# HEAT SHOCK PROTEIN 90, A POTENTIAL BIOMARKER FOR TYPE I DIABETES: MECHANISMS OF RELEASE FROM PANCREATIC BETA CELLS

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**DEDICATION**

This work is dedicated to my family and friends for all of your needs and intentions.

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Gail Jean Ocaña

# HEAT SHOCK PROTEIN 90, A POTENTIAL BIOMARKER FOR TYPE I DIABETES: MECHANISMS OF RELEASE FROM PANCREATIC BETA CELLS

Heat shock protein (HSP) 90 is a molecular chaperone that regulates diverse cellular processes by facilitating activities of various protein clients. Recent studies have shown serum levels of the alpha cytoplasmic HSP90 isoform are elevated in newly diagnosed type I diabetic patients, thus distinguishing this protein as a potential biomarker for pre-clinical type I diabetes mellitus (TIDM). This phase of disease is known to be associated with various forms of beta cell stress, including endoplasmic reticulum stress, insulitis, and hyperglycemia. Therefore, to test the hypothesis that HSP90 is released by these cells in response to stress, human pancreatic beta cells were subjected to various forms of stress in vitro. Beta cells released HSP90 in response to stimulation with a combination of cytokines that included IL-1β, TNF-α, and IFN-γ, as well as an agonist of toll-like receptor 3. HSP90 release was not found to result from cellular increases in *HSP90AA1* gene or HSP90 protein expression levels. Rather, cell stress and ensuing cytotoxicity mediated by c-Jun N-terminal kinase (JNK) appeared to play a role in HSP90 release. Beta cell HSP90 release was attenuated by pre-treatment with tauroursodeoxycholic acid (TUDCA), which has been shown previously to protect beta cells against JNK-mediated, cytokine-induced apoptosis. Experiments here confirmed TUDCA reduced beta cell JNK phosphorylation in response to cytokine stress. Furthermore pharmacological inhibition and siRNA-mediated knockdown of JNK in beta cells also attenuated HSP90 release in response to cytokine stress. Pharmacological inhibition of HSP90 chaperone function exacerbated islet cell stress during the development of TIDM in vivo; however, it did not affect the overall incidence of disease.

Together, these data suggest extracellular HSP90 could serve as a biomarker for pre-clinical TIDM. This knowledge may be clinically relevant in optimizing treatments aimed at restoring beta cell mass. The goal of such treatments would be to halt the progression of at-risk patients to insulin dependence and lifelong TIDM.

Janice S. Blum, Ph.D., Chair

**TABLE OF CONTENTS**

## LIST OF TABLES

**LIST OF FIGURES**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 17-AAG | 17-allylamino-17-demethoxygeldanamycin |
| 17-DMAG | 17-dimethylaminoethylamino-17-demethoxygeldanamycin |
| ANOVA | Analysis of variance |
| AP-1 | Activator protein-1 |
| APC | Antigen presenting cell |
| ASK1 | Apoptosis signal-regulating kinase 1 |
| ATF | Activating transcription factor |
| BiP | Binding immunoglobulin protein |
| BMI | Body mass index |
| BSA | Bovine serum albumin |
| cDNA | Complimentary DNA |
| CHOP | CCAAT/enhancer-binding protein homologous protein |
| CMRL | Connaught Medical Research Laboratories |
| CREB | cAMP response element binding |
| Ctrl | Control |
| Cyt | Cytokine |
| DAMP | Damage-associated molecular pattern |
| DMEM | Dulbecco's modified Eagle medium |
| DMOG | Dimethyloxaloylglycine |
| DNA | Deoxyribonucleic acid |
| DPBS | Dulbecco's phosphate-buffered saline |

| | |
|---|---|
| ds | Double-stranded |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| ERAD | ER-associated degradation pathway |
| FBS | Fetal bovine serum |
| GAD | Glutamic acid decarboxylase |
| Glc | Glucose |
| HBSS | Hanks' balanced salt solution |
| HMGB1 | High mobility group box 1 |
| HRP | Horseradish peroxidase |
| HSP | Heat shock protein |
| IFN | Interferon |
| IL | Interleukin |
| IL-1R1 | Type 1 IL-1 receptor |
| iNOS | Inducible nitric oxide synthase |
| IRE1 | Inositol requiring enzyme 1 |
| IRF | Interferon regulatory factor |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| MAPK | Mitogen-activated protein kinase |
| MHC | Major histocompatibility complex |
| MMP-2 | Matix metalloproteinase-2 |
| NAC | *N*-actetylcysteine |
| ND | Not diabetic |
| NF-κB | Nuclear factor κ-light-chain-enhancer of activated B cells |
| NK | Natural killer |
| NOD | Non-obese diabetic |
| ns | Not significant |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| PBST | Phosphate-buffered saline containing 0.05% Tween-20 |
| PERK | dsRNA-dependent protein kinase R (PKR)-like ER kinase |
| PHD | Prolyl hydroxylase |
| PIC | Polyinosinic-polycytidylic acid |
| PRR | Pattern recognition receptor |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| RIDD | Regulated IRE1-dependent decay |
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| RPMI | Roswell Park Memorial Institute |

| | |
|---|---|
| SDS | Sodium dodecyl sulfate |
| SERCA | Sarco-endoplasmic reticulum $Ca^{2+}$ ATPase |
| siRNA | Small interfering RNA |
| SLE | Systemic lupus erythematosus |
| SREC-I | Scavenger receptor expressed by endothelial cells |
| STAT | Signal transducer and activator of transcription |
| TIDM | Type I diabetes mellitus |
| TBST | Tris-buffered saline containing 0.05% Tween-20 |
| Tg | Thapsigargin |
| Th | T helper |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TNF-R1 | TNF receptor type 1 |
| TRAF2 | TNF receptor-associated factor 2 |
| Treg | T regulatory |
| TUDCA | Tauroursodeoxycholic acid |
| UD | Undetected |
| UPR | Unfolded protein response |
| XBP1 | X-box binding protein 1 |

## INTRODUCTION

### I. Overview of the Immune System

The human immune system has evolved a number of intricate mechanisms over the millennia to protect our species from infection with pathogenic microorganisms. Certain components of the immune system are also involved in healing tissue damage induced by these infectious agents as well as other environmental insults. Our immune systems can even target and kill cancerous cells. In order to recognize an invading pathogen, the immune system must have the ability to distinguish self from non-self. To be fully protective, the immune system must also be able to differentiate benign substances in our environment from those that are harmful, as well as healthy cells from those that are malignant. The immune system is traditionally thought to orchestrate these processes through two distinct branches: the innate immune system and the adaptive immune system (1).

### II. Innate Immune Mechanisms

The innate immune system is the human body's first line of defense against pathogen invasion. It serves to prevent pathogen entry or to detect and destroy an invading microorganism within minutes or hours of its breach. The body's epithelial surfaces act as barriers that prevent pathogens from entering and establishing an infection. If a pathogenic organism does manage to break through these barriers, other innate mechanisms are standing by to prevent propagation of the pathogen throughout the host.

#### *A. Phagocytes*

Phagocytes are specialized immune cells that recognize and engulf, or phagocytose, infectious microorganisms. These cells also ingest debris and dead cells to promote tissue repair and wound healing. Phagocytic cells include neutrophils, the short-lived first responders to a site of infection; macrophages, long-lived tissue-resident

phagocytes that mature from circulating monocytes; and dendritic cells, central players in both activation of adaptive immunity as well as antiviral responses.

During infection, pathogens are destroyed when the phagosome, the vacuole containing the phagocytosed microorganism, fuses with a lysosome, thus forming the phagolysosome. Within the phagolysosome, pathogens are destroyed through the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Neutrophils and macrophages produce high levels of ROS and RNS that potently degrade bacterial proteins. Dendritic cells, on the other hand, produce lower levels of ROS and RNS and are therefore better at preserving certain specific epitopes, or antigenic determinants, of bacterial proteins. This feature is essential for later activation of the adaptive immune system (1).

An important feature of phagocytic cells that allows them to distinguish self from non-self is the expression of pattern recognition receptors (PRRs). These receptors recognize conserved features of microorganisms known as pathogen-associated molecular patterns (PAMPs). These are features unique to microorganisms and are not present on host cells.

### *B. Toll-Like Receptors*

One important family of PRRs is the Toll-like receptor (TLR) family. These receptors are thus named because they are homologs of the *Drosophila melanogaster* Toll receptor. This receptor is important for innate immune responses to fungal pathogens in fruit flies. Thus, TLRs represent an ancient, evolutionarily conserved pathogen-recognition system (1). Humans express several TLRs that recognize PAMPs from various types of pathogenic organisms: gram-positive and gram-negative bacteria, mycobacteria, fungi, and viruses. For example, TLR3 recognizes double-stranded (ds) ribonucleic acid (RNA) encoded by viruses; TLR4 recognizes lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria; and TLR9 recognizes

deoxyribonucleic acid (DNA) with unmethylated CpG, a PAMP expressed by intracellular bacteria and herpesviruses. TLRs are expressed in distinct cellular locations depending on where the pathogenic organism is likely to be found. For example, TLR3 is generally located on endosomal membranes to facilitate the encounter of this TLR with viruses, given these intracellular pathogens often infect cells via delivery into the endocytic pathway. On the other hand, TLR4 is most often located on the plasma membrane because gram-negative bacteria are extracellular pathogens (2).

Cell signaling via TLRs induces expression of cytokines, chemokines, antimicrobial peptides, and type I interferons (IFNs) that normally promote inflammation and pathogen clearance. However, signaling through TLRs can also induce expression of anti-inflammatory cytokines such as interleukin (IL)-10 to counterbalance strong pro-inflammatory responses that could result in tissue damage. These genes are induced in response to a number of signaling pathways that each results in the activation of different transcription factors.

One important transcription factor family activated by TLR signaling is the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) family of transcription factors. TLR signaling can also activate members of the activator protein-1 (AP-1) family through signaling pathways involving mitogen-activated protein kinases (MAPKs). Furthermore TLR signaling can activate members of the interferon regulatory factor (IRF) family. NF-κB and AP-1 both induce expression of pro-inflammatory cytokines and chemokines, while the IRF transcription factors induce expression of the antiviral type-I IFNs, IFN-α and IFN-β (2).

Pro-inflammatory cytokines secreted in response to PRR engagement include IL-1β, tumor necrosis factor (TNF)-α, and IL-6. Pro-inflammatory cytokines promote changes in blood circulation that allow serum proteins and immune cells to access the site of microbial invasion in order to clear an infection and prevent its dissemination

throughout the body. This process is commonly known as an inflammatory response. Additionally PRR engagement can promote IFN responses (1).

### *C. Pro-Inflammatory Cytokines*

IL-1β is largely produced by neutrophils and macrophages but can also be produced by epithelial cells, endothelial cells, and fibroblasts. Mature IL-1β is secreted through an unconventional protein secretion pathway in response to inflammasome activation. Once secreted, this cytokine can act in a paracrine or systemic fashion. IL-1β signals through the ubiquitously expressed type 1 IL-1 receptor (IL-1R1) on target cells, resulting in the activation of NF-κB transcriptional activity as well as the c-Jun N-terminal kinase (JNK) and p38 MAPK signaling pathways, which activate the AP-1 and cAMP response element binding (CREB) transcription factors, respectively. Together these signals induce expression of canonical IL-1 target genes such as *IL6*.

Interestingly, many components of the IL-1 signaling pathway are also part of TLR signaling and other signaling cascades induced by cytotoxic stressors (**Figure 1**) (3). IL-1β activates the local vascular endothelium and increases access of effector immune cells to the site of infection. Specifically, it is a potent chemoattractant for neutrophils. These processes allow the pathogen to be cleared before it can spread to other parts of the body. However, IL-1β also results in fever and can promote local tissue destruction (1).

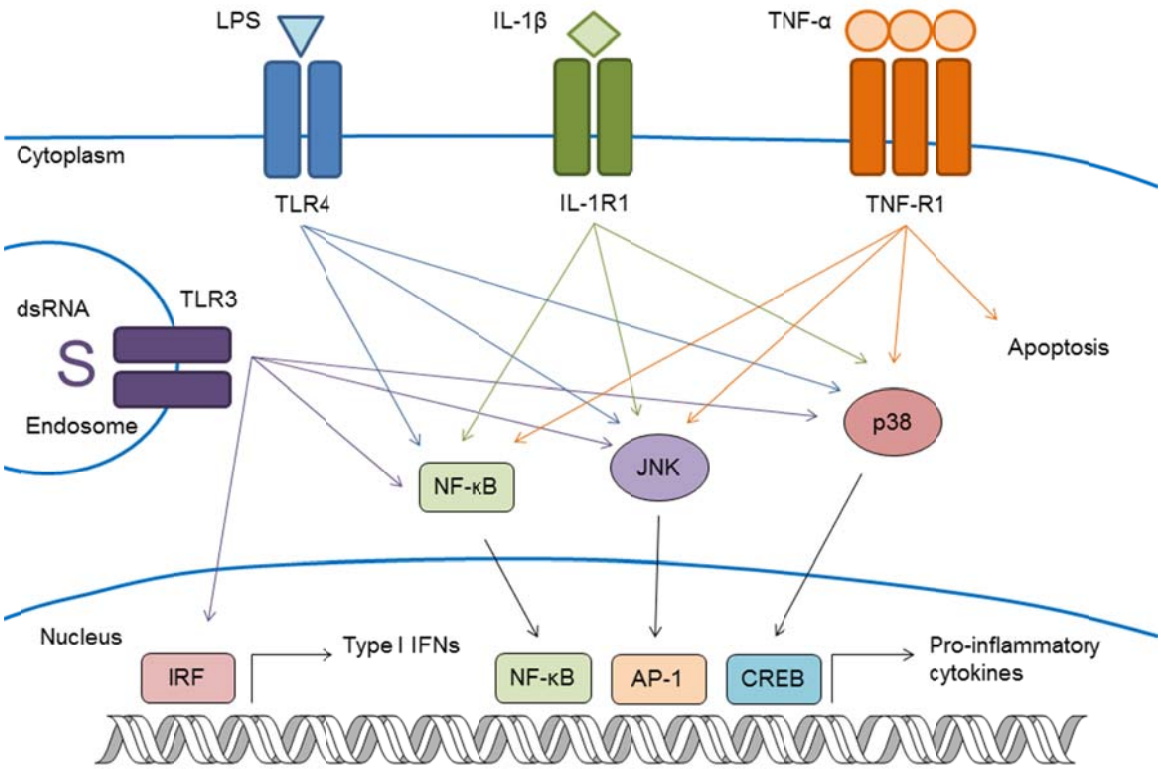


**Figure 1. Crosstalk between TLR and pro-inflammatory cytokine signaling pathways.** TLR4 binds LPS on the cell surface, while TLR3 binds dsRNA within endosomes. Signaling is induced by the ligand-induced dimerization of each receptor. After engaging distinct signaling adaptor molecules, downstream signaling pathways are stimulated that activate NF-κB, JNK, and p38, all of which ultimately lead to the induction of genes encoding pro-inflammatory cytokines. Endosomal TLRs can also stimulate IRF transcription factors to induce type I IFN responses (2). The pro-inflammatory cytokines IL-1β and TNF-α can also induce signaling that results in NF-κB, JNK, and p38 activation, thus amplifying the inflammatory response. In some instances, TNF-α signaling can result in apoptosis.

TNF-α is mainly secreted by macrophages but can also be released by lymphocytes, mast cells, eosinophils, endothelial cells, and fibroblasts in response to LPS and other microbial PAMPs. It is synthesized as a trimeric membrane-bound protein and is released via proteolysis by the TNF-α converting enzyme. This cytokine transduces signals through the TNF receptor type 1 (TNF-R1) which is constitutively

expressed in the majority of tissues. Similarly to IL-1β, TNF-α signaling also results in the activation of NF-κB transcriptional activity as well as the JNK and p38 MAPK signaling pathways; however, TNF-α is also known to induce apoptosis in some situations (**Figure 1**) (4). TNF-α activates the vascular endothelium, induces local vasodilation, and also stimulates endothelial cells to express factors that promote blood clotting and occlusion of local small blood vessels. This activity of TNF-α is important for preventing the dissemination of a pathogen throughout the body. However, if bacteria are present in the bloodstream, a condition known as septicemia results, during which macrophages release massive amounts of TNF-α, causing systemic vasodilation. This vasodilation leads to a dramatic drop blood pressure and a life-threatening condition known as septic shock (1).

IL-6 is produced by macrophages, dendritic cells, B cells, keratinocytes, fibroblasts, and epithelial cells, among other cell types, in response to stimuli such as LPS, IL-1β, and TNF-α. It binds to the specific IL-6 receptor expressed on target cells which utilizes the common signaling component gp130 to initiate signaling through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. The IL-6 receptor is highly expressed by leukocytes and hepatocytes. In the development of an adaptive immune response, IL-6 signaling can promote the development of T helper (Th)17 cells over T regulatory (Treg) cells. It also is extremely important for plasma B cell differentiation (5).

As part of the innate immune response, the cytokines IL-1β, TNF-α, and IL-6 cooperatively activate hepatocyte production of acute-phase proteins. Acute-phase proteins coat the surface of, or opsonize, pathogens, which promotes their clearance by phagocytes. They also stimulate the release of neutrophils from bone marrow, further promoting pathogen clearance. IL-1β, TNF-α, and IL-6 are also endogenous pyrogens,

meaning they act on the hypothalamus to elevate body temperature, which also facilitates pathogen clearance (1).

***D. Interferon Responses***

Type I IFNs include IFN-α, a family of several closely-related proteins, and IFN-β. These cytokines, first identified for their ability to "interfere" with viral replication, are produced largely in response to viral infection, but can also be produced in response to infection with certain types of bacteria (6). Practically all cell types can produce these cytokines, but plasmacytoid dendritic cells are particularly specialized for this function. Once released, these cytokines bind to the type I IFNα/β receptor, expressed by a number of cell types, and initiate signaling via the JAK-STAT pathway. Type I IFN signaling induces the expression of genes encoding antiviral proteins and also upregulates expression of genes involved in the major histocompatibility complex (MHC) class I pathway, which is important for the initiation of adaptive immune responses. In addition to activating macrophages and dendritic cells, type I IFNs activate natural killer (NK) cells to kill virus-infected cells and induce the expression chemokines that recruit lymphocytes to the site of infection. All of these activities of type I IFNs help induce a cellular state in which viral replication is inhibited (1).

IFN-γ is the only member of the type II class of IFNs. During innate immune responses, it is produced by monocytes, macrophages, dendritic cells, and NK cells. During adaptive immune responses, it is secreted by $CD4^+$ Th1 cells as well as $CD8^+$ T cells. IFN-γ binds the IFN-γ receptor, which is expressed by numerous types of immune cells as well as non-immune cells, and activates signaling primarily through the JAK-STAT pathway. Once activated, STAT1 homodimers translocate to the nucleus and bind to promoter sequences containing an IFN-γ activation site. Several IFN-regulated genes are themselves transcription factors, such as IRF-1, that help promote the next wave of transcriptional changes. IRF-1 target genes include *NOS2*, which encodes the inducible

nitric oxide synthase (iNOS), and *IFNB1*, which encodes IFN-β. IFN-γ also upregulates genes involved in the MHC-I and MHC-II antigen presentation pathways and the development of Th1 cell responses, both of which are important elements of the adaptive immune response. IFN-γ also inhibits cell proliferation, regulates apoptosis, and activates the microbicidal effector functions of macrophages and other cell types. Similarly to type I IFNs, IFN-γ is also important for the induction and maintenance of an antiviral state (7).

The innate immune system offers several elegant mechanisms to help clear infections with pathogenic microorganisms and promote wound healing. However, the innate immune system lacks a vast diversity of highly-specific antigen receptors and the ability to form cellular memory, two features that are required for an optimal immune response. These elements are hallmarks of the adaptive immune system.

**III. Adaptive Immune Mechanisms**

The adaptive branch of the immune system is further divided into two components: cellular immunity, which is mediated by $CD4^+$ Th cells and $CD8^+$ cytotoxic T cells working in concert with certain myeloid cells, and humoral immunity, which is mediated by B lymphocytes and the antibodies they secrete.

To initiate an adaptive immune response, antigen presenting cells (APCs), of which dendritic cells are the most potent activators of naïve T cells, acquire antigenic proteins derived from the invading microorganism. These proteins are then processed into smaller peptide fragments and presented on the cell surface to naïve T cells in the context of MHC-I or MHC-II molecules. $CD4^+$ T cells recognize antigen presented in the context of MHC-II (**Figure 2**), while $CD8^+$ T cells recognize antigen presented in the context of MHC-I (8). To be activated, the antigen-specific T cell receptor of a naïve T cell must recognize its specific epitope presented in the context of the correct MHC in the presence of co-stimulation (9).

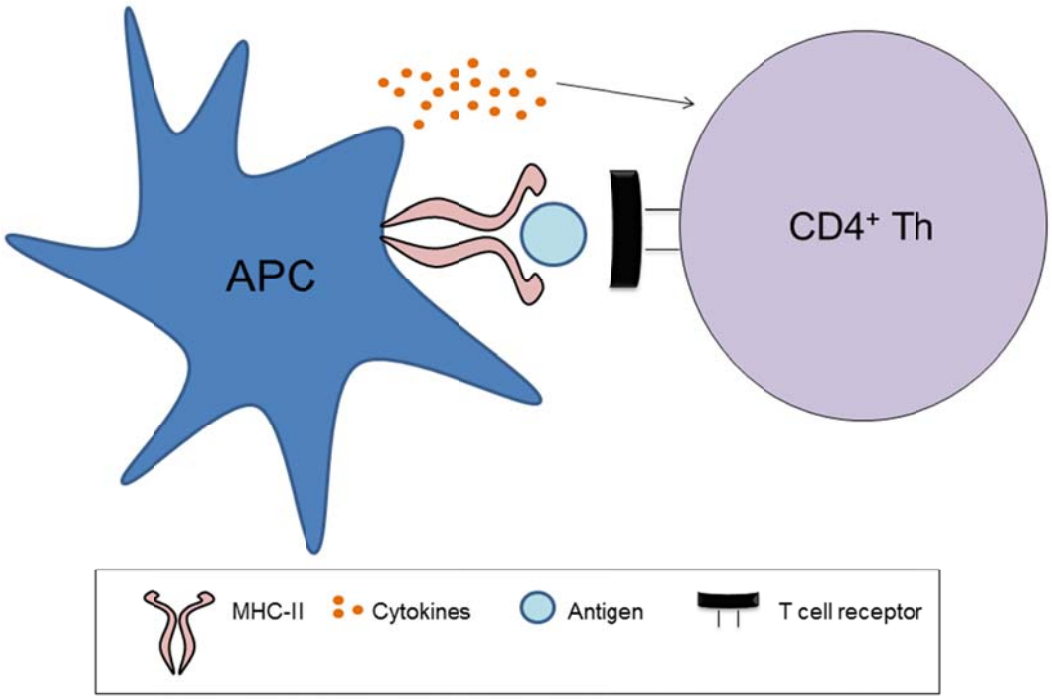


**Figure 2. Initiation of an adaptive immune response.** Professional APCs, which include dendritic cells, macrophages, and B lymphocytes, acquire peptides derived from foreign or self antigens. These antigens are proteolytically processed and presented in the context of MHC-I or MHC-II molecules and presented to $CD8^+$ or $CD4^+$ T cells, respectively. If the APC has been previously activated and expresses the appropriate co-stimulatory molecules, the T cell will become activated and release cytokines to promote an adaptive immune response. APCs also release cytokines that skew development of T cells into specialized subsets of effector cells.

### *A. $CD4^+$ T Helper Cell Responses*

Once stimulated by APCs, naïve $CD4^+$ T cells can differentiate into one of several subsets of Th cells, depending on the cytokine signals it receives from the APC and extracellular environment. For example, the presence of IL-12 and IFN-γ can induce the polarization of $CD4^+$ T cells to the Th1 cell phenotype. Different Th subsets in turn produce cytokines that promote immunity to specific pathogen classes. For example, Th1 cells secrete IFN-γ and TNF-α, two cytokines that are particularly important for

macrophage activation and the clearance of viral infections as well as infections with other intracellular pathogens. During this process, $CD4^+$ T cells can also differentiate into either central memory or effector memory $CD4^+$ T cells (10).

### *B. Cytotoxic $CD8^+$ T Cell Responses*

During the course of an infection, $CD8^+$ T cells are activated by APCs in the lymph nodes and spleen, sometimes with the help of $CD4^+$ T cells, depending on the pathogen (11). Cytotoxic $CD8^+$ T cells then undergo clonal expansion and migrate to peripheral tissues where they recognize infected cells that present antigen in the context of MHC-I and subsequently induce apoptosis of these infected cells (12). $CD8^+$ T cell-mediated induction of apoptosis may be achieved by the delivery of perforin and granzyme to the target cells or by engaging death receptors such as FAS expressed on the target cell surface (11; 12). Similarly to effector $CD4^+$ T cells, cytotoxic $CD8^+$ T cells can secrete cytokines such as IFN-γ and TNF-α in order to activate other components of the immune system. Like $CD4^+$ T cells, $CD8^+$ T cells can differentiate into effector and central memory T cells (12).

### *C. B Cell Responses*

Naïve B lymphocytes express antigen-specific immunoglobulin molecules on their cell surface. These receptors allow B cells to efficiently capture antigen from the extracellular environment. B cells then process and present this antigen in the context of MHC-II to $CD4^+$ T cells in the lymph node. Cytokines, notably IL-4 from Th2 cells, in turn activate the naïve B cells to become antibody-secreting plasma cells. The antibodies secreted by plasma cells are better specialized for targeting a specific antigen due to the processes of immunoglobulin class switching and affinity maturation that occur during plasma cell differentiation (13). Antibodies play a number of roles in immunity including neutralization, opsonization, and complement activation. After infection, antigen-specific memory B cells maintain expression of their high affinity, class-switched antibody (1).

## IV. Overview of Autoimmunity

Normally the innate and adaptive branches of the immune system work exquisitely in concert to efficiently clear an infection and mount a memory response that will prevent the host from becoming ill again should reinfection occur. To accomplish this task, the immune system relies on its ability to distinguish self from non-self. Unfortunately in some instances, undesirable adaptive immune responses can be mounted against the body's own self-antigens, a condition known as autoimmunity.

A number of tolerance mechanisms normally prevent the development of autoimmunity. For example, central tolerance mechanisms promote the deletion of autoreactive T and B cells during their development in the thymus and bone marrow, respectively. Additionally, the activity of Treg cells can suppress the development of autoimmunity in the periphery by secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor-β. Treg cell activity is an example of a peripheral tolerance mechanism. Autoimmunity can ensue when these central and peripheral tolerance mechanisms fail (1).

## V. Type I Diabetes

Type I diabetes mellitus (TIDM), also referred to as insulin-dependent diabetes mellitus, is an organ-specific, T cell-mediated autoimmune disease that results in the targeted destruction of pancreatic beta cells. These cells, located within the pancreatic islets of Langerhans, are responsible for secreting insulin, a hormone that, during a fed state, stimulates the cellular uptake of glucose and other biofuel sources from the blood. During TIDM, the other pancreatic islet cell types, such as glucagon-secreting alpha cells, remain intact. Glucagon is a hormone that promotes fat breakdown in adipose tissue, protein breakdown in muscle and other tissues, and gluconeogenesis in the liver. The loss of insulin during TIDM, with continued secretion of glucagon, effectively results in a state of accelerated starvation. Before the implementation of insulin therapy in the

early 1920s, children with TIDM suffered an agonizing illness characterized by weight loss, cachexia, and overwhelming acidosis. Most patients passed away within one or two years of diagnosis (14).

### *A. Etiology*

In the United States and Europe, nearly 1 in 600 children will develop TIDM in their lifetime (14). Although the precise sequence of events leading to TIDM development remains unclear, both genetic and environmental risk factors appear to play a role in disease susceptibility. The inheritance of TIDM appears to be polygenic (15). By far the strongest genetic determinant is the MHC genotype of the individual. Certain MHC-II DR3 and DR4 alleles are present in at least 90 percent of cases. Other genes known to contribute to TIDM susceptibility include *PTPN22*, *IL2RA*, and *CCR5*, all of which play a role in the immune system. Also the *INS* gene, which encodes insulin, is itself a candidate causal gene (16).

Although strong genetic associations with TIDM have been identified, the concordance rate of TIDM in monozygotic twins is only 50 percent, suggesting environmental risk factors also play an important role (15). Environmental risk factors thought to contribute to TIDM development include viral infections, specifically infections with coxsackieviruses and other enteroviruses, and dietary risk factors, such as early exposure to cow's milk (17).

### *B. Pathogenesis*

Although genetic and environmental risk factors are thought to contribute to disease susceptibility, having one or more of these risk factors, or even some degree of autoimmunity, does not necessarily mean an individual will develop TIDM. Rather pancreatic beta cell mass must decrease to a certain threshold for clinical symptoms of the disease to manifest. By the time overt diabetes sets in, some patients have lost up to 90 percent of their original beta cell mass (**Figure 3**) (18). However, the precise

contributions of infiltrating immune cells and the beta cells themselves to this pathogenic loss of beta cell mass remain unclear.

Regarding the immune system, it has long been proposed that an ill-defined environmental trigger (**Figure 3**), such as a viral infection, results in the release of beta cell autoantigens, such as insulin and glutamic acid decarboxylase (GAD), into the extracellular environment. These autoantigens are then scavenged by APCs and presented in the context of MHC-II to autoreactive $CD4^+$ T cells, which have previously escaped selection in the thymus, in nearby lymph nodes. These $CD4^+$ T cells in turn activate B cells to produce autoantibodies and cytotoxic $CD8^+$ T cells to directly kill beta cells by secreting perforin and granzyme and engaging the death receptor FAS (19).

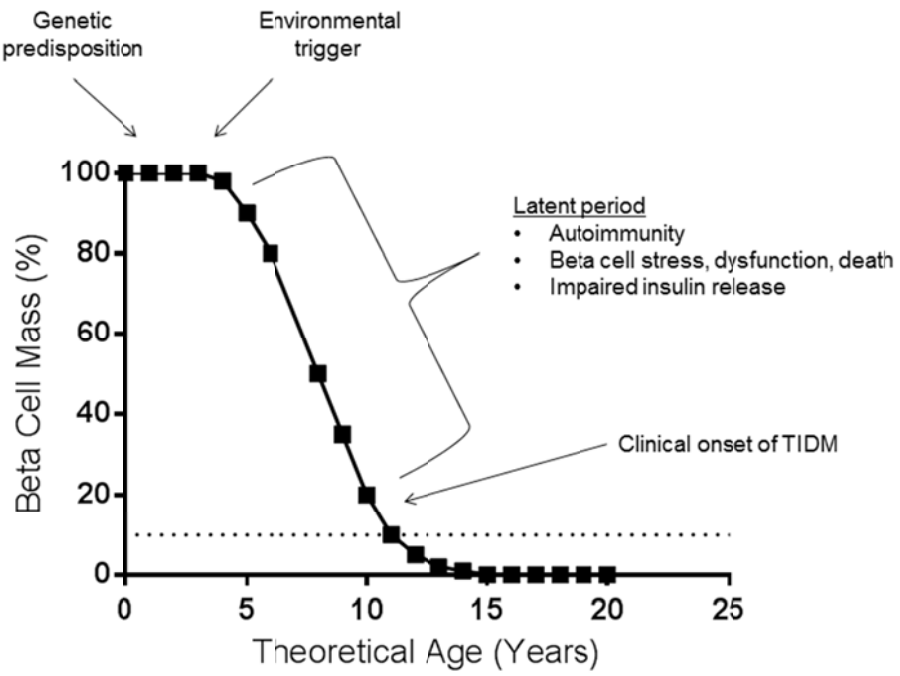


**Figure 3. Progression to TIDM.** In genetically predisposed individuals, an environmental trigger is thought to initiate the progressive loss of beta cell mass that ultimately leads to TIDM. The latent period of TIDM is characterized by progressive autoimmunity, beta cell stress, dysfunction, and death, as well as impaired insulin release. The clinical onset of TIDM does not occur until up to 90 percent of the original beta cell mass has been lost.

In addition to this role of immune cells, a growing body of evidence supports the notion that beta cells themselves contribute substantially to their own demise. For example, these cells are particularly vulnerable to stress-induced changes that may occur during an immune response that could result in beta cell death. For example, beta cells display remarkable sensitivity to cytokine-mediated killing by the pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ. Beta cells are vulnerable to these stress-induced changes because of their exceptional sensitivity to endoplasmic reticulum (ER) stress and ROS-mediated oxidative stress (19).

ER stress occurs when the protein folding requirements in the cell exceed the maximum folding capacity of the ER. As a result, misfolded proteins begin to

accumulate. Beta cells are particularly sensitive to ER stress due to the high basal level of protein synthesis that occurs in these cells. Twenty percent of total protein synthesized in beta cells is proinsulin, the precursor of insulin. Thus, additional demands in protein synthesis resulting from immune-mediated stress may result in ER stress and beta cell death. Beta cells are particularly sensitive to ROS-mediated oxidative stress due to their unique lack of catalase, an enzyme required to breakdown $H_2O_2$. Beta cells paradoxically produce high basal levels of $H_2O_2$ due to their unique metabolic programming. Unlike other cell types, in which pyruvate generated during glycolysis can be shuttled to a number of different metabolic pathways, most of the pyruvate produced in beta cells is channeled to the tricarboxylic acid cycle in the mitochondria. This process generates high levels of oxygen radicals, which are converted to $H_2O_2$ by the enzyme superoxide dismutase 2. Thus, any additional ROS produced in response to stress-induced signaling is likely to be detrimental to beta cells (19).

Hyperglycemia resulting from the progressive loss of beta cell mass can further exacerbate these problems, thus leading to even more beta cell death. High levels of glucose in the blood will increase the demand for proinsulin synthesis by the remaining beta cells, thus contributing to a possible increase in ER stress (19; 20). Furthermore, elevated levels of glucose will result in more pyruvate being shuttled to the tricarboxylic acid cycle and increased generation of ROS. Due to these unique beta cell-specific vulnerabilities to immune-mediated stressors, a current view purports that TIDM results from immune-mediated, albeit self-assisted, beta cell death (19).

***C. Clinical Significance***

Currently TIDM can be well-managed with a combination of recombinant human insulin, designer insulin analogues, high-tech insulin pumps, and state-of-the-art insulin sensors. The discovery and clinical implementation of these therapeutic advancements represent remarkable achievements in modern medicine (21). Patients with TIDM now

survive and live their lives with a disease that, less than a century ago, was fatal; however, TIDM is still an incurable, progressive disease with significant, if not life-threatening, complications. Some of these complications include diabetic retinopathy, nephropathy, neuropathy, foot ulcers, and cardiovascular diseases (20; 21).

Because TIDM is a chronic illness that requires lifelong management, this disease is a great financial burden to both individual patients and our country as a whole. A patient living with TIDM can incur up to $10,000 dollars per year in standard healthcare costs, while each year TIDM costs our country over $14.4 billion dollars in medical costs and lost income (22). Thus, eliminating TIDM remains a critically important objective. Understanding processes such as ER stress that contribute to beta cell death and TIDM progression will be necessary to develop better therapies and prevention strategies for this chronic disease.

**VI. The Unfolded Protein Response**

To combat ER stress, mammalian cells have evolved a three-pronged, cytoprotective signal transduction pathway known as the unfolded protein response (UPR). To induce the UPR, three ER-associated transmembrane proteins are utilized: inositol requiring enzyme 1 (IRE1), dsRNA-dependent protein kinase R (PKR)-like ER kinase (PERK), and activating transcription factor (ATF) 6. Normally, these proteins are rendered inactive by their association with the ER chaperone binding immunoglobulin protein (BiP). During ER stress, BiP dissociates from IRE1, PERK, and ATF6 in order to assist with protein folding, thus allowing these proteins to initiate signal transduction. The goal of UPR activation is to restore ER homeostasis by increasing the expression of chaperones to assist with protein folding, while simultaneously halting translation of proteins not involved in mitigating ER stress. The UPR also activates the ER-associated degradation pathway (ERAD), which targets misfolded proteins to the proteasome for degradation (23).

The UPR sensor IRE1 is a transmembrane protein that has both endonuclease and kinase activities. IRE1 autophosphorylation activates its endonuclease activity, which is responsible for the unconventional, cytosolic splicing of *XBP1* mRNA. *XBP1* mRNA encodes the transcriptional repressor X-box binding protein 1 (XBP1). Splicing of *XBP1* to *XBP1s* produces a transcript encoding the activating transcription factor XBP1s, which induces the expression of genes encoding ER chaperones as well as ERAD components. IRE1 endonuclease activity also promotes the degradation of mRNAs in a process known as regulated IRE1-dependent decay (RIDD) (23). IRE1 kinase activity is less well understood; however, it is known to result in the activation of JNK. JNK activation may promote cell survival by inducing autophagy or induce apoptosis in cells with unsurmountable levels of ER stress (**Figure 4**) (24).

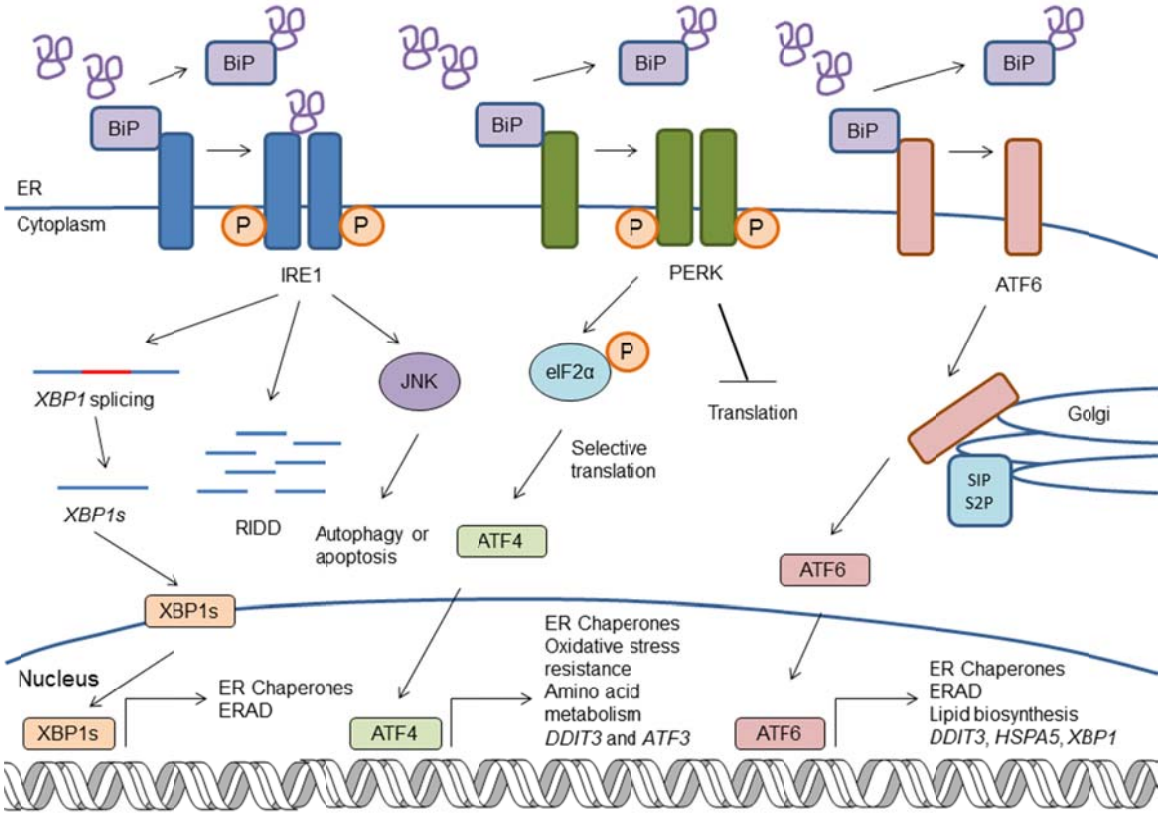


**Figure 4. Overview of UPR activation.** When unfolded proteins begin to accumulate in the ER, BiP dissociates from the ER transmembrane sensors IRE1, PERK, and ATF6 to assist with protein folding, thus allowing the initiation of UPR signaling. Endonuclease activity of the IRE1 sensor protein mediates *XBP1* splicing as well as RIDD, the degradation of ER-associated mRNA. The kinase activity of IRE1 mediates JNK activation, which may induce autophagy to promote cell survival, or alternatively lead to apoptosis in cells with unsurmountable levels of ER stress. Activated PERK phosphorylates the translation initiation factor eIF1α. Once phosphorylated, this protein inhibits global translation while simultaneously promoting translation of ATF4. In response to ER stress, ATF6 translocates from the ER membrane to the Golgi where it is cleaved by site proteases before translocating to the nucleus. Together the transcription factors XBP1s, ATF4, and ATF6 activate transcription of UPR target genes.

The UPR sensor PERK, activated by transphosphorylation following BiP dissociation, phosphorylates the translation initiation factor eIF2α. Phosphorylation of eIF2α inhibits synthesis of new secretory proteins, while simultaneously promoting translation of ATF4, a transcription factor that induces expression of genes involved in amino acid metabolism and oxidative stress resistance, in addition to genes encoding ER chaperones (23). In the case of unresolvable ER stress, ATF4 promotes the transcription of *DDIT3*, which encodes CCAAT/enhancer-binding protein homologous protein (CHOP), a transcription factor that upregulates genes involved in apoptotic cell death. ATF4 also promotes transcription of *ATF3*, which encodes a transcription factor known to suppress the expression of genes encoding pro-inflammatory cytokines (**Figure 4**) (24; 25).

Activation of the UPR sensor ATF6 results in its translocation to the Golgi apparatus where it is cleaved by site proteases S1P and S2P. Truncated ATF6 then translocates to the nucleus where it induces expression of genes involved in protein folding and transport, ERAD, and lipid biosynthesis. Here ATF6 also induces transcription of *DDIT3*, *HSPA5*, which encodes BiP, and *XBP1*, thus amplifying the UPR (**Figure 4**) (23).

**VII. Pro-Inflammatory Cytokines and Beta Cell ER Stress and Death**

As mentioned above in the discussion of TIDM pathogenesis, the pro-inflammatory cytokines IL-1β, TNF-α, and IFN-γ are known to contribute to ER stress and beta cell death. These cytokines are released by activated macrophages, NK cells, and T cells during the course of insulitis, the TIDM-associated infiltration of immune cells into the pancreatic islets (**Figure 5**) (26). Pro-inflammatory cytokines contribute to the induction of ER stress in beta cells via different mechanisms, depending on the species of origin. Studies in rat beta cells have shown that pro-inflammatory cytokines contribute to ER stress and apoptosis through NO-dependent downregulation of sarco-

endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) 2b, an ER calcium pump. In this study, decreased expression of SERCA2b resulted in ER $Ca^{2+}$ depletion and ER stress induction (27). However, more recent studies have shown that cytokine-induced apoptosis does not require the production of NO in mouse beta cells or human islet cells. Rather, cytokine-induced ER stress was found to mediate apoptosis of human beta cells, at least in part, by activation of JNK (28).

ROS production can contribute to ER stress by targeting chaperones as well as ER $Ca^{2+}$ channels, the latter of which could result in the leakage of $Ca^{2+}$ from the ER to the cytosol. The leakage of $Ca^{2+}$ from the ER contributes to ER stress because the concentration of ER $Ca^{2+}$ is critical for maintaining proper chaperone function and protein folding in the ER. Calcium released from the ER eventually becomes concentrated in the mitochondria where it disrupts electron transport, thus promoting even more ROS generation (29). Thus, although NO was found to be dispensable for ER stress induction in human beta cells, other ROS may contribute to ER stress induction in these cells.

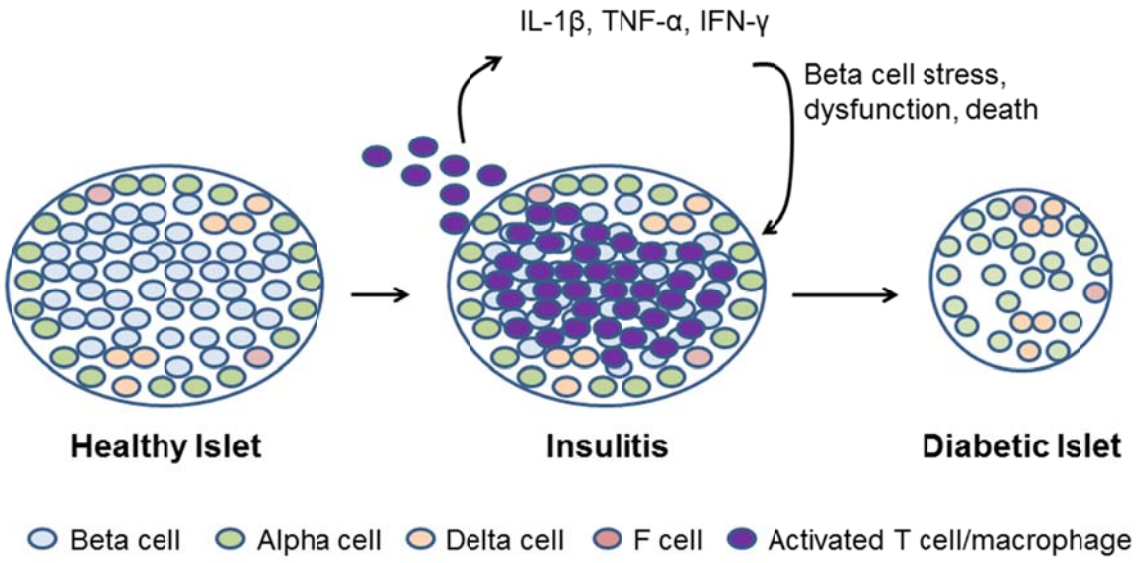


**Figure 5. Insulitis preceding the development of TIDM.** TIDM is a T cell-mediated autoimmune disorder that results in the targeted destruction of insulin-producing pancreatic beta cells. The clinical onset of TIDM is known to be preceded by insulitis, the infiltration of inflammatory cells into the pancreatic islets. During insulitis, activated macrophages, NK cells, and T cells secrete IL-1β, TNF-α, and IFN-γ. In addition to direct killing by $CD8^+$ T cells and NK cells, these pro-inflammatory cytokines also contribute to beta cell stress, particularly ER stress; dysfunction, marked by impaired insulin production; and ultimately death. ER stress may result from dysregulated ER $Ca^{2+}$ levels induced by ROS produced in response to pro-inflammatory cytokine stimulation.

**VIII. Prevention of TIDM**

As discussed previously, TIDM is thought to be initiated in genetically predisposed individuals by an unknown environmental trigger. This trigger initiates an immune response that directly targets beta cells for destruction, while simultaneously promoting stress responses in the beta cells themselves that further lead to their demise (19). This period of beta cell stress, dysfunction, and death is known as the latent period in the progression to TIDM (**Figure 3**). In some patients, this preclinical stage lasts only a few months, while in others it can last many years. During this latent period, autoimmunity and beta cell loss have begun; however, sufficient numbers of insulin-

secreting beta cells remain to inhibit overt hyperglycemia and other clinical symptoms of disease (21).

A major obstacle in the road to developing effective prevention strategies for TIDM is the lack of known, specific biological markers that could detect individuals undergoing this clinically latent period of beta cell stress (21). If such markers could be identified, the implementation of TIDM prevention strategies, such as the use of anti-CD3 or anti-CD20 monoclonal antibodies, could be optimized, perhaps through better timing or the co-administration of drugs to preserve beta cell function (19). Anti-CD3 and anti-CD20 therapies, which deplete T and B lymphocytes, respectively, have shown promise in preventing TIDM in a number preclinical and clinical studies (30).

**IX. Current Biomarkers for TIDM**

Currently a number of biomarkers for TIDM have been identified. For example, serum autoantibody markers have been established for the following beta cell autoantigens: insulin, GAD, tyrosine phosphatase-like insulinoma antigen 2, C-terminal epitope of IA2, and zinc transporter 8 (**Table 1**). These autoantibody markers are used to confirm TIDM diagnosis in the clinic (21). In addition to serum autoantibodies, other biomarkers for TIDM include serum cytokines, such as CXCL1; beta cell-specific epigenetic changes, such as unmethylated insulin DNA; and beta cell stress-induced miRNAs and proteins (21). Some have speculated markers of ER stress, such as CHOP, could be used as biomarkers for beta cell destruction in the clinic, but detecting changes in expression levels of beta cell proteins in vivo remains challenging (20; 21).

Recently collaborative studies between our laboratory and others have shown that proinsulin to C-peptide ratios are elevated in patients with newly diagnosed TIDM compared to body mass index (BMI), age, and gender matched control subjects. This result suggests that proinsulin is not processed correctly to C-peptide and mature insulin in newly diagnosed patients with TIDM. This lack of optimal post-translational processing

would be expected to occur in stressed beta cells. Furthermore, this same study found that serum levels of heat shock protein (HSP)90 were elevated in patients newly diagnosed with TIDM relative to controls (31). However, whether the presence of this protein in the serum is indicative of beta cell stress characteristic of latent TIDM is unclear.

| Antigen | Association with TIDM |
|---|---|
| Insulin (IAAs) | Detected in 60% of newly diagnosed children |
| Glutamic acid decarboxylase (GAD) | Detected in 70-80% of newly diagnosed individuals |
| Tyr phosphatase-like insulinoma antigen 2 (IA2) | Detected in 60% of newly diagnosed individuals |
| C-terminal epitope of IA2 (ICA512) | Detected in 70-80% of newly diagnosed individuals |
| Zinc transporter 8 (ZnT8) | Detected in 60% of newly diagnosed individuals |

**Table 1. Established serum autoantibody markers used in the diagnosis of TIDM.** The presence of any of the autoantibodies listed above in patient serum is used to distinguish TIDM from type II diabetes in the clinic. However, TIDM can still be present in autoantibody negative individuals. Other TIDM biomarkers that can be identified include serum cytokines, beta cell-specific epigenetic changes, miRNAs, and proteins. However, a critical need exists for the identification of new biomarkers that could distinguish individuals in the latent period of TIDM (**Figure 3**). If patients could be identified before beta cell mass is completely obliterated, preventative therapies could be more successfully implemented to preserve beta cell mass and prevent the progression to TIDM.

### X. HSP90

HSP90 is a highly conserved member of the heat shock protein family of molecular chaperones. These proteins, upregulated in response to heat, are an essential component of the heat shock response. HSP90, in cooperation with co-chaperone proteins, assists a wide variety of protein clients in the acquisition of their active conformations by using energy derived from ATP binding and hydrolysis. HSP90 client proteins are involved in numerous cellular processes including signal transduction,

protein trafficking, receptor maturation, as well as innate and adaptive immunity. Human beings express four isoforms of HSP90: GRP94, the ER isoform; TRAP1, the mitochondrial isoform; and two cytoplasmic isoforms, HSP90α and HSP90β. Cytoplasmic HSP90 is one of the most abundant proteins in the cell, comprising 1-2 percent of total protein levels (32).

***A. HSP90 Associations with Human Autoimmune Disease***

Our laboratory became interested in studying HSP90 in the context of TIDM due to numerous associations of this protein with autoimmune disease. For example, skin and peripheral blood mononuclear cell (PBMC) levels of HSP90 were found to be elevated in patients with bullous pemphigoid, an autoimmune skin blistering disease, compared to control subjects (33). Another study revealed elevated levels of HSP90 in PBMCs from patients with systemic lupus erythematosus (SLE). These authors also found that increased levels of intracellular HSP90 in PBMCs correlated with increased levels of IL-6 and IgG autoantibodies to HSP90 in the serum of patients with SLE (34).

Furthermore another study found that although both control subjects and patients with TIDM had circulating autoantibodies to HSP90, patients with TIDM had higher levels of circulating IgG1 and IgG3 class-switched autoantibodies to this protein. These isotypes of antibody are most commonly produced during Th1-mediated immune responses, which predominate the autoimmune response in TIDM (35). This data led us to question whether or not HSP90 protein could be released from cells during the development of TIDM.

***B. HSP90 Inhibition and Murine Models of Autoimmune Disease***

Interestingly, pharmacological inhibition of HSP90 chaperone function has shown beneficial effects in a number of animal models of autoimmune disease. In the MRL/lpr mouse model of SLE, animals treated with the HSP90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) exhibited reduced

proteinuria, serum anti-dsDNA autoantibodies, and follicular B cells (36). In a mouse model of epidermolysis bullosa acquisita, a skin blistering disease associated with autoimmunity to type VII collagen, 17-DMAG treatment reduced total splenic B cells while increasing splenic regulatory B cells. Mice treated with 17-DMAG also displayed lower levels of circulating autoantibodies in this study (37). Application of another inhibitor of HSP90, SNX-4414, resulted in reduced inflammation, swelling, and cartilage damage in rat models of rheumatoid arthritis (38). Similarly, the HSP90 inhibitor EC144 suppressed inflammatory responses in two rodent models of autoimmune arthritis (39).

In addition to these effects observed in models of autoimmune diseases mediated by pathogenic autoantibodies, HSP90 inhibition has also shown beneficial effects in models of T cell-mediated autoimmune diseases. Treatment with 17-allylamino-17-demethoxygeldanamycin (17-AAG), another HSP90 inhibitor, prevented the onset of experimental autoimmune encephalomyelitis, the murine model of multiple sclerosis. This study also found that T cells from 17-AAG-treated mice produced less IL-2 in response to in vitro re-stimulation with peptide antigen (40). Others have shown in vitro that 17-DMAG inhibits T cell proliferation, decreases $CD4^+$ T cell expression of IFN-γ and IL-17, and reduces secretion of IFN-γ, TNF-α, and IL-17 in anti-CD3 stimulated human PBMC cultures (41).

Taken together, these studies suggest HSP90 may play a role in the pathogenesis of autoimmune diseases. However, precisely how HSP90 could be regulating immune responses is not yet clear. Nevertheless, other studies have suggested specific roles for this protein in modulating immune responses.

### *C. HSP90 and Immune Responses*

A role for HSPs, including HSP90, in immunity was revealed nearly thirty years ago with the discovery that HSPs purified from cancer cells could illicit immunity to tumors, while HSP preparations from normal tissues could not. This immunogenicity was

found to result from the association of HSPs with tumor-specific antigens. These HSP-antigen complexes could be taken up by APCs through association with the HSP receptor CD91. This receptor-mediated uptake of HSP-antigen complexes both promoted antigen processing within the APCs and increased presentation of tumor-associated antigens to T cells in the context of MHC molecules (42). Interestingly, our laboratory discovered a potential mechanism by which HSP90 could promote antigen presentation in the context of TIDM. In human B lymphoblasts, HSP90 was found to associate with and promote MHC-II presentation of the TIDM-associated autoantigen GAD (43).

Other studies have pointed to a role for HSPs in promoting innate immune responses. HSPs were found to promote both dendritic cell migration to lymph nodes as well as dendritic cell maturation, measured by cell surface expression of MHC-II, CD86, and CD40. HSPs have also been shown to promote the production of pro-inflammatory cytokines such as IL-1β and TNF-α as well as the induction of iNOS and subsequent production of NO in APCs. These activities are remarkably similar to the effects of LPS activation on dendritic cells and macrophages (42).

Researchers have suggested that HSPs are able to activate the immune system by functioning as damage-associated molecular patterns (DAMPs), molecules that activate an immune response via mechanisms similar to PAMPs. However, whether or not DAMPs contain specific molecular patterns that are recognized by the immune system is unclear at present. Rather these molecules can be thought of as danger signals of non-microbial origin. Instead of infection, DAMPs alert the immune system to danger brought on by non-physiological cell death, damage, or stress. These molecules are normally contained within the cytoplasm of live cells, but upon cell stress, damage, or death, are released into the extracellular environment where they can activate the immune system (44).

Indeed beta cell death and the subsequent release of DAMPs are thought to contribute to autoimmunity in animal models of TIDM, including the non-obese diabetic (NOD) mouse model (44). NOD mice spontaneously develop a T cell-mediated disease that shares many similarities with human TIDM, including the early development of insulitis and production of beta cell-specific autoantibodies (45). Treatment of NOD mice with a pan-caspase inhibitor to prevent apoptosis blocked the activation of diabetogenic T cells (46). In another study, inducing beta cell death using multiple, small doses of streptozotocin was found to induce pancreatic insulitis and subsequent diabetes in mice (47).

Furthermore one study found that HSP90, GRP94, and HSP70 are released during necrotic cell death, and that purified preparations of these proteins can deliver maturation signals to dendritic cells, resulting in activation of the NF-κB pathway and secretion of pro-inflammatory cytokines. This study made use of an LPS antagonist Rslp to rule out the effects of contaminating endotoxin on dendritic cell stimulation (48).

Nevertheless, although numerous studies have identified a role for DAMPs, such as high mobility group box 1 (HMGB1) protein, in activating the innate immune system, the ability of HSPs to trigger an innate immune response remains unclear (44). Contrary to the study described above, other studies have shown that highly purified, endotoxin-free HSPs are unable to activate APCs (49; 50). Further experiments testing the effects of HSP depletion on the adjuvant activity of dead cells are needed to clarify these contradictory results. As numerous HSPs could possibly be working together to produce these effects, such experiments will not be trivial (44).

***D. Extracellular HSP90***

Although whether extracellular HSP90 functions as a DAMP to activate APCs and initiate innate immune responses is still unclear, several studies suggest that this protein is released from cells into the extracellular environment during stress. In

response to heat shock, B lymphoblastoid cell lines were found to release elevated levels of HSP90 in extracellular vesicles known as exosomes (51). Exosomes are cell-derived vesicles formed by the inward budding of multivesicular body membranes. Multivesicular bodies are cellular compartments of endocytic origin that release extracellular vesicles out of the cell into the extracellular environment. Once released exosomes are known to perform functions in intercellular communication and biological signaling (52). However, B cell-derived exosomes in the study described above were unable to trigger dendritic cell maturation (51).

Another study reported that oxidative stress results in HSP90 secretion from vascular smooth muscle cells. These authors proposed that HSP90 was responsible for subsequent activation of ERK1/2 in these cells (53), and this pathway is known to be required for cell growth (54). Furthermore, HSP90 was found to be secreted by human fibroblasts in response to hypoxia and HIF-1α activation. In these studies, HSP90 was found to promote fibroblast migration and wound healing (55). Although HSP90 has been shown to be secreted by normal cells during various forms of stress, tumor cells are known to secrete HSP90 constitutively (54).

In cancer, extracellular HSP90 is widely thought to contribute to cancer cell motility and metastasis. Although the specific mechanisms leading to HSP90 secretion from tumor cells are not well understood, HSP90 release is known to be stimulated by environmental stressors and growth factors (56). At the molecular level, HSP90 secretion is regulated by post-translational modifications such as phosphorylation and acetylation (56; 57). Extracellular HSP90 can stimulate cancer cell invasiveness by activating matrix metalloproteinase-2 (MMP-2) (58). It can also stimulate migration through binding the cell surface receptor CD91 independently of its ability to bind and hydrolyze ATP (56).

## XI. Research Goals

In a recent study carried out by our laboratory and others, HSP90 was found to be elevated in the serum of newly diagnosed patients with TIDM relative to control subjects, thus identifying this protein as a potential biomarker of beta cell stress (31). Class-switched anti-HSP90 autoantibodies are also elevated in patients with TIDM, suggesting this protein may be released extracellularly during TIDM development (35). Furthermore many other studies have shown HSP90 is secreted in response to cellular stress (54). In light of this information, we hypothesized HSP90 may be released from pancreatic beta cells in response to cellular stress events associated with the latent period of TIDM.

The first goal of this project was to understand what stimuli could induce release of HSP90 from pancreatic beta cells. To investigate this aim, two human pancreatic beta cell lines, βLox5 and 1.1B4, were used. Experiments were also carried out using the murine insulin-secreting cell line MIN6 and primary human cadaveric islets isolated from non-diabetic donors. To specifically test the hypothesis that pancreatic beta cells release HSP90 in response to cellular stress, cells were treated in vitro with thapsigargin, pro-inflammatory cytokines, high glucose growth medium, and TLR agonists for 24 hours, and HSP90 release was measured by ELISA.

The next goal for this project was to elucidate the intracellular signaling events that contributed to HSP90 release from pancreatic beta cells in response to specific stressors. Various pharmacological inhibitors and other drugs were used with our in vitro cell culture systems in an attempt to block HSP90 release. The goal of these experiments was to determine a specific signaling pathway that was important for driving HSP90 release. As part of these first two aims, various stress pathways were characterized by measuring expression levels of specific genes and proteins associated with these pathways.

The final goal of this work was to investigate the role of HSP90 activity in the development of TIDM in vivo. Female NOD mice were treated with the HSP90 inhibitor 17-DMAG over the course of several weeks, and TIDM development was monitored via weekly measurements of body weight and blood glucose. Insulitis in these mice was characterized by histology, while stress responses in islet cells by were analyzed by measuring expression levels of stress-associated genes.

The overall objective of this work was to confirm that extracellular HSP90 is a marker of pancreatic beta cell stress associated with the latent period of TIDM. If true, serum levels of HSP90 could potentially be measured in the clinic as a biomarker to identify patients in this pre-clinical period of disease. Thus, the implementation of treatments aimed at TIDM prevention could be more accurately applied, better timed, and optimized with other treatments aimed at restoring and preserving beta cell mass. The outcome of these therapies would be to halt the progression of at-risk patients to insulin dependence and lifelong TIDM.

## MATERIALS AND METHODS

### *Cell culture*

The human pancreatic beta cell line βLox5 (59) was kindly provided by Dr. Clayton E. Mathews (University of Florida). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 1 g/L D-glucose and 110 mg/L sodium pyruvate (Gibco). Media was supplemented with 10% characterized fetal bovine serum (FBS) (HyClone), 15 mM HEPES buffer (Corning), 50 U/mL penicillin and 50 µg/mL streptomycin (Corning), 2 mM L-glutamine (Corning), 1X minimal essential medium non-essential amino acids (Gibco), and 0.02% bovine serum albumin (BSA) (Sigma-Aldrich). To passage, media was aspirated and monolayers were washed twice with Hanks' balanced salt solution (HBSS) (Corning). Cells were then incubated with 0.25% trypsin with 2.21 mM ethylenediaminetetraacetic acid (EDTA) (Corning) for 8 minutes at 37°C. Detached cells were collected and washed with complete medium, and cell viability was determined by trypan blue staining. 1.25-5 x $10^5$ cells were seeded into 100 mm tissue culture plates (Corning) containing 10 mL complete medium. Cells were grown at 37°C with 7% $CO_2$. Media was replaced every 2-3 days. Cells were passaged at 100% confluency and discarded after eight passages.

The human pancreatic beta cell line 1.1B4 (60) was also provided by Dr. Clayton E. Mathews (University of Florida). 1.1B4 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media with L-glutamine (Corning) supplemented with 10% characterized FBS, 50 U/mL penicillin and 50 µg/mL streptomycin, and 2 mM L-glutamine. To passage, media was aspirated and monolayers were washed twice with HBSS. Cells were then incubated with 0.25% trypsin with 2.21 mM EDTA for 10 minutes at 37°C. Detached cells were collected and washed with complete medium, and viability was determined by trypan blue exclusion. 2.5-5 x $10^5$ cells were seeded into 100 mm tissue culture plates containing 10 mL complete medium. Cells were grown at 37°C with

7% $CO_2$. Media was replaced every 2-3 days. Cells were passaged at 90% confluency and discarded after eight passages.

Human primary cadaveric islets were obtained from the Integrated Islet Distribution Program (City of Hope). Minimal health information was provided with islet preparations; however, donors were adults ranging from 28-52 years of age with no medical history of type II diabetes (**Table 2**). Upon receipt islets were rested by incubation for one hour at 37°C to allow recovery after transit. Whole islets were sorted by microscopic inspection and hand-picking from debris and then maintained in Connaught Medical Research Laboratories (CMRL) 1066 media (Gibco) supplemented with 10% FBS (HyClone), 50 U/mL penicillin and 50 µg/mL streptomycin, and 2 mM L-glutamine. Islets were cultured in 35 mm petri dishes (Celltreat) at 37°C with 7% $CO_2$. Following in vitro stress treatments, approximately 20 whole islets were washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Corning) and digested into a single cell suspension with 0.25% trypsin containing 2.21 mM EDTA for 15 minutes at 37°C. Cell viability was then determined by trypan blue staining.

| Donor | Sex | Age | BMI |
|---|---|---|---|
| 1 | M | 40 | 38.9 |
| 2 | M | 36 | 33.8 |
| 3 | F | 39 | 45.2 |
| 4 | F | 47 | 22.5 |
| 5 | M | 28 | 22.9 |
| 6 | M | 48 | 31.2 |
| 7 | M | 43 | 36.5 |
| 8 | F | 39 | 25.9 |
| 9 | F | 52 | 31.4 |

**Table 2. Human islet donor sex, age, and BMI data.** Human islets isolated from non-diabetic cadaveric donors were obtained from the Integrated Islet Distribution Program (City of Hope). Islets from male and female donors aged 28-52 were used for in vitro stress treatments.

The murine insulinoma cell line MIN6 was kindly provided by Dr. Debbie Thurmond (City of Hope). MIN6 cells were maintained in DMEM containing 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning) supplemented with 10% characterized FBS, 50 U/mL penicillin and 50 µg/mL streptomycin, 2 mM L-glutamine, and 55 µM 2-mercaptoethanol (Gibco). To passage, media was aspirated and monolayers were washed twice with DPBS. Cells were then incubated with 0.25% trypsin with 2.21 mM EDTA for 10 minutes at 37°C. Detached cells were collected and washed with complete medium. Cells were then split 1:5 into new 100 mm tissue culture plates. Cells were grown at 37°C with 7% $CO_2$. Media was replaced every 2-3 days. Cells were passaged at 80-90% confluency and discarded after eight passages.

***Cryopreservation***

βLox5 and 1.1B4 cells were suspended at 3 x $10^5$ – 1 x $10^6$ cells/mL in freezing medium composed of 10% dimethyl sulfoxide (Corning), 40% characterized FBS, and 50% βLox5 or 1.1B4 tissue culture media. Aliquots of 1 mL were then transferred to CryoTube vials (Thermo Scientific). Vials were placed at -80°C in Nalgene cryo 1°C

freezing containers filled with 500 mL isopropanol to achieve a cooling rate of -1°C per minute. After 24 hours, vials were transferred to liquid nitrogen tanks.

***In Vitro Stress Treatments of Beta Cell Lines and Primary Human Cadaveric Islets***

For all in vitro stress treatment experiments, βLox5 and 1.1B4 cells were treated at approximately 90% confluency, MIN6 cells at 70-80% confluency, and primary human cadaveric islets at a density of 200 islets/1.5 mL, unless otherwise noted in a particular experiment. As a control, cells were treated with media alone unless otherwise noted.

To test the effects of cytokine stress on human beta cell lines and primary cadaveric islets, cells were treated for various time points with a 100X cocktail of pro-inflammatory cytokines prepared in DPBS + 0.01% BSA. Specifically, human beta cell lines and primary cadaveric islets were treated with final concentrations of 5 ng/mL human recombinant IL-1β (eBioscience), 10 ng/mL human recombinant TNF-α (PeproTech), and 100 ng/mL human recombinant IFN-γ (PeproTech). In βLox5 cells, the effects of individual cytokines were also tested by treating cells with identical concentrations of cytokine, either individually or in pairs. MIN6 cells were treated with final concentrations of 5 ng/mL murine recombinant IL-1β (eBioscience), 10 ng/mL murine recombinant TNF-α (PeproTech), and 100 ng/mL murine recombinant IFN-γ (PeproTech).

To induce ER stress in beta cell lines, βLox5, 1.1B4, and MIN6 cells were treated in vitro with 1 μM thapsigargin (Cayman) for 6 hours or 0.1 μM thapsigargin for 24 hours.

To test the effects of glucotoxicity on beta cell lines, βLox5, 1.1B4, and MIN6 cells were treated for 6 or 24 hours with their respective culture medium supplemented to a final D-glucose (Sigma) concentration of 6 g/L (33.3 mM).

To stimulate TLR3 signaling, βLox5 cells were treated with 0.1 μg/mL of the synthetic dsRNA analogue polyinosinic-polycytidylic acid (PIC) (Santa Cruz Biotechnology, Inc.) for 24 hours. Transfection of PIC into cells was achieved using

Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, PIC was diluted to 0.002 μg/μL in serum and BSA-free tissue culture media. Lipofectamine 2000 was diluted to 40 μL/mL in serum and BSA-free media, mixed with an equal volume of diluted PIC, and incubated for 5 minutes at room temperature. A volume of 100 μL PIC/Lipofectamine 2000 was added to βLox5 cells in 12-well tissue culture plates containing 900 μL complete tissue culture medium, for a final concentration of 0.1 μg/mL PIC and 2 μL/mL Lipofectamine 2000. As a control, βLox5 cells were treated with 100 μL serum/BSA-free medium containing Lipofectamine 2000 alone.

To initiate TLR4 signaling, βLox5 cells were treated with 1 μg/mL LPS (Sigma-Aldrich) for 24 hours.

***Inhibitor Studies***

To block various stress-associated responses in βLox5 cells, several inhibitors were used. Cells were treated with inhibitors for 6 hours prior to 24 hour stress treatment with pro-inflammatory cytokines or PIC. Optimal concentrations of inhibitors were determined in pilot experiments assessing cell viability and drug effectiveness. βLox5 cells were treated with 100 μM 1400W (Cayman) to inhibit iNOS activity, 10 nM chetomin (Cayman) to inhibit HIF-1α activity, 100 μM dimethyloxaloylglycine (DMOG) (Sigma-Aldrich) to stabilize HIF-1α, 0.2 mM and 1 mM *N*-acetylcysteine (NAC) (Sigma-Aldrich) pH 7.4 to scavenge ROS, 0.2 mM and 1 mM tauroursodeoxycholic acid (TUDCA) (Millipore) to mitigate ER stress, 10 μM and 50 μM 4μ8c (Millipore) to inhibit IRE1 endonuclease activity, 10 μM SP600125 (Santa Cruz Biotechnology, Inc.) to inhibit JNK signaling, and 5 μM and 10 μM SB202190 (Sigma-Aldrich) to inhibit p38 signaling.

***Lactate Dehydrogenase (LDH) Cytotoxicity Assay***

βLox5 cell viability in response to in vitro stress treatments was determined using a commercially-available LDH cytotoxicity assay kit (Pierce). Volumes of 50 μL conditioned media were assayed in triplicate in 96-well plates according to the

manufacturer's instructions. After background absorbance values at 680 nm were subtracted from absorbance values at 490 nm, viability was calculated according to the following equation:

Viability = 100 – ((Sample A490 – Control A490)/(Maximum A490 – Control A490))*100

***Enzyme-Linked Immunosorbent Assay (ELISA) Kits***

After stress treatments, conditioned media was harvested from cells and spun at 2000 rpm at 4°C in a tissue culture centrifuge to remove debris. Supernatants were transferred to clean microfuge tubes and stored at -20°C for analysis. Commercially-available ELISA kits for human HSP90α (Enzo), human HSC70 (Cloud-Clone Corp.), human HSP70 (Enzo), human IFN-α (PBL Assay Science), and murine HSP90α (Biomatik) were used according to the manufacturers' instructions.

***ELISA for Human IL-6***

After stress treatments, conditioned media was harvested and stored as described above. To detect extracellular IL-6, 96-well enzyme-linked immunosorbent assay/radioimmunoassay plates (Corning) were coated with 50 µL per well of 2 µg/mL anti-human IL-6 capture antibody (Invitrogen) in 0.1 M carbonate buffer (pH 9.2) and incubated overnight at 4°C. Plates were washed 4 times with DPBS (Gibco) containing 0.05% Tween 20 (PBST) (Fisher Scientific) and blocked 1 hour at room temperature with DPBS + 1% BSA blocking buffer. A standard curve of 0-4 ng/mL recombinant human IL-6 (PeproTech) and sample dilutions were prepared in tissue culture medium. Volumes of 100 µL standards and samples were added to wells and incubated overnight at 4°C. Plates were washed 4 times with PBST and incubated 1 hour at room temperature with 50 µL per well of 1 µg/mL anti-human IL-6 biotin conjugate (Invitrogen) in blocking buffer. Plates were washed 4 times with PBST and incubated 30 minutes at room temperature with 50 µL per well of 0.2 µg/mL streptavidin-horseradish peroxidase (HRP) (Thermo Scientific) in blocking buffer. Plates were washed 4 times with PBST and

incubated for approximately 30 minutes with 100 µL per well of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) substrate (SurModics). Absorbance at 405 nm was measured, and IL-6 concentration was determined by interpolating absorbance values of samples into the known recombinant IL-6 standard curve.

***RNA Isolation and Complimentary DNA (cDNA) Synthesis***

βLox5 cells, 1.1B4 cells, MIN6 cells, and primary human cadaveric islets were harvested and washed 3 times with DPBS. After a final spin, DPBS was aspirated and cell pellets were frozen and stored at -80°C prior to RNA isolation. Murine islets were harvested by the Indiana University School of Medicine Islet and Physiology Core (Indianapolis, IN), washed 3 times with DPBS, and processed immediately for RNA isolation. Total cellular RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Cells were lysed in buffer RLT (Qiagen) containing 1% 2-mercaptoethanol (Sigma Aldrich) and homogenized using QiaShredder spin columns (Qiagen). On-column DNase digestion was also performed using the RNase-free DNase Set (Qiagen). Next cDNA was synthesized from 0.05-1 µg of RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions.

***Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)***

To quantitate relative gene expression levels, 5.5 µL master mix containing 0.5 µL of a specific TaqMan Gene Expression Assay (Applied Biosystems) and 5 µL of TaqMan Fast Universal PCR Master Mix (Applied Biosystems) was mixed with 100 ng of cDNA in 4.5 µL diethylpyrocarbonate-treated water (Invitrogen) in MicroAmp Fast Optical 96-well Reaction Plates (Applied Biosystems). Each condition was run in duplicate or triplicate. Amplification of cDNA was achieved by qRT-PCR using the 7500 Fast Real-Time PCR System (Applied Biosystems). Samples were initially denatured for 20 seconds at 95°C and then put through 40-45 cycles of 15 seconds at 95°C and 1 minute

at 60°C. Commercially available assays for human *ACTB*, *ATF3*, *ATF4*, *B2M*, *CA9*, *DDIT3*, *GAPDH*, *HIF1A*, *HSPA5*, *HSP90AA1*, *HSP90AB1*, *HSP90B1*, *IFI27, IFNA1*, *IFNB1*, *IL6*, *ISG15*, *NOS2*, *TLR2*, *TLR3*, *TLR4*, *TLR9*, *TRAP1*, and *VEGFA* were used (**Table 3**). Additionally, a custom assay for human *XBP1s* was designed to detect the spliced version of this transcript (**Table 4**). Commercially available assays for murine *Actb*, *Ddit3*, *Hif1a*, *Hmgb1*, *Hspa1b*, *Hspa5*, *Hsp90aa1*, *Hsp90ab1*, *Hsp90b1*, *Il6*, *Ins1*, *Map1lc3a*, *Nos2*, *Sqstm1*, and *Trap1* were also used (**Table 5**). Gene expression was presented as target mRNA levels detected relative to transcripts for endogenous control genes (delta $C_T$ method). These control or housekeeping transcripts were identified by screening beta cell lines and islets for abundant, broadly expressed transcripts which minimally varied with cell stress induction. Typically these control transcripts displayed less than one cycle of variation between untreated and stressed cell samples. For βLox5 and 1.1B4 cells, *GAPDH* was used as an endogenous control transcript, while *ACTB* was used as a control for primary cadaveric islets. *Actb* was also used for MIN6 cells as well as murine islets. Genes that exhibited $C_T$ values greater than 35 were considered undetected (UD).

| Gene | Description | Assay Product Number |
|---|---|---|
| *ACTB* | β-actin | 4333762-1108032 |
| *ATF3* | Activating transcription factor 3 | Hs00231069_m1 |
| *ATF4* | Activating transcription factor 4 | Hs00909569_g1 |
| *B2M* | $\beta_2$-microglobulin | 4333766-1006021 |
| *CA9* | Carbonic anhydrase IX | Hs00154208_m1 |
| *DDIT3* | C/EBP homologous protein (CHOP) | Hs00358796_g1 |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | Hs02758991_g1 |
| *HIF1A* | Hypoxia-inducible factor 1, alpha subunit | Hs00153153_m1 |
| *HSPA5* | Binding immunoglobulin protein (BiP) | Hs00607129_gH |
| *HSP90AA1* | Heat shock protein 90, α cytosolic isoform | Hs00743767_sH |
| *HSP90AB1* | Heat shock protein 90, β cytosolic isoform | Hs01546478_g1 |
| *HSP90B1* | Heat shock protein 90, ER isoform (GRP94) | Hs00427665_y1 |
| *IFI27* | Interferon, alpha-inducible protein 27 | Hs01086373_g1 |
| *IFNA1* | Interferon-α 1 | Hs00855471_g1 |
| *IFNB1* | Interferon-β 1 | Hs01077958_s1 |
| *IL6* | Interleukin-6 | Hs00985639_m1 |
| *ISG15* | Interferon-stimulated gene 15 | Hs01921425_s1 |
| *NOS2* | Nitric oxide synthase, inducible (iNOS) | Hs01075529_m1 |
| *TLR2* | Toll-like receptor 2 | Hs01872448_s1 |
| *TLR3* | Toll-like receptor 3 | Hs01551078_m1 |
| *TLR4* | Toll-like receptor 4 | Hs00152939_m1 |
| *TLR9* | Toll-like receptor 9 | Hs00152973_m1 |
| *TRAP1* | Heat shock protein 90, mitochondrial isoform | Hs00212476_m1 |
| *VEGFA* | Vascular endothelial growth factor A | Hs00900055_m1 |

**Table 3. Human TaqMan gene expression assays.**

| Component | Sequence |
|---|---|
| Forward Primer | 5’ CCTGGTTGCTGAAGAGGAG 3’ |
| Reverse Primer | 5’ AGTCAATACCGCCAGAATCC 3’ |
| Probe | 5’ CCTGCACCTGCTGCGGACTC 3’ |

**Table 4. Custom *XBP1s* TaqMan gene expression assay sequences.**

| Gene | Description | Assay Product Number |
|---|---|---|
| *Actb* | β-actin | 4352933-1112036 |
| *Ddit3* | C/EBP homologous protein (CHOP) | Mm01135937_g1 |
| *Hif1a* | Hypoxia-inducible factor 1, alpha subunit | Mm00468869_m1 |
| *Hmgb1* | High mobility group box 1 | Mm00849805_gH |
| *Hspa1b* | Heat shock protein 70 (HSP70) | Mm03038954_s1 |
| *Hspa5* | Binding immunoglobulin protein (BiP) | Mm00517690_g1 |
| *Hsp90aa1* | Heat shock protein 90, α cytosolic isoform | Mm00658568_gH |
| *Hsp90ab1* | Heat shock protein 90, β cytosolic isoform | Mm00833431_g1 |
| *Hsp90b1* | Heat shock protein 90, ER isoform (GRP94) | Mm00441926_m1 |
| *Il6* | Interleukin-6 | Mm00446190_m1 |
| *Ins1* | Insulin | Mm01950294_s1 |
| *Mapk1lc3a* | Microtubule-associated protein 1 light chain 3 α (LC3) | Mm00458725_g1 |
| *Nos2* | Nitric oxide synthase, inducible (iNOS) | Mm00440502_m1 |
| *Sqstm1* | Sequestome 1 (p62) | Mm00448091_m1 |
| *Trap1* | Heat shock protein 90, mitochondrial isoform | Mm00446003_m1 |

**Table 5. Murine TaqMan gene expression assays.**

***Exosome Isolation***

To prepare cell culture media with serum for βLox5 exosome production, DMEM containing 1 g/L D-glucose and 110 mg/L sodium pyruvate was supplemented with 20% characterized FBS and centrifuged at 100,000 *x g* overnight at 4°C to remove serum-derived bovine exosomes. The following morning, DMEM and additional supplements were added to yield complete βLox5 tissue culture medium containing 10% characterized FBS (see *Cell Culture* methods).

To isolate exosomes released in the media from βLox5 cells, complete media was aspirated and monolayers were washed twice with HBSS. Exosome production medium was added, and cells were treated and incubated as required per experiment. Following incubation, media was harvested into 15 mL conical tubes, and exosomes were precipitated from 4 mL of tissue culture media using ExoQuick-TC (System

Biosciences) according to the manufacturer's instructions. To liberate exosome proteins for ELISA, Exosome Binding Buffer (System Biosciences) was used following the manufacturer's instructions. HSP90 levels in exosome and soluble fractions were assayed by ELISA, and concentrations were multiplied by sample volume to give total amount of HSP90 in ng.

***Antibodies***

The following antibodies were used to detect the abundance of specific proteins by immunoblotting. HSP90 was detected with the mouse monoclonal antibody clone AC88 (Enzo) diluted 1:1000 or with the rat monoclonal antibody clone 16F1 (Abcam) diluted 1:500. Phospho-SAPK/JNK (Thr183/Tyr185) was detected with the rabbit monoclonal antibody clone 81E11 (Cell Signaling Technology) diluted 1:500, while total SAPK/JNK was detected using a rabbit polyclonal antibody (Cell Signaling Technology) diluted 1:500. Phospho-p38 MAPK (Thr180/Tyr182) was detected with the rabbit monoclonal antibody clone D3F9 (Cell Signaling Technology) diluted 1:500, while total p38 MAPK was detected with a rabbit polyclonal antibody (Cell Signaling Technology) diluted 1:1000. Actin and GAPDH were immunoblotted as loading controls. Actin was detected with the mouse monoclonal antibody clone ACTN05 (C4) (NeoMarkers) diluted 1:1000, while GAPDH was detected using the mouse monoclonal antibody clone 6C5 (Millipore) diluted 1:2000. Primary antibodies were detected using peroxidase-conjugated AffiniPure F(ab')$_2$ fragment goat anti-mouse or anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:5000 or peroxidase-conjugated AffiniPure F(ab')$_2$ fragment goat anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:10,000.

***Immunoblotting***

Human beta cell lines were harvested and washed three times with DPBS. After a final spin, DPBS was aspirated, and cell pellets were stored at -80°C. Whole cell

lysates were prepared from pellets by lysing for 30 minutes on ice in 10 mM Tris-HCl pH 6.8, 150 mM NaCl, and 1% Triton-X 100 containing 2X protease inhibitor cocktail (Sigma-Aldrich). When a phosphorylated protein was to be detected, 1X phosphatase inhibitor cocktail (Cell Signaling Technology) was also added to the lysis buffer. Debris and nuclei were pelleted by centrifuging for 10 minutes at 14,000 rpm at 4°C. Clarified lysates were transferred to clean microfuge tubes and placed on ice.

Protein concentrations were determined via microtiter protein assay (Bio-Rad) by interpolating absorbance at 570 nm into a known BSA standard curve (Pierce). Volumes of lysate containing 20-80 µg protein, depending on lysate concentration and well volume of stacking gel, were mixed with 5X reducing sample buffer and boiled 5 minutes.

Samples were loaded into gels and resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis for approximately 40 minutes at 60 mA using 8% or 10% gels. Proteins were then transferred to 0.2 µm nitrocellulose membranes (GE Healthcare Life Sciences) for 60 minutes at 100 V at 4°C. Membranes were blocked for 1 hour at room temperature or overnight at 4°C in a blocking buffer containing 5% milk in PBST. Incubations with primary antibodies diluted in blocking buffer were performed overnight at 4°C on a nutator. Alternatively, incubations with primary antibody to GAPDH were performed for 2 hours at room temperature.

Membranes were washed 4 times for 5 minutes with tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated with the proper secondary antibody for 30 minutes at room temperature. Streptactin-HRP (Bio-Rad) was also included to detect protein standards (Bio-Rad). Membranes were washed again, and proteins were detected with Luminata western HRP substrates (Millipore).

To determine relative protein expression levels, densitometry was performed using Fiji software (National Institutes of Health). Expression levels for experimental samples were normalized to that of the control sample.

As an internal control, levels of phospho-JNK and total JNK as well as phospho-p38 and total p38 were visualized using the same membrane. After detection of phospho-proteins and loading controls, membranes were stripped for 20 minutes at 55°C in 0.1 M Tris-HCl pH 7.4, 2% SDS, and 0.7% 2-mercaptoethanol. Membranes were washed twice in deionized water for 5 minutes and twice in TBST for 10 minutes and blocked as described above before incubation with the second primary antibody.

***Transfection of βLox5 Cells with Small Interfering RNA (siRNA)***

βLox5 cells were grown in 12-well tissue culture plates in antibiotic-free βLox5 medium to 90% confluency. Cells were then transfected with 100 nM control or SAPK/JNK siRNA (Cell Signaling Technology) for 72 hours using Lipofectamine 2000. Briefly, siRNA was diluted to 2 μM in antibiotic, serum, and BSA-free βLox5 medium in polystyrene tubes, while Lipofectamine 2000 was diluted to 80 μL/mL. These mixtures were vortexed gently and incubated for 6 minutes at room temperature. Equal volumes of siRNA and Lipofectamine 2000 were mixed together and incubated for 20 minutes at room temperature. Volumes of 100 μL siRNA/Lipofectamine 2000 were added dropwise to wells containing 900 μL antibiotic-free βLox5 medium and swirled gently. Wells contained a final concentration of 100 nM siRNA and 4 μL/mL Lipofectamine 2000. Media was replaced with fresh antibiotic-free βLox5 media after 24 hours, and cells were allowed to recover for 48 hours at 37°C. After this 72 hour transfection period, one well of control and JNK siRNA-treated cells were harvested, and protein knockdown was confirmed by immunoblotting. Media was aspirated from remaining wells, and cells were treated for 24 hours with media alone or cytokine cocktail. After 24 hours, media was harvested and stored at -20°C for ELISA.

### *Mice*

Five week-old female NOD/ShiLtJ (NOD) mice were purchased from the Jackson Laboratory and housed in a specific pathogen-free environment within the Indiana University School of Medicine Laboratory Animal Research Care Facility (Indianapolis, IN). Mice were allowed to acclimate for one week after shipping prior to the initiation of an experiment. All animal experiments were performed in accordance with protocols approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (Indianapolis, IN).

### *Treatment with 17-DMAG*

Six week-old female NOD mice were injected intraperitoneally every third day with PBS or 2 mg/kg 17-DMAG (InvivoGen) for 18 weeks (61). The development of TIDM was monitored by measuring body weight and non-fasting blood glucose levels once per week. Mice were considered diabetic after two consecutive weekly blood glucose readings above 300 mg/dl. Blood glucose measurements were taken via tail bleed using a glucometer (Abbott). Moribund animals were sacrificed at approximately 19 weeks of age, and pancreata were isolated for histology. Surviving animals were sacrificed at 21 and 24 weeks of age, and pancreatic islets were isolated for qRT-PCR.

### *Collection of Serum*

Blood samples were collected from a cohort of PBS and 17-DMAG-treated mice once per week by facial bleed into Microvette CB 300 K2E non-heparinized tubes (Sarstedt). Serum was isolated by centrifugation and used for HSP90 ELISA.

### *Histology*

Pancreata from female NOD mice were fixed in formalin overnight and then placed in 70% ethanol for 24 hours. Histological sections were prepared and stained with hematoxylin and eosin by the Indiana University School of Medicine Histology Core (Indianapolis, IN). Insulitis was scored as previously described by a single observer (62).

Briefly, grade 1 = no mononuclear cell infiltrates associated with islets; grade 2 = peri-insulitis affecting less than 50% of the islet circumference with no evidence of intra-islet invasion; grade 3 = peri-insulitis affecting greater that 50% of the islet circumference with no evidence of intra-islet invasion; grade 4 = invasion of mononuclear cells into islets.

***Statistics***

For comparisons between two groups, statistical significance was determined using a two-tailed, unpaired *t* test. For qRT-PCR experiments in which two different treatments were tested on samples from the same individual human donor, a two-tailed, ratio paired *t* test was used. To determine statistical significance among three or more groups, a one-way analysis of variance (ANOVA) was used with Dunnett's test to correct for multiple comparisons, each experimental mean being compared to the control mean. In experiments with two independent variables, statistical significance was determined using a two-way ANOVA with Tukey's test to correct for multiple comparisons between groups. Survival curves were analyzed using a Mantel-Cox test. A p-value less than 0.05 was considered statistically significant with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. Statistics were calculated using Prism 6.04 (GraphPad Software, Inc.).

## RESULTS

### I. Pancreatic Beta Cell HSP90 Release in Response to Stress

#### *A. Effects of Cell Density on Beta Cell HSP90 Release*

To investigate the effects of cellular stress on HSP90 release from pancreatic beta cells, two human pancreatic beta cell lines, βLox5 and 1.1B4, were used. βLox5 cells were previously generated by retroviral transduction of adult beta cells with the floxed SV40 T antigen, $Ras^{val12}$, and hTERT oncogenes (59). 1.1B4 cells were previously generated by electrofusion of adult cadaveric islet cells with the immortalized PANC-1 epithelial cell line (60). As our lab had no prior experience growing these cells, preliminary optimization experiments were performed to determine the appropriate cell density at which to treat these cells with various stress-inducing stimuli.

βLox5 and 1.1B4 cells grown to medium and high density were treated with a cocktail of pro-inflammatory cytokines containing 5 ng/mL human recombinant IL-1β, 10 ng/mL human recombinant TNF-α, and 100 ng/mL human recombinant IFN-γ for 24 hours at 37°C. This combination of cytokines was chosen because it was found to produce the most cytotoxic effect, relative to other combinations of cytokines, in primary human cadaveric islets isolated from adult pancreata (63). Following treatment, supernatants were harvested, and levels of the alpha cytoplasmic isoform of HSP90 were measured by ELISA. HSP90 release in response to cytokine stress was more pronounced in βLox5 and 1.1B4 cells when treated at high density (**Figure 6 A-B**). These results may be due to increased levels of stress in the more confluent cells. In light of these results and given that beta cells in vivo are tightly clustered within the islets of Langerhans, cells grown to high density were treated with stress-inducing stimuli in all subsequent experiments.

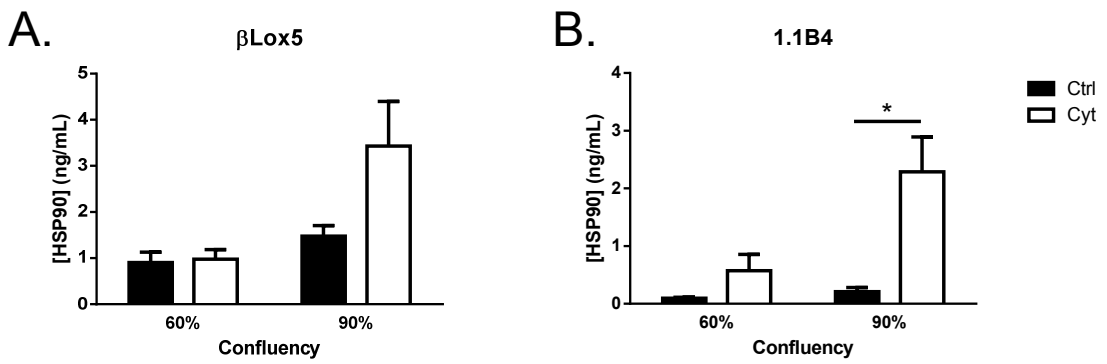


**Figure 6. Human beta cell lines release more HSP90 in response to pro-inflammatory cytokines when treated at high density.** βLox5 (A) and 1.1B4 (B) cells grown to 60% or 90% confluency were treated with media alone (Ctrl) or a cytokine cocktail containing 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt) for 24 hours at 37°C. Culture supernatants were harvested, and HSP90 levels were assayed by ELISA. Data are mean + SEM from N=3 experiments. *$p < 0.05$ Two-tailed, unpaired *t* test.

### *B. Beta Cell HSP90 Release in Response to Various Stressors*

As discussed previously, beta cell stress is a major factor that contributes to the development of TIDM, and several cellular stress events are known to be associated with these cells during the latent period of TIDM. For example, ER stress has been shown to precede the development of TIDM in NOD mice (64), and elements of ER stress are known to be upregulated in the islets of humans with TIDM (65). As beta cell mass starts to wane during this pre-clinical stage of disease, beta cells are particularly vulnerable to ER stress due to an increased demand for insulin production.

Insulitis, the infiltration of immune cells into the islets of Langerhans prior to the onset of TIDM, is another source of beta cell stress. Treating beta cell lines with a cocktail of IL-1β, TNF-α, and IFN-γ is used to model insulitis in vitro. These cytokines are secreted by activated macrophages and T cells during insulitis and are known to contribute to beta cell ER stress, dysfunction, and death (66).

As beta cell mass decreases and insulin production is impaired, blood glucose levels rise. This condition, known as hyperglycemia, is another source of beta cell stress during the latent period of TIDM (19). Because beta cells utilize the majority of glycolysis-generated pyruvate in the tricarboxylic acid cycle, which produces ROS, hyperglycemia results in increased ROS production by beta cells. These elevated levels of ROS can further exacerbate ER stress (19), possibly by altering $Ca^{2+}$ levels in the ER (29). This condition is known as glucotoxicity.

To test whether ER stress could result in HSP90 release from pancreatic beta cells, βLox5 and 1.1B4 cells were treated with thapsigargin, an inhibitor of SERCAs. Thapsigargin inhibits the transport of $Ca^{2+}$ from the cytosol to the ER, resulting in the dysfunction of ER chaperones and the development of ER stress. To model insulitis, cells were again treated with a cocktail of pro-inflammatory cytokines containing IL-1β, TNF-α, and IFN-γ. To test whether glucotoxicity could result in HSP90 release from pancreatic beta cells, βLox5 and 1.1B4 cells were treated with culture media containing 33.3 mM glucose. Interestingly both βLox5 and 1.1B4 cells released HSP90 in response to cytokine stress but not in response to thapsigargin or high glucose (**Figure 7 A-B**). These results suggest HSP90 may be released in response to cytokines independently of their ability to induce ER stress.

To determine whether these results are also true for other species, this experiment was repeated with the murine insulinoma cell line MIN6. This cell line was originally developed from a transgenic mouse expressing the large SV40 T antigen in pancreatic beta cells and has been shown to exhibit characteristics of glucose metabolism and insulin secretion that are similar to those of normal islets (67). Contrary to what was observed with human beta cell lines, MIN6 cells released HSP90 in response to all three stimuli: thapsigargin, pro-inflammatory cytokines, and high glucose (**Figure 7C**). However, the strongest response was produced in response to cytokine

cocktail. These differences between MIN6 cells and the human beta cell lines could be due to the different species of origin. Indeed, pro-inflammatory cytokines have been shown to give rise to ER stress by different mechanisms in mouse, rat, and human beta cell lines (28), suggesting the stress responses in beta cells from these three species may be different. For these reasons, human beta cell lines were used as a model for the majority of experiments in this work.

The ability of cytokine stress to promote HSP90 release in human beta cells was confirmed by treating primary human cadaveric islets with cytokine cocktail. Similarly to beta cell lines, primary human cadaveric islets also released HSP90 in response to cytokine stress (**Figure 7D**).

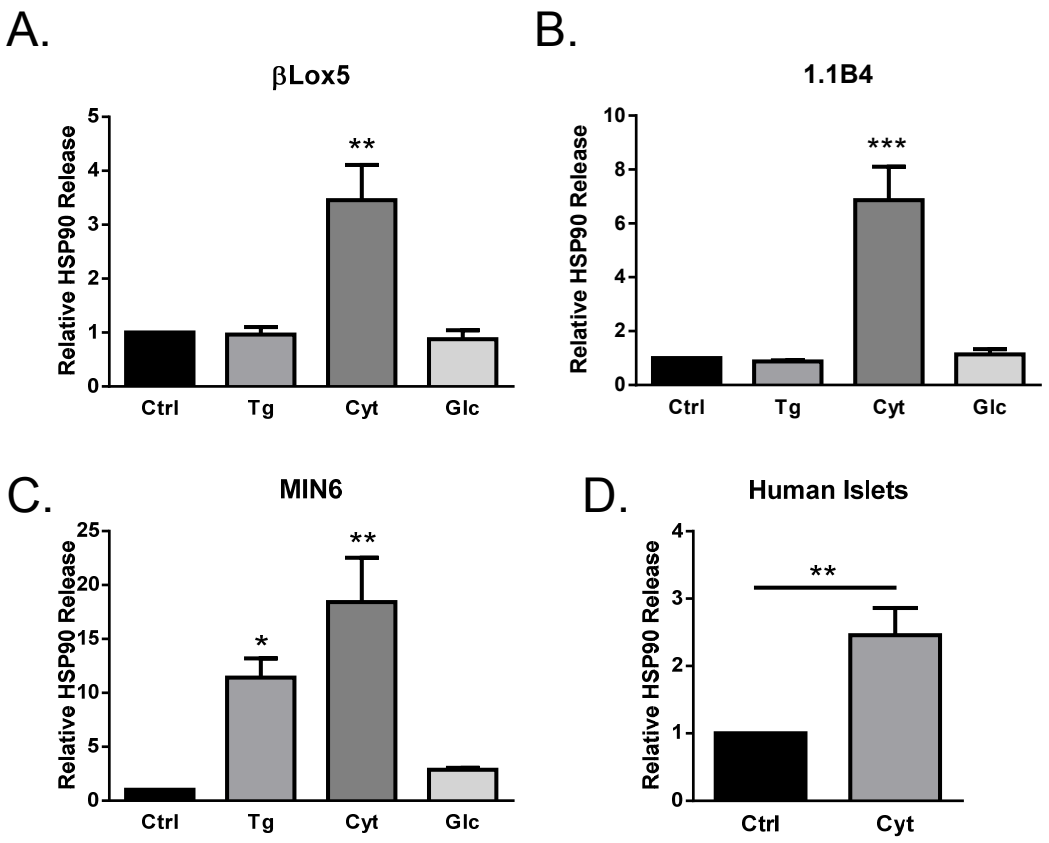


**Figure 7. HSP90 is released by human beta cells in response to cytokine stress.** Human beta cell lines βLox5 (A) and 1.1B4 (B) were treated for 24 hours at 37°C with media alone (Ctrl); 0.1 μM thapsigargin (Tg) to induce ER stress; a cytokine cocktail containing 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt) to mimic insulitis; or culture media containing 33.3 mM glucose (Glc) to mimic glucotoxicity. Culture supernatants were harvested, and HSP90 levels were assayed by ELISA. The same experiment was performed in the murine MIN6 cell line (C) using recombinant murine cytokines. Results in A and B were confirmed by treating primary human cadaveric islets with human recombinant cytokine cocktail (D). Data are presented as relative to control. Actual values of HSP90 were detected between 0.7-4.9 ng/mL for βLox5, 0.9-6.4 ng/mL for 1.1B4, 0.3-19.9 ng/mL for MIN6, and 0.9-20.1 ng/mL for human islets. Data are mean + SEM of N=3 experiments or N=9 islet donors. For cell line experiments *p <0.05, **p < 0.01, ***p < 0.001 One-way ANOVA with Dunnett's correction for multiple comparisons, each mean compared to control mean. For human islets, **p<0.05 Two-tailed, unpaired *t* test.

### *C. Mechanism of Release*

In vitro treatment with pro-inflammatory cytokines is known to result in apoptosis of human beta cell lines, particularly after 48 hours (28). However, in these experiments cells were treated with cytokines for a shorter 24 hour time period to avoid the possibility that HSP90 may passively leak out of cells as a result of increased cell membrane permeability following cell death. To confirm that stress treatments were not affecting cell viability, trypan blue exclusion was performed, and no differences in viability were observed in βLox5, 1.1B4, or primary human islet cells (**Figure 8 A-C**).

To confirm these results, LDH cytotoxicity assays were performed in βLox5 cells. This colorimetric assay quantitatively measures the activity of LDH, a cytosolic enzyme, released non-specifically into culture media as a result of cell death. As in experiments with trypan blue, no changes in viability were observed in stressed βLox5 cells relative to the control sample (**Figure 8 D**). LDH assays of βLox5 cells treated with cytokines for various time points confirmed that significant cytotoxicity did not occur until 48 hours post-treatment (**Figure 8E**), thus confirming that HSP90 was not passively released as a result of cell death at earlier time points.

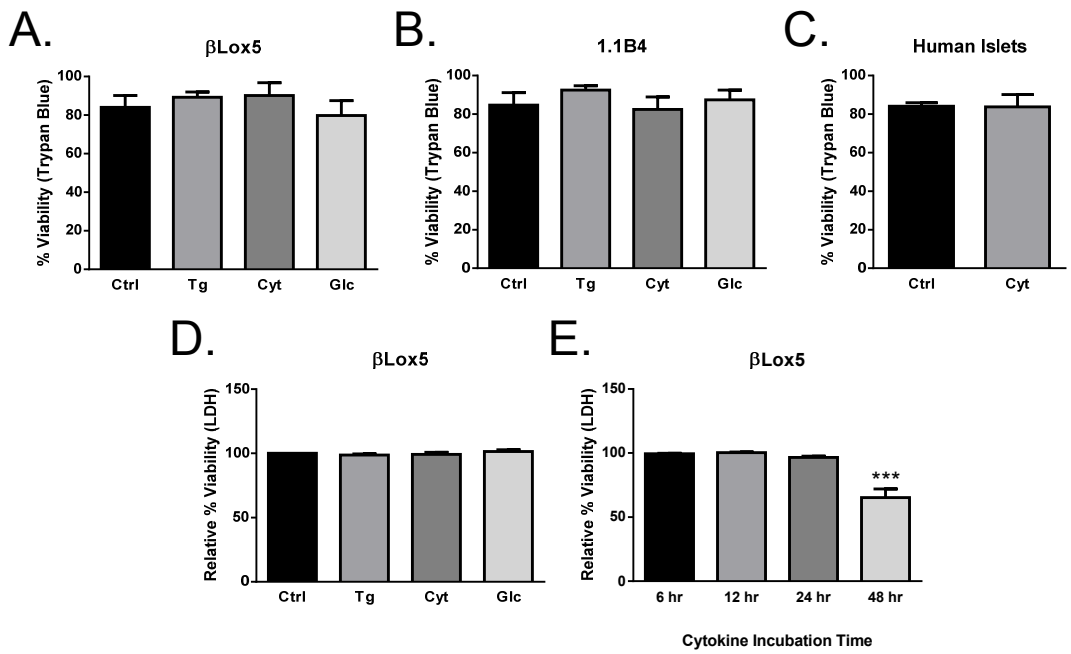


**Figure 8. Human beta cell 24 hour stress treatments are not cytotoxic.** βLox5, 1.1B4, and human islets were treated as in Figure 7, and viability was determined by trypan blue exclusion (A-C). Results were confirmed in βLox5 cells by LDH assay (D). Here viability is calculated as percent relative to the control sample. βLox5 cells were treated with cytokine cocktail for various time points, and LDH assays were performed (E). Here viability for each time point is presented as a percent relative to control cells treated with media alone for the same amount of time. Data are mean + SEM of N=3 experiments (A-B) and (D-E) or N=4 islet donors (C). ***$p < 0.001$ One-way ANOVA with Tukey's correction for multiple comparisons.

Although elevated levels of HSP90 were clearly released in response to cytokine stress in beta cells (**Figure 7**), the mechanism of release into the extracellular environment remained unclear. LDH assays suggested HSP90 was not passively released as a result of cell death (**Figure 8**).

To further address this question, ELISAs for HSP70 and HSC70, two other cytosolic chaperone proteins, were performed on culture media from control and cytokine-treated βLox5 cells. No extracellular HSP70 or HSC70 was detected in either experiment, suggesting cytosolic proteins were not passively leaking from cells in

response to cytokine-mediated cell death (data not shown). Together the data presented here suggest HSP90 was released by an active secretory mechanism.

Interestingly several studies have shown HSP90 is released in response to cell stress (53; 55; 58), and some studies have shown HSP90 is present in extracellular vesicles known as exosomes (68). Furthermore, HSP90-containing exosomes were found to be secreted by B cells in response to heat shock stress (51). Exosomes are known to play important roles in intercellular communication and biological signaling (52), and may even have implications for TIDM, as one study showed horizontal transfer of exosomal microRNAs could transfer apoptotic signals between beta cells (69).

To determine whether beta cells release HSP90 in association with exosomes, a commercially available exosome-precipitation solution was used to isolate βLox5 exosomes released after 24 hours of cytokine stress. These exosomes were lysed, and exosomal HSP90 levels were assayed by ELISA together with the soluble HSP90 levels present in supernatants following exosome precipitation. A good control experiment to pursue in the future would be to confirm exosome purity obtained with this solution using nanoparticle tracking analysis or immunoblotting for exosome markers such as CD63.

Nevertheless, experiments here showed HSP90 levels were significantly increased in exosomes isolated from cytokine-treated βLox5 cells compared to control-treated cells (**Figure 9**). In fact, the relative increase in exosomal HSP90 with cytokine stress was much greater than the increase in soluble HSP90 (≈eight-fold versus two-fold). This result may indicate an increase in the quantity of exosomes released, or alternatively, it could signify a change in the protein content of beta cell exosomes during cytokine stress. Thus, exosomal HSP90 may be a more sensitive marker of beta cell stress than soluble HSP90.

However, exosome isolation and analysis can be difficult with small volumes of biological fluids. Thus, exosomes represent a challenging source material for biomarker

analysis, particularly in children, who are the primary candidates for TIDM pre-screening. Therefore, we elected to continue to focus these studies on detection of soluble HSP90 given our success in using this form of the chaperone in predicting TIDM onset in young children (31).

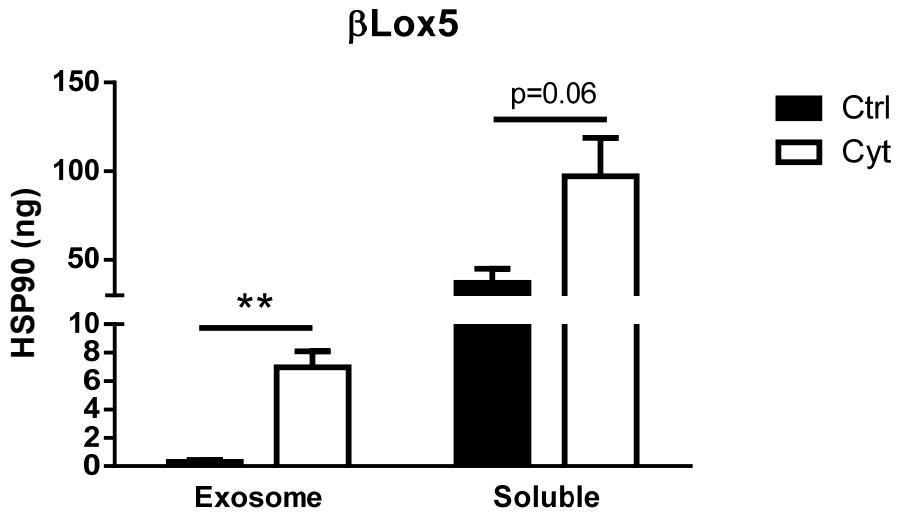


**Figure 9. HSP90 released from beta cells in response to cytokine stress is mostly soluble and not associated with exosomes.** Exosome-free βLox5 media was prepared by ultracentrifugation and used to treat βLox5 cells for these experiments. βLox5 cells were treated with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ for 24 hours at 37°C. Media was collected and βLox5 exosomes were precipitated using a commercially available exosome precipitation solution. Exosomes were lysed and HSP90 levels were assayed by ELISA. HSP90 concentrations were multiplied by the final sample volume to give total HSP90 in ng. The soluble fraction is the supernatant left after exosome precipitation. Data are mean + SEM of N=3 experiments. **$p < 0.01$ Two-tailed, unpaired *t* test.

To determine whether IL-1β, TNF-α, or IFN-γ alone or whether some combination of these cytokines was responsible for HSP90 release, βLox5 cells were treated with all possible permutations of IL-1β, TNF-α, and IFN-γ for 24 hours at 37°C. Although IL-1β alone and each pair of cytokines induced a slight elevation in HSP90 release relative to control-treated cells, by far the most robust release of HSP90 was

observed when cells were treated with all three cytokines (**Figure 10**). These results are consistent with previous studies showing this combination of cytokines induces the most stress in human islets (63).

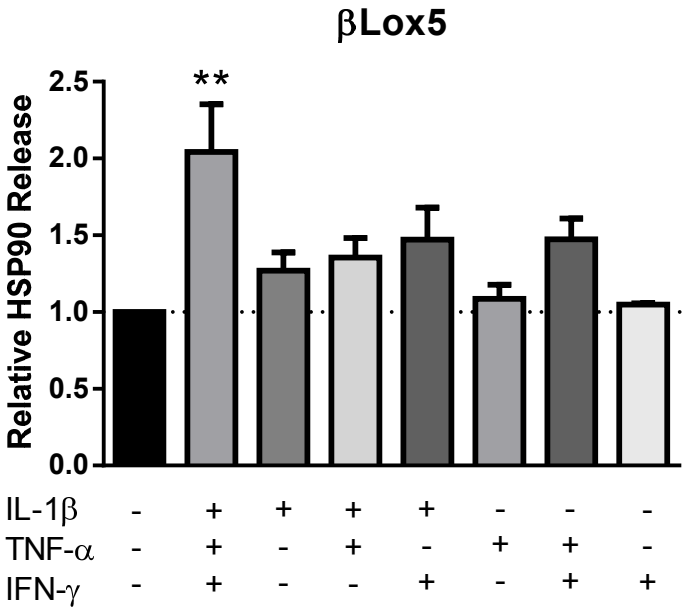


**Figure 10. Beta cells optimally release HSP90 in response to a combination of IL-1β, TNF-α, and IFN-γ.** βLox5 cells were treated with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ alone or in various combinations for 24 hours at 37°C. Media was harvested after incubation, and HSP90 levels were measured by ELISA. Data are presented as relative to control. Actual values of HSP90 were detected between 3.4-30.9 ng/mL. Data are mean + SEM of N=3 experiments. **p<0.01 One-way ANOVA with Dunnett's correction for multiple comparisons, each mean compared to no cytokine control.

To determine whether increased HSP90 release in response to cytokine stress was a result of changes in gene or protein expression, qRT-PCR and immunoblotting experiments were performed. Expression levels of *HSP90AA1*, which encodes the alpha cytoplasmic isoform of HSP90, were measured by qRT-PCR using samples from βLox5, 1.1B4, and MIN6 cells treated with thapsigargin, cytokine cocktail, or high glucose for 6 or 24 hours. No notable increases in *HSP90AA1* expression levels were observed in these cell lines at either time point (**Figure 11 A-F**). These results suggest increased HSP90 release did not occur as a result of increased *HSP90AA1* gene expression. These results were confirmed by performing qRT-PCR on samples from primary human cadaveric islets treated for 24 hours with cytokine cocktail (**Figure 11 G**).

Detecting changes in *HSP90AA1* gene expression in human islets is complicated by the presence of alpha cells and potentially other exocrine and endocrine cell types present in these structures. Thus, the current studies focused on relative changes in gene expression with cell stress rather than absolute values. Cellular stress can in some cases also alter housekeeping gene expression in cells, thus the current studies were analyzed by examining several endogenous genes as controls. For all human qRT-PCR studies in this work, *GAPDH* was ultimately used as an endogenous control for βLox5 and 1.1B4 cells, while *ACTB* was used for primary human islets.

To determine whether elevated levels of HSP90 were released in response to cytokine stress due to changes in protein level, we measured HSP90 levels by immunoblotting in βLox5 and 1.1B4 cells treated with cytokine cocktail for 24 hours. No increases in HSP90 protein level were observed in cytokine-treated βLox5 (**Figure 12 A-B**) or 1.1B4 cells (**Figure 12 B-D**). In fact cytokine-treated βLox5 cells actually exhibited a slight but statistically significant decrease in HSP90 protein level (**Figure 12 A-B**). This result is consistent with cytoplasmic HSP90 being released from these cells.

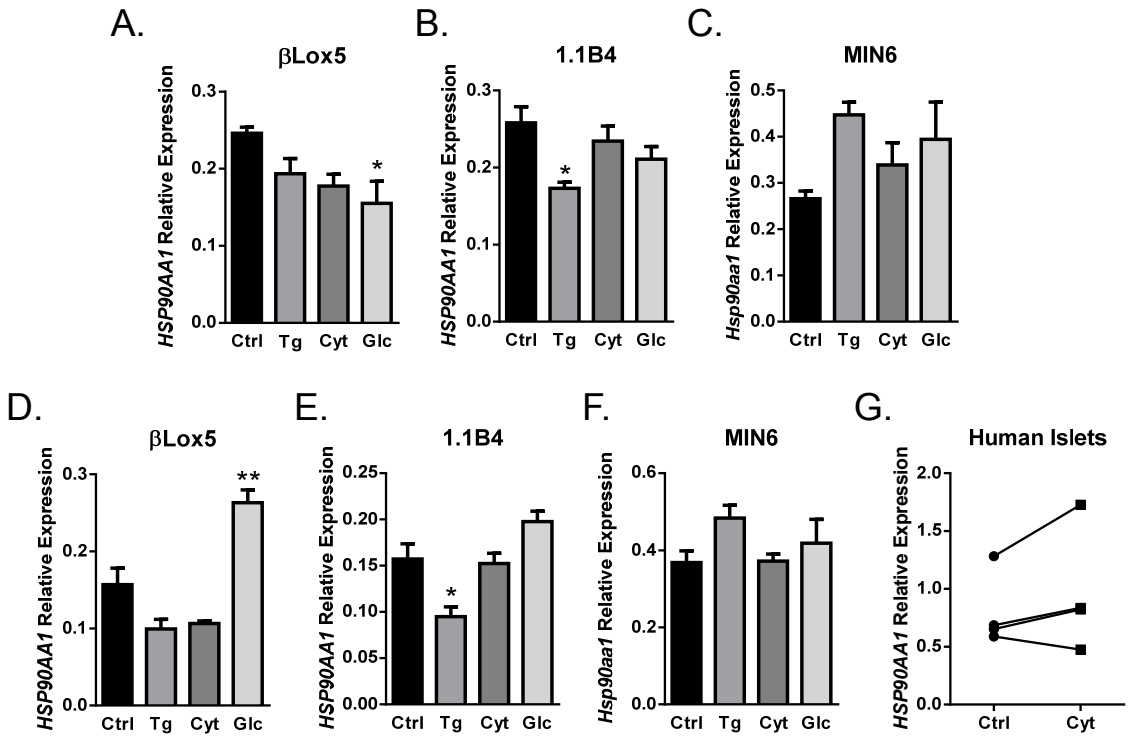


**Figure 11. Cytokine stress does not increase *HSP90AA1* gene expression levels in beta cells.** βLox5 (A), 1.1B4 (B), and MIN6 (C) cells were treated with for 6 hours with media alone (Ctrl); 1 μM thapsigargin (Tg); a cytokine cocktail containing 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt); or culture media containing 33.3 mM glucose (Glc) for 6 hours at 37°C. *HSP90AA1* gene expression levels were measured by qRT-PCR. *HSP90AA1* levels were also assayed in the same cell lines (D-F) as well as human islets (G) treated for 24 hours as in Figure 7. Data are mean + SEM of N=3 experiments (A-F) or actual values for N=4 islet donors (G). *$p < 0.05$, **$p < 0.01$ One-way ANOVA with Dunnett's correction for multiple comparisons, each mean compared to the control mean.

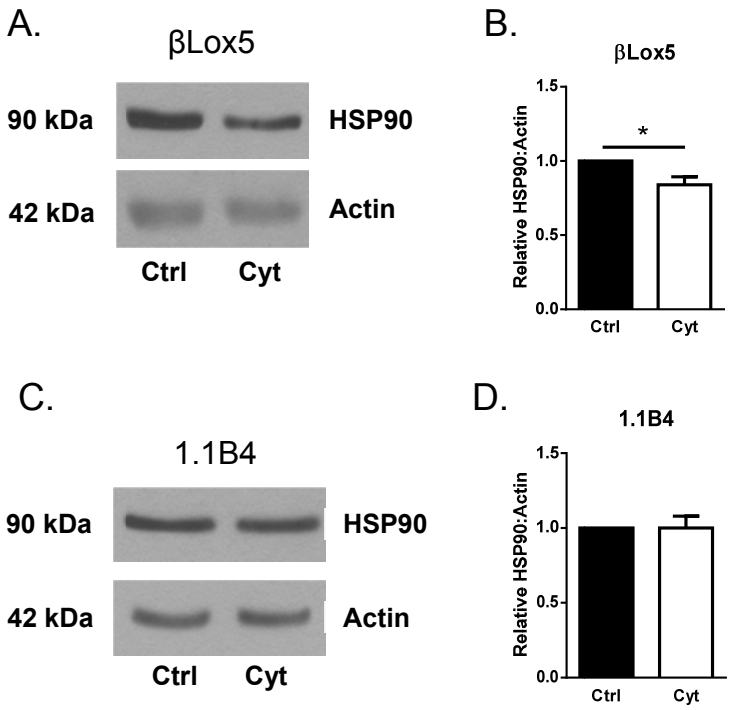


**Figure 12. Cytokine stress does not increase HSP90 protein expression levels in beta cells.** HSP90 protein levels were assessed by immunoblotting in βLox5 (A-B) and 1.1B4 (C-D) cells treated with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ for 24 hours at 37°C. Panels A and C are representative images, while panels B and D are densitometry results from N=3 experiments. For βLox5 densitometry, *$p < 0.05$ Two-tailed, unpaired *t* test.

### *D. Expression Levels of HSP90 Isoform Genes*

The relative abundance of multiple heat shock proteins can shift in response to distinct cell stressors. Thus, qRT-PCR experiments were performed to determine whether expression levels of other HSP90 isoform genes were increased with stress. No significant differences in *HSP90AB1*, which encodes the beta cytosolic isoform of HSP90, were observed in βLox5, 1.1B4, or MIN6 cells following 6 hours (**Figure 13 A-C**) or 24 hours (**Figure 13 D-F**) of incubation with thapsigargin, cytokine cocktail, or high glucose media.

Levels of *HSP90B1*, which encodes the ER isoform of HSP90 GRP94, were significantly increased with thapsigargin treatment at both time points in all three cell lines examined (**Figure 14 A-F**). This result is expected given this gene is upregulated in response to ER stress and UPR activation (70).

Next, levels of *TRAP1*, which encodes the mitochondrial isoform of HSP90, were measured in βLox5, 1.1B4, and MIN6 cells treated with cytokine cocktail for 24 hours. No significant increases in *TRAP1* expression were observed, again suggesting that cytokine stress did not induce upregulation of other HSP90 isoform genes (**Figure 15 A-C**).

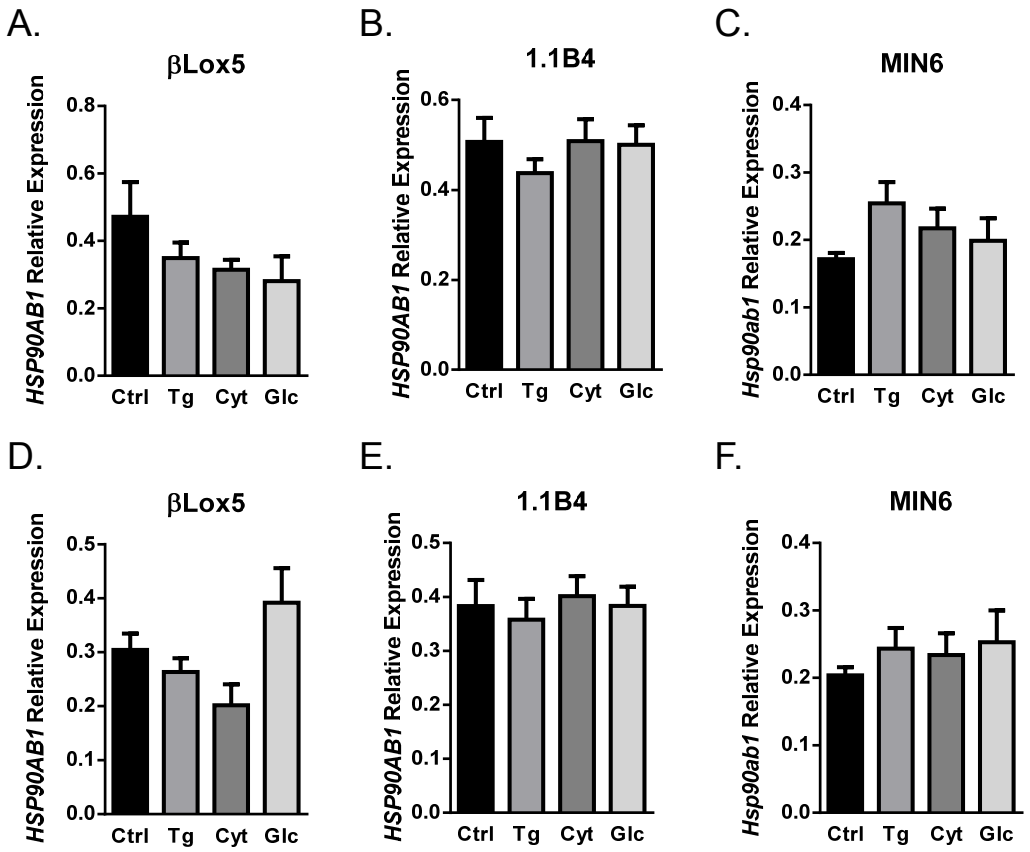


**Figure 13. *HSP90AB1* gene expression levels are unaltered in stressed beta cells.** Beta cells were treated as described in Figure 11. Expression levels of *HSP90AB1*, which encodes the beta cytosolic isoform of HSP90, were measured by qRT-PCR at 6 hours (A-C) and 24 hours (D-F) post-treatment. Data are mean + SEM of N=3 experiments and were analyzed by one-way ANOVA with Dunnett's correction for multiple comparisons, each mean compared to the control mean.

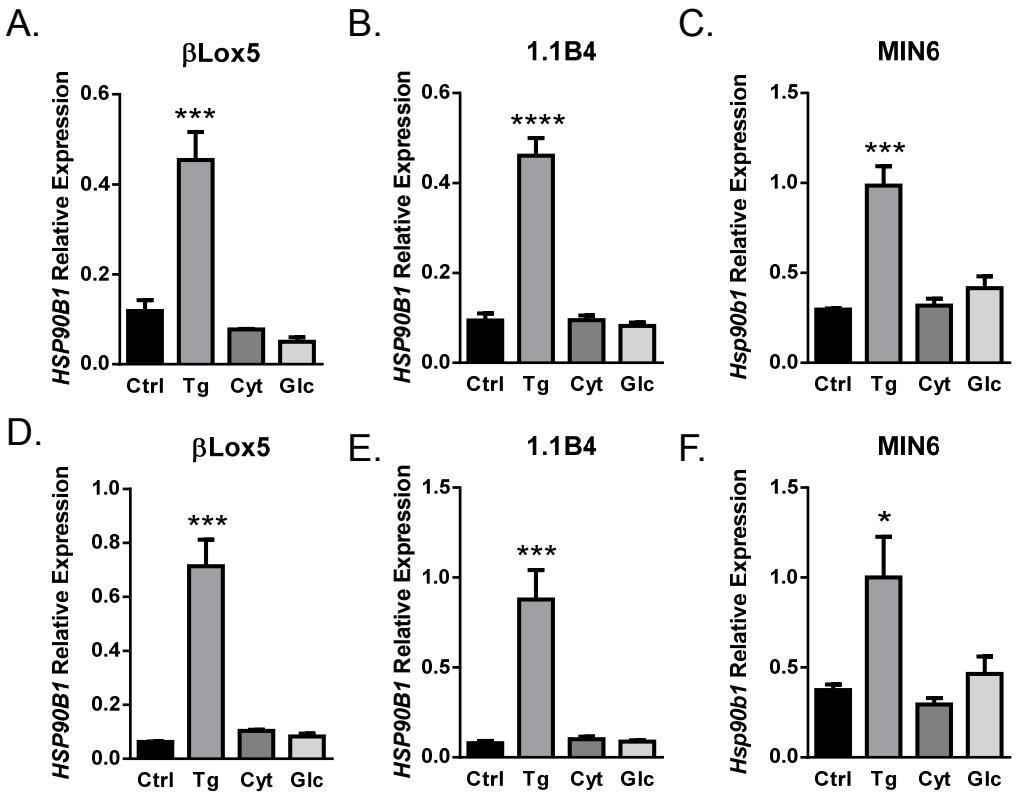


**Figure 14. Thapsigargin treatment increases *HSP90B1* gene expression levels in beta cells.** Beta cells were treated as described in Figure 11. Expression levels of *HSP90B1*, which encodes the ER isoform of HSP90 GRP94, were measured by qRT-PCR at 6 hours (A-C) and 24 hours (D-F) post-treatment. Data are mean + SEM of N=3 experiments. *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ One-way ANOVA with Dunnett's correction for multiple comparisons, each mean compared to the control mean.

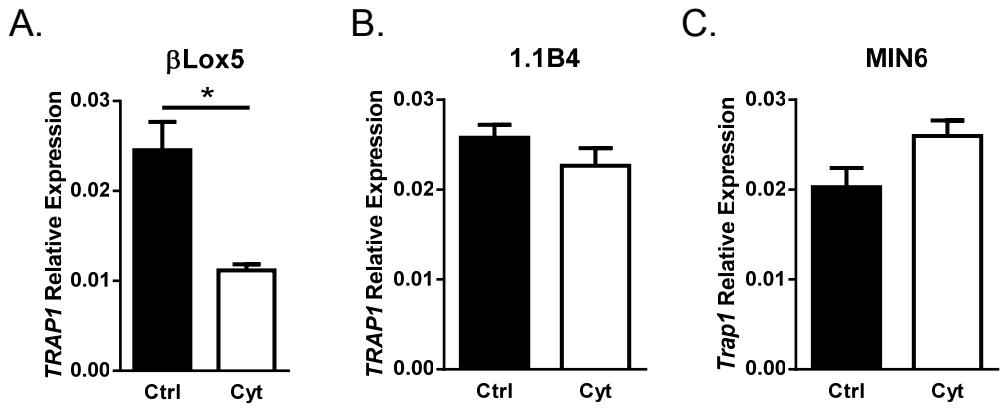


**Figure 15. *TRAP1* gene expression levels in beta cells treated with pro-inflammatory cytokines.** βLox5 (A), 1.1B4 (B), and MIN6 (C) cells were treated with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ for 24 hours at 37°C. Following incubation, cell pellets were harvested for RNA extraction and cDNA synthesis. Expression levels of *TRAP1*, which encodes the mitochondrial isoform of HSP90, were measured by qRT-PCR. Data are mean + SEM of N=3 experiments. *$p < 0.05$ Two-tailed, unpaired *t* test.

Expression levels of *HSP90AB1*, *HSP90B1*, and *TRAP1* were also measured in human islets treated with cytokine cocktail for 24 hours. Patterns of expression similar to those seen in βLox5 cells were observed. No significant differences in *HSP90AB1* expression were detected (**Figure 13 D and Figure 16 A**). *HSP90B1* expression increased about two-fold in both βLox5 cells and primary islets treated with cytokine (**Figure 14 D and Figure 16 B**); however, the result seen in βLox5 cells was not statistically significant when compared to the robust response of the thapsigargin-treated cells. These results suggest this cytokine cocktail of IL-1β, TNF-α, and IFN-γ may be inducing ER stress in beta cells, but not to the extent that thapsigargin does. *TRAP1* levels were reduced by one-half in both βLox5 cells and primary islets (**Figure 15 A and Figure 16 C**). Taken together, the results of these qRT-PCR experiments suggest that of the cell lines tested, βLox5 cells respond to stress most similarly to primary islet cells. The relative increase in HSP90 release observed in βLox5 cells by ELISA was also the most similar to the HSP90 release observed with primary human islets (**Figure 7 A and**

**D**), approximately a two to three-fold increase. For these reasons, βLox5 cells were used as a model of pancreatic beta cell stress for the majority of experiments in this work.

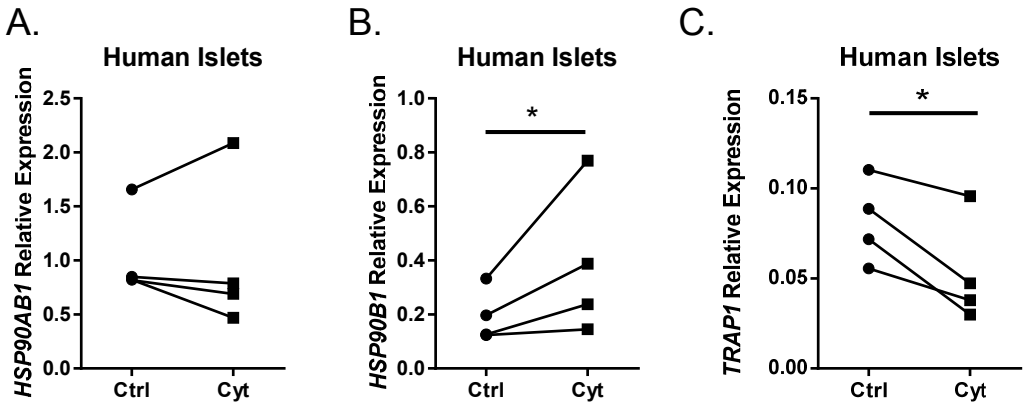


**Figure 16. HSP90 isoform gene expression levels in primary islet cells treated with pro-inflammatory cytokines.** Primary human cadaveric islets were treated with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ for 24 hours at 37°C. Following incubation, cells were harvested for RNA isolation and cDNA synthesis. Expression levels of *HSP90AB1* (A), *HSP90B1* (B), and *TRAP1* (C) were measured by qRT-PCR. Data are actual values for N=4 islet donors. *p < 0.05 Two-tailed, ratio paired *t* test.

### *E. Beta Cell HSP90 Release in Response to TLR Stimulation*

Given HSP90 was released by beta cells in response to pro-inflammatory cytokines but not thapsigargin or high glucose (**Figure 7**) and that this change was not the result of decreased cell membrane integrity (**Figure 8**) or changes in gene or protein expression levels (**Figures 11-12**), we next hypothesized that HSP90 may be released as part of a unique cell-autonomous response to cytokine stress.

Cell-autonomous immunity is defined as the response of host cells, specifically non-immune cells, to invading infectious agents. Components of cell-autonomous immunity include antimicrobial proteins, specialized degradative compartments, and programmed host cell death. These responses are often initiated in response to pathogen sensing through PRRs and danger receptors (71; 72). Because so much crosstalk exists between TLR and pro-inflammatory cytokine signaling pathways (**Figure**

**1**), we wondered if HSP90 was being released in response to some form of cell-autonomous innate immune signaling in beta cells.

In one study involving murine cells, differential cell-autonomous responses in murine pancreatic alpha and beta cells were shown to determine the outcome of coxsackievirus infection. Alpha cells were able to induce a stronger STAT1-mediated antiviral response and thus thwart the deleterious effects of the virus more efficiently than beta cells. These authors postulated that less efficient beta cell-autonomous immune responses may play an important role in beta cell death, the initiation of insulitis, and ultimate progression to TIDM in response to viral infection (73).

Mouse and human pancreatic islets have been reported to express TLR2, TLR3, TLR4, and TLR9, and TLR3 expression has been reported to increase with IL-1β and IFN-γ stimulation. Extracellular dsRNA derived from dying cells has even been shown to bind beta cell TLR3 and trigger apoptosis (26). Thus, beta cell-autonomous immune responses may be initiated through stimulation of these receptors.

Expression levels of *TLR2*, *TLR3*, *TLR4*, and *TLR9* mRNA were measured by qRT-PCR in βLox5 and primary islet cells treated with media alone or IL-1β, TNF-α, and IFN-γ for 24 hours. Both βLox5 and primary islets expressed *TLR2*, *TLR3*, and *TLR4*, while no *TLR9* was detected (**Figure 17 A-B**). Expression of *TLR9* in islet cells reported in other studies may have been due to the presence of infiltrating immune cells. Expression levels of *TLR2*, *TLR3*, and *TLR4* were similar in βLox5 cells and increased slightly with cytokine stimulation. However in primary islets, expression levels of *TLR2* and *TLR3* were similar without cytokine stimulation and increased following treatment; however, *TLR3* expression was most highly expressed with cytokine stimulation in primary islet cells (**Figure 17 B**). Expression levels of *TLR4* in primary islet cells were lower than *TLR2* and *TLR3* (**Figure 17 B**).

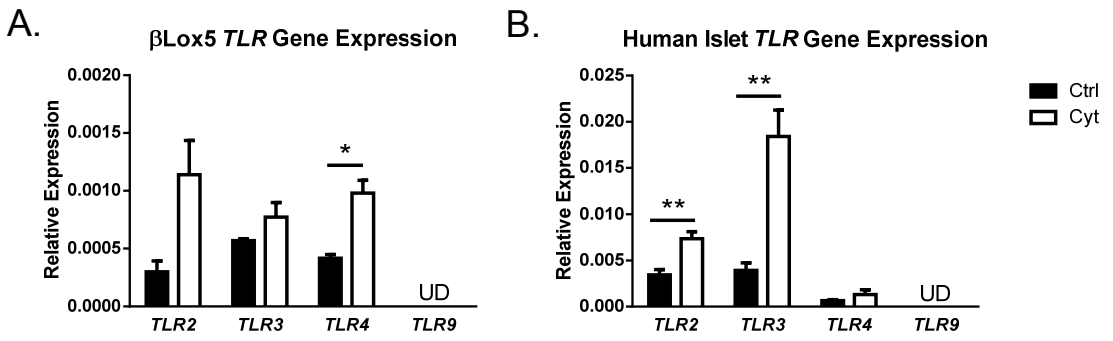


**Figure 17. TLR gene expression levels in βLox5 and primary human islet cells.** βLox5 (A) and primary islet (B) cells were treated with media alone or 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ for 24 hours at 37°C. Following incubation, cells were harvested for RNA isolation and cDNA synthesis. Expression levels of *TLR2*, *TLR3*, *TLR4*, and *TLR9* were measured by qRT-PCR (UD, undetected). Data are mean + SEM of N=3 experiments or N=3-4 islet donors. *p < 0.05, **p < 0.01 Multiple *t* tests with multiple comparisons corrected using the Sidak-Bonferroni method.

In one study, TLR3 stimulation was found to induce diabetes in B6/RIP-B7.1 mice, while stimulation of TLR2, TLR4, and TLR9 did not (74). Thus, to determine the effects of TLR stimulation on pancreatic beta cells, stress responses were measured in response to TLR3 and TLR4 stimulation. Human βLox5 cells were stimulated with PIC to stimulate TLR3 and LPS to stimulate TLR4 for 24 hours. As a positive control for cell activation by each TLR ligand, IL-6 levels were measured by ELISA, and both TLR3 and TLR4 stimulation resulted in similar levels of IL-6 production (**Figure 18 A-B**). Interestingly, however, elevated levels of HSP90 were released in response to TLR3 stimulation with PIC, but not in response to TLR4 stimulation with LPS (**Figure 18 C-D**). PIC treatment was slightly cytotoxic to cells, while LPS was not (**Figure 19 A-B**). Additionally treatment with PIC or LPS did not alter gene expression levels of *HSP90AA1* (**Figure 19 C-D**).

These results suggest TLR3 stimulation in response to a viral infection may be relatively more stressful and cytotoxic to pancreatic beta cells than TLR4 stimulation in

response to a bacterial infection. Thus these results may provide insight as to why viral infections might be associated with beta cell death and the development of TIDM.

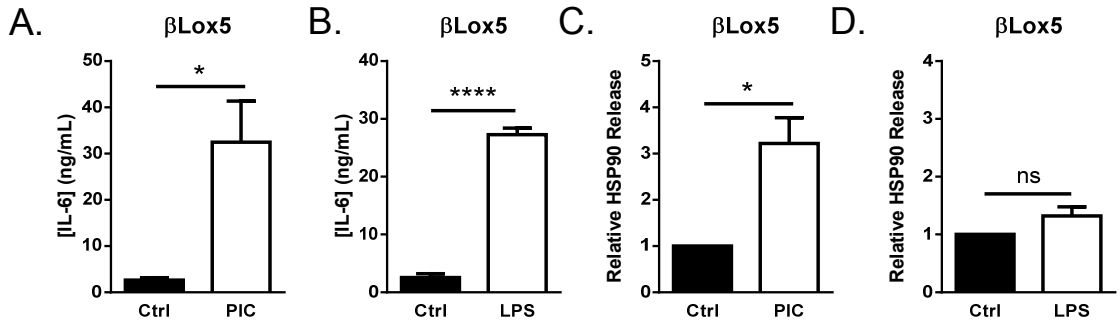


**Figure 18. Beta cells release HSP90 in response to TLR3, but not TLR4 stimulation.** Human βLox5 cells were transfected with 0.1 μg/mL PIC in Lipofectamine 2000 to stimulate TLR3 (A and C) and treated with media containing 1 μg/mL LPS to stimulate TLR4 (B and D) for 24 hours at 37°C. For PIC stimulation, cells were treated with βLox5 media containing Lipofectamine 2000 alone as a control. For LPS stimulation, cells were treated with βLox5 media alone as a control. After incubation, culture media was harvested and IL-6 (A-B) and HSP90 (C-D) levels were assayed by ELISA. For HSP90 ELISAs, data are presented as relative to control. Actual values were detected between 8.9-84.4 ng/mL for PIC experiments and 10.0-52.2 ng/mL for LPS experiments. Data are mean + SEM of N=3 experiments. *$p < 0.05$, ****$p < 0.0001$ Two-tailed, unpaired *t* test (ns, not significant).

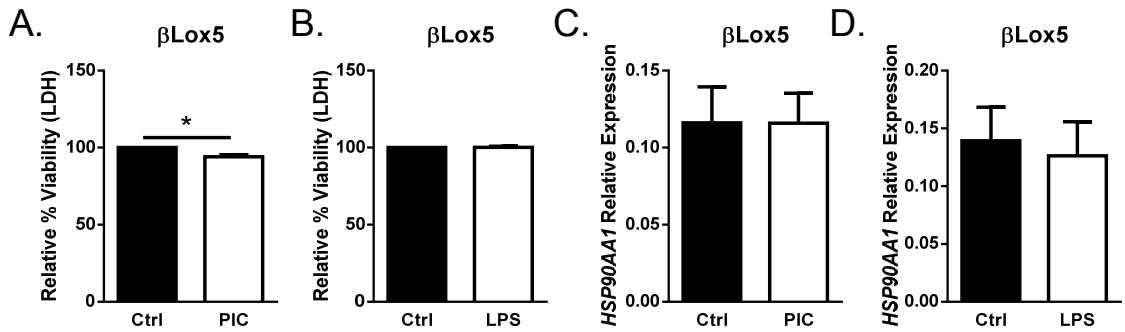


**Figure 19. Beta cell viability and *HSP90AA1* gene expression levels in response to TLR3 and TLR4 stimulation.** Human βLox5 cells were treated with PIC and LPS as described in Figure 18. LDH assays were performed with culture supernatants (A-B). Data are presented as percent viability relative to control samples. Cells were also harvested after treatment for RNA isolation and cDNA synthesis. Expression levels of *HSP90AA1* were measured by qRT-PCR (C-D). Data are mean + SEM of N=3 experiments. *$p < 0.05$ Two-tailed, unpaired *t* test.

Thus far, results of these studies have shown that pancreatic beta cells release HSP90 in response to stimulation with a cocktail of pro-inflammatory cytokines containing IL-1β, TNF-α, and IFN-γ as well as TLR3 stimulation with PIC. However, human beta cells do not release HSP90 in response to stimulation with thapsigargin, high glucose media, or LPS. HSP90 release was not associated with dramatic decreases in cell viability or changes in HSP90 gene or protein expression levels. Thus, these results suggest HSP90 may be actively released in response to a specific innate immune stress in these cells, which may possibly occur in vivo during the pre-clinical latent period of TIDM, thus supporting a role for this protein as a biomarker of latent TIDM. Our next aim for this work was to understand the intracellular signaling events that contribute to cytokine- and PIC-induced HSP90 release from beta cells.

## II. Beta Cell HSP90 Release and Oxidative Stress

### *A. Beta Cell Cytokine Stress and iNOS Activity*

Although pro-inflammatory cytokines have been shown to induce ER stress in pancreatic beta cells (28; 66), here HSP90 was not released by beta cells in response to thapsigargin, a SERCA inhibitor that induces ER-stress (**Figure 7**). Thus, we hypothesized that some other stress response induced in response to cytokines may contribute to HSP90 release in pancreatic beta cells, rather than ER stress.

In rat beta cells, pro-inflammatory cytokine stimulation was shown to result in production of the RNS NO. In this study, NO production downregulated *SERCA2b* expression and resulted in the depletion of ER $Ca^{2+}$ stores, development ER stress, and subsequent cell death (27). NO can be produced by the enzyme iNOS, which is encoded by the gene *NOS2*. Here, 24 hour cytokine stress was shown to induce expression of *NOS2* in βLox5, MIN6, and primary human islets (**Figure 20 A-C**). However, expression of *NOS2* was not induced in response to cytokine stress in 1.1B4 cells (data not shown). This difference could be due to the fact that 1.1B4 is an islet-epithelial fusion cell line (60).

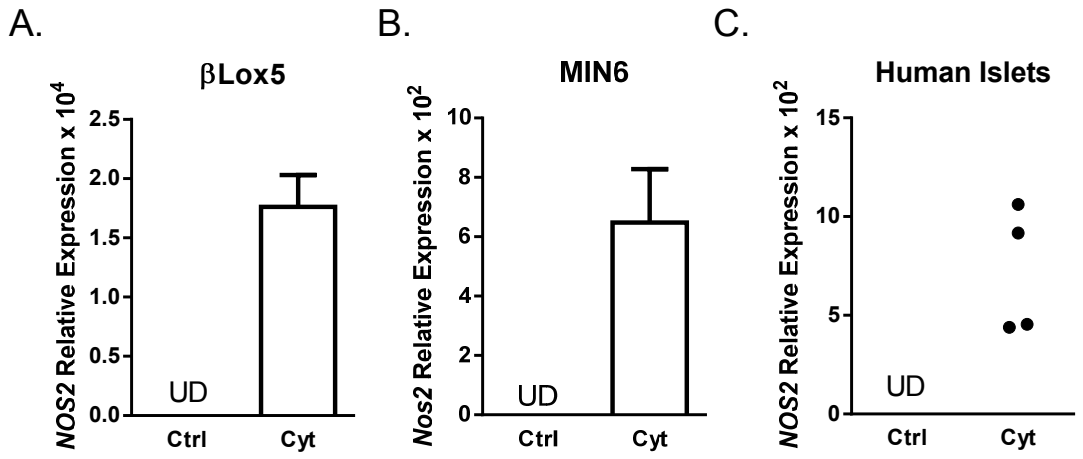


**Figure 20**. **Expression levels of *NOS2* in pancreatic beta cells treated with pro-inflammatory cytokines.** βLox5 (A), MIN6 (B), and primary human islet cells (C) were treated for 24 hours at 37°C with media alone (Ctrl) or a cytokine cocktail containing 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt). Following incubation cells were harvested for RNA isolation and cDNA synthesis, and *NOS2* expression was measured by qRT-PCR (UD, undetected). Data are mean + SEM of N=3 experiments (A-B) or actual values of N=4 islet donors (C).

Given that HSP90 has been shown to be released from vascular smooth muscle cells in response to oxidative stress (53) and that *NOS*2 was shown to be induced by pro-inflammatory cytokines in pancreatic beta cells in our studies, we hypothesized that HSP90 may be released in response to NO production in these cells. To determine whether or not NO production was required for HSP90 release in response to cytokine stress, βLox5 cells were pre-treated for 6 hours with 1400W, an iNOS inhibitor, prior to 24 hour stress treatment with IL-1β, TNF-α, and IFN-γ. HSP90 levels were then measured by ELISA, while viability was measured by LDH assay. Pre-treatment with 1400W did not affect HSP90 release in response to cytokine stress in βLox5 cells (**Figure 21 A**). Additionally, no difference in viability was observed by LDH assay in βLox5 cells treated with cytokine and 1400W compared to cells treated with cytokine alone (**Figure 21 B**). Together these results suggest that production of NO is not

required for HSP90 release in these cells. Indeed the expression levels of *NOS2* in βLox5 cells and primary islets in response to cytokine stress shown in Figure 20 were relatively low (approximate $C_T$ = 32 in both cell types).

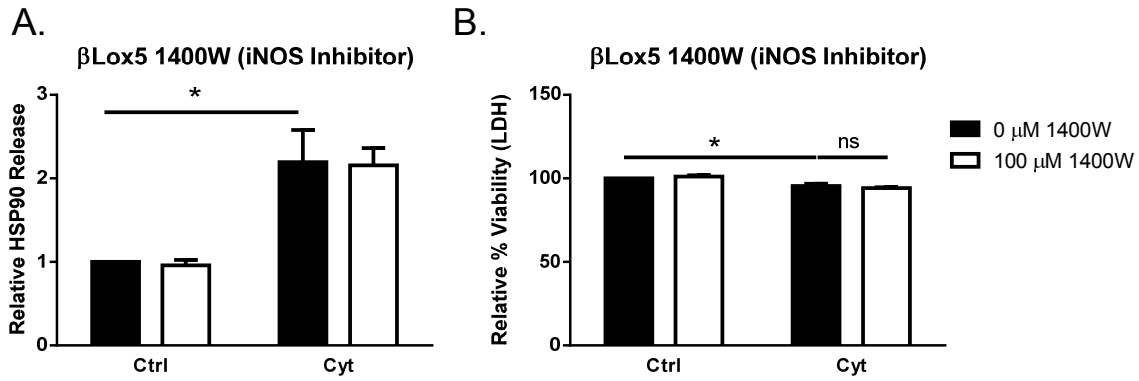


**Figure 21**. **Pharmacological inhibition of iNOS does not affect beta cell HSP90 release or viability in response to cytokine stress.** βLox5 cells were treated for 6 hours at 37°C with media alone or 100 μM 1400W followed by 24 hour stimulation with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ. Following incubation, media was harvested, and HSP90 levels were detected by ELISA (A). Data are presented as relative to control cells without 1400W. Actual values of HSP90 were detected between 10.0-34.6 ng/mL. Cell viability in response to treatment was determined by LDH assay (B). Data are presented as percent viability relative to control cells without 1400W. Data are mean + SEM of N=3 experiments. *$p < 0.05$ Two-way ANOVA with Tukey's correction for multiple comparisons (ns, not significant).

### *B. Beta Cell Cytokine Stress and HIF-1α Activity*

Although NO production was found to be dispensable for HSP90 release in response to cytokine stress, some other source of oxidative stress could be contributing to HSP90 release in human beta cells. Interestingly, some studies have shown that activity of the transcription factor HIF-1α can be regulated by oxidative stress (75).

Usually under normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylase (PHD) enzymes. This hydroxylation targets HIF-1α for polyubiquitination and degradation by the proteasome. However under hypoxic conditions, HIF-1α is not

hydroxylated and is able to translocate to the nucleus to induce expression of HIF target genes with its transcriptional partner HIF-1β (76) (**Figure 22**). Furthermore, some studies have shown that ROS can inhibit PHD enzymes, thus resulting in stabilization of HIF-1α and induction of HIF-1α transcriptional activity, even under normoxic conditions (75) (**Figure 22**).

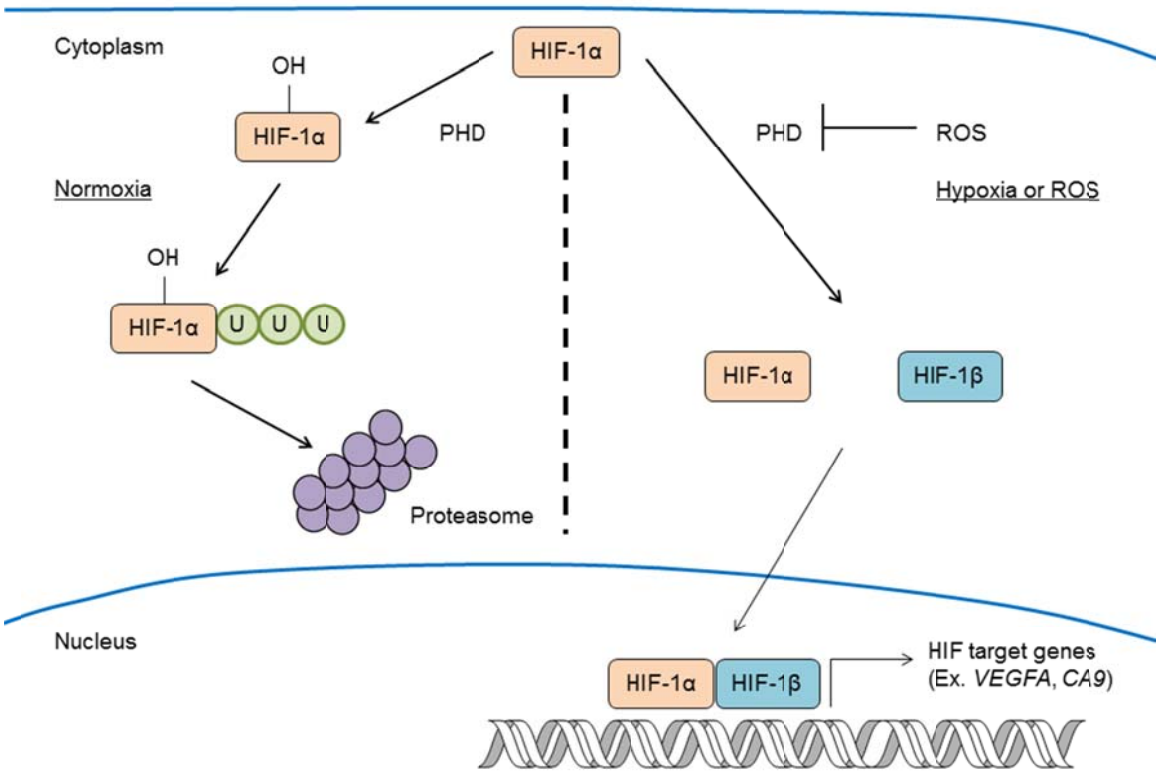


**Figure 22. HIF-1α regulation by normoxia, hypoxia, and ROS.** During normoxia (left), the transcription factor HIF-1α, located in the cytoplasm, is hydroxylated by PHD enzymes. This hydroxylation marks HIF-1α for polyubiquitination and degradation by the proteasome. During hypoxia (right), HIF-1α is not hydroxylated and is thus free to translocate to the nucleus with HIF-1β to induce expression of HIF target genes (76). Some studies have shown that PHD enzymes can be inhibited by ROS, thus resulting in HIF-1α stabilization and transcriptional activity even under normoxic conditions (75).

We hypothesized that increased oxidative stress and HIF-1α activity may promote HSP90 release from beta cells. This hypothesis was based on a recent study using human fibroblast cells which demonstrated that hypoxia can induce secretion of HSP90 through the activation HIF-1α and that secreted HSP90 is important for promoting fibroblast motility and wound healing (55). However, how a transcription factor such as HIF-1α could promote secretion of HSP90 is currently unclear. In the present study, *HIF1A* expression was upregulated in response to 24 hour treatment with IL-1β, TNF-α, and IFN-γ in βLox5 and 1.1B4 cells (**Figure 23 A-B**) as well as primary islet cells from three of four donors (**Figure 23 C**). This change was not observed in MIN6 cells (**Figure 23 D**), but this discrepancy may be due to inter-species differences.

These results are not surprising given *HIF1A* is a target of NF-κB and thus would be expected to increase in response to in vitro stimulation with pro-inflammatory cytokines. Sites of tissue inflammation, induced in response to infection or autoimmunity, are generally hypoxic and contain a high concentration of free oxygen radicals. Thus, the HIF-1α transcriptional program is often induced at these sites in vivo. For example, evidence of HIF-1α activation has been observed in affected tissues from patients with inflammatory disorders such as rheumatoid arthritis. Furthermore, HIF-1α has been shown to promote the production of pro-inflammatory cytokines by dendritic cells (77).

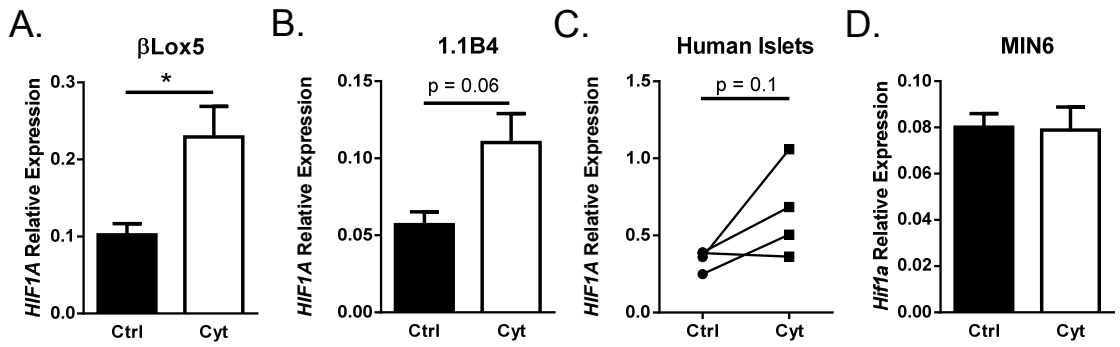


**Figure 23. *HIF1A* gene expression levels in beta cells treated with pro-inflammatory cytokines.** βLox5 (A), 1.1B4 (B), primary human islet (C), and MIN6 (D) cells were treated with media alone (Ctrl) or with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt) for 24 hours at 37°C. Following incubation, cells were harvested for RNA isolation and cDNA synthesis. Expression levels of *HIF1A* were measured by qRT-PCR. Data are mean + SEM of N=3 experiments or actual values from N=4 islet donors. For cell line data in panels A, B, and D, *p < 0.05 Two-tailed, unpaired *t* test. Human islet data in panel C were analyzed by two-tailed, ratio paired *t* test.

To determine whether HIF-1α transcriptional activity was induced in pancreatic beta cells in response to cytokine stress, expression levels of *VEGFA* and *CA9*, two HIF-1α target genes, were examined. *VEGFA* and *CA9* expression levels were significantly increased in βLox5 cells in response to cytokine stress (**Figure 24 A-B**). However, *VEGFA* was not increased in 1.1B4 cells (**Figure 24 C**), while *CA9* was not detected in this cell line (data not shown). *VEGFA* expression was increased in three of 4 islet donors (**Figure 24 D**); however, *CA9* was not detected in these cells (data not shown). These results suggest HIF-1α may be more transcriptionally active in βLox5 and human islet cells than in 1.1B4 cells.

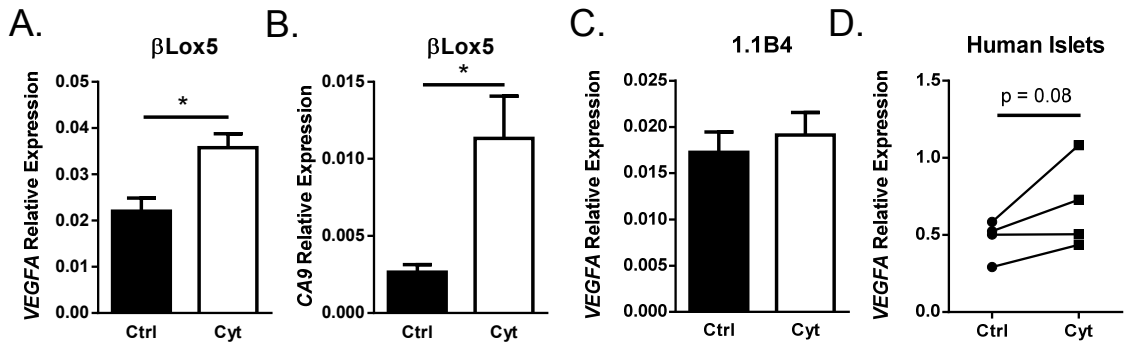


**Figure 24. Beta cell HIF-1α target gene expression in response treatment with pro-inflammatory cytokines.** βLox5 (A-B), 1.1B4 (C), and primary human islet (D) cells were treated with media alone (Ctrl) or 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt) for 24 hours at 37°C. Following incubation, cells were harvested for RNA isolation and cDNA synthesis. Expression levels of *VEGFA* and *CA9* were measured by qRT-PCR. Data are mean + SEM of N=3-4 experiments or individual values from N=4 islet donors. For cell line data in panels A-C, *p < 0.05 Two-tailed, unpaired *t* test. Human islet data in panel D were analyzed by two-tailed, ratio paired *t* test.

To determine whether or not HIF-1α activity is required for HSP90 release in response to cytokine stress in pancreatic beta cells, βLox5 cells were pre-treated with chetomin, a HIF-1α inhibitor, for 6 hours prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. Following incubation, cell pellets were harvested for RNA isolation and cDNA synthesis, and culture media was harvested for LDH assay and HSP90 ELISA. To determine whether or not chetomin treatment was effective, expression levels of the HIF-1α target *CA9* were measured by qRT-PCR. Pre-treatment with chetomin decreased expression levels of *CA9* in cytokine-treated cells back to control levels, suggesting HIF-1α activity was inhibited with this drug (**Figure 25 A**). However, contrary to our hypothesis, HIF-1α inhibition further increased HSP90 release relative to control cells (**Figure 25 B**).

LDH assays revealed that the difference in viability between cytokine-treated cells with and without chetomin was not statistically significant (**Figure 25 C**). We next examined expression levels of stress-associated genes in order to determine whether

chetomin treatment resulted in increased beta cell stress. These studies revealed that pre-treatment with chetomin increased expression levels of *HSP90AA1* and *NOS2* in cytokine-treated cells (**Figure 26 A-B**), suggesting that pre-treatment with chetomin may be placing additional stress on beta cells. Taken together, these results suggest HIF-1α activity is not required for HSP90 release in pancreatic beta cells in response to cytokine stress, and perhaps, HIF-1α activity may be important for mitigating stress responses in cytokine-treated beta cells.

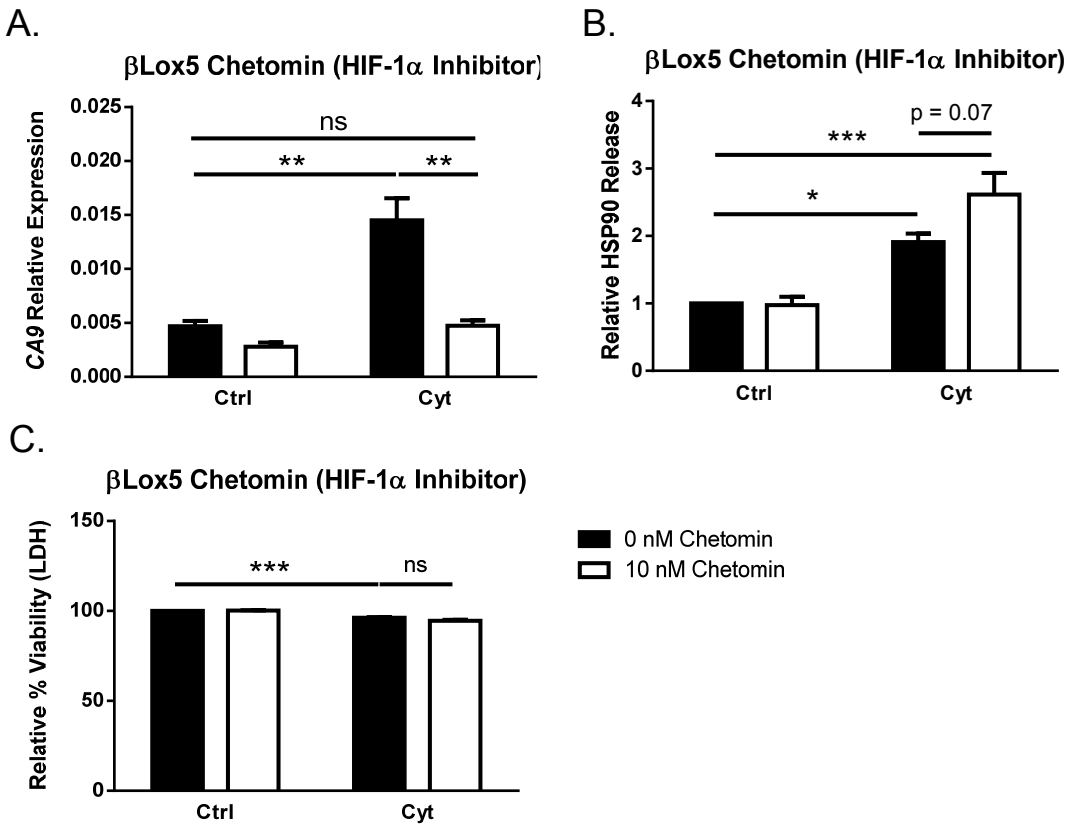


**Figure 25. HIF-1α activity is not required for HSP90 release in pancreatic beta cells in response to treatment with pro-inflammatory cytokines.** βLox5 cells were treated with media alone or 10 nM chetomin for 6 hours at 37°C followed by 24 hour stimulation with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ. Following incubation, cell pellets were harvested for RNA isolation and cDNA synthesis, while media was harvested for LDH assay and HSP90 ELISA. Expression levels of *CA9* were measured by qRT-PCR (A). HSP90 levels were measured by ELISA (B). Data are presented as relative to control cells without chetomin. Actual values were detected between 1.4-46.5 ng/mL. Cell viability was measured by LDH assay (C). Data are presented as percent viability relative to control cells without chetomin. Data are mean + SEM of N=3-4 experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Two-way ANOVA with Tukey's correction for multiple comparisons (ns, not significant).

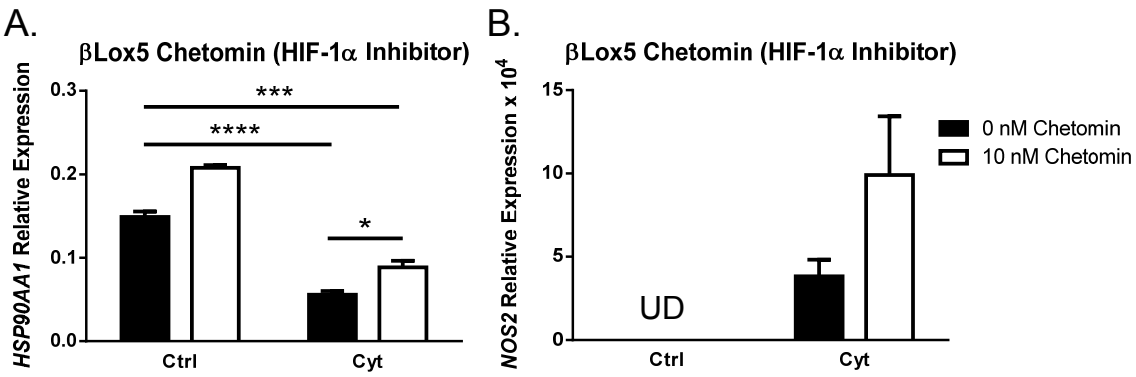


**Figure 26. Effects of HIF-1α pharmacological inhibition and pro-inflammatory cytokine treatment on *HSP90AA1* and *NOS2* gene expression levels in pancreatic beta cells.** Human βLox5 cells were treated as in Figure 25. Following incubation, cell pellets were harvested for RNA isolation and cDNA synthesis. Expression levels of *HSP90AA1* and *NOS2* were measured by qRT-PCR (A-B) (UD, undetected). Data are mean + SEM of N=3 experiments. *p < 0.05, ***p < 0.001, ***p < 0.0001 Two-way ANOVA with Tukey's correction for multiple comparisons.

To confirm that HIF-1α activity was not required for HSP90 release from pancreatic beta cells in response to cytokine stress, βLox5 cells were pre-treated with DMOG for 6 hours prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. DMOG is a prolyl hydroxylase inhibitor and thus functions as a pharmacological stabilizer of HIF-1α. Following incubation, cell pellets were harvested for RNA isolation and cDNA synthesis, while media was harvested for HSP90 ELISA. LDH assays were also performed. To determine whether or not DMOG treatment was effective, expression levels of *CA9* were monitored by qRT-PCR. Expression levels of *CA9* were increased in both control and cytokine-treated cells that had been pre-treated with DMOG, suggesting this drug was effective at the concentration used (**Figure 27 A**).

However, pre-treatment with DMOG did not significantly affect HSP90 release in control or cytokine-treated cells. HSP90 release in response to DMOG plus cytokine stress was slightly increased compared to the release observed in cells treated with cytokine alone, relative to control cells without DMOG (**Figure 27 B**). However, similar changes were observed by LDH assay, suggesting the slight increase observed in HSP90 release in cytokine-treated cells with DMOG may relate to cytotoxicity (**Figure 27 C**). Overall, these results are consistent with those presented in Figure 25. Especially given that DMOG had no effect on HSP90 release in the control-treated cells, the results presented here suggest that increased HIF-1α activity does not result in increased HSP90 release from pancreatic beta cells.

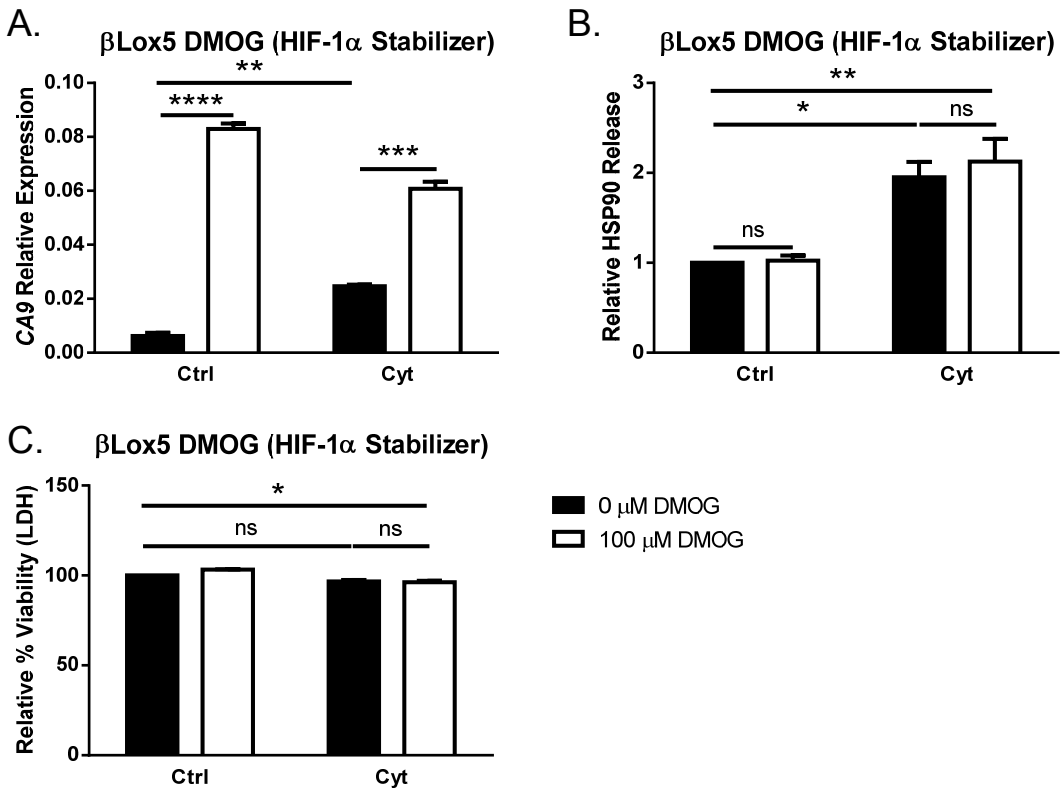


**Figure 27. HIF-1α stabilization does not promote HSP90 release from beta cells.** βLox5 cells were pre-treated with media alone or 100 μM DMOG for 6 hours at 37°C prior to 24 stimulation with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ. Following incubation, cell pellets were harvested for RNA isolation and cDNA synthesis. Expression levels of *CA9* were measured by qRT-PCR (A). Media was also harvested, and HSP90 levels were measured by ELISA (B). Data are presented as relative to control cells without DMOG. Actual values of HSP90 were detected between 3.0-23.9 ng/mL. Cell viability was also assessed by LDH assay (C). Here data are presented as percent viability relative to control cells without DMOG. Data are mean + SEM from N=2-3 experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 Two-way ANOVA with Tukey's correction for multiple comparisons (ns, not significant).

### *C. Beta Cell HSP90 Release in Response to Cytokine Stress and NAC*

To rule out a more general role for oxidative stress in promoting HSP90 release in pancreatic beta cells in response to cytokine stress, beta cells were treated with the antioxidant NAC prior to cytokine stress. Human βLox5 cells were treated for 6 hours with media alone, 0.2 mM, or 1 mM NAC for 6 hours at 37°C prior to 24 hour stimulation

with IL-1β, TNF-α, and IFN-γ. Following incubation, media was harvested for HSP90 ELISA and LDH assay. Interestingly, while 0.2 mM NAC had no effect on HSP90 release, pre-treatment with 1 mM NAC resulted in increased HSP90 release in both control and cytokine-treated cells (**Figure 28 A**). Meanwhile no changes in viability were observed by LDH assay (**Figure 28 B**). These results suggest that scavenging cellular ROS does not appear to inhibit HSP90 release. Yet unexpectedly, the use of a strong reductant to promote a redox shift within the environment of the cell did promote HSP90 release.

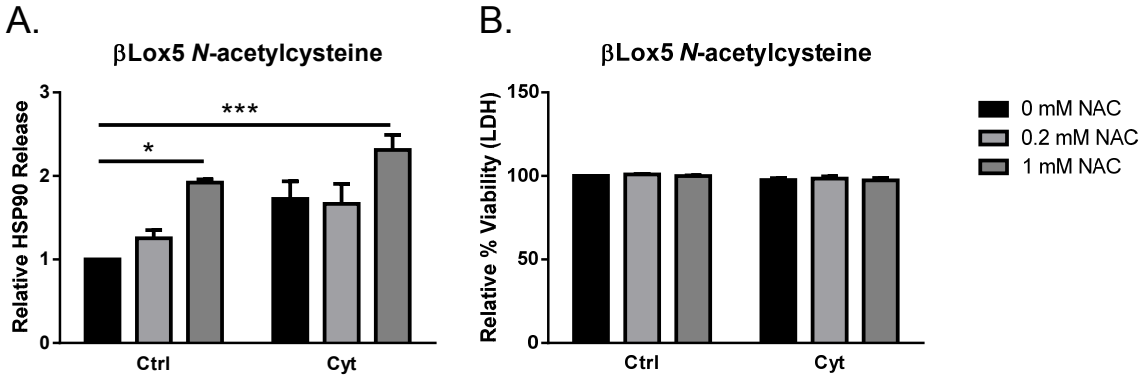


**Figure 28. Effects of NAC on HSP90 release and viability in beta cells treated with pro-inflammatory cytokines.** βLox5 cells were pre-treated with media alone, 0.2 mM, or 1 mM NAC for 6 hours at 37°C prior to 24 stimulation with 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ. Following incubation, media was harvested, and HSP90 levels were measured by ELISA (A). Data are presented as relative to control cells without NAC. Actual values of HSP90 were detected between 15.5-44.4 ng/mL. Cell viability was also assessed by LDH assay (B). Here data are presented as percent viability relative to control cells without NAC. Data are mean + SEM from N=3 experiments. *$p < 0.05$, ***$p < 0.001$, Two-way ANOVA with Tukey's correction for multiple comparisons.

Although expression levels of *NOS2* were increased in cytokine-treated beta cells, results of these studies suggest iNOS activity is not required for HSP90 release from pancreatic beta cells, as inhibition of iNOS with 1400W had no effect on HSP90 release in beta cells treated with IL-1β, TNF-α, and IFN-γ. Additionally, these results do not support a role for oxidative stress-induced HIF-1α activity in HSP90 release from beta cells in response to treatment with pro-inflammatory cytokines. Although *HIF1A* and HIF-1α-dependent gene expression levels were increased in beta cells treated with IL-1β, TNF-α, and IFN-γ, chetomin, a HIF-1α inhibitor, did not prevent HSP90 release from beta cells treated with these cytokines. Similarly, stabilization of HIF-1α with DMOG did not promote HSP90 release from beta cells. Experiments with the potent reductant NAC, however, suggest that the overall redox environment in the cell may impact the propensity of beta cells to release HSP90. The reducing micro-environment within the ER is important for protein folding and induction of the UPR. In light of this information, we decided to revisit the possibility that HSP90 release may be related to cytokine-induced ER stress.

**III. Beta Cell HSP90 Release in Response to JNK Activation**

***A. Expression Levels of UPR Markers in Stressed Beta Cells***

To determine whether or not ER stress plays a role in HSP90 release by beta cells, expression levels of five ER stress-associated genes, *XBP1s*, *ATF4*, *ATF3*, *DDIT3*, and *HSPA5,* were examined in cells treated with cytokine cocktail or PIC, the two stimuli shown to induce pancreatic beta cell HSP90 release in these studies. As a positive control, expression levels of ER stress-associated genes were examined in cells treated with thapsigargin. Additionally, expression levels of ER stress-associated genes were examined in LPS-treated beta cells.

As part of the UPR, *XBP1s* is activated downstream of IRE1 signaling, while *ATF4* is activated downstream of PERK signaling. *ATF3* expression is induced by ATF4

transcriptional activity (24). *DDIT*3 and *HSPA5*, which encode CHOP and BiP, respectively, are induced downstream of ATF6 signaling, although *DDIT3* can also be induced by ATF4 (**Figure 4**) (23; 24).

In βLox5 cells, 24 hour stimulation with IL-1β, TNF-α, and IFN-γ resulted in a modest increase in the expression levels of *XBP1s* and *ATF3* relative to control; however, these results were not significant when compared to the more robust responses of thapsigargin-treated cells (**Figure 29 A, C**). A similar result was observed previously with *HSP90B1* expression (**Figure 14 D**). No differences were observed, however, in expression levels of *ATF4*, *DDIT3*, and *HSPA5* (**Figure 29 B, D-E**). Together, these results suggest that cytokine stress may induce a mild or non-canonical ER stress in pancreatic beta cells after 24 hours of stimulation. Others have shown, however, that these transcripts can be induced to a greater extent in human pancreatic beta cells after 48 hours of cytokine stress (28). Thus, full induction of an ER stress response may require longer incubation times with pro-inflammatory cytokines. The current analysis focused on the first 24 hours after cytokine exposure, as cell death was minimal compared to 48 hours (**Figure 8**).

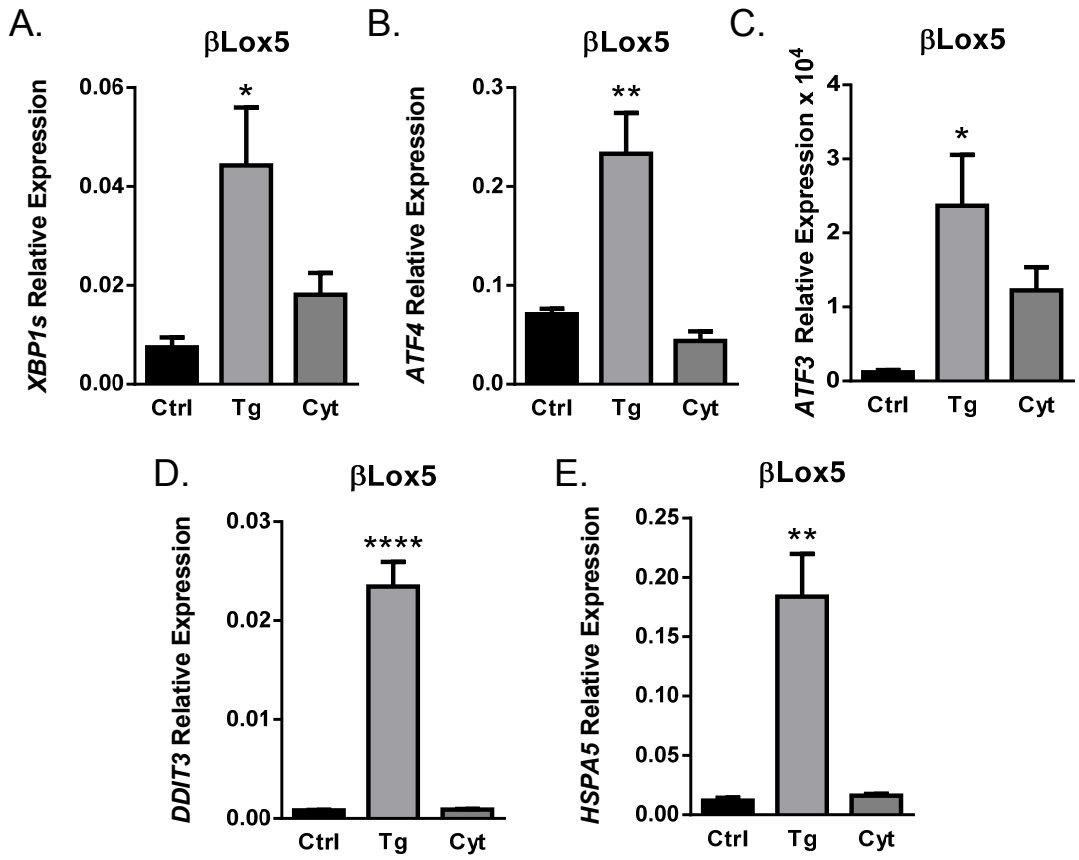


**Figure 29. UPR gene expression in beta cells treated with thapsigargin and pro-inflammatory cytokines.** Human βLox5 cells were treated with media alone (Ctrl), 0.1 μM thapsigargin (Tg), or 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt) for 24 hours at 37°C. Following incubation cells were harvested for RNA isolation and cDNA synthesis. Expression levels of the following ER stress markers were measured by qRT-PCR: *XBP1s*, *ATF4*, *ATF3*, *DDIT3* (CHOP), and *HSPA5* (BiP). Data are mean + SEM of N=3 experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ One-way ANOVA with Dunnett's correction for multiple comparisons, each mean compared to control.

In human islets, 24 hour stimulation with IL-1β, TNF-α, and IFN-γ did not result in substantial changes in *XBP1s* or *ATF4* expression in three of four donors. In one donor, expression of these genes increased (**Figure 30 A-B**). All four donors, however, showed increased expression of *ATF3* with cytokine stimulation (**Figure 30 C**). Modest increases in *DDIT3* and *HSPA5* expression were observed in two of four donors for *DDIT3* (**Figure 30 D**) and three of four donors for *HSPA5* (**Figure 30 E**). More substantial increases in expression levels of these genes were observed in the fourth donor (**Figure 30 D-E**). These results suggest that ER stress may be induced to different degrees in human islets in response to cytokine stress, depending on the donor. Again, full induction of ER stress may require more than 24 hours of stimulation with cytokines.

One interesting observation is that *ATF3* expression was induced in response to cytokine stress in βLox5 and primary islet cells, but not *ATF4*. This result may be explained by the fact that PERK signaling directly results in ATF4 translation (**Figure 4**), rather than *ATF4* transcription. Thus, *ATF3* transcription may be induced before *ATF4* transcription. Interestingly, ATF3 has been shown to negatively regulate transcription of *IL6* and other NF-κB target genes (25). Furthermore, transcription of *ATF3* can be upregulated by increased levels of ROS, as the ROS-sensitive transcription factor NRF2 can also induce *ATF3* expression (78). Thus, *ATF3* expression may be induced by ER stress as well as other cellular stress events that elevate ROS as a mechanism to negatively regulate inflammation.

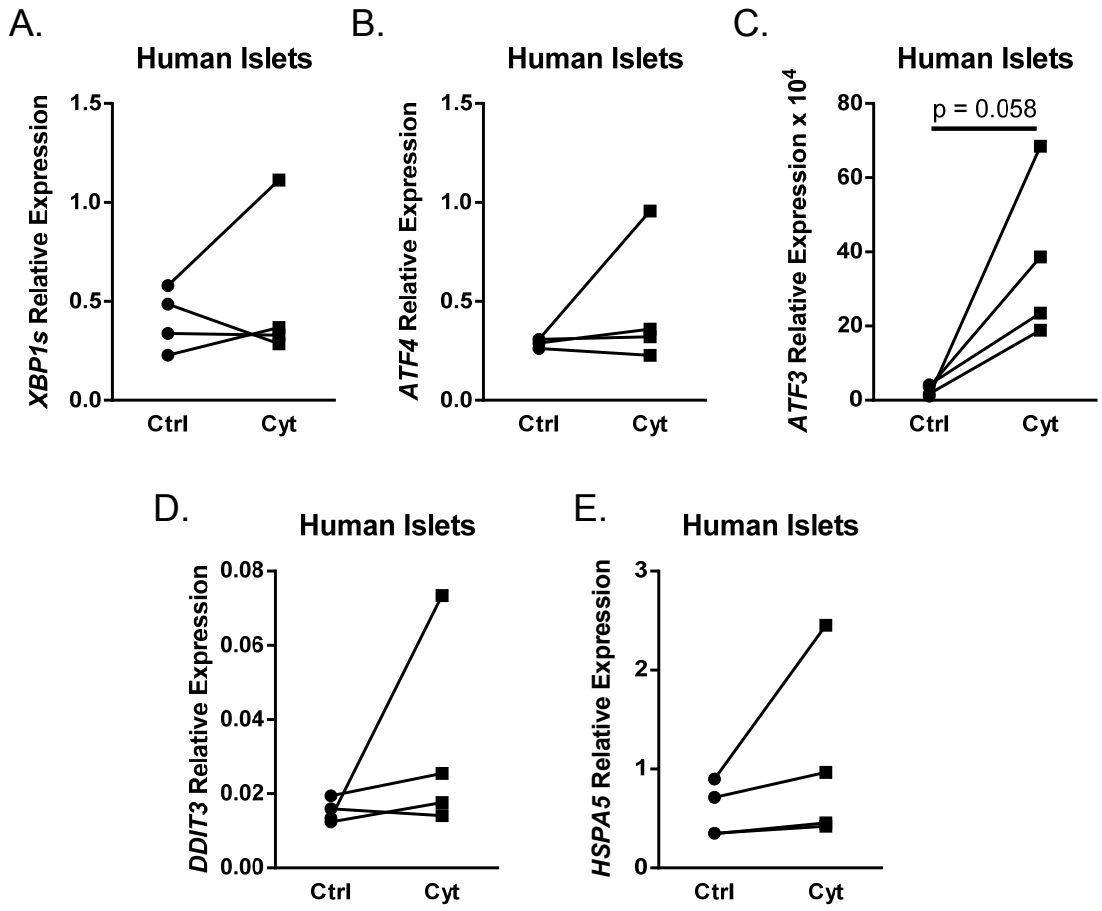


**Figure 30. UPR gene expression in primary human cadaveric islets treated with pro-inflammatory cytokines.** Primary human cadaveric islets were treated with media alone (Ctrl) or 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt) for 24 hours at 37°C. Following incubation cell pellets were harvested for RNA isolation and cDNA synthesis. Expression levels of *XBP1s* (A), *ATF4* (B), *ATF3* (C), *DDIT3* (D), and *HSPA5* (E) were measured by qRT-PCR. Data are actual values from N=4 islet donors. Data were compared by two-tailed, ratio paired *t* test.

Stimulation of beta cells with PIC resulted in increased expression of all five ER stress-associated genes (**Figure 31 A-E**). These results are consistent with previous studies in primary rat beta cells showing PIC transfection results in the induction of ER stress markers in these cells (79). However, the increases were less robust than those observed with thapsigargin (**Figure 29**). LPS stimulation had no marked effect on ER stress marker expression in βLox5 cells, although modest increases in *XBP1s* and *ATF3* expression were observed (**Figure 31 F-J**).

Taken together, these results suggest 24 hour stimulation with IL-1β, TNF-α, and IFN-γ may induce a mild or non-canonical form of ER stress in pancreatic beta cells. PIC, however, induces a response more reminiscent of traditional ER stress.

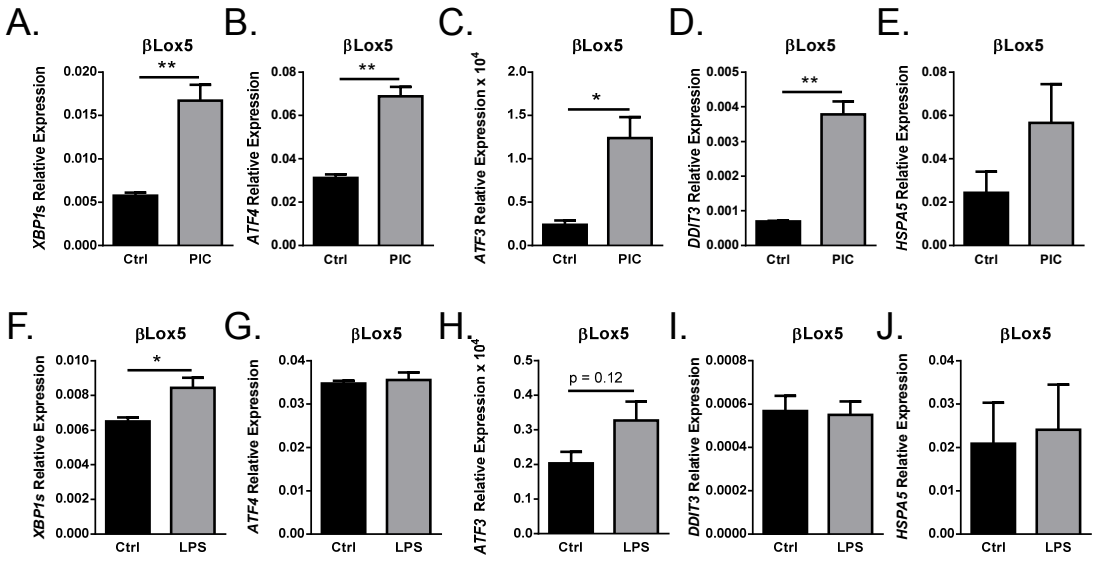


**Figure 31. UPR gene expression in pancreatic beta cells treated with PIC and LPS.** Human βLox5 cells were treated with PIC (A-E) or LPS (F-J) for 24 hours as described in Figure 18. Following incubation cells were harvested for RNA isolation and cDNA synthesis. Expression levels of *XBP1s*, *ATF4*, *ATF3*, *DDIT3*, and *HSPA5* were measured by qRT-PCR. Data are mean + SEM of N=3 experiments. *p < 0.05, **p < 0.01 Two-tailed, unpaired *t* test.

### *B. Effects of TUDCA on Beta Cell Stress*

Since some increases in ER stress marker expression were observed in pancreatic beta cells in response to cytokine stress, we hypothesized that ER stress may be contributing to HSP90 release in these cells. To test this hypothesis, βLox5 cells were pre-treated for 6 hours with TUDCA prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ or PIC. TUDCA is a bile acid that functions as a chemical chaperone and helps restore homeostasis during ER stress. Although humans naturally express very low levels of this bile acid, hibernating animals such as black bears express very high levels of TUDCA, and black bear bile has been used in traditional Chinese medicine for over three millennia (80). Studies have shown that this compound has potent anti-apoptotic effects, and it has been used successfully in the clinic to treat a variety of liver diseases (80; 81). In one study, administration of TUDCA during the pre-diabetic stage of TIDM dramatically reduced diabetes incidence in two mouse models of disease (82).

Here, pre-treatment with TUDCA substantially inhibited HSP90 release in βLox5 cells in response to cytokine stress (**Figure 32 A**) and partially blocked HSP90 release in response to PIC stimulation (**Figure 32 B**). TUDCA did not improve cell viability in response to cytokine stress as measured by LDH assay (**Figure 32 C**); however, TUDCA pre-treatment slightly preserved viability in response to PIC stimulation (**Figure 32 D**). Thus, the protective effects of TUDCA may differ mechanistically in cytokine and PIC-treated cells.

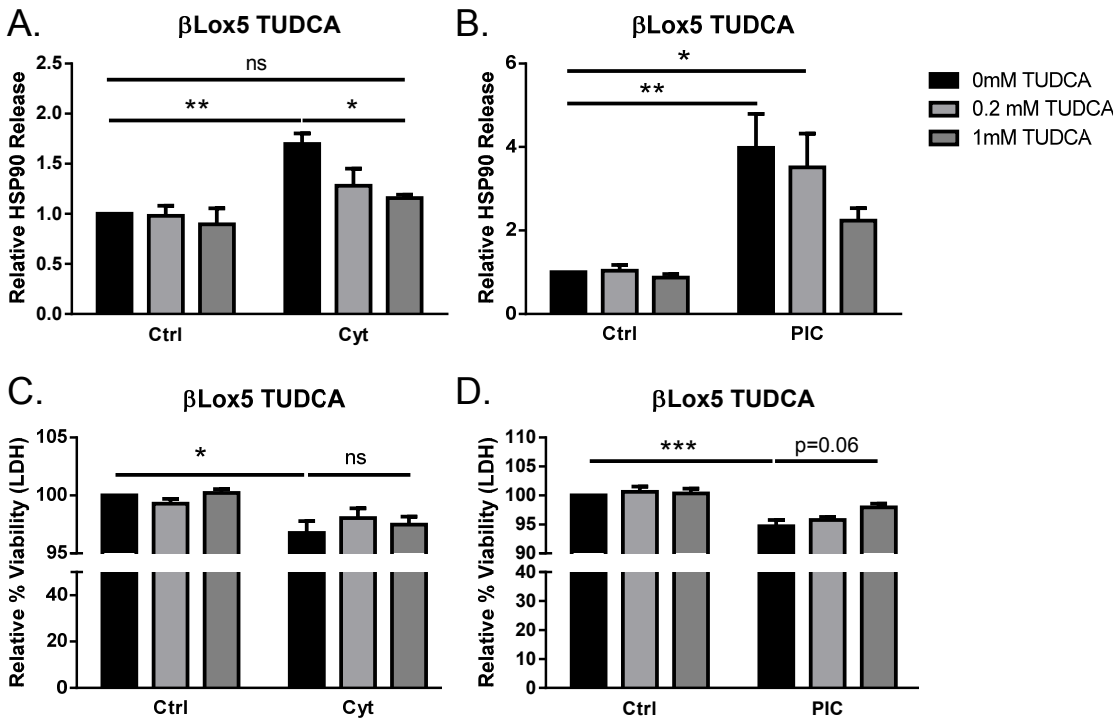


**Figure 32. TUDCA inhibits HSP90 release in pancreatic beta cells treated with pro-inflammatory cytokines or PIC.** Human βLox5 cells were treated with media alone, 0.2 mM, or 1 mM TUDCA for 6 hours at 37°C prior to 24 hour stimulation with cytokine cocktail or PIC as described in Figures 7 and 18, respectively. Following incubation, media was harvested and HSP90 ELISAs (A-B) and LDH assays (C-D) were performed. Data are mean + SEM of N=3-4 experiments. HSP90 ELISA data are presented as relative to control cells without TUDCA. Actual values were detected between 5.0-20.5 ng/mL for panel A and 4.0-51.7 ng/mL for panel B. LDH data are presented as percent viability relative to control cells without TUDCA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ Two-way ANOVA with Tukey's correction for multiple comparisons (ns, not significant).

In support of the conclusion that the protective effects of TUDCA may differ mechanistically in cytokine and PIC-treated cells, TUDCA did not inhibit IL-6 production in βLox5 cells treated with cytokine cocktail (**Figure 33 A**); however, pre-treatment with TUDCA partially inhibited IL-6 production in βLox5 cells treated with PIC (**Figure 33 B**).

These results are consistent with the fact that PIC treatment resulted in a more traditional ER stress response than treatment with pro-inflammatory cytokines (**Figures 29 and 31**). ER stress has been shown in several studies to promote NF-κB activation

through all three branches of the UPR (83). Thus, in PIC-stimulated beta cells, a portion of IL-6 may be produced in response to NF-κB activation mediated by the UPR. This IL-6 production can be inhibited by TUDCA, which alleviates ER stress (**Figure 33 B**). Alternatively, this slight reduction in IL-6 production observed in the PIC-stimulated βLox5 cells pre-treated with TUDCA may not be biologically meaningful.

Since cytokine treatment does not promote a robust activation of the UPR (**Figure 29**), IL-6 production in response to cytokine treatment is likely driven largely by activation of AP-1 and NF-κB downstream of pro-inflammatory cytokine receptor activation, and this activation is not inhibited by TUDCA (**Figure 33 A**). Nevertheless, given that some components of the UPR seem to be activated in response to pro-inflammatory cytokine stimulation and that TUDCA can inhibit HSP90 release in response to cytokine-mediated stress, ER stress likely plays some role in HSP90 release from pancreatic beta cells in response to pro-inflammatory cytokines.

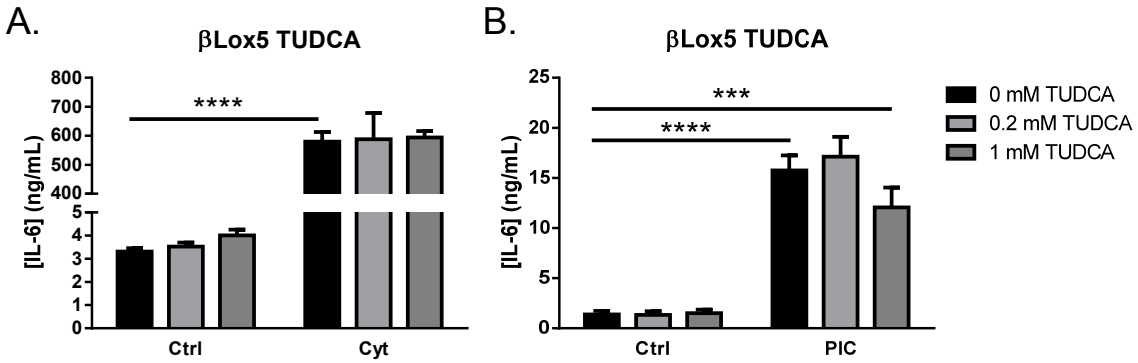


**Figure 33. TUDCA partially inhibits IL-6 release in beta cells treated with PIC.** Human βLox5 cells were treated as in Figure 32. Following incubation, media was harvested and IL-6 ELISAs (A-B) were performed. Data are mean + SEM of N=3-4 experiments. ***p < 0.001, ****p < 0.0001 Two-way ANOVA with Tukey's correction for multiple comparisons.

To determine the signaling events promoting HSP90 release in response to stimulation with pro-inflammatory cytokines, expression patterns of beta cell stress-associated genes were examined to determine which genes, upregulated in response to cytokine stress, could be modulated by TUDCA. In βLox5 cells, TUDCA partially blocked upregulation of the NF-κB and IRF target *NOS2* (**Figure 34 A**), as well as the ER stress marker *ATF3* (**Figure 34 B**). These genes were also upregulated with cytokine stress in human islets (**Figure 20 C** and **Figure 30 C**). Upregulation of the ER stress marker *XBP1s* and the pro-inflammatory cytokine *IL6* were slightly blocked with 1 mM TUDCA, although not significantly (**Figure 34 C-D**). Expression of *XBP1s* was only upregulated in two of four islet donors in response to cytokine stress (**Figure 30 A**). Expression levels of other stress associated markers examined in this work thus far, including *HSP90AA1*, *HIF1A*, *CA9*, and *VEGFA* were not altered by pre-treatment with TUDCA in cytokine-treated cells (data not shown).

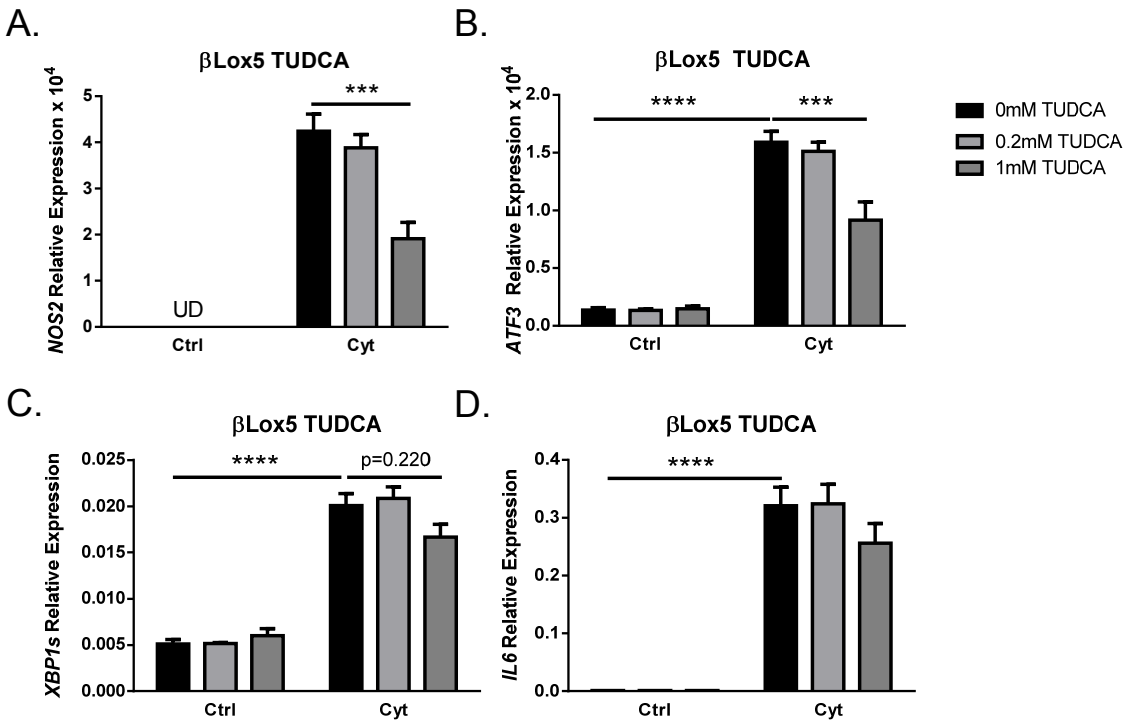


**Figure 34. Pre-treatment with TUDCA modulates the upregulation of specific cytokine-induced genes in pancreatic beta cells.** Human βLox5 cells were treated with media alone, 0.2 mM, or 1 mM TUDCA for 6 hours at 37°C prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. Following incubation, cells were harvested for RNA isolation and cDNA synthesis. Gene expression levels of *NOS2* (A), *ATF3* (B), *XBP1s* (C), and *IL6* (D) were analyzed by qRT-PCR (UD, undetected). Data are mean + SEM of N=3 experiments. ***p < 0.001, ****p < 0.0001 Two-way ANOVA with Tukey's correction for multiple comparisons.

Interestingly, several IFN regulated genes were upregulated in response to cytokine stress in beta cell lines (**Figure 35 A-C**). TUDCA significantly blocked upregulation of the interferon-stimulated genes *ISG15* and *IFI27* (**Figure 35 A-B**); however, pre-treatment with TUDCA did not significantly affect upregulation of the interferon-regulated gene *B2M* (**Figure 35 C**), suggesting the inhibitory effects of TUDCA are gene-specific rather than global. *ISG15*, *IFI27*, and *B2M* were also upregulated in primary islet cells in response to cytokine stress (**Figure 36 A-C**).

ISG15 is a 17 kDa protein induced in response to type I IFN signaling, pathogen infection, and cellular stress. Free ISG15 can be secreted from cells where it functions as both cytokine and chemokine. Alternatively, ISG15 can be conjugated to other proteins in a process termed ISGylation, which is similar to ubiquitination. Although a universal function of ISGylation has not been described, it is thought to play a role in diverse cellular processes, including antiviral immunity (84). Less is known about IFN-α-inducible protein 27, encoded by *IFI27*, but it is known to be upregulated in the PBMCs of SLE patients and may promote apoptosis (85; 86). The gene *B2M* encodes β-2-microglobulin, which is part of the MHC-I complex and is known to be upregulated with IFN-γ stimulation.

*IFNA1* or *IFNB1*, which encode an IFN-α isoform and IFN-β, respectively, were not detected in βLox5 cells treated with IL-1β, TNF-α, and IFN-γ (data not shown). Furthermore, no IFN-α was detected in cytokine-treated βLox5 cell supernatants by an ELISA kit designed to detect eight variations of this protein (data not shown). Thus, these results suggest *ISG15* and *IFI27* expression levels were not regulated by autocrine effects of IFN-α and IFN-β produced in response to stimulation with pro-inflammatory cytokines.

On the other hand, regulation of *ISG15* and *IFI27* may have been partially mediated by ER stress. Several studies have demonstrated crosstalk between ER stress and pro-inflammatory pathways in cells. In particular, the IRE1 branch of the UPR signals through TNF receptor-associated factor 2 (TRAF2), which leads to activation of JNK and NF-κB. Furthermore IRE1 signaling initiates RIDD, which generates mRNA fragments that can stimulate intracellular innate immune receptors. This process, along with the transcription factor XBP1s, also activated by IRE1, can upregulate IRF transcription factors (23). Thus, the changes in interferon stimulated gene expression

observed with TUDCA (**Figure 35**), may be due to modulation of ER stress, particularly IRE1 signaling.

Interestingly, studies have shown IRE1 signaling can be activated independently of PERK and ATF6 (87). This activation has been shown to occur in macrophages following TLR2 and TLR4 stimulation and was found to be required for pro-inflammatory cytokine production (88). If IRE1 were activated in cytokine-treated pancreatic beta cells but not PERK or ATF6, differential activation could explain why TUDCA was effective in blocking HSP90 release, while at the same time *DDIT3* and *HSPA5* were not upregulated with 24 hours of cytokine stress in these experiments (**Figure 29**).

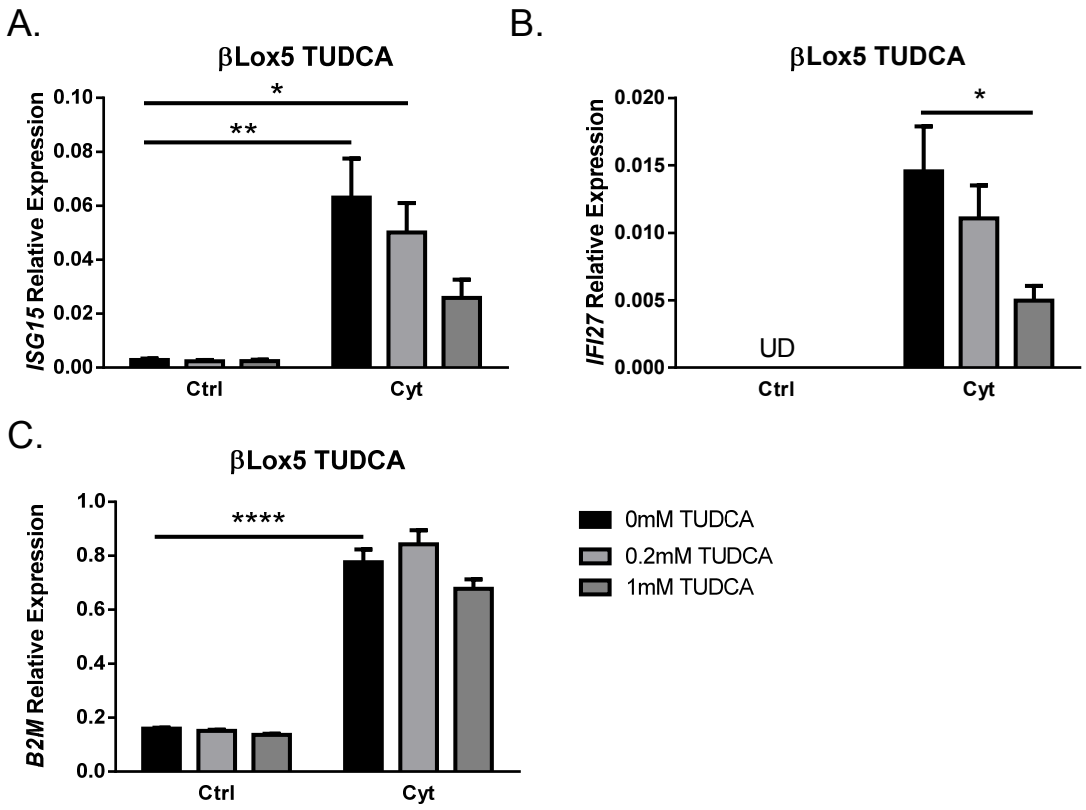


**Figure 35. TUDCA inhibits expression levels of specific interferon-regulated genes in pancreatic beta cells treated with pro-inflammatory cytokines.** Human βLox5 cells were treated with media alone, 0.2 mM, or 1 mM TUDCA for 6 hours at 37°C prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. Following incubation, cells were harvested for RNA isolation and cDNA synthesis. Expression levels of *ISG15* (A), *IFI27* (B), and *B2M* (C) were analyzed by qRT-PCR (UD, undetected). Data are mean + SEM of N=3 experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ Two-way ANOVA with Tukey's correction for multiple comparisons.

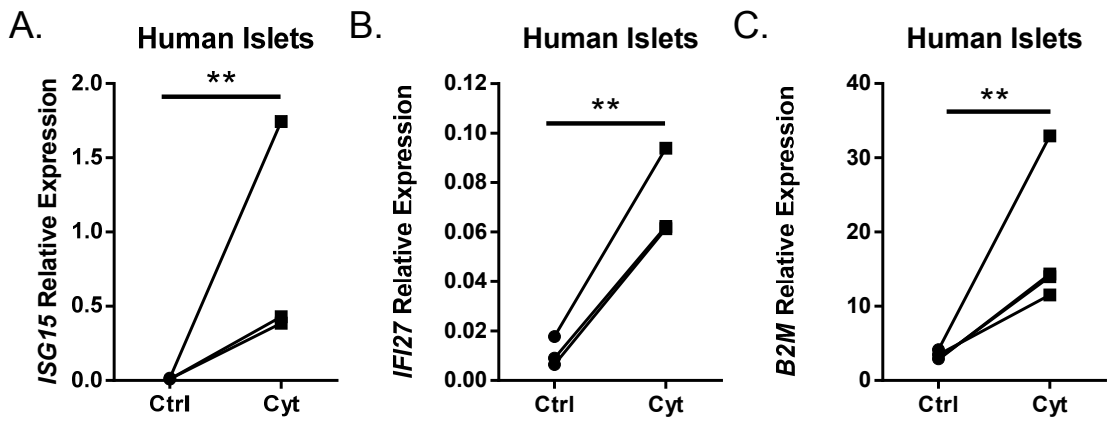


**Figure 36. Expression levels of interferon-regulated genes in primary cadaveric islet cells treated with pro-inflammatory cytokines.** Primary human cadaveric islets were also treated for 24 hours with IL-1β, TNF-α, and IFN-γ at 37°C. Following incubation, cells were harvested for RNA isolation and cDNA synthesis. Expression levels of *ISG15* (A), *IFI27* (B), and *B2M* (C) were analyzed by qRT-PCR. Data are actual values of N=3-4 islet donors. **p < 0.01 Two-tailed, ratio paired *t* test.

### *C. Beta Cell Cytokine Stress and IRE1 Endonuclease Activity*

To determine whether IRE1 signaling was responsible for cytokine-induced HSP90 release in pancreatic beta cells, βLox5 cells were pre-treated with a pharmacological inhibitor of IRE1 endonuclease activity, 4μ8C, for 6 hours at 37°C prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. We hypothesized that IRE1 inhibition would inhibit HSP90 release in response to cytokine stress in these cells. However, contrary to our hypothesis, βLox5 cells pre-treated with 4μ8c released significantly more HSP90 in response to cytokine stress as measured by ELISA (**Figure 37 A**). This increase in HSP90 release was not found to be the result of increased cell permeability, as LDH assays demonstrated improved viability with 4μ8c treatment (**Figure 37 B**). Thus, IRE1 signaling would appear to limit HSP90 release in response to cytokine stress.

However, 4μ8c specifically inhibits the endonuclease activity of IRE1, which is responsible for *XBP1* splicing and RIDD. As mentioned previously, IRE1 also exhibits a

less well understood kinase activity that is thought to be important for JNK activation (24). Thus, IRE1 kinase activity and subsequent JNK activation may be important for HSP90 release in pancreatic beta cells in response to cytokine stress. Inhibiting IRE1 endonuclease activity with 4μ8c may have caused an increase in IRE1-mediated JNK activation, thus increasing HSP90 release in response to cytokine stress.

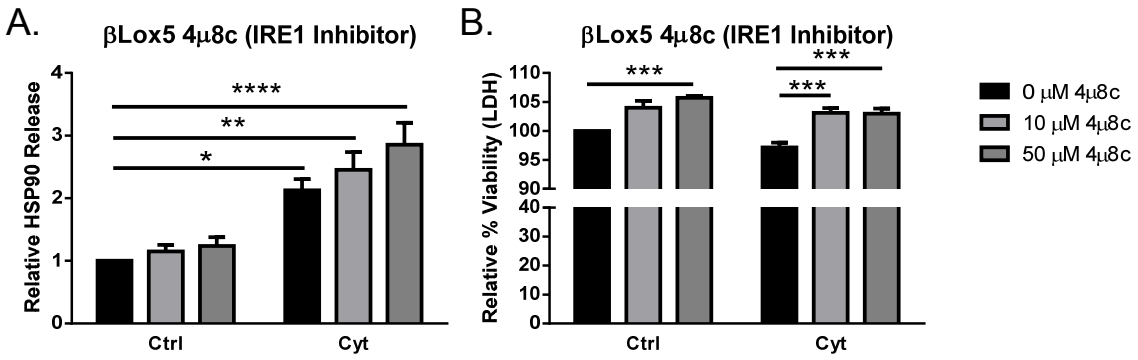


**Figure 37. Pharmacological inhibition of IRE1 endonuclease activity increases HSP90 release and viability in response to cytokine stress in pancreatic beta cells.** Human βLox5 cells were treated with media alone, 10 μM, or 50 μM 4μ8c for 6 hours at 37°C prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. After incubation, media was harvested for HSP90 ELISA (A) and LDH assay (B). Data are mean + SEM of N=4 experiments. HSP90 ELISA data are presented as relative to control cells without 4μ8c. Actual values of HSP90 were detected between 3.2-43.0 ng/mL. LDH assay data are presented as percent viability relative to control cells without 4μ8c. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ Two-way ANOVA with Tukey's correction for multiple comparisons.

### *D. Beta Cell Cytokine Stress and JNK Activity*

Recent studies in human beta cells have shown cytokine-induced apoptosis is mediated by JNK activation downstream of IRE1, rather than NO production, as has been shown to occur in rat beta cells. This cytokine-induced JNK activation could be mitigated by pre-treatment with TUDCA (28). Thus, levels of JNK phosphorylation were examined in pancreatic beta cells in response to cytokine stress following pre-treatment with TUDCA. Human βLox5 cells were treated for 6 hours with 1 mM TUDCA prior to

stimulation with IL-1β, TNF-α, and IFN-γ, and phospho-JNK levels were analyzed by immunoblotting. TUDCA inhibited JNK phosphorylation in response to cytokine stress following 30 minutes (**Figure 38 A-B**) and 24 hours (**Figure 38 C-D**) of cytokine treatment. These results suggest that TUDCA may inhibit HSP90 release in pancreatic beta cells by limiting JNK activation in response to cytokine stress.

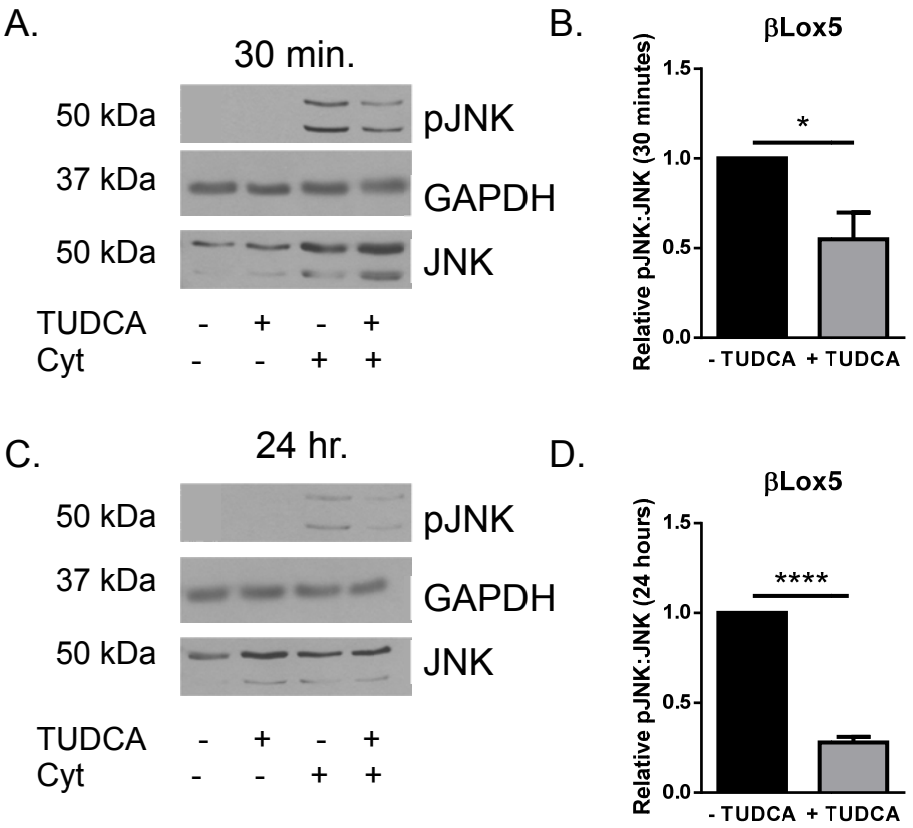


**Figure 38. TUDCA inhibits JNK phosphorylation in response to cytokine stress in pancreatic beta cells.** Human βLox5 cells were pre-treated with media alone or 1 mM TUDCA for 6 hours prior to 30 minute (A-B) or 24 hour (C-D) stimulation with IL-1β, TNF-α, and IFN-γ (Cyt). Following incubation, cell pellets were harvested to prepare protein lysates, and cellular levels of phospho-JNK were detected by immunoblotting. GAPDH was detected as a loading control. Blots were then stripped and total JNK was detected. Panels A and C are representative images, while panels B and D are densitometry results of phospho-JNK to total JNK ratios in cytokine-treated cells, with and without TUDCA pre-treatment for N=3 blots. *$p < 0.05$, ****$p < 0.0001$ Two-tailed, unpaired *t* test.

To confirm that JNK activation was playing a role in HSP90 release from pancreatic beta cells in response to cytokine stress, βLox5 cells were treated with a JNK inhibitor prior to cytokine stimulation. Human βLox5 cells were treated for 6 hours at 37°C with the JNK inhibitor SP600125 prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. This inhibitor partially blocked HSP90 release in response to cytokine stress, suggesting that JNK activation is, at least in part, important for HSP90 release in pancreatic beta cells in response to cytokine stress (**Figure 39 A**). JNK inhibition also slightly improved viability in cytokine-treated pancreatic beta cells as measured by LDH assay, suggesting that inhibiting JNK activity may protect beta cells from cytokine-induced cell death (**Figure 39 B**). We also confirmed the concentrations of SP600125 used in these experiments were effective by performing IL-6 ELISAs. Indeed, IL-6 release in response to cytokine stress was significantly inhibited by pre-treatment with SP600125 (**Figure 39 C**).

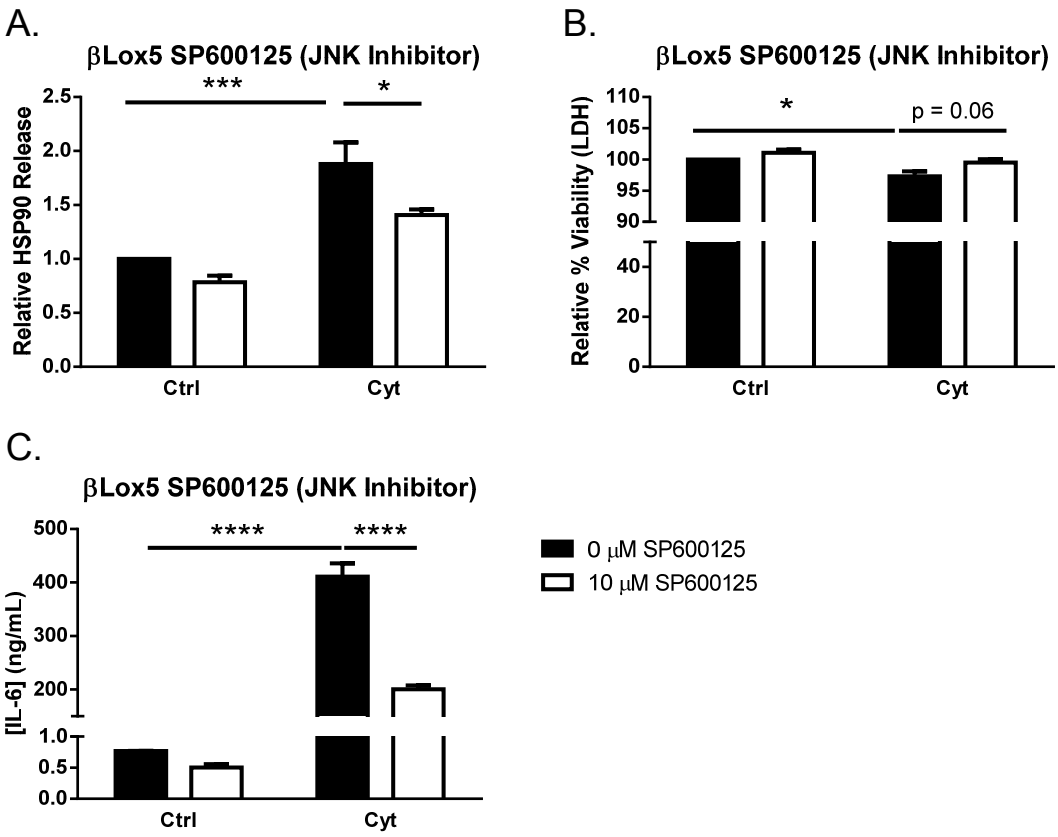


**Figure 39. Pharmacological inhibition of JNK partially inhibits HSP90 release from pancreatic beta cells in response to cytokine stress.** Human βLox5 cells were treated with media alone or 10 μM of the JNK inhibitor SP600125 for 6 hours at 37°C prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. Following incubation, media was harvested for HSP90 ELISA (A), LDH assay (B), and IL-6 ELISA (C). HSP90 ELISA data are presented as relative to control cells without SP600125. Actual values of HSP90 were detected between 6.8-77.1 ng/mL. LDH data are presented as percent viability relative to control cells without SP600125. Data are mean + SEM of N=3-4 experiments. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$ Two-way ANOVA with Tukey's correction for multiple comparisons.

### *E. Beta Cell Cytokine Stress and p38 Activity*

Although JNK inhibition was shown to be important for HSP90 release in pancreatic beta cells in response to cytokine stress, whether or not this JNK activation occurred downstream of IRE1 remained unclear. As mentioned previously, JNK is also activated by MAPK signaling pathways that are initiated downstream of pro-inflammatory cytokine receptor stimulation. Thus, to determine whether MAPK signaling was important for HSP90 release in response to cytokine stress, pancreatic beta cells were treated with a p38 inhibitor. Like JNK, p38 is a signal transducer in MAPK signaling pathways that are activated in response to pro-inflammatory cytokine stimulation.

Human βLox5 cells were treated with the p38 inhibitor SB202190 for 6 hours at 37°C prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. Following incubation, media was harvested for HSP90 ELISA and LDH assay. Rather than inhibit HSP90 release, pre-treatment with the p38 inhibitor SB202190 actually increased HSP90 release in response to cytokine stress (**Figure 40 A**), similarly to pre-treatment with 4μ8c. This increase in HSP90 release was found not to be due to an increase in cell permeability, because p38 inhibition had no effect on cell viability, as measured by LDH assay (**Figure 40 B**). Inhibition of p38 may have resulted in increased signaling through JNK, thus promoting increased HSP90 release. Nevertheless, HSP90 release does not seem to result from synergistic MAPK signaling downstream of cytokine receptor activation. Rather, HSP90 release in response to cytokine stress appears to be specific to JNK activation.

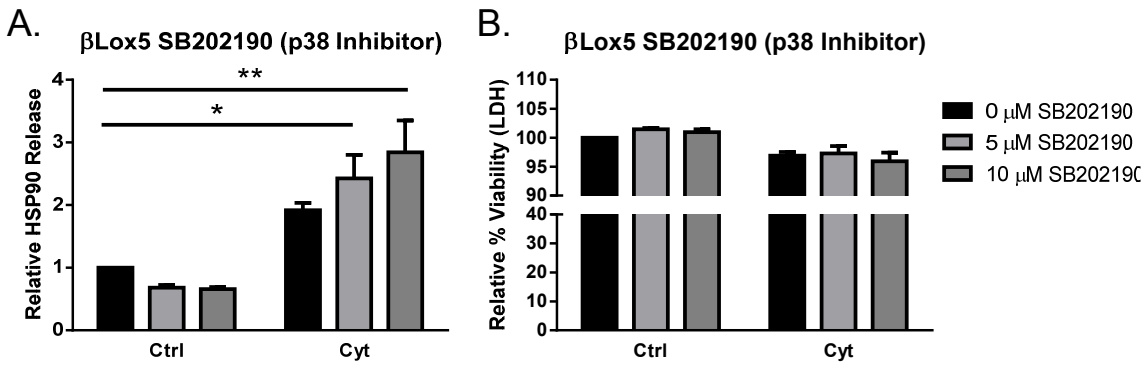


**Figure 40. Pharmacological inhibition of p38 increases HSP90 release in pancreatic beta cells in response to cytokine stress without affecting viability.** Human βLox5 cells were pre-treated with media alone, 5 μM, or 10 μM of the p38 MAPK inhibitor SB202190 for 6 hours at 37°C prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. Following incubation, media was harvested for HSP90 ELISA (A) and LDH assay (B). ELISA data are presented as relative to control cells without SB202190. Actual values of HSP90 were detected between 2.8-34.1 ng/mL. LDH assay data are presented as percent viability relative to control cells treated without SB202190. Data are mean + SEM of N=4 experiments. *$p < 0.05$, **$p < 0.01$ Two-way ANOVA with Tukey's correction for multiple comparisons.

In support of this conclusion, pre-treatment with TUDCA, which inhibited HSP90 release in response to cytokine stress, had no effect on p38 phosphorylation levels, as measured by immunoblotting, at both 30 minutes (**Figure 41 A-B**) and 24 hours (**Figure 41 C-D**) following cytokine stimulation.

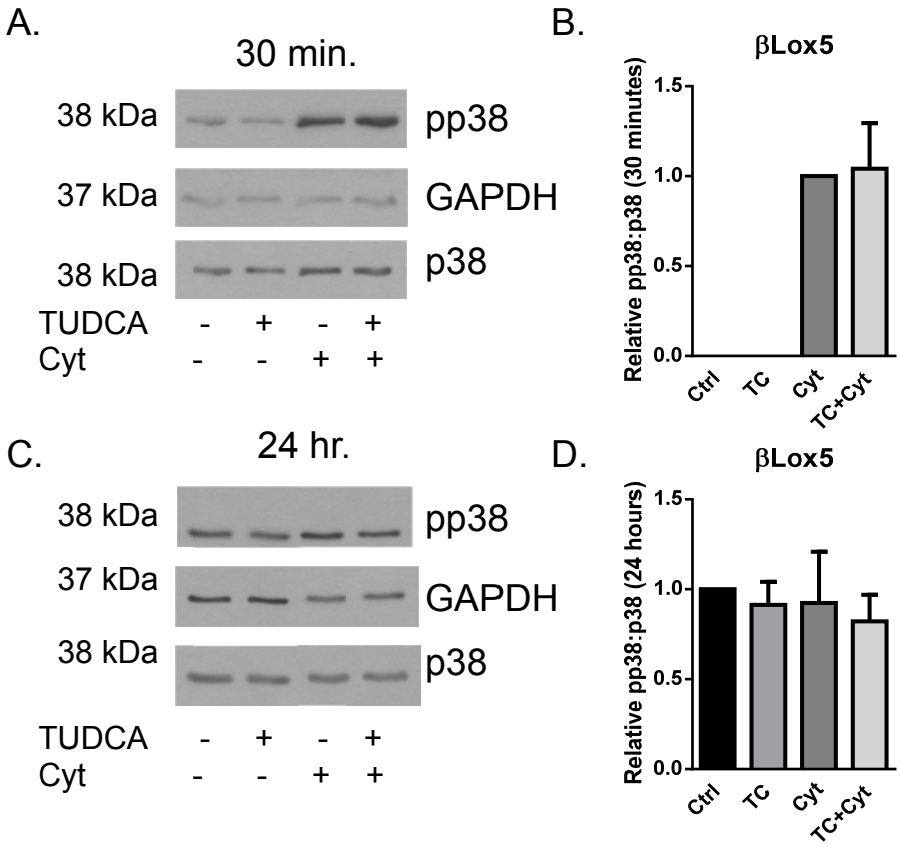


**Figure 41. TUDCA does not affect p38 phosphorylation in response to cytokine stress in pancreatic beta cells.** Human βLox5 cells were pre-treated with media alone or 1 mM TUDCA for 6 hours prior to 30 minute (A-B) or 24 hour (C-D) stimulation with IL-1β, TNF-α, and IFN-γ (Cyt). Following incubation, cell pellets were harvested to prepare protein lysates, and cellular levels of phospho-p38 were detected by immunoblotting. GAPDH was detected as a loading control. Blots were then stripped and total p38 was detected. Panels A and C are representative images, while panels B and D are densitometry results of phospho-p38 to total p38 ratios for N=3 blots.

### *F. Confirming a Role for JNK in HSP90 Release*

To further confirm that beta cell release of HSP90 was mediated by JNK activity, siRNA-mediated knockdown of JNK was performed in βLox5 cells. Human βLox5 cells were transfected with a control or JNK-specific siRNA for 72 hours at 37°C using Lipofectamine 2000. Following 72 hours of transfection, JNK protein expression was assessed by immunoblotting, and a 25 percent decrease in JNK protein expression was observed in cells transfected with JNK siRNA relative to cells transfected with control siRNA (**Figure 42 A-B**). After 72 hours of transfection, cells were treated with media alone or IL-1β, TNF-α, and IFN-γ for an additional 24 hours at 37°C. Following incubation, media was harvested, and HSP90 levels were assayed by ELISA. HSP90 release in response to cytokine stress was markedly reduced in cells transfected with JNK siRNA relative to cells transfected with control siRNA (**Figure 42 C**). These results further support a role for JNK activity in promoting HSP90 release from pancreatic beta cells in response to cytokine stress.

Given that the relative increase in exosomal HSP90 was much greater than the increase in soluble HSP90 observed with cytokine stress (Figure 9), another potential future experiment would be to look at exosome HSP90 levels released from beta cells treated with SP600125 or JNK siRNA in response to pro-inflammatory cytokine stress. This experiment would determine whether or not exosome-associated HSP90 is released by a similar mechanism as soluble HSP90.

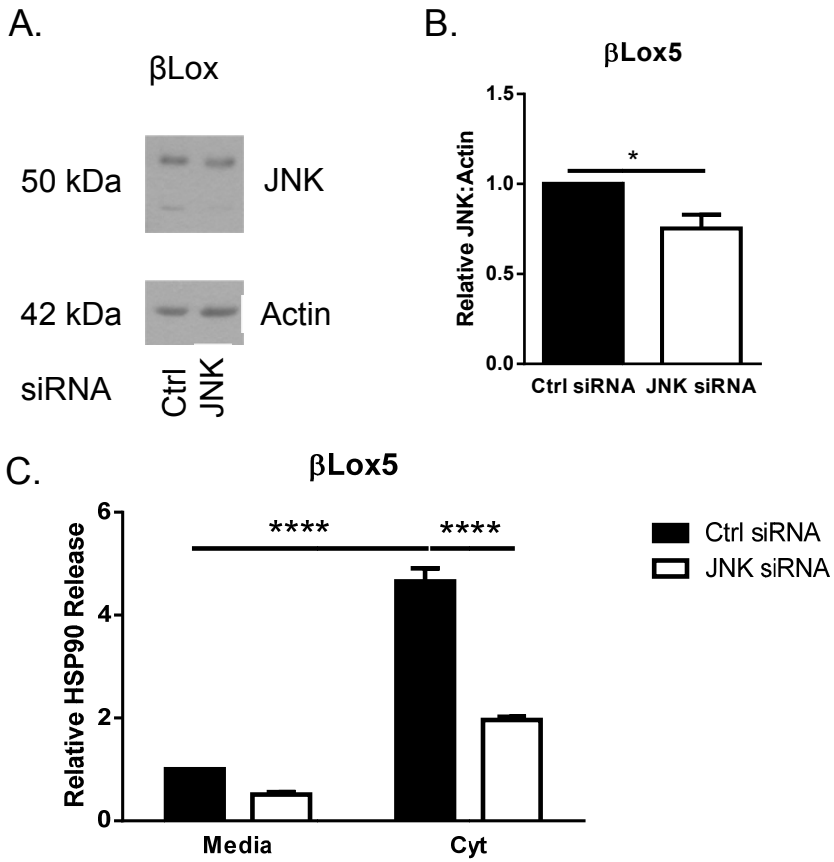


**Figure 42. JNK knockdown with siRNA inhibits HSP90 release from beta cells in response to stimulation with pro-inflammatory cytokines.** Human βLox5 cells were transfected for 72 hours with 100 nM control or JNK siRNA using Lipofectamine 2000, replacing media after the first 24 hours. After 72 hours, one well of control and JNK siRNA-transfected cells were harvested, and expression levels of JNK were examined by immunoblotting. Panel A is a representative image, while panel B is densitometry quantitation of JNK:Actin ratios in cells transfected with JNK siRNA relative to control siRNA. After 72 hours of transfection, control and JNK siRNA-transfected cells were treated with media alone or 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ for 24 hours at 37°C. Following incubation, media was harvested and HSP90 levels were assayed by ELISA (C). Data are presented as relative to control siRNA-transfected cells treated with media alone. Actual values of HSP90 were detected between 13.1-178.5 ng/mL. Data in B and C are mean + SEM of N=4 experiments. For B, *$p < 0.05$ Two-tailed, unpaired *t* test. For C, ****$p < 0.0001$ Two-way ANOVA with Tukey's correction for multiple comparisons.

If HSP90 was released in response to JNK activation downstream of IRE1 signaling, the reason why thapsigargin-treated human beta cells did not release HSP90 (**Figure 7**) remained unclear, given that this chemical inducer of ER stress should activate the IRE1 pathway. Results of *XBP1s* qRT-PCR in thapsigargin-treated cells (**Figure 29 A**) suggest that indeed this pathway was induced by thapsigargin. Thus, levels of JNK phosphorylation were examined in thapsigargin-treated βLox5 cells by immunoblotting. Levels of phospho-JNK were not increased in beta cells treated with thapsigargin for 24 hours compared to cells treated with media alone (**Figure 43**). This result differs from that observed with pro-inflammatory cytokine treatment, which resulted in increased levels of JNK phosphorylation (**Figure 43**). These results may suggest the ER stress response induced by thapsigargin is different from the ER stress response induced by pro-inflammatory cytokines in pancreatic beta cells. This conclusion is supported by qRT-PCR analysis of ER stress markers in response to these two treatments (**Figure 29**). Thus, these results also offer an explanation as to why thapsigargin-treated beta cells did not release HSP90.

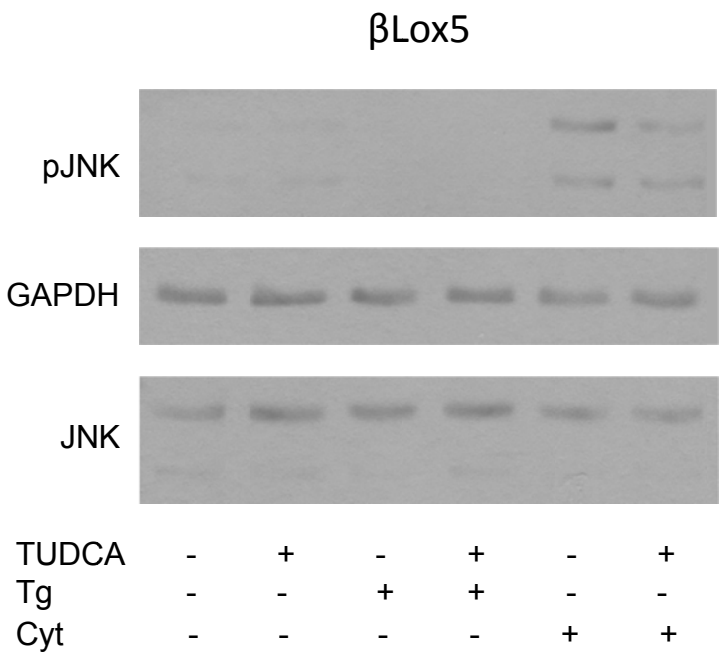


**Figure 43. Thapsigargin treatment does not induce JNK phosphorylation in pancreatic beta cells.** Human βLox5 cells were treated with media alone or 1 mM TUDCA for 6 hours at 37°C prior to 24 hour stimulation with 0.1 μM thapsigargin (Tg) or 5 ng/mL IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ (Cyt). Following incubation, cells were harvested, and phosho-JNK levels were examined by immunoblotting. GAPDH was blotted as a loading control. Membranes were then stripped, and total JNK levels were detected. Representative image of N=3 experiments.

Together, these results suggest that some components of the UPR are activated in pancreatic beta cells in response to cytokine stress (**Figures 29-30**), although the responses are not as robust as those observed with thapsigargin (**Figure 29**) and PIC (**Figure 31**). Thus, the ER stress responses induced by these three stimuli are likely distinct. However, HSP90 release is likely related to this ER stress response, as pre-treatment with TUDCA was able to block release of HSP90 in response to cytokine stress (**Figure 32**).

Our results were consistent with previous studies which showed that TUDCA was able to inhibit JNK phosphorylation induced by cytokine stress and IRE1 activation (**Figure 38**) (28). Thus, we hypothesized that HSP90 release in response to cytokine

stress may be related to JNK activation downstream of IRE1. Pharmacological inhibition of IRE1 endonuclease activity did not prevent HSP90 release in response to cytokine stress (**Figure 37**); however, JNK activation is known to result from IRE1 kinase activity (24). Pharmacological inhibition of JNK (**Figure 39**), but not p38 (**Figure 40**), slightly decreased, while siRNA-mediated knockdown of JNK markedly decreased HSP90 release in response to cytokine stress (**Figure 42**). Thapsigargin likely did not induce HSP90 release (**Figure 7**) as treatment with this compound did not result in beta cell JNK phosphorylation (**Figure 43**).

Thus, the results presented here suggest JNK activation promotes HSP90 release from pancreatic beta cells in response to cytokine stress; however, further studies are needed to confirm whether or not this same mechanism contributes to HSP90 release in response to PIC stimulation. Furthermore, these experiments provide in vitro evidence that beta cell stress during latent TIDM may also promote HSP90 release in vivo, further suggesting this protein could serve as a biomarker of beta cell stress associated with pre-clinical TIDM.

**IV. Pharmacological Inhibition of HSP90 during TIDM in Vivo**

Although HSP90 was found to be released from pancreatic beta cells in response to inflammatory stimuli known to be present during the latent period of TIDM, the in vivo role of HSP90 during the development of TIDM is unknown. Thus, the next objective of this work was to determine whether or not HSP90 inhibition could affect the development of TIDM in vivo.

The NOD mouse model, perhaps the most extensively studied model of TIDM, was utilized for these experiments. The spontaneous development of TIDM in these mice is very similar to that which occurs in humans. For example, NOD mice express diabetogenic MHC-II alleles as well as several non-MHC genetic risk loci. They also

develop insulitis, antibodies that target islet cell autoantigens, and ultimately an autoimmune diabetes mediated by autoreactive $CD8^+$ T cells (45).

HSP90 inhibition, particularly inhibition with 17-DMAG, has shown protective effects in a number of autoimmune disease models, including SLE, by modulating immune responses (36; 37; 41). Thus, we hypothesized that HSP90 inhibition with 17-DMAG may inhibit the development of TIDM in vivo. 17-DMAG, a semisynthetic derivative of the naturally occurring anti-tumor antibiotic geldanamycin, inhibits HSP90 chaperone function by binding the ADP/ATP binding pocket, thus inhibiting client protein maturation and folding. These immature, unfolded client proteins are ultimately ubiquitinated and targeted for degradation by the proteasome (89; 90).

In these studies, female NOD mice were injected with PBS or 17-DMAG (2 mg/kg) for 18 weeks, beginning at six weeks of age. This dosage was found to reduce inflammatory responses in a mouse model of atherosclerosis (61). Only female NOD mice were used in this study as female mice have a much greater rate of diabetes incidence (80-90%) than males (10-40%). Of note, this gender bias is not observed in humans (45).

The development of TIDM was monitored over the course of the study by measuring body weight and blood glucose levels once per week. Serum was collected once per week from a cohort of mice to monitor HSP90 levels by ELISA. A large cohort of moribund mice were sacrificed around 19 weeks of age and used for histology. Finally at 21 and 24 weeks of age, cohorts of the surviving mice were sacrificed for qRT-PCR analysis of islet gene expression (**Figure 44**).

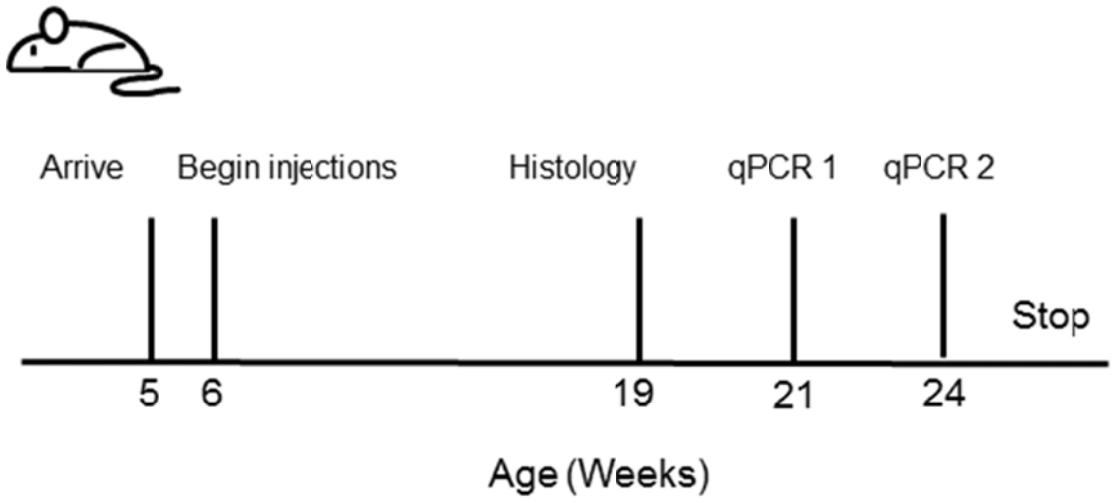


**Figure 44. Effects of 17-DMAG treatment on diabetes development in NOD mice – experimental timeline.** Five-week old female NOD mice were purchased from the Jackson Laboratory. 17-DMAG (2 mg/kg) was injected every third day for approximately 18 weeks beginning at age six weeks, and TIDM progression was monitored by weekly measurements of weight and blood glucose. Moribund animals were sacrificed at approximately 19 weeks of age and used for histology. At 21 and 24 weeks of age, a cohort of mice was sacrificed for analysis of islet gene expression by qRT-PCR.

To determine whether 17-DMAG treatment was effective at inhibiting HSP90 chaperone function, expression levels of the gene *Hspa1b* were measured by qRT-PCR using cDNA samples prepared from the islet cells of NOD mice. The gene *Hspa1b* encodes another cytoplasmic chaperone, HSP70. When active, cytosolic HSP90 inhibits the transcription factor HSF1, which is required for the transcription of *Hspa1b*. Thus, if HSP90 is inhibited, expression of *Hspa1b* will increase due to increased HSF1 activity (37).

At age 21 weeks, several 17-DMAG-treated mice exhibited increased expression of *Hspa1b*, although overall, the increase observed in the 17-DMAG-treated group was not statistically significant from the PBS-treated group (**Figure 45 A**). However, in the cohort of mice sacrificed at age 24 weeks, all mice showed an increase in *Hspa1b* expression, and the increase overall observed in the 17-DMAG-treated group was statistically significant compared to the PBS group (**Figure 45 B**). No differences in *Hspa1b* expression were observed in non-diabetic (ND) mice versus mice with TIDM for PBS or 17-DMAG-treated groups (**Figure 45 C-D**). Thus overall, 17-DMAG did appear to effectively inhibit HSP90 function in vivo, although perhaps not in some mice from the cohort sacrificed at 21 weeks.

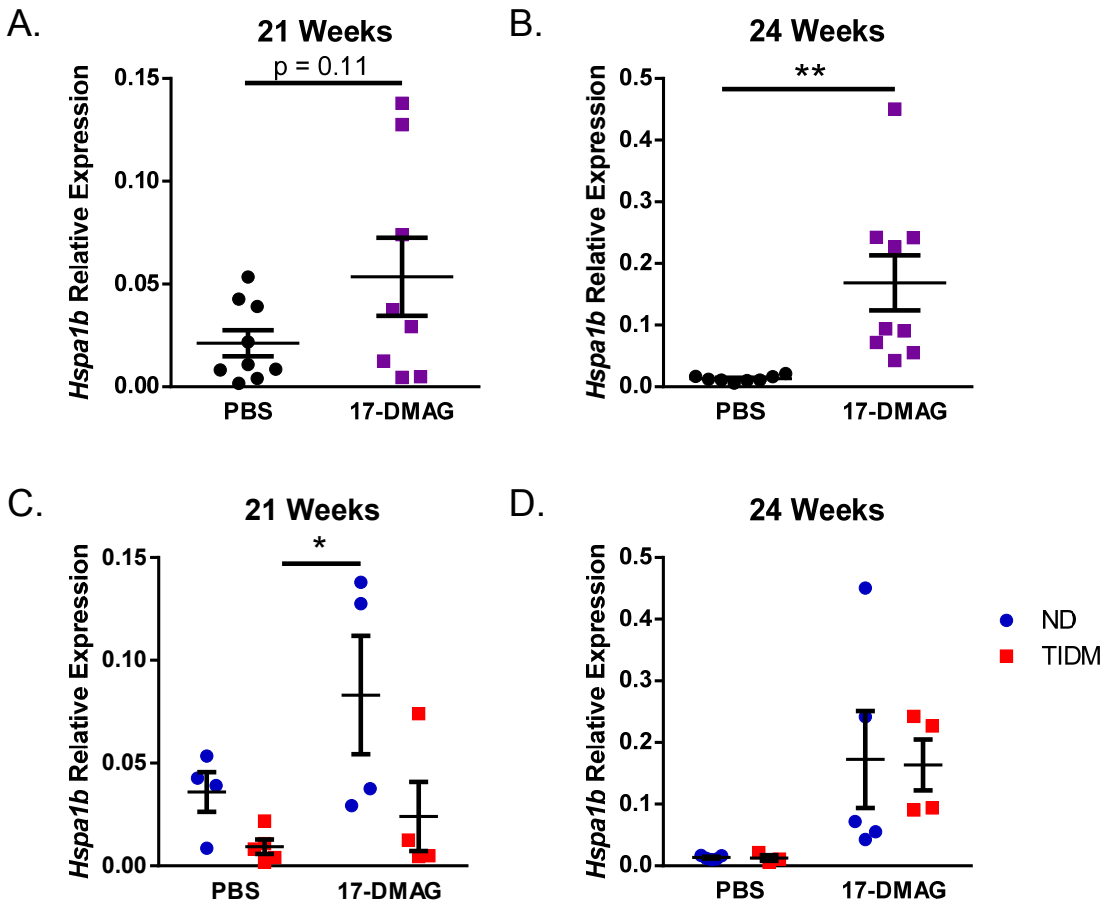


**Figure 45. Expression levels of *Hspa1b* in islet cells from NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS and 17-DMAG as described in Figure 44. Whole pancreatic islets were harvested from mice sacrificed at age 21 (A and C) and 24 weeks (B and D) for RNA isolation and cDNA synthesis. Expression levels of *Hspa1b* were measured by qRT-PCR. Data presented in the bottom two panels are the same PBS and 17-DMAG data presented in the top panels, but relative expression values from non-diabetic (ND) mice and mice with TIDM have been distinguished. Data in A and B were analyzed by two-tailed, unpaired *t* test. Data in C and D were analyzed by two-way ANOVA with Tukey's correction for multiple comparisons. *$p < 0.05$, **$p < 0.01$.

Serum was collected once per week from a cohort of mice during the course of the study, and HSP90 levels were measured by ELISA. Although elevated levels of HSP90 were detected as the mice approached fifteen weeks of age, no differences were observed in serum HSP90 levels between the 17-DMAG and PBS-treated groups (**Figure 46**). These results suggest that inhibition of HSP90 chaperone function with 17-DMAG did not affect the release of cytoplasmic HSP90 from cells into the serum.

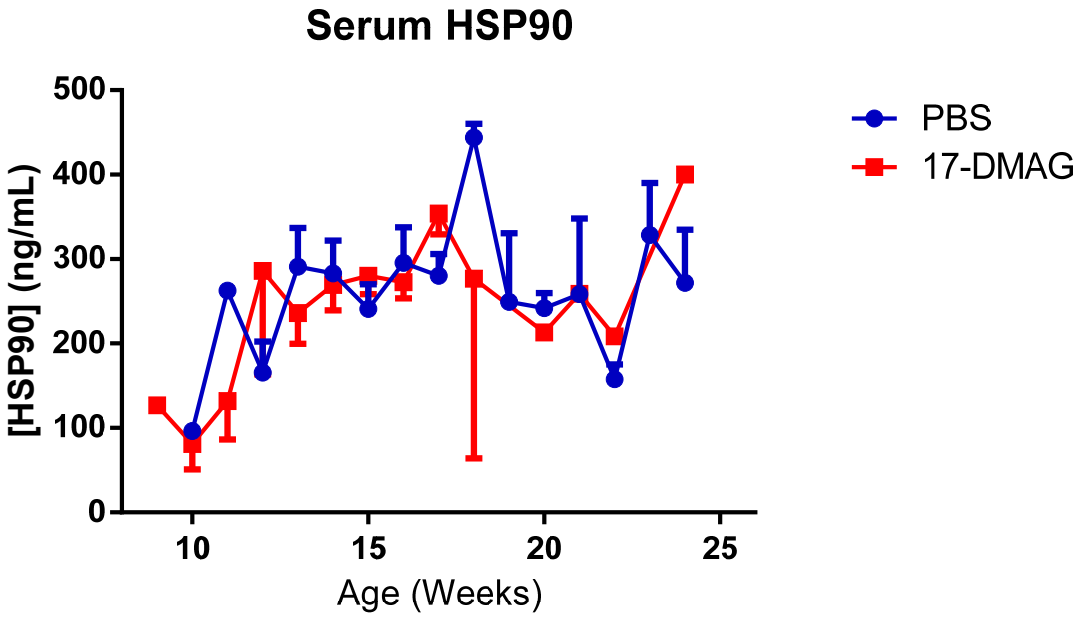


**Figure 46. Serum levels of HSP90 in NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Serum was collected once per week from a cohort of mice, and HSP90 levels were measured by ELISA. Data are mean ± SEM of N=4 mice per group.

As mentioned previously, pharmacological inhibition of HSP90 has been shown to limit the development of autoimmune diseases by attenuating inflammatory responses (36-41). Treatment with 17-DMAG was also found to inhibit inflammatory responses in a model of atherosclerosis (61). Thus, we hypothesized that HSP90 inhibition would attenuate inflammatory responses in NOD mice.

Islet gene expression levels of three inflammatory genes, *Nos2*, *Il6*, and *Hmgb1* were measured by qRT-PCR. No difference in *Nos2* expression was observed between PBS and 17-DMAG-treated mice at 21 weeks of age (**Figure 47 A**); however, at 24 weeks of age, a trend toward decreased expression of *Nos2* was observed in 17-DMAG treated mice (**Figure 47 B**). These variations may reflect an absence of infiltrating immune cells at this time point, possibly because the beta cells have already been largely destroyed. No differences in *Il6* expression were observed in either cohort of mice (**Figure 47 E-H**). Expression levels of *Hmgb1*, which encodes another inflammatory mediator, were slightly upregulated with 17-DMAG treatment at both time points (**Figure 47 I-J**).

By histology, insulitis scores were slightly worse in 17-DMAG-treated diabetic mice than in PBS-treated diabetic mice sacrificed at 19-20 weeks of age (**Figure 48**). Taken together, these results suggest that, contrary to our hypothesis, HSP90 inhibition with 17-DMAG did not dampen inflammatory responses in the NOD mouse model of TIDM, but rather may have exacerbated inflammation. To confirm this result, we plan on looking at transcription of other inflammation and immune cell markers by qRT-PCR.

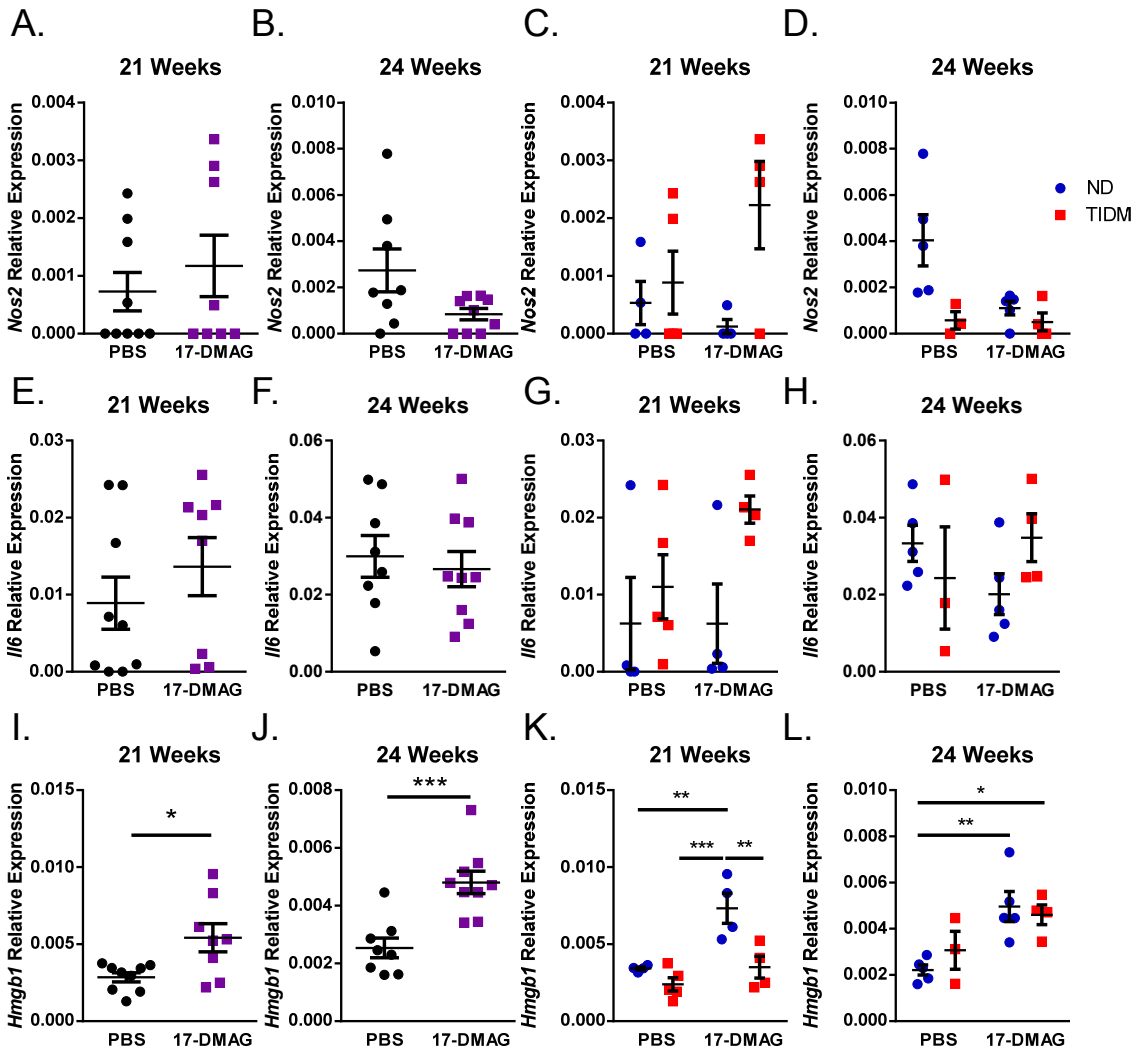


**Figure 47. Expression levels of *Nos2*, *Il6*, and *Hmgb1* in islet cells from NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Whole pancreatic islets were harvested from mice sacrificed at age 21 and 24 weeks for RNA isolation and cDNA synthesis. Expression levels of *Nos2* (A-D), *Il6* (E-H), and *Hmgb1* (I-L) were measured by qRT-PCR. Data presented in the right two columns are the same PBS and 17-DMAG data presented in the left two columns, but relative expression values from non-diabetic (ND) mice and mice with TIDM have been distinguished. Mice with undetectable levels of expression were assigned a value of 0, and statistics were not performed (*Nos2* and *Il6*). Data in the left two columns were analyzed by two-tailed, unpaired *t* test, while data in the right two columns were analyzed by two-way ANOVA with Tukey's correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 (*Hmgb1*).

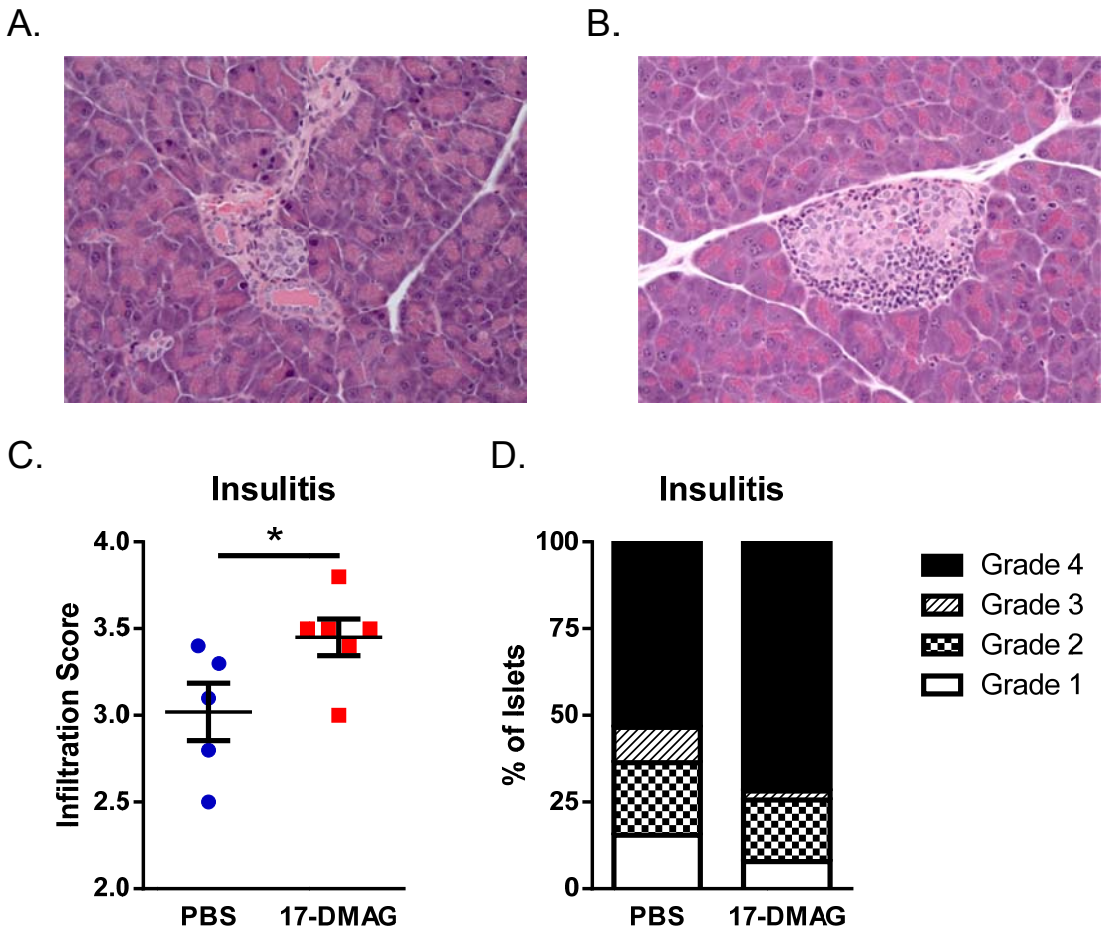


**Figure 48. Insulitis in NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Moribund diabetic mice were sacrificed at 19-20 weeks of age, and pancreata were harvested for histology. Insulitis was scored as follows: Grade 1 = no mononuclear cell infiltrates associated with islets; Grade 2 = peri-insulitis affecting less than 50% of the islet circumference with no evidence of intra-islet invasion; Grade 3 = peri-insulitis affecting greater that 50% of the islet circumference with no evidence of intra-islet invasion; Grade 4 = invasion of mononuclear cells into islets. Panel A is a representative image from a PBS-treated mouse (Grade 3), while panel B is a representative image from a 17-DMAG-treated mouse (Grade 4). Panel C shows the average infiltration score for all mice (PBS N=5, 17-DMAG N=6, 10-30 islets counted per mouse), while panel D shows the distribution of insulitis scores for each group. *$p < 0.05$ Two-tailed, unpaired *t* test.

In addition to insulitis and markers of inflammatory stress, other parameters of TIDM were examined over the course of the present study. As PBS and 17-DMAG-treated mice began to develop diabetes, 17-DMAG-treated diabetic mice began to lose weight at a faster rate as they approached 19 weeks of age (**Figure 49**). These results suggest that 17-DMAG-treated diabetic mice may have been getting sicker than the PBS-treated diabetic mice, perhaps due to an increase in some form of metabolic stress in the context of diabetes. To confirm this conclusion, future experiments are needed in which control and NOD mice are each treated with 17-DMAG. Nevertheless, these data are also consistent with the increased insulitis scores that were observed in the 17-DMAG-treated diabetic mice.

Consistent with the data described above, gene expression levels of *Ins1*, which encodes insulin, were elevated in 17-DMAG-treated non-diabetic mice relative to PBS-treated non-diabetic mice (**Figure 50**). Both PBS and 17-DMAG-treated diabetic mice expressed very low levels of *Ins1* (**Figure 50**), as the beta cell mass in these mice was likely over 90 percent depleted (18). These results suggest that beta cells in 17-DMAG-treated mice may actually have been experiencing a greater degree of stress than beta cells from PBS-treated mice, as the demand for insulin release from the beta cells of the 17-DMAG-treated mice appeared to be higher.

On the other hand, these results may suggest that 17-DMAG treatment promoted the development of insulin resistance in these mice, as insulin resistance is known to be closely related to hyperinsulinemia (91). Several anti-cancer therapies such as glucocorticoids, chemotherapy, hormonal therapies, and other targeted drugs can actually induce insulin resistance (92); however, to our knowledge, this effect has not been shown with HSP90 inhibitors. In fact, the HSP90 inhibitor AUY922 actually improved insulin sensitivity in the diet-induced obese mouse model of insulin resistance

(93). Nevertheless, the results presented here suggest 17-DMAG treatment may have increased levels of pancreatic beta cell stress.

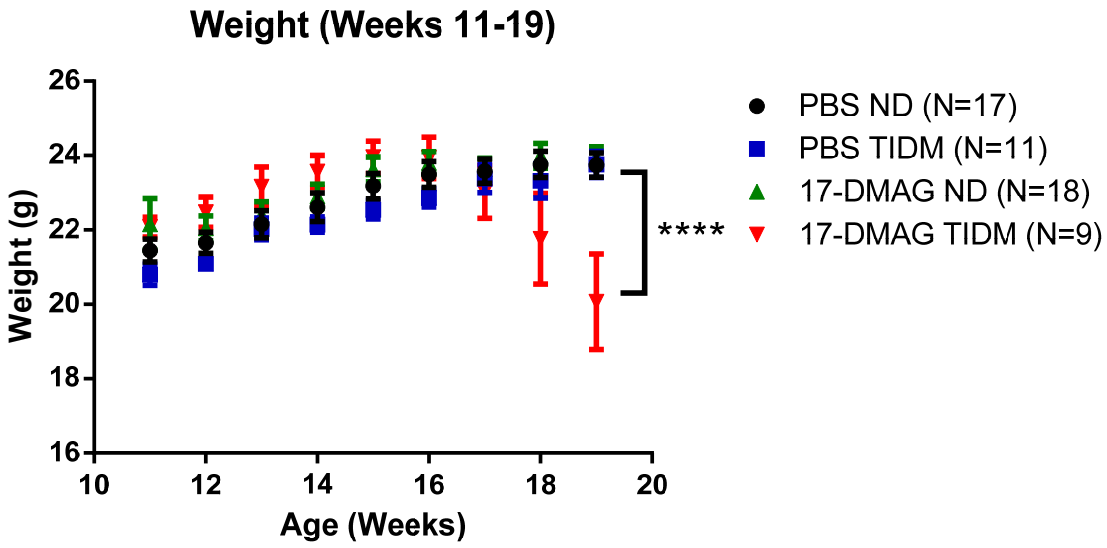


**Figure 49. Average weight of NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Body weights were measured once per week during the course of the study. ND = not diabetic. TIDM = type I diabetes mellitus. Data are mean ± SEM. ****p < 0.0001 One-way ANOVA.

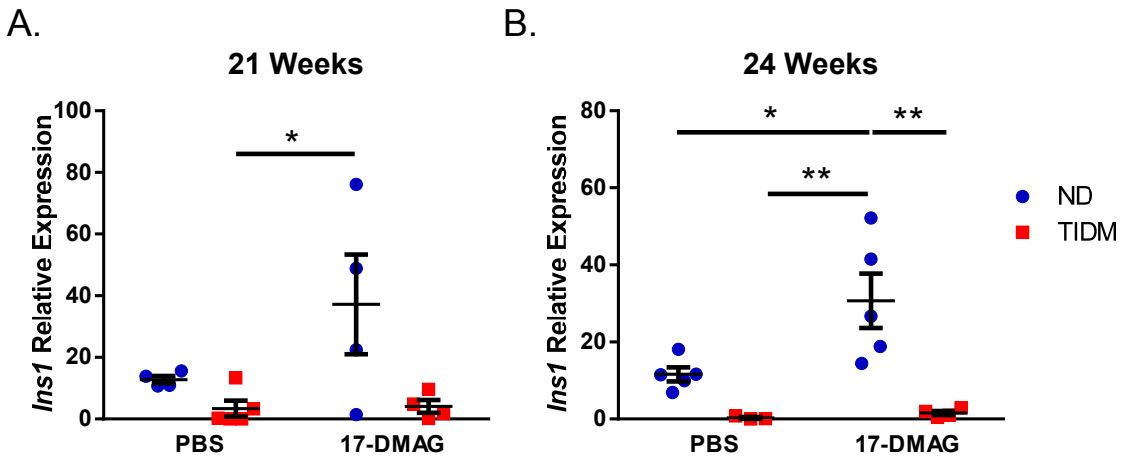


**Figure 50. *Ins1* gene expression levels in islet cells from NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Whole pancreatic islets were harvested from mice sacrificed at age 21 (A) and 24 weeks (B) for RNA isolation and cDNA synthesis. Expression levels of *Ins1* were measured by qRT-PCR. Data are mean ± SEM. *p < 0.05, **p < 0.01 Two-way ANOVA with Tukey's correction for multiple comparisons.

To further characterize the islet cell stress response in NOD mice treated with 17-DMAG, expression levels of ER stress-associated genes were examined by qRT-PCR. ER stress is known to precede the development of TIDM in the NOD mouse model (64). Expression levels of *Ddit3*, which encodes CHOP, were elevated in 17-DMAG-treated mice relative to PBS-treated mice, although not significantly at 21 weeks of age (**Figure 51 A**). This increase became statistically significant at 24 weeks of age (**Figure 51 B**). Expression levels of *Hspa5*, which encodes BiP, were elevated in 17-DMAG-treated mice at 21 and 24 weeks of age compared to PBS-treated mice (**Figure 51 E-F**). Furthermore, expression levels of *Hsp90b1*, which encodes GRP94, were increased in 17-DMAG-treated mice at 21 weeks of age (**Figure 51 I**), and this increase was more pronounced at 24 weeks of age (**Figure 51 J**). Overall, the diabetic mice in each treatment group expressed lower levels of these genes, likely because beta cells were mostly depleted or approaching apoptosis in these animals.

Together, these results suggest that islet cells from 17-DMAG-treated mice experienced an exacerbated ER stress response compared to islet cells from the PBS-treated mice. These results are consistent with those shown in Figure 50, as the non-diabetic 17-DMAG-treated mice were making more insulin mRNA than non-diabetic PBS-treated mice, suggesting the insulin protein demand was higher in 17-DMAG-treated mice.

In addition to cytoplasmic HSP90α and HSP90β, 17-DMAG has been shown to inhibit GRP94 and TRAP1 (94). Thus, 17-DMAG inhibition of the ER chaperone GRP94 in NOD mice may have further promoted ER stress responses in islet cells of 17-DMAG-treated mice.

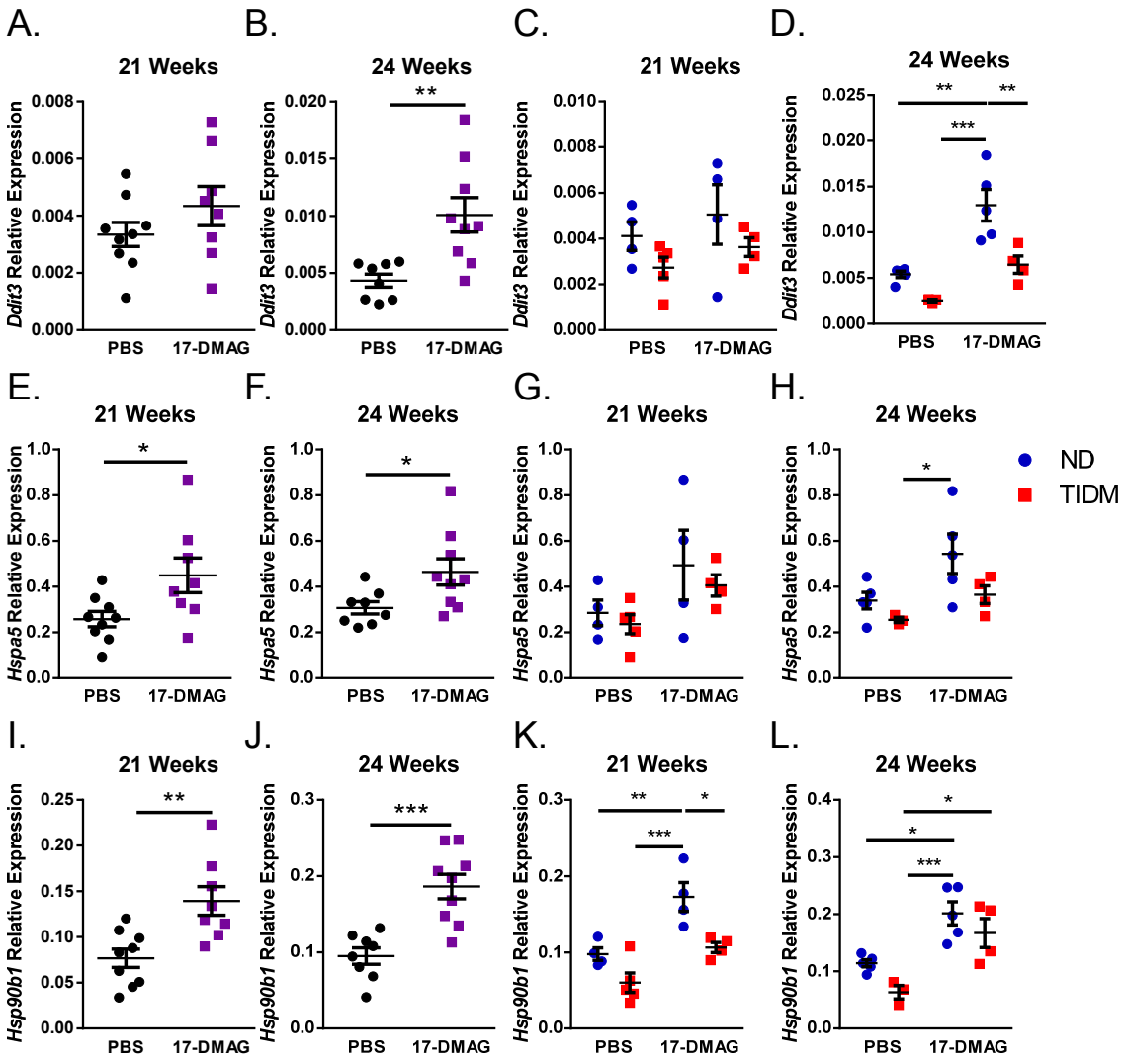


**Figure 51. Expression levels of UPR genes in islet cells from NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Whole pancreatic islets were harvested from mice sacrificed at age 21 and 24 weeks for RNA isolation and cDNA synthesis. Expression levels of *Ddit3* (A-D), *Hspa5* (E-H), and *Hsp90b1* (I-L) were measured by qRT-PCR. Data presented in the right two columns are the same PBS and 17-DMAG data presented in the left two columns, but relative expression values from non-diabetic (ND) mice and mice with TIDM have been distinguished. Data in the left two columns were analyzed by two-tailed, unpaired *t* test, while data in the right two columns were analyzed by two-way ANOVA with Tukey's correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

Because levels of *Ins1* and ER stress-associated genes were elevated in 17-DMAG-treated mice, we hypothesized that other stress-associated genes may have been affected by 17-DMAG treatment. Expression levels of *Hsp90aa1* and *Hsp90ab1*, which encode the HSP90α and HSP90β cytosolic isoforms of HSP90, respectively, were examined by qRT-PCR. Results of these experiments showed that expression levels of both of these genes were upregulated in mice treated with 17-DMAG at 24, but not 21, weeks of age (**Figure 52 A-B, E-F**). No differences in upregulation were observed in non-diabetic and diabetic mice treated with 17-DMAG (**Figure 52 C-D, G-H**).

These results are somewhat surprising, given that *Hsp90aa1* and *Hsp90ab1* should both be induced in response to HSF1 activation (95), which is known to occur with administration of 17-DMAG. These results are consistent with those presented in Figure 45, in which *Hspa1b* expression was not induced in all 17-DMAG treated mice at 21 weeks of age. These results suggest 17-DMAG may have taken slightly longer to have an effect in vivo. However, these mice had been injected for a total of 15 and 18 weeks, respectively. Alternatively, the dosage of 17-DMAG used may not have had an effect on a cohort of the mice sacrificed at 21 weeks.

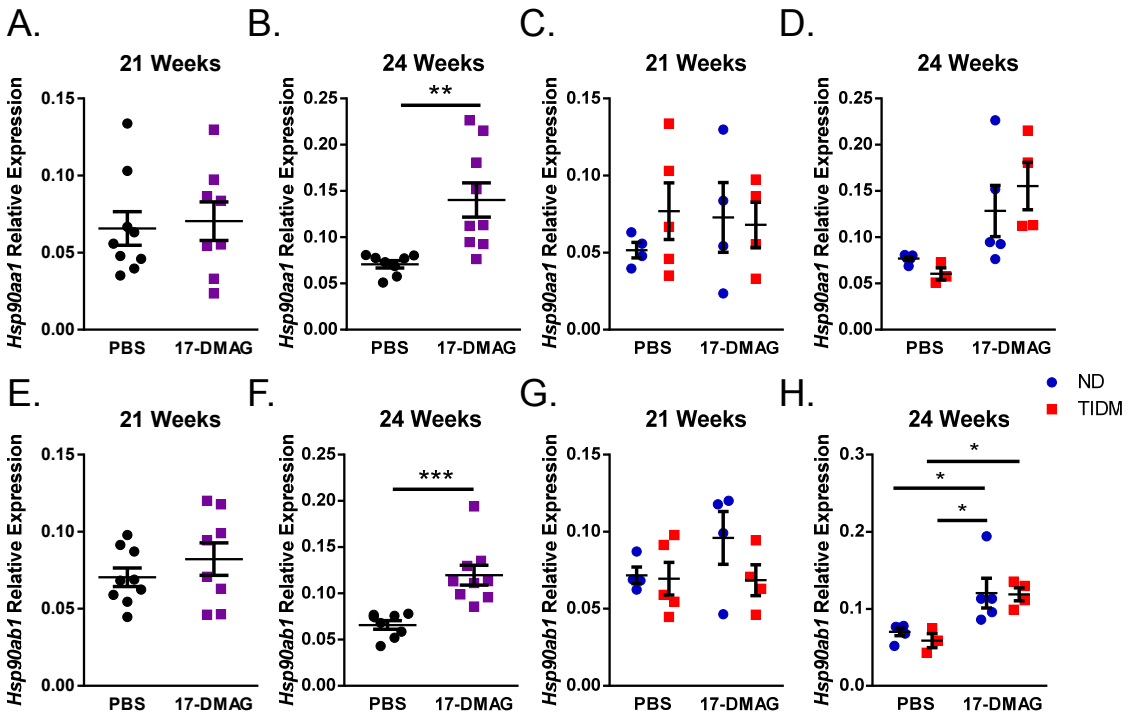


**Figure 52. Expression levels of *Hsp90aa1* and *Hsp90ab1* in islet cells from NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Whole pancreatic islets were harvested from mice sacrificed at age 21 and 24 weeks for RNA isolation and cDNA synthesis. Expression levels of *Hsp90aa1* (A-D) and *Hsp90ab1* (E-H) were measured by qRT-PCR. Data presented in the right two columns are the same PBS and 17-DMAG data presented in the left two columns, but relative expression values from non-diabetic (ND) mice and mice with TIDM have been distinguished. Data in the left two columns were analyzed by two-tailed, unpaired *t* test, while data in the right two columns were analyzed by two-way ANOVA with Tukey's correction for multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

Recent studies have also shown that in addition to activating the UPR and promoting apoptosis, ER stress can induce macroautophagy, a stress-induced process in which eukaryotic cells recycle macromolecules and organelles from their own cytoplasm (96). Thus, expression levels of two genes involved in macroautophagy, *Sqstm1*, which encodes SQSTM1/p62, and *Map1lc3a*, which encodes LC3, were measured by qRT-PCR. At 21 and 24 weeks of age, *Sqstm1* and *Map1lc3a* expression levels were both increased in 17-DMAG-treated mice compared to PBS-treated controls (**Figure 53 A-B, E-F**). However, no significant differences were observed between non-diabetic and diabetic mice (**Figure 53 C-D, G-H**). Together, the results presented in Figures 51 and 53 are consistent with previous studies that have shown *Sqstm1* and *Map1lc3a* are upregulated downstream PERK signaling and ATF4 activation (97).

Overall, 17-DMAG treatment did not have an effect on diabetes incidence in NOD mice (**Figure 54 A**). A similar trend was observed in the mice that survived past 19 weeks of age that were sacrificed at 21 and 24 weeks of age for qRT-PCR (**Figure 54 B**). If two 17-DMAG-treated mice from the qRT-PCR groups that had extremely low expression of *Hspa1b*, the remaining 17-DMAG-treated mice appeared to have a slightly lower incidence of TIDM (**Figure 54 C**); however, this result was still not significant.

Thus, our overall conclusion is that HSP90 inhibition does not affect the incidence of TIDM in vivo. However, given that 17-DMAG-treated mice that developed diabetes had worse insulitis than PBS-treated diabetic mice (**Figure 48**), lost more weight than PBS-treated mice (**Figure 49**), and that islet cells from 17-DMAG treated mice express higher levels of stress-associated genes than PBS-treated mice (**Figures 50-53**), HSP90 inhibition appears to exacerbate islet cell stress during the course of TIDM in vivo. Alternatively, the increased weight loss observed in the 17-DMAG treated diabetic mice may have been due to increased stress responses in other tissues such as muscle or adipose tissue. Nevertheless, HSP90 inhibition would likely not prevent TIDM

in human patients at risk for developing the disease. Rather, HSP90 inhibition may have adverse effects on beta cell stress, dysfunction, and death.

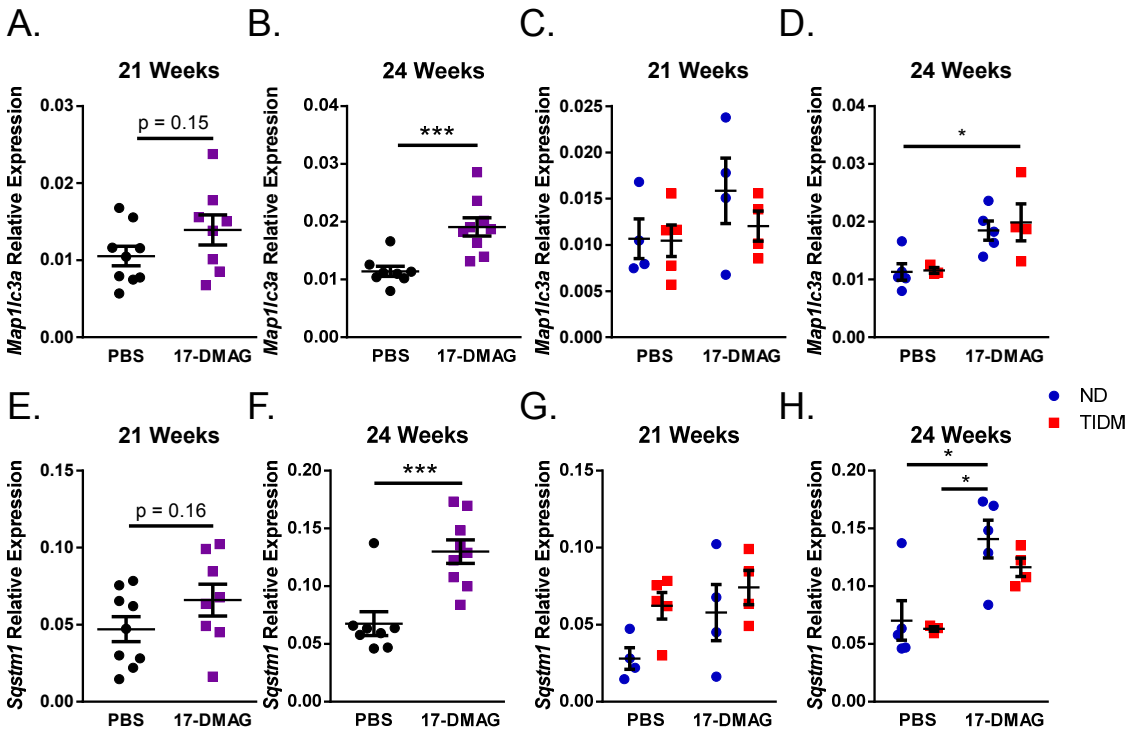


**Figure 53. Expression levels of *Map1lc3a* and *Sqstm1* in islet cells from NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Whole pancreatic islets were harvested from mice sacrificed at age 21 and 24 weeks for RNA isolation and cDNA synthesis. Expression levels of *Map1lc3a* (A-D) and *Sqstm1* (E-H) were measured by qRT-PCR. Data presented in the right two columns are the same PBS and 17-DMAG data presented in the left two columns, but relative expression values from non-diabetic (ND) mice and mice with TIDM have been distinguished. Data in the left two columns were analyzed by two-tailed, unpaired *t* test, while data in the right two columns were analyzed by two-way ANOVA with Tukey's correction for multiple comparisons. *p < 0.05, ***p < 0.001.

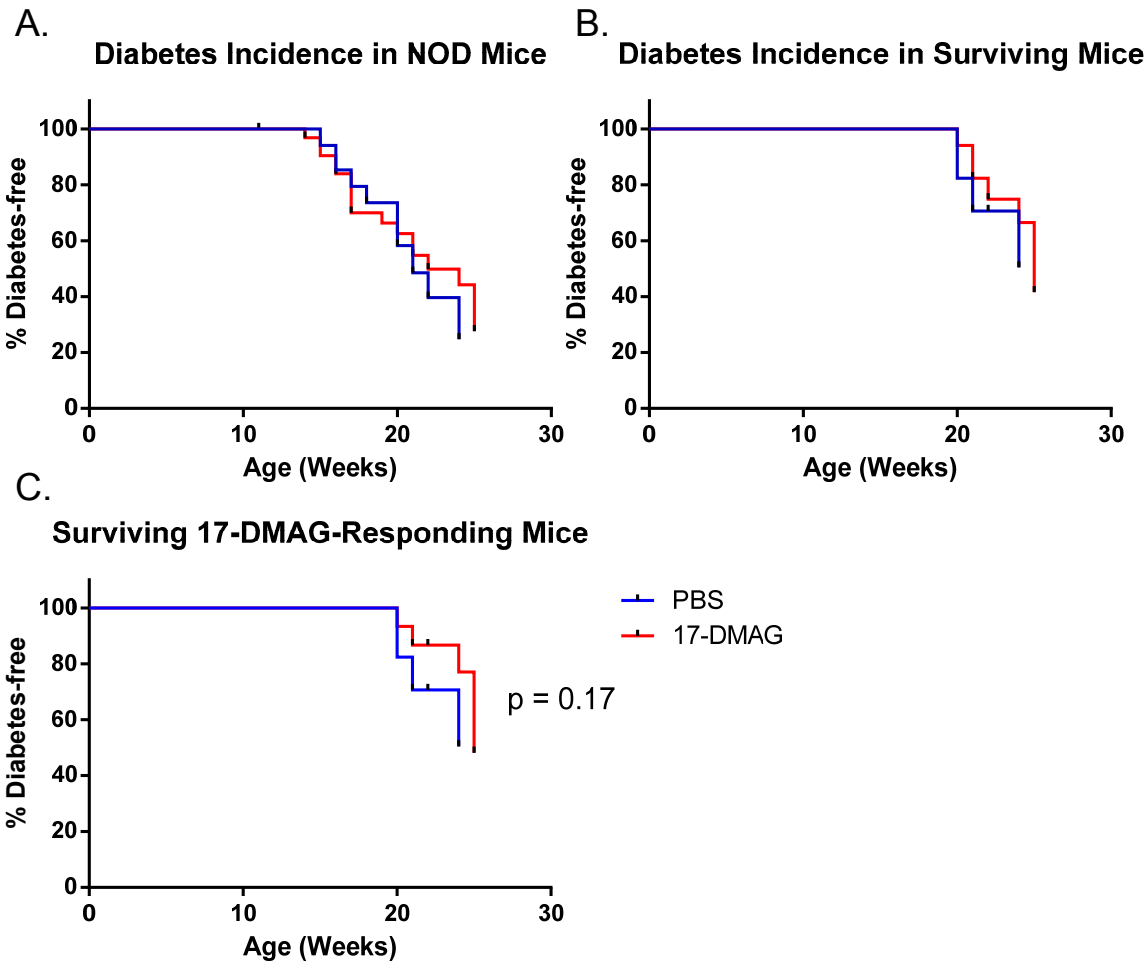


**Figure 54. Diabetes incidence in NOD mice treated with 17-DMAG.** Female NOD mice were treated with PBS or 17-DMAG as described in Figure 44. Blood glucose levels were measured once per week. Mice were considered diabetic after two consecutive blood glucose readings above 300 mg/dl. Data in panel A are for all mice (PBS N=34, 17-DMAG N=33), while data in panel B are the surviving mice sacrificed for qRT-PCR at 21 and 24 weeks of age (PBS N=17, 17-DMAG N=17). Data in panel C are the same mice as in B, but two mice that had extremely low expression level values of *Hspa1b* were excluded (PBS N=17, 17-DMAG N=15). Data were analyzed by Mantel-Cox test.

## DISCUSSION

### I. Rationale

Currently, a major obstacle on the road to TIDM prevention is a lack of known biological markers that could identify patients in the pre-clinical, latent phase of TIDM development. Recent collaborative studies from our lab and others have shown serum levels of the alpha cytoplasmic isoform of HSP90 are elevated in newly diagnosed TIDM patients relative to control subjects (31). Furthermore, other studies have shown patients with TIDM have elevated levels of circulating class-switched IgG1 and IgG3 autoantibodies to HSP90 compared to control subjects. These antibody isotypes are produced during Th1-mediated immune responses, which predominate the autoimmune response in TIDM (35). Together, these results suggest HSP90 may be released extracellularly during the development of TIDM. Thus, HSP90 may be able to serve as a predictive biomarker of beta cell stress and latent TIDM. If true, serum HSP90 levels could be measured in the clinic to identify patients at risk for progressing to TIDM, and treatments aimed at disease prevention could be more appropriately implemented.

### II. Release of HSP90 from Pancreatic Beta Cells

In light of this information, we hypothesized HSP90 may be released from pancreatic beta cells during periods of beta cell stress associated with latent TIDM. HSP90 has previously been shown to be released from cells in response a number of different stress events, including heat shock, oxidative stress, and hypoxia (54). Studies here using the human pancreatic beta cell lines βLox5 and 1.1B4 demonstrated that HSP90 was released in response to stimulation with a combination of pro-inflammatory cytokines that included IL-1β, TNF-α, and IFN-γ, but not in response to stimulation with thapsigargin or high glucose treatment (**Figure 7 A-B**).

The results obtained with these human beta cell lines differed from those obtained with the murine insulinoma cell line MIN6, which released HSP90 in response

to all three stimuli, although the most robust release was observed with pro-inflammatory cytokines (**Figure 7 C**). These incongruities may be due to interspecies differences between human and mouse. To support this conclusion, a recent study has shown that cytokines induce ER stress in mouse, rat, and human beta cells via different mechanisms (28), suggesting beta cells from different species may respond differently to various stress treatments. Alternatively, MIN6 cells may be more sensitive to thapsigargin and high-glucose treatment, as this insulinoma cell line is likely producing high levels of insulin relative to the human beta cell lines and may therefore reach ER processing capacity more quickly with additional stress. For these reasons, the majority of experiments in this work were performed with human cell lines.

Next, we confirmed that primary human cadaveric islet cells also release HSP90 in response to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ (**Figure 7 D**). Human βLox5 cells were used as a model in the majority of experiments in this work because they responded to cytokine stimulation most similarly to human islets in terms of gene expression of HSP90 isoforms (**Figures 11, 13-16**), oxidative stress markers (**Figures 20, 23-24**), ER stress markers (**Figures 29-30**), and interferon-stimulated genes (**Figures 35-36**). The 1.1B4 cell line may have responded differently (**Figures 20 and 24**) due to the fact that this cell line was derived by electrofusion of adult cadaveric islet cells with the immortalized PANC-1 epithelial cell line (60). Thus, this cell line may exhibit characteristics of epithelial cells as well as beta cells. Additionally, electrofusion can generate aneuploidy in cells, which could also potentially affect gene expression.

Interestingly in addition to pro-inflammatory cytokines, βLox5 cells also released HSP90 in response to 24 hour stimulation with an agonist for TLR3 but not TLR4 (**Figure 18 C-D**), even though these cells expressed both receptors (**Figure 17 A**). Thus, HSP90 appeared to be released in response to distinct inflammatory stimuli.

**III. Mechanism of HSP90 Release**

We next wanted to understand the mechanism by which HSP90 was released from beta cells. The combination of pro-inflammatory cytokines used in these experiments has been shown to be cytotoxic to primary islet cells (63). Thus, HSP90 may have been released non-specifically from cells as a result of compromised cell membrane integrity associated with cell death (**Figure 55 A**). Indeed, others have shown HSP90 and other DAMPs are released by necrotic, but not apoptotic, cells (48). Results here showed a decrease in viability by LDH assay of approximately two to five percent in human βLox5 cells treated with IL-1β, TNF-α, and IFN-γ for 24 hours relative to control-treated cells. However, significant decreases in viability were not observed until 48 hours post-treatment (**Figure 8 E**). These results are consistent with other studies showing increased beta cell death in response to stimulation with cytokines for 48 hours (28; 98). Thus, HSP90 does not appear to be passively released as a result of cell death after 24 hours of cytokine treatment. However, HSP90 may be passively leaking from cells in response to cytokine stress at later time points associated with significant levels of cell death.

One limitation associated with the LDH assay is cell confluency. A more confluent monolayer of cells will naturally exhibit higher LDH activity than a less confluent monolayer. Thus, if any stress treatment or pharmacological agent utilized in these experiments were to affect cell growth or confluency, the LDH assay results could be skewed accordingly. To confirm the results obtained here with LDH assays, beta cell apoptosis and necrosis could be examined via microscopy (28).

Nevertheless, to further support the conclusion that cytokine stress does not result in non-specific release of HSP90 at 24 hours, no extracellular HSC70 or HSP70 was detected by ELISA following 24 hours of stimulation with IL-1β, TNF-α, and IFN-γ (data not shown). These results suggest cytosolic proteins were not passively leaking

out of cells in response to stimulation with pro-inflammatory cytokines. Moreover, changes in HSP90 release were not always consistent with changes in LDH activity. For example, pre-treatment with TUDCA inhibited HSP90 release in response to stimulation with pro-inflammatory cytokines and PIC (**Figure 32 A-B**); however, pre-treatment with TUDCA did not affect viability in response to 24 hour cytokine stimulation (**Figure 32 C**), while it slightly improved viability in response to TLR3 stimulation (**Figure 32 D**). These results suggest a portion of the HSP90 released in response to 24 hour stimulation with PIC, but not pro-inflammatory cytokines, may have resulted from or been associated with cell death. However, this result does not definitively prove that HSP90 was passively leaking from cells as a result of cell death. Furthermore, pre-treatment with the IRE1 endonuclease inhibitor 4μ8C increased levels of HSP90 release in response to cytokine stress, while simultaneously increasing viability by LDH assay (**Figure 37 A-B**).

Other studies have shown HSP90 can be released from cells in the context of exosomes (51; 68). Results here suggest that while some extracellular HSP90 was associated with exosomes, the majority of HSP90 released in response to 24 hour cytokine stimulation was soluble (**Figure 9**) (**Figure 55 B**). Thus, results here demonstrate that in response to 24 hour stimulation with pro-inflammatory cytokines, the majority of HSP90 is released specifically by an as of yet unknown secretory mechanism (**Figure 55 C**). Together, these results are consistent with previous studies suggesting HSP90 can be actively released from cells in response to stress (53; 55; 58). Secretion of HSP90 has been demonstrated in a number of studies: however, the underlying secretory mechanism remains elusive (56; 57).

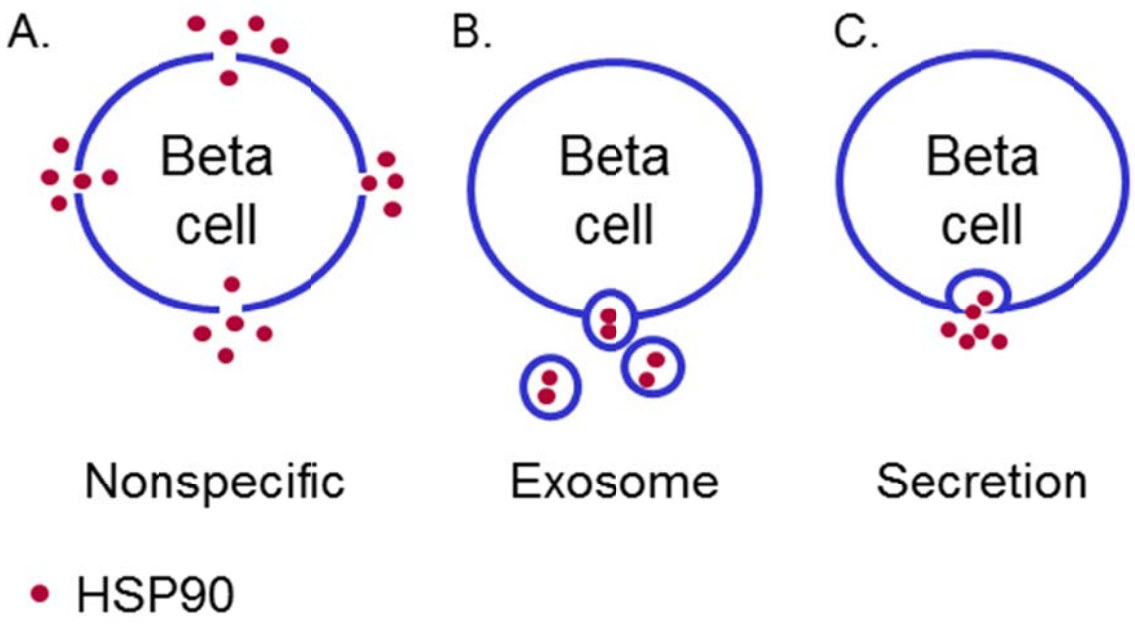


**Figure 55. Potential avenues of HSP90 release from pancreatic beta cells.** Studies here have demonstrated that in response to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ, HSP90 is likely not passively released as a result of impaired cell membrane integrity associated with cell death (A). This mechanism likely becomes more relevant after longer periods of time associated with significant levels of cell death. A portion of extracellular HSP90 is associated exosomes (B); however, the majority of HSP90 is released by an unknown secretory mechanism (C).

A recent study using the breast cancer cell line MCF-7 showed that secreted HSP90α is a truncated form of the protein in which the C-terminal EEVD motif has been removed and in which residue Thr-90 has been phosphorylated (57); however, the authors did not identify the protease required for cleavage of the EEVD motif. Future studies are needed to determine whether or not secretion of HSP90 from beta cells in response to pro-inflammatory cytokine stimulation is regulated by similar post-translational modifications of HSP90.

Another recent study has demonstrated that various proteins involved in inflammation, cytoprotection, and tissue repair are secreted by an unconventional, rather than ER/Golgi-dependent, secretion mechanism that requires cleavage by caspase-1 (99), and HSP70 has been shown to be secreted by a similar mechanism (100). HSP90 does not contain a classical N-terminal secretion signal peptide, and therefore may also be secreted by such an unconventional mechanism (57). More work is needed to understand the specific secretory mechanism by which HSP90 is secreted.

**IV. HSP90 Release and Oxidative Stress**

We next sought to understand the intracellular signaling events that promoted HSP90 release from human pancreatic beta cells in response to stimulation with pro-inflammatory cytokines and PIC. Because HSP90 was not released in response to treatment with thapsigargin, a SERCA inhibitor that induces ER stress, we hypothesized that ER stress did not contribute to HSP90 release, even though pro-inflammatory cytokines and PIC have both been shown previously to induce ER stress in pancreatic beta cells (28; 66; 79).

In pancreatic beta cells, pro-inflammatory cytokine stimulation induced expression of *NOS2*, which encodes the NO-producing enzyme iNOS (**Figure 20**); therefore, we hypothesized oxidative stress mediated by NO may play a role in HSP90 release. Oxidative stress is caused by the accumulation of RNS and ROS within cells.

RNS and ROS contain unpaired electrons and are therefore highly reactive molecules that can cause significant damage to various cellular structures (29). In pancreatic islet cells, NO has been shown to induce detrimental effects on islet function and viability by damaging mitochondria and mitochondrial proteins (101). Interestingly, a recent study showed that nitration of HSP90 in response to oxidative stress could induce cell death in motor neurons (102). Thus, we hypothesized that NO production in pancreatic beta cells may promote oxidative stress and HSP90 release. However, pre-treatment of βLox5 cells with the iNOS inhibitor 1400W did not affect HSP90 release in response to pro-inflammatory cytokine stimulation (**Figure 21**).

Expression levels of *NOS2* were low in both βLox5 and primary islet cells (approximate $C_T$ = 32) (**Figure 20**). Consistent with these results, others have reported βLox5 cells do not produce NO in response to cytokine stimulation (98), and similar results were shown with the human beta cell line EndoC-βH1 (103). Furthermore, studies from two independent groups have shown NO production is dispensable for cytokine-mediated apoptosis in EndoC-βH1 cells (28; 103). Thus, primary human pancreatic beta cells may not produce a significant amount of NO. However, primary human pancreatic islet cells are known to produce NO in response to stimulation with pro-inflammatory cytokines, and this NO is a major mediator of cytokine-induced damage of human islet cells (101; 103). Given the presence of multiple cell types within islets, the NO detected in these studies may have arisen from another endocrine cell type such as alpha cells. More research is therefore needed to determine whether or not primary human beta cells produce NO and whether or not it is cytotoxic to these cells. This work will hopefully be possible in the future with the development of reagents to sort human beta cells from mixed islet cell populations by flow cytometry. In the meantime, determining whether or not pre-treatment with 1400W could inhibit HSP90 release in response to cytokine stress in primary human islet cells may be worthwhile.

Another oxidative stress factor that was induced in human beta cells in response to cytokine stress was *HIF1A* (**Figure 23**). Expression of *HIF1A* can be induced by the transcription factor NF-κB (77), which is activated in response to pro-inflammatory cytokine stimulation (**Figure 1**). In the presence of ROS, HIF-1α can be stabilized and transcriptionally active, even under normoxic conditions (**Figure 22**). Here cytokine stress increased the expression of HIF-1α target genes in beta cells (**Figure 24**), suggesting HIF-1α transcriptional activity increased with cytokine treatment in the present study. Interestingly, human fibroblast cells have been shown to secrete HSP90 in response to hypoxia and HIF-1α activation (55). Thus, we hypothesized that increased HIF-1α activity in beta cells may promote HSP90 release. However, pharmacological inhibition of HIF-1α with chetomin did not inhibit HSP90 release in response to cytokine stress (**Figure 25**). Moreover, pharmacological stabilization of HIF-1α with DMOG did not promote HSP90 release from beta cells (**Figure 27**). These results suggest that, unlike in fibroblasts, HIF-1α activity in beta cells does not promote HSP90 release.

At this point, HSP90 release did not appear to be regulated by cytokine-induced oxidative stress mediated through iNOS or HIF-1α. To determine once and for all whether any source of oxidative stress could promote HSP90 release in response to pro-inflammatory cytokine stimulation, βLox5 cells were pre-treated with the antioxidant NAC for 6 hours prior to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. Surprisingly, 1 mM NAC actually increased levels of HSP90 release in both control- and cytokine-treated cells (**Figure 28**). These unexpected results suggest that a more reducing environment in the cell may promote HSP90 release.

Protein folding in the ER requires oxidizing conditions for the formation of intramolecular and intermolecular disulfide bonds (29). Therefore, NAC treatment may have placed additional stress on protein folding in cells, and this stress may have resulted in increased HSP90 release. More work is needed to understand how pro-

inflammatory cytokine stimulation affects the redox environment in beta cells and what role the redox environment plays in beta cell stress responses and HSP90 release.

**V. Pro-Inflammatory Cytokine Stimulation and Subsequent Activation of a Non-Canonical UPR in Pancreatic Beta Cells**

In light of the results discussed above, we next decided to revisit the possibility that HSP90 release from pancreatic beta cells may be related to cytokine-induced ER stress. In response to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ, βLox5 expression levels of the ER stress-associated markers *HSP90B1* (GRP94) and *XBP1s* were slightly increased relative to control cells, while *ATF3* expression increased approximately ten-fold; however, these increases were not as significant as the robust responses induced by thapsigargin treatment (**Figure 14, 29**). Similar results were observed with primary human islet cells (**Figure 16, 30**), although the results obtained with these cells were more variable and may reflect genetic differences among the individual donors. Overall these results suggest pancreatic beta cells may require a longer incubation with pro-inflammatory cytokines in order to fully induce the UPR. Others have shown significant increases in EndoC-βH1 expression levels of *XBP1s*, *DDIT3* (CHOP), and *HSPA5* (BiP) after 48 hours of treatment with IL-1β, TNF-α, and IFN-γ; however, these increases were all less than five-fold relative to control-treated cells (28).

Another possibility is that stimulation with IL-1β, TNF-α, and IFN-γ may induce a non-canonical form of the UPR in pancreatic beta cells. Classically, the UPR is thought of as a response to ER stress that increases the protein-folding capacity of the ER by upregulating expression of ER chaperones, such as GRP94 and BiP, while simultaneously blocking translation of proteins not involved in alleviating ER stress. However, this view is quickly becoming outdated, as more and more studies are identifying new roles for the UPR in regulating a variety cellular processes, including

metabolism, inflammation, and apoptosis (104). One such “non-canonical” UPR pathway is the activation of JNK through phosphorylation mediated by the UPR sensor IRE1 (**Figure 56**) (104).

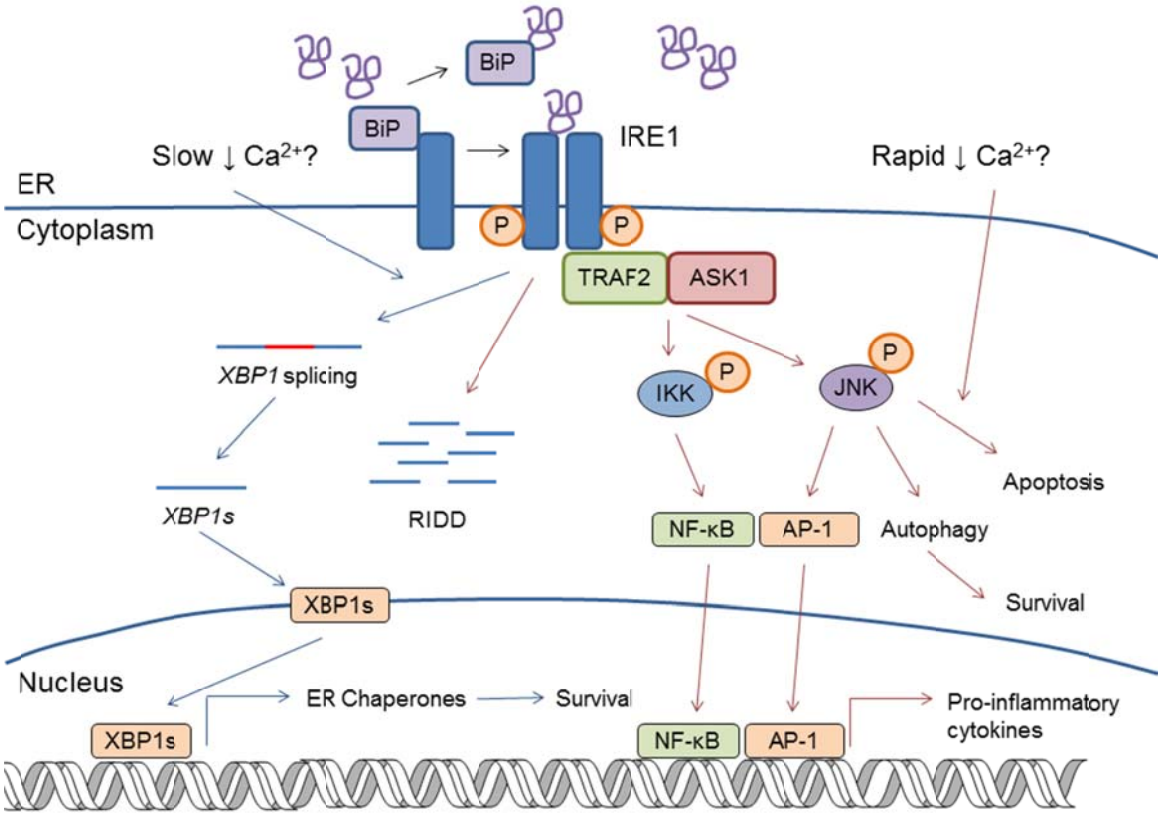


**Figure 56. Canonical and non-canonical IRE1 signaling.** Canonical IRE1 signaling, the unconventional, cytosolic splicing of *XBP1* mRNA by IRE1 endonuclease activity, is outlined with blue arrows. *XBP1* splicing results in translation of the XBP1s transcription factor, which promotes the transcription of genes encoding ER chaperones that restore ER homeostasis and promote survival during periods of ER stress. Non-canonical IRE1 signaling pathways are outlined in red arrows. These pathways include: mRNA degradation via RIDD, which is also mediated by IRE1 endonuclease activity; phosphorylation of IKK, which is mediated by TRAF2 and ASK1, and the subsequent activation of NF-κB and transcription of pro-inflammatory cytokine genes; and phosphorylation of JNK, also mediated by TRAF2 and ASK1. In the context of the UPR, JNK phosphorylation has been shown to promote the upregulation of pro-inflammatory cytokine genes through the activation of AP-1, survival via the induction of autophagy, and apoptosis. The different conditions that promote one signaling pathway over another are not understood but could include changes in ER $Ca^{2+}$ levels.

Once activated, IRE1 binds TRAF2, an adaptor protein that facilitates JNK activation through apoptosis signal-regulating kinase 1 (ASK1). In the context of the UPR, JNK activation has been shown to initiate inflammatory responses via AP-1 activation as well as promote survival by initiating autophagy. On the other hand, JNK activation downstream of IRE1 has also been shown to promote apoptosis in cells with unsurmountable levels of ER stress (**Figure 56**) (23; 24). Interestingly, a recent study showed that pro-inflammatory cytokine-mediated apoptosis of EndoC-βH1 cells was facilitated, at least in part, by activation of JNK. In this study, the authors also showed that siRNA-mediated knockdown of IRE1 reduced JNK phosphorylation in response to cytokine stress, suggesting that JNK was activated downstream of IRE1. In this same study, the authors showed that pre-treating EndoC-βH1 cells with the chemical chaperone TUDCA resulted in decreased levels of JNK phosphorylation and protection from cytokine-mediated apoptosis (28).

TUDCA, a bile acid that functions as a chemical chaperone and restores homeostasis during periods of ER stress, has been shown to prevent beta cell death and inhibit the development of TIDM in two mouse models of disease (82). In the present study, pre-treatment with TUDCA inhibited beta cell HSP90 release in response to cytokine stress (**Figure 32**). Therefore, these results suggest ER stress likely plays some role in HSP90 release from pancreatic beta cells in response to pro-inflammatory cytokine stimulation.

We next sought to determine whether or not TUDCA could inhibit JNK phosphorylation in response to cytokine stress, as was observed in the study by Brozzi, *et al* (28). We chose to look at JNK phosphorylation at two time points: 30 minutes and 24 hours following the addition of cytokines. We hypothesized that TUDCA would not inhibit JNK phosphorylation at the 30 minute time point, which we thought would be associated with JNK activation that occurs downstream of IL-1R1 and TNF-R1 signaling

(**Figure 1**), but that TUDCA would inhibit JNK phosphorylation 24 hours after the addition of cytokines, which we thought would be associated with the induction of ER stress and IRE1 activity (**Figure 4**). Surprisingly, pre-treatment with TUDCA inhibited JNK phosphorylation at 30 minutes and 24 hours following stimulation with IL-1β, TNF-α, and IFN-γ (**Figure 38**), suggesting that TUDCA may inhibit HSP90 release in response to cytokine stress by inhibiting JNK phosphorylation at both early and late time points.

Furthermore, pharmacological inhibition and siRNA-mediated knockdown of JNK inhibited HSP90 release in response to cytokine stress (**Figure 39, 42**). These results confirm that HSP90 release in response to pro-inflammatory cytokine stimulation was mediated, at least in part, by JNK activity in beta cells. However, whether or not JNK is activated downstream of IRE1 in response to cytokine stress is still unknown. The fact that pre-treatment with TUDCA can inhibit JNK phosphorylation in response to cytokine stress does suggest JNK activation and HSP90 release may occur downstream of UPR activation.

However, signaling through IL-1R1 and TNF-R1, the receptors for IL-1β and TNF-α, respectively, can also activate JNK activity (**Figure 1**). Signaling through these receptors can also activate other MAPK signal transducers, including p38. However, pre-treatment of βLox5 cells with SB202190, a pharmacological inhibitor of p38, did not inhibit HSP90 release in response to 24 hour stimulation with pro-inflammatory cytokines (**Figure 40**). Furthermore, pre-treatment with TUDCA did not affect p38 phosphorylation in response to 30 minute or 24 hour stimulation with IL-1β, TNF-α, and IFN-γ, although p38 phosphorylation was not sustained 24 hours after treatment as was JNK phosphorylation (**Figure 41**). Together, these results suggest HSP90 was not released in response to activation of p38 MAPK.

Stimulation with pro-inflammatory cytokines has been shown to deplete ER calcium stores in pancreatic beta cells (105). As discussed previously, the concentration

of ER $Ca^{2+}$ is critical for maintaining proper chaperone function and protein folding in the ER; thus, depletion of ER $Ca^{2+}$ results in ER stress and induction of the UPR (29). A potential future experiment would be to determine the kinetics of ER $Ca^{2+}$ depletion in response to cytokine stress in βLox5 cells and primary islets. If cytokines are able to deplete ER $Ca^{2+}$ and induce ER stress within 30 minutes, this result would perhaps explain why TUDCA was able to inhibit JNK phosphorylation following 30 minutes of cytokine stimulation. One could speculate that such a rapid depletion of ER $Ca^{2+}$ stores may promote an alternative, or non-canonical, activation of the UPR, perhaps mediated by JNK (**Figure 56**) (24).

Severe or prolonged UPR activation is known to favor apoptosis over cell survival. Some have postulated that differential activation of IRE1, PERK, and ATF6 may determine whether or not a cell undergoes apoptosis in response to ER stress. For example, reduced *XBP1* splicing via IRE1 endonuclease activity, which promotes survival, in favor of JNK activation via IRE1 kinase activity may promote apoptosis in the face of severe or prolonged ER stress (106). In the case of prolonged ER stress, CHOP is known to play an important role in ER-stress induced apoptosis (107). However, cytokine-mediated apoptosis in the rat beta cell line INS-1E was found to occur independently of the PERK-ATF4-CHOP axis of the UPR (108). Thus, stimulation with high concentrations of pro-inflammatory cytokines may promote a rapid depletion of ER $Ca^{2+}$ levels that induces an acute ER stress that favors IRE1-JNK-mediated apoptosis, rather than upregulation of canonical UPR mediators and cell survival (**Figure 56**). Another piece of evidence that supports this assertion is that stimulation of βLox5 cells with thapsigargin, which did induce expression of canonical UPR markers (**Figure 14, 29**), did not induce JNK phosphorylation (**Figure 43**).

Hence our current hypothesis is that pancreatic beta cells release HSP90 in response to an acute ER stress that favors JNK activation downstream of IRE1.

However, exactly how JNK activation promotes HSP90 release is still unclear at present. HSP90 release may be associated with the induction of autophagy or apoptosis, both of which occur downstream of JNK activation in the UPR (24). Alternatively, JNK activation may promote the upregulation of genes involved in unconventional pathways of protein secretion.

However, as mentioned previously, more experiments are needed to determine whether JNK activation occurs downstream of IRE1 activity in response to cytokine stimulation. To our knowledge, there is no pharmacological agent or mutation known to inhibit IRE1 kinase activity and subsequent JNK phosphorylation (107). Pre-treatment of βLox5 cells with the IRE1 endonuclease inhibitor 4μ8c increased the levels of HSP90 released in response to 24 hour stimulation with IL-1β, TNF-α, and IFN-γ. These results further suggest that canonical UPR signaling through the IRE1-XBP1s pathway does not promote HSP90 release, but rather, inhibiting IRE1 endonuclease activity with 4μ8c may have shifted downstream signaling in favor of IRE1-mediated JNK activation, thus increasing HSP90 release in response to cytokine stress. Future studies will be conducted to determine the effect of siRNA-mediated knockdown of *ERN1*, which encodes IRE1, on cytokine-induced JNK phosphorylation and HSP90 release from pancreatic beta cells.

Another piece of evidence that suggests pro-inflammatory cytokine stimulation promotes a non-canonical activation of the UPR in pancreatic beta cells is that pre-treatment with TUDCA, which alleviates ER stress, inhibited the upregulation of the interferon stimulated genes *ISG15* and *IFI27* (**Figure 35**). These results suggest transcription of these interferon stimulated genes is, at least in part, mediated by ER stress. Indeed several studies have established links between ER stress and subsequent activation of inflammatory pathways via the UPR (**Figure 56**) (23; 104).

However, how transcription of these genes is upregulated in response to stimulation with IL-1β, TNF-α, and IFN-γ (**Figure 35-36**) is presently unknown.

Alternatively, TUDCA may have had off target effects on beta cells, rather than specifically inhibiting of ER stress. For example, TUDCA may have blocked upregulation of *ISG15* and *IFI27* by interfering with STAT1 activation. Furthermore, TUDCA may have inhibited JNK phosphorylation independently of UPR activation and IRE1 activity. Future studies are needed to determine what other effects TUDCA may have on pancreatic beta cells.

One major drawback of using any pharmacological inhibitor is that these drugs may produce off-target effects within cells. Thus, HSP90 release may have increased further in response to cytokine stimulation with the addition of chetomin, 4μ8C, and SB202190 (**Figures 25, 37, and 40**) due to an association of these drugs with off-target proteins, rather than HIF-1α, IRE1, and p38, respectively. Future experiments in which these proteins are knocked down or overexpressed could be used to confirm the results obtained with these and other pharmacological inhibitors used in the present study.

Overall, results of these experiments have shown that HSP90 is released by human pancreatic beta cells in response to stimulation with a combination of IL-1β, TNF-α, and IFN-γ, as well as PIC, an agonist of TLR3, but not in response to thapsigargin, high glucose, or LPS, an agonist of TLR4. The chemical chaperone TUDCA was able to inhibit HSP90 release in response to stimulation with pro-inflammatory cytokines and PIC, suggesting HSP90 release was, at least in part, associated with ER stress. TUDCA also inhibited JNK phosphorylation in response to stimulation with pro-inflammatory cytokines. Both pharmacological inhibition and siRNA-mediated knockdown of JNK also inhibited HSP90 release in response to pro-inflammatory cytokine stimulation, suggesting HSP90 is also released in response to JNK activation in these cells (**Figure 57**). Our current hypothesis posits that JNK is activated downstream of a non-canonical

activation of the UPR sensor IRE1. More experiments are needed to determine whether PIC stimulation induces HSP90 release via a similar mechanism.

Thus, these experiments have provided a link between HSP90 release and a stress response known to induce apoptosis in pancreatic beta cells. Therefore, these results suggest HSP90 may be released from pancreatic beta cells during beta cell stress and latent TIDM in vivo and thus may serve as a biological marker of this pre-clinical phase of disease.

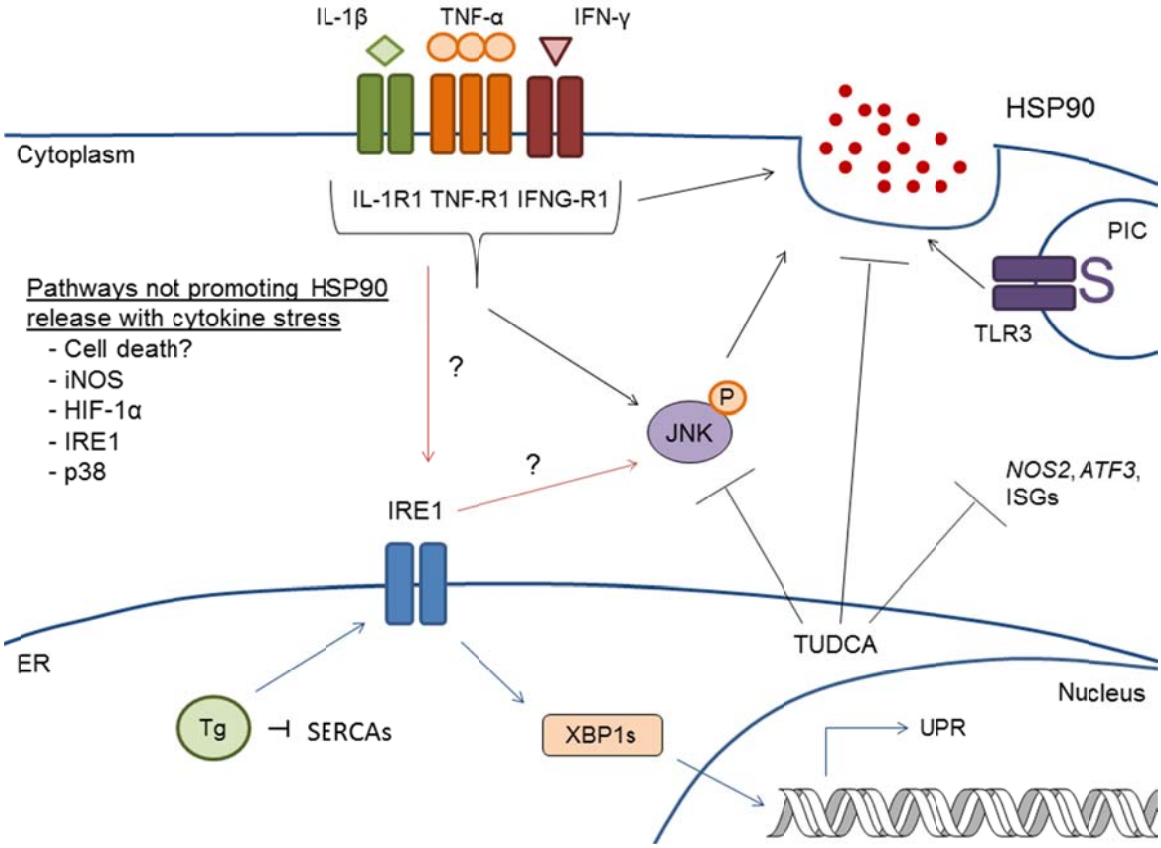


**Figure 57. Model of HSP90 release from pancreatic beta cells.** Studies here have shown HSP90 is released from pancreatic beta cells in response to stimulation with IL-1β, TNF-α, and IFN-γ as well as PIC. TUDCA, an ER stress-mitigating chemical chaperone, inhibited HSP90 release in response to these stimuli. TUDCA also inhibited JNK phosphorylation in response to stimulation with pro-inflammatory cytokines as well as the induction of *NOS2*, *ATF3*, and ISG expression. Pharmacological inhibition and siRNA-mediated knockdown of JNK also inhibited HSP90 release in response to pro-inflammatory cytokine stimulation. These results suggest HSP90 release was mediated, at least in part, by ER stress and JNK activation. Our current hypothesis is that JNK is activated downstream of a non-canonical activation of the UPR sensor IRE1 (red arrows). Treatment of beta cells with thapsigargin did not promote JNK phosphorylation or HSP90 release, as this SERCA inhibitor likely induces canonical IRE1 signaling and activation of XBP1s (blue arrows). Cell death is not thought to contribute to passive HSP90 leakage from cells, at least following 24 hours of stimulation with pro-inflammatory cytokines. Similarly, the activity of iNOS, HIF-1α, IRE1 endonuclease, and p38 do not promote HSP90 release from beta cells in response to cytokine stress.

**VI. Pros and Cons of Using HSP90 as a Biomarker of Latent TIDM**

One potential drawback of using serum HSP90 as a biomarker of pre-clinical TIDM is that it may not be a specific biomarker of *beta cell* stress, as this protein is expressed by all cell types. Elevated serum HSP90 may therefore represent a general indicator of cell stress, including malignant transformations associated with cancer. However, HSP90 is a highly abundant protein and can constitute up to one to two percent of the total protein level in cells. Thus, if a protein were to be detected in the serum as a marker of beta cell stress and death, HSP90 would likely be more readily detectable than GAD or some other beta cell-specific protein whose expression is much lower. Thus, HSP90 would likely be a good candidate for use in a TIDM biomarker panel with other markers that may be more difficult to detect but more beta cell-specific, such as proinsulin to C-peptide ratios.

**VII. Biological Function of Extracellular HSP90 in the Context of TIDM**

Our results here have shown that HSP90 can be released from pancreatic beta cells in response to stress responses known to be associated with latent TIDM in vivo. However, the biological function of extracellular HSP90 during beta cell stress and latent TIDM is unknown.

As discussed previously, nearly 30 years ago, HSPs purified from cancer cells were found to elicit immunity to tumors, and this immunogenicity was found to result from the non-covalent association of HSPs with tumor-specific antigens. These HSP-antigen complexes could be taken up by APCs through association with the HSP receptor CD91. This receptor-mediated uptake of HSP-antigen complexes both promoted antigen processing within the APCs and increased presentation of tumor-associated antigens to T cells in the context of MHC molecules (42).

More recently, association of HSP90 with antigen has been shown to enhance both MHC-I and MHC-II presentation to T cells by promoting antigen uptake through

scavenger receptor expressed by endothelial cells (SREC-I) (109; 110). SREC-I is a common HSP receptor that is expressed on the surface of dendritic cells that avidly binds HSP70, HSP90, GRP94, HSP110, and GRP170 with or without associated antigens (111). Moreover, studies from our lab were the first to demonstrate that HSP90 can associate with the TIDM autoantigen GAD and enhance its presentation (43); however, whether or not HSP90 can enhance autoantigen presentation and the development of TIDM in vivo remains unknown, but may represent an interesting avenue of future research.

Furthermore, our studies here suggest exosome secretion by pancreatic beta cells is increased with pro-inflammatory cytokine stimulation (**Figure 9**), and unpublished studies from Baekkeskov and colleagues have demonstrated similar findings. Exosomes are known to play important roles in intercellular communication and biological signaling (52). Interestingly, exosomes isolated from insulinoma cells have been shown to contain TIDM-associated autoantigens and to trigger autoimmunity in NOD mice (112). However, whether or not exosome-associated HSP90 can promote antigen presentation and autoimmunity in the context of TIDM remains unclear, but may represent another potential avenue of future research.

In the context of cancer, extracellular HSP90 has been shown to activate MMP-2, thus promoting tumor cell invasiveness (58). Interestingly, urine and plasma levels of MMP-2 were found to be elevated in patients with TIDM in studies from two independent groups (113; 114). However, one can only speculate the combined role of HSP90 and MMP-2 in the context of TIDM. Together, these proteins could promote immune cell infiltration (extravasation), islet remodeling, or immune cell exit from the islets (intravasation) (57). Future studies looking into these mechanisms would certainly merit investigation.

**VIII. HSP90 Chaperone Function in Vivo in the Context of TIDM**

Despite the fact that we have shown HSP90 is released by pancreatic beta cells in response to pro-inflammatory cytokine and PIC stimulation, the in vivo function of this protein in the context of disease progression is still unknown. Thus, to answer this question, we treated female NOD mice with the HSP90 inhibitor 17-DMAG and monitored the progression of TIDM over the course of eighteen weeks. Although treatment with 17-DMAG has previously shown beneficial effects in a number of autoimmune disease models (36; 37; 41), here 17-DMAG treatment had no effect on the incidence of TIDM in NOD mice (**Figure 54**). Furthermore, 17-DMAG treatment did not affect serum HSP90 levels in these animals. Although serum HSP90 levels increased as mice aged, both PBS- and 17-DMAG-treated animals exhibited similar levels of serum HSP90 throughout the course of the study (**Figure 46**). This result may reflect the islet stress detected in mice treated with 17-DMAG and may suggest HSP90 ATPase function is not required for its secretion.

Results of qRT-PCR experiments measuring *Hspa1b* expression levels indicated that some animals, although not the majority, may not have responded well to 17-DMAG treatment (**Figure 45 A**). However, 17-DMAG-treated mice that progressed to TIDM lost more weight than PBS-treated mice and 17-DMAG-treated mice that did not develop diabetes (**Figure 49**), suggesting 17-DMAG-treated diabetic mice may have been getting sicker than the other groups of mice. In the future, control CD1 mice could be treated with 17-DMAG to confirm that this effect was specific to diabetic mice.

Furthermore, islet cells from 17-DMAG-treated mice showed higher expression levels of stress-associated genes encoding insulin (**Figure 50**), ER stress markers (**Figure 51**), heat shock proteins (**Figure 52**), and autophagy proteins (**Figure 53**), suggesting pancreatic beta cell stress may have been more severe in 17-DMAG-treated

mice. Together, these results suggest HSP90 inhibition may have adverse effects on beta cell stress, dysfunction, and death in vivo during latent TIDM.

However, 17-DMAG treatment may have had adverse effects on other systems or cell types in the mouse rather than just the pancreatic islet cells alone. This assertion is supported by the fact that 17-DMAG-treated diabetic mice lost more weight than PBS-treated and 17-DMAG-treated non-diabetic mice. This weight loss could be indicative of some form of metabolic stress in these mice. Such metabolic stress could have been caused by adverse effects of 17-DMAG on the nervous, digestive, endocrine, hepatic, musculoskeletal, or other organ systems. For these reasons, HSP90 inhibition is likely a poor candidate for a potential TIDM preventative therapy.

Rather, these results suggest promoting HSP90 chaperone activity could be beneficial in the context of diabetes. A potential future experiment would be to overexpress HSP90 in NOD mice to determine whether or not HSP90 expression is able to inhibit the development or severity of TIDM in these animals.

We originally hypothesized that HSP90 inhibition with 17-DMAG would inhibit the development of TIDM in vivo by attenuating immune responses, as had been shown in other models of autoimmune disease (36; 37; 41). The authors of these studies proposed several mechanisms by which HSP90 inhibition could potentially attenuate autoimmune responses. For example, HSP90 is known to chaperone a number of molecules involved in intracellular inflammatory signaling cascades including phosphoinositide 3-kinase, mammalian target of rapamycin, and Akt as well as the transcription factor NF-κB. Thus, HSP90 inhibition may prevent signaling through these molecules during immune cell activation (36; 41).

Furthermore, HSP90 chaperone activity is required for activation of the non-receptor tyrosine kinase Lck following stimulation of the T cell receptor. Therefore, HSP90 inhibition may block T cell receptor signaling and subsequent T cell proliferation

(36; 41). Finally, inhibition of HSP90 is known to increase HSP70 expression through activation of the transcription factor HSF1. For this reason, HSP70 expression is commonly used as a marker for effective HSP90 inhibition. Interestingly, HSP70 has also been shown to have a negative feedback effect on NF-κB signaling; therefore, HSP90 inhibition may indirectly attenuate NF-κB-mediated inflammatory responses (36; 37; 41).

All of these proposed effects are mediated by inhibition of intracellular HSP90 within immune cells. However in TIDM, beta cell stress and death, particularly ER stress, is known to precede the development of disease in NOD mice (64). In the present study, HSP90 inhibition appeared to exacerbate beta cell stress responses, and these effects may have outweighed any immunomodulatory effects in the context of this particular disease model. Treatment with 17-DMAG may have exacerbated beta cell stress responses by inhibiting the ER chaperone GRP94, the ER-resident isoform of HSP90. Thus, treatment with 17-DMAG may have exacerbated ER stress responses in NOD mice. This conclusion is supported by increased expression of *Ddit3* (CHOP), *Hspa5* (BiP), and *Hsp90b1* (GRP94) in the islet cells of 17-DMAG-treated mice (**Figure 51**). An alternative approach to try in the future may be to use a drug that does not inhibit GRP94, such as TAS-166, a selective inhibitor of cytosolic HSP90α and HSP90β (94).

Given expression levels of various stress markers were increased with 17-DMAG treatment in the present study, one might expect that the incidence of TIDM should have been higher in 17-DMAG-treated mice compared to PBS-treated controls. The reason increased TIDM incidence was not observed with 17-DMAG treatment in light of these results is not clear at present. Treatment with 17-DMAG may possibly have exacerbated beta cell stress responses without affecting beta cell death and progression to TIDM.

Given that studies here have shown HSP90 is released from pancreatic beta cells during stress responses known to be associated with latent TIDM, supporting a

potential role for this protein as a biomarker of pre-clinical disease, another potential future approach would be to inhibit extracellular HSP90, but not intracellular, during the development of TIDM in vivo.

In the context of cancer, blocking extracellular HSP90 with antibodies or cell-impermeable inhibitors was shown to block cancer cell motility and invasion in vitro as well as tumor metastasis in vivo (56; 57). Extracellular HSP90 stimulation of cell migration in this context occurs through its interaction with CD91, and this property is independent of its ability to bind and hydrolyze ATP (56). This fact is not surprising, given concentrations of ATP outside the cell are quite low relative to concentrations inside the cell. In the context of TIDM, the function of extracellular HSP90 likely also does not depend on its ability to utilize ATP. Thus in the present study, 17-DMAG may not have had a significant effect on extracellular HSP90, as this drug inhibits the ability of HSP90 to bind and hydrolyze ATP (89; 90). Inhibition of extracellular HSP90, rather than intracellular, may have a different effect on the development of TIDM in vivo as it is currently unclear whether or not extracellular HSP90 is a risk factor for TIDM development in vivo.

**IX. Conclusion**

In vitro studies here are the first to show that HSP90 is released from pancreatic beta cells in response to specific inflammatory stimuli known to be associated with latent TIDM. Mechanistic studies further demonstrated that HSP90 release occurs downstream of acute ER stress and JNK activation. Therefore, these studies provide mechanistic evidence that pancreatic beta cells can release HSP90 in response to stress, thus supporting a role for this protein as a potential biological marker of latent TIDM. In vivo studies demonstrated that inhibition of HSP90 chaperone activity does not affect the overall incidence of TIDM in NOD mice, but rather exacerbates islet cell stress, suggesting inhibitors of HSP90 chaperone activity likely do not represent a viable

therapeutic option for the prevention of TIDM. Rather, a better approach may be to preserve HSP90 chaperone function in individuals at risk for developing TIDM.

## ONGOING STUDIES AND FUTURE DIRECTIONS

### I. HSP90 Release in Response to Cytokine Stress

Studies here have shown that HSP90 is released from pancreatic beta cells in response to stimulation with a combination of pro-inflammatory cytokines that included IL-1β, TNF-α, and IFN-γ. HSP90 release was found to occur downstream of non-canonical ER stress and JNK activation. To further elucidate the molecular pathways leading to HSP90 release during cell stress, studies are currently underway to determine whether or not JNK activation in response to cytokine stress occurs downstream of IRE1 activity. Human βLox5 cells will be transfected with siRNA targeting *ERN1*, which encodes IRE1, to knockdown expression of this protein. Transfected cells will be treated with pro-inflammatory cytokines, and levels of JNK phosphorylation will be analyzed by immunoblotting. Additionally, HSP90 release will be measured by ELISA. These studies may reveal a target upstream of JNK which could be disrupted to modulate beta cell stress.

A role for IRE1 and JNK in non-canonical UPR activation will then be confirmed by monitoring expression levels of *ATF3*, *XBP1s*, *NOS2*, *IL6*, *ISG15*, and *IFI27* by qRT-PCR in cytokine-treated βLox5 cells treated with siRNA targeting JNK or IRE1. These experiments will determine whether or not knockdown of these UPR signaling components affects expression levels of these genes in a manner similar to TUDCA, thus confirming a role for this ER stress pathway in regulating beta cell responses to pro-inflammatory cytokines.

Given that experiments with beta cell lines may not always accurately recapitulate what occurs in primary cells, ideally, experiments performed here examining the effects of TUDCA, JNK inhibition, and siRNA-mediated knockdown of JNK and IRE1 on HSP90 release, cell signaling, and gene expression in response to stimulation with IL-1β, TNF-α, and IFN-γ should also be performed with primary human cadaveric islet

cells. These experiments would confirm the results obtained here with human beta cell lines, thus ensuring the biological and translational significance of this mechanistic pathway (59).

**II. Mechanism of HSP90 Release**

Studies here have demonstrated that HSP90 released from beta cells in response to cytokine stress is mostly soluble and not associated with exosomes. Furthermore, soluble HSP90 was released prior to detection of significant levels of cell death, as measured by LDH assay, suggesting HSP90 may be released by a specific secretory mechanism. One study recently showed that HSP70 is secreted from tumor cells by a mechanism independent of de novo HSP70 synthesis and cell death, similarly to what was observed here with HSP90 (100).

In this study, ammonium chloride, which inhibits lysosome function by raising lysosomal pH, inhibited HSP70 release from prostate carcinoma cell lines in response to heat shock. Furthermore, HSP70 secretion from these cells correlated with the appearance of the lysosomal marker LAMP-1 on the cell surface. Thus, these authors proposed that HSP70 was secreted from endolysosomal compartments (100). Studies with ammonium chloride in βLox5 cells will be performed to determine whether or not HSP90 release in response to cytokine stress occurs via a similar mechanism. Additionally, surface levels of LAMP-1 will be examined by flow cytometry.

**III. HSP90 Release in Response to PIC**

Thus far, studies here have shown HSP90 is released from pancreatic beta cell lines in response to stimulation of TLR3 with PIC. Pre-treatment with TUDCA for 6 hours partially inhibited HSP90 release in response to PIC stimulation, suggesting that HSP90 release in response to PIC was mediated, at least in part by ER stress, as was observed with pro-inflammatory cytokine stimulation. Based on these results, we hypothesize that HSP90 is released in response to JNK activity downstream of UPR activation with PIC

stimulation, as was observed with pro-inflammatory cytokine stress. Similar studies that were performed with cytokine treatment are needed to confirm whether or not HSP90 release in response to PIC occurs via this same mechanism. The effect of TUDCA on JNK phosphorylation in response to PIC stimulation will be examined by immunoblotting in βLox5 cells. Similarly, HSP90 release in response to PIC treatment will be measured by ELISA with pharmacological inhibition and siRNA-mediated knockdown of JNK. As with cytokine treatment, results obtained with PIC treatment of βLox5 cells should also be confirmed with primary human cadaveric islet cells.

**IV. HSP90 as a Biomarker of TIDM in Vivo**

Previous studies from our lab have shown that serum levels of HSP90 are elevated in newly diagnosed patients with TIDM (31). Our studies here helped confirm that this protein may be able to serve as a biological marker of latent TIDM by demonstrating that HSP90 is released by pancreatic beta cells in response to stress stimuli known to be associated with latent TIDM.

More in vivo studies are needed, however, to confirm that HSP90 can function as a biomarker of latent TIDM. Currently, serum is being collected from NOD mice as well as control CD1 mice over time for HSP90 ELISA as the NOD mice progress toward TIDM. Results of these experiments will determine whether or not HSP90 levels are increased in the serum of animals that develop TIDM, similarly to what was observed in humans (31). Islet cell HSP90 levels will also be examined in pancreatic sections from these animals by immunohistochemistry staining. These experiments will help confirm that HSP90 is being released from islet cells over time as the NOD mice progress toward TIDM.

If serum HSP90 levels are found to be increased in NOD mice that develop diabetes compared to non-diabetic CD1 controls, we next would like to examine the effect of blocking diabetes development in NOD mice with TUDCA on serum HSP90

levels. Treatment with TUDCA was previously shown to inhibit the development of TIDM in NOD mice (82). If preventing TIDM in NOD mice with TUDCA was shown to lower serum HSP90 levels, these results would help confirm that this protein can serve as a biomarker for latent TIDM in mice.

If true, these results will next need to be confirmed in human subjects. Currently, clinical trials are underway to test the effects of TUDCA treatment on TIDM development in patients with new onset TIDM. We would like to obtain serum samples from these patients in order to determine the effects that TUDCA treatment has on serum HSP90 levels in human subjects in order to confirm that HSP90 can function as a biomarker of latent TIDM. If true, serum HSP90 levels could be measured in the clinic to identify individual patients at risk for progressing to TIDM, and treatments aimed at preventing this disease could be more appropriately administered.

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109. Murshid A, Gong J, Calderwood SK: Heat shock protein 90 mediates efficient antigen cross presentation through the scavenger receptor expressed by endothelial cells-I. Journal of immunology (Baltimore, Md : 1950) 2010;185:2903-2917

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**CURRICULUM VITAE**

**GAIL J. OCAÑA**

## EDUCATION

| | |
|---|---|
| 2011-2016 | Doctor of Philosophy, Indiana University (IUPUI)<br>Major: Microbiology and Immunology<br>Minor: Life Sciences |
| 2007-2011 | Bachelor of Science, Indiana University (Bloomington)<br>Major: Microbiology<br>Minors: Chemistry, Spanish |

## RESEARCH AND TRAINING EXPERIENCE

2011-2016 Graduate student, Indiana University (IUPUI)

Dissertation title: Heat Shock Protein 90, a Potential Biomarker for Type I Diabetes: Mechanisms of Release from Pancreatic Beta Cells

Mentor: Janice S. Blum, Ph.D.

Thesis committee: Mark Kaplan, Ph.D., C. Henrique Serezani, Ph.D., Jie Sun, Ph.D.

Summary: The main focus of my dissertation research was aimed at understanding stress responses in human pancreatic beta cells that contribute to the development of type I diabetes mellitus. We were particularly interested in understanding why beta cells release heat shock protein 90 in response to inflammatory stimuli. We believe serum levels of this protein could be used as a biomarker for pre-diabetes in the clinic.

2009-2011 Undergraduate researcher, Indiana University (Bloomington)

Mentor: Daniel B. Kearns, Ph.D.

Summary: My undergraduate research utilized genetic approaches to investigate swarming motility in the soil bacterium *Bacillius subtilis*.

## PUBLICATIONS

1. **Ocaña, G.J.**, Guindon, L., Pérez, L., Evans-Molina, C., and J.S. Blum. (2016). Inflammatory stress of pancreatic beta cells drives the release of extracellular HSP90, a biomarker for type I diabetes. *In preparation.*
2. **Ocaña, G.J.**, Guindon, L., and J.S. Blum. (2016). Pharmacological inhibition of

heat shock protein 90 exacerbates islet cell stress during the development of type I diabetes in vivo. *In preparation.*

3. Pérez, L., McLetchie, S., **Gardiner, G.J.**, Deffit, S.N., Zhou, D., and J.S. Blum. (2016). LAMP-2C inhibits MHC class II presentation of cytoplasmic antigens by disrupting chaperone-mediated autophagy. *J Immunol*.
4. **Gardiner, G.J.**, Deffit, S.N., and Blum, J.S. (2015). Antigen processing. In: eLS. John Wiley & Sons, Ltd: Chichester.
5. Yang X., Zhu J., Tung C.Y., **Gardiner G.**, Wang Q., Chang H.C., and Zhou, B. (2014). Lunasin alleviates allergic airway inflammation while increases antigen-specific tregs. *PLoS One*.
6. **Gardiner, G.J.**, Deffit, S.N., McLetchie, S., Perez, L., Walline, C.C., and Blum, J.S. (2013). A role for NADPH oxidase in antigen presentation. *Front Immunol*.
7. Wu, Q., **Gardiner, G.J.**, Berry, E., Wagner, S.R., Lu, T., Clay, B.S., Moore, T.V., Ferreira, C.M., Williams, J.W., Luster, A.D., Medoff, B.D., Cannon, J.L., Sperling, A.I., and Shilling, R.A. (2013). ICOS-expressing lymphocytes promote resolution of CD8-mediated lung injury in a mouse model of lung rejection. *PLoS One*.

**CONFERENCES ATTENDED**

1. **Ocaña, G.J.**, Guindon, L., Pérez, L., Evans-Molina, C., and J.S. Blum. (2016). Heat shock protein 90, a potential biomarker for type I diabetes, is released by human pancreatic beta cells in response to cytokine stress. Midwest Islet Club. Indianapolis, IN. Poster presentation.
2. **Gardiner, G.J.**, Guindon, L., Evans-Molina, C., and J.S. Blum. (2015). Heat shock protein 90, a potential biomarker for type I diabetes, is released by human pancreatic beta cells in response to cytokine stress. IU Center for Diabetes and Metabolic Diseases Diabetes Symposium. Indianapolis, IN. Poster presentation.
3. **Gardiner, G.J.**, Guindon, L., Pérez, L., Evans-Molina, C., and J.S. Blum. (2015). Heat shock protein 90, a potential biomarker for type I diabetes, is released by human pancreatic beta cells in response to cytokine stress. AAI Annual Meeting. New Orleans, LA. Poster presentation.
4. **Gardiner, G.J.**, Pérez, L., Dinauer, M.C., and J.S. Blum. (2014). Leukocyte NADPH oxidase modulates MHC-II antigen presentation in B lymphocytes. Gordon Research Conference – Nox Family NADPH Oxidases. Barga, IT. Poster presentation.
5. **Gardiner, G.J.**, Pérez, L., Dinauer, M.C., and J.S. Blum. (2014). Leukocyte NADPH oxidase modulates MHC-II antigen presentation in B lymphocytes. Gordon Research Seminar – Nox Family NADPH Oxidases. Barga, IT. Oral and poster presentation.
6. **Gardiner, G.J.**, Wu, Q., Wagner, S.R., and R.A. Shilling. (2013). ICOS-expressing Treg are required for resolution of CD8-mediated lung injury. AAI Annual Meeting. Honolulu, HI. Poster presentation.

7. **Gardiner, G.J.**, Wu, Q., Wagner, S.R., and R.A. Shilling. (2012). ICOS expression promotes proliferation, but not survival, of regulatory T cells. Great Lakes Transplant Immunology Forum. Madison, WI. Poster presentation.

## HONORS, AWARDS, FELLOWSHIPS

| | |
|---|---|
| 2013-2016 | NIH Pre-Doctoral Fellowship T32 “Basic Sciences on Gene Therapy of Blood Diseases” (P.I. – Hal E. Broxmeyer, Ph.D.) |
| 2015 | Indiana University Diabetes Symposium Poster Award |
| 2015 | Harold Raidt Graduate Student Teaching Award, Department of Microbiology and Immunology, Indiana University (IUPUI) |
| 2015 | Graduate-Professional Educational Grant Travel Award, Indiana University (IUPUI) |
| 2015 | University Graduate School Travel Fellowship, Indiana University (IUPUI) |
| 2014 | GRS Nox Family NADPH Oxidases Travel Award |
| 2013-2014 | Indiana University Simon Cancer Center Merilyn Hester Scholarship |
| 2013 | Best First Time Presenter – Research in Progress Awards, Department of Microbiology and Immunology, Indiana University (IUPUI) |
| 2011 | University Fellowship, Indiana University (IUPUI) |
| 2010 | Phi Beta Kappa, Indiana University (Bloomington) |
| 2010 | L.S. McClung Scholarship in Microbiology, Department of Biology, Indiana University (Bloomington) |
| 2007 | Outstanding Student in C117 – Principles of Chemistry and Biochemistry, Department of Chemistry, Indiana University (Bloomington) |
| 2007 | Hutton Honors College, Indiana University (Bloomington) |
| 2007 | Lilly Endowment Community Scholarship (Fayette County, IN) |

## PROFESSIONAL EXPERIENCE

| | |
|---|---|
| 2010 | Summer Intern in Research and Development, The Procter and Gamble Company, Cincinnati, OH |

| | |
|---|---|
| 2011 | Undergraduate Research Associate, Department of Biology, Indiana University (Bloomington) |

## TEACHING EXPERIENCE

| | |
|---|---|
| 2014 | Graduate Teaching Assistant, Department of Microbiology and Immunology, Indiana University (IUPUI) |
| 2009-2010 | Undergraduate Teaching Assistant, Department of Biology, Indiana University (Bloomington) |

## MEMBERSHIPS

| | |
|---|---|
| 2013-2016 | American Association of Immunologists (AAI) |

## UNIVERSITY SERVICE

| | |
|---|---|
| 2013-2016 | Volunteered as a student ambassador during campus visits for interviewing graduate students |
| 2012-2015 | Presented *Think, Talk, and Study like a Graduate Student* workshop to incoming graduate students |
| 2012-2015 | Volunteered as a peer mentor for incoming graduate students |

## COMMUNITY SERVICE

| | |
|---|---|
| 2014-2016 | Volunteered with Operation Leftover providing food, clothing, and fellowship to our homeless neighbors in downtown Indianapolis |
| 2014-2016 | Volunteered with Gennesaret Free Clinics at St. Vincent de Paul Food Pantry and St. Mary's Catholic Church helping provide free medical care to those in need |