THE ROLE OF HSPs IN MHC CLASS II PRESENTATION OF SELECT ANTIGENS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Microbiology and Immunology,
Indiana University

April 2009

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

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This work is dedicated to my father and mother whose sacrifices and unconditional love have made this all possible.

ACKNOWLEDGEMENTS

I would like to extend my heartfelt gratitude to the many people who have helped me make this dream a reality. I would like to thank God for His many blessings and love; He has always provided for me in every way. I would also like to thank my family and friends who have been a constant stream of love and support over the years. Their unwavering faith never failed to restore my own. Thanks for helping me keep my perspective and never forget who I really am. My love for you all is more than I can ever express. I would like to extend special thanks to my husband Justin. I am eternally grateful for his assurance, strength, and love. I look forward to traveling this journey of life with him by my side. My deepest appreciation goes to my parents who have taught me that anything can be achieved with hard work, dedication, perseverance, and faith. I would like to thank my grandparents for their steadfast love and comfort which has nourished my soul. I am extremely grateful to my big sis, Traci, who has always been the first to have my back regardless. She has been right beside me during the good and the bad times, and has always encouraged me to keep on keepin' on. To Steve, I am thankful for his comic relief that never fails to make me laugh. To Morgan and Tyler, I send a big thanks for keeping me forever 21. To my fellow MP's (Jennifer, Andrea, Brandi, Alicia, and Misty) who have been my girls since 1995, thanks for all the laughter and good times. To Tom, thanks for all the great memories.

I would also like to extend my sincere gratitude to my mentor, Dr. Janice Blum, for her guidance, support, advice, and friendship. She has continually encouraged me to be brave, strong, and believe in my abilities. I would also like to thank the members of my graduate advisory committee (Drs. Brutkiewicz, Klemsz, and Thurmond) for all their support, encouragement, and guidance that they have shown me throughout my graduate career. I am also thankful for my labmates who have provided me with constructive criticism, advice, support, and friendship all these years. And finally I would like to thank my fellow grad student friends, especially Kristy whose always been there for me with a cup of coffee and a listening ear, Joanna whose humor and faith have always lifted me up, and Nan whose late night pep talks and camaraderie have meant so much.

ABSTRACT

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THE ROLE OF HSPs IN MHC CLASS II PRESENTATION OF SELECT ANTIGENS

The function of major histocompatability complex (MHC) class II molecules is to present antigenic peptides to CD4+ T cells. Typically, MHC class II molecules present peptides derived from exogenous sources. Yet, certain endogenous antigens (Ags) have been found to be presented by class II molecules. Studies suggest that specific heat shock protein family members may play a role in Ag processing and subsequent class II presentation. The studies presented here using B lymphoblasts demonstrate the importance of HSP90α, HSP90β, and possibly HSP70 in selectively regulating MHC class II presentation.

Inactivation of HSP90 function using pharmacological inhibitors inhibited class II presentation of exogenous and endogenous GAD, but did not perturb the presentation of several other intra- and extracellular Ags. Individual knockdown of HSP90 isoforms using isoform specific siRNA selectively inhibited GAD Ag presentation. These results demonstrate a requirement for HSP90α and HSP90β in regulating MHC class II presentation of select Ags.

Studies to explore mechanistically the roles of HSP90α and HSP90β in regulating GAD Ag presentation were pursued. The pathways of exogenous and endogenous MHC class II presentation of GAD Ag are distinct yet converge with shared terminal processing of GAD within endosomal/lysosomal vesicles. The effect of HSP90 manipulation on various shared components of the MHC class II pathway was examined. The studies presented here suggest that HSP90α and HSP90β regulate MHC class II presentation of GAD Ag at discrete steps most likely involving HSP90 binding to GAD Ag rather than perturbing overall MHC class II function.

Studying the role of HSP90 in MHC class II presentation in B cells revealed the potential requirement for HSP70 in the presentation of select Ags. The studies presented here demonstrate a possible role for HSP70 in the presentation of Ags such as SMA or Ig kappa by MHC class II molecules.

Also included in this work is a study of a rare case of diabetes caused by type B insulin resistance due to development of insulin receptor autoantibodies during the treatment of hepatitis C with interferon alpha and ribavirin. Clinical and laboratory findings in the case are presented.

Janice S. Blum, Ph.D.

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ABBREVIATIONS

Ab	Antibody
AEP	Asparagine endopeptidase
Ag	Antigen
AMC	Aminomethylcoumarin
APC	Antigen presenting cells
β2M	β_2 microglobulin
β-ME	β mercaptoethanol
BiP	Binding immunoglobulin protein
B-LCL	B-lymphoblastoid cell line
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHO	Chinese hamster ovary cells
CLIP	Class II-associated invariant chain peptide
CMA	Chaperone mediated autophagy
CPM	Counts per minute
DC	Dendritic cell
ddH_2O	Double distilled water
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Epichemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum

ERAD	ER-associated degradation
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GA	Geldanamycin
GABA	Gamma-aminobuturic acid
GAD	Glutamate decarboxylase
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HSA	Human serum albumin
HSC	Heat shock constitutive
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IR	Insulin receptor
LAMP	Lysosome-associated membrane protein
LCMV	Lymphocytic choriomeningitis virus
LIP	Leupeptin induced peptide
mAb	Monoclonal antibody
MBP	Myelin basic protein
MHC	Major histocompatibility complex

MIIC	MHC class II compartment
OVA	Ovalbumin
NaCl	Sodium chloride
NaN_3	Sodium azide
NK	Natural killer
NOD	Non-obese diabetic
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PGS	Protein G Sepharose
PMN	Polymorphonuclear
PMSF	Phenylmethylsulfonylflouride
RA	Radicicol
SDS	Sodium dodecyl sulfate
SLIP	Small leupeptin induced peptide
TAP	Transporter associated with antigen processing
TEC	Thymic epithelial cell
TID	Type I diabetes
TNF	Tumor necrosis factor
TLCK	Nα-p-tosyl-l-lysinechloromethyl ketone
UPR	Unfolded protein response

INTRODUCTION

The immune system

The human body is constantly challenged by numerous foreign agents such as bacteria, viruses, fungi, and parasitic worms. Some of these invading microorganisms are infectious or pathogenic, and their own survival can lead to the death of the individual host. To ensure protection from these invading pathogens, the human body has evolved a defense network known as the immune system to identify and destroy potential threats. The immune system consists of multiple, biological effector mechanisms, but is generally divided into two branches, the innate immune system and the adaptive immune system.

The innate immune system is widely known as the first line of defense against invading pathogens. A foreign invader first encounters the host's barrier defenses, which are key to innate immunity. Most simply, structures such as the skin and mucous coated membranes provide a physical barrier which the invading agent must breach to gain entry into the body. Additionally, the innate immune system employs chemical barriers to disarm disease causing agents. In the stomach, gastric acid lowers the pH and inhibits the growth of many microorganisms while lysozyme in mucous, tears, and saliva can damage bacterial cell walls. If pathogens do breach the body's physical and chemical barriers, the innate immune system can launch an immediate, but non-specific response. Immune cells such as natural killer (NK) cells, macrophages, and neutrophils are recruited to the primary infection site. These cells function to capture and destroy invading pathogens as well as to clear damaged or infected host cells. These innate immune cells can efficiently clear pathogens or keep them in check until more specialized reinforcements arrive.

Following the action of the innate response, the more specialized cells of the adaptive immune system are recruited. A hallmark of the adaptive immune system is the ability to recognize antigens (Ags) found on or within pathogens. One group of specialized cells of the adaptive immune system responsible for this pathogen specific recognition are called lymphocytes. Lymphocytes are divided into B and T cells based on their distinct locations for development and their response to internal and external signals. Both B and T cells recognize specific pathogenic targets via cell surface receptors. The B cell

receptor is a cell surface bound antibody (Ab) and can recognize either whole or processed Ag. In contrast, the T cell receptor can only recognize processed Ags when coupled with a self major histocompatability complex (MHC) molecule.

B cells function as professional antigen presenting cells (APCs), processing whole Ag into antigenic fragments and displaying those fragments via MHC molecules to T cells. In addition to Ag processing and presentation, B cells are responsible for Ab secretion. Secreted Abs identify and neutralize extracellular foreign Ags or tag them for attack by other immune cells. B cells also play key roles in the memory response to Ags. Upon pathogenic infection, certain memory B cells are generated and retained in the body after the infection is cleared. Each memory B cell recognizes antigenic epitopes on a specific pathogen. Upon re-infection with that same pathogen, subsets of memory cells recognizing these epitopes will rapidly proliferate and differentiate into effector cells able to rapidly clear the pathogenic infection (1).

T cells are key players in the immune response functioning in Ag recognition, targeted cell destruction, and regulating the immune system. T cells are further divided into two major groups, CD4+ and CD8+ T cells, based on their surface co-receptors. Both these groups recognize their target antigenic peptides in the context of different classes of MHC molecules. Naïve CD4+ T cells recognize specific antigenic peptides in the context of MHC class II molecules, while CD8+ T cells recognize specific antigenic peptides in the context of MHC class I molecules. Upon antigenic recognition, naïve CD4+ T cells differentiate into effector CD4+ T cells. As effector cells, CD4+ T cells secrete cytokines that stimulate B cells to differentiate and produce specific antibodies against extracellular pathogens and activate macrophages to specifically kill infected cells. Effector CD8+ T cells are cytotoxic T cells that search for and kill infected cells.

Two other types of APCs that play critical roles in the immune system are known as dendritic cells (DCs) and macrophages. Macrophages are a key component of the innate immune system. They are widely distributed in the body and are immediately available to combat a variety of pathogens without requiring prior exposure. At the site of

infection, they function as key APCs, initiate inflammation, and recruit additional immune cells. DCs play a key role in bridging the gap between innate and adaptive immunity. Immature DCs migrate from the blood into various tissues and specialize in phagocytosis. When they encounter an Ag, they rapidly mature and migrate to lymphoid tissues where they specialize in Ag presentation to T cells.

Autoimmune type I diabetes and GAD

The goal of the immune system is to protect and defend the human body from foreign invaders. This requires the ability to distinguish self from non-self. However, sometimes the immune system loses this ability and begins to recognize self Ags as non-self, resulting in the body attacking itself. This loss of self-recognition results in the development of autoimmunity. Currently, there are over 70 autoimmune diseases ranging from well known disorders such as multiple sclerosis to lesser known diseases such as scleroderma. Interestingly, studies show that autoimmune diseases are much more common among women than men (2). Research has yet to determine the exact events that result in the loss of self-tolerance and the development of autoimmune diseases. Studies suggests that there are genetic components involved, as well as the possibility of microbial triggers (3, 4).

Type I diabetes (TID) is one of the most common autoimmune disorders affecting approximately 1 in every 300 children (5). The rising incidence of TID worldwide suggests the need for a better understanding of the pathogenesis of TID in order to develop prevention and treatment strategies. Current studies are attempting to develop cell based therapies based on either replacement of pancreatic islet cells with islet-like cells derived from embryonic or adult stem cells or re-establishing immunological tolerance to self Ags using regulatory T cells and other tolerance promoting cells (6). The only effective therapy available involves the lifetime requirement for daily injections of exogenous insulin, which treats the symptoms of the disease, but is not a cure. An alternative therapy is whole pancreas or islet transplantation, but this treatment is reserved only for severely ill patients and does not typically cure disease.

TID is characterized by the selective destruction of the insulin-producing, pancreatic β cells by the body's own immune system. The pathogenesis of TID has been extensively studied; however, the exact mechanisms involved in the initiation and progression of the disease is unclear. One hallmark of disease progression is the presence of insulitis, the infiltration of the pancreas by immune cells. Animal studies indicate that macrophages and DCs are among the first infiltrating cells (7). Autoreactive CD4+ and CD8+ T cells have also been shown to infiltrate the pancreatic β cells and mediate islet destruction (5, 7-9). One of the earliest signs of disease progression is the appearance of islet-reactive antibodies (10-12). Studies have found B lymphocytes play an important role in the pathogenesis of TID, functioning as APCs in the autoimmune response to islet cell Ags (13). In both rodents and humans, genetic susceptibility and also resistance to TID is strongly linked to the inheritance of certain MHC class II alleles (14). However, it is still somewhat controversial whether resident as well as recruited MHC class II-positive cells function to present islet cell Ags in the islets of TID (14).

Glutamic acid decarboxylase (GAD) has been identified as a key target autoantigen in the development of TID (10, 12). GAD is an enzyme involved in the synthesis of gamma-aminobuturic acid (GABA), a key downregulating neurotransmitter in the central nervous system. There are two isoforms of GAD differentiated by their molecular weight, GAD65 and GAD67. Both isoforms are detected in the brain, but GAD65 appears to be the predominant form within the pancreas. Research has clearly determined the role of GAD and GABA in the brain, but the role of GAD in the pancreas has yet to be understood (10). Within β cells and neural cells, the GAD protein associates with intracellular membranes due to palmitoylation occurring at the N-terminus (15).

GAD was originally identified as a diabetes autoantigen when studies showed that 85% of newly diagnosed TID patients contain antibodies directed against this protein (16). Since this first observation, much research has focused on determining a role for GAD in TID. Unfortunately, it is controversial as to how central a role GAD plays in TID pathogenesis. Some studies in young NOD mice found that immunization with purified GAD65 protein can induce tolerance preventing insulitis and diabetes while

immunization with other autoantigens conferred only partial protection (17, 18). However, another study found that immunization with GAD Ag resulted in only delayed onset of diabetes (19). Studies have identified specific epitopes within the GAD protein that are highly immunogenic and can activate T lymphocytes (20-22). Moreover, GAD-reactive CD4+ Th1 cells were shown to induce diabetes in NOD/SCID mice (23). While the majority of studies conclude that GAD plays a key role in TID development, there are a few studies that suggest otherwise. One study found isolated GAD-specific T cell clones that do not induce diabetes (24). Another study found in NOD mice that β-cell specific suppression of GAD expression prevented diabetes progression (25). Moreover, an additional study found that even GAD65 knockout mice developed diabetes (26). It has been speculated that the controversy in determining the importance of GAD in TID pathogenesis is due to differences in laboratory techniques, substrains of NOD mice, and the preparation of GAD Ag, GAD peptide, and GAD-reactive T cell clones (27). Although there are a few studies that disagree, a majority of studies support a role for GAD in TID. However, the exact role GAD plays in pathogenesis has yet to be defined.

Previous studies in our lab have found that GAD Ag can be processed and presented by B cells. Current and future studies are focused on determining the molecular mechanisms involved in GAD Ag processing by B cells. Understanding how APCs process and present GAD to CD4+ reactive T cells could provide clues as to how GAD is involved in TID.

MHC class I and class II molecules

MHC class I molecules are surface, transmembrane glycoproteins consisting of an α heavy chain associated with a small subunit known as β_2 microglubulin (β2M) (Fig. 1). MHC class I molecules are expressed on all nucleated cells. The peptide binding groove of MHC class I molecules is closed; thus the peptides presented by MHC class I molecules typically range between 8-12 amino acids in length. There are two anchor residues within the binding groove located at the carboxyl terminus and at an internal residue (28).

MHC class II molecules are also transmembrane glycoproteins, but they are comprised of an α heavy chain and a β heavy chain (Fig. 1). Unlike MHC class I molecules, MHC class II molecules are selectively expressed by only certain cell types. Primarily, MHC class II molecules are expressed on professional APCs which include B cells, macrophages, and DCs. However, their expression on additional cell types can be induced. The binding groove of MHC class II molecules is open-ended allowing for the binding of longer peptides up to 24 amino acids in length (29).

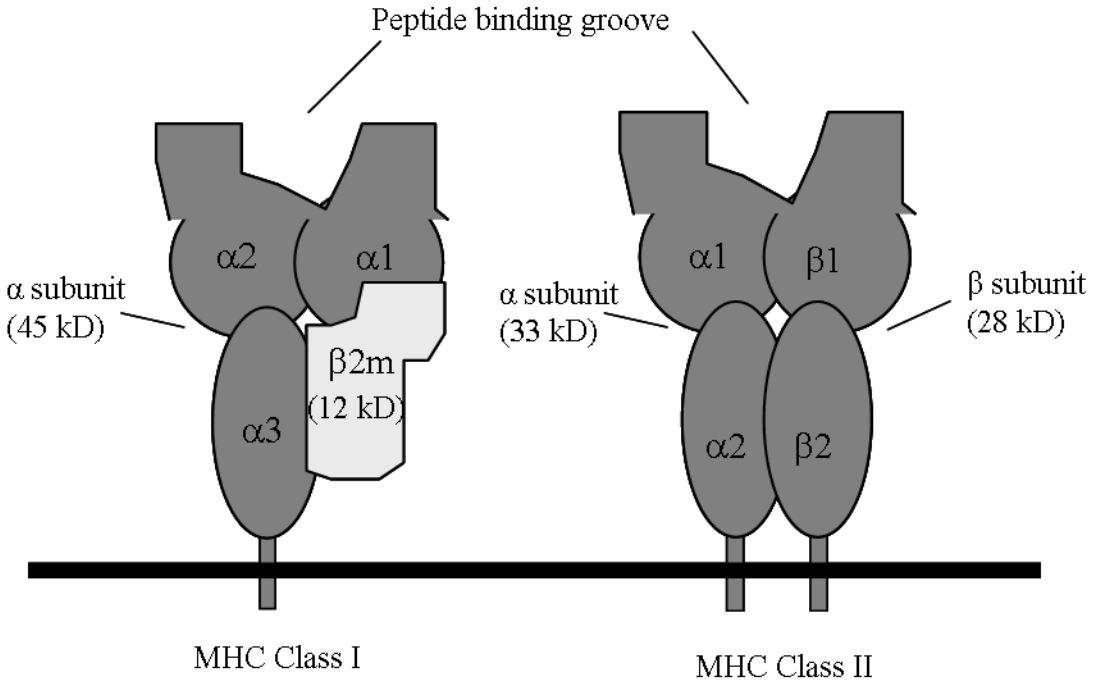


Figure 1. The basic structure of MHC molecules. *Left,* The MHC class I molecule is a heterodimer composed of a 45 kD α subunit consisting of 3 domains and a 12 kD β_2 microglobulin (β2M) subunit. Within the α subunit, the N-terminus α1 and α2 domains form the membrane-distal region of the complex and the peptide binding groove. The C-terminal α3 domain forms the membrane-proximal domain linked to the transmembrane, and cytoplasmic regions of the complex. *Right,* The MHC class II molecule is a heterodimer composed of a 33 kD α subunit and a 28 kD β subunit. The peptide binding groove is formed by the N-terminal regions of both subunits, α1 and β1 domains. In contrast to MHC class I molecules, the C-terminal domains of both subunits, α2 and β2, are linked to the transmembrane and cytoplasmic regions of the MHC class II molecule.

In humans, both MHC class I and II molecules are encoded by genes within the MHC locus on chromosome 6. Human MHC class I molecules are encoded by three predominant allele families: HLA-A, HLA-B, and HLA-C. Human MHC class II molecules are encoded by three sets of genes: HLA-DR, HLA-DQ, and HLA-DP. MHC molecules are expressed in a co-dominant manner. The genes encoding the MHC molecules are the most polymorphic genes known to date with hundreds of alleles already characterized (30). The polymorphism and co-dominant expression of MHC molecules results in great genetic diversity among humans with regards to their HLA type.

Classical MHC class I and class II presentation pathways

Classically, MHC class I molecules have been recognized as presenting peptides derived from intracellular sources. A schematic representation of this pathway is depicted in Figure 2. MHC class I heterodimers formed by the association of an α heavy chain and a small β2M subunit are assembled in the ER. The α chain is partially folded and associated with calnexin until it binds the β2M subunit. Upon formation of the MHC class I α:β2M heterodimer and release from calnexin, the complex binds to multiple chaperone proteins including calreticulin and ERp57 and is tethered to the transporter associated with antigen processing (TAP) via tapasin. Concurrently, cytosolic proteins are degraded to peptides via the multicatalytic proteasome and other cytosolic proteases. Peptide fragments are then delivered into the lumen of the ER through TAP and selectively loaded onto MHC class I molecules. Folding of the MHC class I molecule is completed upon peptide binding at the binding groove. MHC class I:peptide complexes are then exported from the ER via the Golgi secretory pathway. Once the complexes reach the cell surface, they can interact with surveying CD8+ T cells and initiate a T cell response. Clonal expansion of specific CD8+ T cells results in the generation of cytotoxic effector T cells as well as memory T cells (31, 32).

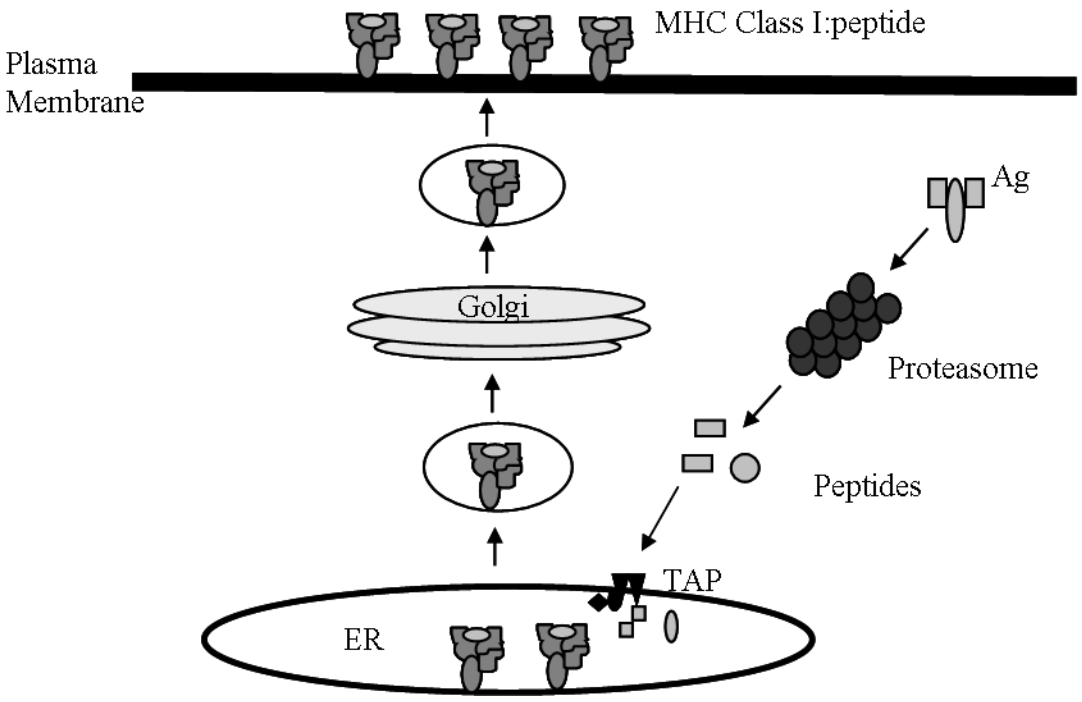


Figure 2. Classical MHC class I presentation of endogenous antigens. Endogenous, cytoplasmic Ags are digested into antigenic peptide fragments by cytoplasmic proteases such as the proteasome. The antigenic peptides generated are transported into the ER by TAP1/2 heterodimers. With the help from additional ER proteins such as tapasin, calreticulin, and Erp57, these peptides are loaded onto the peptide binding grooves of MHC class I molecules. MHC class I:peptide complexes dissociate from these ER proteins, transit through the Golgi secretory network, and are displayed on the cell surface for screening by circulating CD8+ T cells.

In contrast to MHC class I molecules, MHC class II molecules classically present peptides derived from extracellular sources. Figure 3 schematically depicts this classical pathway. DCs and macrophages internalize extracellular pathogens by either pinocytosis or phagocytosis while B cells internalize extracellular pathogens by either receptor mediated endocytosis or less efficiently, pinocytosis. Once internalized, extracellular Ags transit through the endosomal/lysosomal network from early to late endosomes, lysosomes, and the MHC class II rich compartment (MIIC). Within these low pH

compartments, engulfed Ags are degraded by various proteases and denaturing reactions into smaller peptide fragments capable of binding MHC class II dimers (33-36).

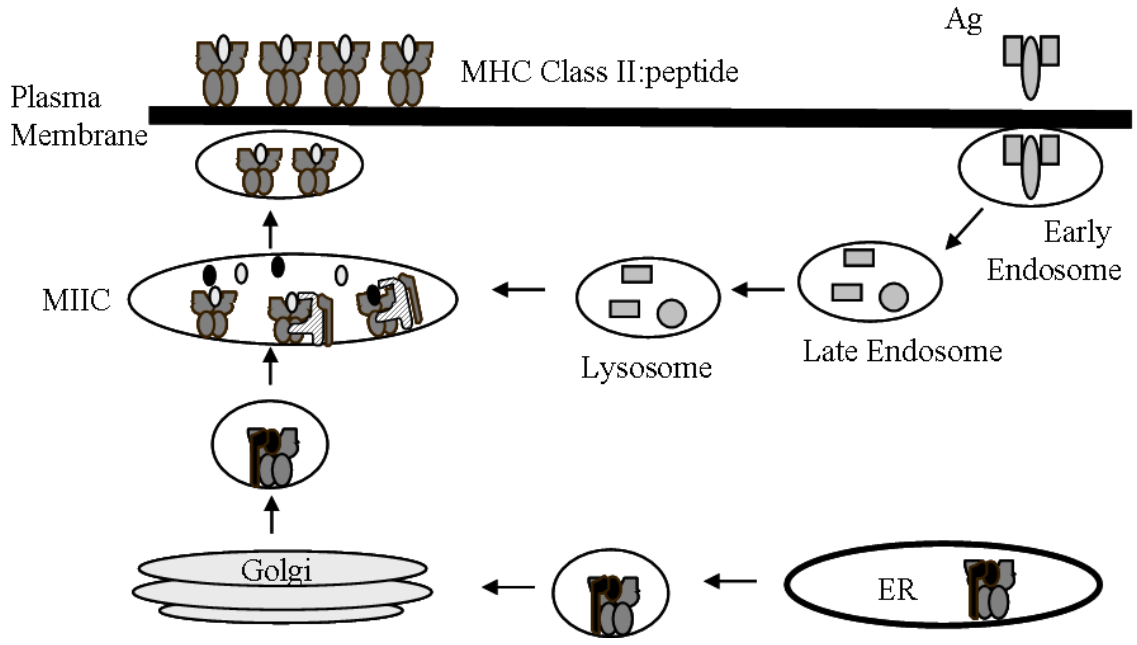


Figure 3. Classical MHC class II presentation pathway. Exogenous Ags are internalized by APCs and degraded into peptide fragments while they are sorted through the endosomal/lysosomal network. MHC class II molecules are assembled in the ER and associated with the chaperone invariant chain (Ii). MHC class II:Ii complexes transit through the secretory network. During transit, Ii is sequentially degraded into smaller peptide fragments, ultimately leaving the final CLIP fragment in the peptide binding groove of MHC class II molecules. Vesicles from the endosomal/lysosomal network containing antigenic peptide fragments fuse with secretory vesicles containing MHC class II:Ii complexes, resulting in the formation of MHC class II containing compartments (MIIC). In this compartment, HLA-DM and HLA-DO catalyze the dissociation of CLIP fragment and the loading of antigenic peptide onto MHC class II molecules. MHC class II:peptide complexes continue to transit to the cell surface to be displayed to circulating CD4+ T cells.

In the ER, newly synthesized MHC class II α and β chains are assembled into heterodimers. The chaperone molecule invariant chain (Ii) associates with the MHC class II dimers and performs various chaperone functions (37, 38). The physical binding of Ii within the peptide binding groove of the MHC class II dimers prevents premature peptide loading of the dimers. A targeting sequence within the cytoplasmic tail of Ii guides the Ii:MHC class II complexes to the secretory pathway and mediates intracellular sorting. While progressing through the secretory network, Ii undergoes stepwise degradation by proteases to a final fragment called the class II-associated invariant chain peptide (CLIP), which remains associated with the MHC class II peptide binding groove (31, 39-47). A schematic of Ii degradation is shown in Figure 4. When MHC class II:CLIP complexes reach the MIIC compartment, the non-classical class II molecule HLA-DM catalyzes the removal of CLIP from the peptide binding groove and the capture of an antigenic peptide by a MHC class II molecule (39, 42, 43, 48-51). HLA-DM activity is regulated by its interaction with another non-classical class II molecule HLA-DO (51-55). A schematic of the activity of HLA-DM and HLA-DO in the removal of CLIP and peptide loading of MHC class II molecules is depicted in Figure 5. MHC class II molecules have been found to bind to peptides at different points in the endosomal pathway; however, the major point of association appears to be in the MIIC, a mature endosomal or prelysosomal vesicle (39). The MHC class II:peptide complex is then trafficked to the cell surface where it can be surveyed by CD4+ T cells resulting in T cell activation and an immune response (31, 39-44, 47-49). Recycling of MHC class II:peptide complexes can occur by reinternalization of surface MHC class II:peptide complexes via the endocytic network. Within recycling early endosomes, MHC class II molecules can exchange their peptides for new peptides, resulting in further diversification of the spectrum of ligands bound to MHC class II molecules (56-58).

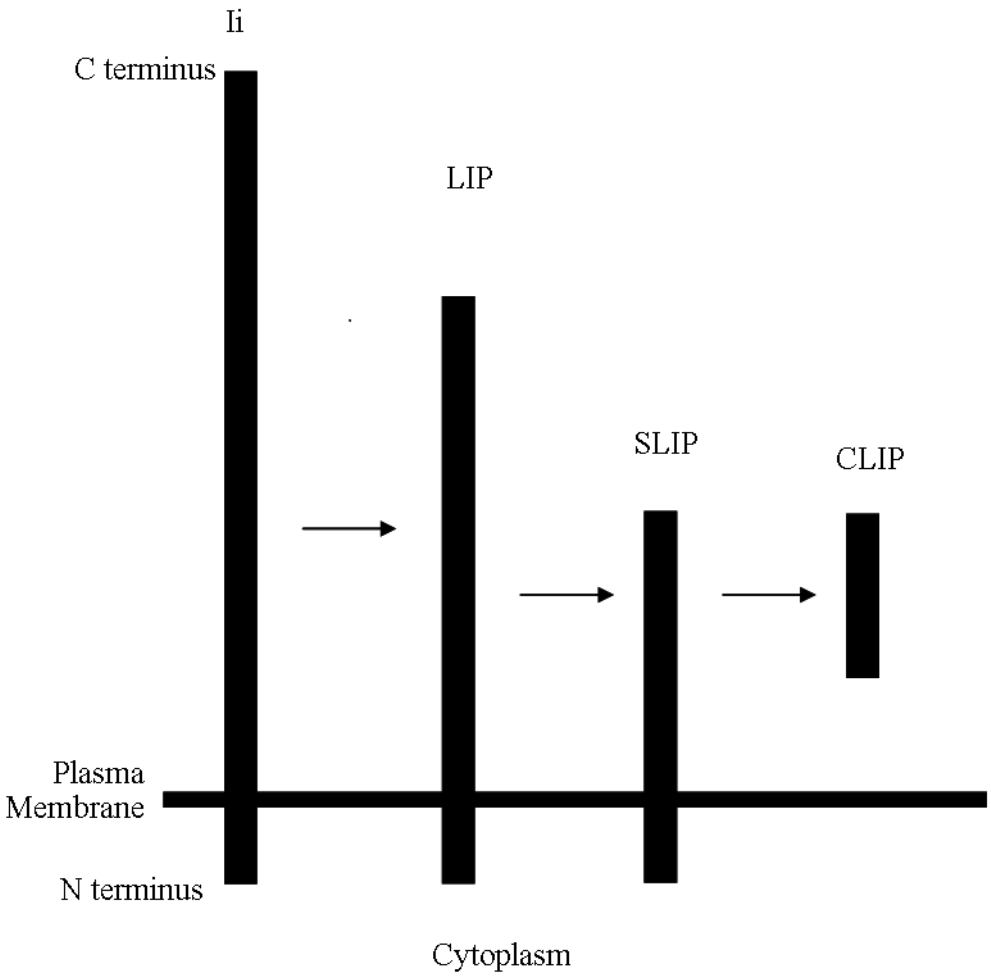


Figure 4. Schematic of Ii processing in B cells. In B cells, Ii binds to MHC class II molecules in the ER and chaperones MHC class II dimers as they transit through the secretory network. During transit, Ii is sequentially degraded into smaller peptide fragments. The C-terminal of Ii is cleaved leaving the leupeptin induced peptide, LIP. Further processing on the C-terminus cleaves LIP into the small leupeptin induced peptide, SLIP. Ultimately, the N-terminus anchoring Ii to the vesicle membrane is cleaved resulting in the final Ii fragment called class II-associated invariant chain peptide**,** CLIP. CLIP remains in the MHC class II peptide binding groove until HLA-DM/HLA-DO remove it.

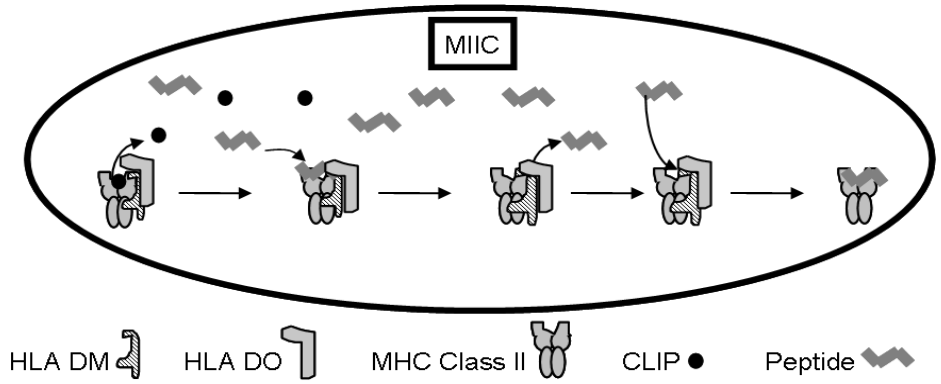


Figure 5. Schematic of HLA-DM and HLA-DO function in B cells. In the MIIC, HLA-DM functions to remove CLIP from the MHC class II binding groove and facilitate the binding of antigenic peptides. HLA-DO regulates the activity of HLA-DM.

MHC class II presentation of cytoplasmic Ags

Classically, MHC class I and class II molecules were viewed as having distinct and non-overlapping functions with MHC class I molecules presenting peptides derived from intracellular Ags and MHC class II molecules presenting peptides derived from internalized, extracellular Ags. In recent years, it has been determined that there is significant crosstalk between these two classical pathways of Ag presentation. It has been well established that MHC class I molecules can present peptides derived from exogenous sources by a process called cross-presentation (59, 60). It is also well established that that MHC class II molecules are capable of binding peptides derived from endogenous sources and presenting them to CD4+ T cells (36, 47, 61-74).

Biochemical approaches such as peptide-elution from MHC class II molecules in combination with peptide sequencing via tandem mass spectrometry have detected MHC class II ligands originating from endogenous proteins. While the majority of these cytoplasmic peptides are derived from endogenous proteins found at the cell surface or within endocytic and secretory vesicles, a significant number were found to originate from cytosolic proteins (36, 47, 61-74). Analysis of the peptides bound by MHC class II molecules in mice also revealed the presence of many cytosolic-derived epitopes. One such study found the majority of MHC class II ligands to be derived from self-proteins

such as transmembrane and secretory proteins. However, 21% of the ligands detected were peptides derived from cytosolic proteins such as actin, tubulin, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (68). Another study in human B cells detected greater than 85% of the MHC class II ligands to be derived from endogenous proteins including cytosolic proteins (75).

Additionally, studies with viruses and tumor cells have also shown that epitopes from cytoplasmic Ags access MHC class II molecules and trigger T cell responses (61, 73, 74, 76, 77). Viral Ags such as the matrix and nucleocapsid proteins from the measles virus and the M1 matrix protein of the influenza virus were all shown to be presented by MHC class II molecules, and viral-specific T cell responses were detected (78, 79). Epitopes derived from two different human melanoma tumor Ags, a mutated form of the human melanoma Ag CDC27 and a mutated version of triosephosphate isomerase, were both detected as ligands for MHC class II-restricted presentation and recognized by tumor specific CD4+ T cells (77). Studies also suggest that during T cell development in the thymus, MHC class II restricted presentation of cytosolic Ags is a requirement for complete elimination of autoreactive T cells. For example, in mice transgenic for either endogenous or cell surface pigeon cytochrome c, both forms of the Ag were presented by MHC class II molecules and induced CD4+ T cell tolerance (80).

Many cell types appear capable of presenting cytoplasmic Ags via MHC class II molecules. To date, B cells, DCs, MHC class II-transfected tumor and epithelial cells, thymic epithelial cells (TECs), and myeloid progenitor cells have all been shown to present cytoplasmic Ags via MHC class II molecules. However, these different cell types vary in their epitope repertoire as well as their efficiency of cytoplasmic Ag presentation.

Functional and biochemical studies have found that depending upon the specific HLA-DR allele analyzed, the contribution of cytosolic peptides to the ligand repertoire varies (61, 65, 67, 75, 77, 78, 81-83). Moreover, not all cytoplasmic proteins appear to be a source of MHC class II peptide ligands (66, 78, 84). For example, endogenously synthesized membrane associated viral glycoprotein (GP) from the lymphocytic

choriomeningitis virus (LCMV) can be efficiently presented by MHC class II molecules, but the LCMV nucleoprotein (NP) cannot (66). Moreover, *in vivo* studies with LCMV infected animals determined that CD4+ T cells can recognize LCMV-GP, but not LCMV-NP (84).

Taken together, it appears that MHC class II molecules have evolved mechanisms to sample select antigenic peptides from the cytoplasm and present them to CD4+ T cells in order to enhance helper T cell responses to intracellular pathogens and tumors and to maintain self tolerance (61, 66, 67, 69, 73, 74, 77, 80). However, the mechanisms involved in cytoplasmic Ag presentation by MHC class II molecules remain rudimentary.

Possible pathways of MHC class II presentation of endogenous Ags

Endogenous Ags may access MHC class II molecules for presentation to CD4+ T cells via different routes. One possibility is that endogenous Ags may be released during cell death and presented by bystander APCs via the classical MHC class II pathway (Fig. 3). While studies have shown that APCs can present Ags released from apoptotic or lysed cells, studies also suggest few cytoplasmic epitopes are released by viable cells (67, 69, 70, 74, 85). This pathway may be engaged during cytopathic viral infections when damage and death of infected cells are quite common. Another possible route is via autophagy which involves the sequestering of cytoplasmic and nuclear Ags into autophagosomes prior to Ag processing and MHC class II presentation (Fig. 6). Bulk autophagy can be subdivided into two types of autophagy, macroautophagy and microautophagy, distinguishable by chemical inhibitors and microscopy. In macroautophagy, ER membranes bud and randomly enclose nuclear and cytoplasmic material within vacuoles termed autophagosomes that eventually fuse with lysosomes for content degradation. While macroautophagy can occur at low levels in healthy cells, it can be up-regulated with serum starvation (86). In contrast, microautophagy has only been observed in yeast and is regulated by starvation. This pathway involves the direct sequestering of proteins into lysosomes for protein degradation. The role of macroautophagy in MHC class II presentation is controversial. Some studies using chemical agents blocking macroautophagy suggest that macroautophagy is involved in

the MHC class II presentation of some nuclear and cytoplasmic Ags (74, 83, 87). However, other studies found MHC class II presentation of cytoplasmic Ags unaffected by inhibition of macroautophagy (69, 70, 73, 85). A third possible route of MHC class II presentation of endogenous Ags involves the processing of cytoplasmic Ags within the cytoplasm and the selective translocation of the resulting cytoplasmic peptides into MHC class II-rich organelles (Fig. 6). Chaperone proteins direct the processing and translocation of Ags through this pathway; thus, the pathway is termed chaperone mediated autophagy (CMA).

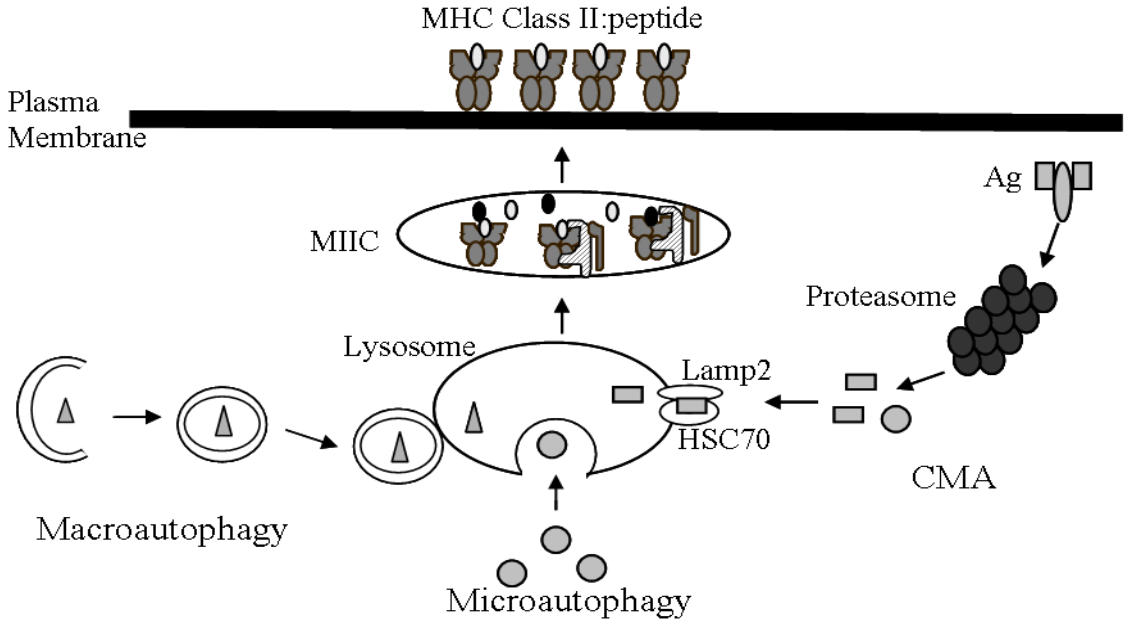


Figure 6. Alternative pathways of MHC class II presentation of cytoplasmic Ags. Endogenous, cytoplasmic Ag can gain access to the MHC class II machinery via different routes of autophagy. In macroautophagy, ER membranes bud and randomly enclose cytoplasmic material forming intracellular vesicles called autophagosomes. Autophagosomes can directly fuse with lysosomes, empting their contents into the lysosomes for degradation. In microautophagy, random cytosolic proteins are directly sequestered into the lysosomes for protein degradation by lysosomal proteases. In contrast, selective degradation of cytosolic proteins occurs in chaperone mediated autophagy (CMA). Two proteins, lamp2 and HSC70, have been shown to play critical roles in this pathway by facilitating entry of antigenic peptides into the lysosomes.

Key players in MHC class II presentation

Invariant chain (Ii)

As mentioned previously, it is well established that Ii functions as an MHC class II chaperone by facilitating proper MHC class II αβ folding, stabilizing and targeting dimers through the secretory network, and preventing premature peptide loading. Research suggests Ii expression alters MHC class II expression. Ii deficiency in Ii knockout mice resulted in low MHC class II expression in APCs (37, 88). Loss of Ii chain in NOD mice resulted in decreased surface MHC class II expression and the retention of MHC class II aggregates in the ER (89).

Studies suggest that Ii plays a role in MHC class II presentation of exogenous and endogenous Ags. Our lab recently implicated Ii in MHC class II recycling and peptide presentation (90). APCs from Ii knockout mice exhibited decreased exogenous Ag presentation, but enhanced presentation of peptides due to their ability to bind directly to MHC class II molecules (91). Studies in Ii transfected fibroblast cells also correlated Ii expression levels with Ag presentation; the amount of exogenous Ag required to induce a T cell response was inversely correlated with the level of Ii expression (92). However, low Ii expression is thought to promote the presentation of endogenous Ags. The presentation of a cytosolic peptide from the influenza virus matrix protein was efficiently presented by Ii deficient cells and inhibited by the expression of Ii (79). MHC class II expressing myeloid progenitor cells are efficient at presenting cytosolic peptides (81). Another study found that loss of Ii in MHC class II cancer vaccines resulted in enhanced activation of tumor reactive T cells (93).

Alterations in Ii expression have been shown to alter the immune response. In mice, Ii deficiency altered the Th-2 immune response resulting in a preferential Th-1 immune response (94). Another study found Ii deficient mice to have an impaired ability to mount cellular and humoral immunity upon viral infections (95). Ii may play a role in the development of autoimmunity as suggested by the finding that loss of Ii in NOD mice prevented the onset of TID (96).

While Ii expression is typically linked to MHC class II expression and function, there are some circumstances where this is not the case. There is evidence that MHC class II proteins are expressed even in the absence of Ii (89, 97-101). This may reflect that different MHC class II alleles are more or less dependent on Ii expression as well as variation in MHC class II biosynthesis in different cell types. Studies suggest the existence of Ii independent pathways of MHC class II presentation (102). Ii deficiency in NOD mice did not substantially alter Ag presentation. Yet, taken together, studies overall suggest that regulation of Ii expression may provide a way to modulate MHC class II Ag presentation. Thus, understanding the role of Ii in MHC class II presentation may prove important for the development of novel immunotherapeutics against malignant diseases, viral infections, as well as autoimmune disorders.

Cathepsins

The endosomal/lysosomal network contains numerous hydrolases including proteases. The most well known endosomal/lysosomal proteases are the cathepsins. In humans, there are 11 known cysteine proteases, cathepsins B, C, F, H, K, L, O, S, V, X, and W. There are also three aspartic proteases, cathepsins D, cathepsin E, and AEP, and one serine protease, cathepsin G. Cathepsin family members have been shown to be involved in numerous biological functions including the immune response and Ag processing (103). Cathepsins B, C, D, X, H, L, and S have all been shown to have clear roles in the immune system, and cathepsins S and L are the only ones shown to have non-redundant roles (103). In general, these cysteine proteases are optimally active within the slightly acidic, reducing environment of endosomes and lysosomes.

Cathepsin S is primarily expressed in phagocytic APCs and is believed to be the major cysteine protease involved in MHC class II Ag processing and presentation (104-106). Various studies in cathepsin S null mice support this theory. The APCs from cathepsin S deficient mice exhibited a severe decrease in Ii degradation and alterations in MHC class II-bound Ii fragments and MHC class II presentation of exogenous Ags (46, 107). Inhibition of cathepsin S in B cells prevented complete proteolysis of Ii (44). Studies also found cathepsin S to be required for the normal trafficking, maturation, and peptide

loading of MHC class II molecules (108, 109). Inhibition of cathepsin S with small molecule inhibitors resulted in diminished Ag presentation by MHC class II molecules (104, 107). Cathepsin S deficiency has also been shown to decrease the presentation of certain epitopes, suggesting that cathepsin S can directly affect the processing of select Ags (107, 110). In contrast to other cathepsins, cathepsin S retains considerable stability and proteolytic activity over a broad pH range, functioning in both endosomes and lysosomes (103, 109, 111, 112). In human DCs and B cells, the selective inhibition of cathepsin S resulted in the accumulation of incompletely processed Ii fragment, suggesting that cathepsin S plays a non-redundant role in Ii processing (44, 113). While studies have analyzed various Ii fragments, the exact cathepsin S cleavage sites on Ii were unknown until recently (114). This discovery may prove helpful in identifying cathepsin S cleavage sites within Ags and determining the relevance of this protease in generating antigenic peptides for MHC class II presentation. Studies also suggest that cathepsin S is an immunomodulator of immune diseases such as rheumatoid arthritis and bronchial asthma; thus, cathepsin S inhibitors are currently being evaluated as potential therapeutics for certain immune disorders (115, 116).

Cathepsin L is primarily expressed in cortical thymic epithelial cells (cTECs) and minimally in professional APCs. Cathepsin L deficient mice exhibit decreased numbers of CD4+ T cells and defective Ag presentation by MHC class II molecules in their cTECs due to incomplete Ii processing (45). Thus, cathepsin L is thought to regulate positive selection in the thymus. Additional studies identified cathepsin L as an essential enzyme for the development of TID in NOD mice (117). Studies suggest that Cathepsin L mediates the final cleavage of Ii to the CLIP fragment (40, 103). As shown with cathepsin S, cathepsin L deficient APCs show decreased Ag presentation of select Ags. This suggests that like cathepsin S, cathepsin L may also mediate MHC class II presentation of select Ags (110). Interestingly, a splice variant of Ii, p41, has been shown to inhibit cathepsin L activity, suggesting a negative feedback control mechanism (112).

Studies from our own lab and others suggest a role for the cathepsins B and D in both Ii processing and MHC class II presentation (35, 67, 118-120). Treatment of APCs with

specific cathepsin B and D inhibitors resulted in decreased Ag presentation and Ii expression (35, 118, 119). Treatment of B cells with cathepsin B inhibitor II slightly inhibited both endogenous and exogenous GAD presentation, while treatment with a cathepsin D inhibitor only altered exogenous GAD presentation (67). Our results suggest that cathepsins B and D may play roles in endogenous and exogenous GAD Ag presentation by MHC class II molecules. Recent studies found activation of cathepsin D to be required for the processing and presentation of a mycobacterial Ag in macrophages (120). However, some evidence suggests their activity may be redundant and dispensable for MHC class II presentation. One study found that cathepsin B and D deficiency in knockout mice only moderately shifted the efficiency of Ag presentation of select epitopes, but did not perturb the overall capacity of APCs (121). Another study found cathepsin D deficiency actually enhanced the presentation of certain peptides and suggests some redundancy among the lysosomal proteases (122). Taken together, these results suggest that like cathepsin S, cathepsin B and D may be required for the presentation of select Ags for MHC class II presentation.

Asparagine endopeptidase (AEP)

AEP is an asparagine-specific cysteine protease found within endosomes and lysosomes. Although it is a cysteine protease, AEP is not grouped with cathepsins S and L as it is homologous with the caspases (123). Unlike the other lysosomal cysteine proteases, AEP is leupeptin insensitive (124, 125). Evidence suggests that AEP is involved in MHC class II presentation. In B cells, AEP was found to initiate the processing of the exogenous Ag tetanus toxin (125). Moreover, disruption of a single AEP cleavage site in the microbial Ag tetanus toxin C fragment resulted in a dramatic decrease in its ability to be processed and presented to T cells (126). The role of AEP in Ii processing remains controversial. A study with B cell lines and monocyte-derived DCs showed that AEP can initiate Ii processing and that inhibition of AEP results in delayed Ii processing (127). However, another study in AEP-deficient mice found Ii processing, MHC class II maturation, and Ag presentation unaffected by AEP deficiency (128).

AEP activity has been linked to autoimmunity as well as to neuronal injury during neuroexcitotoxicity or ischemia. In reference to autoimmunity, studies found an AEP processing site within the myelin basic protein (MBP) autoantigen. Moreover, the presentation of MBP was found to be inversely proportional to the amount of cellular AEP activity. It is suggested that the destruction of epitopes within MBP may limit its display in the thymus and prevent central tolerance to this autoantigen (129). A current study found AEP to play a crucial role in mediating kainic acid or stroke elicited neurotoxicity (130). In this study, AEP was found to be activated under acidic conditions, responsible for the cleavage of neuronal protein, and the cause of DNA damage in the brain. Thus, understanding the role of AEP may have implications on immune tolerance and neuronal cell death.

HLA-DM and HLA-DO

Our lab and others have found HLA-DM and HLA-DO to play critical roles in MHC class II mediated Ag presentation in APCs. HLA-DM is mainly localized in the endosomal/lysosomal vesicles of APCs (51). In these compartments, HLA-DM assists the peptide loading of MHC class II molecules by catalyzing the exchange of CLIP for antigenic peptide fragments within endocytic compartments (39, 42, 43, 48-51). Additionally, HLA-DM edits the repertoire of peptides loaded onto MHC class II molecules by preferentially loading peptides with high affinity to MHC class II (51). Studies in mice with an HLA-DM deficiency in APCs resulted in almost all the surface MHC class II molecules loaded with CLIP (131). Studies in our lab found that MHC class II presentation of endogenous and exogenous GAD was affected by the expression level of HLA-DM; high HLA-DM levels inhibited GAD presentation (132). The function of HLA-DO is still controversial as it may function as a positive or negative regulator of HLA-DM (51-55). Studies also suggest that HLA-DM and HLA-DO function may have implications in autoimmune disorders (133). Certain MHC class II alleles confer protection and susceptibility to certain autoimmune disorders. It is suggested that this is possibly due differences in MHC class II affinity for CLIP and peptides. Since HLA-DM and HLA-DO regulate the binding and release of CLIP based on MHC class II affinity, manipulation of HLA-DM and HLA-DO function is considered

a possible therapeutic target. Taken together, studies suggest that HLA-DM and HLA-DO function together to influence the repertoire of epitopes presented by MHC class II molecules.

Cytoplasmic proteases: proteasome and calpain

While exogenous Ags are processed by proteases within endosomes/lysosomes for MHC class II presentation, cytoplasmic Ags for MHC class I presentation are primarily processed by the cytoplasmic protease proteasome within the cytosol. Both cytosolic and endo/lysosomal proteases may be required for MHC class II presentation of endogenous Ags. Studies in our lab and others found Ag processing by cytoplasmic proteases to be a requirement for MHC class II presentation of cytoplasmic Ags (67, 69). Treatment of PriessGAD B cells with chloroquine, an anti-malarial drug that raises the pH of endo/lysosomes, completely inhibited MHC class II presentation of exogenous GAD Ag, but only slightly decreased presentation of cytoplasmic GAD Ag (67). Treatment of PriessGAD cells with calpeptin, an inhibitor of the cytoplasmic protease calpain, completely inhibited MHC class II presentation of cytosolic GAD Ag, but did not affect exogenous GAD Ag presentation (67). Moreover, treatment with lactacystin or epoxomicin, proteasome inhibitors, selectively inhibited cytoplasmic GAD presentation (67 and data not shown). MHC class II molecules have been shown to present peptides from Ags introduced into the cytosol via hyperosmotic lysis of pinosomes (69). MHC class II presentation of these Ags was blocked by inhibition of the proteasome by inhibitors such as lactacystin (69). Taken together, these results suggest that the cytoplasmic proteases calpain and the proteasome are required for MHC class II presentation of endogenous Ags. However, further trimming of those peptides within the endosomes/lysosomes may also be required.

Heat shock proteins are potential players in MHC class II presentation

Links between heat shock proteins (HSPs) and the immune system have been suggested. Roles for HSPs have been identified in immune responses, tumor surveillance, and Ag presentation (134-136). Research by our laboratory and others suggests that HSPs can act as chaperones to facilitate Ag processing for MHC class I and II presentation (85, 137-144). HSPs are a group of highly conserved proteins that primarily serve as molecular chaperones. They are grouped into families based on their molecular size, sequence homologies, and antigenic cross reactivities (145). HSPs have been found to play various intracellular roles including assisting in protein folding, assembly, and degradation, scavenging for peptides produced during Ag proteolysis, and facilitating protein translocation (146-148). The term HSP originated due to the inducibility of some of these proteins upon heat or cellular stress. However, many of these proteins are expressed constitutively, sometimes at high levels, suggesting a critical role in the everyday functions of a cell (145).

HSC70

The constitutively expressed heat shock cognate protein HSC70, also known as HSC73, is a member of the HSP70 family and makes up 1% of the total cellular protein content (149). Unlike other HSPs, HSC70 is only slightly induced by heat shock even though the gene contains functional heat-shock regulatory elements (150). The structure of HSC70 is typical of an HSP70 family member, an ATP-binding domain at the amino-terminus followed by a peptide binding domain in the middle (151). The carboxyl-terminal domain provides binding sites for cofactors whose role is to modulate HSC70 activity (152). HSC70 is depicted schematically with key protein domains labeled in figure 7. Like other HSPs, peptide binding by HSC70 is ATP-dependent; in the ATP bound state, HSC70 binds peptides, but upon ATP hydrolysis to ADP, HSC70 releases bound peptides (151). HSC70 recognizes short peptide motifs with a minimal size of 5-7 residues via a ligand binding groove, but is preferential to ligands with hydrophobic and basic residues, originally termed the KFERQ motif (153, 154). Further research has revised this sequence to include more flexibility (155).

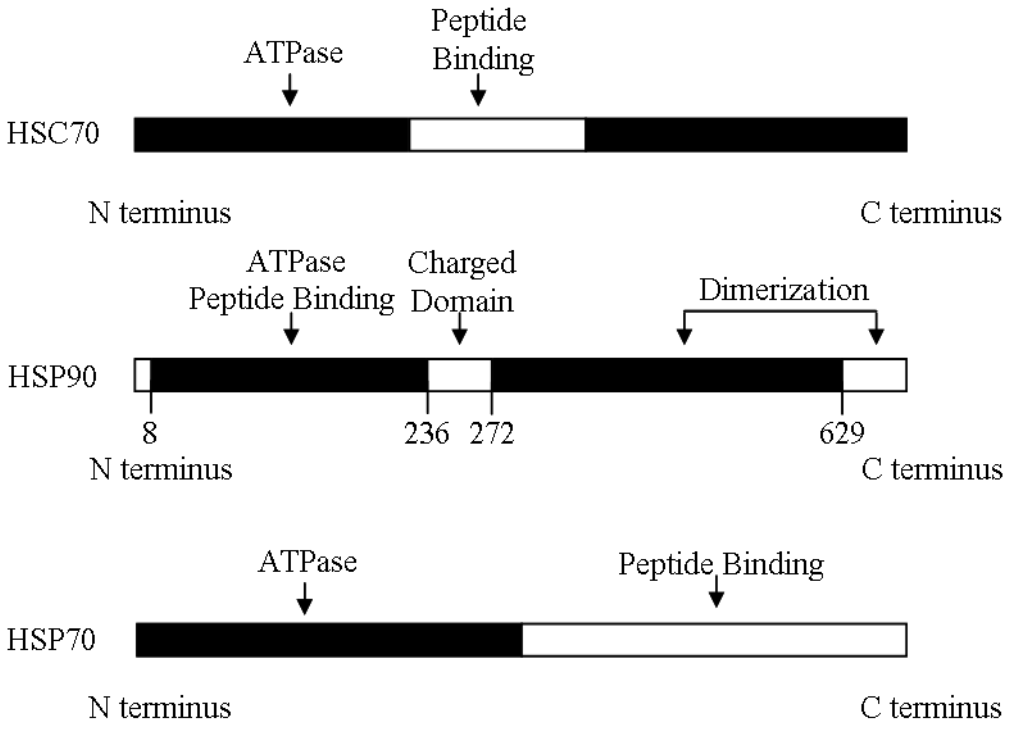


Figure 7. Schematic diagram of HSPs. The structure of HSC70, HSP90, and HSP70 are schematically represented above. The N-terminus of HSC70, HSP90, and HSP70 contains an ATPase domain. A peptide binding domain is located within the center of the HSC70, at the N-terminus of HSP90, and at the C-terminus of HSP70. The C-terminus of HSP90 contains a dimerization domain, allowing the formation of HSP90 homodimers.

Endogenous HSC70 is predominately localized in the cytoplasm (156). In the cytosol, HSC70 operates as part of a molecular chaperone complex. This complex includes cofactors such as Hip, Hop, HSP40, BAG-1, and HSP90. Hip functions to stimulate the assembly of HSC70 with HSP40 and its substrate, while BAG-1 functions to uncouple the binding of substrate proteins from the ATPase cycle of HSC70 (157-160). HSP40 stimulates the ATPase activity of HSC70. HSP90 is linked to HSC70 by the co-chaperone Hop (157, 161). HSC70 is also localized on the luminal and cytoplasmic face of endosomes and lysosomes (162, 163). It is proposed that this localization allows HSC70 to assist in the transport of cytoplasmic peptides to these organelles (164). While

luminal HSC70 does not associate with any of its cytoplasmic chaperones, HSC70 on the cytoplasmic face acts in concert with the lysosomal associated membrane protein-2a (lamp2a) (162, 163). Lamp-2a is a transmembrane endosomal/lysosomal protein that assists in the translocation of peptides into lysosomes (165, 166). Studies in our lab and others indicate that HSC70 is required for peptide transport into the lysosome (85, 163). Our lab has previously shown in B cells that alteration of either HSC70 or Lamp-2a can disrupt MHC class II presentation of numerous cytoplasmic autoantigens leading one to deduce that CMA may regulate immunity (85).

HSP90

HSP90 is also found within the cytoplasmic multi-chaperone complex (167). HSP90 is a highly conserved, constitutively expressed cytosolic chaperone protein. In eukaryotes, HSP90 is a critical protein chaperone as indicated by studies finding HSP90 null mutations lethal. Under normal conditions, HSP90 constitutes 1-2% of the total cellular protein content, but under stressed conditions such as heat shock, HSP90 levels can increase to 4-6% (168). HSP90 functions include controlling protein folding, turnover, and trafficking. While HSP90 has been linked to almost 100 different client proteins, many of the known HSP90 substrates are signal transduction molecules such as the glucocorticosteroid receptor (143, 151, 168, 169). Thus, the best characterized example of HSP90 chaperone activity is the HSP90 dependent signaling pathway of steroid hormone receptors (169). Typically, HSP90 binds to client proteins that are in a near native state or late stages of folding, recognizing unfolded regions within the proteins (155, 170). Like HSC70, HSP90 is an ATP-dependent chaperone in that HSP90 chaperone activity requires both binding and hydrolysis of ATP (171-173). The structure of HSP90 is similar to other HSPs and is depicted schematically in Figure 7 with key domains labeled. The N-terminus houses the ATPase and peptide binding domains, a charged domain is in the middle, and a dimerization domain is found at the C-terminus (174).

In the cell, HSP90 functions in a multi-chaperone complex in association with other heat shock proteins such as HSC70, HSP70, and HSP40 as well as co-factors such as BAG-1,

HOP, CHIP, and HIP (152, 167, 175, 176). Through interactions with its co-factors, HSP90 is linked to the ubiquitin-proteasome pathway (152). While HSP90 null mutations have proven lethal, conditional inhibition of HSP90 by the use of pharmacological inhibitors has proven useful in understanding HSP90 structure and function. The benzoquinon ansamycin antibiotic geldanamycin (GA) and the macrocyclic drug radicicol (RA) are two such HSP90 inhibitors that bind to the ATP-binding site of HSP90 and inhibit the ATPase activity of HSP90, thus inhibiting HSP90 chaperone activity (177).

In humans, HSP90 is expressed as two closely related isoforms, HSP90α and HSP90β, which are both constitutively expressed and induced by cellular stress (170, 174, 178). Assays using pharmacological inhibitors such as RA and GA fail to distinguish between the function of these two isoforms, with both proteins inactivated by these compounds (174). Therefore, until recently, both isoforms were referred to simply as HSP90. While both isoforms bind similarly to their intracellular co-chaperones, in some cases HSP90α and HSP90β were found to behave differently with respect to substrate interactions (178). HSP90 has been implicated in regulating both direct and cross-presentation pathways for MHC class I molecules. HSP90 ligands include peptides which can bind MHC class I molecules, and inhibition of HSP90α expression disrupts cytoplasmic Ag presentation by MHC class I molecules (141). HSP90 also appears to regulate human DC functions including cell maturation, MHC class I and II expression, and MHC class II presentation (143). However, the role of HSP90 in MHC class II Ag presentation in B cells as well as the contribution of the individual HSP90 isoforms has yet to be determined.

HSP90 has been shown to play a key role in chaperoning client proteins in a variety of cellular processes including cell proliferation, differentiation, and apoptosis (174). Manipulation of HSP90 activity has been used to modulate protein folding and to induce the proteolysis of misfolded or mutant proteins in a variety of disease conditions including a wide range of malignancies and neurological disorders including Alzheimer's disease, Parkinson's disease, autoimmune encephalomyelitis, and polyglutamine diseases (179-182). HSP90 inhibitors have also been tested in human clinical trials to promote

tumor regression (181). Studies also suggest that HSP90 and MHC class II presentation may play a role in TID. B lymphocytes play an important role in the pathogenesis of TID, functioning as APCs in the autoimmune response to islet cell Ags such as GAD (13). However, it is still somewhat controversial whether resident as well as recruited MHC class II-positive cells function to present β cell Ags in the islets of TID patients (183). Notably, in TID, β cell stress results in an induction of HSP90 expression (184). An increase in select anti-HSP90 Ab isotypes has also been noted in type I diabetics and family members, suggesting the potential release of this HSP from cells (185). Thus, understanding the role of HSP90 isoforms in MHC class II presentation may yield mechanistic insights regarding the presentation of autoantigens such as GAD.

HSP70

Another HSP found to interact with both HSP90 and HSC70 is HSP70. HSP70 is an inducible HSP found in the cytosol and in lysosomal compartments (166). As with HSC70 and HSP90, HSP70 also contains an amino-terminal ATPase subunit and a carboxyl-terminal peptide binding domain, as depicted schematically in figure 7 (186). HSP70 has been shown to perform a variety of cellular functions including stabilizing newly synthesized or unfolding polypeptides, facilitating translocation of nascent chains across membranes, mediating assembly or disassembly of multimeric protein complexes, and targeting proteins for degradation within lysosomes (187-189). Due to its wide variety of functions, HSP70 recognizes and binds a wide variety of client proteins in a promiscuous manner. However, studies suggest that HSP70 recognizes and binds hydrophobic regions of polypeptides that are typically buried within proteins and exposed during misfolding or denaturation (140, 153).

In response to stress, HSP70 is secreted from a variety of cell types (190-195). However, it is still controversial as to the mechanisms involved in the HSP70 secretion pathway. Some studies suggest that HSP70 is released by a non-specific process such as cell lysis while others suggest specialized membranes such as lipid rafts or exosomes are involved (190, 192, 193, 196). Studies in tumor cells found that HSP70 was secreted by tumor cells through a pathway involving lysosomal endosomes (197). Studies suggest that

extracellular HSPs including HSP70 may play important immunoregulatory functions (198-206). For example, treatment of monocytes and DCs with recombinant HSP70 stimulated cytokine production, upregulated co-stimulatory molecules, and enhanced MHC class II Ag presentation (200, 204).

HSP70 has been highly studied as a key player in the pathogenesis of amyloidosis. Amyloidosis is a group of diseases characterized by the assembly of protein fibrils that are deposited extracellulary in various organs and tissues. HSP70 has been shown to promote the proteasomal degradation of the proteins that make up these fibrils, potentially regulating fibril accumulation (207). Studies have detected extracellular HSP70:peptide complexes in MHC class II-enriched compartments after receptor-mediated endocytosis (208). Interestingly, HSP70 has been shown to interact with MHC class II molecules (209). Studies also suggest HSP70 mediated enhancement of MHC class II restricted peptide presentation (137, 209). And relevant to autoimmunity, HSP70 has been shown to promote myelin autoantigen presentation by MHC class II molecules (139). Thus, a better understanding of the role of HSP70 in amyloidosis and autoimmunity may have implications on developing better therapeutics for these types of diseases.

ER chaperones and MHC class II presentation

The ER is a specialized environment where newly synthesized polypeptides are translocated, properly folded into functional proteins, and transported to their final destinations. Nearly one third of all eukaryotic cellular proteins are translocated into the ER for processing (210). Additional ER chaperones, folding enzymes, and resident proteins are also abundant in the ER at even higher levels than newly synthesized polypeptides. Thus, the protein concentration in the ER lumen can be greater than 100 mg/ml (211).

Within the ER, there are quality control mechanisms that maintain homeostasis. While properly folded proteins are exported from the ER, misfolded proteins are retained in the ER and selectively degraded. Under some conditions, the cellular demand on ER

protein-folding activities exceeds the ER's capacity. This situation of imbalance, termed ER stress, results in the accumulation of misfolded proteins and the triggering of intracellular signaling pathways called the unfolded protein response (UPR). The UPR temporarily halts protein translocation, degrades the misfolded proteins, and induces ER chaperones and folding enzymes in order to increase ER folding and degradation capacity (212, 213). If proteins cannot be folded correctly, they are targeted for removal from the folding pathway via the ER-associated degradation (ERAD). Through ERAD, proteins are exported from the ER into the cytosol for proteasomal degradation.

ER chaperones play critical roles within the ER under both homeostatic conditions and ER stress. Two main ER chaperones, grp94 and BiP, are chaperones of the HSP families. Grp94 is an ER homolog of cytosolic HSP90 and is expressed at high levels within the ER. The ER chaperone binding immunoglobulin protein (BiP or grp78) is a homolog of cytosolic HSP70 and is one of the most abundant ER chaperones. Parallels are being drawn between the grp94/BiP chaperone complex in the ER and HSP90/HSP70 in the cytosol. BiP functions in both protein folding and in ERAD. Similar to HSP70, BiP recognizes hydrophobic regions that are exposed in misfolded or unassembled proteins and functions in an ATP-dependent manner. However, additional research indicates that HSP90 may also be linked to the cellular transcriptional response to ER stress, suggesting that chaperones on both sides of the ER are involved in the ER stress response (214).

Studies suggest that ER chaperones play critical roles in mammalian development and are linked to a wide variety of diseases. ER chaperones are linked to the prevention of protein misfolding in neurodegenerative diseases, cancer progression and tumor immunity, atherosclerosis, type II diabetes, and autoimmune disease such as TID (215). In reference to autoimmune diseases, BiP has been shown to associate with autoantigens suggesting a possible mechanism for triggering autoimmunity (216). Additional research has shown that in Ii deficient cells, MHC class II molecules can bind grp94 (217). This suggests that in the absence of Ii, ER stress chaperones such as grp94 may bind to MHC class II molecules and retain them in the ER. This process could prevent endogenous peptide loaded MHC class II molecules from exiting the ER and minimize the

autoimmune responses to endogenous self Ags. Taken together, ER chaperones play critical roles in protein assembly and degradation under both normal and stressed conditions and may prove crucial to the maintenance of homeostasis in the immune system.

Research summary

As previously stated, studies suggest that HSPs such as HSC70 play important roles as chaperones in MHC class II presentation of exogenous and endogenous Ags. I hypothesize that in addition to HSC70, both HSP90 and HSP70 can modulate MHC class II presentation of select Ags in B cells. To determine if HSP90 is involved in MHC class II presentation, two pharmacological inhibitors of HSP90, GA and RA, were used to inhibit HSP90 chaperone activity. The effect of HSP90 inhibition on MHC class II presentation of a variety of endogenous and exogenous Ags was measured. Manipulation of HSP90 isoform expression was performed by using isoform specific siRNA. The effect of inhibition of HSP90 isoform expression on MHC class II presentation of a variety of endogenous and exogenous Ags, including the autoantigen GAD, was measured. Studies were performed to identify a possible mechanism for HSP90 involvement in MHC class II presentation of select Ags. A role for HSP70 in MHC class II presentation was also explored using additional endogenous and exogenous Ags. These studies should provide insights into the involvement of HSP90 and HSP70 in MHC class II presentation of select Ags such as GAD and may prove helpful in understanding the link between GAD, MHC class II, HSPs, and autoimmunity.

This work also includes a study of a rare case of diabetes caused by type B insulin resistance due to development of insulin receptor autoantibodies during treatment for hepatitis C with interferon α and ribavirin. Clinical and laboratory findings in the case are presented. Literature on type B insulin resistance and interferon-induced autoimmunity is reviewed. This case demonstrated that type B insulin resistance can occur as a complication of interferon α therapy. This was the first case in the United States of type B insulin resistance with insulin receptor autoantibodies during treatment with interferon α.

MATERIALS AND METHODS

Cell lines

The human B lymphoblastoid cell line (B-LCL) Priess is homozygous for HLA-DR4 (DRA1*0101,DRB1*0401) expression. Retroviral transduction of Priess cells with GAD65 cDNA resulted in constitutive endogenous expression of GAD65 yielding PriessGAD cells (67). Another human B-LCL, Frev, expresses both the HLA-DR4 (DRA*0101, DRB1*0401) and HLA-DR1 (DRA*0101, DRB1*0101) class II alleles. Transfection of Frev with a CMV promoter driven plasmid encoding SMA, a mutant form of immunoglobulin (Ig) light chain κ4, followed by drug selection with 500 μg/ml hygromycin resulted in constitutive endogenous expression of SMA yielding FrevSMA cells (207). These B-LCL cells were maintained in Iscove's MEM (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 100 μ/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL Life Technologies, Grand Island, NY).

The T cell hybridoma 33.1 recognizes the $GAD_{273-285}$ epitope within the context of HLA-DR4 (L. Wicker, Merck Research Laboratories, Rahway, NJ). The 1.21 T cell hybridoma recognizes the $\kappa II_{145-159}$ (kappa II) epitope of Ig κ within the context of HLA-DR4 (P. Whitley, Merck). The T cell hybridoma 2.18 recognizes the $\kappa I_{188-203}$ (kappa I) epitope of Ig κ within the context of HLA-DR4 (P. Whitley, Merck). The 17.9 T cell hybridoma recognizes the HSA_{64-76} epitope from human serum albumin (HSA) within the context of HLA-DR4 (Merck Research Laboratories, Rahway, NJ). The HT-2 cell line is an IL-2 dependent T cell hybridoma (Merck Research Laboratories, Rahway, NJ). All T cell hybridomas were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 50 μM β mercaptoethanol (βME) (Gibco BRL Life Technologies, Grand Island, NY), 100 μ /mL penicillin, and 100 μg/mL streptomycin. For HT2 cells, an additional 20% T-Stim (BD Biosciences, Bedford, MA) was added to the T cell media.

CHO, Chinese hamster ovary cells, and CHO/IR cells (Jeffrey Pessin, University of Iowa, Iowa City, Iowa) were cultured in α MEM (Hyclone, Logan, UT) with 10% heat-inactivated calf serum.

Antibodies

The following antibodies were used in various immunoprecipitations, Western immunoblots, and flow cytometry assays in order to study HSP90 and HSP70 function in MHC class II presentation. All of the following primary antibodies were to human proteins. The rabbit anti-human kappa light chain pAb and the rabbit anti-GAD65 pAb were purchased from Sigma Aldrich (St. Louis, MO). The mouse anti-GAPDH mAb was purchased from Chemicon (Temecula, CA). The mouse anti-actin mAb, rabbit anti-HSP90α pAb, rabbit anti-HSP90β pAb were all purchased from Neomarkers/Thermo Fisher (Fremont, CA). The mouse anti-KDEL mAb, mouse anti-HSP90 mAb, rabbit anti-HSP40 pAb, mouse anti-HSP70 mAb, and rat anti-HSC70 mAb were all purchased from Assay Designs (Ann Arbor, MI). The mouse anti-MHC class II DRα (DA6.147) and mouse anti-MHC class II dimers (L243) were provided by Peter Cresswell (Yale). The GAD6 Ab (mouse anti-GAD65) was obtained from the Developmental Studies Hybridoma Bank, Univ. Iowa (Iowa City, IA). Various secondary antibodies were used in these studies. The goat anti-rabbit IgG-HRP Ab, goat anti-rat IgG-HRP Ab, and goat anti-mouse IgG-HRP Ab were all purchased from Jackson Laboratories (West Grove, PA). The goat anti-mouse IgG-PE was purchased from Dako (Carpinteria, CA). To detect ovalalbumin protein, specific rabbit anti-serum from Cappel Laboratoris was used (Downington, PA).

For the co-immunoprecipitation assays performed in the case study of type B insulin resistance, the following antibodies were used. The rabbit anti-insulin receptor α (N-20): sc-710 pAb and the rabbit anti-insulin receptor β (C-19): sc-711 pAb were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The goat anti-β actin pAb was purchased from Abcam (Cambridge, MA).

Antigens, peptides, and proteins

A variety of Ags, peptides, and proteins were used in the following study. The human GAD65 Ag was provided by J. Elliott (University of Alberta) or purchased from Diamyd (Pittsburgh, PA). The HSA Ag was purchased from Sigma Aldrich (St. Louis, MO). The synthetic peptides $GAD_{273-285}$ (IAFTSEHSHFSLK), kappa $I_{188-203}$ (KHKVYACEVTHQGLSS), kappa $II_{145-159}$ (KVQWKVDNALQSGNS), and HSA_{64-76} (VKLVNEVTEFAKTK) were generated using FMOC technology, HPLC purified, and mass analyzed to confirm purity and structure. Purified native, human HSP90 protein was purchased from Assay Designs (Ann Arbor, MI). Ovalbumin (OVA) protein was purchased from Sigma Aldrich (St. Louis, MO). Kappa Ag isolated from human Bence Jones protein was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY).

Pharmacological inhibitors

The HSP90 inhibitors, geldanamycin (GA) and radicicol (RA), were purchased from EMD (Rahway, NJ) and solubilized in DMSO. GA was used at concentrations ranging from 0.09 to 1.8 μM, and RA was used at concentrations ranging from 1-5 μM.
The lysosomal protease inhibitor leupeptin was solubilized in PBS and used at a concentration of 50 μM (Sigma Aldrich, St. Louis, MO). The toxicity of all inhibitors was tested to ensure a typical cell viability at $\geq$ 85% following cell treatments.

Amaxa siRNA nucleofection

Target specific siRNA was used to specifically knockdown HSP90α and HSP90β expression. HSP90α and HSP90β siRNA oligomers as well as RISC-free siRNA controls were produced by Dharmacon (Lafayette, CO). As previously described, the target sequences for hsp90α knockdown were 5′-AAAGCGUUCAUGGAAGCUUUG-3′ and 5′-AAGGCUGACUUGAUCAAUAAC-3′ while the siRNA specific for hsp90β was 5′-AAGGCCAAGCACGACAAGTAC-3′. All oligonucleotides had 3′ dTdT overhangs (141). HSP90α, HSP90β, and RISC-free control siRNA were used at concentrations between 20-200 pMol. RISC-free siRNA lacks a signal for nuclear translocation and functions as a negative control siRNA for nucleofection. Nucleofection of B cells with

siRNA and a GFP-expression plasmid was performed using the Lonza (Gaithersburg, MD) nucleofection kit V and program Y-01. After nucleofection, B cells were incubated for 48 hours.

Flow cytometry

Flow cytometry was used to detect expression of MHC class II, CLIP, Ii, HLA-DM, and HLA-DO. For cell surface expression, 5 x 10^5 B cells were incubated with primary Ab for 60 min on ice, washed with ice cold phosphate buffered saline (PBS), and incubated with either FITC- or PE-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (1:200 dilution) prior to aldehyde fixation. For total expression, 5 x 10^5 B cells were fixed in 1% paraformaldehyde (10 min, 25°C) and permeabilized with PBS + 1% bovine serum albumin (BSA) + 0.1% sodium azide (NaN_3) + 0.1% saponin for 30 minutes on ice prior to Ab staining. Flow cytometry was performed on a FACScan™ and data analyzed using CELLQuest software.

Antigen presentation assay

To measure MHC class II presentation of endogenous Ags, treated B cells were washed in cold PBS, fixed in 0.5% paraformaldehyde (10 min, 25°C), and washed in cold B cell media to remove the paraformaldehyde. For exogenous Ag or peptide presentation, B cells were pre-incubated with serially diluted Ags or peptides for 16 hours at 37°C prior to fixation. Variable numbers of B cells (ranging from 1-5 x 10^4 cells) were added to peptide specific T cells (1 x 10^4) and incubated for 24 hours at 37°C. The IL-2 dependent T cell line, HT2, was used to measure IL-2 production during the APC/T cell incubation. HT2 cells (5 x 10^3 cells) were incubated with supernatants from the APC/T cell co-cultures for 18 hours at 37°C. [^{3}H]-thymidine (2 µCi/well) was added to the HT-2 cells and incubated for another 8 hours at 37°C. The cells were then harvested using a 96-well plate cell harvester (Skatron, Sterling, VA), and [^{3}H] -thymidine incorporation was measured by liquid scintillation counting using a Wallac Microbeta plate reader (Gaithersburg, MD). Data is expressed as the average counts per minute (CPM) of triplicate samples.

Immunoprecipitations

For co-immunoprecipitation assays with B cells, 1 x 10^7 cells per sample were lysed in 500 µl of either 1% Triton-X lysis buffer (10 mM Trizma base, 150 mM NaCl, 0.2 mM PMSF, 0.1 mM TLCK, 1% Triton-X 100, pH = 7.4) or 1% N-octyl-β-glucopyranoside lysis buffer (10 mM Trizma base, 150 mM NaCl, 0.2 mM PMSF, 0.1 mM TLCK 1% N-octyl-β-glucopyranoside, pH = 7.4) for 15 minutes on ice. Lysates were first centrifuged at 1000 RPM for 5 minutes, then re-centrifuged at 14,000 RPM for 10 minutes. Pre-clearing of the lysates was performed to remove any non-specific binding. Normal rabbit serum (1:1,000 dilution) was added to the lysate and incubated at 4°C for 30 minutes while rocking. Blocked, cold PBS-washed protein G sepharose beads (Sigma-Aldrich, St. Louis, MO) were added and incubated for an additional 15 minutes at 4°C while rocking. The protein G sepharose beads were centrifuged at 7000 RPM at 4°C and the supernatant was collected. Prior to use, protein G sepharose beads were blocked with a 30 minute incubation at 4°C with rocking in TNNB buffer (50 mM Trizma base /HCL with pH= 8.0, 250 mM NaCl, 0.1% BSA, 0.02% NaN_3, 0.2 mM PMSF, 0.1 mM TLCK), then washed and resuspended 1:1 with cold PBS. Specific Abs or isotype control Abs were incubated with blocked, PBS-washed protein G sepharose beads overnight at 4°C while rocking. After incubation, Ab-PGS complexes were washed with cold PBS and resuspended 1:1 with cold-PBS. Pre-cleared cell lysates were added to the Ab-PGS complexes and incubated at 4°C for 18 hours while rocking. The beads were then washed three times with lysis buffer without protease inhibitors. Ag-Ab complexes were eluted with 50 µl 2X reducing sample buffer, boiled for 5 minutes, and briefly centrifuged. Elutes were resolved on 10% SDS-PAGE and Western immunoblotting was performed as described below.

For the co-immunoprecipitation assays CHO and CHO/IR cells were collected by scraping cells from plates with 10 mM Trizma Base, 150 mM NaCl, 0.2 mM PMSF, 0.1 mM TLCK (pH = 7.4) buffer. Cells were then homogenized with Daunce homogenizer and passed through a 20 gauge needle. Following ultracentrifugation, cells were lysed in 1% Triton-X 100, 10 mM Trizma Base, 150 mM NaCl, 0.2 mM PMSF, 0.1 mM TLCK (pH = 7.4). Cell lysates were then pre-cleared, and co-immunoprecipitation was

performed. Cell lysates were pre-cleared with SACI. Patient or control serum was added to cell lysates at 4°C overnight, followed by protein G sepharose for 1 hour at 4°C. Ag-Ab complexes were collected and washed prior to elution and analysis by 10% SDS-PAGE and Western immunoblotting.

Gel electrophoresis and Western immunoblotting

For cell lysates, 5 x 10^6 cells were lysed in 250 ul of 1% Triton-X lysis buffer (10 mM Trizma Base, 150 mM NaCl, 0.2 mM PMSF, 0.1 mM TLCK, pH = 7.4) on ice for 15 minutes. Lysates were first centrifuged at 1000 RPM for 5 minutes, then re-centrifuged at 14,000 RPM for 10 minutes. Cellular protein concentrations were determined by BioRAD protein assay (Hercules, CA). Equal amounts of cell protein (50-100 µg) in either non-reducing or reducing sample buffer was resolved by either 10% or 12% SDS-PAGE and transferred onto nitrocellulose membranes (BioRAD, Hercules, CA). Reducing sample buffer contained βME and was used in all cases except for the detection of invariant chain and MHC class II dimers. Membranes were blocked in Blotto overnight at 4°C and incubated with specific antibodies. HRP-conjugated secondary antibodies and enhanced chemiluminescence from Pierce (Rockford, IL) was used to visualize antibodies on membranes. Densitometry was performed using the Quantity One program and electronic imaging (BioRAD, Hercules, CA).

For analysis of additional proteins on the blots, the blots were stripped and reprobed with additional antibodies of interest. Following development of the blot, any residual ECL was rinsed with TBS-T. The blots were then stripped of bound antibodies by incubating the blots for 30 minutes at 65°C in a stripping solution containing 100 mM βME and 2% SDS. After washing to remove any residual stripping solution, the blots were blocked in Blotto and probed with additional antibodies. This procedure could be repeated at least 3 times with reproducible results.

Media concentration for Western analysis

Fresh and conditioned media was concentrated from 13 ml to approximately 50 µl using Centricon centrifugal filter devices with 50,000 and 30,000 NMWL Ultracel YM

membranes (Millipore, Billerica, MA). Cultured cells were centrifuged at 1000 RPM for 5 minutes to separate the cells from the media. The supernatant was removed and centrifuged at 5000 x g max for 60 minutes in a Centricon filter device with 50,000 NMWL membrane. The concentrated media was removed and recentrifuged at 5000 x g for 30 minutes in a Centricon filter device with 30,000 NMWL membrane.

Semi-quantitative RT-PCR

Total RNA was extracted from treated B cells with an RNEasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. cDNA was generated from RNA using an Advantage RT for PCR kit from BD Biosciences. Primers for PCR amplification were designed using the Custom Primers - OligoPerfect™ Designer software (Invitrogen, Grand Island, NY). The primers used for human Ii were 5'-GCT GTC GGG AAG ATC AGA AG-3' (sense) and 5'-GCC ATA CTT GGT GGC ATT CT-3' (antisense); for HLA-DRα 5'-CAA AGA AGG AGA CGG TCT GG-3' (sense) and 5'-AGC ATC AAA CTC CCA GTG CT-3' (antisense); for human cathepsin S 5'-GGA TCA CCA CTG GCA TCT CT-3' (sense) and 5'-CCA GCT TTC CTG TTT TCA GC-3' (antisense); for human cathepsin B 5'-GCT ATC CTG CTG AAG CTT GG-3' (sense) and 5'-CAT TGT CAC CCC AGT CAG TG-3' (antisense); for human cathepsin D 5'-AGC TGG TGG ACC AGA ACA TC-3' (sense) and 5'-CTC TGG GGA CAG CTT GTA GC-3' (antisense); and for human cathepsin L 5'-TGT GGT TCT TGT TGG GCT TT-3' (sense) and 5'-CAG GCC TCC ATT ATC CTG AA-3' (antisense). GAPDH primers were obtained from the Advantage RT for PCR kit. Amplification reactions were performed using 1.1×ReddyMix™ PCR Master Mix (ABgene/Thermo Fisher, Rockford, IL) with different cycle times in a MJ Research thermal cycler. The number of amplification cycles for analysis was 20, 28, 30, 36, or 50 cycles. The cycling parameters used were: 95°C, 15 sec; 50°C, 30 sec; and 68°C, 1 min. PCR products (10 µl) were electrophoresed on 1% agarose gels, stained with SYBR® safe DNA gel stain (Invitrogen, Grand Island, NY), and detected with UV transillumination using ChemiDoc™ XRS (BioRad, Hercules, CA).

Cathepsin activity assays

The following protocol for measuring cathepsin activity was adapted from the published protocol described by Kirschke and Wiedernanders (218). For these assays, 5 ml glass tubes were used. For each sample, 2 x 10^7 cells were suspended in 0.5 ml of 20 mM Hepes, 0.5% Triton-X 100, pH = 6.5 with HCL and incubated for 3 minutes on ice. Cells were homogenized with a Dounce homogenizer for 20 strokes on ice. Homogenates were centrifuged at 1000 RPM for 5 minutes at 4°C. Samples were serially diluted (1:2, 1:4, and 1:10) in diluent. Buffer/Activator (50 µl) for each assay was added to the diluted samples and incubated for 2 minutes at room temperature. 50 µl of substrate solution was added and incubated for exactly 30 minutes for cathepsin B, 10 minutes for cathepsin S/L, and 60 minutes for cathepsin S assays. After incubation, 200 µl of stopping buffer was added and mixed. For each sample, 200 µl was transferred to a 96 well plate and analyzed for fluorescence with excitation at 360 nm and emission at 460 nm. Samples were compared to a 0.5 µM 7-amino-4-methylcoumarin (AMC) standard. The instrument was set to read 1000 arbitrary fluorescence units (FU) for the 0.5 µM AMC standard. Cathepsin activity was calculated as the change in sample fluorescence units (FU) after 30 minutes. Here, the observed FU for samples with protease at 30 minutes was corrected for background substrate hydrolysis by subtracting the FU of a control or blank sample lacking enzyme. For cathepsins, international convention holds that 1 mU of enzyme activity can catalyze a change of 1000 FU per minute. Thus, for a 30 minute assay, the following equation was used: (X FU/30 minutes) x (1 min/1000 FU) = Y microunits of protease (µU). The BioRad protein assay was performed to determine the total protein concentration of each sample. Using the protein concentration, the total cathepsin activity per mg protein was calculated using the equation: (Y µU/ml lysate added)/ (mg/ml) protein concentration = X µU/mg.

For all assays, the stop solution was 100 mM sodium monochloracetate ($CH_2ClCOONa$), 30 mM sodium acetate ($NaCH_3COO$), 70 mM acetic acid (CH_3COOH), pH = 4.3. The 10 mM AMC standard reconstituted in DMSO was diluted to a working concentration of 0.1 µM with a 1:1 mixture of assay buffer:stopping reagent each day of use. The diluent, buffer/activator, and substrates for each assay is listed as follows. For cathepsin B, the

diluent was 0.1% Brij 35 detergent (Sigma), the buffer/activator was 352 mM monopotassium phosphate (KH_2PO_4), 48 mM disodium phosphate (Na_2HPO_4), 4 mM EDTA, pH = 6.0 with 8mM dithiothreitol (DTT) added on day of use, and the stock substrate solution was 1mM Z-Arg-Arg-AMC in DMSO, diluted to a working solution of 20 uM in ddH_20 on day of use. For cathepsin S/L assays, the diluent was 0.1% Brij 35 detergent (Sigma), the buffer/activator was 340 mM sodium acetate ($NaCH_3COO$), 60 mM acetic acid (CH_3COOH), 4 mM EDTA, pH = 5.5 with 8mM DTT added on day of use, and the stock substrate solution was 1mM Z-Phe-Arg-AMC in DMSO, diluted to a working solution of 20 µM in ddH_20 on day of use. For cathespin S, the diluent was 0.01% Triton-X-100, .1M potassium phosphate buffer, mM EDTA, pH=7.5; the buffer/activator was 0.1M potassium phosphate buffer/5mM disodium EDTA with 5mM DTT added on day of use; and the stock substrate solution was 10mM Z-Val-Val-Arg-AMC in DMSO diluted to a working solution of 12.5 µM in ddH_20 on day of use.

RESULTS

Chapter 1. HSP90 modulates GAD antigen presentation in B cells

In order to study MHC class II presentation of GAD, two model B cell lines, Priess and PriessGAD, were utilized. Both Priess and PriessGAD are EBV transformed human B cell lines homozygous for the diabetes susceptible MHC class II allele HLA-DR4. PriessGAD cells were generated by retroviral transduction of Priess with cDNA encoding the 65 kD form of human GAD (67). Within the cytoplasm of cells, GAD is primarily associated with the outer face of vesicular organelles. Previous studies from our lab showed that similarly in PriessGAD cells, GAD is also primarily associated with the cytoplasmic face of intracellular membranes (67). Effective MHC class II presentation of the immunodominant T cell epitope $GAD_{273-285}$ resulted from the expression of human GAD within the cytoplasm of PriessGAD cells (219). Western immunoblotting of conditioned media from PriessGAD cells failed to detect any secreted GAD Ag suggesting that endogenous GAD is processed via an endogenous pathway (67). When pulsed with exogenous human GAD Ag, Priess cells can present the immunodominant $GAD_{273-285}$ epitope via MHC class II molecules (219). Thus, the autoantigen GAD can be efficiently presented by MHC class II molecules either by cytoplasmic or extracellular (exogenous) routes.

Pharmacological inhibition of HSP90 specifically decreases MHC class II Presentation of GAD

HSP90 is constitutively expressed by all cells including islet beta cells and APCs. In addition to cytoplasmic GAD, PriessGAD cells also express endogenous HSP90 at relatively high levels. Previous studies in our laboratory implicated HSC70 as a player in MHC class II presentation of endogenous GAD. HSC70 has been shown in the literature to interact with HSP90 (162). Therefore, experiments were performed to determine whether HSP90, like HSC70, is involved in MHC class II presentation of GAD. In order to explore the role of HSP90 in GAD Ag processing and presentation in B cells, we utilized two HSP90 inhibitors, GA and RA. GA, a benzoquinone ansamycin antibiotic, and RA, a macrocyclic drug, have been shown to bind to HSP90 and inhibit its chaperone activity in cell lines and animals (177). PriessGAD cells were treated for 18 hours with

increasing concentrations of GA and RA and analyzed for MHC class II presentation of endogenous, intracellular GAD. Both GA and RA treatment of PriessGAD cells resulted in a dose-dependent decrease in MHC class II presentation of endogenous GAD (Fig. 8). Treatment of cells with GA and RA for 18 hours did not alter cell viability as assessed by trypan blue staining (data not shown).

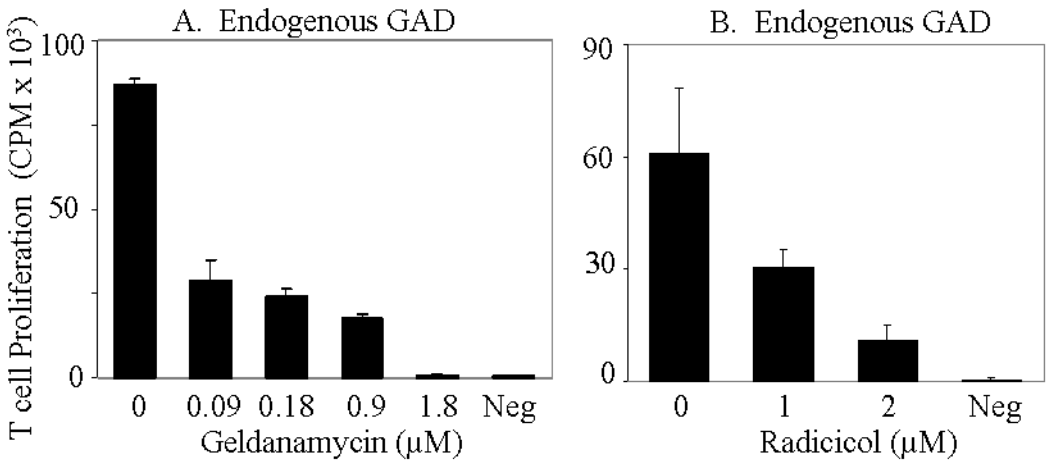


Figure 8. Pharmacological inhibition of HSP90 reduces MHC class II presentation of endogenous GAD Ag. PriessGAD cells were treated with increasing concentrations of HSP90 inhibitors GA (A) or RA (B) for 18 hours prior to paraformaldehyde fixation. MHC class II presentation of endogenous GAD was analyzed by T cell assays. Multiple control assays were carried out including analysis of the production of IL-2 by APCs or T cells cultured alone. The negative control indicated here represents proliferation of HT-2 cells without addition of IL-2. Results are representative of at least 3 separate experiments.

Drug-treated PriessGAD cells were also analyzed for their capacity to present epitopes from another endogenous Ag, the kappa light chain of human Ig Ab. The kappa light chain subunit is produced via the secretory pathway, transiting from the ER to the Golgi and routed to endosomes for secretion or to the cell surface via endosomes. Previous studies in our lab using inhibitors of endo/lysosomal Ag processing suggest that kappa light chain is processed in acidic compartments. Further studies have shown that epitopes of kappa light chain Ag are displayed by MHC class II molecules on the cell surface of Priess and PriessGAD cells. PriessGAD cells were treated with increasing concentrations of GA or RA and analyzed for MHC class II presentation of both the dominant kappa I and sub-dominant kappa II epitopes from endogenous Ig kappa light chain. GA or RA treatment did not alter MHC class II presentation of endogenous kappa I or kappa II epitopes (Fig. 9). These results indicate that HSP90 inhibition by either GA or RA appears to perturb MHC class II presentation of select endogenous Ags such as GAD. These results point to a potential selectivity or specificity in HSP90 modulation of MHC class II presentation which may be linked to Ag or Ag localization.

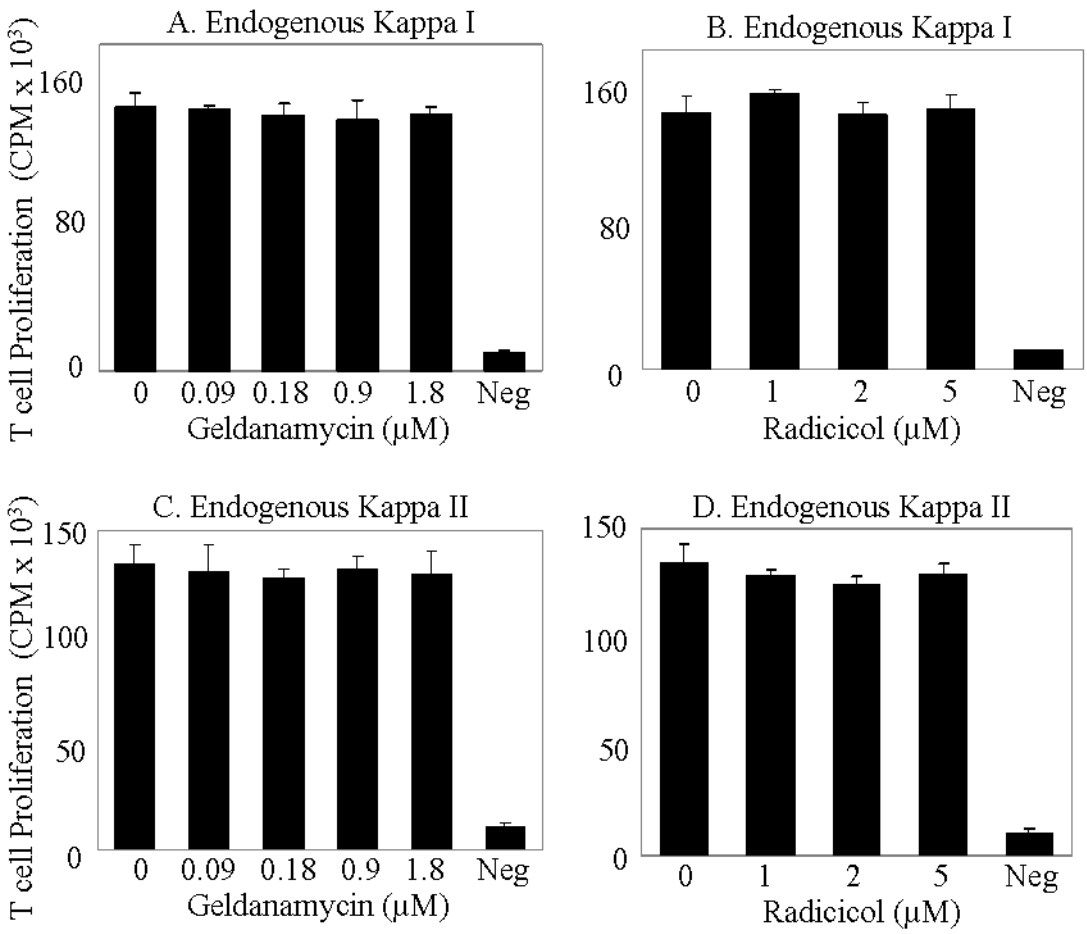


Figure 9. Pharmacological inhibition of HSP90 function does not affect MHC class II presentation of endogenous IgG kappa Ag. PriessGAD cells were treated with increasing concentrations of HSP90 inhibitors GA (A and C) or RA (B and D) for 18 hours. MHC class II presentation of two endogenous peptide epitopes encoded within the Ag IgG, termed kappa I (A and B) and kappa II (C and D) was analyzed by T cell assays. Control assays were carried out as in figure 8. Here, the negative control indicates HT2 cell proliferation without IL-2 addition. Results are representative of at least 3 separate experiments.

The question remained whether HSP90 is involved in the classical exogenous MHC class II pathway or functions only in the endogenous MHC class II presentation pathway. Thus, Priess cells were treated with GA or RA and pulsed with several exogenous Ags and peptides prior to testing for MHC class II presentation. Priess cells were first analyzed for the effect of GA and RA on MHC class II presentation of exogenous GAD Ag (Fig. 10A and B). GA or RA treatment inhibited MHC class II presentation of exogenous GAD Ag by Priess cells. To determine if the effect of GA and RA treatment was specific for Ag processing, GA or RA treated Priess cells were pulsed with the exogenous, synthetic $GAD_{273-285}$ peptide and analyzed for MHC class II presentation of that specific epitope. Neither GA nor RA affected MHC class II presentation of GAD epitope from exogenous GAD peptide (Fig. 10C and D).

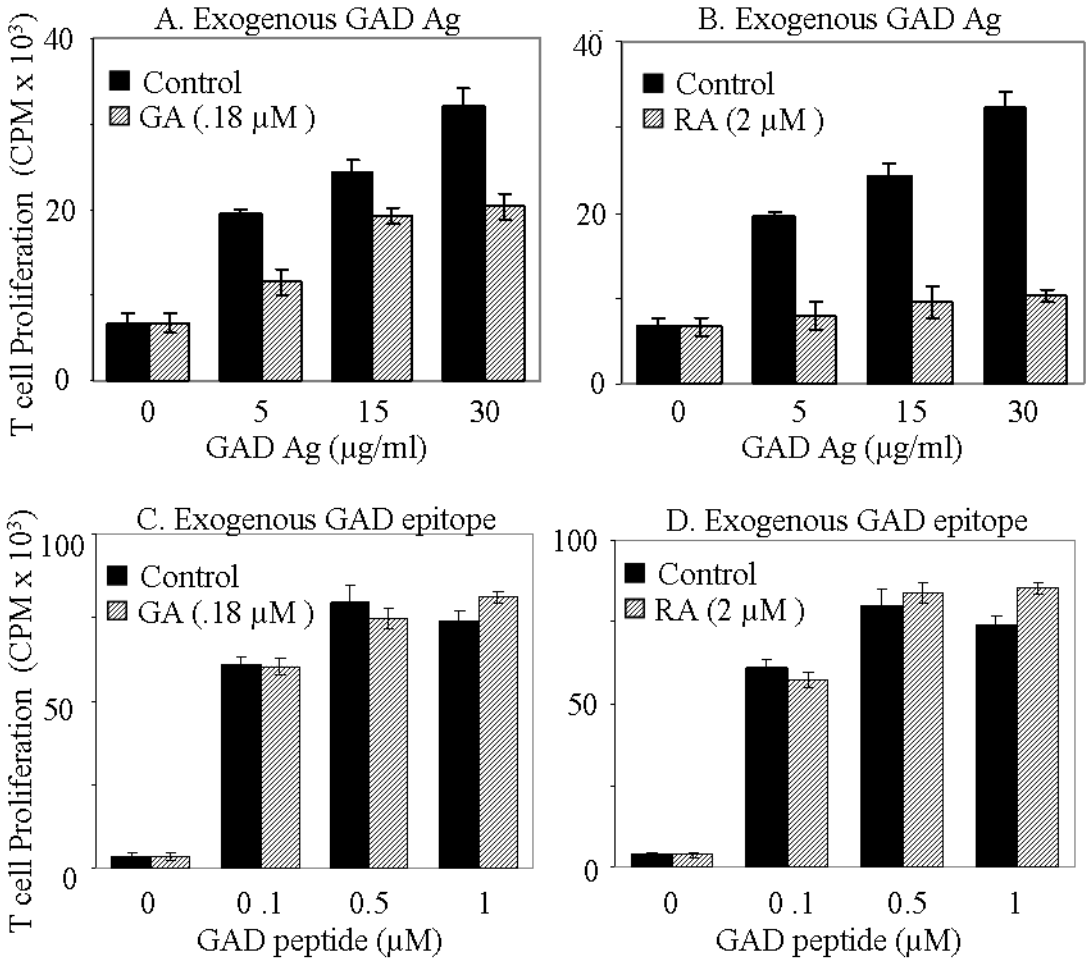


Figure 10. Pharmacological inhibition of HSP90 diminishes MHC class II presentation of exogenous GAD Ag, but not exogenous GAD peptide. Priess cells were treated with either 0.18 µM GA (A and C) or 2 µM of RA (B and D) for 18 hours. After 2 hours of drug treatment, exogenous GAD Ag (A and B) or GAD peptide (C and D) were added to these B cells for an additional 16 hours. MHC class II presentation of exogenous GAD (A and B) and exogenous GAD peptide (C and D) by B cells prior to paraformaldehyde fixation and T cell assays. Control assays were carried out as in Figure 8. Here, the negative control is GA/RA treated Priess cells without exogenous Ag/peptide added. Results are representative of at least 3 separate experiments.

To further address the Ag specificity of GA and RA treatment on MHC class II presentation in B cells, the effect of drug treatment on the presentation of an epitope from human serum albumin (HSA) Ag was examined. PriessGAD cells pulsed with whole HSA Ag can present HSA epitopes including the HSA_{64-76} epitope via MHC class molecules. GA and RA treated PriessGAD cells were pulsed with exogenous HSA Ag and measured for MHC class II presentation of this HSA epitope. Neither GA nor RA treatment of PriessGAD cells affected MHC class II presentation of HSA_{64-76} epitope from exogenous HSA Ag (Fig. 11A and B). GA or RA treated PriessGAD cells were also pulsed with the exogenous, synthetic HSA_{64-76} peptide and analyzed for MHC class II presentation of this epitope. MHC class II presentation of the exogenous HSA_{64-76} peptide was not affected by GA or RA treatment. Taken together, these results suggest that disruption of HSP90 function by GA and RA selectively inhibits MHC class II presentation of GAD epitopes derived from both exogenous and endogenous sources of GAD Ag.

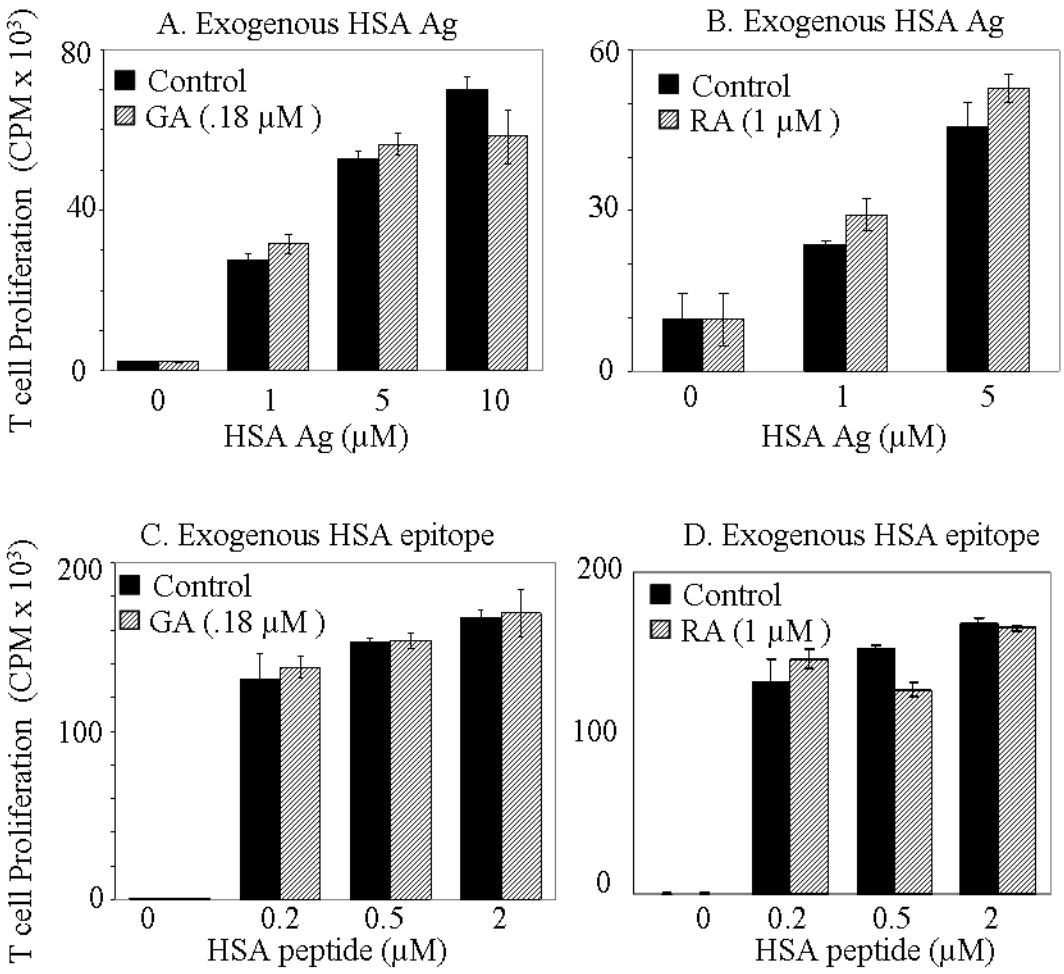
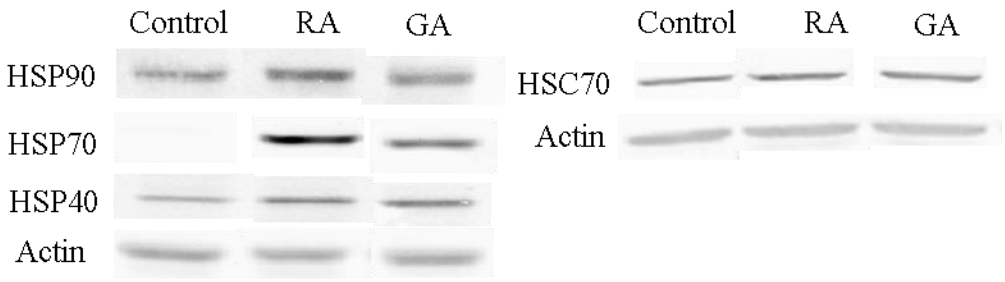


Figure 11. Pharmacological inhibition of HSP90 function does not affect MHC class II presentation of exogenous HSA Ag or exogenous HSA peptide. PriessGAD cells were treated with either 0.18 μM GA (A and C) or 1 μM of RA (B and D) for 18 hours. After 2 hours of drug treatment, exogenous HSA Ag (A and B) or HSA peptide (C and D) were added to PriessGAD B cells. MHC class II presentation of exogenous HSA (A and B) and exogenous HSA peptide (C and D) by these B cells was analyzed by T cell assays. Control assays were carried out as in Figure 9. Here, the negative control is GA/RA treated Priess cells without exogenous Ag/peptide added. Results are representative of at least 3 separate experiments.

Pharmacological inhibition of HSP90 induces a stress response in B cells

Prolonged exposure of cells to HSP90 inhibitors can induce a stress response, as monitored by increased cellular expression of HSP70, HSP90, HSC70, and HSP40 protein levels (179, 220, 221). To determine if RA and GA were inducing a stress response in B cells, levels of intracellular HSP90, HSP70, HSC70, and HSP40 protein were measured by Western immunoblotting. Results show that both RA and GA treatments significantly induce HSP70 expression and to a lesser extent HSP40 (Fig. 12). HSP90 expression is also slightly induced by RA treatment as cells attempt to compensate for loss of HSP90 function as previously reported (Fig. 12) (179, 220, 221). However, statistical analysis determined this induction was not statistically significant. These results indicate that inhibition of HSP90 function by RA and GA treatment induces a stress response in B cells.

A.



B.

Relative Expression of HSP/HSC			
	Control	RA treated	GA treated
HSP90/Actin	100%	154%	96%
HSC70/Actin	100%	117%	129%
HSP70/Actin	100%	5016%	2641%
HSP40/Actin	100%	213%	185%

C.

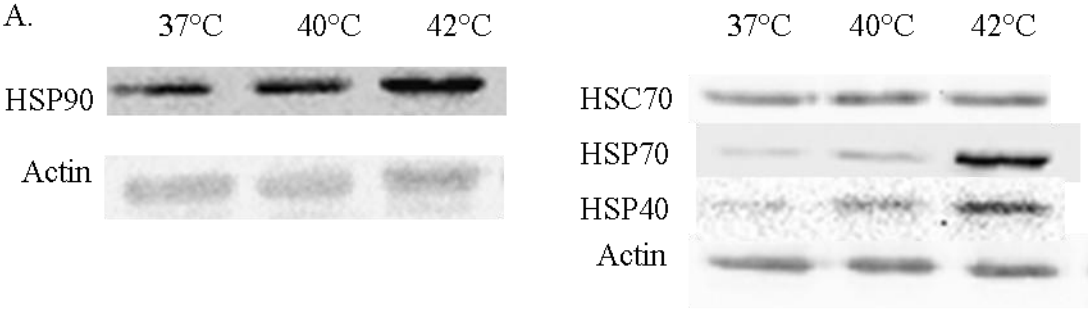
Average Relative Expression of HSP/HSC			
	Control	RA treated	GA treated
HSP90/Actin	100%	160% +/- 6%	109% +/- 13%
HSC70/Actin	100%	155% +/- 38%	132% +/- 3%
HSP70/Actin	100%	2641% +/- 1194% *	1923% +/- 366% *
HSP40/Actin	100%	229% +/- 10% *	255% +/- 10% *

Figure 12. Pharmacological inhibition of HSP90 induces a stress response.

PriessGAD cells were treated with either RA, GA, or DMSO as a control for 18 hours. (A) Whole cell lysates from treated cells were fractionated on 10% SDS-PAGE gel and Western immunoblotted for HSP90, HSC70, HSP70, HSP40, and actin protein expression. (B) Densitometry of the representative experiment in panel A. Results are representative of at least 2 separate experiments. (C) The average relative expression of HSP/HSC +/- SEM as determined by analysis of at least 2 separate experiments. *Statistically significant as determined by student t tests.

Studies have shown that cell stress such as heat shock can promote enhanced Ag processing and MHC class II presentation of some exogenous and endogenous Ags by B cells (222). It is possible that the altered Ag presentation seen in drug treated PriessGAD cells was due to their induction of a stress response rather than HSP90 inhibition. Experiments were performed to determine if cell stress by heat shock altered GAD Ag presentation. PriessGAD cells were heat shocked to mimic the stress response that was observed upon RA/GA treatment. PriessGAD cells were incubated at 37, 40, or 42°C for 20 minutes followed by a 24 hour incubation at 37°C. Exposure of PriessGAD cells to 40 or 42°C resulted in significantly increased HSP90, HSP70, and HSP40 expression confirming an induced stress response (Fig. 13). Previous studies in the lab have shown that this increase in HSP/HSC expression was maintained for up to 48 hours (data not shown). To determine the effect of this stress on Ag presentation, heat stressed PriessGAD cells were assayed for endogenous GAD, exogenous HSA Ag, and HSA peptide presentation. Heat stressing PriessGAD cells did not alter MHC class II presentation of endogenous GAD, exogenous HSA Ag, or exogenous HSA peptide (Fig.14). These results indicate that while RA and GA each induced a stress response and upregulated overall HSP/HSC expression in B cells, cellular stress alone does not contribute to the observed decrease in MHC class II presentation of GAD Ag associated with pharmacological inhibition of HSP90.

A.



B.

Average Relative Expression of HSP/HSC			
	37°C	40°C	42°C
HSP90/Actin	100%	125% +/-11%	101% +/-1%
HSC70/Actin	100%	102% +/- 11%	104% +/- 16%
HSP70/Actin	100%	370% +/- 140% *	1389% +/- 153% *
HSP40/Actin	100%	223% +/- 7% *	409% +/- 27% *

Figure 13. Heat stress induces HSP expression in B cells. PriessGAD cells were incubated for 20 minutes at 37, 40, or 42°C in medium plus 10% heat inactivated serum followed by a 24 hour incubation at 37°C. (A) Whole cell lysates were fractionated on 10% SDS-PAGE gels and Western immunoblotted for HSP90, HSC70, HSP70, HSP40, and actin protein expression. Blot is representative of at least 2 separate experiments. (B) Average relative expression of HSP/HSC +/- SEM as determined by densitometry from at least 2 separate experiments is displayed in the table. *Statistically significant as determined by student t tests.

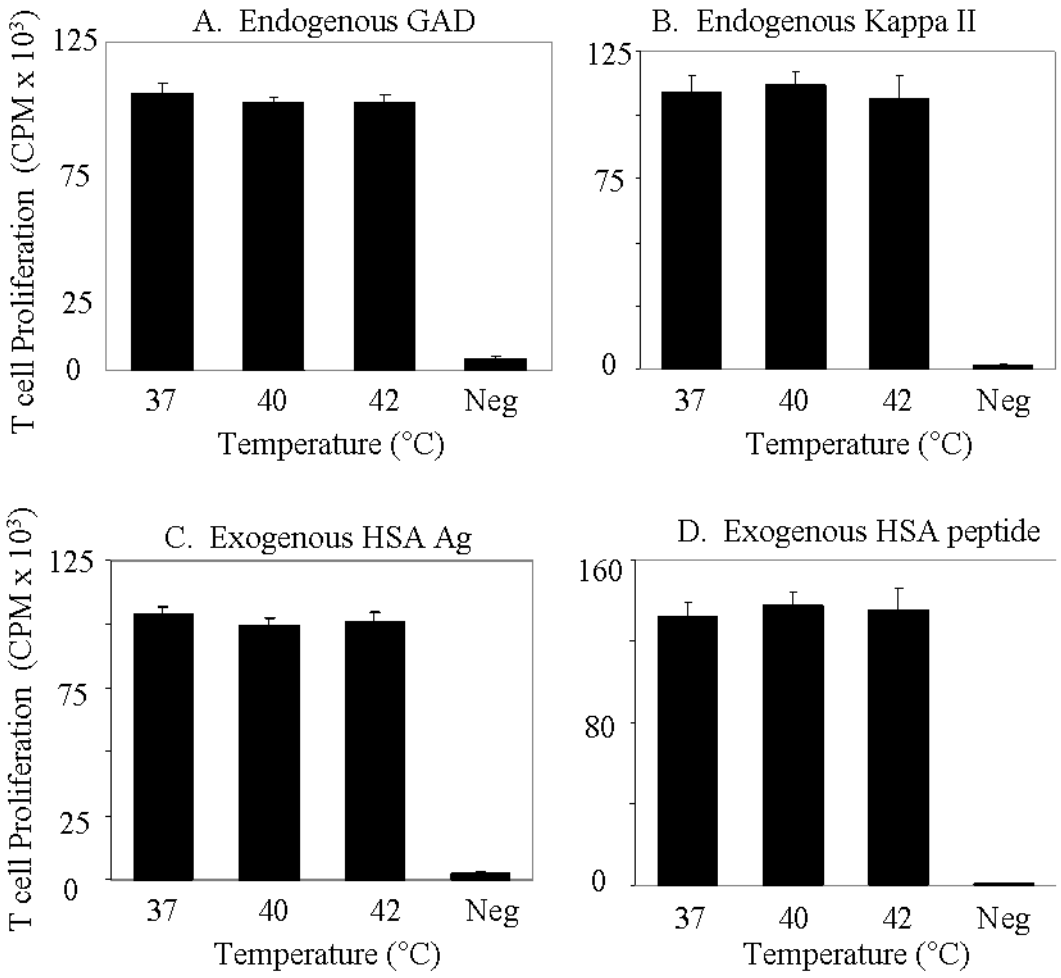
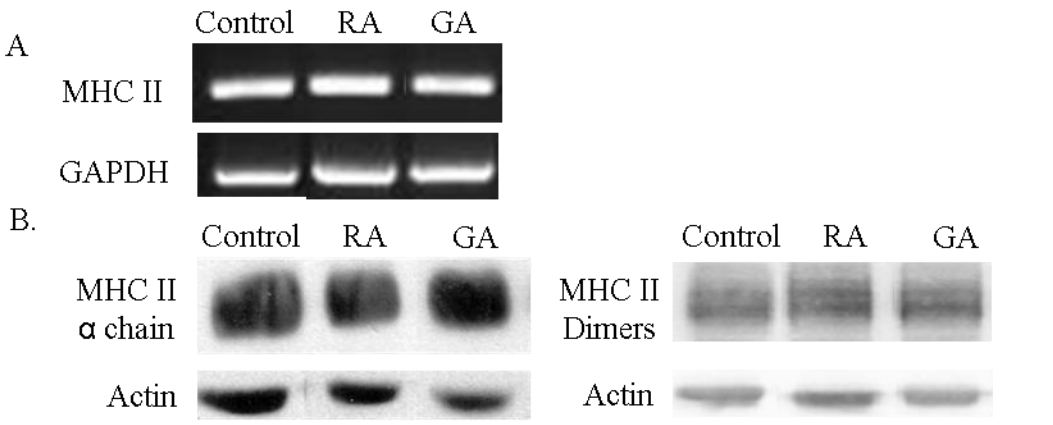


Figure 14. Heat stress does not alter MHC class II presentation of endogenous GAD Ag, endogenous IgG kappa Ag, exogenous HSA Ag, or exogenous HSA peptide. PriessGAD cells were incubated for 20 min at 37, 40, or 42 °C followed by a 24 hr incubation at 37°C with either 5 µM of exogenous HSA Ag (C) or exogenous HSA peptide (D) added after 8 hours. Samples of B cells were divided and assayed by T cell assay for MHC class II presentation of endogenous GAD epitope (A), endogenous kappa II epitope (B), or HSA epitopes from exogenous HSA Ag (B) or HSA peptide (C). Control assays were carried out as in Figure 8. Here, the negative control indicates HT2 cell proliferation without IL-2 addition. Results are representative of at least 3 separate experiments.

Pharmacological inhibition of HSP90 does not alter MHC class II expression

In a study using macrophages, HSP90 overexpression was found to enhance MHC class II dimer stability while GA and RA treatments decreased dimer stability (142). Both HSP90 overexpression and inhibition were reported not to alter surface and total MHC class II protein expression in these murine cells (142). To evaluate whether GA or RA inhibition of HSP90 altered MHC class II expression levels in human B cells, PriessGAD cells were treated with either agent, and then evaluated for total cellular MHC class II mRNA and protein expression as well as MHC class II surface expression. To determine if HSP90 inhibition affects MHC class II transcription, RT-PCR was performed on RA, GA, or control treated cells. HSP90 inhibition by RA or GA did not alter MHC class II RNA expression levels (Fig. 15A). Western immunoblotting was performed on RA, GA, or control treated cells to measure the total cellular content of MHC class II DRα monomers or dimers. Neither GA nor RA significantly altered the total cellular content of MHC class II DRα monomers as detected by Western immunoblotting (Fig. 15B). While MHC class II monomer expression was not affected, dimerization of MHC class II α and β chains could be altered. Therefore, the effect of RA and GA on MHC class II dimerization in B cells was explored. Western immunoblotting for MHC class II dimers showed that steady state levels of MHC class II dimers were also unchanged by GA or RA treatment (Fig. 15B).



C.

Average Relative Expression			
	Control	RA	GA
MHC II α/Actin	100%	100% +/- 27%	90% +/- 6%
MHC II Dimers/Actin	100%	119% +/- 3%	108% +/- 11%

Figure 15. Pharmacological inhibition of HSP90 does not alter MHC class II mRNA or protein expression. PriessGAD cells were treated with DMSO as a control, RA (2 μM), or GA (0.18 μM) for 18 hours. (A) Following treatment, mRNA was prepared and transcribed to cDNA. From each sample, cDNA was subjected to PCR to amplify sequences from MHC II α chain and GAPDH. PCR products were resolved on an agarose gel with SyberSafe stain. Results shown are representative of at least 2 separate experiments. (B) Whole cell lysates from treated cells were analyzed by Western immunoblotting for expression of MHC class II DRα chain (left) or MHC class II DR αβ dimers (right). Results shown are representative of at least 3 separate experiments. (C) The table depicts the average relative expression of MHC class II DRα and Dr αβ dimers +/- SEM. Student t tests determined no significant change in MHC class II expression.

Additionally, flow cytometry of RA and GA treated cells was used to examine total cellular levels of MHC class II dimers. This analysis confirmed no alteration of total MHC class II dimer expression (Fig 16A). The observed decreases in MHC class II GAD Ag presentation could result from decreased surface expression of MHC class II dimers, even though total monomer and dimer expression remained stable. Therefore, surface levels of MHC class II dimers were also examined by flow cytometry. Analysis indicated that GA and RA did not decrease surface MHC class II dimer expression (Fig. 16B). Taken together, these results indicate that the decrease in MHC class II GAD Ag presentation observed with GA or RA treatment is not due to alterations in MHC class II expression or dimer stability in B cells.

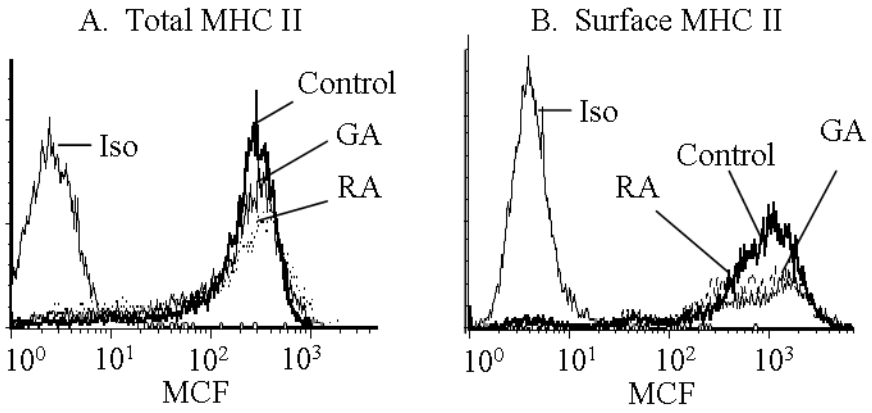


Figure 16. Pharmacological inhibition of HSP90 does not alter total or surface MHC class II expression PriessGAD cells were treated with DMSO as a control, RA (2 μM), or GA (0.18 μM) for 18 hours. (A) Following treatment, cells were fixed in 1% paraformaldehyde, permeabilized, and stained for total MHC class II expression or isotype control (Iso). (B) For surface MHC class II expression, cells were first stained for surface MHC class II expression or isotype control (Iso) and then fixed in 1% paraformaldehyde. MHC class II expression was detected by flow analysis. Thick black line indicates control cells, thin gray line indicates GA treated cells, dashed black line indicates RA treated cells, and thin gray line indicates isotype control staining. Results shown are representative of at least 3 separate experiments.

HSP90 selectively associates with GAD Ag

HSP90 can bind to both proteins and peptides to potentiate intra- and extracellular transport, folding, and proteolysis. HSP90 has been shown to bind peptides for both direct and indirect MHC class I presentation (223). While results thus far suggest HSP90 is involved in MHC class II presentation of GAD Ag, it is unknown whether HSP90 interacts with native GAD Ag. Thus, experiments were performed to determine whether HSP90 naturally associates with cytoplasmic GAD Ag using the human B cell line PriessGAD. Using lysates from PriessGAD cells, immunoprecipitations were performed with either GAD specific or IgG isotype control Abs. Following SDS-PAGE and transfer onto nitrocellulose, blots were probed with an HSP90 specific antibody. The results show that GAD co-immunoprecipitated with HSP90, while an IgG isotype control Ab failed to immunoprecipitate HSP90 confirming specificity (Fig.17A). These results demonstrate intracellular association of HSP90 and GAD in cells co-expressing these proteins. Our results suggest that HSP90 inhibition does not affect MHC class II presentation of endogenous Ig kappa. One explanation for this result is that unlike GAD Ag, Ig kappa Ag may not be a client protein for HSP90. Co-immunoprecipitation experiments were performed on PriessGAD whole cell lysates to determine if Ig kappa Ag associates with HSP90 in PriessGAD cells co-expressing these proteins. Unlike GAD Ag, Ig kappa Ag was not found associated with HSP90 (Fig. 17A).

Intracellularly, HSP90 has been shown to work in concert with other HSPs including HSC70, HSP70, and HSP40 (162, 167). The association of HSP90 with GAD could be due to a direct interaction of these two molecules or an indirect interaction via binding through an additional HSP. In order to determine if HSP90 and GAD can directly interact, purified HSP90 protein was incubated with purified GAD Ag followed by immunoprecipitation with GAD, HSP90, or IgG isotype control Abs. Immunoprecipitates were Western immunoblotted with GAD and HSP90 Abs. HSP90 was detected co-precipitating with GAD, indicating that HSP90 can bind directly to GAD (Fig. 17B). As a control, HSP90 was also incubated with purified Ig kappa light chain and immunoprecipitated with HSP90, Ig kappa light chain, or IgG isotype control Abs. Western immunoblotting did not detect Ig kappa light chain associated with HSP90

confirming that the interaction between HSP90 and GAD is not due to nonspecific binding. These results suggest the association of HSP90 and GAD seen in PriessGAD lysates is likely due to a direct association of these two proteins. HSP90 inhibition by GA was found to decrease MHC class II presentation of exogenous OVA (ovalbumin) Ag in APCs (142). The authors suggested that the effect of HSP90 inhibition on OVA Ag presentation was due to alterations in overall MHC class II expression. However, another possible explanation could be that HSP90 may bind to OVA Ag and selectively chaperone OVA Ag for MHC class II presentation. To determine if HSP90 could directly interact with OVA Ag, purified HSP90 was pre-incubated with purified OVA Ag and immunoprecipitated with HSP90, OVA, or IgG isotype control Abs. HSP90 was found to co-immunoprecipitate with OVA Ag, indicating that HSP90 and OVA Ag can directly interact in vitro (Fig. 17B). These results suggest that the previously published results could be due to selective chaperone activity of HSP90 and not due to overall alterations in MHC class II expression or function.

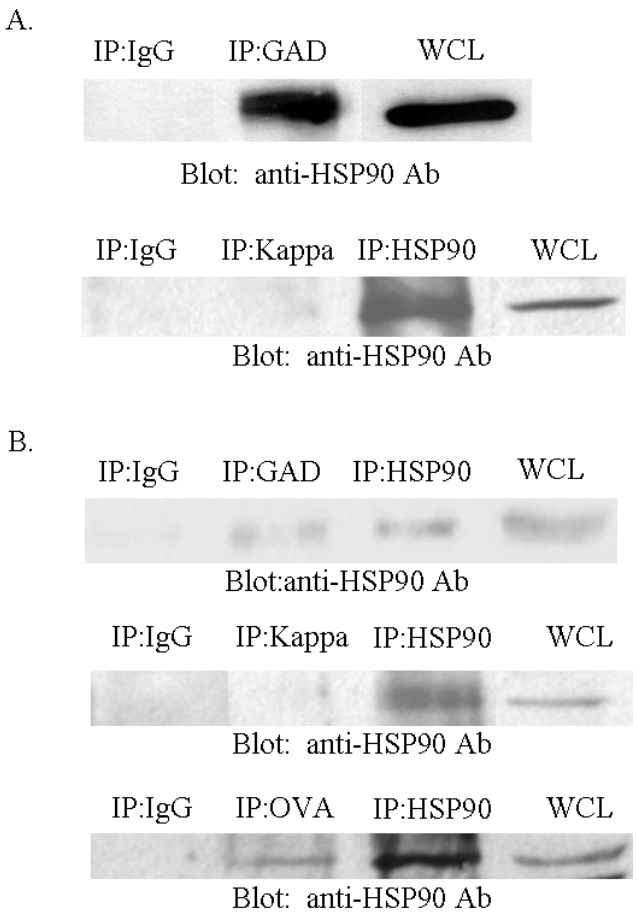


Figure 17. HSP90 selectively associates with GAD Ag. (A and B) PriessGAD whole cell lysates were incubated overnight at 4°C with anti-GAD Ab, anti-HSP90 Ab, anti-kappa light chain, or the isotype control IgG Ab bound to protein-G sepharose beads. Immunoprecipitated proteins eluted with 2X reducing sample buffer were subjected to 10% SDS-PAGE and Western immunoblotting with anti-HSP90 Ab. Whole cell lysate (WCL) was run as a positive control. (B) Purified GAD, kappa light chain, or OVA protein was pre-incubated with HSP90 at a 3:1 molar ratio overnight at 37°C prior to immunoprecipitation with anti-HSP90 Ab, anti-GAD Ab, anti-kappa light chain, anti-OVA Ab, or isotype control IgG Ab bound to protein-G sepharose beads. Immunoprecipitated proteins eluted with 2X reducing sample buffer were subjected to 10% SDS-PAGE and Western immunoblotting with anti-HSP90 Ab. WCL was run as a control for immunoblotting. Gamma settings were adjusted in panel B. Results are representative of at least 3 separate experiments.

HSP90α and HSP90β isoform expression is altered with isoform specific siRNA

In humans, HSP90 is constitutively expressed as two closely related isoforms, HSP90α and HSP90β (170). Assays using pharmacological inhibitors such as RA and GA fail to distinguish between the function of these two isoforms, with both proteins inactivated by these compounds (174). While both isoforms bind similarly to their intracellular co-chaperones, in some cases HSP90α and HSP90β were found to behave differently with respect to substrate interactions (178). Research has implicated HSP90α, but not HSP90β in regulating class I presentation (141). However, a role for HSP0α and HSP90β isoform in MHC class II presentation in B cells has yet to be explored. To investigate the function of HSP90 isoforms in MHC class II presentation of GAD Ag in B cells, a panel of B cells was examined for steady state HSP90α and HSP90β expression. Equal amounts of protein from Priess, PriessGAD, Frev, and FrevSMA whole cell lysates were Western immunoblotted with HSP90α and HSP90β specific antibodies. Both HSP90α and HSP90β isoforms were detected in Priess, PriessGAD, Frev, and FrevSMA (Fig. 18). Moreover, HSP90α and HSP90β isoforms appear to be expressed at measurable levels at steady state conditions in all B cells tested.

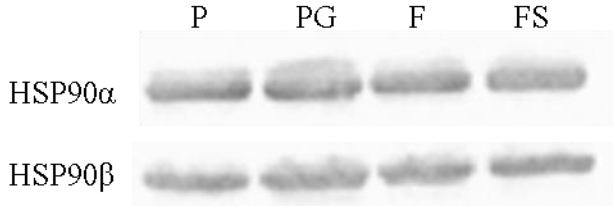
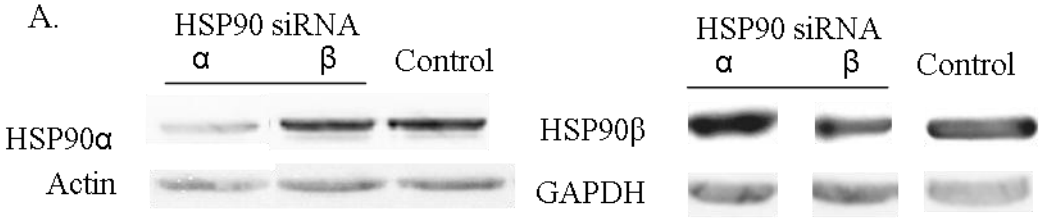


Figure 18. HSP90α and HSP90β are abundantly expressed in B cells. Equal amounts of protein from Priess (P), PriessGAD (PG), Frev (F), and FrevSMA (FS) whole cell lysates were fractionated by 10% SDS-PAGE and Western immunoblotted with either HSP90α or HSP90β specific Abs.

To further evaluate the biological function of these HSP90 isoforms in MHC class II presentation, siRNA specifically designed to disrupt HSP90α and HSP90β mRNA expression were utilized (141). PriessGAD cells were nucleofected with HSP90α or HSP90β siRNA as well as controls such as a RISC-free siRNA. Co-nucleofection with a GFP-containing plasmid was performed as a nucleofection control. Flow cytometry of

nucleofected cells to detect GFP positive cells indicated that DNA transfection efficiency ranged from 45-55% of cells. Total protein expression of HSP90α, HSP90β, actin, and GAPDH was determined by Western immunoblotting. Treatment of B cells with HSP90α siRNA specifically knocked down HSP90α expression, but did not affect HSP90β levels (Fig. 19). Densitometry indicated that HSP90α siRNA treatment resulted in a 66% decease in HSP90α protein expression compared to either HSP90β or control siRNA treated cells. HSP90β siRNA treatment of B cells decreased HSP90β protein expression, but did not consistently affect HSP90α protein abundance (Fig. 19). HSP90β protein expression was decreased to 60% when compared to HSP90α siRNA treated or control siRNA treated cells.

A.



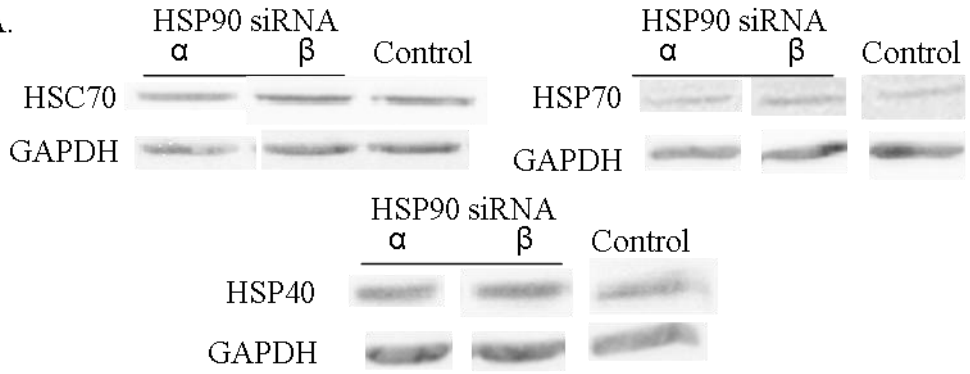
B.

Relative HSP90α or HSP90β Expression			
	HSP90α siRNA	HSP90β siRNA	Control
HSP90α/Actin	34%	100%	100%
HSP90β/GAPDH	100%	60%	100%

Figure 19. HSP90 isotype specific siRNA specifically inhibits HSP90α and HSP90β expression. PriessGAD cells were nucleofected with siRNA for HSP90α, HSP90β, or controls such as RISC-free siRNA as well as the plasmid pMaxGFP and incubated for 48 hr at 37˚C. Flow cytometry of nucleofected cells to detect GFP-positive cells indicated the DNA transfection efficiency ranged from 45-55% cells. (A) Whole cell lysates were analyzed for HSP90α, actin, HSP90β, and GAPDH expression by Western immunoblotting. (B) Densitometry confirmed specific disruption of each HSP90 isoform expression by its respective siRNA. Results are representative of at least 3 separate experiments.

In contrast to HSP90 inhibition by RA and GA, neither HSP90α nor HSP90β siRNA treatment altered HSP70, HSC70, or HSP40 protein expression levels in cells (Fig. 20). This may be due to low levels of residual HSP90α or HSP90β in siRNA treated cells.

A.



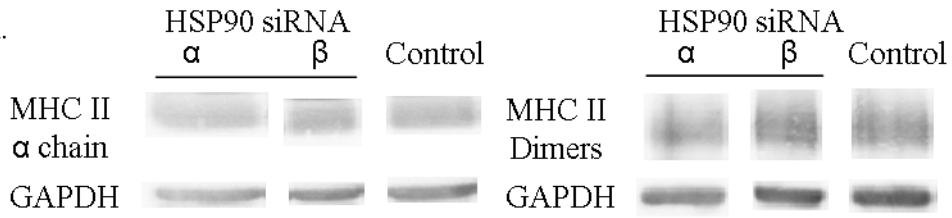
B.

Relative Expression of HSP/HSC			
	HSP90α siRNA	HSP90β siRNA	Control
HSC70/GAPDH	93%	123%	100%
HSP70/GAPDH	98%	101%	100%
HSP40/GAPDH	101%	92%	100%

Figure 20. Inhibition of HSP90α or HSP90β by siRNA does not induce a heat stress response. PriessGAD cells were treated with HSP90α, HSP90β, or control siRNA and incubated for 48 hours as previously described. (A) Whole cell lysates from treated cells were fractionated on 10% SDS-PAGE gel and Western immunoblotted for HSC70, HSP70, HSP40, and GAPDH. (B) Densitometry of the representative experiment. Results are representative of at least 3 separate experiments.

Total cellular MHC class II monomer and dimer protein abundance were unaffected by siRNA treatment as confirmed by Western immunoblotting (Fig. 21A). Flow cytometry of permeabilized and non permeabilized cells indicated no affect of these siRNAs on MHC class II expression (Fig. 21C). Together, these results suggest that HSP90α or HSP90β siRNA treatment specifically knocks down the expression of discrete HSP90 isoforms, respectively, without altering the expression of MHC class II and other HSPs.

A.



B.

Average Relative Expression			
	HSP90α siRNA	HSP90β siRNA	Control
MHC II α/GAPDH	106% +/- 5%	97% +/-3%	100%
MHC II Dimers/GAPDH	100% +/- 0%	99% +/- 0%	100%

C.

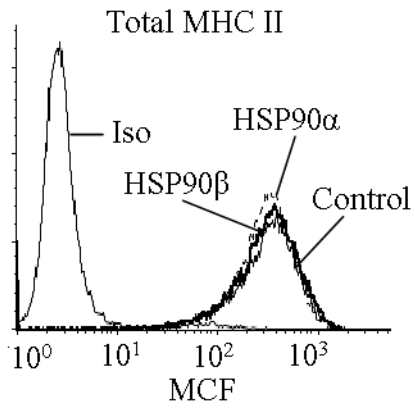


Figure 21. Inhibition of HSP90α or HSP90β by siRNA does not alter MHC class II expression. PriessGAD cells were treated with HSP90α, HSP90β, or control siRNA and incubated for 48 hours as previously described. (A) WCLs of treated cells were analyzed by Western immunoblotting for MHC class II α chain (left) and MHC class II dimers (right). Results are representative of at least 2 separate experiments. (B) The table depicts the average relative expression of MHC class II α and αβ dimers +/- SEM as determined by densitometry of at least 2 separate experiments. (C) PriessGAD cells treated with siRNA were fixed in 1% paraformaldehyde, permeabilized, and stained for total MHC II dimers by flow cytometry. Thick black line indicates control siRNA treated cells, thin black line indicates HSP90α siRNA treated cells, hashed black line indicates HSP90β siRNA treated cells, and thin gray line indicates isotype control staining (Iso). Results are representative of at least 2 separate experiments.

Disruption of HSP90α or HSP90β specifically inhibits MHC class II presentation of GAD

To determine the role of HSP90α and HSP90β in MHC class II presentation, PriessGAD cells were treated with siRNA for HSP90α, HSP90β, or controls such as RISC-free siRNA followed by analysis of MHC class II presentation for several Ags and epitopes. Treatment of PriessGAD cells with either HSP90α or HSP90β siRNA resulted in decreased MHC class II presentation of endogenous GAD (Fig. 22).

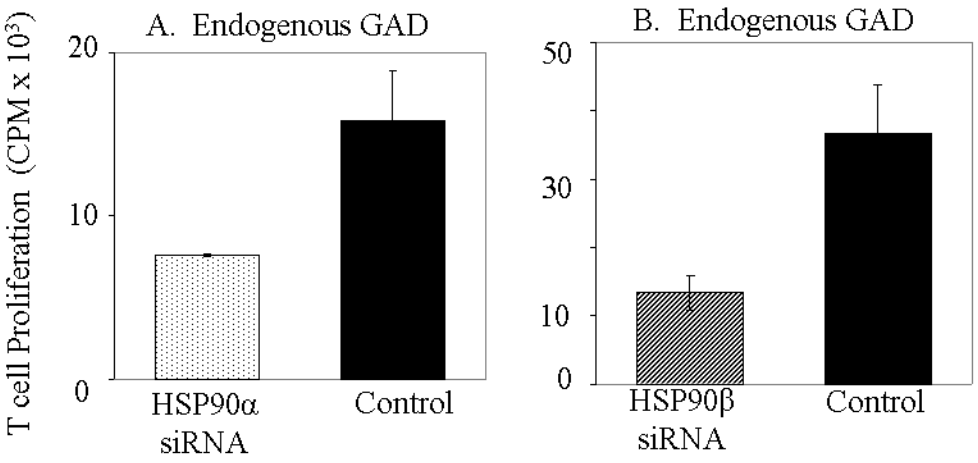


Figure 22. HSP90α and HSP90β siRNA inhibits MHC class II presentation of endogenous GAD Ag. PriessGAD cells were treated with either HSP90α (A), HSP90β (B), or control siRNA (A and B) and incubated for 48 hours as previously described. Following siRNA treatment of B cells, MHC class II presentation of endogenous GAD was measured by T cell assays. In each panel, black bars represent control cells, dotted bars represent HSP90α siRNA-treated cells, and hashed bars represent HSP90β siRNA-treated cells. Control assays were carried out as in Figure 8. Results are displayed as proliferation above background. Results are representative of at least 3 separate experiments.

Neither HSP90α nor HSP90β siRNA affected MHC class II presentation of endogenous kappa I or kappa II epitopes from endogenous IgG kappa light chain (Fig. 23). These results indicate that individual inhibition of HSP90α and HSP90β isoform expression by isoform specific siRNA perturbs MHC class II presentation of select endogenous Ags

such as GAD. These results point to a potential selectivity or specificity for HSP90α and HSP90β modulation of MHC class II presentation which is linked to Ag.

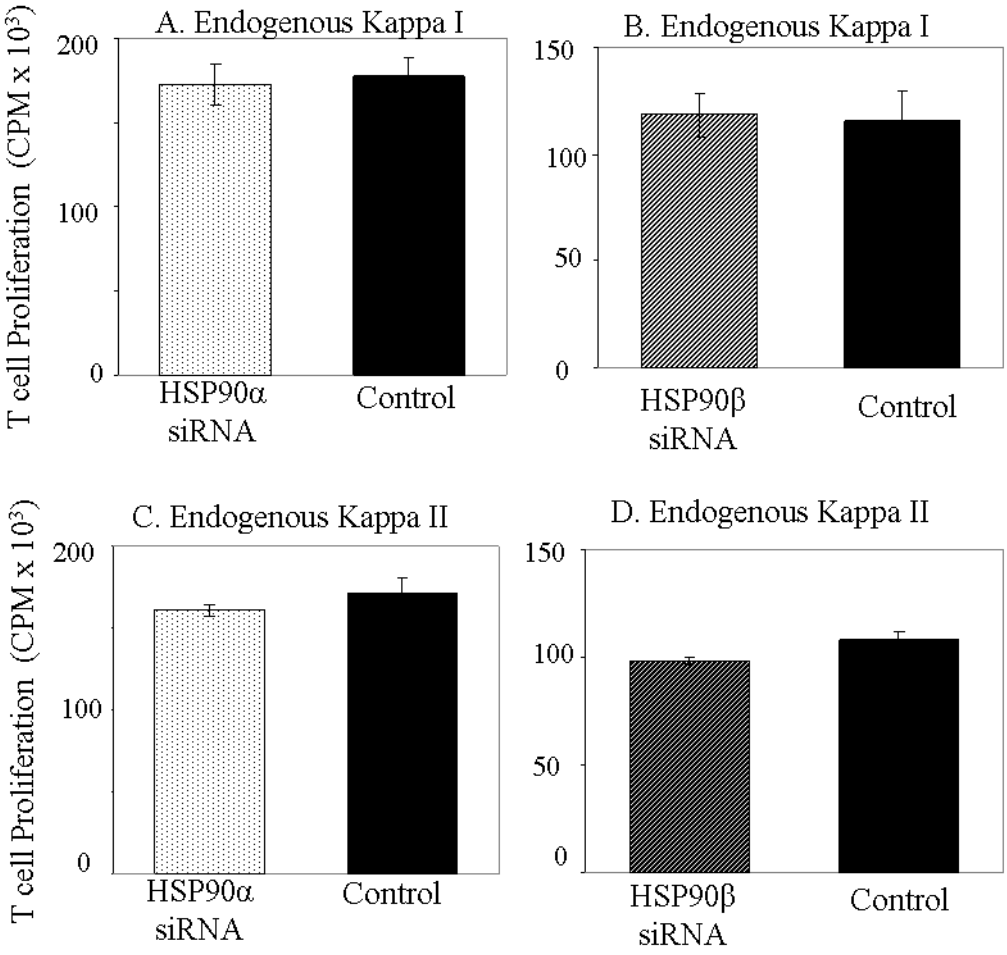


Figure 23. HSP90α and HSP90β siRNA does not alter MHC class II presentation of endogenous kappa epitopes. PriessGAD cells were treated with either HSP90α, HSP90β, or control siRNA and incubated for 48 hours as previously described. Following siRNA treatment of B cells, MHC class II presentation of endogenous kappa I or kappa II epitopes was measured by T cell assays. In each panel, black bars represent control cells, dotted bars represent HSP90α siRNA-treated cells, and hashed bars represent HSP90β siRNA-treated cells. Control assays were carried out as in Figure 8. Results are displayed as proliferation above background. Results are representative of at least 3 separate experiments.

Pharmacological inhibition of HSP90 indicated that HSP90 modulates MHC class II presentation of a select GAD epitope from exogenous GAD Ag, but not exogenous GAD peptide. To determine which HSP90 isoform is involved HSP90α or HSP90β siRNA treated cells were pulsed with either exogenous GAD Ag or synthetic GAD peptide and analyzed for MHC class II presentation of GAD epitope. HSP90α or HSP90β siRNA treatment decreased MHC class II presentation of GAD epitope from exogenous GAD Ag, but not exogenous GAD peptide (Fig 24).

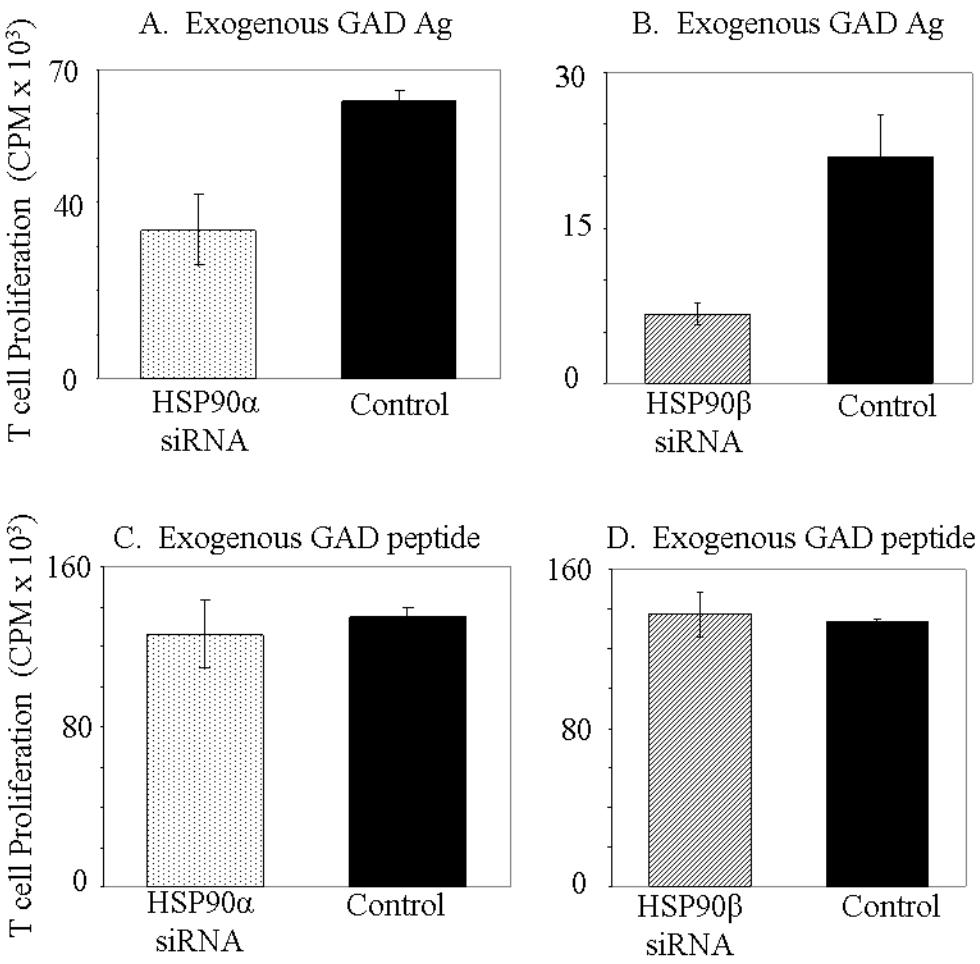


Figure 24. HSP90α and HSP90β siRNA inhibits exogenous GAD Ag but not GAD peptide presentation. Priess cells were treated with either HSP90α, HSP90β, or control siRNA and incubated for 48 hours as previously described. At 32 hours post nucleofection, 10 µg/ml exogenous GAD Ag (A and B) or 2 µM GAD peptide (C and D) was added to cells and incubated an additional 16 hours. At 48 hours post siRNA treatment of these cells, MHC class II presentation of GAD epitopes from either exogenous GAD Ag or GAD peptide was measured by T cell assays. In each panel, black bars represent control cells, dotted bars represent HSP90α siRNA-treated cells, and hashed bars represent HSP90β siRNA-treated cells. Control assays were carried out as in Figure 8. Results are displayed as proliferation above background. Results are representative of at least 3 separate experiments.

HSP90α or HSP90β siRNA treated cells were also pulsed with either HSA protein or HSA peptide and assayed for MHC class II presentation of an HSA epitope. HSP90α or HSP90β siRNA treatment did not alter MHC class II presentation of the HSA epitope using either exogenous HSA Ag or HSA peptide as a source of this epitope (Fig. 25). Taken together, these results indicate that both HSP90α and HSP90β isoforms can regulate MHC class II presentation of GAD Ag. Thus, these studies establish that in contrast to MHC class I Ag presentation, both HSP90α and HSP90β can regulate the MHC class II pathway. Furthermore, HSP90 appears to selectively promote MHC class II presentation of the diabetes autoantigen GAD.

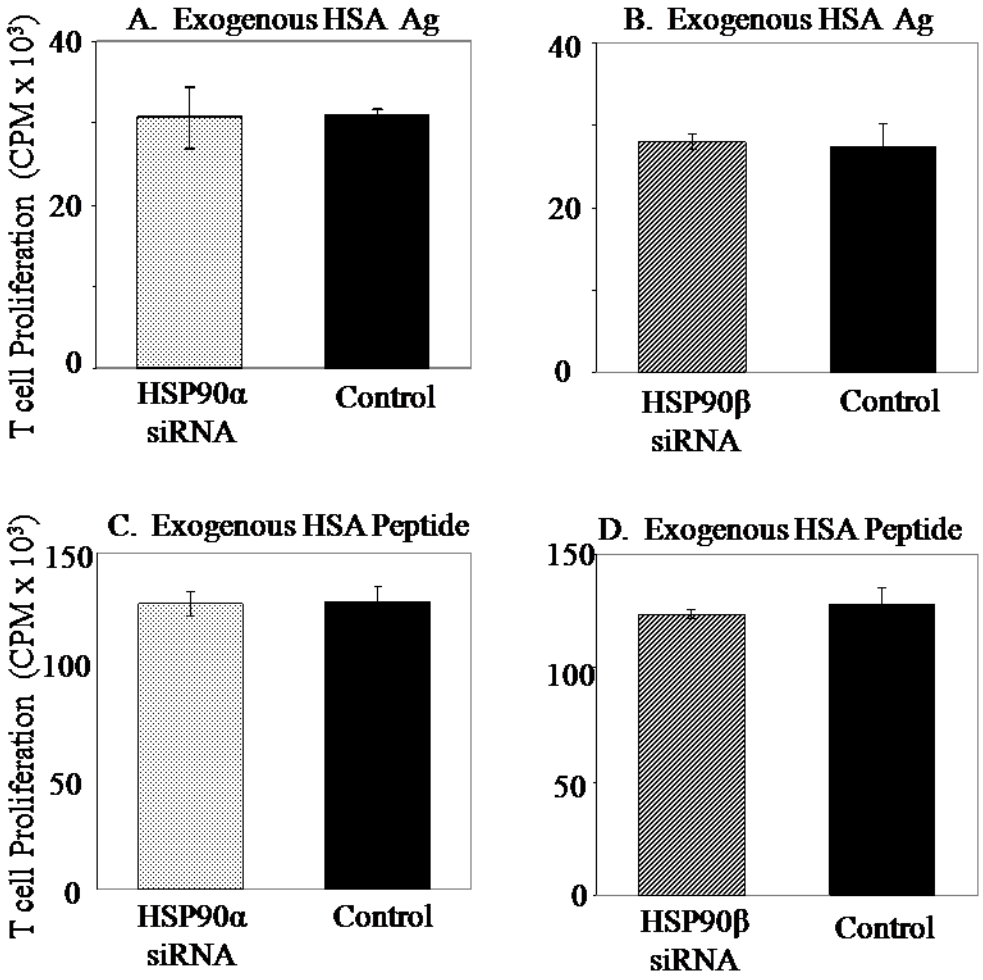


Figure 25. HSP90α and HSP90β siRNA does not affect MHC class II presentation of exogenous HSA Ag or exogenous HSA peptide. PriessGAD cells were treated with either HSP90α, HSP90β, or control siRNA and incubated for 48 hours as previously described. At 32 hours post siRNA treatment, exogenous HSA Ag (5 µM) or HSA peptide (5 µM) was added to nucleofected cells and incubated an additional 16 hours. At 48 hours post siRNA treatment of cells, MHC class II presentation of HSA epitopes from either exogenous HSA Ag or HSA peptide was measured by T cell assays. In each panel, black bars represent control cells, dotted bars represent HSP90α siRNA-treated cells, and hashed bars represent HSP90β siRNA-treated cells. Control assays were carried out as in Figure 8. Results are representative of at least 3 separate experiments.

Chapter 2. Mechanistic analysis of HSP90 regulation of GAD Ag presentation in B cells

HSP90α and HSP90β appear to regulate both endogenous and exogenous presentation of GAD Ag. Analysis of MHC class II presentation of short synthetic GAD epitopes failed to reveal a role for HSP90 in the cell surface loading of MHC class II molecules or T cell recognition. Thus, HSP90 may exert its effect on intracellular GAD Ag processing or MHC class II binding of GAD epitopes. The pathways of exogenous and endogenous MHC class II presentation of GAD Ag are distinct yet converge with shared processing of GAD in endosomal and lysosomal compartments. Studies from our lab and others showed endogenous Ag processing for MHC class II presentation to be dependent upon cytoplasmic proteases including the proteasome and calpain; exogenous Ag processing was not affected by inhibition of the proteasome or calpain (67, 69). Further evidence has implicated HSC70 and lamp2a as chaperones of endogenous GAD presentation by MHC class II molecules (85). Additional studies in our lab have shown that exogenous GAD Ag is processed via the classical MHC class II presentation pathway requiring endo/lysosomal acidification as well as cysteine and aspartyl proteases within those vesicles (67).

However distinct, the two pathways of MHC class II presentation share some characteristics. While endogenous GAD is processed in the cytoplasm by cytosolic proteases into peptide fragments, those peptides must be translocated into membrane organelles for MHC class II presentation. Once translocated into those vesicles, further trimming of the GAD peptides is mediated by acidic proteases within endo/lysosomes (67). Taken together, these studies indicate some overlap between the classical MHC class II presentation pathway for exogenous GAD and the alternative MHC class II presentation of endogenous GAD. With evidence for a role for HSP90 in both of these pathways, further studies were carried out to determine whether HSP90 is affecting a shared component of these two pathways.

Inhibition of HSP90 and invariant chain processing

During transit through the Golgi secretory network, MHC class II molecules are chaperoned by Ii. Ii functions as a chaperone to stabilize MHC class II dimers, target them to secretory vesicles, and prevent premature peptide loading. As shown schematically in Figure 5, while progressing through the endosomal/lysosomal network, Ii undergoes stepwise degradation by lysosomal proteases to the smaller peptide fragments LIP and SLIP and the final fragment called CLIP, which remains associated with the MHC class II peptide binding groove. Ii is proteolytically cleaved by lysosomal proteases during transit (31, 40-44, 47). Studies have shown that alterations in Ii processing result in altered MHC class II presentation (79, 81, 90-93). Therefore, disruption of Ii or its chaperone function could be a possible explanation for decreased GAD presentation by HSP90 inhibition.

To determine if HSP90 inhibition affects Ii processing, PriessGAD cells were treated with RA (2 μM), GA (0.18 μM), or DMSO solvent as a control for 18 hours and analyzed for Ii mRNA expression by semi-quantitative RT-PCR. Neither RA nor GA altered Ii mRNA expression in PriessGAD cells (Fig 26).

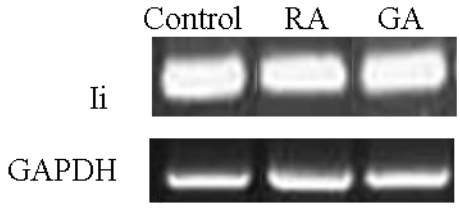


Figure 26. Pharmacological inhibition of HSP90 does not alter Ii mRNA levels.

PriessGAD cells were treated with either DMSO control, RA (2 μM), or GA (0.18 μM) for 18 hours. Following treatment, mRNA was isolated from cells and reverse transcribed to cDNA samples were subjected to PCR to amplify Invariant Chain (Ii) and GAPDH sequences using multiple cycles of amplification. In this figure, 30 cycles was used to amplify Ii and GAPDH sequences. PCR products were resolved on an agarose gel with SyberSafe stain. Results are representative of at least 2 separate experiments.

Control, RA, and GA treated cells were also analyzed for total Ii expression by flow cytometry. Neither RA nor GA affected total Ii expression in PriessGAD cells (Fig. 27A). To further determine if HSP90 inhibition could affect Ii processing, PriessGAD cells were treated with HSP90α, HSP90β, and control siRNA and analyzed for total Ii expression by flow cytometry. Neither inhibition of HSP90α or HSP90β isoforms altered total Ii expression in PriessGAD cells (Fig. 27B).

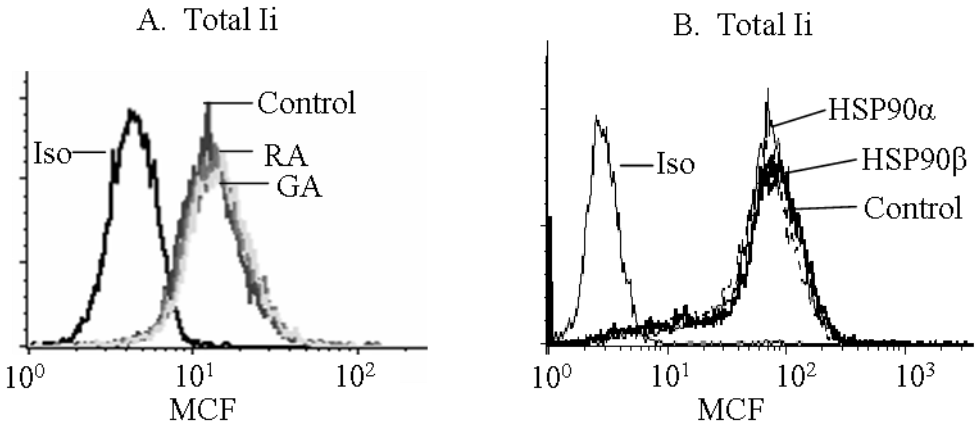


Figure 27. Inhibition of HSP90α and HSP90β does not alter total cellular Ii expression. (A) PriessGAD cells were treated with DMSO as a control, RA (2 μM), or GA (0.18 μM) for 18 hours. (B) PriessGAD cells were treated with HSP90α, HSP90β, or control siRNA as previously described and incubated for 48 hours. Following treatment, cells were fixed in 1% paraformaldehyde, permeabilized, and stained for total Ii expression using Abs and flow cytometry. (A) Black line indicates control cells, dark gray line indicates RA treated cells, light gray line indicates GA treated cells, and thin gray line indicates isotype control. (B) Thick black line indicates siRNA control treated cells, thin black line indicates HSP90α siRNA treated cells, hashed black line indicates HSP90β siRNA treated cells, and thin gray line indicates isotype control staining. Results shown are representative of at least 2 separate experiments.

To determine if HSP90α and HSP90β inhibition influences Ii cleavage to CLIP, drug or siRNA treated PriessGAD cells were analyzed for total and surface CLIP expression by flow cytometry. Total and surface CLIP expression in PriessGAD cells was not affected

by RA or GA treatment (Fig. 28A and B). Additionally, neither HSP90α or HSP90β siRNA treatment altered total CLIP expression in PriessGAD cells (Fig. 28C).

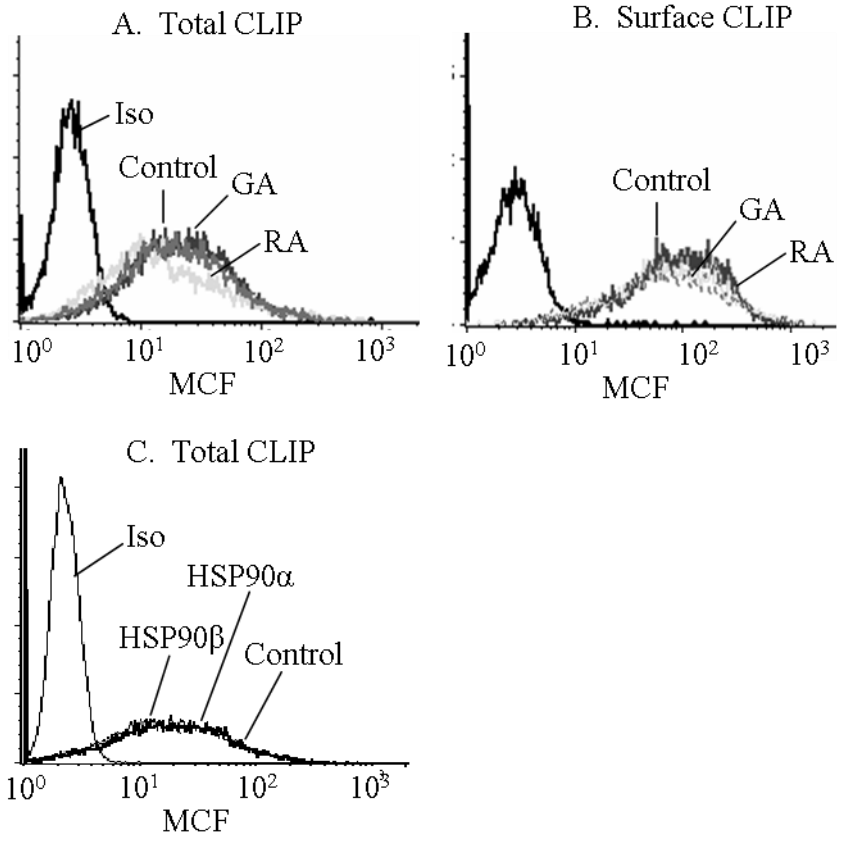
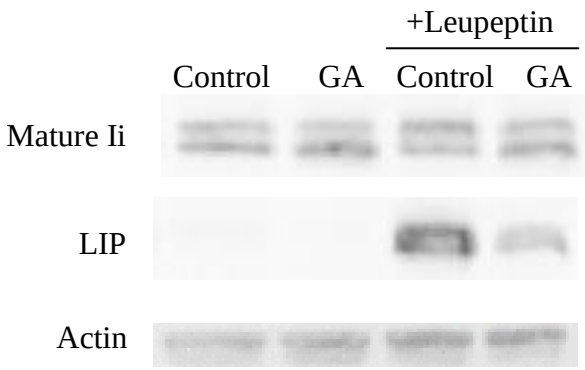


Figure 28. Inhibition of HSP90α and HSP90β does not alter total or surface CLIP expression. (A and B) PriessGAD cells were treated with DMSO as a control, RA (2 μM), or GA (0.18 μM) for 18 hours. (C) PriessGAD cells were treated with HSP90α, HSP90β, or control siRNA and incubated for 48 hours as previously described. Following treatment, cells were fixed in 1% paraformaldehyde, permeabilized, and Ab stained for total CLIP (A and C) or surface CLIP (B) expression by flow cytometry. (A and B) Black line indicates control cells, dark gray line indicates RA treated cells, light gray line indicates GA treated cells, and thin gray line indicates isotype control staining. (C) Thick black line indicates siRNA control treated cells, thin black line indicates HSP90α siRNA treated cells, hashed black line indicates HSP90β siRNA treated cells, and light gray line indicates isotype control staining. Results shown are representative of at least 2 separate experiments.

As previously described, the processing of Ii is dependent upon the activity of lysosomal proteases. Treatment of B cells with the lysosomal protease inhibitor leupeptin results in LIP accumulation (224, 225). To determine if HSP90 inhibition altered Ii processing in a manner similar to leupeptin, PriessGAD lysates were co-treated with or without leupeptin and GA. Following theses treatments, whole cell lysates were Western immunoblotted for mature Ii and LIP fragment expression. In both control and GA treated cells, only mature Ii was detected (Fig. 29). Upon treatment with leupeptin, mature Ii and LIP fragment were detected in both control and GA treated cells (Fig. 29). The average relative expression as determined by densitometry revealed that mature Ii expression was not altered by GA, leupeptin, or GA + leupeptin treatment. However, leupeptin induced LIP accumulation was decreased by 60% in GA treated cells compared to control treated cells. Thus, treatment with these inhibitors appears to alter the steady state abundance of the intermediate LIP but not the final proteolytic product CLIP. Possibly, GA treatment might alter the kinetics of Ii processing by altering cellular cathepsin activity.

A.



B.

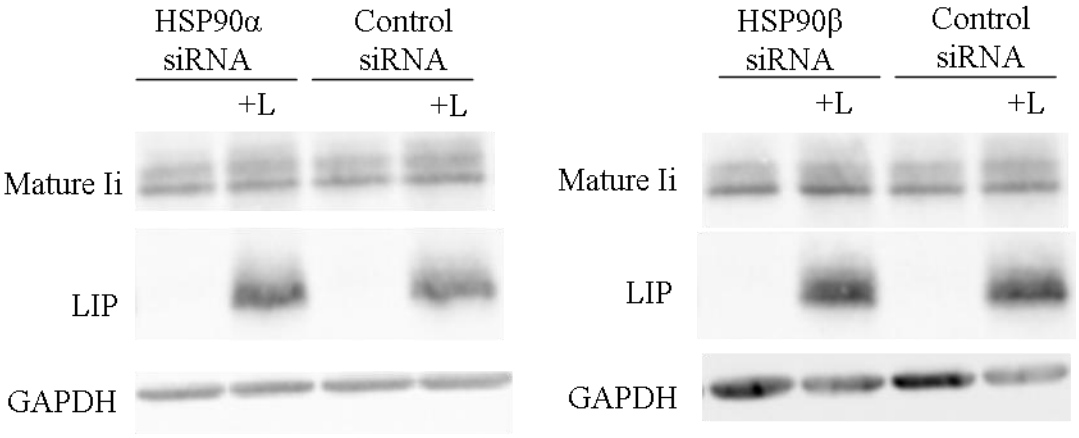
Relative Expression				
			+ Leupeptin	
	Control	GA	Control	GA
Mature Ii/Actin	100%	97%	90%	95%
LIP/Actin	0%	0%	100%	40%

Figure 29. Pharmacological inhibition of HSP90 alters leupeptin induced LIP accumulation. PriessGAD cells were treated with either DMSO control, GA (0.18 µM), DMSO control and leupeptin (50 µM), or GA (0.18 µM) and leupeptin (50 µM) for 18 hours. (A) WCLs of treated cells were fractionated on 12% SDS-PAGE gels and Western immunoblotted for mature Ii, LIP, and actin. Blot is representative of at least 3 separate experiments. (B) Average relative expression of mature Ii and LIP expression as determined by densitometry from 3 separate experiments is displayed in the table.

To further explore this observation, PriessGAD cells were co-treated with leupeptin and either HSP90α or HSP90β siRNA. Following treatment, Western immunoblotting for mature Ii and LIP fragment expression was performed on whole cell lysates. Only mature Ii expression was detected in HSP90α siRNA and control treated cells (Fig. 30A). Upon leupeptin treatment of HSP90α siRNA and control treated cells, both mature Ii expression and LIP accumulation was detected (Fig. 30A). Similarly, treatment with HSP90β siRNA and control alone resulted in detection of only mature Ii expression (Fig. 30A). Upon leupeptin treatment, LIP fragment accumulation was detected in both HSP90β siRNA and control treated cells (Fig. 30A). The average relative expression as determined by densitometry revealed that mature Ii expression was not altered by either HSP90α or HSP90β siRNA treatment wjhen compared to control. Leupeptin treatment of HSP90α siRNA, HSP90β siRNA, or controls did increase mature Ii expression (Fig. 30B). However, leupeptin induced LIP accumulation was unaffected by either HSP90α or HSP90β siRNA compared to controls (Fig. 30).

While leupeptin induced LIP accumulation was affected by GA treatment, neither HSP90α nor HSP90β siRNA treatment appear to alter this step in Ii processing. Previous results found that GA and RA treatment of cells induced a heat shock stress response (Fig. 13). Neither HSP90α nor HSP90β siRNA treatment of B cells induced a heat shock stress response (Fig. 20). While pharmacological inhibition of HSP90 by GA and RA affects MHC class II expression and function similarly to HSP90α and HSP90β siRNA treatment, the siRNA treatment is likely more specific than pharmacological inhibitors and less prone to inducing an overall stress response. Potentially, the affects of GA on leupeptin induced LIP accumulation may be due to the stress response induced by this drug and not inhibition of HSP90. Considering that neither pharmacological or siRNA treatment altered total Ii, total CLIP, or surface CLIP expression as determined by flow cytometry, these results suggest that Ii processing is not radically altered in cells cultured under these conditions (Fig. 27 and 28). Thus, the decreased MHC class II presentation of GAD upon HSP90α and HSP90β inhibition is not likely a result of altered Ii expression or function.

A.



B.

	HSP90α siRNA		Control siRNA	
		+L		+L
Mature Ii/GAPDH	98%	117%	100%	139%
LIP/GAPDH	0%	108%	0%	100%

	HSP90β siRNA		Control siRNA	
		+L		+L
Mature Ii/GAPDH	100%	110%	100%	207%
LIP/GAPDH	0%	105%	0%	100%

Figure 30. HSP90α and HSP90β inhibition does not alter leupeptin induced LIP accumulation. PriessGAD cells were nucleofected with HSP90α and HSP90β siRNA as previously described. After 30 hours, cell samples were split, and half of each set was treated with leupeptin (50 μM) for the remaining 18 hours. (A) WCLs were fractionated on 12% SDS-PAGE gels and Western immunoblotted for mature Ii chain, LIP, and GAPDH. Blot is representative of at least 3 separate experiments. (B) Average relative expression of mature Ii and LIP expression as determined by densitometry from at least 2 separate experiments is displayed in the tables.

Pharmacological inhibition of HSP90 does not alter cathepsin expression, but does alter cathepsin activity

Previous studies have shown that lysosomal acidic proteases systematically cleave Ii (111, 119, 224). For example, cathepsin B and D are required for processing Ii as well as exogenous Ag for MHC class II presentation (224). Alterations in cathepsin expression or protease activity could possibly explain the decreased MHC class II presentation of GAD upon GA and RA treatment. To further explore the role of HSP90 in the MHC class II presentation pathway, the effect of pharmacological inhibition of HSP90 on cathepsin expression was investigated. PriessGAD cells were treated with RA (2 μM), GA (0.18 μM), or DMSO solvent as a control and analyzed for cathepsin S, L, B, and D mRNA expression by RT-PCR. Neither RA nor GA altered cathepsin S, L, B, or D mRNA expression in PriessGAD cells (Fig. 31).

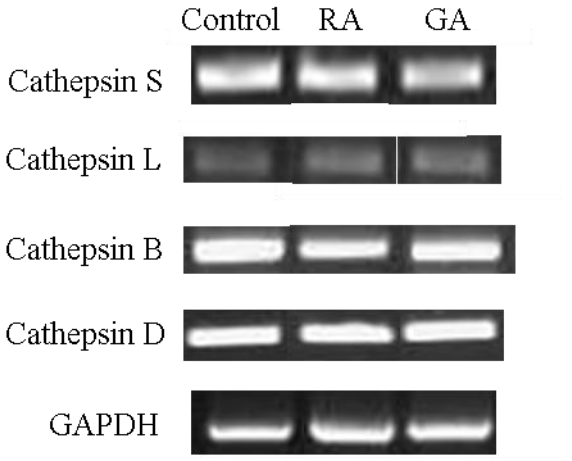


Figure 31. Pharmacological inhibition of HSP90 does not alter cathepsin expression. PriessGAD cells were treated with either DMSO control, RA (2 μM), or GA (0.18 μM) for 18 hours. Following treatment, mRNA was isolated from cells and reverse transcribed to cDNA. From each sample, cDNA was subjected to PCR to amplify cathepsin S, cathepsin L, cathepsin B, cathepsin D, and GAPDH sequences. PCR products were resolved on an agarose gel with SyberSafe stain. Results are representative of at least 2 separate experiments.

In addition to RNA expression, cathepsin S, S/L, and B protease activity in GA, RA, or DMSO treated PriessGAD cells were measured by cathepsin activity assays. The activity of cathepsin S was unaffected by GA or RA treatment (Fig. 32A). Using a substrate that is cleaved by both cathepsin S/L, both GA and RA treatment increased the activity of cathepsin S/L together when compared to DMSO control treatment (Fig. 32B). GA and RA treatment of cells also decreased cathepsin B activity in PriessGAD cells (Fig. 32C). Cathepsin S and L are known to cleave LIP to CLIP during Ii processing. Thus, increased expression of either of these enzymes might lead to more efficient LIP conversion to CLIP, consistent with our results in Figure 29.

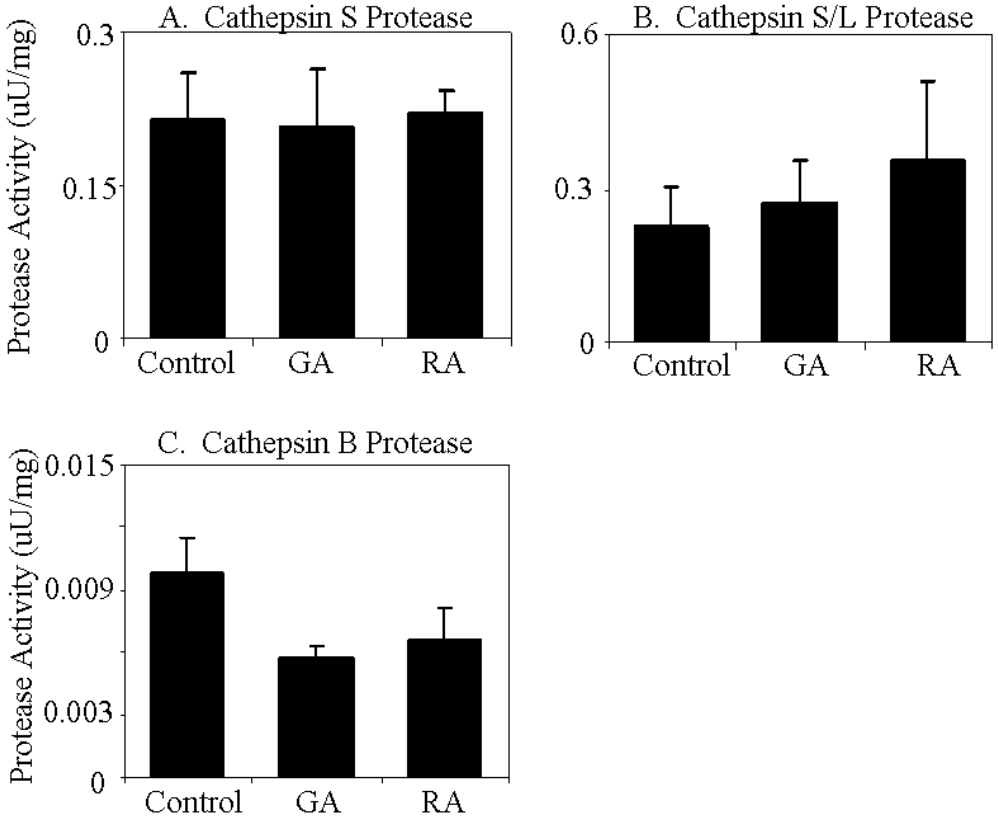


Figure 32. Pharmacological inhibition of HSP90 altered cathepsin activity in B cells. PriessGAD cells were treated with either DMSO control, GA (0.18 µM), or RA (2 µM) for 18 hours. These cells were lysed and detergent extracted whole cell lysates were analyzed for cathepsin S (A), S/L (B), or B (C) protease activity. Results are representative of at least 3 separate experiments.

Inhibition of HSP90α and HSP90β does not alter HLA-DM and HLA-DO function. HLA-DM functions at the terminal stages of MHC class II maturation by catalyzing the removal of CLIP and the subsequent loading of antigenic epitopes onto MHC class II molecules (226). This activity is shown schematically in figure 6. In the presence of HLA-DM, the loading of antigenic epitopes onto MHC class II molecules is selective, favoring peptides with high affinity for MHC class II dimers (227). Previous studies in the lab indicated that HLA-DM regulates MHC class II presentation of both exogenous and endogenous GAD Ag (132). HSP90α and HSP90β inhibition by either the pharmacological inhibitors RA and GA or isotype specific siRNA, could be affecting HLA-DM expression and activity. To determine if HLA-DM expression is affected by HSP90 inhibition, PriessGAD cells were treated with RA, GA, or DMSO solvent as a control and analyzed for HLA-DM expression by flow cytometry. Neither RA nor GA treatment altered HLA-DM protein expression in PriessGAD cells (Fig. 33A). Additionally, PriessGAD cells were also treated with HSP90α, HSP90β, and control siRNA and analyzed for HLA-DM protein expression by flow cytometry. HLA-DM protein levels were not altered by HSP90α or HSP90β siRNA treatment (Fig. 33B).

In B cells, HLA-DM function is regulated by HLA-DO (51-55). This activity is shown schematically in figure 6. Changes in HLA-DO levels due to altered HSP90α and HSP90β function could alter overall MHC class II presentation. To determine if HLA-DO protein expression is affected by HSP90 inhibition, PriessGAD cells were treated with RA, GA, or DMSO solvent as a control and analyzed for HLA-DO expression by flow cytometry. Neither RA nor GA treatment altered HLA-DO protein expression in PriessGAD cells (Fig. 33B). Additionally, PriessGAD cells were also treated with HSP90α, HSP90β, and control siRNA and analyzed for HLA-DO protein expression by flow cytometry. HLA-DO protein expression was not altered by HSP90α or HSP90β siRNA treatment (Fig. 33D). These results indicated that HLA-DM and HLA-DO expression is not altered by either pharmacological inhibition of HSP90 or HSP90α and HSP90β isotype specific siRNA. Figure 28 indicated that total and surface CLIP expression was not affected by either pharmacological inhibitors, RA and GA, or HSP90 specific siRNA. These results indicate that neither HLA-DM nor HLA-DO function is

altered as the level of CLIP bound by surface MHC class II molecules on cells is intimately tied to HLA-DM and HLA-DO function in cells. Therefore, these results indicate that HLA-DM and HLA-DO protein expression and function are not affected by HSP90α and HSP90β inhibition.

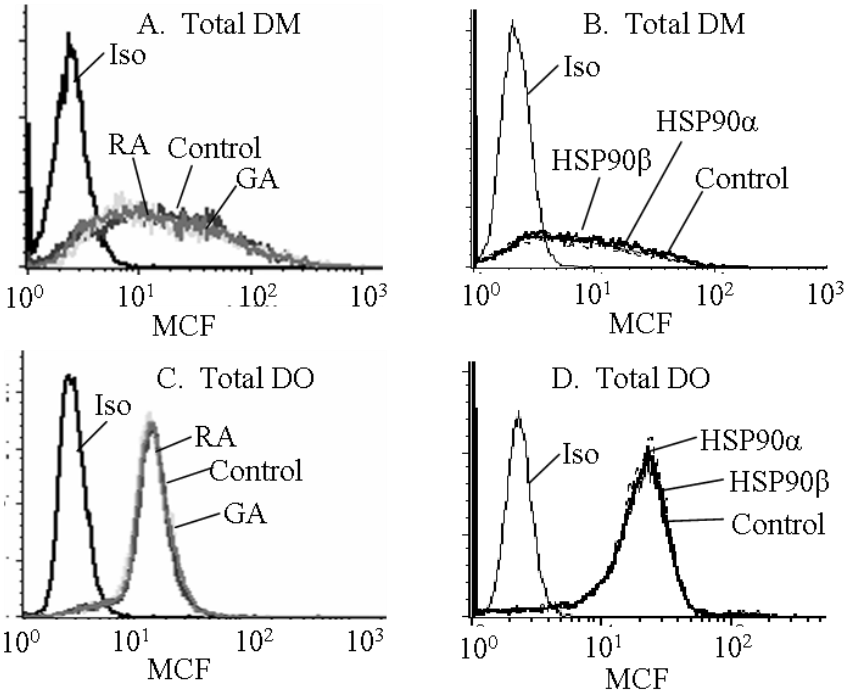


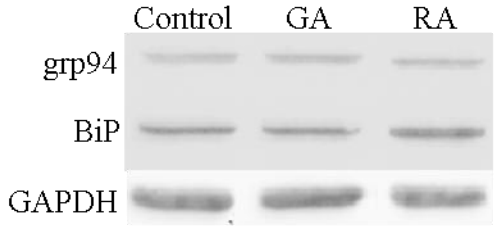
Figure 33. Inhibition of HSP90α and HSP90β does not alter total cellular HLA-DM or HLA-DO expression. PriessGAD cells were treated with DMSO as a control, RA (2 μM), or GA (0.18 μM) for 18 hours (A and C). PriessGAD cells were treated with HSP90α, HSP90β, or control siRNA and incubated for 48 hours as previously described (B and D). Following treatment, cells were fixed, permeabilized, and stained for total HLA-DM (A and B) or total HLA-DO (C and D) expression by flow cytometry. (A and C) Black line indicates control cells, dark gray line indicates RA treated cells, light gray line indicates GA treated cells, and thin gray line indicates isotype control staining. (B and D) Thick black line indicates siRNA control treated cells, thin black line indicates HSP90α siRNA treated cells, hashed black line indicates HSP90β siRNA treated cells, and light gray line indicates isotype control staining. Results shown are representative of at least 2 separate experiments.

Inhibition of HSP90α and HSP90β does not induce ER stress

As previously described, the ER chaperones grp94 and BiP are ER homologs of cytoplasmic HSP90 and HSP70 respectively. Due to the similarity between grp94 and HSP90, GA can bind to both of these chaperones. Using a variety of cell lines, studies indicate that GA treatment can induce an ER stress response represented by an upregulation of ER chaperones (228). This upregulation was due to the unfolded protein response and not due to any affects on HSP90. However, additional research suggests that HSP90 may also be linked to the cellular transcriptional response to ER stress (214). Previous studies indicated that MHC class II molecules can bind to select ER chaperones (217). Thus, induction of ER stress could be one explanation for the observed decrease in MHC class II presentation of GAD upon HSP90 inhibition.

To determine if HSP90 inhibition affects ER stress, the expression of grp94 and BiP was examined by Western immunoblotting whole cell lysates of PriessGAD cells treated with pharmacological inhibitors. Neither GA nor RA induced grp94 or BiP expression in PriessGAD cells with reduced HSP90 activity compared to controls (Fig. 34A). The average relative expression of grp94 and BiP as determined by densitometry of at least 3 separate experiments confirmed there was no significant affect of HSP90 inhibition on ER stress in PriessGAD cells (Fig. 34B).

A.



B.

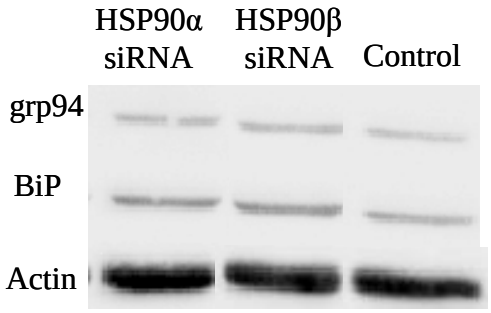
Average Relative Expression			
	Control	GA	RA
grp94/GAPDH	100%	109% +/- 10%	102% +/- 8%
BiP/GAPDH	100%	102% +/- 9%	100% +/- 13%

Figure 34. Pharmacological inhibition of HSP90 does not induce ER stress.

PriessGAD cells were treated with either DMSO control, GA, or RA. (A) WCL from treated cells were analyzed by Western immunoblotting for expression of grp94 and BiP. Blot is representative of at least 3 separate experiments. (B) The average relative expression of grp94 and BiP expression +/- SEM as determined by densitometry of at least 3 separate experiments is displayed in the table. Student t tests determined no significant change in grp94 or Bip expression.

To further explore the effect of HSP90 inhibition on ER stress, grp94 and BiP expression was measured by Western immunoblotting in PriessGAD cells upon HSP90α and HSP90β siRNA treatment. Neither HSP90α nor HSP90β siRNA treatment induced grp94 or BiP expression in PriessGAD cells (Fig. 35A). Densitometry of at least 3 separate experiments indicated that HSP90α and HSP90β inhibition by siRNA did not significantly alter the relative expression of grp94 or BiP (Fig.35B). Taken together, these results indicate that HSP90 inhibition does not induce ER stress in PriessGAD.

A.



B.

Average Relative Expression			
	HSP90α siRNA	HSP90β siRNA	Control
grp94/GAPDH	103% +/- 7%	105% +/- 3%	100%
BiP/GAPDH	107% +/- 10%	111% +/- 10%	100%

Figure 35. HSP90α and HSP90β inhibition by siRNA did not induce ER stress. PriessGAD cells were treated with HSP90α siRNA, HSP90β siRNA, or control siRNA as previously described. (A) WCL from treated cells were analyzed by Western immunoblotting for expression of grp94 and BiP. Blot is representative of at least 3 separate experiments. (B) The average relative expression of grp94 and BiP expression +/- SEM as determined by densitometry from at least 3 separate experiments is displayed in the table. Student t tests determined no significant change in grp94 or BiP expression.

Pharmacological inhibition of HSP90 does not induce HSP90 secretion

While HSP90 is located primarily in the cytoplasm of cells, a study in B cells found that heat stress induced enrichment of HSPs in exosomes, including HSP90 (229). HSP70, which can associate with HSP90, was found to be secreted by tumor cells through a pathway involving lysosomal endosomes (197). Notably, another intracellular chaperone, HSC70 is found both in the cytoplasm and endosomes/lysosomes with studies suggesting it translocates into the endosomal network in an ATP-dependent process (166). Whether HSP90 uses a similar pathway to access the endosomal network is unknown. Several studies have suggested peptide-HSP90 complexes released by tumors can promote MHC class I presentation (137, 140, 144, 223). Notably, islet cell stress including exposure to cytokines such as IL-1β is known to induce HSP90 expression prior to cell death (184). The presence of extracellular HSP90 could explain the requirement for functional HSP90 for MHC class II presentation of exogenous GAD Ag.

To determine if extracellular HSP90 is present in B cell cultures, conditioned B cell media, fresh media, and individual media components were examined for the presence of HSP90. PriessGAD, Priess, Frev, and FrevSMA cells were cultured for 48 hours. After 48 hours, the conditioned media was collected and fractionated on a 10% SDS-PAGE gel along with fresh B cell media, IMDM media, serum, H-SFM media, and WCL from PriessGAD cells as a control. Western immunoblotting did not detect any secreted HSP90 from conditioned B cells (Fig. 36A). Exogenous HSP90 was also not detected in fresh B cell media or its components as indicated by Western immunoblotting (Fig. 36A). These results indicate that HSP90 is not likely secreted from B cells at measurable levels upon cell culture.

While HSP90 is not secreted by B cells during steady state conditions, treatment of cells with pharmacological inhibitors of HSP90 could possibly induce HSP90 secretion. To determine if HSP90 is secreted upon stress conditions in B cells, PriessGAD cells were treated with either GA (0.18 μM), RA (2 μM), or DMSO solvent as a control. Conditioned media from these cell treatments was concentrated and examined for the presence of HSP90 by Western immunoblotting. Neither GA nor RA induced HSP90

secretion in PriessGAD (Fig. 36B). While HSP90 was not secreted by B cells, we cannot rule out that these drugs promoted delivery or retention of HSP90 in endosomes and lysosomes of these cells where it may intersect endogenous GAD.

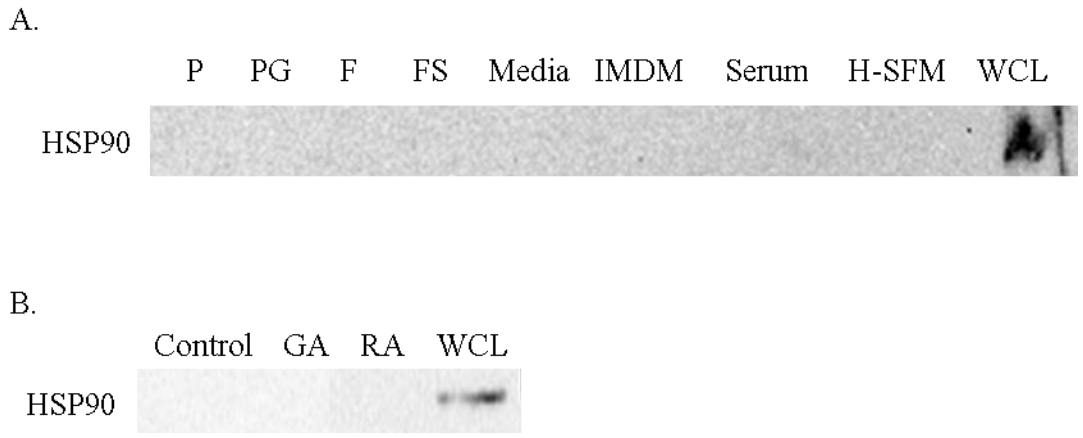


Figure 36. Pharmacological inhibition of HSP90 does not induce HSP90 secretion by B cells. (A) Conditioned media from Priess (P), PriessGAD (PG), Frev (F), and FrevSMA (FS) cells; fresh IMDM cell media plus serum; fresh cell media components IMDM or serum; and an the H-SFM cell media was concentrated from 13 ml into approximately 50 µl. Concentrated samples were ran on 10% SDS PAGE gel along with PriessGAD WCL as a positive control. Blots were probed for HSP90. (B) PriessGAD cells were treated with either DMSO control, GA, or RA for 18 hours. After treatment, 75 µl of media was fractionated on 10% SDS PAGE gels along with PriessGAD WCL as a positive control. Results are representative of at least 2 separate experiments.

As an alternate approach, whether exogenous HSP90 could enhance exogenous GAD presentation was tested. Purified, recombinant human GAD Ag was pre-complexed with purified HSP90 at a 1:1 molar ratio prior to the addition to Priess cells. MHC class II presentation of GAD Ag using HSP90/GAD pre-complexes was compared to presentation of GAD in the absence of exogenous HSP90. Pre-complexing purified HSP90 with exogenous GAD in vitro prior to addition to Priess cells slightly enhanced GAD presentation (Fig. 37). Notably this effect was only seen using low levels of HSP90 and GAD Ag (Fig. 37A). At higher concentrations, the presence of HSP90 did not enhance GAD Ag presentation (Fig. 37B).

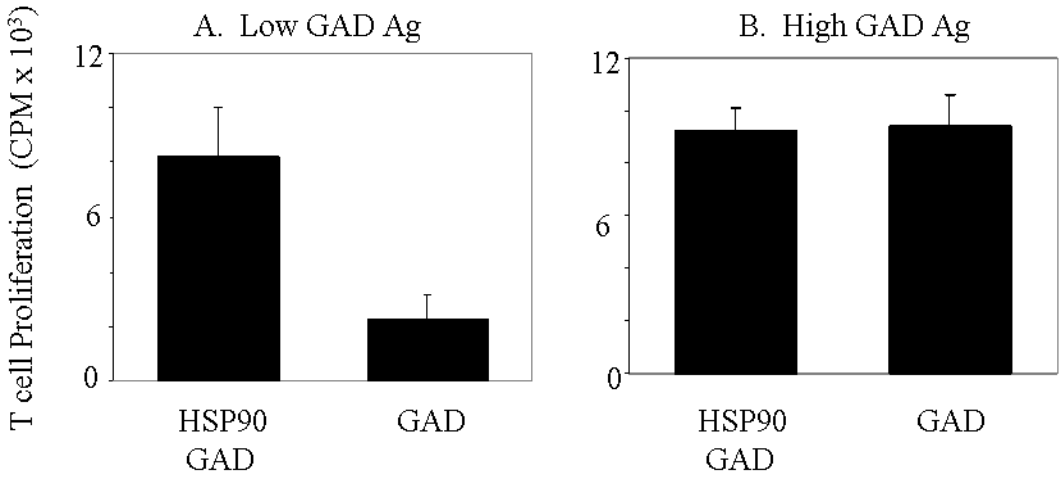


Figure 37. Low concentrations of exogenous HSP90 enhance MHC class II presentation of exogenous GAD Ag. Purified HSP90 and purified GAD Ag (3:1 molar ratio), purified GAD Ag in PBS, or HSP90 in PBS were incubated for 24 hours in order to permit protein complex formation. (A) Priess cells were treated with low concentrations of HSP90/GAD (0.00192μM/ 0.00064 μM), GAD Ag (0.00064 μM), or HSP90 (0.00192 μM) for 16 hours. (B) Priess cells were treated with high concentrations of HSP90/GAD Ag (0.0192 μM/0.0064 μM), GAD Ag (0.0064 μM), or HSP90 (0.0192 μM) for 16 hours. MHC class II presentation of exogenous GAD was measured by T cell assay. Control assays were carried out as in figure 8. Results are displayed as proliferation above background. Results are representative of at least 3 separate experiments.

Chapter 3. Implication of HSP70 as a possible chaperone for SMA in B cells

Studying the role of HSP90 in MHC class II presentation revealed the potential requirement for another HSP, HSP70, in the presentation of some cytoplasmic Ags. The human B-LCL Frev expresses both the HLA-DR4 and HLA-DR1 MHC class II alleles. Unlike Priess cells, Frev cells lack the secretory Ig kappa light chain. Thus, Frev cells were transfected with a plasmid encoding the cDNA for SMA, a mutant cytoplasmic form of the Ig light chain κ4. FrevSMA cells express endogenous, cytoplasmic SMA Ag which can be processed and presented by MHC class II molecules. SMA was isolated from a patient with amyloidosis (207, 230). Amyloidosis is a group of diseases characterized by the assembly of protein fibrils that are deposited extracellulary in various organs and tissues. Almost immediately following SMA protein synthesis, this amyloid protein misfolds and is translocated out of the ER to the cytosol. Once in the cytosol, SMA is ubiquitinated and is either degraded by cytoplasmic proteases or aggregates in inclusion bodies. The molecular events that govern SMA escape from the cell's quality control system and which lead to protein fibril formation are unknown. However, HSP70 has been shown to promote the proteasomal degradation of SMA and potentially regulate fibril accumulation (207). Two SMA peptide epitopes are formed upon SMA proteasome processing and translocated by CMA into endosomes for complexing with MHC class II molecules. These two SMA epitopes are termed $SMA_{188-203}$ and $SMA_{145-159}$ in FrevSMA cells. Similar kappa I and kappa II epitopes are presented by Frev cells incubated with wild type IgG or Ig kappa Ags. Thus like GAD, endogenous and exogenous forms of Ig kappa yield identical peptides for MHC class II presentation despite differences in their intracellular location and initial processing. Studies here examined the role of HSP90 and HSP70 in MHC class II presentation of SMA Ag.

Studies have detected extracellular HSP70:peptide complexes in MHC class II –enriched compartments after receptor-mediated endocytosis (208). HSP70 has been shown to interact with MHC class II molecules (209). Studies also suggest HSP70 mediated enhancement of MHC class II restricted peptide presentation (209, 231). And relevant to

autoimmunity, HSP70 has been shown to promote myelin autoantigen presentation by MHC class II molecules (139).

Pharmacological inhibition of HSP90 enhances MHC class II presentation of SMA

To determine the effect of HSP90 inhibition on MHC class II presentation of endogenous SMA, FrevSMA cells were treated with increasing concentrations of GA and RA for 18 hours. MHC class II presentation of SMA was enhanced in a dose-dependant manner with both GA and RA (Fig. 38). These results are in contrast to endogenous GAD presentation by MHC class II molecules, which was decreased by GA and RA treatment (Fig. 9).

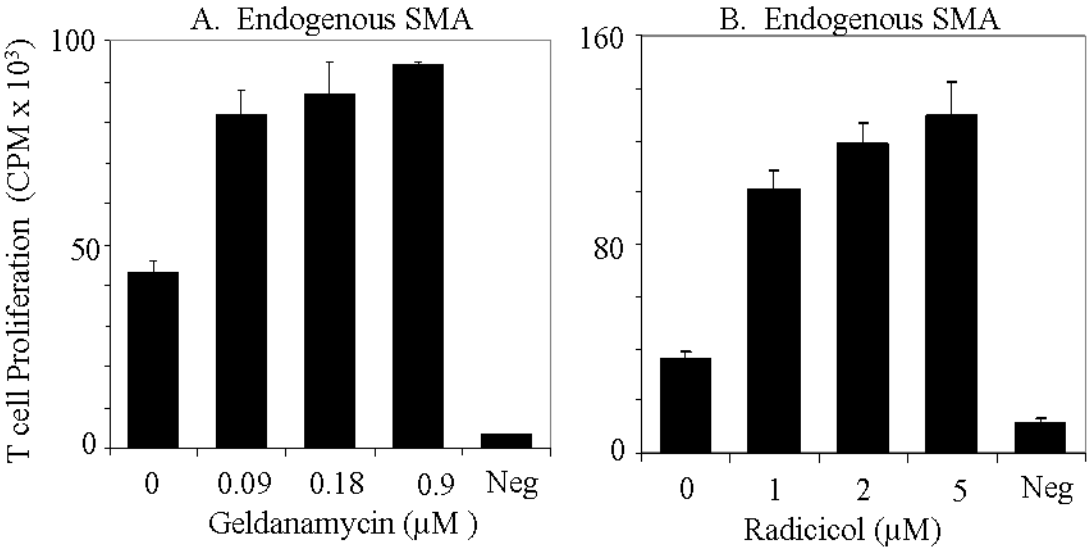


Figure 38. Pharmacological inhibition of HSP90 increases MHC class II presentation of endogenous SMA. FrevSMA cells were treated with DMSO as a negative control or increasing concentrations of GA (A) or RA (B) for 18 hours. MHC class II presentation of endogenous SMA was analyzed by T cell assays. Control assays were carried out as in Figure 8. Here, the negative control indicates HT2 cell proliferation without IL-2 addition. Results are representative of at least 3 separate experiments.

The effect of pharmacological inhibition of HSP90 on MHC class II presentation of epitopes from exogenous whole IgG Ag was also analyzed. Frev cell lines were treated with GA (0.18 μM), RA (2 μM), or DMSO solvent as a control for 18 hours with varying concentrations of exogenous whole IgG Ag added after 2 hours of drug treatment. MHC class II presentation of kappa I or kappa II epitopes from exogenous whole IgG Ag was detected using T cell assays. Both GA and RA increased MHC class II presentation of the subdominant kappa I epitope from exogenous whole IgG Ag (Fig. 39 A and B). MHC class II presentation of the dominant kappa II epitope from exogenous whole IgG Ag was also increased by both GA and RA treatment (Fig.39 C and D).

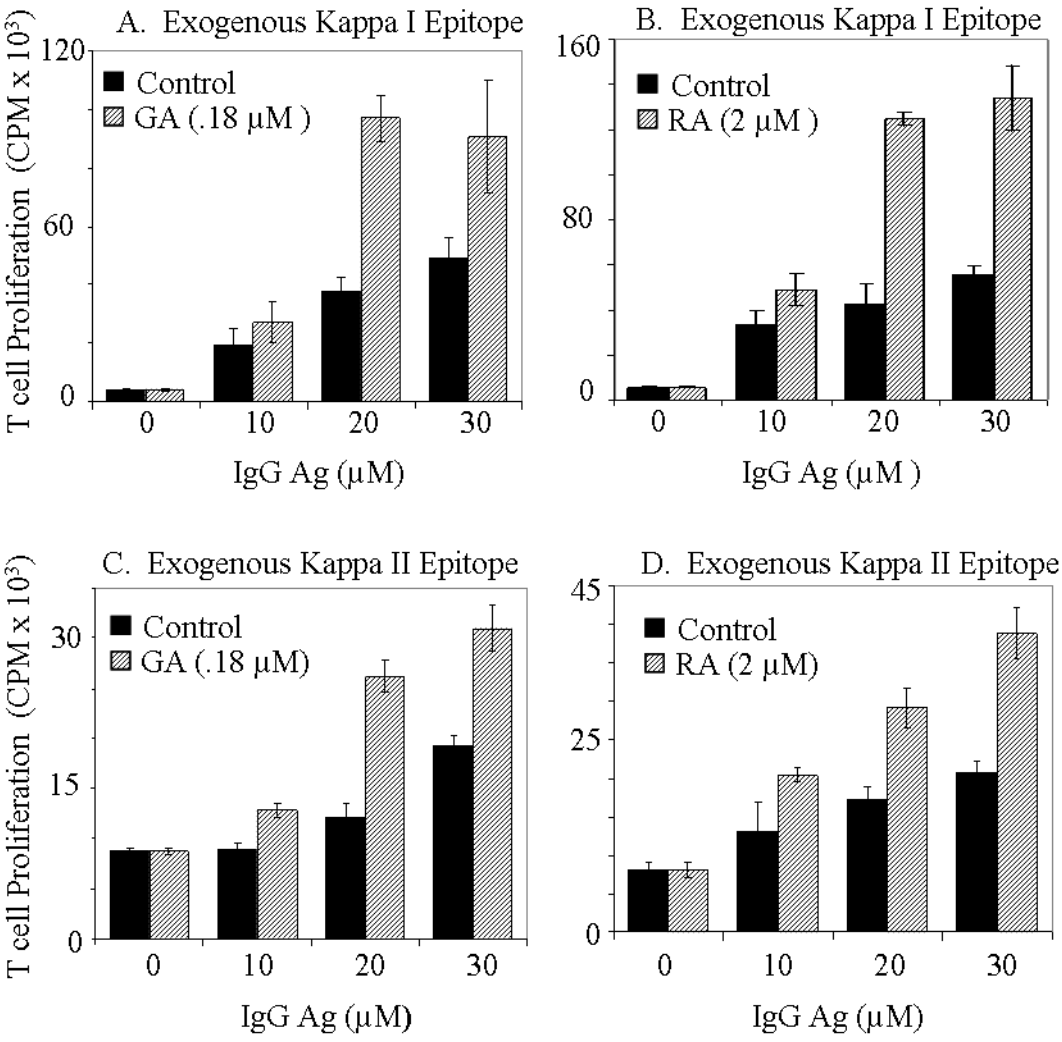


Figure 39. Pharmacological inhibition of HSP90 increases MHC class II presentation of exogenous IgG kappa Ag. Frev cells were treated with DMSO as a control, GA (0.18 µM) (A and C), or RA (2 µM) (B and D) for 18 hours. After 2 hours of treatment, whole IgG kappa Ag was added to B cells at 10, 20, or 30 µM concentrations. After 18 hours, B cell MHC class II presentation of kappa I (A and B) and kappa II (C and D) epitopes from exogenous IgG kappa Ag was measured by T cell assays. Control assays were carried out as in Figure 8. Results are representative of at least 3 separate experiments.

GA and RA treated Frev cells were also pulsed with exogenous kappa I and kappa II peptides and MHC class II presentation of each of these peptides was detected using T cell assays. When low concentrations of exogenous kappa I peptide was added, both RA and GA increased MHC class II presentation of kappa I epitope (Fig. 40 A and B). When higher concentrations of peptide were added, neither RA nor GA affected kappa I epitope presentation (Fig. 40 A and B). MHC class II presentation of kappa II peptide was also increased upon RA and GA treatment at low peptide concentrations (Fig 40 C and D). Again, when higher concentrations of peptide were added, GA and RA did not alter peptide presentation (Fig. 40 C and D).

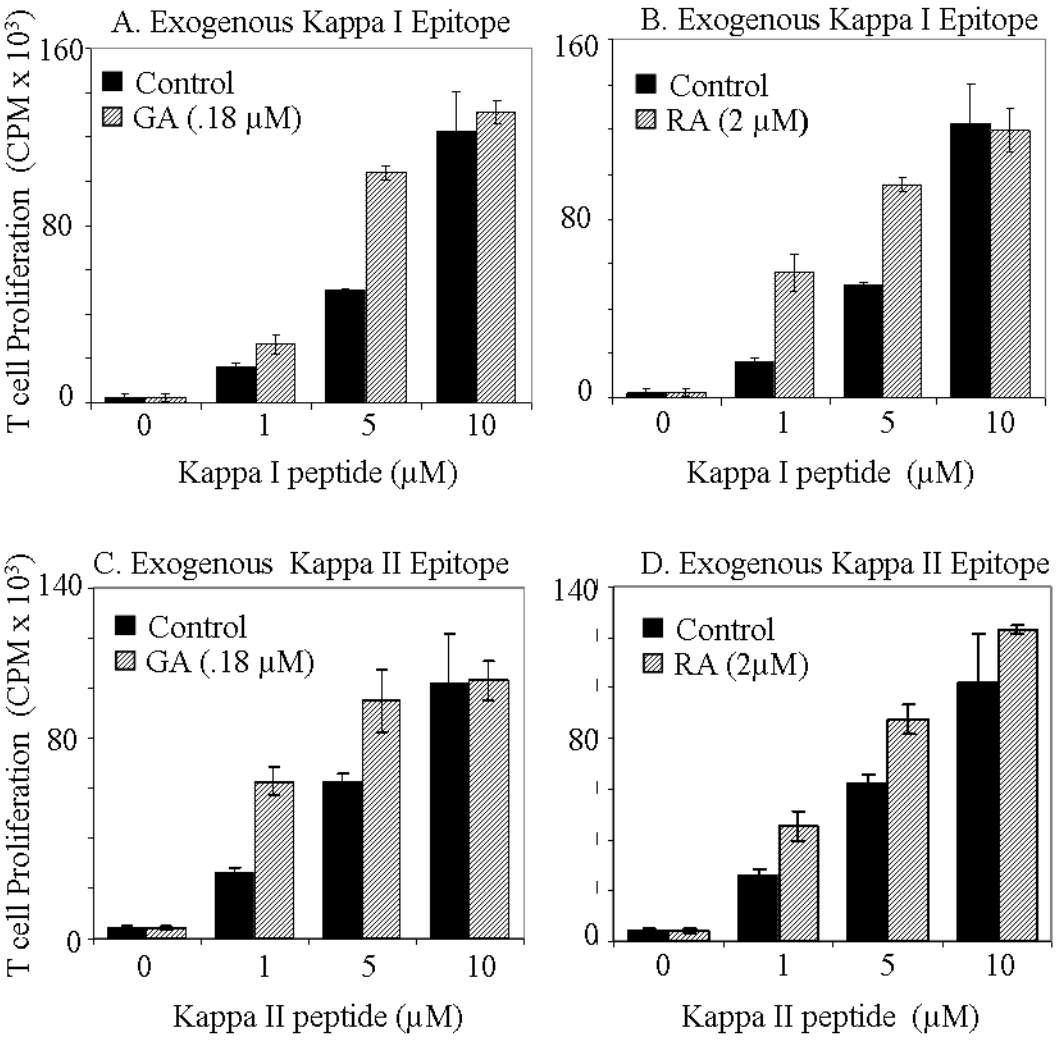
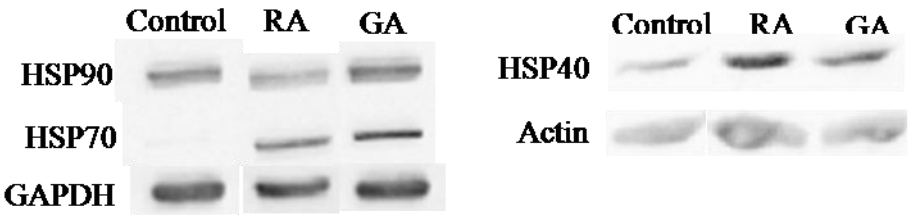


Figure 40. Pharmacological inhibition of HSP90 increased MHC class II presentation of exogenous IgG kappa epitopes. Frev cells were treated with GA (0.18 μM) (A and C), RA (2 μM) (B and D), or DMSO as a control for 18 hours. After 2 hours of treatment, synthethic kappa I and kappa II peptide was added to cells at 1, 5, and 10 μM concentrations. After 18 hours, MHC class II presentation of kappa I (A and B) and kappa II (C and D) epitopes from exogenous kappa peptides was measured by T cell assays. Results are representative of at least 3 separate experiments.

Pharmacological inhibition of HSP90 induces a heat shock stress response

Previous results with PriessGAD cells indicated that both GA and RA induce a heat shock stress response. Both GA and RA induced the expression of HSP70 and HSP40 in PriessGAD cells (Fig. 13). FrevSMA cells were examined for a heat shock stress response upon GA and RA treatment. Similar to the results with PriessGAD cells, both GA and RA induced a heat shock stress response in FrevSMA cells (Fig. 41A). Both RA and GA treatment induced HSP70 and HSP40 expression. Densitometry revealed that HSP70 expression was enhanced by 700% to 1100% (Fig. 41B). Induction of heat shock induced HSP70 and HSP40 expression in B cells (Fig. 41).

A.



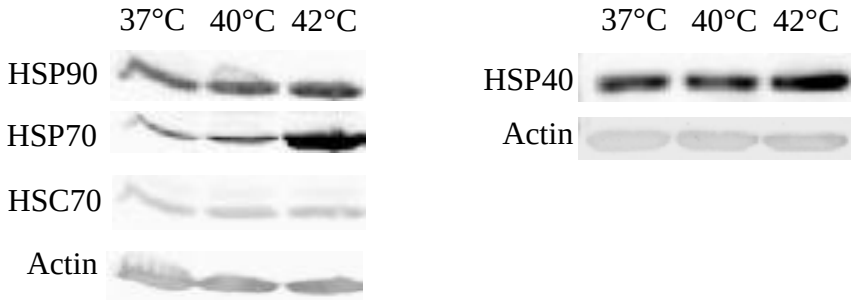
B.

Relative Expression of HSP/HSC			
	Control	RA	GA
HSP90/GAPDH	100%	133%	104%
HSP70/GAPDH	100%	3617%	3421%
HSP40/Actin	100%	273%	260%

Figure 41. Pharmacological inhibition of HSP90 induces a stress response in FrevSMA B cells. FrevSMA cells were treated with either RA (2 μM), GA (0.18 μM), or DMSO as a control for 18 hours. (A) Whole cell lysates from treated cells were fractionated on 10% SDS-PAGE gels and Western immunoblotted for HSP90, HSC70, HSP70, HSP40, and actin protein expression. (B) Densitometry of the representative experiment. Results are representative of at least 3 separate experiments.

To determine if heat shock could mimic the heat shock stress response in FrevSMA cells upon GA or RA treatment. FrevSMA cells were subjected to heat shock and measured for HSP70 and HSP40 expression by Western immunoblotting. Upon heat shock at 40°C and 42°C, both HSP70 and HSP40 expression was induced in FrevSMA cells (Fig. 42). Thus, heat shock can mimic the heat shock stress response upon RA and GA treatment in both PriessGAD and FrevSMA cells.

A.



B.

Average Relative Expression of HSP/HSC			
	37°C	40°C	42°C
HSP90/Actin	100%	122% +/- 19%	125% +/- 30%
HSP70/Actin	100%	205% +/- 45% *	598% +/- 229% *
HSC70/Actin	100%	118% +/- 15%	121% +/- 5%
HSP40/Actin	100%	102% +/- 21%	111% +/- 8%

Figure 42. Heat stress induces HSP expression in FrevSMA cells. FrevSMA cells were incubated for 20 minutes at 37, 40, or 42°C followed by a 24 hour incubation at 37°C. (A) Whole cell lysates from these cells were fractionated on 10% SDS-PAGE gels and Western immunoblotted for HSP90, HSC70, HSP70, HSP40, and actin protein expression. The results are representative of at least 3 separate experiments. (B) The table depicts the average relative expression of HSP/HSC +/- SEM as determined by densitometry of at least 3 separate experiments. * Statistically significant as determined by student t tests.

Subjecting PriessGAD cells to heat shock did not alter MHC class II presentation of endogenous GAD, endogenous kappa, exogenous HSA Ag, and exogenous HSA peptide (Fig. 15). To determine if MHC class II presentation of endogenous SMA is altered by heat shock, FrevSMA cells were incubated at 37 or 42°C for 20 minutes followed by a 24 hour incubation at 37°C. Following heat shock, FrevSMA cells were measured for MHC class II presentation of endogenous SMA by T cell assays. In contrast to endogenous GAD presentation, heat shock enhanced MHC class II presentation of endogenous SMA (Fig. 43).

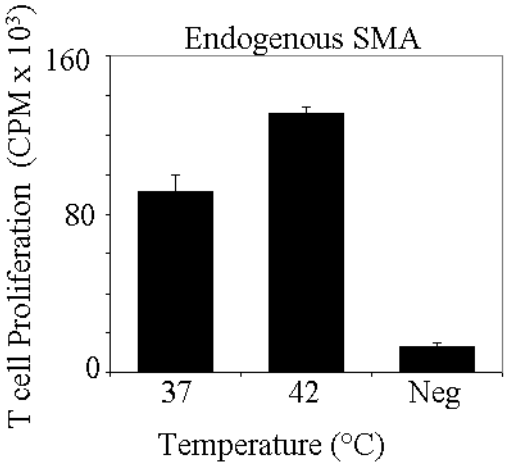


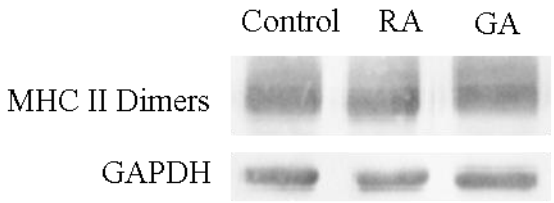
Figure 43. Heat stress enhances MHC class II presentation of endogenous SMA. FrevSMA cells were incubated for 20 min at 37 or 42°C followed by a 24 hr incubation at 37°C. After incubation, MHC class II presentation of endogenous SMA epitope was measured by T cell assay. Results are representative of at least 3 separate experiments**.**

Pharmacological inhibition of HSP90 does not alter MHC class II expression

As previously stated, HSP90 overexpression in macrophages was found to enhance MHC class II dimer stability while GA and RA treatments decreased dimer stability (142). Both HSP90 overexpression and inhibition were reported not to alter surface and total MHC class II protein expression in these murine cells (142). However, Western immunoblotting of PriessGAD cells treated with either GA or RA found that MHC class II monomer expression and dimer stability was not affected by pharmacological

inhibition of HSP90 (Fig. 16). Flow cytometric analysis of GA or RA treated PriessGAD cells confirmed the lack of affect on both surface and total MHC class II levels (Fig. 17). To evaluate whether GA or RA inhibition of HSP90 altered MHC class II expression levels in FrevSMA cells, FrevSMA cells were treated with either GA (0.18 µM), RA (2 µM), or DMSO as a control and Western immunoblotted for MHC class II dimer expression. Neither GA nor RA significantly altered the steady state levels of MHC class II dimers (Fig. 44). These results indicate that the increase in MHC class II SMA Ag presentation observed with GA or RA treatment is not due to alterations in MHC class II dimer expression or stability in B cells.

A.



B.

Average Relative Expression to Actin			
	Control	RA	GA
MHC II Dimers/Actin	100%	101% +/- 20%	107% +/- 12%

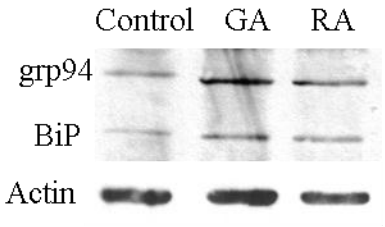
Figure 44. Pharmacological inhibition of HSP90 does not alter MHC class II expression in FrevSMA B cells. FrevSMA cells were treated with either RA (2 µM), GA (0.18 µM), or DMSO as a control for 18 hours. (A) Whole cell lysates from treated cells were fractionated on 10% SDS-PAGE gels and Western immunoblotted for MHC II dimers and GAPDH protein expression. Blot is representative of at least 3 separate experiments. (B) The table depicts the average relative expression of MHC II dimers +/- SEM as determined by densitometry of at least 3 separate experiments. Statistical analysis using the student t tests determined no significant difference in the expression of MHC class II dimers.

Pharmacalogical inhibition of HSP90 induces ER stress in FrevSMA

As previously described, the ER chaperones grp94 ad BiP are ER homologs of cytoplasmic HSP90 and HSP70 respectively. Due to the similarity between grp94 and HSP90, GA can bind to both of these chaperones. Previous studies indicate that GA treatment can induce an ER stress response represented by an upregulation of ER chaperones in certain cell lines (228). Neither GA nor RA treatment induced an ER stress response in PriessGAD cells (Fig. 34). However, it was unknown whether ER stress is induced in FrevSMA cells upon GA and RA treatment. An induction of ER stress could be one explanation for the observed decrease in MHC class II presentation of GAD upon HSP90 inhibition.

To determine if HSP90 inhibition affects ER stress in FrevSMA cells, the expression of grp94 and BiP was examined by Western immunoblotting whole cell lysates of FrevSMA cells treated with pharmacological inhibitors. Both GA and RA significantly induced grp94 and BiP expression in FrevSMA cells with reduced HSP90 activity compared to controls (Fig. 45A). However, RA treatment enhanced the expression of both grp94 and BiP slightly more than GA. The average relative expression of grp94 and BiP as determined by densitometry of at least 3 separate experiments confirmed these results (Fig. 45B). These results indicate that in contrast to PriessGAD cells, HSP90 inhibition by GA and RA may induce ER stress in FrevSMA cells.

A.



B.

Average Relative Expression			
	Control	GA	RA
grp94/GAPDH	100%	143% +/- 5% *	183% +/- 2% *
BiP/GAPDH	100%	117% +/-1% *	167% +/-3% *

Figure 45. Pharmacological inhibition of HSP90 induces ER stress in FrevSMA cells. FrevSMA cells were treated with either GA (0.18 µM) or RA (2 µM), or DMSO control. A. Whole cell lysates from treated cells were analyzed by Western immunoblotting for expression of grp94 and BiP. Blot is representative of at least 3 separate experiments. B. The average relative expression of grp94 and gp78 expression +/- SEM as determined by densitometry of at least 2 separate experiments is displayed in the table. *Statistically significant as determined by student t tests.

Pharmacological inhibition of HSP90 does not induce HSP70 secretion

Studies in tumor cells found that HSP70 was secreted by tumor cells through a pathway involving lysosomal endosomes (197). Our prior study had failed to detect secreted HSP90 following RA and GA treatment. Secretion of HSP70 could potentially explain the enhanced MHC class II presentation of kappa I and kappa II peptides from exogenous Ig Ag upon GA and RA treatment. Western immunoblotting of concentrated conditioned media from Priess, PriessGAD, Frev, and FrevSMA cells failed to detect any HSP90 or HSP70 protein (Fig. 36A and 46A). HSP70 was also not detected in fresh media or media components (Fig. 46A). Neither GA nor RA treatment induced HSP70 secretion by PriessGAD cells (Fig. 36B). Likewise, HSP70 was not detected in the conditioned media of PriessGAD cells upon RA and GA treatment (Fig. 46B). As a control, blots were stained with Ponceau S to confirm the presence of protein (Fig. 46C). These results suggest that GA and RA do not induce HSP70 secretion in B cells.

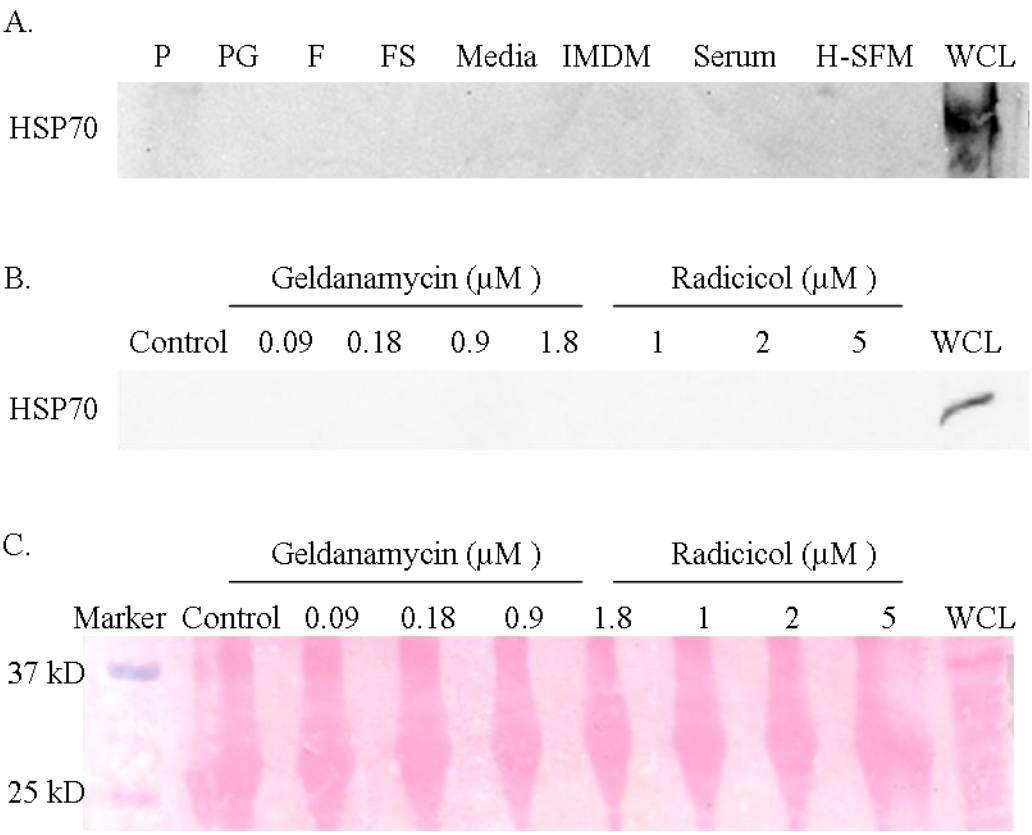
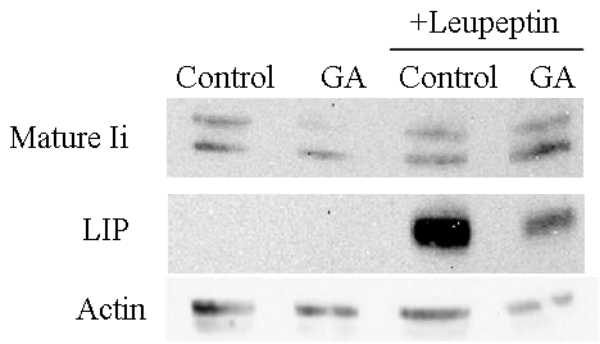


Figure 46. Pharmacological inhibition of HSP90 does not induce HSP70 secretion by B cells. (A) Conditioned media from Priess (P), PriessGAD (PG), Frev (F), and FrevSMA (FS); fresh IMDM cell media plus serum; fresh IMDM media or serum components; and H-SFM media was concentrated from 13 ml into 50 µl. Concentrated samples were fractionated on 10% SDS PAGE gels along with PriessGAD WCL as a positive control. Blots were probed for HSP70. (B and C) PriessGAD cells were treated with either DMSO control, GA, or RA for 18 hours. After treatment, 75 µl of conditioned media was fractionated on 10% SDS PAGE gels along with PriessGAD WCL as a positive control. In panel B, blot was probed for HSP70 and GAPDH. In panel C, blot was stained with Ponceau S for total protein for a control. The results are representative of at least 3 separate experiments.

Pharmacological inhibition of HSP90 alters leupeptin-induced LIP accumulation

In PriessGAD cells, treatment with GA decreased leupeptin induced LIP accumulation (Fig. 29). GA treated FrevSMA cells were also examined for leupeptin induced LIP accumulation. As shown with PriessGAD cells, treatment of FrevSMA cells with GA decreased leupeptin induced LIP accumulation (Fig. 47). These results are similar to those seen with the B cell line PriessGAD.

A.



B.

Relative Expression				
			+ Leupeptin	
	Control	GA	Control	GA
Mature Ii/Actin	100%	101%	254%	244%
LIP/Actin	0%	0%	100%	69%

Figure 47. Pharmacological inhibition of HSP90 alters leupeptin induced LIP accumulation. FrevSMA cells were treated with either DMSO control, GA (0.18 µM), DMSO control and leupeptin (50 µM), or GA (0.18 µM) and leupeptin (50 µM) for 18 hours. (A) WCL of treated cells were ran on 12% SDS-PAGE gel and Western immunoblotted for mature Ii, LIP, and Actin. (B) Densitometry of the representative experiment. Results are representative of at 2 separate experiments.

Chapter 4. Type B insulin resistance developing during interferon α therapy

Interferon α is an inflammatory cytokine belonging to the type I interferon family. The cellular stress of viral infection induces interferon α production by leukocytes. Interferon α has been shown to play key roles in defending the host immune system during a viral infection by binding to cell surface receptors on host cells and making conferring resistance to viral infection (232). Due to its potent anti-viral effects, interferon α has been used either alone or in conjunction with additional anti-viral medications for the treatment of chronic hepatitis infection (233). While interferon α treatment is effective in treating chronic hepatitis C infection, this study indicates that type B insulin resistance can occur due to interferon α therapy.

Introduction

Type B insulin resistance is a rare syndrome caused by the production of autoantibodies to the insulin receptor. These autoantibodies were initially described in patients diagnosed with both diabetes and extreme insulin resistance (234). However, current studies have shown that anti-insulin receptor antibodies can cause abnormalities of glucose homeostasis ranging from profound insulin resistance to life-threatening hypoglycemia (235). Most patients with insulin receptor autoantibodies have an underlying connective tissue disorder such as systemic lupus erythematosis. Research has described autoimmune hypoglycemia with insulin receptor autoantibodies as a paraneoplastic syndrome in Hodgkin's lymphoma and multiple myeloma (236, 237). Autoimmune hypoglycemia has been reported as arising after heterologous bone marrow transplantation (238). The National Institutes of Health evaluated the demographics of a series of 24 patients with type B insulin resistance or autoimmune hypoglycemia and determined that 83% were women and 88% were African Americans (235). Studies confirm that most patients with type B insulin resistance develop acanthosis nigricans, and women of reproductive age usually have ovarian hyperandrogenism (235, 239).

Case report

The patient in this study was a 55 year old African American male diagnosed with hepatitis C, genotype 1b. A biopsy of his liver revealed chronic hepatitis with minimal

activity and mild fibrosis. The patient began treatment with pegylated interferon α-1a and ribavirin. At the beginning of treatment, his hepatitis C viral RNA titer was 3950 KIU/mL. He had no personal history of diabetes mellitus, and his fasting plasma glucose before interferon treatment was 112 mg/dL. Two months after starting therapy, he developed anemia, which was managed with erythropoietin α and a reduction of his ribavirin. After 6 months of treatment, his weight had fallen 16 kg and viral RNA was not detectable. Two months later, he presented with symptoms of polyuria, polydipsia, weakness, blurred vision, and fatigue. Over the preceding month, his weight had fallen another 11 kg, and his weakness was so profound that he was unable to tie his shoes. At this time, he was admitted to the hospital for treatment. Upon admission, laboratory testing was performed on his serum and urine. His serum glucose was 405 mg/dl, CO_2 was 24 mmol/L, and anion gap was 10 mmol/L. Urine ketones were 1+, and his serum hemoglobin A1c was 9.3%. His serum creatinine fell to 0.8 mg/dl with aggressive hydration. His serum bilirubin, AST, ALT, alkaline phosphatase, amylase, and lipase levels were normal. Interferon and ribavirin treatment was discontinued. Subcutaneous insulin was started and increased over three days to a daily dose of 180 units; however, his blood glucose levels still ranged from 300 to over 600 mg/dL. An insulin infusion was started and titrated over two days to 52 units per hour, but his serum glucose levels were still 230-300 mg/dL.

At this point, the patient was transferred to our institution. A physical examination of the patient revealed a thin, African American male with a weight of 68 kg, and a height of 170 cm. He was in no acute distress with a blood pressure was 114/72 mm Hg. His sclerae were anicteric. While his abdomen was soft and nondistended, his right upper quadrant was mildly tender. He had a palpable liver edge 4 cm below the costal margin. The patient did not have acanthosis nigricans, spider angiomas, palmar erythema, or splenomegaly. His insulin infusion was increased to 125 units/hour, but his blood glucose levels still ranged from 170 to 430 mg/dL. Lower values were present after fasting overnight while higher readings were detected through the day. This patient was now suspected of developing type B insulin resistance. After a week on intravenous insulin treatment, he was transitioned to U500 regular insulin at a dosage of 300 units

QID (4 times a day). His blood glucose levels then ranged from 110 to 300 mg/dL. During his third week in the hospital, he developed bilateral facial weakness on the right right side, right-sided facial numbness, and weakness of the right lateral rectus. An MRI of his brain was normal. His cerebrospinal fluid was examined and revealed no white blood cells, nonreactive VDRL, negative viral and bacterial cultures, angiotensin converting enzyme activity 3 units (reference range < 10), glucose 114 mg/dL (reference interval 40 - 70), and protein 70 mg/dL (reference interval 15 - 45). Prednisone, 40 mg daily for 5 days, and acyclovir was used to treat his polycranial neuropathy, and partial improvement of the neuropathy was noted one week later. During his prednisone treatment, he was switched back to an insulin infusion with frequent glucose monitoring because of concern that his insulin requirements might decline dramatically. However, his blood glucose levels and insulin requirement only increased. After 4 weeks, his glucose levels ranged from 70 to 260 mg/dL, and he was discharged home on U500 insulin at a dosage of 400 units QID.

To confirm that the patient developed type B insulin resistance, his serum was assayed for the presence of anti-insulin antibodies using recombinant human insulin receptor in an immunoprecipitation assay (235). Detergent-solubilized membranes of CHO/IR cells expressing human insulin receptor (IR) were the source of insulin receptor used in these assays (240). Patient sera and pooled healthy donor sera used as a control were added to solubilized CHO/IR membranes, and any resulting IR-antibody complexes precipitated. The precipitated human antibodies (pellets) and the remaining soluble cell extracts (supernatants) were probed by Western blotting with commercially obtained antibodies to insulin receptor α-subunit (Fig. 48). Parallel immunoprecipitations of detergent solubilized membranes from parent CHO cells, which do not express insulin receptor, were performed as controls in all cases. An intense band corresponding to insulin receptor α-subunit was present in antibody precipitates formed with patient serum and solubilized CHO/IR membranes (Fig. 48A). The insulin receptor α-subunit was not detected in precipitates with control CHO membranes or precipitates with pooled human serum from healthy donors. The insulin receptor α -subunit was detected by Western immunoblotting of the IP supernatants from CHO/IR membrane incubation with pooled

human sera and to a lesser extent with patient serum (Fig. 48B). Neither the insulin receptor α -subunit nor insulin receptor β-subunit was detected in CHO membrane controls. These results confirmed that the patient did develop auto-antibodies to insulin receptor, thus confirming his diagnosis of type B insulin resistance.

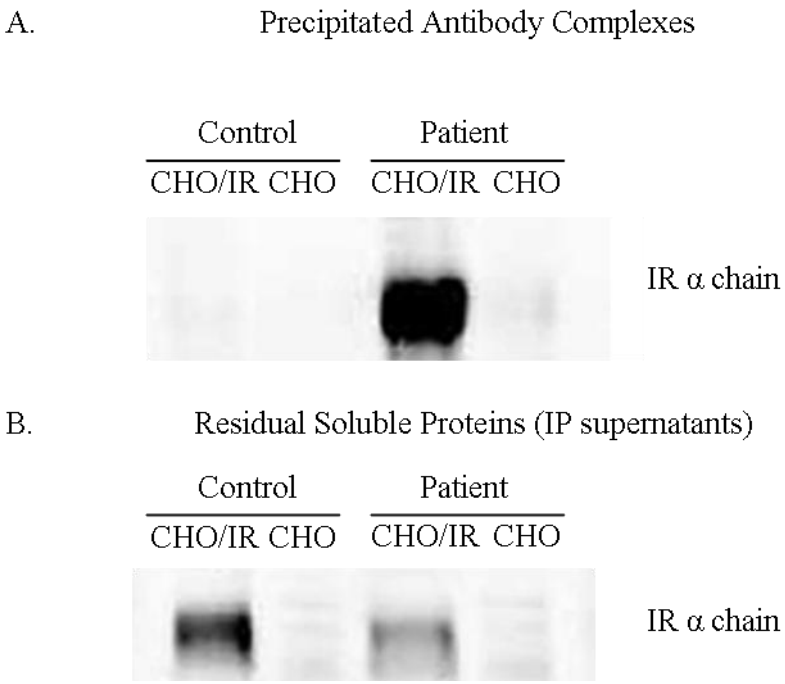


Figure 48. Anti-insulin Abs were detected in original patient serum. Solubilized CHO/IR and CHO cell membrane lysates were incubated overnight at 4°C with 50 µl of either patient sera or control pooled human sera prior to a 1 hour incubation with protein-G sepharose. Precipitated antibody complexes (pellets) (A) and residual soluble proteins (IP supernatants) (B) were analyzed by western blotting with a commercial anti-insulin receptor α Ab. Results representative of at least 3 separate experiments.

During his hospitalization, his sera was assayed for the presence of additional islet cell antibodies and glutamic acid decarboxylase antibodies. Both tests were negative. His C-peptide level was 2.1 ng/mL, and his insulin antibodies levels were 4.2 units/mL (reference range < 5.0). His serum was also analyzed for anti-nuclear antibodies and screened for antibodies to extractable nuclear antigens (SM, RNP, Ro/SS-A, La/SS-B, Scl-70, and Jo-1), single stranded DNA, and double stranded DNA. All results were negative. His erythrocyte sedimentation rate was 32 mm/hr (reference range < 20), and his serum protein electrophoresis was normal. His TSH level was 1.45 µIU/mL with normal free T4 and free T3 levels. Several early morning measurements of his cortisol and ACTH levels did not suggest any pituitary-adrenal abnormality. A fasting lipid panel two weeks after admission showed a cholesterol level of 206 mg/dL, triglyceride level of 68 mg/dL, HDL level of 58 mg/dL, and LDL level of 135 mg/dL. These additional tests further supported a diagnosis of type B insulin resistance.

During the first three months after his discharge from the hospital, he reported glucoses fluctuating between 60 and 300 mg/dL. His insulin injection was reduced to 50 units QID. Four months after his discharge, the patient experienced two episodes of severe hypoglycemia, one of which required medical treatment in an emergency room. His insulin injection was reduced to 30 units/day. Six months after discharge, he had returned to his pre-interferon weight of 96 kg and was only taking 15 units of insulin/day. Nine months after discharge, he reported blood glucose levels ranging between 90 and 190 mg/dL. His hemoglobin A1c level was 5.7%. Two years after discharge, his insulin treatment was discontinued. His hemoglobin A1c level was 5.9%, and his blood glucose levels ranged from 90-160 mg/dL. Over a one year period, his cranial neuropathies had resolved. Eighteen months after discharge, his hepatitis C viral RNA titer had returned to pretreatment levels. It was suspected that the patient's severe insulin resistance had spontaneously resolved.

To confirm a spontaneous recovery, the patient's serum post-treatment was assayed for the presence of anti-insulin receptor antibodies. Pre-treated patient sera, post-treated patient sera, and pooled healthy donor sera were added to solubilized CHO/IR

membranes, and any resulting IR-antibody complexes precipitated. The precipitated human antibodies (pellets) and the remaining soluble cell extracts (supernatants) were probed by Western blotting with commercially obtained antibodies to insulin receptor α-subunit and insulin receptor β-subunit (Fig. 49 A and B). Parallel immunoprecipitations of detergent solubilized membranes from parent CHO cells, which do not express insulin receptor, were performed as controls in all cases. An intense band corresponding to insulin receptor α -subunit and insulin receptor β-subunit was present in antibody precipitates formed with pre-treatment patient serum and solubilized CHO/IR membranes (Fig. 49A). Neither insulin receptor α -subunit nor insulin receptor β-subunit was detected in precipitates with control CHO membranes or precipitates with post-treatment patient sera and pooled human serum from healthy donors. Western immunoblotting of the IP supernatants from CHO/IR membrane incubation with pre-treatment patient sera, post-treatment patient sera, and pooled human sera detected both insulin receptor α-subunit and insulin receptor β-subunit (Fig. 49B). Neither the insulin receptor α-subunit nor insulin receptor β-subunit was detected in CHO membrane controls. These results failed to detect anti-insulin antibodies in the post-treated sera of the patient, suggesting that the patient no longer produced autoantibodies to the insulin receptor. Thus, these results confirm a spontaneous recovery of severe insulin resistance in this patient.

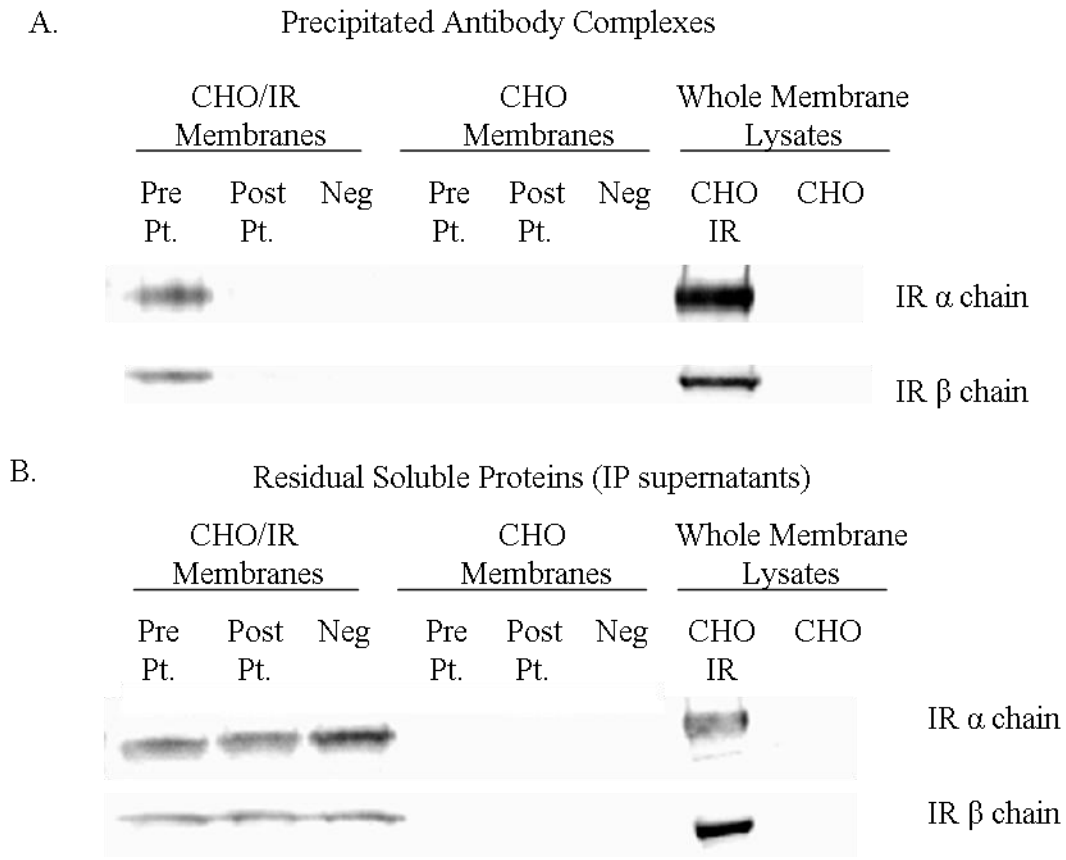


Figure 49. Anti-insulin Abs were detected in original patient serum, but not post-recovery serum. Solubilized CHO/IR and CHO cell membrane lysates were incubated overnight at 4°C with 50 µl of either pre-recovery patient sera (Pre-Pt.) post-recovery (Post-Pt.) patient sera, or control pooled human sera (Neg) prior to a 1 hour incubation with protein-G sepharose. Precipitated antibody complexes (pellets) (A) and residual soluble proteins (IP supernatants) (B) were analyzed by western blotting with anti-insulin receptor α and anti-insulin receptor β Ab. Solubilized CHO/IR and CHO cell membranes were run as a positive control. Results are representative of at least 3 separate experiments.

Discussion

As previously mentioned, interferon α has been used either alone or in conjunction with additional anti-viral medications for the treatment of chronic hepatitis infection and some malignancies (233). However, interferon α treatment is associated with a variety of autoimmune complications, among which, thyroid autoimmunity is most common (241). In several recent studies of hepatitis C patients treated with interferon α, the average incidence of hyper- or hypothyroidism was found to be 6% (242). While some of those patients have destructive thyroiditis without evidence of thyroid autoimmunity, the majority of patients have autoimmune thyroid dysfunction (242, 243). Approximately 50% of patients with interferon-induced autoimmune hypothyroidism and a smaller percentage of those with hyperthyroidism remit after discontinuation of interferon α treatment (242). Numerous other autoimmune conditions have been reported with interferon α therapy; they include TID, systemic lupus erythematosis, myasthenia gravis, celiac disease, autoimmune hepatitis, psoriasis, vitiligo, hemolytic anemia, thrombocytopenia, and sarcoidosis (241, 244, 245). Interferon α -induced autoimmune disease tends to occur in individuals that have a higher baseline risk. Patients are much more likely to develop overt disease while taking interferon α if they are genetically predisposed to thyroid or islet autoimmunity, or with thyroid or islet cell autoantibodies at commencement of therapy (242, 245).

The majority of patients that develop diabetes while receiving interferon α therapy have TID with autoantibodies to islet cell antigens (245). Studies have shown that chronic hepatitis C infection also causes insulin resistance. Patients with hepatitis C have a higher prevalence of type 2 diabetes and impaired fasting glucose (246). Moreover, hepatitis C patients who experience a sustained virologic response to interferon therapy have a lower incidence of glucose abnormalities than those who do not respond (247). However, hepatitis C does not cause insulin resistance of the magnitude seen in this case; the development of type B insulin resistance during interferon therapy is distinctly unusual. There is a case study from Japan of a man who developed diabetes with insulin receptor autoantibodies during interferon-α treatment for hepatitis C (248). Islet autoantibodies were not detected in his serum. His clinical course was characterized by

frequent hypoglycemia during treatment with relatively low doses of insulin. A few months after discontinuation of interferon therapy, insulin receptor antibodies could no longer be detected, but his diabetes did not resolve. Thus, the contribution of these autoantibodies to his diabetes is not clear. A brief report from India describes the case of a woman with type 2 diabetes developing marked hyperglycemia during interferon treatment for chronic hepatitis C (249). Even with insulin doses as high as 700 units/day, the hyperglycemia did not respond to treatment. After discontinuation of interferon therapy, the extreme insulin resistance remitted. Although, measurements of insulin receptor and other autoantibodies were not reported in her case, hyperglycemia refractory to such high insulin doses is consistent with type B insulin resistance.

Insulin receptor autoantibodies have been shown to cause both hyperglycemia and hypoglycemia. This variability in clinical presentation is due to the ability of insulin receptor antibodies to act as either agonists or antagonists of the insulin receptor (235, 250). Insulin receptor antagonism can manifest as severe hyperglycemia refractory to massive doses of insulin. Stimulation of the insulin receptor causes hypoglycemia, which can be life-threatening (235). In some cases, patients with hyperglycemia due to type B insulin resistance can develop severe hypoglycemia later in the course of their illness (235). However, there are also reports of patients with systemic autoimmune disease producing insulin receptor autoantibodies and reporting no obvious abnormality of glucose metabolism (251). It is unknown whether these simply represent antibodies that bind insulin receptors without altering insulin signaling, or whether these patients are at risk of developing autoimmune hyperglycemia or hypoglycemia in the future.

In the United States, type B insulin resistance and autoimmune hypoglycemia are much more common in African Americans and women (235). Most reported cases have occurred in patients with other autoimmune diseases such as systemic lupus erythematosis (250, 252). In the case study presented here, the patient's polycranial neuropathy may have had an autoimmune basis. Cranial neuoropathies can occur in patients with systemic autoimmunity; there are some case reports of Bell's palsy arising during interferon and ribavirin therapy (253-255). There is also an increased incidence of

cranial neuropathies in diabetes (256). It is unknown whether this patient's polycranial neuropathy was caused by autoimmunity, diabetes, or another etiology. While acanthosis nigricans is present in most patients with type B insulin resistance, it was not detected in this patient. Steroids and other immunosuppressive therapies such as azathioprine, cyclophosphamide, cyclosporine, mycophenolate, and rituximab, sometimes in combination with plasmapheresis have all been used as treatment for the underlying autoimmunity in type B insulin resistance (235, 250, 257-259). Type B insulin resistance has a high spontaneous remission rate. Therefore, it is difficult to conclusively attribute improvements in these reports to the immunosuppressive therapy (235). Because of concern that his response to the interferon might be compromised and because his insulin resistance appeared to be improving on its own, there was initial reluctance to use immunosuppressive therapy or plasmapheresis in our patient. When he did eventually receive steroids for cranial polyneuropathy, his glycemic control only worsened. In some cases, patients with type B insulin resistance develop autoimmune hypoglycemia later in the course of their illness. In this study, the patient had two episodes of severe hypoglycemia, which reflect resolution of his insulin resistance, and they did not recur after a sharp reduction of his insulin dose. In this case, the severe insulin resistance remitted spontaneously over a 6 month period after interferon was discontinued.

Conclusion

This case demonstrates that insulin receptor autoantibodies and type B insulin resistance can occur as a complication of interferon α therapy. In patients receiving interferon α therapy who develop new hyperglycemia or hypoglycemia with no evidence for autoimmunity to islet antigens, the possibility of insulin receptor autoantibodies should be considered.

DISCUSSION AND FUTURE CHALLENGES

MHC class II molecules function to present antigenic peptides to CD4+ T cells. Typically, MHC class II molecules present peptides derived from exogenous sources via the classical presentation pathway. In this pathway, exogenous Ags or membrane Ags found at the cell surface are delivered into the endosomal network and processed into peptide fragments by endosomal/lysosomal enzymes prior to loading onto MHC class II molecules. However, some endogenous cytoplasmic and nuclear Ags including tumor, viral, or self proteins have also been found to be sources for peptides presented by MHC II molecules. Such alternative pathways of MHC class II presentation are not well defined or understood. Studies suggest that specific heat shock protein family members may play a role in Ag processing and the subsequent presentation of antigenic peptides by MHC class II molecules. The studies presented here implicate HSP90 as well as HSP70 as regulators of MHC class II presentation for select endogenous and exogenous Ags.

Biological functions of HSP90 and its selective role in Ag presentation

HSP90 has been shown to play a key role in chaperoning client proteins in a variety of cellular processes including cell proliferation, differentiation, and apoptosis (174). Manipulation of HSP90 activity has been used to modulate intracellular protein folding in the cytoplasm and to induce the proteolysis of misfolded or mutant proteins in a variety of disease conditions including malignancies and neurological disorders such as Alzheimer's disease, Parkinson's disease, autoimmune encephalomyelitis, and polyglutamine diseases (179-182). HSP90 inhibitors have also been tested in human clinical trials to promote tumor regression (181). Recent studies have also implicated HSP90 as a potential regulator of both MHC class I and II Ag processing and presentation (142, 143). Studies of the MHC class I pathway suggest HSP90 may guide Ag processing or select epitopes for presentation (141). Yet, whether HSP90 broadly controls MHC class II function or modulates instead the display of select antigenic epitopes has not been dissected.

Here, inhibition of HSP90 in B cells by either pharmacological agents such as GA and RA or siRNA specifically decreased MHC class II presentation of both exogenous and endogenous GAD (Figs. 8, 10A, and 10B). However, MHC class II presentation of several other endogenous and exogenous Ags was unaffected by HSP90 inhibition (Figs. 9, 10C, 10D, 11). Furthermore, disruption of HSP90 function or expression failed to alter exogenous peptide presentation by these human APCs. Thus, HSP90 inhibition appears to selectively affect MHC class II presentation in an Ag specific manner. HSPs have been shown to preferentially recognize certain Ags based on their sequence or structure. For example, HSC70 preferentially recognizes peptides containing a KFERQ like motif, while HSP70 preferentially binds hydrophobic residues within Ags (140, 153, 154).

Studies show that HSP90 exhibits some substrate specificity, although clear motifs recognized by this HSP have yet to be defined (170, 174, 178). HSP90 may also recognize target proteins based on conformation or folding. The autoantigen GAD is well known for its hydrophobic nature and association with lipid membranes via its N-terminus (10, 12). Whether these properties contribute to HSP90 association with GAD remains unclear. However, this study found that HSP90 interacts with both full length GAD Ag as well as an N-terminal truncated form of GAD Ag (Fig. 17 and data not shown). Moreover, HSP90 inhibition decreased MHC class II presentation of both exogenous full length GAD and N-terminal truncated GAD (Figs. 10A, 10B, and data not shown). Studies to examine in vivo T cell responses to GAD Ag in the presence and absence of HSP90 activity could prove useful in revealing if this HSP not only alters the efficiency but also the specificity of GAD presentation and thus the repertoire of peptides displayed on APCs. Epitope profile studies could prove useful in determining if selective inhibition of HSP90 alters GAD processing in a manner that changes the GAD epitope repertoire. Moreover, site directed mutagenesis of the GAD protein could determine which sites within the GAD Ag facilitate HSP90 association and chaperone function.

Association of HSP90 with proteins and links to cell stress

The physical association of HSP90 and the autoantigen GAD in cells co-expressing these molecules was demonstrated (Fig. 17A). HSP90 has been shown in some cases to work in concert with additional HSPs as part of a multi-protein chaperone complex; therefore, HSP90 and GAD association in vivo may involve other HSPs. However, incubating purified HSP90 with purified GAD Ag resulted in the two proteins co-immunoprecipitating (Fig. 17B). Previous studies found HSP90 inhibition to alter MHC class II presentation. Incubation of HSP90 with OVA Ag resulted in the two proteins co-immunoprecipitating (Fig. 17B). Incubation of HSP90 with Ig kappa light chain failed to show any association between these two proteins. Taken together with the specific effect of HSP90 inhibition of MHC class II expression of GAD and OVA Ag, these results suggest that HSP90 may selectively binds to Ags. While these results indicate that HSP90 and GAD Ag can bind directly, it does not rule out the association of GAD-HSP90 complexes with other intracellular HSPs. Potentially, complex formation between HSP90 and GAD may influence GAD proteolytic processing by APCs. The current study suggests the latter, demonstrating that HSP90 modulates MHC class II presentation of select Ags such as the diabetes autoantigen GAD but not other Ags or peptides. Further studies are needed to dissect the role of individual HSP90 isoforms in complexing with additional HSPs and the role of such complexes in regulating Ag presentation.

Prolonged exposure of cells to HSP90 inhibitors has been shown to induce a stress response in some cells as manifested by increased cellular protein expression of HSP70, HSP90, HSC70, and HSP40 (179, 220, 221). In this study, GA/RA treatment of PriessGAD cells resulted in increased expression of HSP70, HSP90, HSC70, and HSP40 suggesting that GA/RA treatment does induce a stress response in B cells (Fig. 12). Studies suggest that cell stress such as heat shock can also promote changes in Ag processing and MHC class II presentation of some exogenous and endogenous Ags by B cells (222). Yet, heat shocking PriessGAD cells failed to affect MHC class II presentation of endogenous GAD Ag, exogenous HSA Ag, or HSA peptide (Fig. 14). These results indicate that while cellular RA and GA treatment induces a stress response

and upregulated overall HSP/HSC expression in B cells, such cellular stress alone does not contribute to the observed decrease in MHC class II presentation of GAD Ag associated with pharmacological inhibition of HSP90. This suggests that HSP90 has a distinct role in MHC class II presentation of GAD Ag.

While the individual functions of HSP90 α and β isoforms have been dissected in terms of MHC class I presentation, this work marks a first step in addressing the role of these HSP90 isoforms in MHC class II presentation. HSP90α alone was found to modulate Ag processing for MHC class I presentation (141). Using HSP90 α and β specific siRNA to modulate HSP90 protein abundance revealed both isoforms were involved in MHC class II presentation of exogenous and endogenous GAD (Figs. 22, 24A, and 24B). These two isoforms may have distinct rather than redundant roles in the Ag processing and presentation pathway. As previously mentioned, HSP90 purifies as a dimer, and in vivo, dimerization is required for HSP90 function (260). Recent studies indicate that HSP90α and HSP90β primarily dimerize as homodimers, but heterdimerization does occur (174). While studies here indicate that both HSP90α and HSP90β are highly expressed in B cells, it remains unknown what form of dimers are primarily found in these cells. The composition of HSP90 dimers in APCs may also influence Ag processing for MHC class II presentation.

Potential steps where HSP90 regulates the MHC class II pathway of Ag presentation

It is interesting that inhibition of HSP90 affects both endogenous and exogenous presentation of GAD Ag. Analysis of MHC class II presentation of short synthetic GAD peptides failed to reveal a role for HSP90 in the cell surface loading of MHC class II molecules or B-T cell interactions. Thus, HSP90 likely exerts its effect on intracellular GAD Ag processing or via unfolding GAD to facilitate MHC class II binding to antigenic epitopes. The pathways of exogenous and endogenous MHC class II presentation for GAD Ag are distinct yet converge with shared processing in endosomal and lysosomal compartments (Figs. 2 and 3). This leads to speculation as to exactly where HSP90 modulates GAD processing and presentation. Studies here examined whether HSP90

affects GAD Ag presentation at one of the shared steps found within the classical and alternate MHC class II presentation pathways.

MHC class II expression and dimer formation

One shared step is loading of MHC class II complexes with peptides within endosomal compartments. This results in both SDS stable MHC class II dimers as well as unstable dimers. In murine macrophages, HSP90 overexpression has been shown to enhance MHC class II dimer stability while GA and RA treatments decrease dimer stability (142). Both HSP90 overexpression and inhibition were reported not to alter surface and total MHC class II protein expression in these murine cells (142). Yet, no change in MHC class II expression or dimer stability was seen with a reduction in HSP90 function in human B cell lines (Figs. 15, 16, 21, and 44). While HSP70 was shown to interact with HLA-DR molecules, it is unknown whether HSP90 can directly interact with HLA-DR molecules (209). Coimmunoprecipitation studies using extracts from human B cells will determine whether HSP90 interacts with MHC class II molecules. In PriessGAD cells, HSP90 inhibition by either GA/RA or HSP90α or HSP90β siRNA did not alter MHC class II expression or dimerization (Figs. 15, 16, and 21). Thus, the decrease in MHC class II GAD Ag presentation observed with GA or RA treatment is not due to alterations in MHC class II expression or dimer stability in B cells (Figs. 8, 10 A and B, 22, and 24 A and B). Moreover, GAD peptide presentation was unaffected by HSP90 inhibition again suggesting no change in MHC class II surface expression or ligand loading (Figs. 10 C and D and 24 C and D). While future studies such as co-immunoprecipitation experiments can determine if HSP90 associates with other components of the MHC class II pathway, the current study suggests that HSP90 is not modulating MHC class II presentation by altering MHC class II protein expression or display at the cell surface.

Ii processing and HSP90

The role of HSP90 in Ii processing was tested using small molecule inhibitors as well as HSP90 specific siRNA. Inhibition of HSP90α and HSP90β expression by siRNA in PriessGAD cells did not affect Ii chain processing in B cells (Fig. 30). However, leupeptin induced LIP accumulation was reduced by GA/RA treatment (Fig. 29). With

both siRNA or pharmacological inhibition of HSP90, surface and total expression of CLIP and Ii were unchanged (Figs. 27 and 28). The expression and function of HLA-DM and HLA-DO were also found to be unaffected by HSP90α and HSP90β inhibition (Fig. 33). While the mRNA expression for several cathepsins was unaffected by RA/GA treatment, cathepsin S, B, and L activity was altered (Figs. 31 and 32). As previously stated, cathepsins S and L have been shown to be essential proteases catalyzing Ii processing and Ag presentation. The enhanced activity of these proteases could reflect a change in endosomal and lysosomal pH or reflect the cellular stress induced by GA/RA treatment of the cells. Still, the effect of GA/RA on cathepsin activity failed to fully explain the overall affect on MHC class II presentation of GAD Ag. While the profile of Ii fragments found in cells treated with GA/RA was similar to control cells or cells treated with HSP90 siRNA, we did note less of the Ii LIP fragment with GA/RA treatment. This could reflect a change in the kinetics of Ii processing, which could be further tested by radiolabeling studies in the future. Notably cellular MHC class II dimers and a final Ii fragment CLIP was not altered by GA/RA. Therefore, I propose that HSP90 functions at an earlier step in the MHC class II pathways, prior to the involvement of these conserved events in the MHC class II pathway.

Potential roles for HSP90 inside and outside of cells

While HSP90 is located primarily in the cytoplasm of cells, a study in B cells found that heat stress induced enrichment of HSPs including HSP90 in exosomes which are released from cells (229). HSP70, which can associate in the cytoplasm with HSP90, is secreted by tumor cells through a pathway involving lysosomal endosomes (197). Notably, another intracellular chaperone, HSC70, is found both in the cytoplasm and endosomes/lysosomes with studies suggesting it translocates into the endosomal network in an ATP-dependent process (166). Whether HSP90 uses a similar pathway to access the endosomal network is unknown. Several studies have suggested peptide-HSP90 complexes released by tumors can promote MHC class I presentation (137, 144, 223). Islet cell stress including exposure to cytokines such as IL-1β is known to induce HSP90 expression prior to cell death (184). Western immunoblotting of concentrated conditioned media from PriessGAD cells failed to detect HSP90 released from these cells

(Fig. 36). Yet, we cannot rule out the retention of HSP90 in endosomes and lysosomes of these cells where it may intersect exogenous Ags such as GAD. Pre-complexing purified HSP90 with exogenous GAD in vitro prior to addition to Priess cells slightly enhanced GAD presentation (Fig. 37). While this enhancement was modest, this could be due to the lack of additional co-factors needed for cellular uptake or functional folding of this purified HSP90. Taken together, these studies suggest that HSP90 may not be accessing endo/lysosomes via extracellular secretion and re-entry, but rather by direct access into those vesicles from the cytoplasm (Fig. 51). Further studies are needed to determine if and how HSP90 is translocated directly into endo/lysosomes and how the regulation of HSP90 compartmentalization might influence Ag presentation. Also, whether HSP90 is found in the endosomes and lysosomes of all cells or only APCs has not been explored.

In the cytoplasm, HSP90 may function to chaperone endogenous GAD Ag for processing by cytoplasmic proteases such as the proteasome or calpain (Fig. 52). This is supported by the observation that HSP90 is associated with cytoplasmic GAD within cell extracts (Fig. 17). GAD Ag has been shown to be ubiquitinated, and previous studies from our lab have determined that GAD Ag MHC class II presentation of endogenous GAD Ag is dependent upon the protease activity of the cytoplasmic proteasome and calpain (67). Moreover, HSP90 is linked to the proteasome via its cofactors such as HSP70. Radiolabeling and immunoprecipitation experiments could be used to determine whether HSP90 inhibition alters GAD processing in B cells. Moreover, co-immunoprecipitation studies could also assess whether GAD-HSP90 association is affected by treatment of PriessGAD cells with specific proteasomal and calpain inhibitors.

Based on our current studies, one also cannot rule out a role for HSP90 in the translocation of GAD peptides into lysosomes. Previous studies indicate that HSC70 plays a key role in cytoplasmic GAD peptide translocation and subsequent MHC class II presentation (85). These studies and work by others indicate that HSC70 facilitates peptide transport into the lysosomes (85, 162). Our lab has previously shown in B cells that altering HSC70 expression can regulate MHC class II presentation of several cytoplasmic autoantigens leading one to deduce that CMA can regulate immunity (85).

In B cells, HSP90 primarily functions as part of several cytoplasmic multi-chaperone complexes containing HSPs such as HSC70 (152, 167, 175, 176). It is unknown whether HSP90 interacts directly with HSC70 to help chaperone antigenic peptide fragments to lysosomes or HSP90 releases peptides to HSC70. Future studies will be needed to determine whether selective HSP90α and HSP90β inhibition affects the translocation and MHC class II presentation of GAD peptides in the cytoplasm of B cells. As one approach, purified HSP90 could be complexed with GAD peptides prior to electroporation into the cell's cytoplasm to determine if this complex enhances peptide translocation and presentation.

The current study supports a role for HSP90 in regulating MHC class II presentation of both exogenous and endogenous GAD Ag by human B cells. Both pharmacological and siRNA targeted disruption of HSP90 specifically inhibited GAD presentation by MHC class II molecules, yet failed to perturb MHC class II presentation of other Ags. Both HSP90 α and β isoforms were found to be involved in GAD Ag presentation by MHC class II molecules. While HSP90 appears to function at the step of Ag binding to promote processing and presentation, future research will be needed to explore the exact sites of HSP90 action. A working model of how HSP90 may function as a chaperone in the MHC class II pathways of exogenous and endogenous presentation is depicted in Figures 50 and 51 respectively. Direct translocation of HSP90 from the cytosol to endo/lysosomes may allow HSP90 to gain access to exogenous Ags with HSP90 chaperoning these Ags for degradation by endo/lysosomal proteases (Fig. 50).

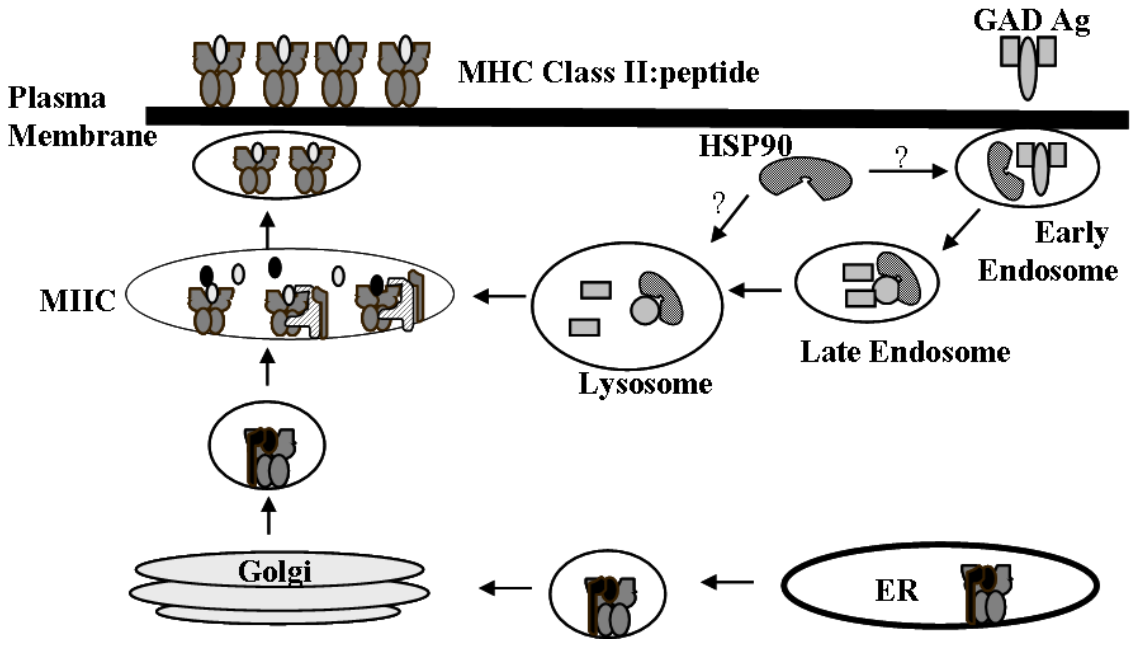


Figure 50. A working model of HSP90 and MHC class II presentation of exogenous GAD Ag. HSP90 may be translocating from the cytosol to endosomes and/or lysosomes. In these vesicles, HSP90 may interact with internalized, exogenous GAD Ag and chaperone its degradation into antigenic peptide fragments.

Likewise, cytoplasmic HSP90 may chaperone cytoplasmic Ags to proteases such as the proteasome or calpain for degradation into antigenic peptides. The resulting peptides might be further chaperoned by HSP90 or transit via HSC70 into the lysosomes for further trimming and loading onto MHC class II molecules (Fig. 51).

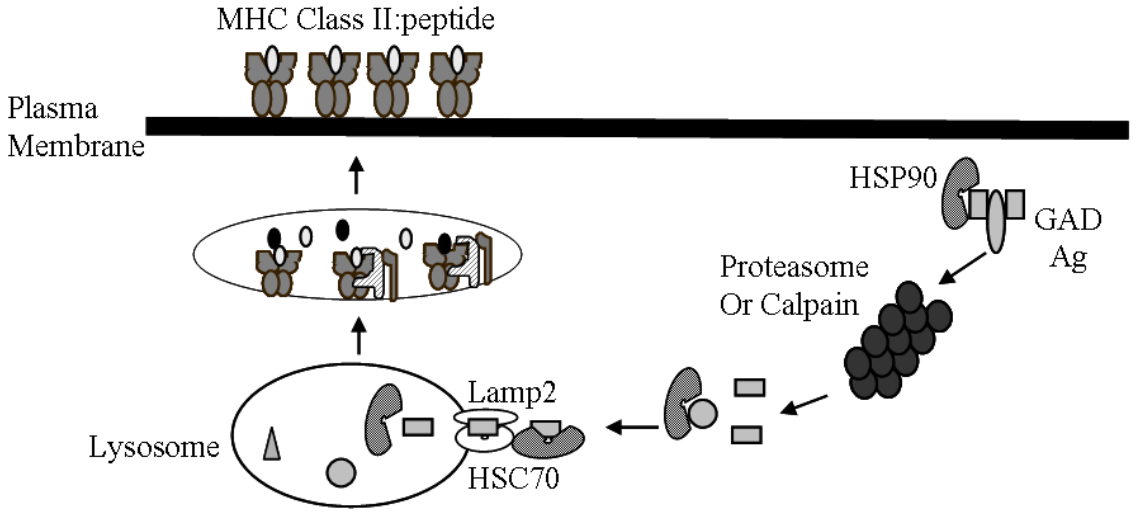


Figure 51. A working model of HSP90 and MHC class II presentation of endogenous GAD Ag. HSP90 may function as a chaperon to GAD Ag pre- and post-degradation by the proteasome or calpain. HSP90 may bind native, endogenous GAD Ag and chaperone GAD Ag to the proteasome or calpain for degradation. The binding of HSP90 to GAD Ag also protect this Ag from premature degradation by other proteases. HSP90 may also bind to GAD peptide fragments generated by cytoplasmic proteases and target them to lysosomes via interacting with HSC70 and Lamp2. Within the lysosomes, GAD peptide fragments may be further trimmed by lysosomal proteases prior to loading onto MHC class II molecules. This may also be mediated by intralysosomal HSPs such as HSP90.

Together, these observations reveal a novel function for HSP90 isoforms and suggest these HSPs may regulate the development of T cell responses to self and potentially foreign Ags. This may prove crucial to understanding the initiation events and pathogenesis of autoimmunity. Further understanding of the role of HSP90 isoforms in GAD Ag processing and presentation by MHC class II molecules may also prove useful in developing new therapeutics to prevent and treat autoimmune diseases such as TID.

HSP70 as a potential player in regulating MHC class II presentation of select antigens

Studying the role of HSP90 in MHC class II presentation revealed a potential requirement for HSP70 in the presentation of select Ags. HSP70 has been shown to perform a variety of chaperone functions including stabilizing newly synthesized or unfolding polypeptides in the cytoplasm, facilitating the translocation of nascent protein chains across membranes, mediating assembly or disassembly of multimeric protein complexes, and targeting proteins for degradation within lysosomes (187-189). Additional studies indicate that HSP70 may play a role in MHC class II presentation of exogenous Ags. Studies have detected extracellular HSP70:peptide complexes in MHC class II enriched compartments after receptor-mediated endocytosis (208). In contrast to HSP90, HSP70 has been suggested to interact with MHC class II molecules (209). Studies also suggest HSP70 mediated enhancement of MHC class II restricted peptide presentation (209, 231). And relevant to autoimmunity, HSP70 has been shown to promote myelin autoantigen presentation by MHC class II molecules (139). The current study suggests that HSP70 not only affects the presentation of select exogenous Ags, but also select endogenous Ags.

HSP70 has been well studied as a key player in amyloidosis, a group of diseases characterized by the assembly of protein fibrils that are deposited extracellulary in various organs and tissues. Rarely are these fibrils deposited intracellularly, but a mutant Ig kappa light chain protein, SMA, does aggregate within the cytoplasm of cells. Studies indicate that HSP70 interacts with SMA and plays an important role in its processing and aggregation. Specifically, overexpression of HSP70 has been shown to decrease the aggregation and promote proteasomal degradation of SMA (207). Therefore, it is suggested that HSP70 can regulate intracellular SMA fibril accumulation. Studies here show also that peptides from endogenous SMA can be presented by MHC class II molecules (Fig. 38). In FrevSMA cells, GA/RA treatment induced a stress response with increased HSP40 and HSP70 protein expression (Fig. 41). The expression of HSP70 was increased 7 to 10 fold in cells treated with GA/RA. Heat shock of FrevSMA cells induced HSP expression comparable to GA/RA treatment (Fig. 42). While heat shock

alone did not affect MHC class II presentation of endogenous and exogenous GAD, heat shock treatment of FrevSMA cells enhanced MHC class II presentation of endogenous SMA (Figs. 15 and 43). Since both GA/RA treatment and heat shock enhanced MHC class II presentation of SMA, inhibition of HSP90 cannot explain the observed enhanced presentation of SMA protein. Yet common to both these treatments was the induction of HSP70 expression. By contrast, HSP40 expression was only slightly enhanced compared to HSP70 expression. These results together with the previous studies indicating HSP70 as a chaperone for SMA, suggest that the enhanced MHC class II presentation of SMA is due to the enhanced HSP70 expression. These results at present only indirectly link HSP70 to MHC class II presentation of SMA, thus further studies using HSP70 specific siRNA and HSP70 overexpressing plasmids are needed to confirm a role for HSP70 in cytoplasmic Ag presentation.

Interestingly, GA/RA treatment induced ER stress as indicated by significantly enhanced grp94 and BiP protein expression in FrevSMA cells, but not PriessGAD cells (Figs. 34, 35, and 45). The induced ER stress may impact and promote SMA degradation and HSP70 chaperone activity. SMA is transported into the ER and fails to exit to the Golgi, but rather is retained in the ER and translocated to the cytosol for proteasomal degradation (207, 261-264). As previously described, both grp94 and BiP play roles in both protein folding in the ER and ERAD. GA and RA can inhibit cytoplasmic HSP90 as well as grp94 in the ER. With misfolded SMA in the ER, ER stress may be induced coupled with grp94 loss of function. Moreover, previous studies indicate that BiP can bind to SMA (207, 230). Enhanced grp94 and BiP expression within the ER may result and enhance SMA translocation from the ER into the cytosol. There cytosolic HSP70 could further assist in chaperoning SMA to the proteasome for degradation. Increased SMA degradation could lead to an increase in the SMA peptides that could be presented by MHC class II molecules. Further studies using grp94 and BiP inhibitors could be carried out to examine the role of ER stress in SMA translocation, degradation, and MHC class II presentation. Further studies using siRNA to block HSP70 expression would be important.

Treatment of Frev cells with GA/RA not only enhanced MHC class II presentation of endogenous SMA, but also MHC class II display of peptides, kappa I and kappa II from exogenous Ig kappa light chain (Fig. 39). While the presentation of synthetic exogenous HSA or GAD peptides was unaffected by GA/RA treatment, this treatment did enhance the MHC class II presentation of exogenous kappa I and kappa II peptides (Fig. 11 C and D, 12 C and D, and 40). As previously stated, HSP70 has been extensively studied as a chaperone for exogenous Ags for both exogenous and endogenous presentation. Moreover, peptides were more efficiently presented by MHC class II molecules when precomplexed with HSP70 (209). In response to stress, HSP70 is secreted from a variety of cell types via an undefined pathway (190-195). Under normal conditions HSP70 is not secreted by FrevSMA cells, and GA/RA treatment did not induce HSP70 secretion (Fig. 46). Thus, secreted HSP70 due to GA/RA treatment is likely not an explanation for enhanced MHC class II presentation of exogenous Ig kappa Ag and kappa I and kappa II peptides. Additionally, this enhancement seems to be Ag and peptide specific as MHC class II presentation of GAD peptide, HSA Ag, and HSA peptide was not unaffected by GA/RA treatment. Further studies are needed to examine the role of HSP70 in the enhancement of MHC class II presentation of select Ags.

While this work implicates HSP70 as a potential modulator of MHC class II presentation of select endogenous and exogenous Ags, further research is needed to confirm these findings and determine specifically how HSP70 modulates these pathways. However, working models of HSP70 chaperone function within the MHC class II pathway of endogenous and exogenous presentation are represented in Figures 52 and 53 respectively. In FrevSMA cells, endogenous HSP70 may chaperone SMA from the ER to the proteasome for proteasomal degradation. HSP70 may also bind SMA peptide fragments and chaperone them to the HSC70/lamp2 complexes at the lysosomal surface for peptide translocation into lysosomes, intersecting the MHC class II pathway (Fig. 52).

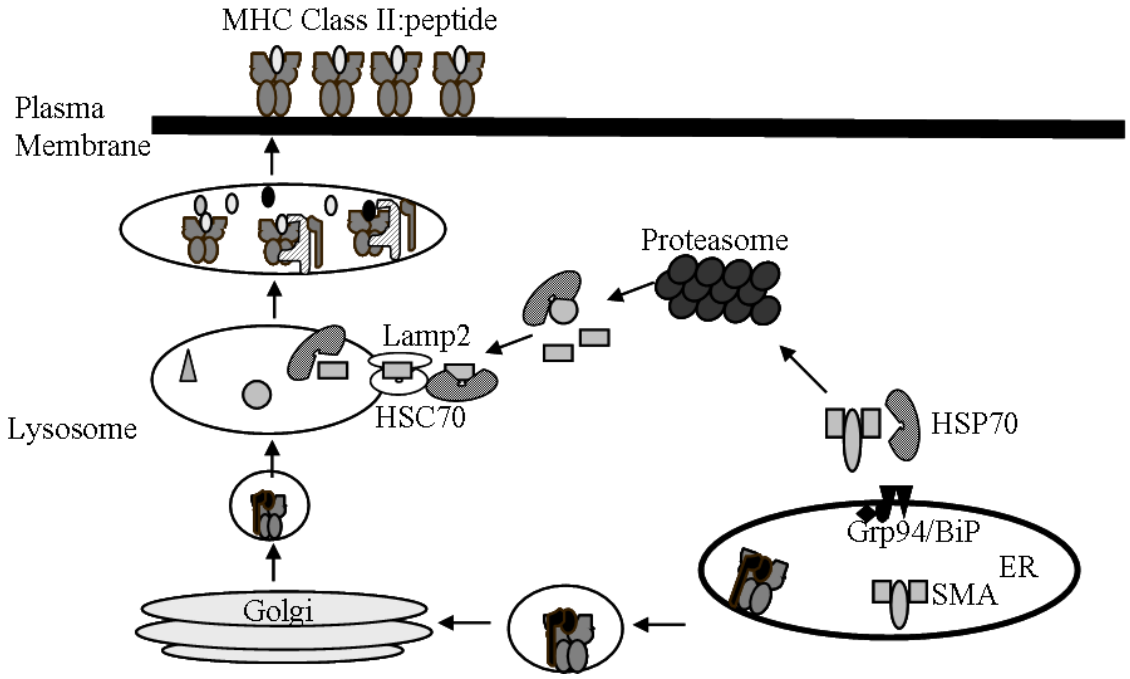


Figure 52. A Working Model of HSP70 and MHC Class II Presentation of Endogenous SMA Ag. Endogenous SMA is translocated from the ER to the cytosol via ER chaperone proteins grp94 and BiP. In the cytosol, HSP70 may bind to cytosolic and chaperone it to the proteasome for degradation into antigenic peptide fragments. HSP70 binding to SMA may also protect it from premature protein degradation. Upon proteasomal degradation, SMA peptide fragments may also be recognized by HSP70 and targeted to the HSC70/Lamp2 complex for translocation into lysosome. HSP70 may also be translocated directly from the cytosol into the lysosomes. Here, HSP70 may mediate further trimming of SMA peptides and/or stabilize them prior to loading onto MHC class II molecules.

For MHC class II presentation of exogenous Ags, HSP70 may gain access to endo/lysosomes and facilitate Ag proteolysis into peptide fragments. Additionally, HSP70 may facilitate peptide loading onto MHC class II molecules (Fig. 53).

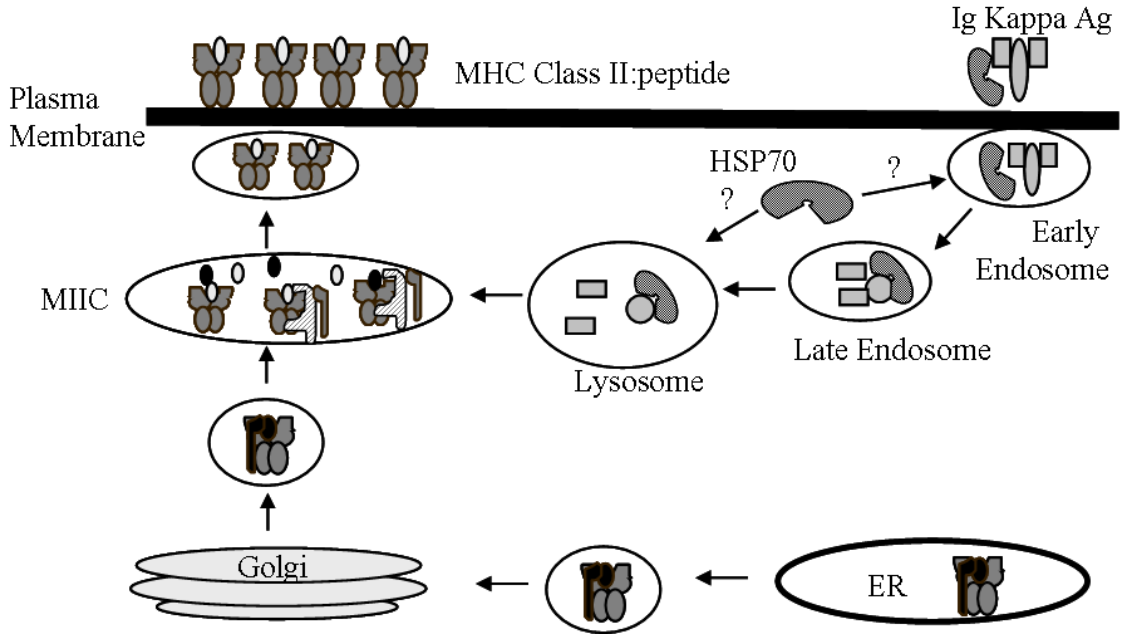


Figure 53. A working model of HSP70 and MHC class II presentation of exogenous Ig kappa Ag. HSP70 may be directly translocated from the cytosol to endosomes and/or lysosomes. Within these vesicles, HSP70 may chaperone internalized, Ig kappa Ag and guide its degradation into antigenic peptide fragments. HSP70 may also assist in the peptide loading of MHC class II molecules.

HSP70 has been shown to perform various intra- and extracellular functions. While studies implicate HSP70 as a chaperone for MHC class I and class II presentation of exogenous Ags, this study suggests a novel role for HSP70 in MHC class II presentation of endogenous Ags. Moreover, this work suggests that HSP70 functions as a chaperone for only select endogenous and exogenous Ags. Research has linked HSP70 chaperone function to protein folding diseases such as amyloidosis and autoimmune diseases such as multiple sclerosis (139, 207). Understanding the role of HSP70 in MHC class II presentation of select Ags may be crucial in understanding the pathogenesis of these diseases as well as developing therapeutics to effectively treat and prevent their onset.

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CURRICULUM VITAE

Josetta Lynn Houlihan

Higher Education

2009	Ph.D.	Microbiology and Immunology, Indiana University Indianapolis, IN
2003	M.S.	Biology, Purdue University Indianapolis, IN
2001	B.A.	Biological and Physical Sciences, Indiana University Kokomo, IN

Employment

2001-2003	Medical Technologist, Mid-America Clinical Laboratories, Indianapolis, IN
2001-2002	Supplemental Instructor, Department of Chemistry, Indiana University, Kokomo, IN
1999-2001	Laboratory Assistant, Mid-America Clinical Laboratories Elwood, IN
1999-2001	Emergency Room Technician, St. Vincent Mercy Hospital Elwood, IN

Research Experience

May 2004-May 2009	Graduate Student- Janice Blum, Ph.D.
Feb. 2004-April 2004	Graduate Rotation- Ann Roman, Ph.D.
Nov. 2003-Feb. 2004	Graduate Rotation- Stanley Spinola, M.D.
Sep. 2003- Nov. 2003	Graduate Rotation- Janice Blum, Ph.D.

University Teaching Experience

Spring 2006	Guest Lecturer for the Department of Microbiology, Indiana University School of Medicine, Indianapolis, IN
Spring 2005	Teaching Assistant for the Department of Microbiology, Indiana University School of Medicine, Indianapolis, IN

2001-2002 Supplemental Instructor for Chemistry Department, Indiana University, Kokomo, IN

Honors and Awards

2005 The Harold Raidt Graduate Student Teaching Award

2001 Inducted into Sigma Alpha Chi National Honor Society

Professional Development

May 2008 Preparing Future Faculty Program

October 2006 NIH Grant Writing Workshop

Publications

Josetta L. Houlihan and Janice S. Blum. "HSP90 Modulates MHC Class II Presentation of Glutamic Acid Decarboxylase." (Being revised for resubmission to The Journal of Immunology).

Amanda McDaniel, **Josetta L. Houlihan**, Jancie S. Blum, and James P. Walsh. "Type B Insulin Resistance Developing During Interferon α Therapy." Endocrine Practice, (In Press)

Ping Li, **Josetta L. Gregg***, Nan Wang, Delu Zhou, Patrick O'Donnell, Janice S. Blum, and Victoria L. Crotzer. "Compartmentalization of class II antigen presentation: contribution of cytoplasmic and endosomal processing." Immunol Rev. 207:206-17, 2005 Oct.

Research Presentations

Josetta L. Houlihan. HSP90 Inhibition Affects MHC Class II Presentation of Glutamic Acid Decarboxylase. November 2008. Autumn Immunology Conference. Chicago, IL

Josetta L. Houlihan. HSP90 Modulates MHC Class II Presentation of Glutamic Acid Decarboxylase. October 2008. Center for Diabetes Research Seminar Conference. Indiana University School of Medicine. Indianapolis, IN

Josetta L. Houlihan. HSP90 Modulates MHC Class II Presentation of Glutamic Acid Decarboxylase. September 2008. Research In Progress. Department of Microbiology and Immunology. Indianapolis, IN

Josetta L. Houlihan. The role of Heat Shock Protein HSP90 in the Presentation of Glutamic Acid Decarboxylase (GAD) by MHC Class II Molecules. May 2008. Immunology Research Conference. Indianapolis, IN

Josetta L. Houlihan. The role of Heat Shock Protein HSP90 in the Presentation of Glutamic Acid Decarboxylase (GAD) by MHC Class II Molecules. October 2007. Immunology Research Conference. Indianapolis, IN

Josetta L. Houlihan. The role of Heat Shock Protein HSP90 in the Presentation of Cytoplasmic Antigens by MHC Class II Molecules. September 2007. Research In Progress. Department of Microbiology and Immunology. Indianapolis, IN

Josetta L. Houlihan. The Role of Heat Shock Proteins HSP90 and HSP70 in the Antigen Presentation by MHC Class II Molecules. April 2007. Immunology Research Conference. Indianapolis, IN

Josetta L. Gregg*. The Role of HSP90 in the Presentation of Cytoplasmic Antigens by MHC Class II Molecules. January 2006. Research In Progress. Department of Microbiology and Immunology. Indianapolis, IN

Josetta L. Gregg*. The Role of HSP90 in the Presentation of Cytoplasmic Glutamic Acid Decarboxylase by MHC Class II Molecules. January 2006. Sigma Xi Research Competition. Indiana University School of Medicine. Indianapolis, IN

Josetta L. Gregg*. The Role of HSP in MHC II Presentation of Cytoplasmic Antigens. January 2005. Research In Progress. Department of Microbiology and Immunology. Indianapolis, IN

Abstracts for Poster Presentations

Josetta L. Houlihan and Janice S. Blum, Ph.D. HSP90 Inhibition Affects MHC Class II Presentation of Glutamic Acid Decarboxylase. November 2008. Autumn Immunology Conference. Chicago, IL

Josetta L. Houlihan and Janice S. Blum, Ph.D. HSP90 Inhibition Affects MHC Class II Presentation of Glutamic Acid Decarboxylase. September 2008. Department of Microbiology and Immunology Poster Session. Indianapolis, IN

Josetta L. Houlihan and Janice S. Blum, Ph.D. HSP90 Inhibition Affects MHC Class II Presentation of Glutamic Acid Decarboxylase. May 2008. Cancer Research Day. IU Simon Cancer Center. Indianapolis, IN

Josetta L. Houlihan and Janice S. Blum, Ph.D. HSP90 Inhibition Affects MHC Class II Presentation of Glutamic Acid Decarboxylase. April 2008. Experimental Biology 2008/The Annual Meeting of The American Association of Immunologists. San Diego, CA

Josetta L. Houlihan and Janice S. Blum, Ph.D. HSP90 Inhibition Affects MHC Class II Presentation of Cytoplasmic Glutamic Acid Decarboxylase. November 2007. Diabetes Summit. Indianapolis, IN

Josetta L. Gregg*, Jennifer J. Metzler, Ph.D., and Janice S. Blum, Ph.D. HSP90 Inhibition Affects MHC Class II Presentation of Cytoplasmic Glutamic Acid Decarboxylase. May 2006. The Annual Meeting of The American Association of Immunologists. Boston, MA.

Josetta L. Gregg* and Janice S. Blum, Ph.D. MHC Class II Presentation of the Cytoplasmic Antigen SMA is Proteasome Dependent. May 2005. Department of Microbiology and Immunology Annual Second Year Graduate Student Poster Session. Indianapolis, IN.

Research Support

2005-2006 Indiana University Diabetes and Obesity Research Training Program, DeVault Fellowship

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