10%

dialyzed FBS, Penicillin and Streptomycin). After selection for 3 weeks, surviving cybrid cells were cultured in DMEM for βLox5, as described above, without pyruvate and uridine. Cybrid cells were cloned using cloning cylinders (Corning, Corning, NY) when visible colonies appeared in the culture.

Cell Death Assays

βLox5, ρ^0 and cybrid cells were seeded in twelve-well Corning Costar culture plates (Fisher Scientific) at a density of $5x10^4$ cells per well in a total of 500 µL and allowed to adhere for 24 h. The cells were then incubated with rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 48 h. βLox5, ρ^0 and cybrid cells were also cultured in the presence of α-Fas activating antibody CH-11 (0.5 µg/mL) with and without rhIFNγ (1000 U/mL). Cell viability was examined using the MTT assay, PI uptake, and externalization of phosphatidylserine (PS) by Annexin V-APC staining.

Percent cell survival after cytokine or α-Fas antibody treatment was measured by determining the ability of the live cells to reduce yellow MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), to insoluble purple formazan crystals. The cells were treated with MTT solution (0.5 mg/mL) for 2 h at 37°C, 5% CO_2. Excess solution was removed and the formazan crystals were then resuspended in acid isopropanol (0.04 N HCl in isopropanol). The optical density of the product was measured at a wavelength of 560 nm and background subtracted at 670 nm.

βLox5 cells were analyzed on a BD LSR-Fortessa flow cytometer using the BD FACSDiva software (BD Biosciences) and FlowJo analysis software (Tree Star, Inc., Ashland, OR). Cellular apoptosis was determined by double staining with PI and Annexin V-APC (dead apoptotic) or single positive Annexin V-APC (live apoptotic),

while necrotic cells were identified as PI single positive cells. Trevigen's CometAssay kit (Trevigen Inc, Gaithersburg, MD) was used to evaluate DNA damage in treated and untreated βLox5 cells based on DNA tail shape and migration pattern. In addition, the ApoGlow Assay Kit (Lonza, Rockland, ME) was employed to distinguish between the different forms of cell death (apoptosis or necrosis), as well as to determine the effects of specific treatments on βLox5 proliferation. The Proteome Profiler Human Apoptosis Array Kit (R&D Systems) was utilized to measure the expression of proteins involved in apoptosis and DNA repair.

Passive release of high-mobility group box 1 (HMGB1) protein by necrotic cells was determined Western Blot. Briefly, βLox5 cells were treated as described and 40 µL of the supernatant of each well was removed without disturbing the attached cells. The supernatant was concentrated by ultracentrifugation (100,000 x g for 30 min at 4°C), separated by SDS-PAGE, and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). HMGB1 was detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate Kit, Thermo Scientific, Waltham, MA) according to the manufacturer's instructions.

Nitric Oxide Detection

The amount of nitric oxide (NO) released by βLox5 cells after cytokine treatment was indirectly measured using the Griess Reaction as previously described [195]. The optical density was read using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

Oxidative Stress Analysis

Glutahione (GSH) levels were detected and quantified with the GSH-Glo Glutathione Assay (Promega, Madison, WI) after a 24 h-incubation with rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL).

Caspase Activity and Inhibition Assays

Caspase 8 and Caspase 3 activities were measured using a commercially available caspase detection kit (Cell Technology, Inc, Palo Alto, CA) as per the manufacturer's instructions. Briefly, Caspase 8 (FAM-LETD-FMK) or Caspase 3 (FAM-DEVD-FMK)-specific carboxyfluorescein (FAM) labeled peptide fluoromethyl ketone (FMK) caspase inhibitors were incubated with 48 h cytokine-treated, 24 h and 48 h α-Fas-treated, or untreated control βLox5 cells for 1 h at 37°C. Cells containing bound inhibitor were analyzed by flow cytometry on the FL1 channel. In some cases, the cells were treated with 50 µM of the pan-caspase inhibitor (Z-VAD-FMK), purchased from Calbiochem (San Diego, CA), for 1 h prior to treatment. Z-VAD-FMK was also added after 24 h of incubation to maintain caspases inactive. Pretreatment for 1 h with the specific inhibitor CA 074 (20 µM & 5 µM) (Sigma) was used to inhibit the lysosomal protease Cathepsin B. Bax translocation into the mitochondria was inhibited with 100 µM of the peptide V5 (Calbiochem).

Flow Cytometry

Cytokine-treated and untreated βLox5 cells were analyzed for Fas surface expression by standard flow techniques. In brief, βLox5 cells were treated with increasing concentrations of rhIFNγ (250, 500, and 1000 U/mL) overnight, stained for 1 h at 4°C and washed to remove excess unbound antibody before analysis.

Immunofluorescence

βLox5 cells were incubated with cytokines as described. Cells were fixed with 2% paraformaldehyde (PFA) for 10 minutes at room temperature (RT), permeabilized with 100% ice-cold methanol for 10 minutes, then blocked with 10% Normal Goat Serum (NGS) for 40 minutes at RT with single PBS washes between each step and two washes before adding the antibody. βLox5 cells were conjugated with AIF antibody (R&D Systems) for 1 h at 37°C, washed 3 times, then stained with FITC conjugated anti-rabbit IgG (1:200) for 60 minutes in the dark. Before visualization, cells were washed and slides were covered using 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium. A Zeiss Axioskop Microscope was used to visualize and image the cells. Images were analyzed using ImageJ/Fiji (National Institute of Health).

Statistical Analysis

Unless stated otherwise, data are shown as mean ± SEM. Significance was determined by a *t* test for two group comparisons (GraphPad Prism 5 for Mac OS X, La Jolla, CA).

Results

Agonistic Activation of Fas Kills βLox5 Cells by Caspase-Dependent Apoptosis

βLox5 cells were incubated for 48 h with an α-Fas monoclonal antibody (CH-11) alone or in combination with rhIFNγ. The combination of CH-11 and rhIFNγ induced death of βLox5 cells (Fig. 2-1A), while neither CH-11 nor rhIFNγ were alone effective. rhIFNγ was required for Fas-induced cell death, as it increased Fas expression on the cell surface, even at the lowest level of IFNγ tested (Fig. 2-1B). Because βLox5 cells treated with rhIFNγ had a reduction in absorbance with the MTT assay, the ApoGlow

assay was performed to distinguish between inhibition of proliferation and cell death. By measuring the amount of available ATP within the cells, as well as the relative ADP:ATP ratio and comparing the results to untreated controls, the ApoGlow assay can distinguish between healthy, metabolically active cells (no change or higher ATP levels and no increase in ADP levels), apoptotic cells (lower ATP levels and increase in ADP:ATP ratio), necrotic cells (lower ATP levels and marked increase in ADP:ATP ratio), and cells that are not proliferating similar to untreated control (lower ATP levels with no change in ADP:ATP ratio). rhIFNγ treatment of βLox5 cells lowered ATP levels with little or no change in the ADP:ATP ratios corresponding with arrested proliferation (Fig. 2-2).

To determine the mechanism of α-Fas-induced killing of βLox5 cells, caspase activity was assayed. Caspases 8 and 3 were shown to be active after only 24 h of treatment (Fig. 2-3A). The pan-caspase inhibitor Z-VAD-FMK (50 μM) was added to cells 1 h prior to and again 24 h after initiation of rhIFNγ and CH-11 treatment. When compared to rhIFNγ control samples, caspase inhibition increased cell survival to control levels (Fig. 2-3B) and eliminated DNA damage (Fig. 2-3C). These data clearly implicate caspase-induced apoptosis as the necessary pathway in Fas-mediated killing of βLox5 cells.

Proinflammatory Cytokine-Induced Killing of βLox5 Cells Occurs Through Caspase-Dependent and –Independent Apoptosis and Necrosis

βLox5 cells were also susceptible to proinflammatory cytokine-mediated cell death. Treatment of these cells with the combination of rhTNFα and rhIFNγ for 48 h caused significant killing (Fig. 2-4); however neither of these cytokines alone was sufficient to kill βLox5 cells. Similar to NOD derived NIT-1 β cells, the addition of IL-1β

to the combination is dispensable [202,203]. Consequently, NO was not detected in the supernatant when measured indirectly via the Greiss Reaction (Data Not Shown). In addition, both untreated and cytokine-treated βLox5 cells were found to contain high levels of Heat Shock Proteins (HSPs), specifically HSP70 and HSP27 (Fig. 2-5), which have been shown to protect β cells against proinflammatory cytokine induction of iNOS and subsequent production of NO [72,190,191,196,204].

Because rhIFNγ was shown to inhibit βLox5 proliferation, the effect of rhTNFα on the proliferation of these cells was tested using a tritiated thymidine (^{3}H-TdR) incorporation assay. In contrast to rhIFNγ, TNFα did not affect proliferation, as rhTNFα-treated cells (5000 U/mL) incorporated the same amount of ^{3}H-TdR after 48 h compared to untreated cells (Fig. 2-6).

Treatment of βLox5 cells with rhIFNγ and rhTNFα resulted in the activation of Caspases 8 and 3 (Fig. 2-4B). Confirmed pan-caspase inhibition failed to completely prevent death (Fig. 2-4C & D), suggesting that cytokines kill these cells by multiple pathways. Accordingly, flow cytometry and Comet assay analyses of cytokine-induced βLox5 killing indicated that these cells die by apoptosis and necrosis (Fig. 2-4A & C). Although caspase inhibition significantly improved βLox5 viability when measured by the MTT assay, levels did not reach rhIFNγ control (Fig. 2-4D) and DNA damage was still observed (Fig. 2-4C).

Analyses of changes in the expression of proteins involved in apoptosis and DNA repair demonstrated that the protein levels of phosphorylated p53 (S15) increased with pan-caspase inhibition (Fig. 2-7), indicating an enhanced effort to repair DNA damage. Cytokine-mediated cell death in βLox5 cells was preceded by an increase in the

proapoptotic protein SMAC/Diablo (Fig. 2-7). SMAC/Diablo contributes to the caspase cascade by binding to inhibitors of apoptosis (IAPs), such as XIAP [205]. As a result, Pro-caspase 3 levels decrease, while cleaved effector Caspase 3 levels increase (Fig. 2-7).

Apoptotic and necrotic βLox5 cell deaths after cytokine treatment and caspase inhibition were further analyzed by the ApoGlow assay and detection of passively released High Mobility Group 1 (HMGB1). The ApoGlow assay showed signatures of apoptosis, as measured by 1) reductions in ATP levels of cytokine treated groups with and without pan-caspase inhibition compared to rhIFNγ controls, and 2) a significant increase in the ADP:ATP ratios that did not differ in rhTNFα and rhIFNγ treated cells with and without caspase inhibition (Fig. 2-8A). These results indicate that the improved viability measured by the MTT assay after pan-caspase inhibition was not biologically significant and apoptosis was still taking place via caspase-independent mechanisms. In contrast, HMGB1 has been demonstrated to only be released during primary necrosis [139,206,207]. Thus, cytokine-induced necrosis was confirmed by the presence of HMGB1 only in the supernatant of cytokine-treated βLox5 cells, compared to untreated and rhIFNγ control cells (Fig. 2-8B). Expectedly, caspase inhibition did not prevent necrotic cell death, as observed by the persistent release of HMGB1 in Z-VAD-FMK-treated cells (Fig. 2-8B).

To examine the contribution of caspase-independent, proapoptotic molecules, Apoptosis Inducing Factor (AIF) localization was visualized by immunofluorescence. AIF is normally found within intact mitochondria and translocates to the nucleus to effect apoptosis independent of the caspase cascade. Compared to rhIFNγ controls, more

AIF was present in the nucleus of rhIFNγ and rhTNFα-treated cells (Fig. 2-9). This suggests that AIF is involved in proinflammatory cytokine-mediated βLox5 cell death. Inhibition studies to further understand the mechanisms of βLox5 cell death showed that these do not depend on Bax translocation, or Cathepsin B activity (Fig. 2-10). Therefore, cytokines trigger apoptotic cell death pathways in βLox5, mediated through the activation of caspase-dependent mechanisms as well as caspase-independent mechanisms. In addition, cytokines also lead to necrotic βLox5 cell death.

Mitochondrial-DNA Deficient βLox5 Cells are Resistant to Cytokine Killing but Sensitive to Fas

To study the role of the mitochondria in cytokine-induced killing of βLox5 cells, the cell line was depleted of mtDNA (βLox5 ρ^0) using low levels of EtBr. PCR and confocal imaging of the cells confirmed successful depletion of the mtDNA (Fig. 2-11). Treated and untreated βLox5 cells amplified primers specific for the human Catalase gene (Fig. 2-11A), but only untreated cells amplified mtDNA-specific primers (Fig. 2-11B). A mitochondrial marker, TMRM, was used to identify the mitochondria, and PicoGreen was used as a DNA dye to identify cytoplasmic and nuclear DNA. Co-localization of the red TMRM and green PicoGreen in the untreated cells was indicative of the presence of DNA in the mitochondria (Fig. 2-11C). However, EtBr treated cells did not co-localize the fluorescent dyes (Fig. 2-11D).

Viability of βLox5 ρ^0 cells after cytokine treatment was measured by the MTT assay and confirmed by flow cytometry analysis of PI exclusion and negative Annexin V staining. Although the MTT assay correlates viability and cell number with succinate dehydrogenase activity, a mitochondrial protein complex, this member of the electron

transport chain is comprised of four-nuclear encoded proteins. Thus, the assay functions normally in mtDNA-deficient cells, as previously described [208,209]. βLox5 ρ^0 cells were found to be resistant to cytokine-mediated cell death but sensitive to Fas-induced killing (Fig. 2-12A), supporting activation of the extrinsic pathway by Fas versus the intrinsic pathway by proinflammatory cytokines. mtDNA sufficient βLox5 Cybrid cells were sensitive to Fas ligation and add-back of mtDNA resulted in regained susceptibility to proinflammatory cytokines (Fig. 2-12A). Pan-caspase inhibition was able to prevent Fas-induced cell death in ρ^0 and cybrid cells (Fig. 2-12A). Because the mitochondrial electron transport chain is the main source of ROS in cells, the redox state of the parental cell line, βLox5, after cytokine treatment was analyzed. Reductions in available GSH were observed after 24 h of cytokine treatment of βLox5 cells (Fig. 2-12B) when no changes in cell survival have been noted.

Discussion

A human β cell line that can be expanded and maintained indefinitely would be a useful tool for advancing our understanding of the autoimmune pancreatic β cell destruction that precedes T1D development in man. Such a model cell would also provide an *in vitro* system to test pharmacological inhibitors or genetic manipulations intended to block killing by autoimmune effectors, as well as non-invasively study the impact of immunosuppressive agents, hyperglycemia, or hyperlipidemia. To date, there have been publications detailing the production of six human derived β cell lines. These lines are NAKT-15 [57], CM and HP62 [63-68,73,75,76], NESY2 [210], EndoC-βH1 [58] and βLox5 [56,92,211-214]; however, only βLox5 is readily available to the scientific community. This study sought to determine the value of this already established,

human pancreatic β cell-derived line, βLox5, in assays aimed at elucidating the role of mitochondria in human β cell apoptosis induced by immune insults.

The Fas/FasL pathway has been associated with the development of T1D in animal models and in humans [3,31,215-217]. Although autoreactive T cell clones from transgenic mice lysed Fas-deficient islets, presumably due to perforin release, perforin-deficient T cells had similar diabetogenic potential as the wild type clones when transferring disease to immunodeficient NOD mice [3,39]. These findings suggest that redundant mechanisms eliminate β cells during autoimmune attack. To mimic direct killing by diabetogenic effectors, the cell line was incubated with an activating α-Fas monoclonal antibody for 48 h. The 48 h treatment period was chosen to obtain a significant amount of cell death while still having the required cell numbers to perform functional assays, such as measuring caspase activity in the apoptotic and live cells. In addition, because βLox5 cells proliferate well, longer incubation times in 12-well plates leads to death even in untreated cells.

Similar to primary islets from human and mouse as well as rodent-derived β cell lines, βLox5 cells required IFNγ priming for sufficient surface Fas expression and subsequent ligation by the antibody (Fig. 2-1), supporting the role of proinflammatory cytokines in providing an environment favorable for cell killing [218]. Moreover, Fas-dependent apoptosis in βLox5 cells was found to be caspase-mediated (Fig. 2-3). mtDNA deficient βLox5 ρ^0 cells, which are deficient in the electron transport chain subunits of Complexes I, III, and IV that are encoded by the mtDNA, were sensitive to Fas ligation due to the extrinsic activation of caspases (Fig. 2-12A). This is in accordance with the extrinsic Type I model of Fas-mediated apoptosis that proceeds

independent of the mitochondria [219], and with Fas-mediated killing mechanisms previously identified in the NIT-1, NOD insulinoma cells, and primary NOD islets [118,220]. These results indicate that βLox5 cells are susceptible to direct killing by immune effectors and die by a relevant pathway in T1D.

Proinflammatory cytokine exposure of primary rat or mouse pancreatic islets, as well as the RIN and INS1 β cell lines established from rat, results in functional inhibition and death that is highly dependent upon the production of NO [194], with small contributions of apoptosis only after long-term culture with cytokines [138]. In mouse β cell lines, killing due to IFNγ□ TNFα□ and IL-1β treatment is less dependent on NO production and in some cell lines is NO-independent [221,222]. In human islets, timing of treatment as well as cytokine combination and dose are critical [193,198]. To test if βLox5 cells are also vulnerable to proinflammatory cytokines, the cells were cultured with IFNγ, TNFα □ and IL-1β□ individually or in combination. The combination of IFNγ and TNFα led to the most significant level of βLox5 cell death by both apoptosis and necrosis (Fig. 2-4). Cytokines promoted both necrosis and caspase-dependent and –independent apoptosis of βLox5 cells that was independent NO, potentially due to the presence of HSP27 and HSP70 (Fig. 2-5), confirming that cytokines can activate a range of pro-death mechanisms in β cells [118,122,125,131,138,192,198,220,221,223-227].

Beta cell mitochondria play a key role in insulin secretion [228] and may be important in β cell death. Apoptosis-inducing stimuli result in the mitochondrial membrane permeability transition (PT) that leads to the release of cytochrome c (Cyt c) and other proapoptotic molecules. PT and Cyt c release generally precede the

disruption of mitochondrial inner membrane potential ($\Delta\Psi m$) and mitochondrial function [229]. In addition, mitochondrial release of proapoptotic molecules potentiates the activation cascade of caspases [230]. Although caspases are involved in the killing of βLox5 cells by proinflammatory cytokines (Figs. 2-4 & 2-8), other pathways are also implicated, as the pan-caspase inhibitor, Z-VAD-FMK, failed to completely prevent death in these cells (Figs. 2-4C&D, & 2-8A). Necrosis was shown to contribute to βLox5 cytokine-induced death (Figs. 2-4A & 2-8B) and could have accounted for the above results; however, DNA damage, which is indicative of apoptosis, was still observed with pan-caspase inhibited cells (Figs. 2-4C & 2-7). Therefore, caspase-independent mechanisms of apoptosis were investigated. Apoptosis Inducing Factor (AIF) is a caspase-independent, proapoptotic molecule that acts through its release from the mitochondria and subsequent translocation to the nucleus, where it binds DNA and causes chromatin condensation [231,232]. In order to determine if AIF is involved in cytokine killing of βLox5 cells, treated cells were visualized for AIF localization. The ratio of nuclear localized AIF to cytoplasmic AIF was significantly increased in cytokine-treated cells (Fig. 2-9). This difference persisted in Z-VAD-FMK pre-treated βLox5 cells, supporting a role for AIF in IFNγ and TNFα cytotoxicity.

Other caspase-independent proapoptotic pathways were studied for their role in cytokine-mediated βLox5-cell death. Cathepsin B has been shown to contribute to TNFα-induced apoptosis in other cell types [233]; however, Cathepsin B inhibition with CA 074 failed to rescue βLox5 survival after cytokine treatment. In fact, at the published concentration of 20 μM [233], CA 074 exacerbated cytokine killing and caspase activation (Data Not Shown). At the highest non-toxic concentration of 5 μM, CA 074

did not increase survival (Fig. 2-10). Recently, Bax-dependent mitochondrial permeabilization was identified as a proapoptotic signal in human islets after cytokine treatment [198]; nonetheless, preventing Bax translocation into the mitochondria was insufficient to prevent death in βLox5 cells (Fig. 2-10).

mtDNA deficient βLox5 ρ^0 cells were not killed by IFNγ and TNFα treatment but mtDNA sufficient βLox5 Cybrid cells were sensitive to cytokine-induced cell death (Fig. 2-12A). This is consistent with a recent report that demonstrated intrinsic apoptosis was activated during cytokine treatment of human islets [198]. Therefore, because functional mitochondria were found to be required for rhIFNγ and rhTNFα killing and mitochondria are a major source of ROS, the cells were tested for signs of oxidative stress. GSH levels after cytokine treatment indicated oxidative stress in βLox5 (Fig. 2-12B), demonstrating that cytokine treatment tilts the redox balance towards oxidation, likely due to increased mtROS production.

The susceptibility of isolated human islets to killing *in vitro* by proinflammatory cytokines has been the focus of significant hypothesis testing, while study of the mechanisms of Fas killing of human islets has been less intense [234]. *Postmortem* histological analysis of pancreas from patients with T1D have demonstrated that within the insulitis, $CD8^+$ T cells express cell surface FasL suggesting a role for Fas in β cell destruction during disease development [235]. Information on how Fas kills islets has been derived from studies using mouse islets with no clear published mechanism using human islets. Mouse studies indicated Fas activated caspases. The enclosed studies are the first demonstration of a mechanism for Fas mediated apoptosis of human β cells, and clearly indicate that Fas activates the extrinsic pathway for apoptosis in βLox5

cells (Figs. 2-3 & 2-12). It remains to be investigated whether this is a shared mechanism with primary human islets.

The ultimate effector molecule resulting from a cytokine attack on primary islets is NO [193]. In contrast to primary human islets, βLox5 cells do not produce NO when exposed to the combination of IL-1β, TNFα, and IFNγ. In the absence of NO production, IL-1β, TNFα, and IFNγ can activate a range of pro-death pathways, including caspase-dependent apoptosis and necrosis, in isolated primary human islets [198,234]. βLox5 cells undergo both necrosis and caspase-dependent apoptosis after treatment with TNFα and IFNγ. However, IL-1β is required to kill primary human islets yet, its addition is superfluous for killing of βLox5 cells.

In summary, βLox5 is a partially dedifferentiated β cell line that produces lower levels of insulin than primary islets and has blunted glucose stimulated insulin secretion. This cell line and its derivative line, βLox5 ρ^0, have been established as unlimited sources of human β cells that are inappropriate to study mechanisms of β cell function; however, they can be used for the study of autoimmune β cell death as well as mitochondrial contributions to death. These cells are primed by proinflammatory cytokines for Fas-induced caspase-dependent apoptosis and are susceptible to cytokine-mediated apoptosis and necrosis through mitochondrial mechanisms that are both caspase-dependent and –independent. In conclusion, these cells will likely be beneficial when analyzing methods of killing employed by autoimmune effector cells

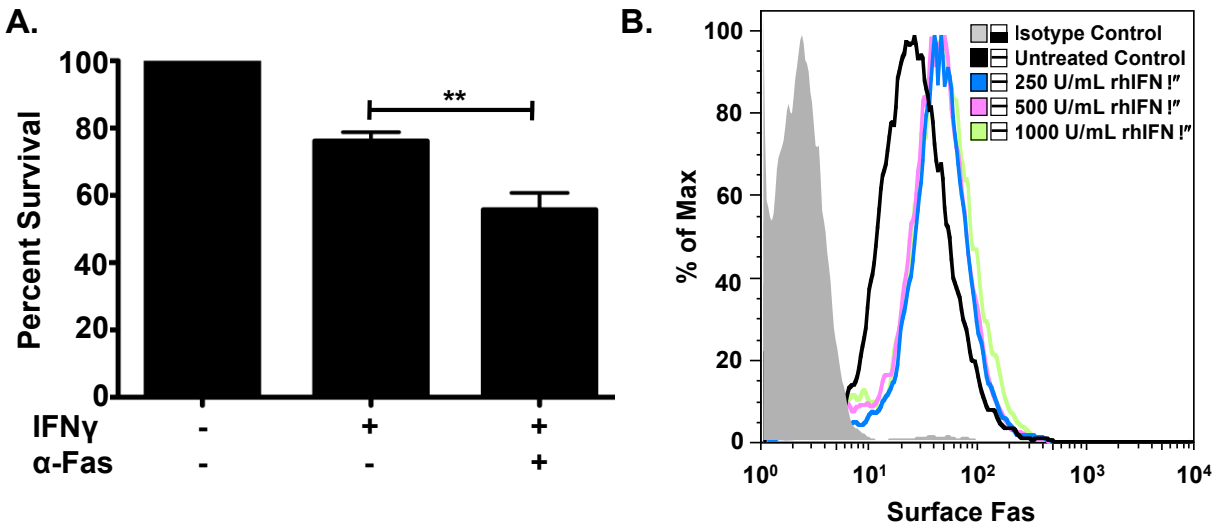

Figure 2-1. βLox5 cells are susceptible to α-Fas monoclonal antibody after rhIFNγ priming. A) βLox5 cells were treated with rhIFNγ alone or the combination of α-Fas antibody clone CH-11 (0.5 µg/mL) and rhIFNγ (1000 U/mL) for 48 h. Viability was measured by the MTT assay. ** denotes statistical significance, $P < 0.005$. B) Overnight priming of βLox5 cells with rhIFNγ increases the expression of surface Fas similarly with 250 U/mL (blue line), 500 U/mL (magenta line), or 1000 U/mL (green line) of rhIFNγ compared to untreated control cells (black line).

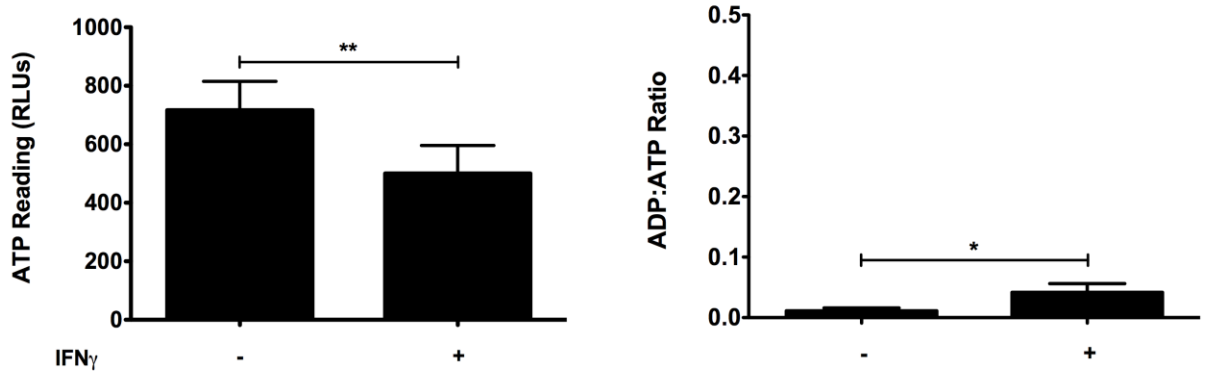

Figure 2-2. rhIFNγ alone causes arrested proliferation. βLox5 cells were treated with rhIFNγ (1000 U/mL) for 48 h. Cell death profile was analyzed by the ApoGlow assay. * denotes statistical difference $P < 0.05$, and **$P < 0.005$.

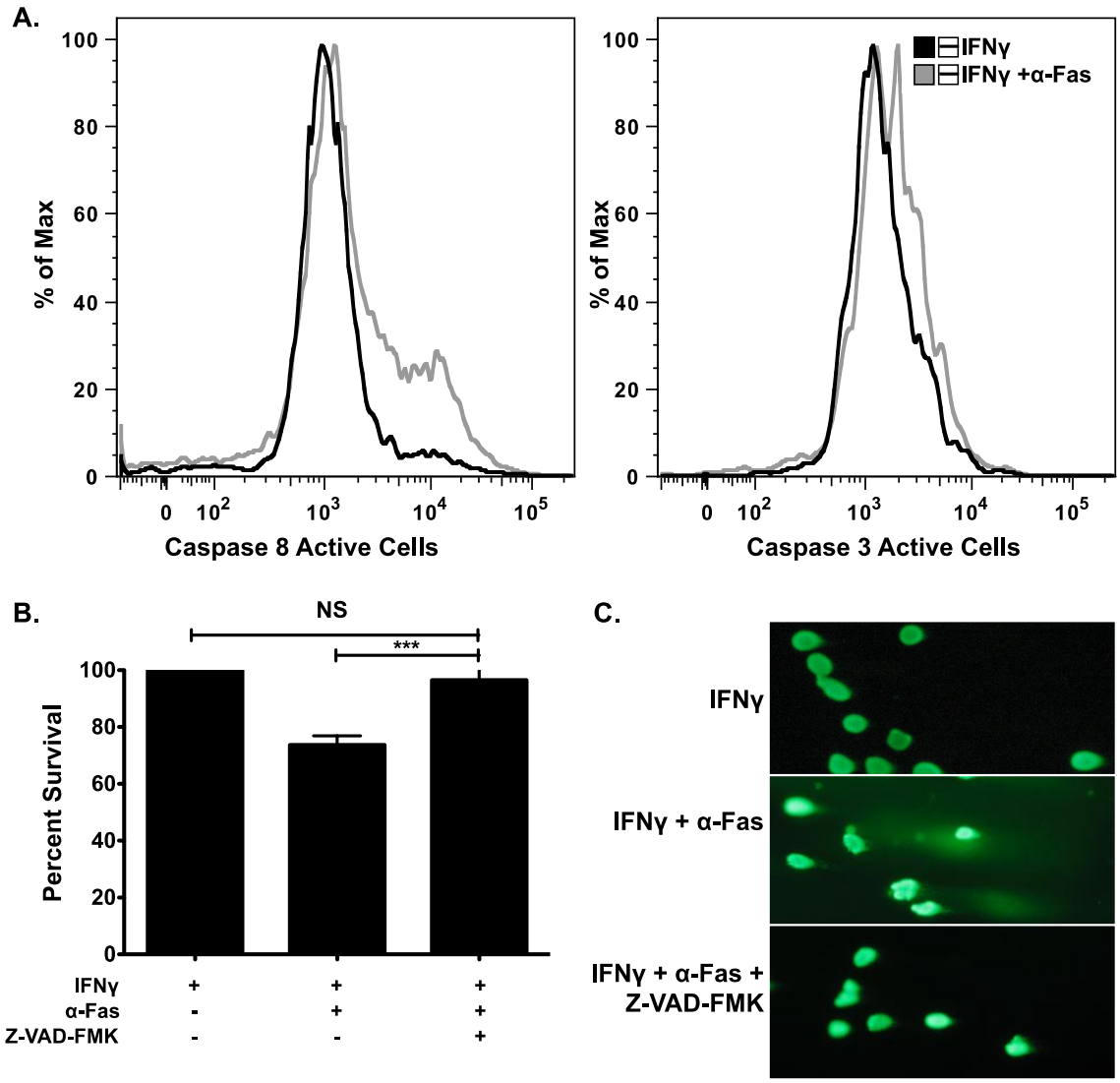

Figure 2-3. Fas-induced killing is caspase-dependent. A) βLox5 cells were primed overnight with rhIFNγ (1000 U/mL) then left untreated or treated with α-Fas antibody clone CH-11 (0.5 µg/mL) for an additional 24h before the activities of Caspases 8 and 3 were measured by FACS analysis. Increased Caspase 8 and 3 activities were noted after only 24 h of Fas stimulation. A representative plot is shown. (B & C) βLox5 cells were treated with rhIFNγ alone or the combination of α-Fas antibody clone CH-11 (0.5 µg/mL) and rhIFNγ (1000 U/mL) for 48 h with and without pan-caspase inhibition with Z-VAD-FMK (50 µM x 2). Viability was measured by the MTT assay B). *** denotes statistical significance with a P value < 0.0001. NS denotes no statistical difference. DNA damage after treatment with and without caspase inhibition was assessed by the Comet Assay C).

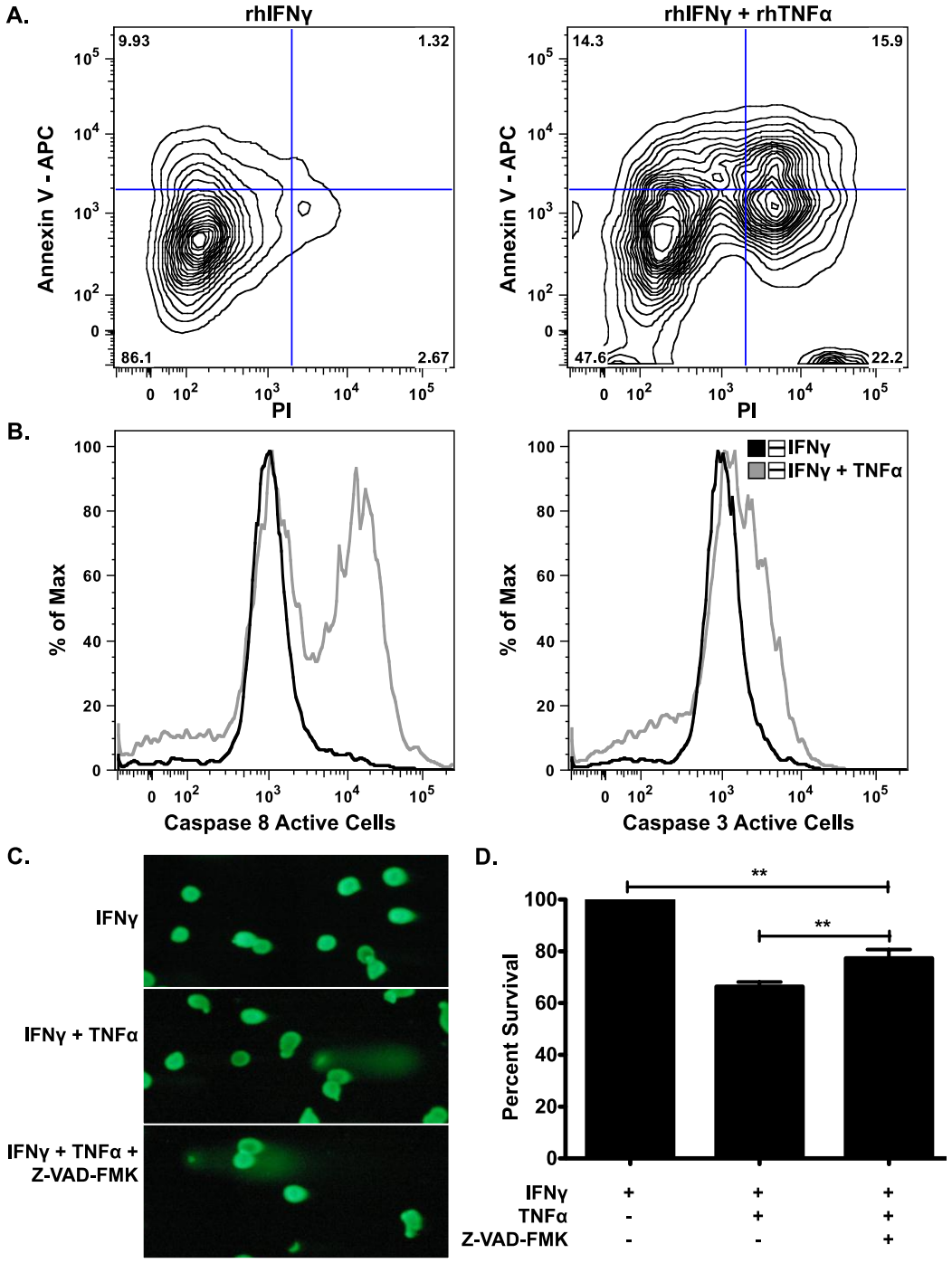

Figure 2-4. Cytokine-induced cell death is partially caspase-dependent. (A, B, C & D) βLox5 cells were treated with the combination of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 48 h with and without pan-caspase inhibition with Z-VAD-FMK (50 µM x 2). The cell death profile (A), as well the activities of Caspases 8 and 3 (B), were measured by FACS analysis. A representative plot is shown. DNA damage and death were assessed via the Comet Assay (C) and the MTT Assay (D), respectively. ** denotes statistical significance with a P value < 0.005.

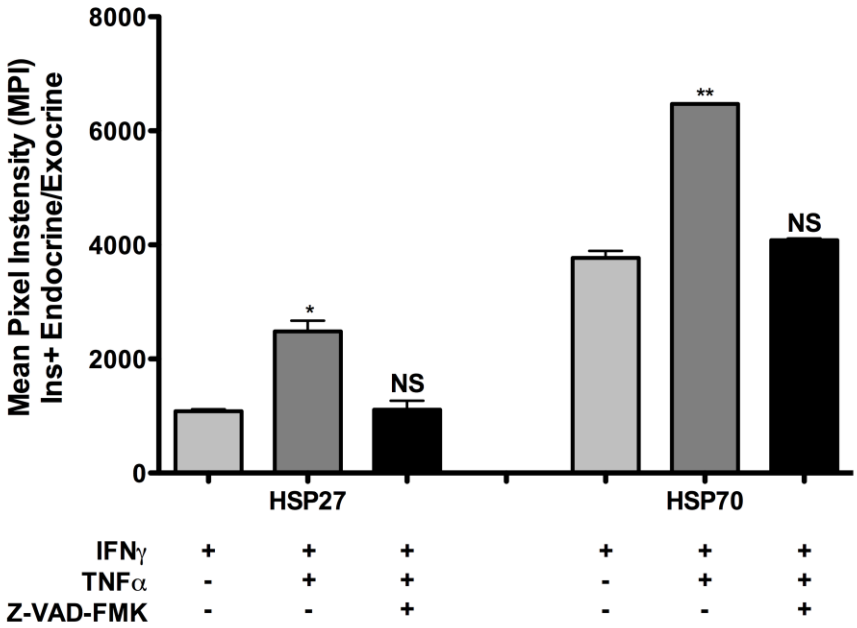

Figure 2-5. Cytokine treatment of βLox5 cells induces the expression of Heat Shock Proteins. βLox5 cells were treated with the combination of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 24 h with and without pan-caspase inhibition with Z-VAD-FMK (50 µM). The Proteome Profiler Human Apoptosis Array Kit was used for protein detection. * denotes statistical significance with a P value < 0.05, * P < 0.05, and NS denotes no statistical difference.

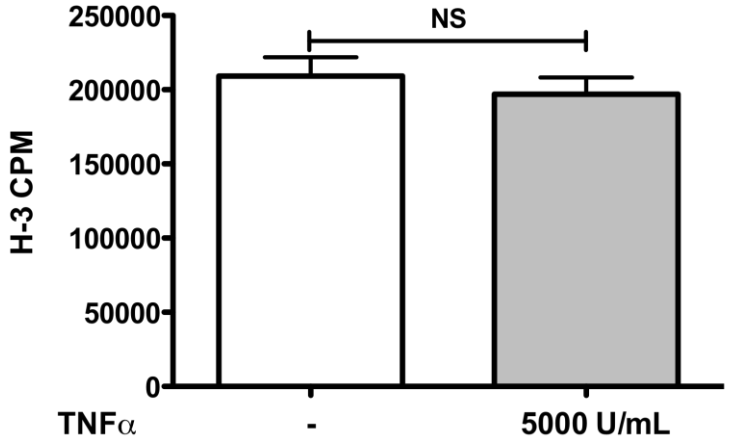

Figure 2-6. rhTNFα alone does not inhibit proliferation of βLox5 cells. βLox5 cells were treated with rhTNFα (5000U/mL) for 48 h. Proliferation was determined by the amount of tritiated thymidine (^{3}H-TdR) incorporated by the cells. NS denotes no statistical difference.

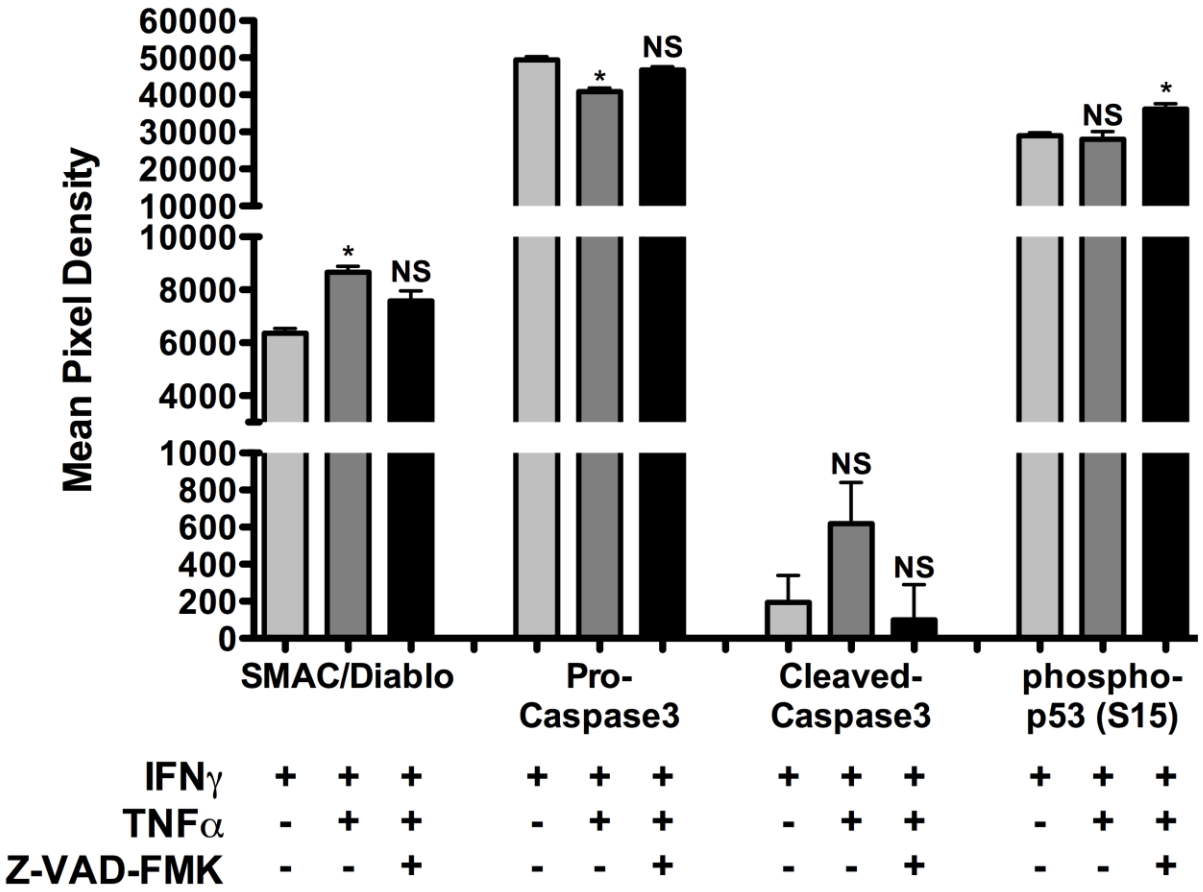

Figure 2-7. Apoptosis and DNA repair protein expression of cytokine-treated βLox5 cells. βLox5 cells were treated with the combination of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 24 h with and without pan-caspase inhibition with Z-VAD-FMK (50 μM). The Proteome Profiler Human Apoptosis Array Kit was used for protein detection. Proteins of interest are shown. * denotes statistical significance with a P value < 0.05, * P < 0.05, and NS denotes no statistical difference.

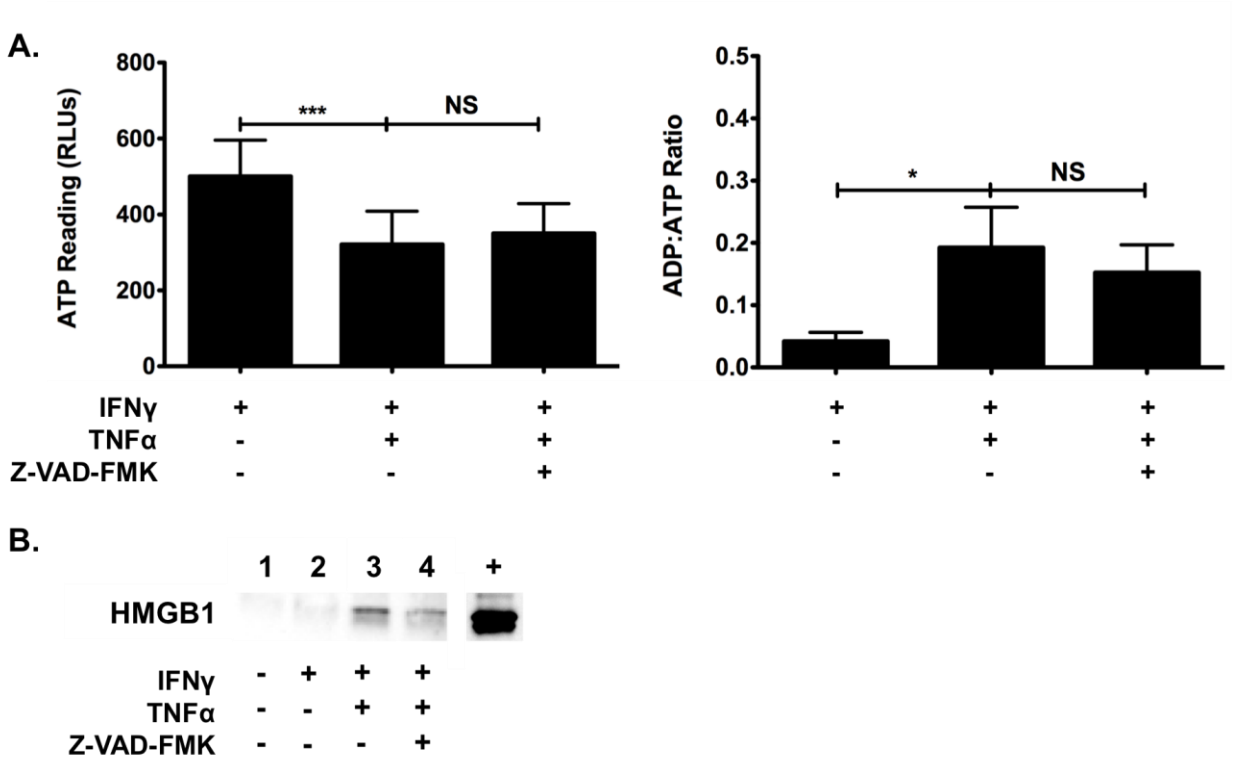

Figure 2-8. βLox5 cells die by apoptosis and necrosis after cytokine treatment with and without pan-caspase inhibition. (A & B) βLox5 cells were treated with the combination of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 48 h with and without pan-caspase inhibition with Z-VAD-FMK (50 µM x 2). A) Viability was analyzed by the ApoGlow Assay. * denotes statistical significance with a P value < 0.05, ***P < 0.0005. NS denotes no statistical difference. B) The supernatant was analyzed for passive HMGB1 release. +: βLox5 cell lysate/positive control, 1: Untreated Control, 2: rhIFNγ, 3: rhIFNγ + rhTNFα, 4: rhIFNγ + rhTNFα + Z-VAD-FMK.

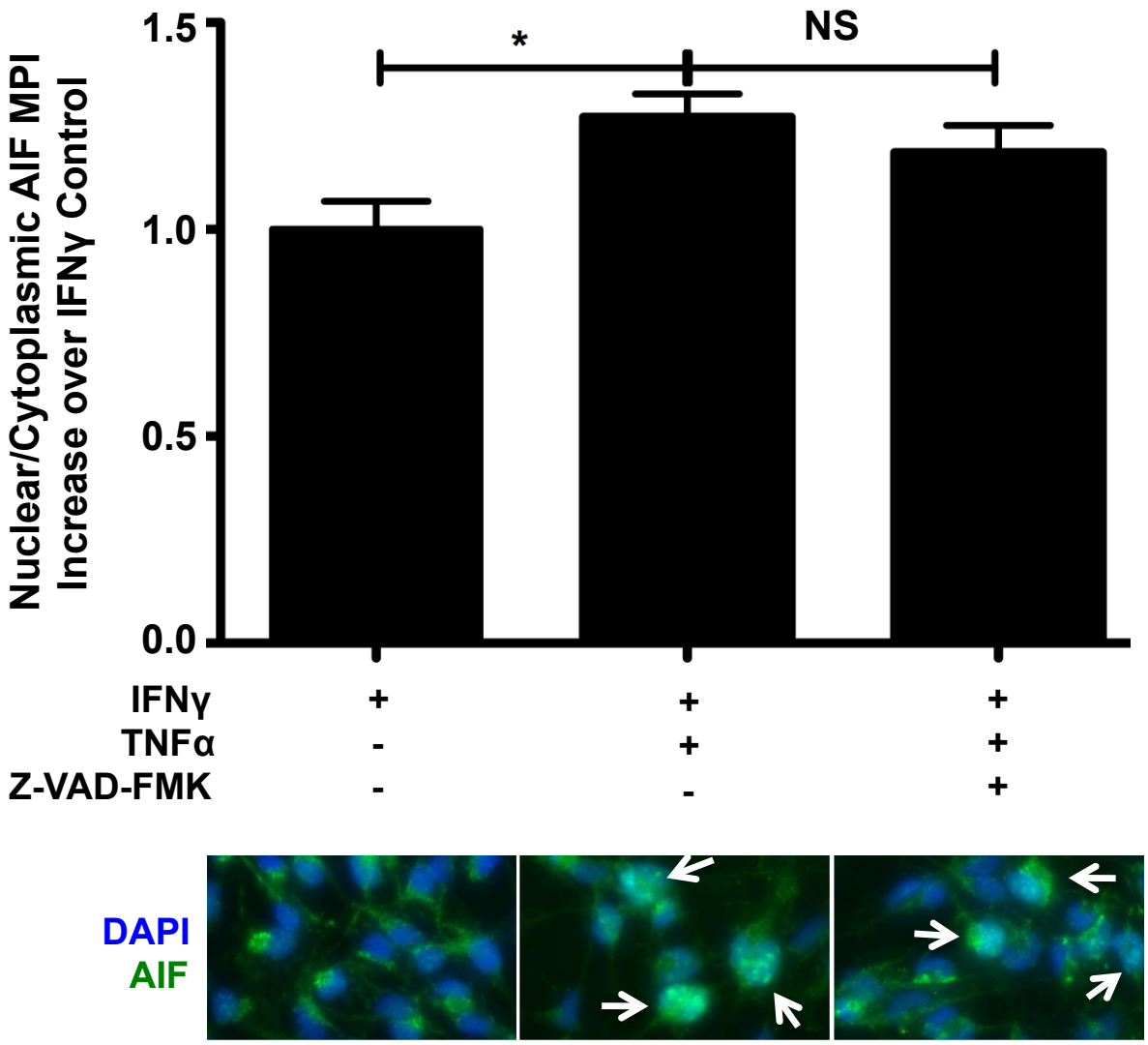

Figure 2-9. Cytokine treatment of βLox5 promotes nuclear translocation of Apoptosis Inducing Factor. βLox5 cells were treated with the combination of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 48 h with and without pan-caspase inhibition with Z-VAD-FMK (50 µM x 2). Immunofluorescence (IF) analysis shows increased AIF translocation to the nucleus in cytokine-treated cells. Representative images are shown. White arrows indicate cells with high nuclear AIF staining. *denotes statistical significance with a P value < 0.05. NS denotes no statistical difference.

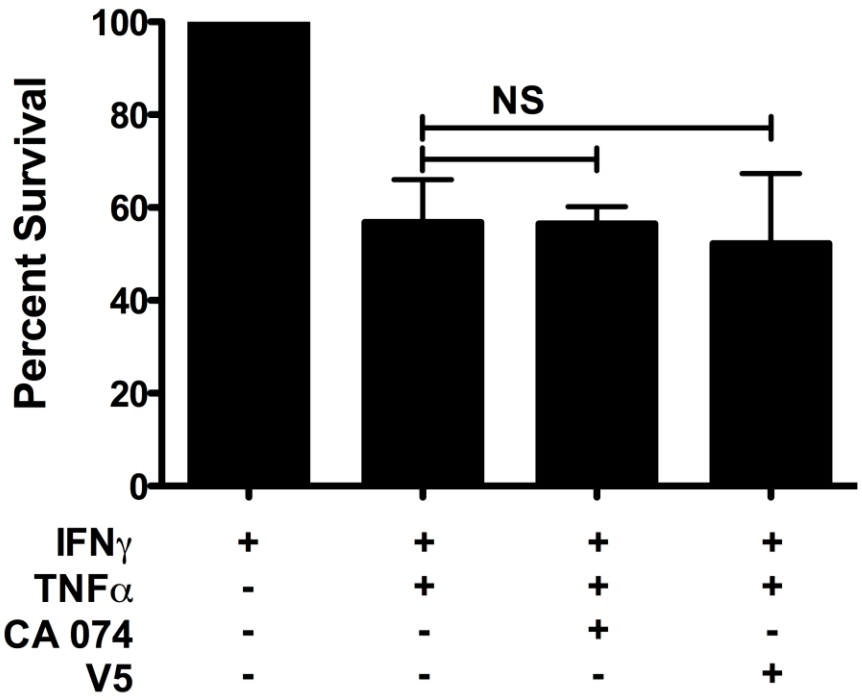

Figure 2-10. Inhibition of Cathepsin B or Bax translocation does not prevent cytokine-mediated βLox5 cell death. βLox5 cells were treated with the combination of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 48 h with and without Cathepsin B inhibition with CA 074 (5 μM) or inhibition of Bax translocation with peptide V5 (100 μM). NS denotes no statistical difference.

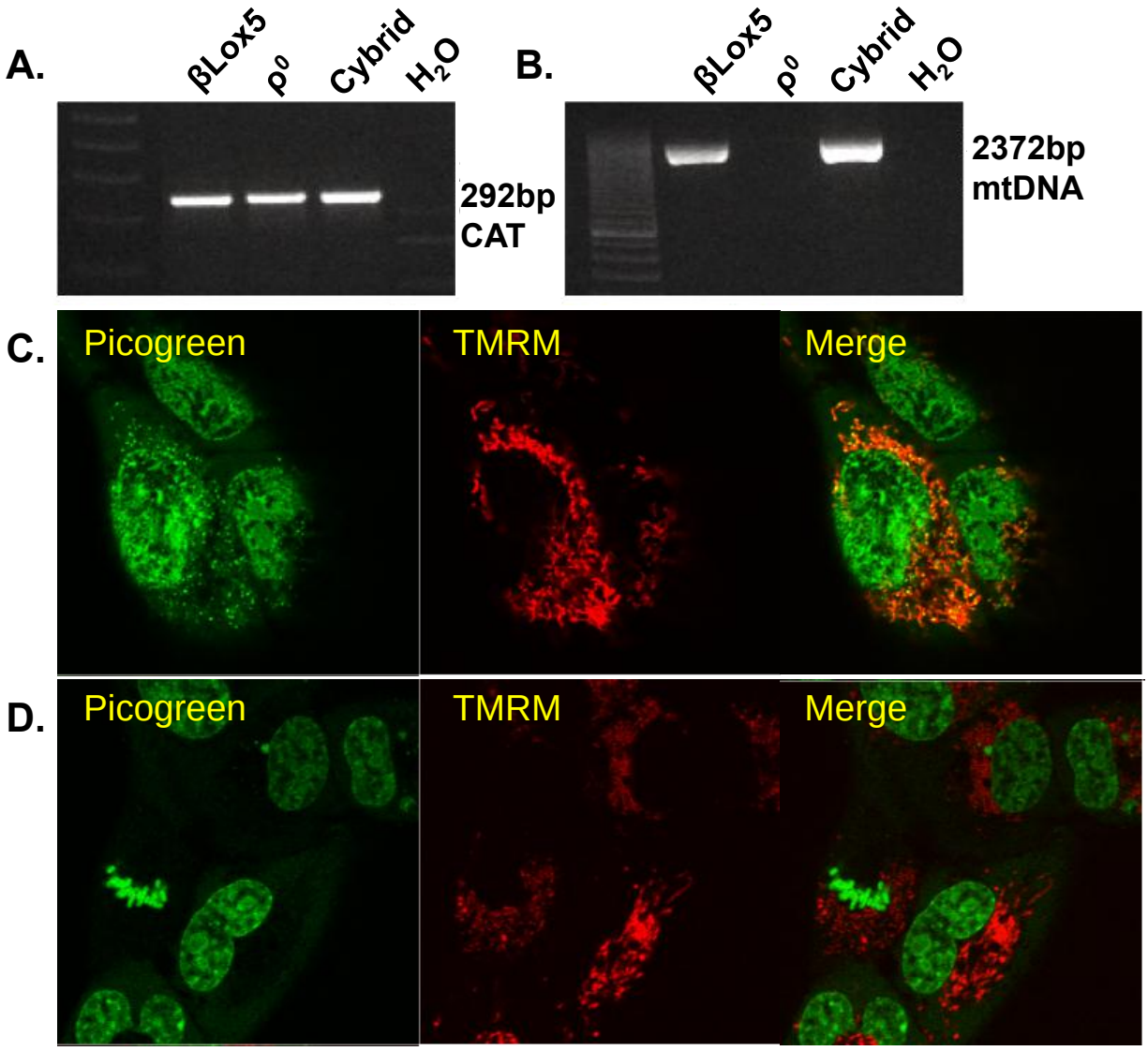

Figure 2-11. Confirmation of mtDNA depletion in βLox5 ρ^0 cells. A) PCR primers specific for a segment of the Catalase gene (CAT) were used as a positive control. Genomic DNA from both EtBr (100 ng/mL) treated and untreated cell cultures exhibited robust amplification with the CAT primer pair (product length: 292 bp). B) Using primers that are specific for the human mtDNA, no mtDNA amplification was seen in the EtBr treated cells, while the untreated cells produced a band of the appropriate size (product length: 2372 bp). C) Confocal images of untreated βLox5 cells using the fluorescent probes PicoGreen (Green-DNA) and TMRM (Red-mitochondrial membrane potential). Untreated cells exhibit co-localization (Orange) of these dyes in the cytoplasm. D) Confocal images of EtBr treated (100 ng/mL) βLox5 cells (βLox5 ρ^0) using the fluorescent probes PicoGreen (Green-DNA) and TMRM (Red-mitochondrial membrane potential). These treated cells exhibit mitochondrial membrane potential but no cytoplasmic positivity for PicoGreen.

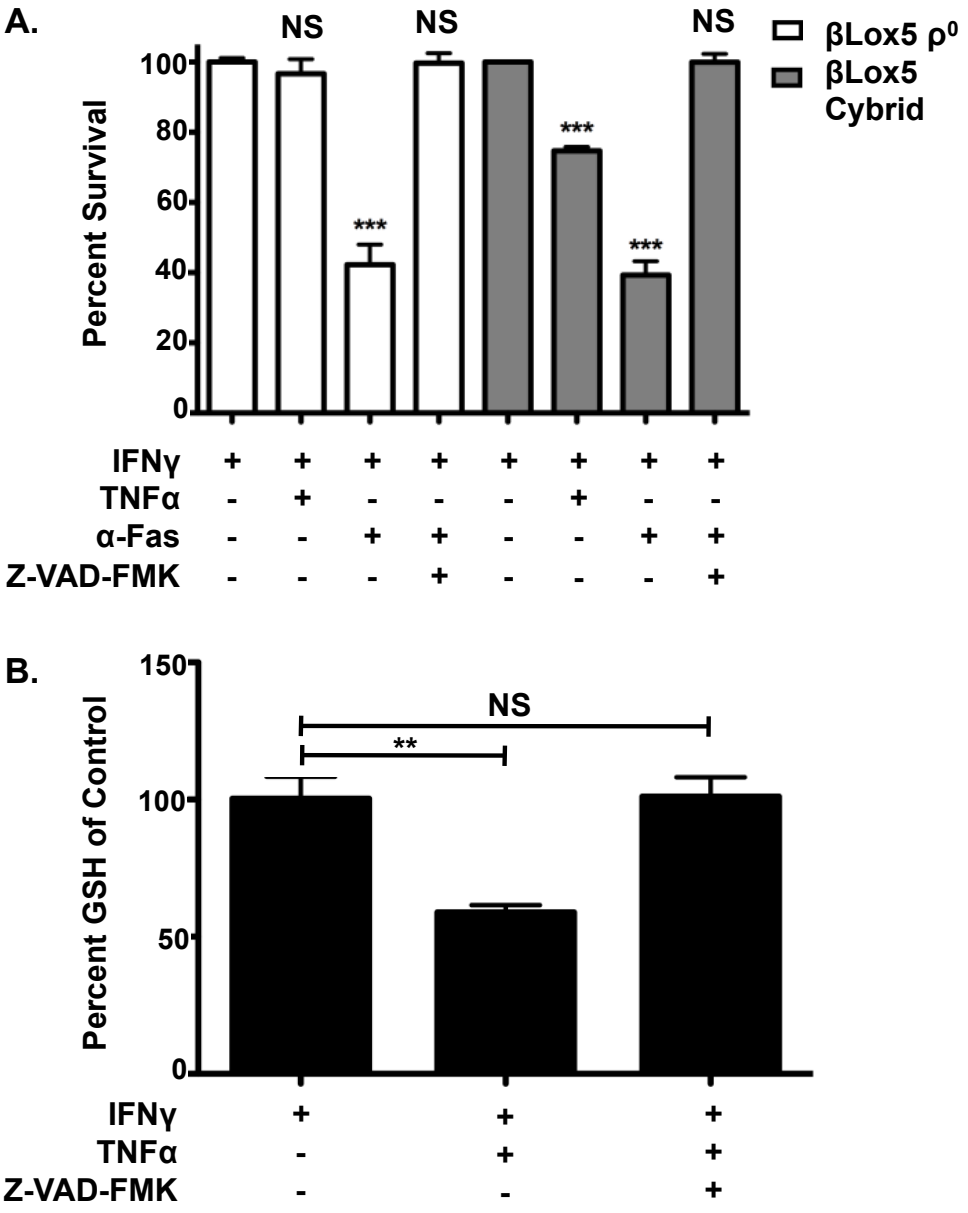

Figure 2-12. Functional mitochondria are required for cytokine killing of βLox5. A) βLox5 ρ^0 cells (empty bars) and βLox5 cybrid cells (gray bars) were treated with rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL), or with CH-11 (0.5 µg/mL) and rhIFNγ (1000U/mL) for 48 h. Z-VAD-FMK (50 µM x 2) was used to inhibit caspase activity. Viability was measured by the MTT assay. Only mtDNA-sufficient cells were killed by cytokines. *** denotes statistical significance with a P value < 0.0001 compared to rhIFNγ control. NS denotes no statistical difference when compared to rhIFNγ control. B) Changes in GSH levels were measured after a 24 h-incubation period with rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL), with and without pan-caspase inhibition. ** denotes statistical significance with a P value < 0.01 compared to rhIFNγ control.

CHAPTER 3
REDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION AND INCREASED RESISTANCE TO CELL DEATH SIGNALS AFFORDED BY *mt-ND2*^{a}

Introduction

Insulin-dependent type 1 diabetes (T1D) is a complex autoimmune disorder modulated by genetic susceptibility and environmental triggers. Clinical studies as well as those using animal models have strongly linked genetic factors with T1D [236,237]. In addition to susceptibility loci in the nuclear genome such as in HLA Class II and the insulin gene (*INS*) [171,236], a mitochondrial polymorphism has been linked to autoimmune diabetes [49,181]. Mutations in mitochondrial DNA (mtDNA) can lead to diabetes resulting from β cell dysfunction and impaired insulin secretion [238]; however, only a single nucleotide polymorphism (SNP) in the mitochondrial gene for NADH dehydrogenase 2 (*mt-ND2*) has been associated with autoimmune diabetes in humans and in T1D-prone NOD mice [49,181,186,239].

In humans, *mt-ND2*^{c} is present at a higher frequency in T1D patients than in controls [181]. The C to A SNP causes an amino acid change from leucine to methionine. Previously, the corresponding C to A transversion (and Leu to Met substitution) in the mouse *mt-Nd2* gene was found to result in lower mitochondrial ROS production [47], rather than increased resistance to oxidative damage as originally proposed [181]. Additionally, immune cells are not affected by the SNP; instead, the β cells exhibit enhanced resistance to T cell-mediated lysis [49]. Due to the similarities of the human and mouse genotypes, it might be expected that the protective phenotype of *mt-ND2*^{a} is also a consequence of reduced mitochondrial ROS production by the β cells.

In contrast to the *mt-ND2* SNPs, the known nuclear DNA loci influence T1D risk through direct or indirect modulation of the immune response. For instance, among the non-HLA-susceptibility regions, polymorphisms in the *INS* gene [240] and the gene encoding lymphoid-specific phosphatase LYP (*PTPN22*) [241] contribute most to T1D sensitivity. Allelic differences within the variable number of tandem repeats (VNTR) locus of the *INS* gene determine the level expression of *INS* mRNA in the thymus [182,183]. The protective class III VNTR correlates with higher levels of *INS* message in the thymus, where central tolerance to self-antigens is established; thus, the risk allele may not allow efficient negative selection of insulin-reactive thymocytes to take place due to the lower expression of *INS* mRNA. In the case of *PTPN22*, the pathogenic variant is also associated with other autoimmune disorders [242]. The gain-of-function mutation of LYP results in decreased T cell receptor (TCR) signaling [184,243], which could then lead to thymocyte hyporesponsiveness during development and, consequently, inefficient deletion of autoreactive T cells in the thymus.

Mitochondrial mutations are commonly studied using cytoplasmic hybrid (cybrid) technology [244]. Cybrids are developed by fusion of mtDNA-deficient (ρ^0) cell lines with platelets of donors harboring the mutation of interest. Here, cybrid cell technology was employed to study the impact of the *mt-ND2*^{c} and *mt-ND2*^{a} alleles in β cell death. βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cell lines were generated from the human β cell line, βLox5 [29,56,91]. The present study confirmed the protective phenotype of *mt-ND2*^{a} in human β cells and tested the hypothesis that resistance results from lower levels of mitochondrial ROS generation. βLox5-*ND2*^{a} cells resisted killing by proinflammatory cytokines and death receptor activation compared to βLox5-*ND2*^{c}. Similar to what was

found in the mouse [47,49], the data presented suggest that protection is attributed to a failure of mitochondria to increase ROS production in response to proapoptotic stimuli.

Materials and Methods

Cell Line and Reagents

The βLox5 cell line was kindly provided by Dr. Fred Levine (Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA). βLox5 cells were maintained as described before in low glucose (1 mg/mL) DMEM (Cellgro, Manassas, VA), supplemented with 10% FBS (HyClone, Fisher Scientific, Pittsburgh, PA), 1% MEM non-essential amino acids (Cellgro), 1% penicillin-streptomycin (Gemini Bio-Products, West Sacramento, CA) solution, 0.02% BSA (Sigma, St. Louis, MO) and 15 mM HEPES (Cellgro) (VC-DMEM) [29]. Recombinant human IFNγ was purchased from BD Biosciences (San Jose, CA). Recombinant human TNFα was acquired from R&D Systems (Minneapolis, MN). Fas agonistic antibody (Clone CH-11) was purchased from Millipore (Temecula, CA). Annexin V-APC, propidium iodide (PI), MitoSox Red, $DiOC_6$, Quant-iT PicoGreen dsDNA reagent, and MitoTracker Deep Red 633 were purchased from Invitrogen (Carlsbad, CA).

Generation of βLox5-*ND2*^{c} and βLox5-*ND2*^{a} Cells

βLox5 ρ^0 cells were generated and maintained as previously described [29]. Briefly, mitochondrial DNA (mtDNA) was depleted by culturing cells in βLox5 medium supplemented with 100 ng/mL Ethidium Bromide (EtBr) for 6 months. Depletion of mtDNA was confirmed by failure to amplify an mtDNA PCR product, confocal microscopy imaging, and failure of βLox5 ρ^0 cells to survive in pyruvate- and uridine-free medium.

From βLox5 ρ^0 cells, cybrid βLox5 cells were generated as described before [201]. In short, cybrid cells were made by fusion of βLox5 ρ^0 cells with mtDNA donor platelets from healthy individuals, selected for highest possibility to express either the C or A allele of *mt-ND2* (Caucasian and Chinese, respectively), under the presence of 50% (W/V) polyethylene glycol 1500 (Roche). Cells were cultured in the medium for βLox5 ρ^0 cells (pyruvate- and uridine-supplemented) during the first 3 days after the fusion and then in selective medium (uridine- and pyruvate-free DMEM supplemented with 10% dialyzed FBS, Penicillin and Streptomycin). After selection for 3 weeks, surviving cybrid cells were cultured in DMEM for βLox5 without pyruvate and uridine. Cybrid cells were cloned using cloning cylinders (Corning, Corning, NY) when visible colonies appeared in the culture. Reconstitution of mtDNA was confirmed by confocal microscopy, and ability to expand the cells in the absence of pyruvate and uridine supplementation, and PCR amplification of a region of the mtDNA.

For confocal visualization, cells were stained with MitoTracker Deep Red (10 μM) in phenol red-free media at 37°C for 30 minutes. PicoGreen (1 μL/mL) was added for 2 minutes to stain DNA. After washing, βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were maintained in phenol red-free media for the duration of the experiment.

To determine the *mt-ND2* allotype, DNA was extracted from cells. A fragment was amplified using PCR primer pair 4451F (GGTTATACCCTTCCCGTACTA) - 6029R (CCAGCTCGGCTCGAAT) and visualized with an agarose DNA gel. The DNA bands were cut from the gel and purified using the Purelink kit (Invitrogen). Amplified DNA fragments were sequenced using the 4451F - 6029R primers. The cybrid cell line developed from the Caucasian platelet donor expressed the C allele and was

designated βLox5-*ND2*^{c}. The cybrid cell line developed from the Chinese platelet donor expressed the A allele and was designated βLox5-*ND2*^{a}.

Cell Death Assays

βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were seeded into twelve-well Corning Costar culture plates (Fisher Scientific) at a density of $5x10^{4}$ cells per well in a total of 500 µL and allowed to adhere overnight. The cells were then incubated with rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL) for 48 h. βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were also cultured with α-Fas activating antibody CH-11 (0.5 µg/mL) and rhIFNγ (1000 U/mL), as IFNγ priming of the parental cell line was previously found to be required for adequate surface Fas expression [29]. In some experiments, the cells were treated with 50 µM of the pan-caspase inhibitor (Z-VAD-FMK), purchased from Calbiochem (San Diego, CA), which was added 1 h prior to treatment. Z-VAD-FMK was also added after 24 h of incubation to maintain caspases inactive. Cell viability was examined by propidium iodide (PI) uptake and externalization of phosphatidylserine (PS) by Annexin V-APC staining.

The antioxidant capacity of the cybrid cell lines βLox5-*ND2*^{c} and βLox5-*ND2*^{a} was challenged by seeding the cells as described above followed by treatment with increasing concentrations of exogenous H_2O_2 (Sigma, St. Louis, MO) for 48 h. The following concentrations were tested: 0, 100, 200, 250, 300, 350, 400, 500, 1000, and 5000 µM. Viability was also measured by PI uptake and PS externalization by Annexin V-APC staining.

βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were analyzed on a BD LSR-Fortessa flow cytometer using the BD FACSDiva software (BD Biosciences) and FlowJo analysis

software (Tree Star, Inc., Ashland, OR). Cellular apoptosis was determined by double staining with PI and Annexin V-APC (dead apoptotic) or single staining by Annexin V-APC (live apoptotic), while necrotic cells were identified as PI single positive cells.

Detection of Mitochondrial Reactive Oxygen Species Production

βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were incubated with rhTNFα and rhIFNγ, or agonistic Fas antibody and rhIFNγ, as described above. After 48 h, MitoSox Red was added to the cells at a final concentration of 2.5 μM and allowed to detect mitochondrial ROS (mtROS) for 20 minutes at 37°C. $DiOC_6$ dye (20 nM final) was also added to the cells during the last 15 minutes of culture to determine the mitochondrial membrane potential of the cells. After washing, the fluorescence of the dyes was measured by flow cytometry on the BD LSR-Fortessa. MitoSox Red is released from the mitochondria of dead cells and binds to nuclear DNA resulting in very strong fluorescence; therefore, only live cells were selected to quantify MitoSox Red fluorescence [245]. To do this, $DiOC_6$ positive, or live cells with mitochondrial membrane potential, were gated and the MitoSox Red mean fluorescence intensity was then measured.

Statistical Analysis

Unless stated otherwise, data are provided as mean ± SEM. Significance was determined by a *t* test for two group comparisons (GraphPad Prism 5 for Mac OS X, La Jolla, CA); when appropriate, paired *t* tests were performed.

Results

***mt-ND2*^{c} *and mt-ND2*^{a} Alleles in the βLox5 Nuclear Background**

Two cybrid cell lines generated from different platelet donors and mtDNA-deficient βLox5 ρ^{0} cells were selected for sequencing. The DNA base pair at position 5,178 within the NADH dehydrogenase subunit 2 gene (*mt-ND2*) demonstrated that one of the

cell lines harbored the C allele, while the other cell line encoded the A allele (Fig. 3-1). The cell lines were then named βLox5-*ND2*^{c} or βLox5-*ND2*^{a}, respectively, according to the *mt-ND2* allotype. Confocal imaging of the cybrid cell lines confirmed that both contained similar mtDNA per mitochondria as the parental cell line (Fig. 3-2). Thus, viability assays with βLox5-*ND2*^{c} or βLox5-*ND2*^{a} were performed to determine the contribution of each allele in the response to cell death signals.

mt-ND2^{a} Protects Human β Cells from Immune-Mediated Destruction

The *mt-ND2*^{c} and *mt-ND2*^{a}-encoding cell lines were treated with IFNγ and TNFα for 48 h using methods identical to those performed with the parental cell line [29]. Cell viability was reduced in both βLox5-*ND2*^{c} and βLox5-*ND2*^{a} after proinflammatory cytokine exposure (Fig. 3-3A). However, over 80% of βLox5-*ND2*^{a} cells survived the combination of IFNγ and TNFα, in contrast to the 44% killing observed in βLox5-*ND2*^{c} cells. Similarly, when compared with βLox5-*ND2*^{c} cells, less βLox5-*ND2*^{a} cells were killed by CH-11 with IFNγ priming (Fig. 3-3A).

Mitochondrial function is dependent on its membrane potential. Changes in mitochondrial membrane potential of βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells after incubation with proinflammatory cytokines or α-Fas antibody were measured with $DiOC_6$ staining. No significant changes in the mitochondrial membrane potential of βLox5-*ND2*^{a} cells after IFNγ and TNFα were observed. On the other hand, βLox5-*ND2*^{c} cells experienced a significant drop in mitochondrial membrane potential after IFNγ and TNFα treatment (Fig. 3-3B). Reductions in both cell lines were measured after Fas killing; however, after treatment, a significantly greater percentage of βLox5-*ND2*^{a} cells were able to maintain higher membrane potential when compared to βLox5-*ND2*^{c} (Fig. 3-3B).

Lower Reactive Oxygen Species Production by βLox5-*ND2*^{a} Cells in Response to Proinflammatory Cytokines and Fas Receptor Activation

Mitochondrial ROS production, as measured by MitoSox Red staining, was quantified in proinflammatory cytokine or CH-11-treated, $DiOC_6$ positive ($DiOC_6$ High) cybrid cells. Cells with high $DiOC_6$ fluorescence staining were found to be mostly Annexin V and PI negative (Fig. 3-4A). In live cells, mitochondrial ROS production was observed in both βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells after IFNγ and TNFα incubation, but a significantly lower induction was measured in treated βLox5-*ND2*^{a} cells (Fig. 3-4B). Similarly, higher levels of mtROS were produced by βLox5-*ND2*^{c} cells after Fas killing compared to βLox5-*ND2*^{a} cells (Fig. 3-4B). These data suggest that mtROS production precedes the loss of mitochondrial membrane potential in β cells.

Of note, basal mtROS generation was also found to be 30% higher in βLox5-*ND2*^{c} than in βLox5-*ND2*^{a} *(*Fig. 3-4B). This is consistent with studies in the mouse where mitochondria isolated from mice expressing *mt-Nd2*^{c} produced 30% more mtROS than mitochondria from the other strains tested [48]. The finding further supports the hypothesis that *mt-ND2*^{a} and *mt-Nd2*^{a} result in similar phenotypes in humans and mice, respectively, and that the protection seen in βLox5-*ND2*^{a} is due to the A allele SNP of *mt-ND2*.

mt-ND2^{a} does not Confer Heightened Resistance to Oxidative Stress

To test whether, in addition to lower endogenous mtROS production, the A allele variant of *mt-ND2* also protects against oxidative damage, βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were incubated with increasing concentrations of exogenous H_2O_2. βLox5-*ND2*^{a} cells did not demonstrate elevated resistance to ROS (Fig. 3-5A). In agreement with

the higher levels of mtROS produced by βLox5-*ND2*^{c} with IFNγ alone compared to βLox5-*ND2*^{a} (Fig. 3-4B), when the cybrids were incubated with IFNγ and exogenous H_2O_2 more βLox5-*ND2*^{a} cells survived higher concentrations of H_2O_2 (Fig. 3-5B).

Caspase Inhibition Fully Rescues Survival in Response to Agonistic Fas Ligation but not Proinflammatory Cytokines

As seen with the parental cell line [29], IFNγ and TNFα treatment activates caspases in βLox5-*ND2*^{c} cells, and pan-caspase inhibition partially prevents cytotoxicity (Fig. 3-6A). On the other hand, viability of βLox5-*ND2*^{a} was unchanged after proinflammatory cytokine killing in the presence of Z-VAD-FMK (Fig. 3-6A). Both cell lines were protected from Fas killing when Z-VAD-FMK (Fig. 3-6B) was also included in the culture media. Like in βLox5 cells [29], activation of the Fas pathway leads to caspase-dependent cell death in βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells.

Discussion

More than 40 chromosomal loci have been associated with T1D susceptibility or resistance [171]. Although some regions that determine risk have been narrowed to include only a single gene, understanding the impact of a given gene to the disease is very difficult due to possible interactions between multiple loci. To date, the only mitochondrial gene correlated with autoimmune diabetes encodes NADH dehydrogenase 2 (*mt-ND2*). In mice, two closely related strains were previously crossed to study the interaction of the nuclear and mitochondrial genomes in the diabetogenic process [48,49,186]. ALR mice are resistant to spontaneous and alloxan-induced T1D. *mt-Nd2*^{a} differentiates the mtDNA of ALR mice from the T1D-susceptible NOD mitochondrial genome, which encodes *mt-Nd2*^{c}. Because mtDNA is maternally inherited, NOD mice were mated with ALR females as described before [48] to obtain a

conplastic mouse strain, NOD.mt^{ALR}. In the presence of a fully diabetogenic environment, *mt-Nd2*^{a} did not prevent spontaneous T1D onset [49]. However, the kinetics of T1D transfer by an autoreactive CD8^{+} T cell clone was altered by *mt-Nd2*^{a}, and autoreactive CD4^{+} T cells did not transfer disease [49]. Furthermore, β cells from the NOD.mt^{ALR} mice exhibited resistance to lysis in cell mediated lymphotoxicity (CML) assays [49]. These data demonstrated that, in mice, protection is at β cell level and nuclear-mitochondrial genome interactions dictate T1D susceptibility.

To understand the contribution of the human *mt-ND2* variants, cybrid cell lines with identical nuclear genomes but differing in their mtDNA were generated from a human β cell line (Figs. 3-1 and 3-2). βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were exposed to pro-death factors that are cytotoxic to the parental βLox5 cell line (Fig. 3-3) [29]. Consistent with the previous observation that IFNγ and TNFα killing is dependent on functional mitochondria [29], βLox5-*ND2*^{a} cells were very well protected from proinflammatory cytokine-induced death (Fig. 3-3A). Less than 20% of the *mt-ND2*^{a}-encoding cells were killed by this treatment, whereas more than 40% of βLox5-*ND2*^{c} cells did not survive incubation with IFNγ and TNFα (Fig. 3-3A). On the other hand, because mtDNA-deficient βLox5 ρ^{0} cells were susceptible to Fas killing, Fas-mediated β cell death in the parental cell line was believed to occur extrinsically, or independent of the mitochondria [29]. Surprisingly, βLox5-*ND2*^{a} cells were also better protected from agonistic α-Fas antibody treatment compared to βLox5-*ND2*^{c} cells (Fig. 3-3A). In both βLox5-*ND2*^{c} and βLox5-*ND2*^{a}, the health of the cells correlated with mitochondrial constitution as determined by the ability to resist changes in the membrane potential of the mitochondria (Fig. 3-3B).

Animal studies indicate that *mt-Nd2*^{a} protects β cells because basal and stimulated mitochondrial ROS production is reduced in cells of this allotype compared to cells expressing *mt-Nd2*^{c} [48]. Mitochondrial ROS generation in live cells can be determined with the indicator MitoSox Red with an emission wavelength of 580 nm (red) when it reacts with mtROS, but MitoSox Red is also highly fluorescent when bound to DNA [245]. To determine the amount of mtROS produced in βLox5-*ND2*^{c} and βLox5-*ND2*^{a}, MitoSox Red fluorescence was measured in cells that maintained mitochondrial membrane potential (Fig. 3-4A), with and without treatment. As hypothesized, βLox5-*ND2*^{a} cells generated significantly lower mtROS in response to both proinflammatory cytokines and Fas receptor activation (Fig. 3-4B). In addition, basal levels of mitochondrial ROS were 30% lower in βLox5-*ND2*^{a} compared to βLox5-*ND2*^{c} (Fig. 3-4B). This is identical to the differences observed in mice; mitochondria isolated from conplastic ALR.mt^{NOD} produce 30% more basal mtROS than NOD.mt^{ALR} mitochondria [48].

As βLox5-*ND2*^{a} cells were more resistant to mitochondrial membrane potential reductions, the cell lines were assayed for susceptibility to exogenous ROS to test if *mt-ND2*^{a} also modifies the antioxidant capacity of β cells. βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were exposed to H_2O_2 for 48 h and viability measured. *mt-ND2*^{a} did not confer increased resistance to exogenous H_2O_2 (Fig. 3-5A). Both cell lines responded equally to H_2O_2 alone, but more βLox5-*ND2*^{a} cells survived when cultured with H_2O_2 and IFNγ (Fig. 3-5B), suggesting that the mtROS produced by βLox5-*ND2*^{c} with IFNγ treatment (Fig. 3-4B) has an additive effect with the exogenously added ROS. These results

indicate that *mt-ND2*^{a} controls mitochondrial ROS production but does not elevate resistance to oxidative stress.

Despite the observation that Fas-mediated βLox5 cell death was seemingly due to extrinsic activation of the caspase cascade [29], βLox5-*ND2*^{a} cells were better protected than βLox5-*ND2*^{c} cells from Fas killing (Fig. 3-3A), suggesting that in mtDNA-sufficient β cells mitochondria may also play a role in Fas-induced death. To elucidate whether the cell death mechanisms activated in the cybrid cell lines differ from those identified in βLox5 [29], βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were killed by proinflammatory cytokines and α-Fas antibody in the presence or absence of the pan-caspase inhibitor, Z-VAD-FMK. As it was noted with caspase inhibition of cytokine-treated βLox5 cells [29], βLox5-*ND2*^{c} were killed by the combination of IFNγ and TNFα through caspase-dependent and –independent mechanisms (Fig. 3-6A). Adding Z-VAD-FMK to the cytokine-containing media significantly rescued viability of βLox5-*ND2*^{c} but not to IFNγ control levels. However, pan-caspase inhibition did not improve cell survival of βLox5-*ND2*^{a} (Fig. 3-6A), indicating that the small percentage of βLox5-*ND2*^{a} cell death measured after IFNγ and TNFα treatment is only a consequence of caspase-independent mechanisms of death. On the other hand, both cell lines were similarly rescued from Fas killing with pan-caspase inhibition (Fig. 3-6B).

Proinflammatory cytokines have been shown to activate multiple pathways of cell death in β cells that vary with the model system and specific treatment parameters [24,29,148-150,198,246]. Proinflammatory cytokine combinations and cell culture conditions that favor the involvement of the mitochondria in β cell death can either induce the intrinsic (mitochondria dependent) apoptotic pathway via the activation of

pro-death molecules that permeabilize the mitochondria and allow the release of cytotoxic factors [198], or amplify extrinsic signals, i.e. TNF receptor activation, through the cleavage of Bid [24]. Furthermore, increasing the antioxidative defense capacity of β cell mitochondria prevents proinflammatory cytokine-induced damage to the mitochondria and cell death [29], implicating mtROS as the initiating signal in mitochondrial-dependent β cell apoptosis.

Fas killing of β cells appears to be more straightforward. β cells were previously demonstrated to behave as Type II cells, in which amplification of the caspase cascade through Bid is required for death induction [24]. However, in the absence of functional mitochondria, human β cells are killed by Fas through the action of caspases [29]. Together with the aforementioned observations, the results presented here contribute to our understanding of the sequence of events that culminate in β cell death in response to pro-death signals.

In summary, when human β cells encoding *mt-ND2*^{c} are incubated with IFNγ and TNFα, mtROS are produced and mitochondrial damage occurs, resulting in activation of the caspase cascade (Fig. 3-7). However, the protective A allele of *mt-ND2* dampens mitochondrial ROS production in response to proinflammatory cytokines, and the caspase cascade does not become activated (Fig. 3-7). Therefore, the signaling cascade with proinflammatory cytokine treatment is proposed to be as follows: 1) intrinsic pathway activation, 2) induction of mtROS production, 3) mitochondrial damage, and 4) caspase cascade activation. Feedback mechanisms likely promote the amplification of this process. In addition, concurrent damage also takes place via caspase-independent cell death pathways and necrosis [29]. As a result of the lower

levels of mtROS in βLox5-*ND2*^{a}, the cells are also more resistant to Fas killing because amplification of the caspase cascade through the mitochondria is weakened (Fig. 3-7). Thus, Fas receptor-mediated β cell death transpires in this sequence: 1) extrinsic pathway activation, 2) activation of caspase cascade, 3) cleavage of Bid and mitochondrial signaling, 4) mtROS production, and 5) amplification of the caspase cascade. These studies demonstrate that a SNP in the mitochondrial genome can modify human β cells to resist killing by insults associated with T1D progression by suppressing mtROS generation.

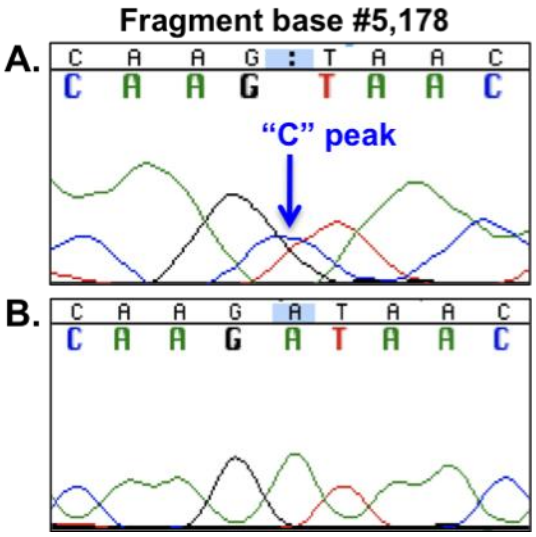

Figure 3-1. Sequencing data of βLox5 cybrid cell clones. Cybrid cell lines developed from two healthy platelet donors in the βLox5 background were sequenced for their *mt-ND2* allotype. One of the cybrid cell lines expressed the C allele (A), and the other the A allele (B).

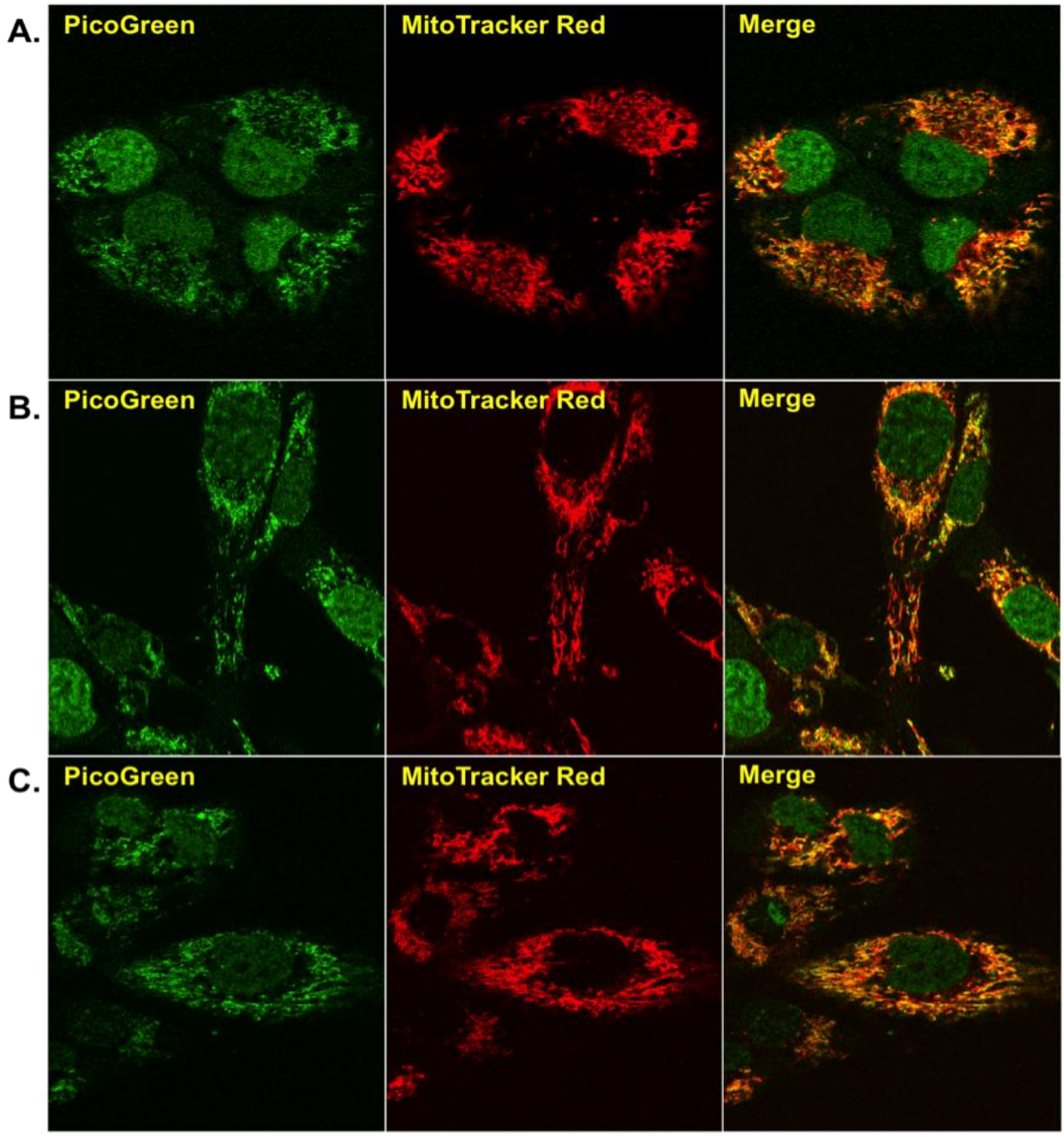

Figure 3-2. Confirmation of mtDNA reconstitution in βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells. Confocal images of untreated βLox5 cells (A) using the fluorescent probes PicoGreen (Green-DNA) and MitoTracker Red (Red-mitochondrial membrane potential). Like the parent cell line, βLox5-*ND2*^{c} (B) and βLox5-*ND2*^{a} (C) cells exhibit co-localization (Orange) of these dyes in the cytoplasm.

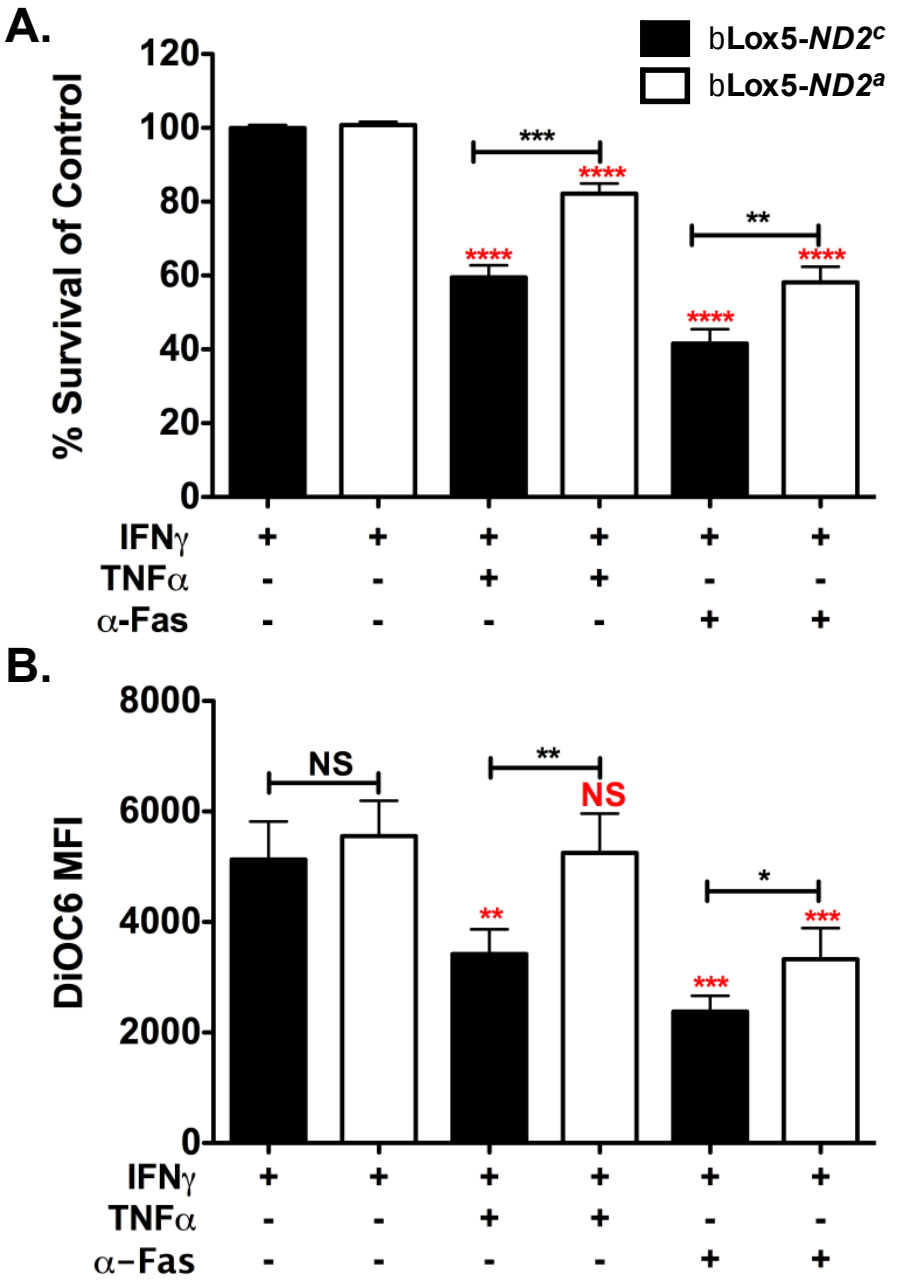

Figure 3-3. *mt-ND2*^{a} protects against proinflammatory cytokine and death receptor-mediated cell death. A) βLox5-*ND2*^{c} (black bars) and βLox5-*ND2*^{a} (white bars) cells were treated with the combinations of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL), or rhIFNγ (1000 U/mL) and CH-11 (0.5 µg/mL) for 48 h. Viability was measured by flow cytometry. B) Mitochondrial membrane potential was determined by the staining intensity of the dye $DiOC_6$. **** denotes statistical significance with a P value < 0.0001, *** P value < 0.0005, ** P value < 0.001, and * P value < 0.05. NS denotes no statistical difference. Red asterisks represent statistical comparison of the specified cell line to its IFNγ control, while black asterisks are used to compare the two cell lines.

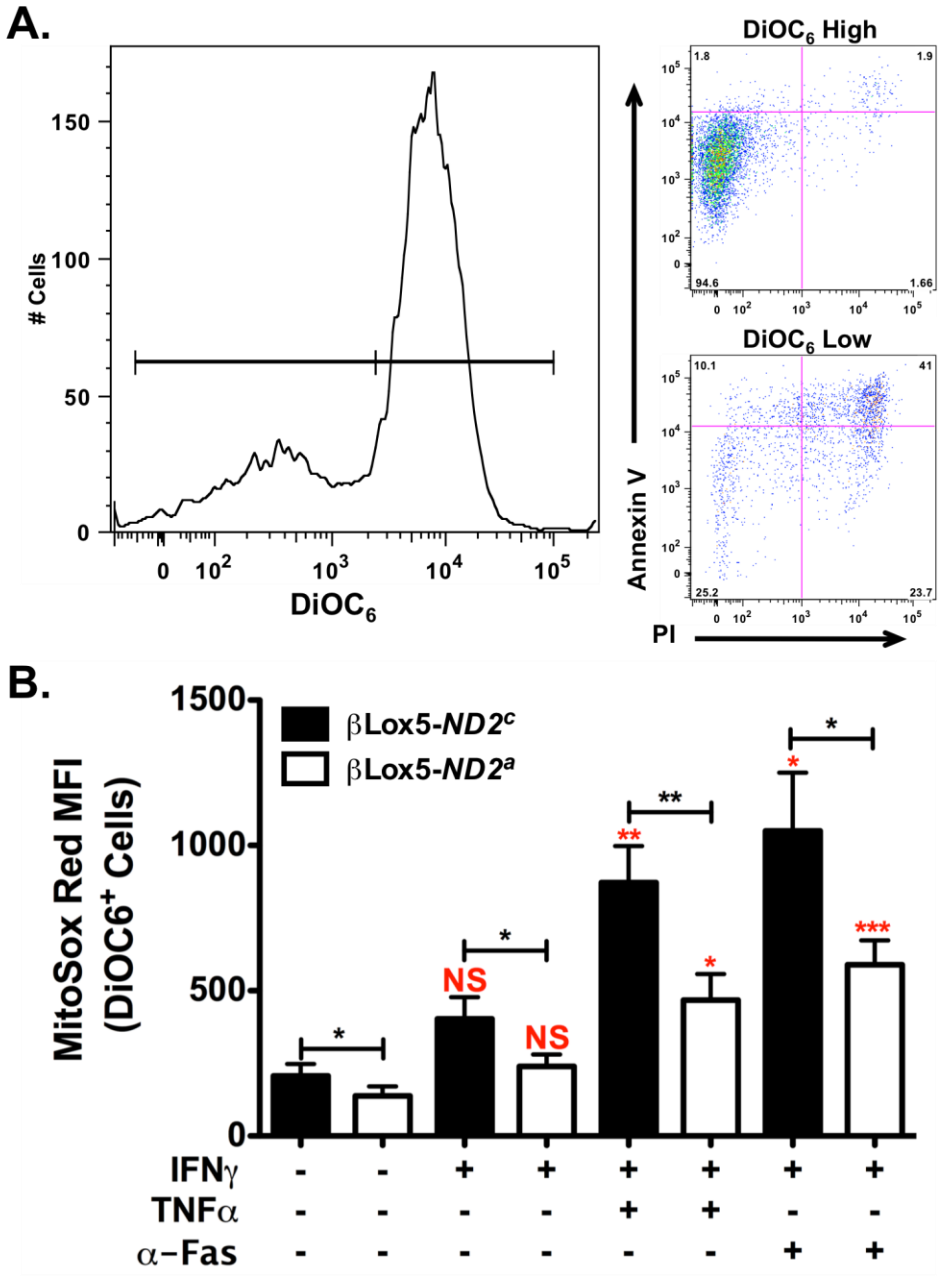

Figure 3-4. Reduced mitochondrial ROS production in βLox5-*ND2*^{a} cells in response to pro-death signals. βLox5-*ND2*^{c} (black bars) and βLox5-*ND2*^{a} (white bars) cells were treated with the combinations of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL), or rhIFNγ (1000 U/mL) and CH-11 (0.5 µg/mL) for 48 h. Mitochondrial ROS production was measured by flow cytometry analysis of MitoStox Red staining (B) in $DiOC_6$ positive, live cells (A). *** denotes statistical significance with a P value < 0.0001, ** P value < 0.005, and * P value < 0.05. NS denotes no statistical difference. Red asterisks are used for statistical comparison of the specified cell line to its untreated control, while black asterisks are used to compare the two cell lines.

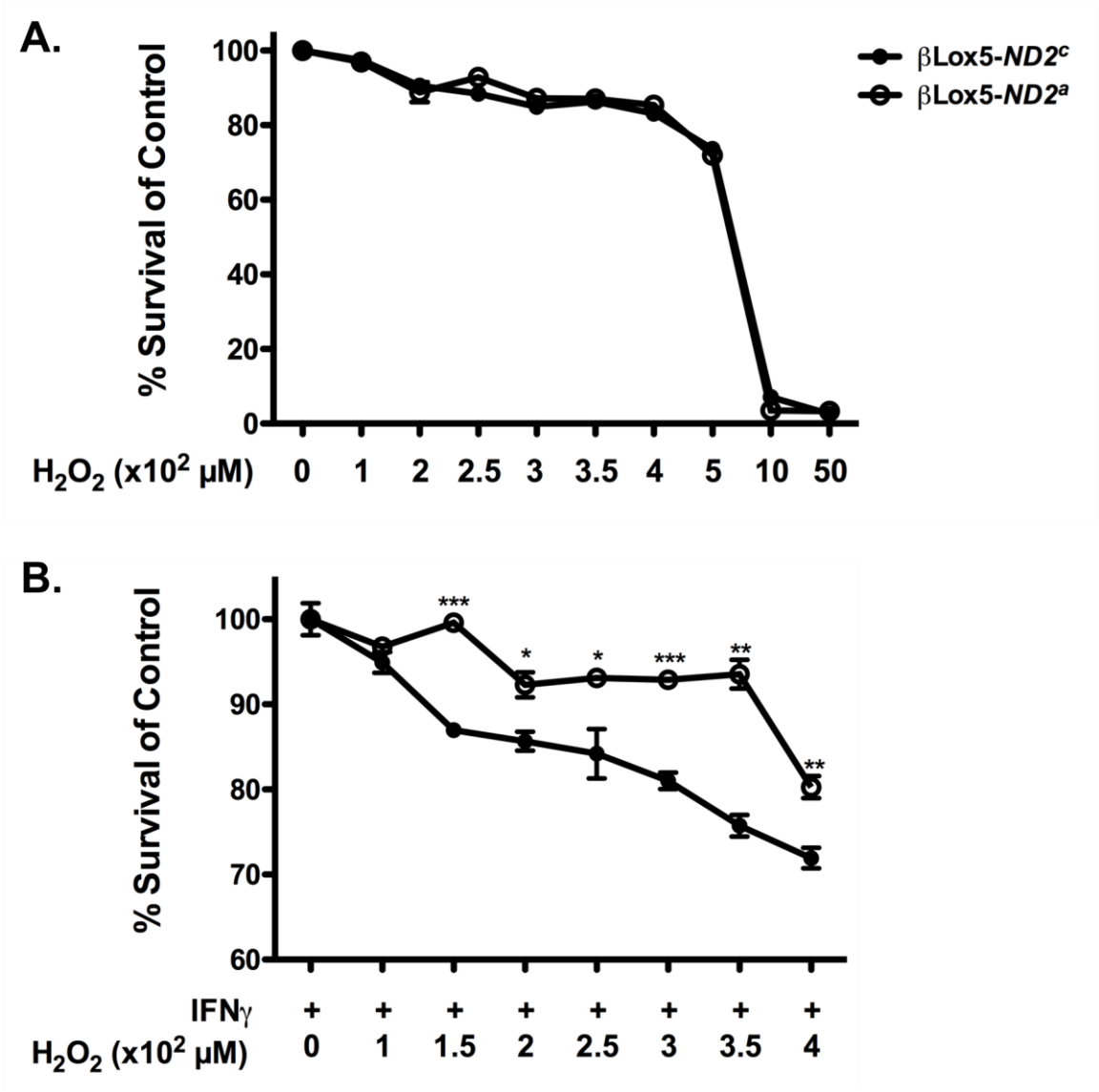

Figure 3-5. *mt-ND2*^{a} does not enhance the antioxidant capacity of βLox5-*ND2*^{a} cells. A) βLox5-*ND2*^{c} (filled circles) and βLox5-*ND2*^{a} (empty circles) cells were treated with increasing concentrations of H_2O_2 for 48 h and viability was measured by flow cytometry. B) Viability was also measured with the addition of rhIFNγ (1000 U/mL). *** denotes statistical significance with a P value < 0.0005, ** P value < 0.01, and * P < 0.05.

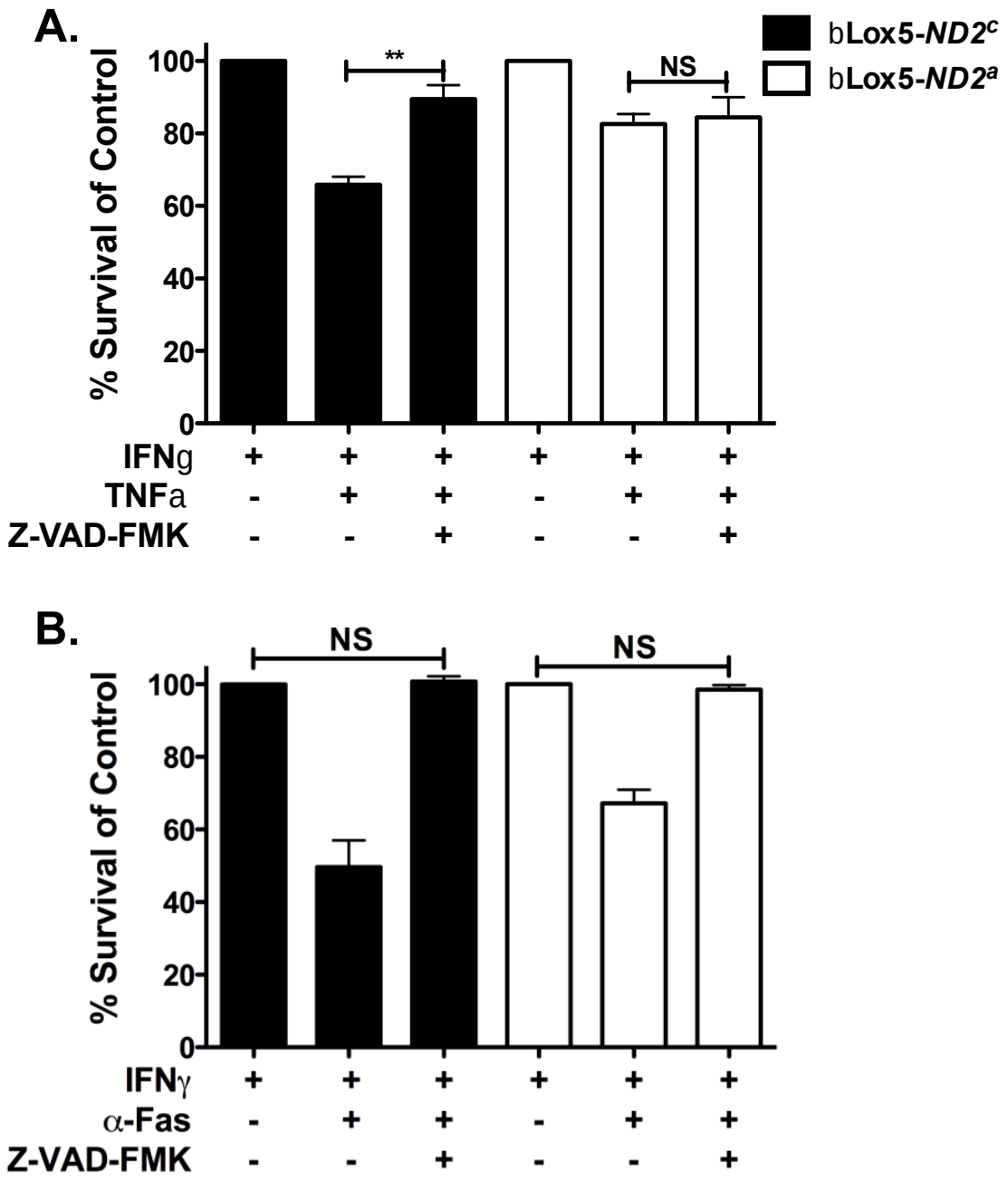

Figure 3-6. Pan-caspase inhibition fails to rescue βLox5-*ND2*^{a} cells from proinflammatory cytokine-mediated cell death but protects against Fas receptor activation. A) βLox5-*ND2*^{c} (black bars) and βLox5-*ND2*^{a} (white bars) cells were treated with the combinations of rhTNFα (2000 U/mL) and rhIFNγ (1000 U/mL), or (B) rhIFNγ (1000 U/mL) and CH-11 (0.5 µg/mL) for 48 h, with and without pan-caspase inhibition with the inhibitor Z-VAD-FMK (50 µM x 2), and cell death was measured by flow cytometry. ** denotes statistical significance with a P value < 0.005. NS denotes no statistical difference.

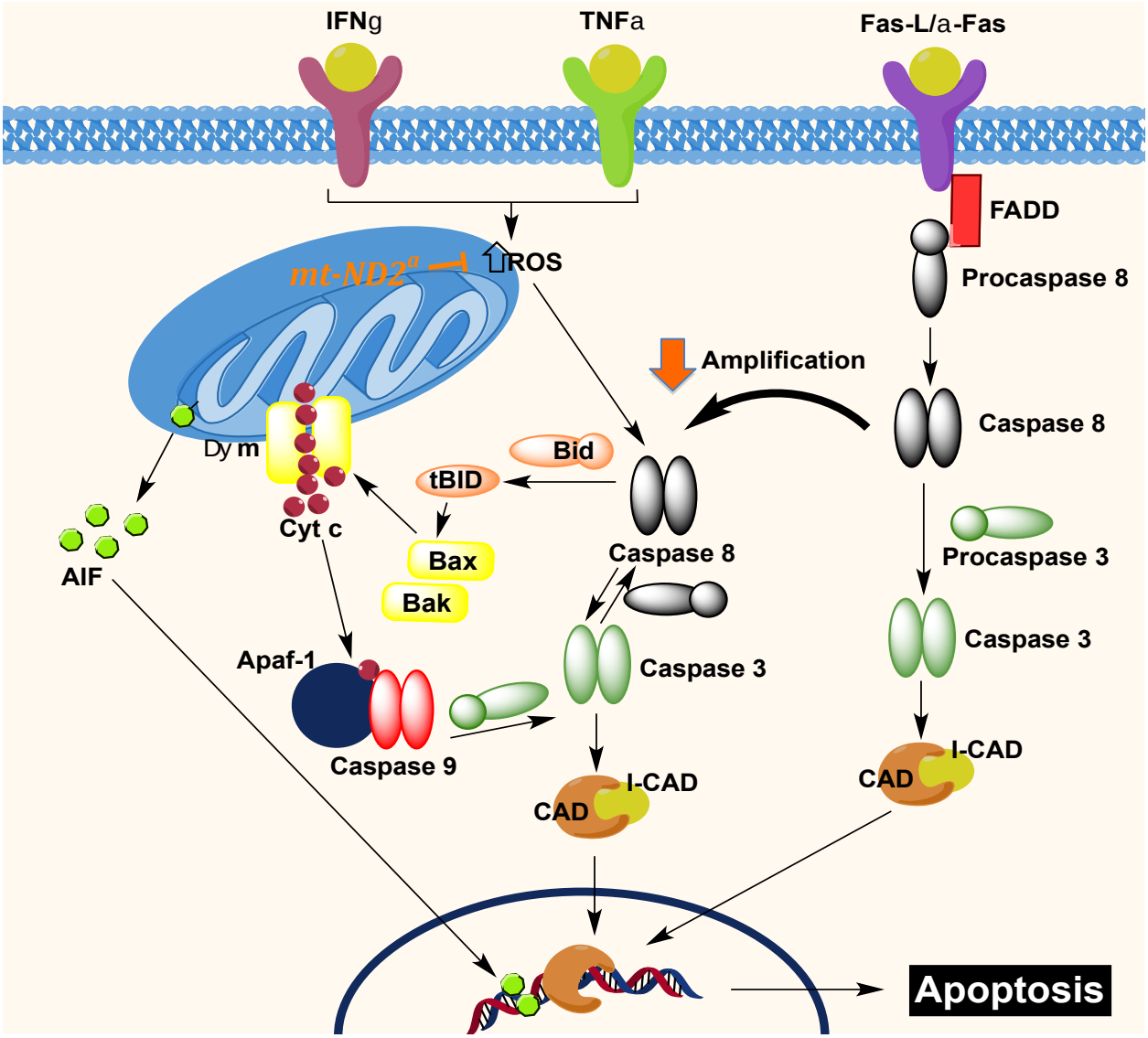

Figure 3-7. Proposed mechanism of protection conferred by *mt-ND2*^{a} in human β cells. Proinflammatory cytokines lead to an increase in mitochondrial ROS production, which activates the caspase cascade as well as caspase independent mechanisms (i.e. AIF translocation to the nucleus [29]). In human β cells expressing *mt-ND2*^{a}, mitochondrial ROS generation is blunted, thus, the caspase cascade fails to be activated and only caspase-independent mechanisms kill the β cells. Conversely, while human β cell death mediated by activation of the Fas death receptor can occur in the absence of functional mitochondria [29], amplification of the caspase cascade through the mitochondria takes place in mtDNA-sufficient cells. Lower mitochondrial ROS production in *mt-ND2*^{a}-encoding human β cells allows for elevated mitochondrial health and stability, thereby diminishing the amplification step and increasing resistance to Fas killing when compared to cells that express *mt-ND2*^{c}. © 2012 Yaíma Luzardo Lightfoot

CHAPTER 4
AUTOREACTIVE CYTOTOXIC T-LYMPHOCYTE-MEDIATED KILLING OF HUMAN β CELLS *IN VITRO*

Introduction

Autoimmune diabetes is believed to be a T cell mediated disorder in man as well as the NOD mouse and BB-DP rat [247]. Specifically, cytotoxic $CD8^+$ T cells are considered the final effectors in the progression to type 1 diabetes (T1D). T1D-prone NOD mice that are deficient in major histocompatibility complex (MHC) class I do not develop T1D or insulitis [248]. In humans, the common HLA-A2 and HLA-B27 haplotypes associate with T1D risk [175,177]. Moreover, when *HLA-A0201* is transgenically expressed in NOD mice, T1D onset is accelerated [249]. While direct evidence for the impact of T cells in T1D development only exists in mice [10-12,250], autoreactive effector $CD8^+$ T cells that recognize β cell-derived antigens can be detected in humans [6,178]. One of these epitopes, $IGRP_{265–273}$ (islet-specific glucose 6 phosphatase catalytic subunit related protein), elicits a T cell response in NOD mice and in humans [251,252]. Understanding how diabetogenic, or T1D-causing, cytotoxic T lymphocytes (CTLs) recognize and target human β cells is essential to the advancement of the field. Identification of mechanisms of cell death specific to β cells could lead to the development of immunotherapies that halt β cell dysfunction and declines in β cell mass, yet are not globally suppressive. However, detection and isolation of autoreactive $CD8^+$ T cells for *in vitro* cell-mediated lymphocytotoxicity (CML) assays is difficult due to the low frequency of these cells in the periphery as well as their potential low affinity for self-peptides [253].

CTL killing mechanisms of human islets have been previously analyzed using viral-specific CTLs and peptide-pulsed dispersed islets [37]. Although the islet cells were specifically lysed, peptide pulsing does not allow for β cell specificity, as all the cells within the islets will present the peptide of interest. In addition, it is likely that the affinity of these CTLs for viral peptides does not represent the weak interaction between autoreactive T cell receptors (TCRs) on CTLs and β cell antigens [253].

The system tested here represents an *in vitro* model to study CTL mechanisms important in T1D development. In this study, βLox5 cells were shown to express relevant T1D autoantigens, including IGRP, and the class I molecule HLA-A0201. To circumvent the difficulties of obtaining diabetogenic human CTLs and to ensure killing of β cells only, the human β cell line βLox5 [29,56,91] was exposed to human $CD8^+$ T cells transfected to express a TCR that recognizes the T1D autoantigen $IGRP_{265–273}$ presented in the context of HLA-A02 [251]. Consequently, these transfected CTLs specifically lysed βLox5 cells. Additionally, genetic manipulation of βLox5 cells can be used to determine the impact of polymorphisms expressed at the β cell level. To this end, βLox5 cells encoding the protective *mt-ND2*^{a} allele or the susceptibility *mt-ND2*^{c} allele [181] were also used as targets in CML assays. Mouse β cell lines that harbor the protective allele (*mt-Nd2*^{a}), resist killing by autoreactive CTLs [49]. As expected from the studies in mice [49] and from previous work using the cybrid cell lines (Chapter 3 and [49]), βLox5-*ND2*^{a} cells were significantly protected from killing by $IGRP_{265–273}$-reactive CTLs.

Materials and Methods

Cell Line and Reagents

The βLox5 cell line was kindly provided by Dr. Fred Levine (Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA). Cybrid cell lines were derived from βLox5 ρ^0 cells (Chapter 3 and [29]). βLox5, βLox5-*ND2*^{c}, and βLox5-*ND2*^{a} cells were maintained as described before [29]. IGRP-transfected and mock-transfected $CD8^+$ T cells were obtained in collaboration with Dr. Todd Brusko (Diabetes Center of Excellence, University of Florida, Gainesville, FL). Briefly, $CD8^+$ T cells sorted from human peripheral blood mononuclear cells (PBMCs) were transfected with either a non-TCR-encoding lentiviral vector or a multicistronic lentivirus encoding the α and β chains of a diabetogenic TCR specific for $IGRP_{265-273}$ in the context of HLA-A0201 [251]. TCR expression is optimized by linking the α and β chains with furin/2A self-cleaving peptides. The lentiviral vectors also contain a GFP-reporter gene that allows for post-transfection sorting and enrichment of the $CD8^+$ T cells of interest. CTLs were expanded and activated *in vitro* before cryopreservation. These effector cells were thawed immediately before use. Recombinant human IFNγ and FITC-conjugated HLA-A2 antibody were purchased from BD Biosciences (San Jose, CA). $IGRP_{265-273}$ peptide was synthesized by EZBiolab (Carmel, IN).

HLA Typing and Autoantigen Expression of βlox5

HLA typing of βLox5 was performed by Dr. Massimo Trucco (Children's Hospital of Pittsburgh Histocompatibility Center, Pittsburgh, PA), using the SSP UniTray High Resolution Kit (Dynal Biotech, Invitrogen), and LABType SSo Typing Tests Kit (One Lambda, Inc., Canoga Park, CA), as per the manufacturers' instructions. The

expression of T1D autoantigens in βLox5 cells was compared to that of human islets by reverse transcriptase PCR (RT-PCR). Aliquots of snap frozen human islets were obtained from the Islet Cell Resource Center at the University of Alabama School of Medicine. RNA was isolated from human islet preparations (HP58, HP60) as well as βLox5 cells. cDNA was synthesized by RT-PCR. Autoantigen expression was assessed using primers purchased from SuperArray Bioscience (Frederick, MD). The primer pairs used for this analysis were specific for Glucose-6-phosphatase, catalytic, 2 (*G6PC2* or IGRP), Insulin (*INS*), Dystrophia myotonica-protein kinase (*DMPK*), Islet cell autoantigen 1 (*ICA1* or ICA69), protein tyrosine phosphatase, receptor type, N (*PTPRN* or IA-2), Solute carrier family 30, member 8 (*SLC30A8*, or ZnT8), as well as glutamic acid decarboxylase 1 and 2 (*GAD1* and *GAD2*). A primer set for GAPDH demonstrated strong amplification for all three samples.

Flow Cytometry

IFNγ-treated and untreated βLox5 cells were analyzed for HLA-A2 expression by standard flow techniques. In brief, βLox5 cells were treated with low doses of rhIFNγ (50, 100, and 200 U/mL) overnight, stained for 1 h at 4°C and washed to remove excess unbound antibody before analysis. βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were analyzed on a BD LSR-Fortessa flow cytometer using the BD FACSDiva software (BD Biosciences) and FlowJo analysis software (Tree Star, Inc., Ashland, OR).

Chromium Release Assay

Cell lines that were IFNγ primed overnight or left untreated were loaded with 10 μCi/mL sodium chromate (^{51}Cr) for 3 h at 37°C in culture media. In some cases, ^{51}Cr loaded cells were pulsed with $IGRP_{265-273}$ peptide for 30 minutes and excess peptide

removed by washing in DMEM media. Transfected CTLs were thawed and diluted to the desired effector:target (E:T) ratio. The CTLs were incubated with 2 x 10^4 target cells per well in triplicate for 16 h. The supernatant was harvested and reactivity counted with a gamma counter [Wizard 1470 (Perkin Elmer)]. The remaining target cells were lysed with 2% SDS, harvested, and reactivity counted with the gamma counter. Total ^{51}Cr was determined by: supernatant counts plus counts in the SDS lysate. The percentage specific ^{51}Cr release was calculated by the following equation: % specific lysis = [^{51}Cr counts$_{supernatant}$/(^{51}Cr counts$_{supernatant}$ + ^{51}Cr counts$_{SDS\ lysate}$) * 100] – spontaneous lysis. Spontaneous lysis was calculated by the %^{51}Cr release in the absence of effector cells. To compare the susceptibility of βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells to antigen specific lysis by IGRP-reactive CTLs, % specific lysis by mock-transfected CD8$^+$ T cells was subtracted from % specific lysis by IGRP-transfected CTLs.

Statistical Analysis

Unless stated otherwise, data are shown as mean ± SEM. Significance was determined by a *t* test for two group comparisons (GraphPad Prism 5 for Mac OS X, La Jolla, CA); when appropriate, paired *t* tests were performed.

Results

βLox5 Cells Express T1D Autoantigens and Encode the Common HLA-A*0201 Allele

The HLA typing of βLox5 demonstrated that the cells encode the HLA alleles HLA-A0201/2501, HLA-B0801/3801, C0701/1203, and DRB0301/1001. PCR amplification with primer sets specific for Glucose-6-phosphatase, catalytic, 2 (*G6PC2* or IGRP), Insulin (*INS*), Dystrophia myotonica-protein kinase (*DMPK*), Islet cell autoantigen 1

(*ICA1* or ICA69), protein tyrosine phosphatase, receptor type, N (*PTPRN* or IA-2), Solute carrier family 30, member 8 (*SLC30A8*, or ZnT8), as well as glutamic acid decarboxylase 1 and 2 (*GAD1* and *GAD2*) demonstrated that, similar to primary human pancreatic islet samples (HP58 and HP60), βLox5 cells expressed autoantigens relevant in the pathogenesis of T1D (Fig. 4-1A). Strong expression of all antigens, save insulin, was observed in βLox5 cells. Interestingly, autoantigen expression in the primary islets was inconsistent (Fig. 4-1A).

Antigen-specific recognition of target cells by activated CTLs requires peptide presentation in the context of HLA Class I molecules. To test if βLox5 cells can be primed for CTL recognition, the cell line was treated with low concentrations of IFNγ and the expression level of HLA-A02 was measured by flow cytometry. At all the IFNγ doses tested (50, 100, and 200 U/mL), the HLA Class I expression of βLox5 cells was significantly induced (Fig. 4-1B).

Diabetogenic CTLs Recognize and Kill βLox5 Cells

βLox5 cells were incubated for 16 h with IGRP-reactive HLA-0201-restricted TCR-transduced human $CD8^+$ T cells. Compared to non-antigen specific lysis of the cell line, CTLs transfected to recognize $IGRP_{265-273}$ in the context of HLA-A0201 were significantly more cytotoxic towards βLox5 cells (Fig. 4-2A). The percent lysis of IGRP-specific CTLs peaked at the highest E:T ratio (25:1) and was reduced at lower E:T ratios (Fig. 4-2A and 2B). Overnight IFNγ priming of βLox5 cells enhanced killing, whereas pulsing the cells with $IGRP_{265-273}$ did not significantly increase lysis of IFNγ-primed βLox5 cells.

mt-ND2^{a} Protects Human β Cells from CTL Killing

A cybrid cell line, developed from βLox5 cells and encoding the T1D-resistance associated *mt-ND2*^{a} allele, was previously shown to be less susceptible to proinflammatory cytokines and to Fas receptor activation (Chapter 3). To test whether *mt-ND2*^{a} also protects human β from killing by autoreactive CTLs, βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were used as targets in a chromium release assay as described above with the parental cell line. Again, a 25:1 E:T ratio demonstrated significant IGRP-specific lysis for both cell lines (Fig. 4-3). However, βLox5-*ND2*^{a} cells were consistently less sensitive to killing than βLox5-*ND2*^{c} at all E:T ratios (Fig. 4-3).

Discussion

To date, no cure exists for T1D in humans. However, the most effective treatments, in terms of slowing loss of β cell mass and function after T1D, are represented by global immunosuppressive therapies or regimens that target either T cells (anti-CD3) or B cells (anti-CD20) [18,254-256]. The rationale behind conducting these trials has been greatly supported by studies using the well-established NOD model to understand the pathogenesis of the disease [237], as well as the success of immunotherapies that deplete or modulate adaptive immune cells in preventing or reversing T1D in animal systems [12,13,257,258]. In humans, a pathological lesion, termed insulitis, can sometimes be identified in *postmortem* examination of recent onset patients [259], providing indirect evidence for the influence of immune cells in T1D. Detailed analyses of the insulitic infiltrates indicate that $CD8^{+}$ T cells are the most abundant immune cell type present during insulitis [5,6]. Nonetheless, functional data proving that $CD8^{+}$ T cells gain cytotoxic, autoreactive function in humans is lacking.

Given the specificity of β cell destruction in autoimmune diabetes, considerable efforts have focused on identifying the β cell proteins recognized by self-reactive immune effectors [52]. A better understanding of why and how tolerance to these autoantigens is lost, along with the mechanisms employed by CTLs to destroy β cells is key to the development of tissue-specific immunotherapies. Here, a human β cell line that expresses several of the known T1D autoantigens (Fig. 4-1A) was used as a target for cytotoxic $CD8^+$ T cells that recognize $IGRP_{265–273}$ in the context of HLA-A0201 [178,251,252]. IGRP-specific $CD8^+$ T cells are not only present in the peripheral blood of T1D patients [178], but also within the islets of recent-onset and longstanding T1D patients [6]. These data suggest that IGRP is an important autoantigen in T1D.

Previously, expression of the death receptor Fas was detected on the cell surface of βLox5 cells [29]. The level of Fas was inducible by IFNγ [29], indicating that the cells could be primed for heightened immune surveillance and potentially lysed by CTLs. In addition to upregulation of death receptors, inflammation promotes MHC Class I hyperexpression in βLox5 (Fig. 4-1B), which is associated with viral and non-viral T1D in humans [6,260]. Because βLox5 cells express IGRP and present antigens in the context of HLA-A0201 (Fig. 4-1A & B), $IGRP_{265–273}$-reactive CTLs effectively lysed these cells. Killing was significantly greater when βLox5 was incubated with the IGRP-transfected CTLs versus the mock-transfected T cells (Fig. 4-2A), suggesting that the autoreactive CTLs lysed βLox5 in an antigen-specific manner.

As βLox5 were efficiently lysed in the CML assays, the system was expanded to include β cell lines that were genetically modified to harbor mitochondrial haplotypes that contain *mt-ND2*^{a} or *mt-ND2*^{c} (Chapter 3). From mitochondrial DNA-depleted βLox5

ρ^0 cells, two cybrid cell lines, βLox5-*ND2*^{a} and βLox5-*ND2*^{c}, were developed to study the contribution of a mitochondrial single nucleotide polymorphism (SNP) in the gene encoding NADH dehydrogenase 2 (*mt-ND2*). The C5178A transversion in *mt-ND2* correlates with T1D protection in mice and humans (Chapter 3 and [49,181,186,239]). When βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were incubated with IGRP-specific CTLs, robust killing of βLox5-*ND2*^{c}, similar to that observed in the parental βLox5 line, was measured (Fig. 4-3). In contrast, CML assays where βLox5-*ND2*^{a} was combined with IGRP-reactive CTLs confirmed that, in agreement with *mt-Nd2*^{a} in mouse [49], *mt-ND2*^{a} prevented CTL lysis of human β cells (Fig. 4-3). Individual analysis of each cell line showed that, like with the parental cell line, CTL killing was more pronounced with IGRP-transfected cells than with mock-transfected CTLs (Data Not Shown).

CTL lysis of human β cells could occur through one, or a combination, of the following mechanisms: 1) FasL on the CTLs activating the Fas receptor on the target cells, 2) cytotoxic granule release of perforin, granzyme, and granulysin molecules into the β cells, 3) production of proinflammatory cytokines (soluble or membrane bound) by the CTLs, or 4) damage-induced reactive oxygen species (ROS) production within the β cells. Previous mechanistic studies with βLox5 and its derivative cell lines provide some indications as to what the most likely pathways activated during CTL killing at the time point measured are (Chapter 3 and [29]).

With the exception of cytotoxic granule killing, the other mechanisms of cell death have been explored with βLox5. Significant cell death of βLox5, βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells is observed after 48 h of proinflammatory cytokine treatment as well as with agonistic antibody activation of the Fas receptor in combination with IFNγ

(Chapter 3 and [29]). Fas-induced cell death of βLox5 can be measured as early as 24 h after treatment provided that the cells have been previously primed overnight with IFNγ (Data Not Shown). However, with incubation times shorter than a 48 h period, only live apoptotic cells are detected with proinflammatory cytokines. Therefore, it is unlikely that the 16 h CML assay allows for these pathways to promote significant death in the β cells. In fact, analysis of ^{51}Cr release by βLox5 incubated with α-Fas antibody and IFNγ for 16 h did not show measurable differences over untreated controls (Data Not Shown). Nonetheless, the involvement of proinflammatory cytokines cannot be ruled out, as production of these soluble effectors by the CTLs at very close proximity to the β cells could increase the local concentration to levels greater than those used in the *in vitro* assays (Chapter 3 and [29]), thereby causing more β cell damage and death.

Perforin is required for the proapoptotic actions of granzyme [261]. Human islets incubated with perforin and granzyme B for 16 h show signs of apoptosis as measured by DNA fragmentation, while perforin alone appears to induce necrotic cell death of mouse islets [30]. ROS are implicated in the cytotoxicity of granzyme. Data from studies using the chronic myelogenous leukemia cell line K562 suggest that granzyme A induces mitochondrial ROS (mtROS) production and caspase-independent cell death [262]. In this study, βLox5-*ND2*^{a} cells were better protected the βLox5-*ND2*^{c} cells from CTL killing (Fig. 4-3). Resistance to triggers of cell death in βLox5-*ND2*^{a} was found to be due to decreased mtROS production within the cells (Chapter 3); therefore, IGRP-specific lysis of the cell lines may be due to perforin/granzyme. Consistent with the results reported, assuming that both granzyme A and B contribute equally to cytotoxicity, βLox5-*ND2*^{a} cells are not expected to be completely resistant to killing but

the lower levels of mtROS should result in higher viability compared to βLox5-*ND2*^{c} cells. These cell lines provide an excellent tool to further study the cytotoxic mechanisms important in T1D.

Although only IGRP$_{265–273}$-reactive CD8^{+} T cells were used to kill the human β cell line βLox5, other antigen-specific effector cells can also be tested because βLox5 cell express several relevant T1D autoantigens. In conclusion, βLox5 can be utilized as a target for diabetogenic CTLs not only in studies aiming to understand mechanisms of killing, but also in high-throughput assays that test protective molecules, as well as in studies analyzing the contribution of the β cell to its own demise.

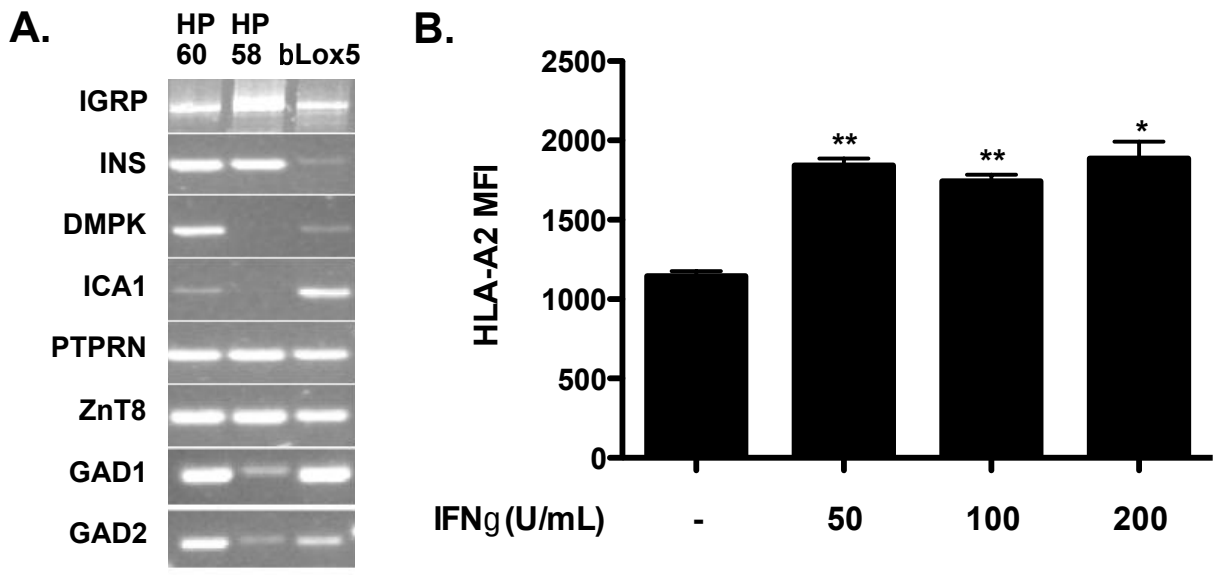

Figure 4-1. The human β cell line βLox5 expresses T1D autoantigens and is primed for immune surveillance. A) Expression of T1D autoantigens was analyzed in βLox5 cells as well as in equivalent amounts of human islet preparations (HP60, HP58). B) Overnight priming of βLox5 cells with low levels of rhIFNγ increases the expression of HLA-A2 molecules compared to untreated control cells. ** denotes statistical significance with a P value < 0.005, * P value < 0.05.

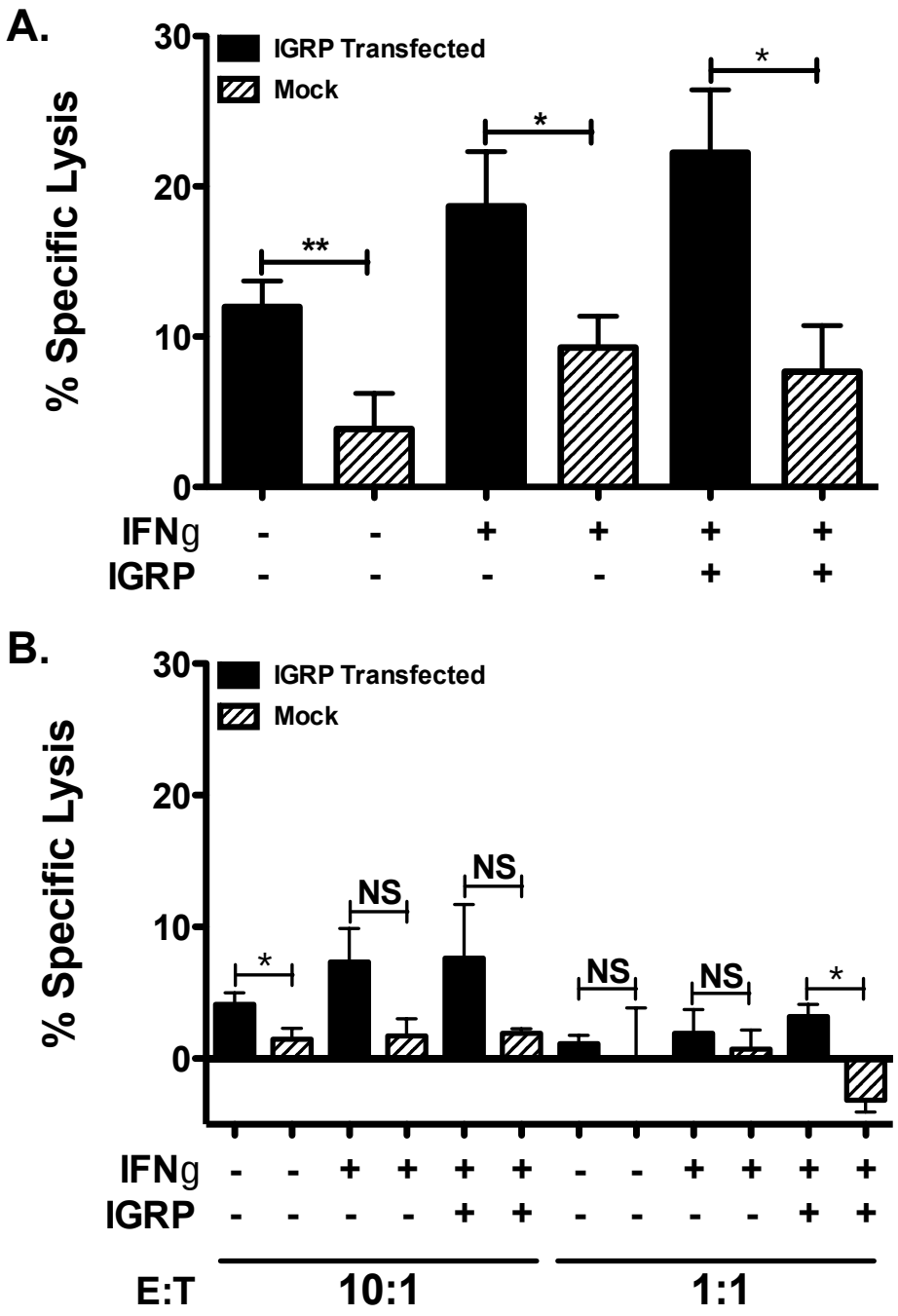

Figure 4-2. βLox5 cells are susceptible to cytotoxic T cell killing by HLA-A0201-restricted IGRP-reactive $CD8^+$ T cells. A) βLox5 cells were incubated with purified $CD8^+$ T cells that were transfected to express a TCR specific for the T1D autoantigen IGRP at a 25 to 1 effector to target ratio. In some cases, βLox5 were primed overnight with rhIFNγ and/or pulsed with IGRP peptide for 30 minutes prior to killing. B) Effector to target ratios (E:T) of 10 to 1 and 1 to 1 were also tested. ** denotes statistical significance with a P value < 0.01, * P value < 0.05. NS denotes no statistical difference.

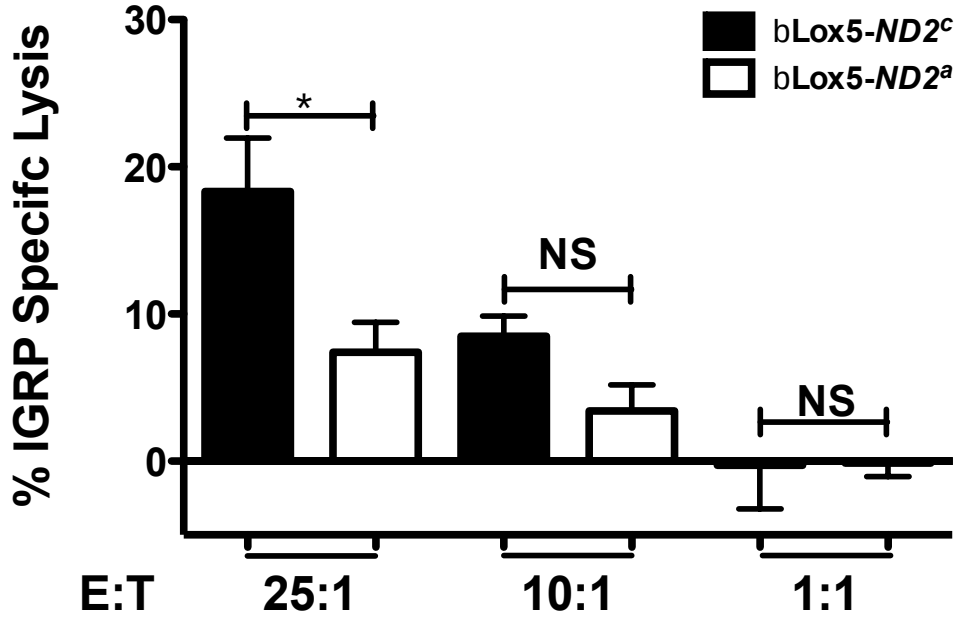

Figure 4-3. *mt-ND2*^{a} protects against cytotoxic T cell killing by HLA-A0201-restricted IGRP-reactive CD8^{+} T cells. βLox5-*ND2*^{c} (black bars) and βLox5-*ND2*^{a} (white bars) cells were incubated with purified CD8^{+} T cells that were transfected to express a TCR specific for the T1D autoantigen IGRP at 25:1, 10:1, and 1:1 effector to target ratios (E:T). Shown here is killing without rhIFNγ priming or IGRP peptide pulsing. Non-specific killing by mock-transfected T cells was subtracted from total killing by the IGRP-transfected CTLs. * denotes statistical significance with a P value < 0.05. NS denotes no statistical difference.

CHAPTER 5
CONCLUSION AND SIGNIFICANCE

Type 1 diabetes (T1D), characterized by insulin deficiency resulting from an autoimmune-mediated loss of β cell mass and/or function, is clinically manifested as chronic hyperglycemia and accompanying metabolic disorders. A primary goal in the field of T1D research has been to identify the mechanisms by which the insulin-secreting pancreatic islet β cells are functionally inhibited and killed, as well as susceptibility and protective genetic components that may alter disease progression in at-risk populations. Because the pathological process begins years before clinical diagnosis, early detection of risk is crucial in the prevention of deadly complications, such as ketoacidosis [263]. Equally important is determining genetic factors that modify β cell function and fate in the face of immune insults occurring during disease progression and after islet replacement therapy. Genetic remodeling of β cells to resist destruction prior to transplantation could enable long-term graft function and minimize the need for, and toxicity associated with systemic immunosuppression, thereby extending the promise of insulin independence to patients other than those with end-stage disease [264].

Mitochondria are the main sources of energy in eukaryotic cells and are also key regulators of the cell death process. Not surprisingly, mutations in the mitochondrial genome are associated with a range of diseases, including diabetes [264]. However, naturally occurring polymorphisms in mitochondrial DNA (mtDNA) can also lead to desirable phenotypes like longevity and resistance to T1D [181,265-267]. A single nucleotide polymorphism (SNP) in the mitochondrially encoded gene NADH dehydrogenase subunit 2 (*mt-ND2*) has been linked with a reduced incidence of T1D in

humans [181]. The C/A nucleotide change at position 5178 of *mt-ND2* causes a leucine to methionine substitution and is found at a lower frequency in T1D patients than in controls [181]. The A allele in the orthologous *mt-Nd2* mouse gene also protects β cells from death induced by immune mediators and toxic compounds [49,268]. Previous *in vitro* and *in vivo* studies suggest that resistance to T1D in the mouse is due to lower oxidative stress in response to pro-death stimuli [268].

The data presented herein indicate that, similar to the observation with murine systems, *mt-ND2*^{a} protects human β cells from destruction by decreasing endogenous mitochondrial reactive oxygen species (mtROS) production when signaled for death. Cytoplasmic hybrid cells, or cybrids, can be created by reintroducing mtDNA into cells previously depleted of native mtDNA. This technology has been a central tool in understanding the effects of disease-specific mtDNA mutations. Here, the impact of *mt-ND2* allotypes in the context of T1D has been investigated through the use of cybrid cell technology. From mtDNA-depleted βLox5 cells, two cybrid cell lines, βLox5-*ND2*^{c} and βLox5-*ND2*^{a}, were developed.

The parental β cell line was first found to be susceptible to proinflammatory cytokine and Fas-induced death [29]. While Fas-mediated β cell death occurred in the absence of mtDNA, functional mitochondria were required for optimal proinflammatory cytokine killing; suggesting that these organelles are especially important in the cellular response to soluble mediators that are believed to exert their cytotoxicity through the induction of ROS generation within the target cell. Indeed, signs of oxidative stress were evident in early time points in IFNγ and TNFα-treated βLox5 cells before death could be detected [29].

Using the same experimental conditions as with βLox5 cells, βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were tested for susceptibility to cytokine and Fas-induced death. While, similar to the C allele-containing parental cell line, βLox5-*ND2*^{c} cells were significantly killed by IFNγ and TNFα (>40% cell death), βLox5-*ND2*^{a} cells were more resistant to cytokine-induced death (~15% killing). Measurements of mitochondrial ROS production after incubation with IFNγ and TNFα demonstrated that the protective allele resulted in diminished ROS production and resistance to changes in mitochondrial membrane potential. Reduced mitochondrial membrane potential is indicative mitochondrial damage, which may result in the release of apoptogenic factors and loss of oxidative phosphorylation.

In agreement with the finding that Fas-FasL signaling in βLox5 can occur independent of the mitochondria [29], both βLox5-*ND2*^{c} and βLox5-*ND2*^{a} cells were killed by Fas receptor activation. However, when compared with βLox5-*ND2*^{c} cells, βLox5-*ND2*^{a} cells were more resistant to Fas killing and generated a significantly lower level of mitochondrial ROS. These results suggest that in the presence of pro-death factors capable of signaling extrinsically, polymorphisms in the mitochondria determine whether amplification of the death pathway takes place, consequently increasing susceptibility or resistance of the cell to the insult. Moreover, *mt-ND2*^{a} was also protective against autoreactive $CD8^{+}$ T cells, which may utilize a range of cytotoxic mechanisms.

Since the classification of T1D as an autoimmune disorder, most studies have focused on the abnormalities of the immune system that lead to β cell destruction. The work described here establishes an active role of the β cell in T1D development and

encourages further identification of genetic components that change how β cells respond to autoimmune attack. Defining these protective pathways may lead to the design of superior β cells suitable for transplantation with minimal or no immunosuppression. In addition, therapies that mimic the downstream effects of the protective mechanisms identified may prevent progression to T1D in individuals at risk.

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BIOGRAPHICAL SKETCH

Yaíma Luzardo Lightfoot was born in Camagüey, Cuba to Isaac C. Luzardo and María A. Acosta. In 1997 she moved with her father, stepmother, Blanca, and brother, Isaac, to Miami, Florida. In 2003, Yaíma graduated from South Miami Senior High School in the top ten of a graduating class of over 500 students. The fall semester of that same year, she began her undergraduate degree program in microbiology and cell science at the University of Florida as a Florida Academic Scholar. During this time, Yaíma also worked as a substitute teacher at Baby Gator Child Development and Research Center and as a technician in the laboratory of Dr. Robert A. Burne in the Oral Biology Department of the College of Dentistry.

In the summer semester of her junior year, Yaíma was selected into the University Scholars Program, which provided funding for her undergraduate research project in the Burne laboratory focused on investigating the mechanisms governing *Streptococcus mutans* gene expression and virulence. She continued to work in Dr. Burne's lab until graduating with honors in the spring of 2007. Due to the excellent mentorship and opportunities to present and publish data in the Burne lab, Yaíma remained interested in research and wished to continue to develop as a scientist by joining the Interdisciplinary Program (IDP) in Biomedical Sciences in the College of Medicine at the University of Florida.

Upon acceptance into the program in the fall of 2007, Yaíma was awarded the Alumni Graduate Fellowship, which provides Graduate Research Assistant support for four years. While in the IDP, she joined the laboratory of Dr. Clayton E. Mathews to investigate the pathogenesis of type 1 diabetes. By pursuing a Ph.D. studying

autoimmune diabetes, Yaíma redirected her career path to focus on her long-lasting interest in the disease that has affected her mother since the age of eleven. During her graduate studies, Yaíma married Joseph A. Lightfoot, whom she met while the two attended the University of Florida as undergraduate students. After graduation, Yaíma plans to remain in academic research and hopes to become a leading investigator in the field of type 1 diabetes.