A ROLE FOR HSC70 IN REGULATING ANTIGEN TRAFFICKING AND PRESENTATION DURING MACRONUTRIENT DEPRIVATION

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A ROLE FOR HSC70 IN REGULATING ANTIGEN TRAFFICKING AND PRESENTATION DURING MACRONUTRIENT DEPRIVATION

Globally, protein malnutrition remains problematic, adversely affecting several systems including the immune system. Although poorly understood, protein restriction severely disrupts host immunity and responses to infection. Induction of high-affinity, long-lasting immunity depends upon interactions between B and T lymphocytes. B lymphocytes exploit several pathways including endocytosis, macroautophagy, and chaperone-mediated autophagy to capture and deliver antigens to the endosomal network. Within the endosomal network antigens are processed and loaded onto major histocompatibility complex (MHC) class II molecules for display and recognition by T lymphocytes. To examine the effect of macronutrient malnutrition on MHC class II antigen presentation, we grew B lymphocytes in media containing amino acids, sugars and vitamins but lacking serum, which contains several types of macronutrients. Our studies show macronutrient stress amplified macroautophagy, favoring MHC class II presentation of cytoplasmic antigens targeted to autophagosomes. By contrast, macronutrient stress diminished MHC class II presentation of membrane antigens including the B cell receptor (BCR) and cytoplasmic proteins that utilize the chaperone-mediated autophagy pathway. The BCR plays a critical role in MHC class II antigen presentation, as it captures exogenous antigens leading to internalization and degradation within the endosomal network. While intracellular

protease activity increased with macronutrient stress, endocytic trafficking and proteolytic turnover of the BCR was impaired. Addition of high molecular mass macronutrients restored endocytosis and antigen presentation, evidence of tightly regulated membrane trafficking dependent on macronutrient status. Cytosolic chaperone HSC70 has been shown to play a role in endocytosis, macroautophagy, chaperone-mediated autophagy and proteolysis by the proteasome, potentially connecting distinct routes of antigen presentation. Here, altering the abundance of HSC70 was sufficient to overcome the inhibitory effects of nutritional stress on BCR trafficking and antigen presentation suggesting macronutrient deprivation alters the availability of HSC70. Together, these results reveal a key role for macronutrient sensing in regulating immune recognition and the importance of HSC70 in modulating distinct membrane trafficking pathways during cellular stress. These results offer a new explanation for impaired immune responses in protein malnourished individuals.

Janice S. Blum, Ph.D., Chair

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List of abbreviations

Ab	Antibody
ABTS	2,2'-azino-bis
AEP	Asparagine endopeptidase
Ag	Antigen
APC	Antigen presenting cell
BCR	B cell receptor
BSA	Bovine serum albumin
CAT	Cathepsin
CHX	Cycloheximide
CLIP	Class II-associated invariant chain peptide
CMA	Chaperone-mediated autophagy
CPM	Counts per minute
CQ	Choloroquine
CytoPG	Priess cells transduced with mutated cytoplasmic GAD
DM	HLA-DM
DMSO	Dimethyl sulfoxide
DO	HLA-DO
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCS	Fetal calf serum

GAD	Glutamic acid decarboxylase 65
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HBSS	Hanks balanced salt solution
HEL	hen egg lysozyme
HSA	Human serum albumin
HSP	Heat shock protein
Ii	Invariant chain
Ig	Immunoglobulin
IMDM	Isocove's modified Dublecco's media
MA	Macroautophagy
MHC	Major histocompatibility complex
MFI	Mean fluorescence intensity
MOI	Multiplicity of infection
MP1	Influenza A matrix protein 1
OVA	ovalbumin
PG	PriessGAD
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
qRTPCR	Quantitative real time polymerase chain reaction
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide gel electrophoresis
TrF	Transferrin receptor

Introduction

The immune system

The human body is under constantly exposed to pathogens and commensal microbes, which utilize nutrients to further their survival. The immune system has evolved to defend against these pathogens. The immune system can be divided into two main branches, the innate and the adaptive immune responses. The innate immune response occurs rapidly and is rather non-specific, recognizing generic molecular patterns associated with pathogens. This rapid response is critical for initial suppression and containment of an infection and for recruiting immune cells to the infection; however, this response does not lead to the development of long-lasting, protective immunity. The adaptive immune response is slower to develop but is highly specific and leads to long lasting immunity through the production of memory cells.

The immune system is dependent on the ability of cells to distinguish foreign pathogens from self. Both the innate and adaptive immune responses depend on specific receptors to recognize foreign pathogens or invaders. Innate immune receptors on human cells can recognize pathogen-associated molecular patterns. The adaptive immune system relies on antigen (Ag) presentation, a process by which cells display or present peptides derived from processed protein Ags bound to receptor-like major histocompatibility complex (MHC) molecules. MHC molecules are constantly presenting peptides on the cell

surface, with the majority of these peptides derived from self-proteins. These MHC:peptide complexes act as a biosensor, as changes in the peptides presented during infection or disease results in activation or tolerance of the adaptive immune response. There are two classes of MHC molecules: MHC class I, which are present on all nucleated cells, and MHC class II, which are present on specialized immune cells.

MHC class II antigen presentation pathway

MHC class II molecules are heterodimers composed of α and β subunits. The HLA-DR, -DQ, and -DP loci encode the human class II α and β subunits (*1*). MHC class II molecules are detected at high levels on professional Ag presenting cells (APCs): dendritic cells, B cells, and macrophages as well as some endothelial, epithelial and tumor cells (*2, 3*). MHC class II molecules fold in the endoplasmic reticulum (ER) and are trafficked to the endosomal/lysosomal network where they bind to processed protein Ags. Once bound to processed peptides, MHC class II molecules are shuttled to the cell surface to interact with CD4^{+} T cells (**Figure 1**). T cell receptors and accessory molecules on CD4^{+} T cells recognize the specific MHC class II:peptide complex and co-stimulatory molecules on APCs. Upon this recognition, CD4^{+} T cells are activated, secreting cytokines and initiating an immune response.
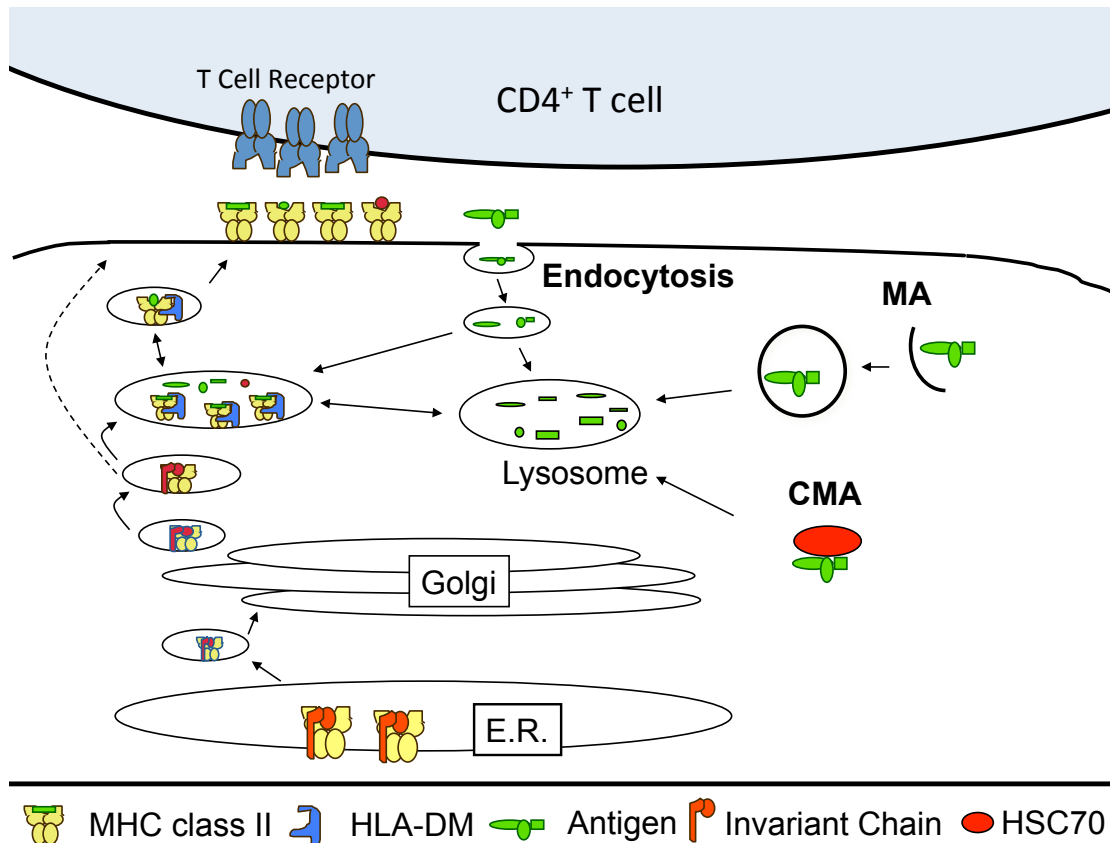
Figure 1. MHC class II antigen presentation pathway. Exogenous Ags are internalized via clathrin-mediated endocytosis and delivered to the endosomal/lysosomal network. Endogenous Ags can be delivered by autophagy pathways such as macroautophagy (MA) and chaperone-mediated autophagy (CMA). Once delivered into the endosomal/lysosomal network, acidic proteases within these compartments process the Ag into small peptides. MHC class II molecules assemble and associate with the chaperone invariant chain in the ER. These complexes are sorted through the Golgi and into the endosomal/lysosomal network. Lysosomal acidic proteases sequentially cleave invariant chain, leaving the invariant chain fragment CLIP in the MHC class II peptide binding groove. HLA-DM catalyzes the removal of CLIP and MHC class II binding of antigenic peptides. MHC class II:peptide complexes are shuttled to the surface to be displayed for recognition by CD4$^+$ T cells

Classically, MHC class II molecules present exogenous Ags that have been

engulfed and processed through the endosomal network (*4*). Within the

endosomal network pH dependent proteases, including cathepsins, play critical

roles in Ag processing and the formation of short (9-25 amino acids) peptides capable of binding MHC class II molecules (*5*). MHC class II molecules can also present peptides derived from self-proteins that gain access to the lysosomal network. Membrane proteins can gain access to endosomes and lysosomes while being shuttled to the surface or when endocytosed. While a majority of the peptides presented by MHC class II molecules follow this classical pathway, roughly 10-30% of the peptides bound to MHC class II are derived from nuclear or cytoplasmic Ags, including viral and tumor Ags (*4, 6-8*). Cytoplasmic and nuclear Ags gain access to the endosomal network through autophagy pathways including the macroautophagy (MA) and chaperone-mediated autophagy (CMA) pathways (**Figure 1**) (*9*). These Ags may be processed by cytoplasmic enzymes such as the proteasome and calpains as well as by enzymes within endosomal compartments (*10, 11*). Activation of CD4^{+} T cells in response to MHC class II presentation of membrane, cytoplasmic and nuclear Ags results in effective immune responses to intracellular pathogens, tumors and the development of autoimmune diseases.

Key players in MHC class II antigen presentation

Invariant chain

MHC class II molecules assemble in the ER where they bind invariant (Ii) chain (*12*). Ii chain stabilizes the MHC class II heterodimer, prevents premature loading of peptides in the ER, and directs the trafficking of MHC class II molecules through the Golgi and into the endosomal network (*13-15*). Ii chain is

systematically cleaved by acidic proteases generating degradative intermediates LIP, SLIP and CLIP: the latter fragment of Ii chain is bound to the MHC class II binding groove (**Figure 2**) (*12, 16-21*). The first cleavage of Ii is mediated by aspartyl cathepsin proteases to yield the fragment known as LIP. After the intial cleavage, cathepsins S and L mediate the final two cleavages yielding the fragments SLIP and finally CLIP (*21, 22*).
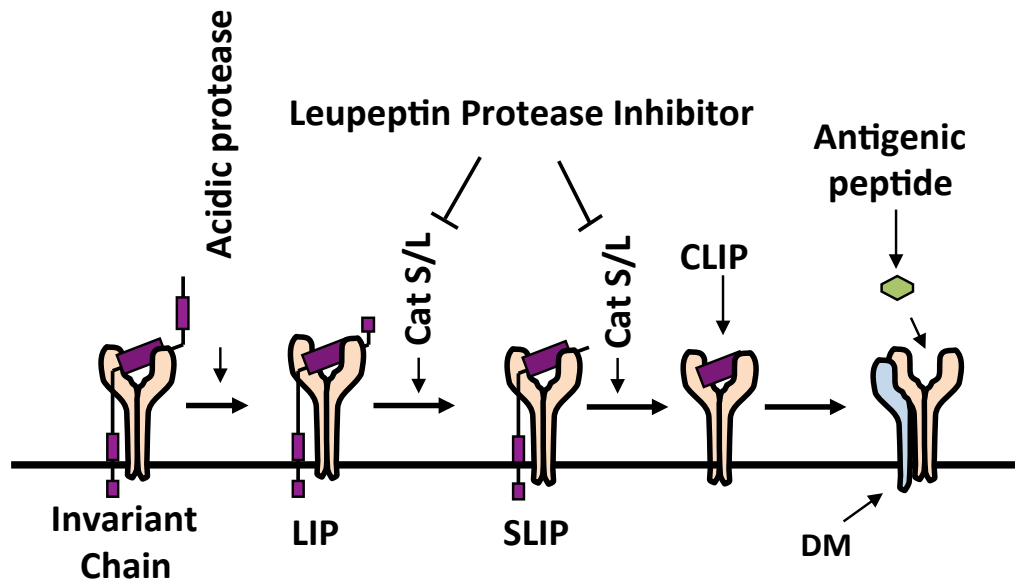
Figure 2. Invariant chain degradation.
Ii chain is sequentially degraded by acidic proteases upon its delivery with MHC class II to the endosomal/lysosomal network. The degradative intermediates LIP (22 kDa) and SLIP (10 kDa) accumulate upon inhibition of cathepsin (Cat) S and L activity by the pharmacological leupeptin allowing detection of these fragments by immunoblot analysis (J. Lich, 2000).

Ii chain is critical for MHC class II presentation of both exogenous and endogenous Ag. The level of Ii chain expression is inversely correlated with the amount of exogenous Ag required to activate T cells (*23*). Furthermore, APCs from Ii chain knockout mice have a reduced capacity to present exogenous Ag to

T cells (*24*). In contrast, MHC class II presentation of endogenous Ags is enhanced by low Ii chain levels. For example, the cytosolic influenza virus matrix protein was presented by MHC class II molecules in Ii chain deficient cells, but inhibited in cells expressing Ii chain (*25*). These studies as well as studies in mice show alterations in Ii chain expression can modify the MHC class II Ag presentation pathway (*26*).

HLA-DM and -DO

HLA-DM (DM), a nonclassical MHC class II protein, catalyzes the removal of the Ii chain fragment CLIP and further edits peptides that bind MHC class II favoring association with high affinity peptides within the ligand binding groove (*19, 27, 28*). DM is a non-polymorphic heterodimer composed of DMα and DMβ (*29*). DM localizes to late endosomes where it associates with MHC class II molecules to remove the CLIP fragment of Ii chain or other low affinity, loosely bound peptides (*30*). As DM is essential in removal of CLIP fragment, the majority of surface MHC class II on APCs from DM-deficient mice are loaded with CLIP (*31*).

B cells and to a lesser extent dendritic cells, express another nonclassical class II protein, HLA-DO (DO). DO also consists of a heterodimer, composed of DOα and DOβ and resides only in endosomal compartments. DO has been shown to inhibit the function of DM, though there has been some controversy regarding this observation with alternate claims that DO enhanced DM function (*32-34*). Recent studies suggest DO binds in a competitive fashion to the same region of

DM as MHC class II molecules (*35*). Initial studies on DO indicated the interaction between DO and DM may be pH dependent with dissociation at low pH, however a recent structural analysis argues against this pH dependence in DM and DO association (*36, 37*).

Lysosomal proteases

Proteases found within lysosomes and endosomes are critical components of the MHC class II pathway. These proteases are required for Ii chain processing as well as degradation of protein Ags into smaller peptides that can be loaded in the ligand binding groove of MHC class II molecules. Cathepsins are a family of acidic proteases within lysosomes and endosomes that have been shown to play critical roles in the MHC class II pathway. Cathepsins B, C, D, X, H, L, and S as well as asparagine endopeptidase (AEP) have been shown to be involved in immune responses (*38*). Several proteases are involved in Ii chain processing including AEP and cathepsin L, S, H, and F (*39-41*). Furthermore, several cathepsins including cathepsin B and D have been shown to play a role in Ag degradation (*42*).

Cathepsin S and L are cysteine endopeptidases exhibiting important non-redundant roles in Ag presentation (*43, 44*). Cathepsin S has been shown to play a critical role in B cells, as well as dendritic cells, where this enzyme is required for complete proteolysis of Ii chain affecting MHC class II trafficking and peptide loading (*21, 42*). Cells deficient in cathepsin S have a reduced capacity to

present certain epitopes suggesting this enzyme is vital in the processing of specific Ags (*5, 43*). Cathepsin S preferentially cleaves peptides N-terminal to Val-Val-Arg motifs, while cathepsin L prefers a very similar motif, hydrophobic/aromatic amino acid-Phe-Arg. Cathepsin L is expressed in thymic epithelial cells and to a lesser extent in professional APCs. In thymic epithelial cells, cathepsin L is responsible for Ii chain degradation, with loss of cathepsin L in these cells disrupting positive selection of T cells in the thymus (*45*). Similar to cathepsin S, cathepsin L-deficient cells exhibit reduced presentation of antigenic epitopes suggesting that individual cathepsins can shape Ag processing and host T cell responses (*5*). Cathepsin B is another cysteine peptidase with carboxypeptidase as well as endopeptidase activity (*46*). Like cathepsins S and L, cathepsin B is involved in MHC class II Ag presentation, influencing both Ii chain degradation and Ag processing (*11, 47, 48*). Inhibiting cathepsin B activity in B cells impairs MHC class II presentation of epitopes derived from endogenous and exogenous Ags (*11*). In some cases these proteases appear to have redundant roles in Ag processing (*49, 50*).

Cytosolic proteases

Cytosolic Ags can be processed by proteases within the cytoplasm prior to transport into MHC class II rich lysosomal/endosomal compartments. The proteasome as well as cytosolic calpains have been shown to play a critical role in the processing of cytosolic Ags for MHC class II presentation (*11, 51*).

The proteasome contains multiple catalytic subunits: the 20S core as well as the

19S and PA28 subunits, which together form the 26S proteasome (*52*). The

proteasome mediates five distinct cleavages: chymotrypsin-like activity where the

enzymatic subunits cleave on the carboxylic side of large hydrophobic residues,

trypsin-like activity in which the enzymes cleave at or near basic residues, and

additional specificities for cleavage sites around acidic, branched and small

neutral amino acids (*53*).

Protein substrates can be preferentially targeted for proteasomal degradation

following their ubiquitination. Ubiquitination generally occurs at specifc lysine

residues within a protein and can impact a protein's fate. Addition of subsequent

ubiquitins on lysine residues within a ubiquitin adduct, leads to the formation of

extended polyubiquitinated chains. Chains of more than 4 ubiquitins, branching

from lysine 48 (K48) of the initial ubiquitin protein conjugate, targets proteins for

destruction by the proteasome (*54*). However, some proteins including

ovalbumin, are targeted to the proteasome in an ubiquitin-independent manner

(*55*). In these latter cases, the mechanisms that regulate the degradation of

these proteins are not well understood.

B cell receptor

B cells are unique in their expression of immunoglobulin (Ig) molecules. Ig is

composed of two heavy and two light chains. There are two loci that express Ig

light chain, Ig λ and Ig κ (*56*). During B cell development, the Ig genes undergo

rearrangement that leads to the expression of one heavy chain rearrangement and one light chain rearrangement that will pair to form the unique Ig protein (*57*). RNA splicing regulates whether Ig is secreted in the form of Ab or membrane-bound (*58, 59*). When membrane-bound, Ig associates with signaling proteins including Igα and Igβ; this is termed the B cell-receptor (BCR) (**Figure 3**) (*60*).
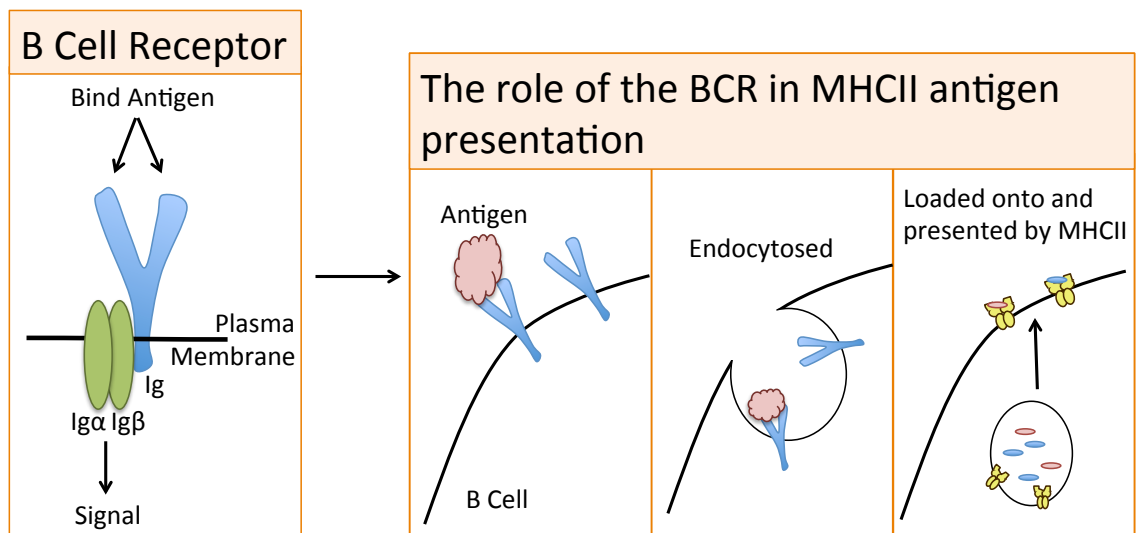
Figure 3. The B cell receptor (BCR). B cells express immunoglobulin (Ig) which is composed of two heavy and two light chains that fold in the ER. In its membrane bound form, Ig associates with signaling molecules Igα and Igβ on the plasma membrane forming the B Cell Receptor (BCR). Surface BCR, alone or complexed with antigen, is constitutively internalized into endosomes. Here, cathepsins digest the BCR and antigens yielding peptides for presentation by MHC class II molecules.

The BCR acts as a signaling molecule as well as a receptor to internalize exogenous Ags. When bound and crosslinked by Ag, the BCR associated Igα and Igβ subunits transmit signals to activate the B cell (*61*). Surface BCR, whether bound to Ag or not, is constitutively internalized and delivered into endosomes and lysosomes for proteolytic processing by cathepsins to yield

peptides for presentation by MHC class II molecules (*62-65*). Trafficking of the BCR:Ag complex to endosomal compartments promotes MHC class II Ag presentation enhancing B and T cell interactions required for antibody (Ab) class switching and affinity maturation, critical aspects of humoral immunity (*66, 67*).

Pathways of antigen delivery

The exact mechanisms that lead to Ag presentation are not fully understood. While classically in this field, MHC class II molecules were thought to only present epitopes derived from membrane and exogenous sources that are internalized via endocytosis, our laboratory as well as others have shown MHC class II molecules present epitopes derived from cytoplasmic and nuclear sources that gain access to the endosomal/lysosomal network (*4, 11, 51, 68-70*). Peptide elution from mouse APCs have shown 20-30% of peptides bound to MHC class II molecules are derived from endogenous cytoplasmic sources such as actin, tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*6*). Peptide elution from MHC class II molecules from human B cells revealed greater than 85% of peptides were derived from endogenous sources the majority being from plasma and endosomal membrane proteins (*7*). These studies reveal that Ags utilize several pathways to encounter MHC class II molecules including endocytosis and autophagy pathways.

Endocytosis

Ags can be internalized via several endocytic pathways including phagocytosis, receptor-mediated endocytosis and macropinocytosis, an actin dependent mechanism to engulf exogenous material. While phagocytosis and macropinocytosis drive the engulfment of large particulates and pathogens, receptor-mediated endocytosis facilitates the internalization of macromolecules (*71, 72*). Clathrin-mediated endocytosis is the process by which cell surface receptor-ligand complexes, such as Ag:BCR, are internalized (*73*). Clathrin-mediated internalization of Ag via the BCR enhances Ag potency by as much as 10^4 fold (*74*). During clathrin-mediated endocytosis, adaptor proteins, including AP2, associate with membrane signaling molecules at the plasma membrane. These adaptor proteins then associate with clathrin which forms the main coat directing membrane invagination to give rise to clathrin-coated vesicles containing receptors, MHC molecules and Ags. More than 60 proteins are involved in clathrin-mediated endocytosis, including HSC70 (*75*). HSC70 is involved in chaperoning clathrin, not only priming it for vesicle formation but also uncoating clathrin from vesicles as these mature into early endosomes (*76*).

Autophagy pathways

Autophagy is the process by which cells degrade unnecessary or dysfunctional cytoplasmic and nuclear components, essentially the process of "eating" oneself. Low levels of autophagy are detected in various cell types including B cells and dendritic cells (*77, 78*). When cells encounter different types of stress, they can

respond by altering the autophagy pathways to promote survival and alleviate the stress (*79-89*). These pathways help cells stabilize during times of stress by degrading modified proteins, eliminating pathogens, and removing toxic products. There are three types of known autophagy in mammalian cells: macroautophagy (MA), chaperone-mediated autophagy (CMA), and microautophagy. MA and CMA have been implicated in delivery of cytoplasmic Ags to the lysosomal network for processing and MHC class II presentation (*77, 78, 90*).

During MA, a crescent shaped membrane is formed in the cytoplasm which can engulf some organelles, large portions of the cytoplasm, or intracellular pathogens during infection. The autophagosome then matures as it fuses with lysosomes and endosomes. Several protein complexes are required for MA, including Ulk1/2 complex, Beclin complex, ATG12/5 conjugation system, and the LC3 conjugation system (*91*). During MA, the LC3 conjugation system converts LC3I to LC3II by addition of the lipid phosphatidylethanolamine, leading to LC3II accumulation on the inside and outside of the autophagosome membranes (*92*). Acidification of autophagosomes by fusion to lysosomes leads to the degradation of internalized cargo protein as well as LC3II localized to the inside of the autophagosome membrane (*92*). While MA is generally thought of as a bulk form of degradation, specific targeting of cytoplasmic proteins to autophagomes in a form of selective autophagy has been demonstrated. The MA receptor p62 has been implicated in this process as it binds ubquitinated targets and links them to the LC3II protein on autophagosome membranes (*93*). While

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polyubiquitin chains branching from the ubiquitin lysine 48 are targeted for proteasomal degradation, chains branching from lysine 63 (K63) of ubiquitin are targeted for p62 recognition and degradation via MA (*94*). Interestingly, HSC70 is known to associate with protein aggregates, inducing ubiquitination of these substrates and targeting them for recognition by p62 (*95*).

While MA can lead to bulk degradation of cytoplasmic proteins, CMA results in the degradation of specific target proteins. In CMA, the cytoplasmic chaperone, HSC70, binds to a pentapeptide (KFERQ-like) motif within proteins, guiding the protein to the lysosomal membrane (*96*). Given HSC70 functions in several different pathways for protein sorting, additional chaperones may be involved in CMA to promote client protein:HSC70 trafficking to lysosomes. Consistent with this, HSP90 has also been shown to be required for CMA (*97, 98*). On the lysosomal membrane a transmembrane protein, LAMP2A, associates with HSC70 and its client protein. By a process not well understood, the client protein is unfolded and translocated into the lysosome for degradation (*98, 99*).

Heat shock chaperones

The heat shock protein (HSP) family is a large group of proteins that function as chaperones to assist in protein folding and transport within cells. The HSPs were given their name based on the induction of many of these chaperones by heat or cell stress. While some of these chaperones can be induced, many are

expressed constitutively, indicating these proteins play important roles within the cell even under low or no stress conditions (*100*).

The HSPs are divided into smaller families based on their molecular weight. Two such protein families are the HSP90 family with molecular weights of 90 kDa and the HSP70 family with molecular weights of 70 kDa (*101*). HSC70, sometimes referred to as HSC73, is a constitutively expressed cytosolic member of the HSP70 family which constitutes approximately 1% of the total protein within a cell (*102*). HSP90 is another cytosolic constitutively expressed HSP that belongs to the HSP90 family. Unlike HSC70 and many other HSP chaperones, HSP90 is not involved in protein folding, but rather appears to regulate signal transduction molecules and protein transport (*103*). Our laboratory has shown both HSC70 and HSP90 are involved in MHC class II presentation of Ags. While experiments have shown addition of exogenous HSP90 binds proteins and assists in the presentation of extracellular Ags, intracellular HSP90 and HSC70 have been implemented in MHC class II presentation of endogenous cytoplasmic Ags (*11, 97*).

Chaperone HSC70

HSC70 is an abundant intracellular chaperone that has numerous roles within cells (*102*). HSC70 is constitutively expressed and is marginally induced by heat shock or other forms of cell stress (*104*). HSC70 functions as an ATPase, binding client proteins dependent on whether ADP or ATP is bound (*102*). As a

chaperone, HSC70 binds to hydrophobic and basic amino acids with a KFERQ-like motif to prevent the unfolding and aggregation of proteins thereby facilitating numerous cellular processes (*105-107*). Unexpectedly, HSC70 is found both in the lumen of the lysosome as well as in the cytoplasm. Given the lack of a known signal sequence for membrane translocation, the mechanism by which HSC70 crosses into the lysosome remains poorly defined.

HSC70 plays an important role in several Ag trafficking pathways. A requirement for the ATPase activity of HSC70 was established for clathrin-mediated endocytosis (**Figure 4**) (*108-110*). Furthermore, cytoplasmic HSC70 has been implicated in the targeting of proteins to the lysosome for CMA, while lumenal HSC70 may function in ratcheting or pulling proteins across the lysosomal membrane through a pore formed by LAMP2A (**Figure 4**) (*106, 111, 112*). This multifaceted protein, HSC70, also guides specific proteins to the proteasome-ubiquitin pathway (*113, 114*). Mirroring its association with lysosomal membranes, HSC70 associates with autophagosome membranes (**Figure 4**) (*115-117*). The purpose or cause of this association has not been well defined though it may be related to the role of HSC70 in selective autophagy.
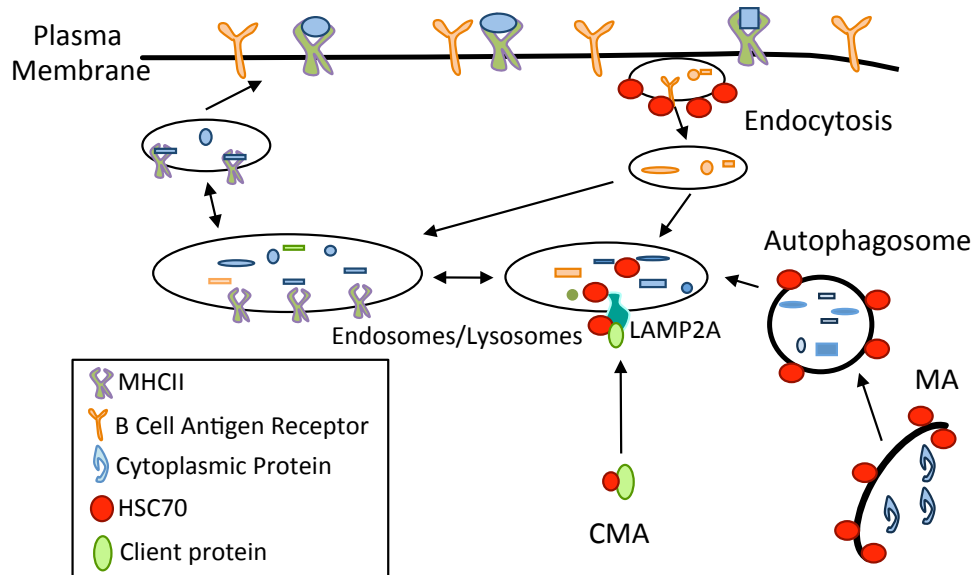
Figure 4. HSC70 plays a critical role in several pathways important for antigen trafficking and presentation. HSC70 is required for clathrin-mediated endocytosis, as the ATPase activity of HSC70 is required for coating and uncoating of vesicles. HSC70 is a key component of CMA, binding to client proteins and guiding them to the lysosomal membrane for interactions with LAMP2A. Furthermore, HSC70 in the lumen of the lysosome is required for translocation of these peptides. While association of HSC70 with autophagsome membranes has been detected, the function of this has not been established though it may be related to selective-autophagy. During selective-autophagy, HSC70 and its co-chaperone BAG3 guide client proteins to p62 and LC3, leading to their degradation via MA.

The exact mechanisms which guide the specificity and the function of HSC70 in these cellular processes remain unknown. While a role for HSC70 association with client proteins with a KFERQ-like motif is important for CMA, whether this specificity is important in other functions of HSC70 is untested. Within cells, HSC70 associates with several cofactors capable of modulating its activity (*118, 119*). Regulation of HSC70 by these cofactors is thought to occur by altering HSC70's intrinsic ATPase activity (*119, 120*). Most of the cytoplasmic cofactors

associate with the carboxy-terminus of HSC70 (*119*). Among the most well studied cofactors for HSC70 is BAG-1. BAG-1 binds the ATPase domain of HSC70 to influence ATPase activity (*121, 122*). With an ubiquitin-like domain, BAG-1 may help recruit HSC70 to the proteasome-ubiquitin pathway (*118*). Furthermore, another BAG family co-chaperone, BAG-3, has been implicated in the association of HSC70 with protein aggregates and the targeting of these aggregates to p62 and selective autophagy (*95*). Two other co-factors known to regulate HSC70, Hip and HSP40, also regulate ATPase activity (*123, 124*). HOP is another HSC70 cofactor. HOP is known to link HSC70 to the highly conserved heat shock protein HSP90, forming a multi-protein complex (*125*). While individual co-chaperones have been implicated in specific functions, overlap occurs complicating assessment of HSC70 regulation. For example, studies have indicated BAG-1 guides client protein:HSC70 complexes to the proteasome, while other studies have revealed BAG-1 associates with LAMP2A, indicating a role for this co-factor in CMA (*126, 127*).

As HSC70 is a critical component of several protein trafficking pathways, its role in MHC class II presentation may not be surprising (Figure 4)(*128-130*). Studies from our laboratory have revealed MHC class II presentation of cytoplasmic autoantigens via CMA is dependent on HSC70 (*78*). Furthermore, within the lumen of the lysosome HSC70 protects peptides from degradation by binding and delivering them to MHC class II molecules for presentation (*131*).

Autoimmunity

In order to defend the body from foreign invaders, it is necessary for the immune system to distinguish self from non-self. The immune system has several mechanisms to facilitate this recognition, however sometimes these processes fail leading the immune system to attack the body in a process called autoimmunity. The exact pathways that lead to the loss of tolerance and the development of autoimmune disease remain elusive, though several factors including genetics and the environment likely contribute (*132*).

Glutamic acid decarboxylase 65 (GAD) is a key autoantigen in type-1 diabetes, though its role in disease progression is not well understood (*133*). GAD is expressed in pancreatic beta cells and can associate with granules where insulin is stored, though the role of this enzyme within these cells has not been determined (*133*). Autoantibodies recognizing the self protein GAD are identified in 85% of newly diagnosed type-1 diabetes patients and studies have identified specific epitopes within the GAD protein can activate T lymphocytes (*134, 135*). It remains unclear how immune cells gain access to GAD as it is a cytoplasmic protein that associates with the luminal face of intracellular membranes, in part due to a pamitoylation of its amino-terminal domain (*136*). Studies within our laboratory have revealed B cells transduced to produce cytoplasmic GAD can display its epitopes on MHC class II molecules to activate T cells. Studies have further revealed that cytoplasmic GAD protein is processed by the chymotrypsin-like activity of the proteasome and associates with HSP90 and HSC70 (*78, 97,*

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137). HSC70 guides GAD through the CMA pathway, requiring LAMP2A for translocation through the lysosomal membrane. Furthermore, efficient presentation of GAD requires processing within the endosomal network by cathepsins (**Figure 5**) (*11, 28*).
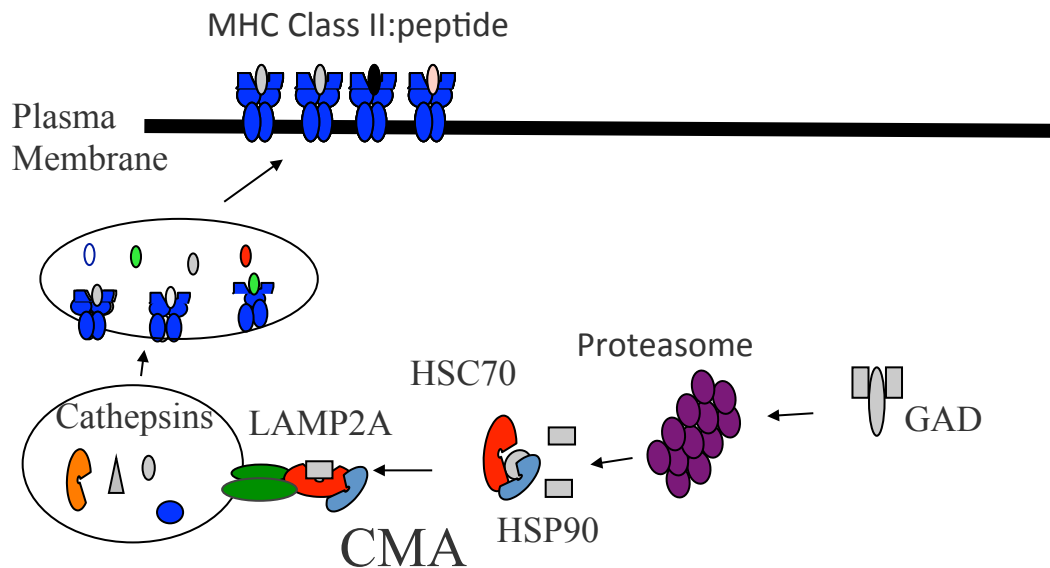
Figure 5. MHC class II presentation of the model autoantigen GAD. Our laboratory discovered that B cells expressing cytoplasmic GAD present epitopes derived from this Ag to activate T cells. Our laboratory has shown GAD is processed in the cytoplasm by the proteasome, associates with HSC70 and HSP90, and is translocated by CMA into lysosomes. Within lysosomes GAD is further processed by cathepsins before loading onto MHC class II molecules for presentation.

Protein malnutrition and immune response

Globally, protein malnutrition remains problematic, affecting 1 in 8 individuals. Protein malnutrition adversely affects the neuroendocrine, cardiac and hematopoietic systems. Although poorly understood, protein restriction severely disrupts host immunity and responses to infection. Nutrition has a profound

influence on host immunity as exemplified by hyper-inflammatory responses associated with high fat diets and connections between immune deficiency and malnutrition (*138, 139*). Furthermore, protein malnutrition has been linked to increased susceptibility to infection (*140*).

Studies have shown protein malnourished children have reduced Ab responses as well as impaired T-cell mediated immunity (*141*). Reduced B cell expansion in response to bacterial infections was also observed in protein malnourished children (*142*). Studies using mice have shown a low protein/high fat diet results in a reduction in circulating Ab, increased instances of autoimmune disease and reduction in life span (*143*). Furthermore, mice receiving low protein/high fat diet have increased susceptibility to influenza infection (*144*). Studies in human B lymphoblasts have shown amino acid starvation reduces cathepsin activity influencing the epitopes presented by MHC class II molecules, raising questions about the ability of B lymphocytes to process Ag during severe stress (*145*). Induction of high affinity, long-lasting humoral immunity is dependent upon interactions between B and T lymphocytes. Taken together these studies indicate protein malnutrition may impair B and T cell mediated immunity, suggesting protein malnutrition may affect MHC class II Ag presentation.

The effect of nutrient deprivation at the cellular level has been extensively studied. Cytoplasmic protein degradation is altered with increased degradation of nonessential proteins to provide nutrients for the cell through the induction of MA

(*146*). MA increases rapidly in cells deprived of amino acids, leading to ordered degradation of organelles, suggestive of a selectivity in degradation (*146, 147*). While MA is induced immediately upon nutrient stress, CMA has only been reported to be induced after 24 h of nutrient deprivation (*106*). Protein deprivation has also been shown to inhibit endocytosis of specific receptors, such as the transferrin receptor, on immune cells and impair the phagocytic capacity of macrophages (*148, 149*). While studies indicate proteasome activity is induced by serum deprivation, the effect of nutrient deprivation on lysosomal proteases is more controversial with some studies indicating increased activity and others suggesting inhibited activity (*145, 150, 151*). These data further suggest protein deprivation may alter the availability of Ags and capacity of MHC class II presentation by affecting Ag trafficking and degradation through these pathways.

Research summary

The adaptive immune response is severely disrupted in individuals who are malnourished. Furthermore, studies have suggested B and T cell interactions may be impaired in malnourished individuals. This led to the development of our hypothesis that protein deprivation alters MHC class II presentation in B cells, affecting B and T cell interactions. To assess this, media containing vitamins, amino acids and glucose but lacking serum, which contains numerous macronutrients, was used to culture cells. Studies assessed the ability of macronutrient deprived human B cell lines to present various model Ags including the BCR and GAD. The effect of macronutrient deprivation on key MHC

class II components was monitored. Studies were performed to identify the effect of macronutrient deprivation on Ag trafficking and degradation. Furthermore, alterations in Ag trafficking and proteolytic activity were assessed in macronutrient deprived primary peripheral blood human B cells. As serum is composed of numerous components, studies assessed the effect of various serum components on Ag trafficking. HSC70 plays an important role in several Ag trafficking pathways. Here, studies assessed the effect of macronutrient deprivation on the accessibility of this conserved chaperone. These studies reveal macronutrient sensing in B cells modulates Ag trafficking and presentation via accessibility of HSC70, offering a novel explanation for altered immune responses in protein malnourished individuals.

Materials and methods

Cell culture

The MHC class II DR4$^+$ (homozygous for DRA1*0101,DRB1*0401) B-lymphoblastoid cell line Priess was previously transduced using retroviral vectors to express wildtype human 65 kDa GAD, creating the PriessGAD (PG) cell line or with a vector coding the cDNA for a truncated form of GAD (CytoGAD) lacking the amino terminal domain provided by S. Baekkeskov UCSF (*152*). This N-terminal deleted form of GAD while localized in the cytoplasm fails to associate with membranes in contrast to the full length GAD protein (*136*). PG cells were stably transfected to express influenza A matrix protein 1 (MP1)-LC3 fusion protein by lentiviral vector transduction provided by C. Münz, University of Zurich (*77*). PG were stably transfected to express HSC70 using the pcDNAZeo$^{(-)}$ plasmid containing cDNA encoding human HSC70 (D. Zhou) (*78*). All B-lymphoblastoid cells were cultured in IMDM (Invitrogen) containing 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco BRL Life Technologies) in the presence or absence of 10% heat inactivated fetal calf serum (FCS) (Hyclone). Cell lines transfected or transduced for ectopic gene expression were cultured for short periods with appropriate drugs to maintain gene expression.

Several hybridoma T cell lines recognizing specific epitopes in the context of HLA-DR4 were used in this work: 2.18α is specific for an epitope encoded by the BCR (Ig $_{\kappa\ 88-203}$), 33.1 is specific for the GAD$_{273-285}$ epitope, 17.9 is specific for a peptide derived from human serum albumin (HSA$_{64-76}$), 7A2-1736 is specific for a

peptide derived from the influenza MP1 (D. Cannaday, Case Western), and 33.4 is specific for an epitope derived from MHC class I molecules; all were maintained in RPMI (Invitrogen) containing 10% fetal bovine serum (FBS) (Hyclone), 2 mM L-glutamine, 50 µM β-mercaptoethanol, 50 U/ml penicillin and 50 µg/ml streptomycin. The IL-2 dependent HT-2 T cell line was maintained in RPMI 1640 (Invitrogen) containing 10% FBS, 50 µM β-mercaptoethanol, 50 U/ml penicillin, 50 µg/ml streptomycin and 20% T-STIM^{TM} (BD Biosciences).

Human whole blood was collected by venipuncture into a Sodium Heparin Vacutainer^{TM} (BD). Institutional approval for human blood collection was obtained for this study. Blood was diluted 1:1 with Phosphate-Buffered Saline (PBS) prior to isolation of human peripheral blood mononuclear cells (PBMC) using a ficoll gradient. Primary B lymphocytes were isolated from fresh PBMC using CD19 Ab coated magnetic beads following manufacturer's directions (Milltinyi Biotech). Isolated human CD19$^+$ B cells were cultured in RPMI 1640, 50 U/ml penicillin and 50 µg/ml streptomycin, in the presence or absence of 10% FBS.

Antigen presentation assay

To examine the effect of serum deprivation on MHC class II presentation, PG cells were used as APCs and co-cultured with T cells. Activation of T cells by APCs was monitored by IL-2 secretion. A bioassay using HT-2 cells was employed to detect IL-2. PG cells were cultured in media with or without serum, or with serum-free media supplemented with 0.01% bovine serum albumin (BSA,

Sigma) for up to 48 h. After incubation, PG cells were harvested and fixed in 0.5% paraformaldehyde (PFA) for 8 min at room temperature and washed with media afterward. To detect exogenous HSA Ag presentation, PG cells were incubated with 20 μM HSA Ag for up to 12 h in media with or without serum prior to fixation. As a control to monitor surface MHC class II function without Ag processing, aldehyde-fixed cells were incubated with the appropriate synthetic peptide (10 μM) at 37^oC for 6 h and then washed in media to remove excess peptide. The synthetic peptides $GAD_{273-285}$ (IAFTSEHSHFSLK) or BCR (Igκ I $_{188-203}$ KHKVYACEVTHQGLSS) were used for these latter studies. After treatment with peptide or Ag, fixed PG cells were washed, seeded at $4*10^4$ cells per well in a 96 well plate and incubated with epitope-specific T cells at $2*10^4$ cells in a total volume of 200 μl for 16 h at 37^oC. T cell activation detected by IL-2 production was monitored using proliferation of the IL-2 dependent HT-2 cell line. HT-2 cells ($5*10^3$ cells per well) were incubated for 18 h at 37^oC with the culture media from APC-T cell co-cultures. [^{3}H]-thymidine (2 μCi/well, PerkinElmer) was used to monitor HT-2 proliferation in response to IL-2 levels. [^{3}H]-thymidine was added to the HT-2 cells followed by an additional 8 h of culture at 37^oC. After this incubation, HT-2 cells were harvested using a 96-well plate harvester (Scatron) and cellular incorporation of [^{3}H] -thymidine into DNA was measured by liquid scintillation counting (Wallac Microbeta). The average counts per minute (cpm) of the isotype by HT-2 cells was recorded and used as a measure of Ag presentation. Results from three or more independent experiments were

determined and averaged. To compare differences in Ag presentation with B cell treatment, the relative fold change between averaged data was graphed.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

B cells were cultured with or without serum, or in media supplemented with 1% BSA for up to 48 h and harvested. RNeasy mini kit and QIAshredder (Qiagen) were used to isolate total cellular RNA following the manufacturer's instructions. High-Capacity cDNA Reverse Transcription kit (Applied Bioscience) was used to generate cDNA. For analysis, TaqMan[TM] Gene Expression Assays (**Table 1**) and TaqMan[TM] Fast Universal PCR Master Mix (Applied Biosciences) were used. Here, we monitored mRNA expression of the DRα, DMβ and DOβ chains of these heterodimeric proteins because Ab regents exist to monitor the protein levels of these chains. The LAMP2 isoform primers were designed using the custom design Taqman interface (Lifescience Technologies). The following sequences were used for Forward (F), Reverse (R) and the Probe (P) to quantitate the mRNA of each isoform (**Table 2**). Samples were amplified by qRT-PCR using the 7500 Real-Time PCR System (Applied Biosystems) for 40 cycles with the following parameters: 95˚C, 15 sec; and 60˚C, 1 m. GAPDH was used as the endogenous cellular mRNA reference and samples were normalized to the serum treated (Cont.) control sample.

Gene	Assay product number
GAD	Hs00609534_m1
Ig κ	Hs01692073_s1
DRα	Hs00219575_m1
DMβ	Hs00157943_m1
DOβ	Hs00157950_m1
Ii	Hs00269961_m1
AEP (LGMN)	Hs00271599_m1
Cathepsin (CAT) A	Hs00264902_m1
CATB	Hs00947433_m1
CATS	Hs00175407_m1
TFEB	Hs00292981_m1
HSC70 (HSPA8)	Hs00852842_m1
HSP90α	Hs00743767_sH
HSP90β	Hs01546478_g1
GAPDH	Hs02758991_m1

Table 1. Taqman gene expression assay product numbers and gene list.

Gene	Primer Set Sequences
LAMP2A	F- AAC TTC CTT GTG CCC ATA GC
	R- AGC ATG ATG GTG CTT GAG AC
	P- CTC CTG CCA AGG CAG CTC CC
LAMP2B	F- AGA GTG TTC GCT GGA TGA TG
	R- TGC CAA TTA CGT AAG CAA TCA
	P- TCA AGC CTG AAA GAC CAG CAC CA
LAMP2C	F- AAG GGT TCA GCC TTT CAA TG
	R- ACA ATT ATA AGG AAG CCC AAG G
	P- CCA CAC CCA CTG CAA CAG GAA TAA GA

Table 2. Primer sequences for LAMP2 isoform Taqman assays. Listed here are the forward (F), reverse (R) and probe (P) sequences for each LAMP2 isoform Taqman probe designed by L. Perez.

Antibodies

The following Abs were used for immunoblots, immunoprecipitations, ELISAs

and flow cytometry assays to determine the effects of serum deprivation on MHC

class II Ag presentation. To detect the MHC class II pathway components by immunoblot, the monoclonal mouse Ab Pin1.1 was used to monitor Ii chain and its fragments and DA6.147 (P. Cresswell, Yale) was used to monitor DRα. For flow cytometry, DR was monitored using a pre-conjugated DR-FITC Ab (BD Pharmingen), surface CLIP:MHC class II complexes were monitored using the pre-conjugated Cer-CLIP-FITC Ab (BD Pharmingen), DM was monitored using the pre-conjugated DM-PE Ab (BD Pharmingen), BCR was detected using the pre-conjugated Ig $_\kappa$-PE Ab (Biosource), CD45R was detected using the pre-conjugated CD45R-AF647 (eBioscience), TrF was detected using the mouse monoclonal B3/25 Ab with the anti mouse Ig-PE secondary (Dako) and DO was monitored using the mouse monoclonal DOβ clone DOB.L1 (Santa Cruz) with the anti mouse Ig-PE secondary. Other Ags detected on immunoblots include: LC3, monitored by the rabbit LC3b Ab (Cell Signaling); GAD, detected using the rabbit Ab to GAD65/67 (Sigma); HSC70, detected using a rat Ab to HSC70 (Stressgen); BCR, detected using a mouse anti-IgG Ab (Jackson ImmunoResearch); MP1, detected using a mouse monoclonal Ab (AbD Serotech); and GAPDH, monitored using the mouse Ab to GAPDH (Millipore). HRP-conjugated secondary Abs recognizing mouse, rabbit or rat were used to detect the above primary Abs on immunoblots (Jackson ImmunoResearch). GAD Ag was immunoprecipitated using the GAD6 Ab (Sigma). For ELISAs, the BCR was detected using the mouse anti-IgG Ab (Jackson ImmunoResearch), MHC class II was detected using the mouse anti-MHC class II 37.1 Ab (L. Wicker), and

transferrin receptor (TrF) was detected using the mouse monoclonal B3/25 Ab (Ian Trowbridge).

Flow cytometry

Human B lymphoblasts or peripheral blood isolated B lymphocytes were cultured in vitro with or without serum or in media supplemented with BSA. These cells were then washed in FACS buffer and fixed with 0.5% PFA for 8 min at room temperature. Cells were further washed in FACS buffer and stained with primary Abs for surface markers for 60 min on ice. For intracellular staining, after fixation cells were permeabilized in 0.1% saponin in FACS buffer for 20 min at room temperature, washed, and blocked with normal rabbit serum (1:1000 dilution) for an additional 10 min at room temperature prior to Ab staining for intracellular proteins for 60 min on ice. Cells were washed in FACS buffer prior to analysis.

Immunoblots

PG cells were harvested and lysed in lysis buffer with 1% Triton-X pH 7.4 (10mM Tris Base, 150mM NaCl, 1% protease inhibitors cocktail (Sigma)) on ice for 10-15 min. The resulting cell lysates were centrifuged at 14,000 rpm to pellet cell debri and nuclei. Protein concentration of cell lysates was determined using the BioRAD protein assay with BSA used as a protein standard. For gel electrophoresis, 50-100 µg of protein from cell lysates were mixed with reducing or non-reducing SDS sample buffer. The reducing SDS sample buffer containing β-mercaptoethanol was used in all cases other than for the detection of invariant

chain and MHC class II:GAD peptide complexes. SDS-PAGE was used to resolve the proteins in cell lysates, followed by the transfer of proteins to nitrocellulose membranes (GE Healthcare). These membranes were blocked using Blotto (5% powdered milk) overnight at $4^{o}C$ prior to membrane incubation with primary Abs. Abs were typically diluted as recommended by the manufacturer. HRP-conjugated secondary Abs (Jackson ImmunoResearch) were used to detect primary Abs using enhanced chemiluminescence (Millipore) to visualize specific protein bands. Densitometry was carried out using ImageJ (NIH) to quantitate protein abundance. Protein levels (band density) were normalized relative to the band density of the same protein detected in serum treated control (Cont.). Results were graphed as the fold change of test samples relative to the serum treated control samples.

Treatment of B cells with pharmaceuticals

Protein turnover was monitored using cycloheximide (CHX) to inhibit protein synthesis. CHX was dissolved in sterile water. PG cells were grown in 10 µg/ml cycloheximide with or without serum for up to 12 h. Cells were harvested and lysed for immunoblot analysis. ImageJ was used to quantitate band density and sample band density was normalized to that of control samples cultured with serum.

To track degradation of the MHC class II chaperone protein Ii chain, cells were cultured with the cysteine protease inhibitor leupeptin. Leupeptin (Sigma) was

solubilized in PBS and cells were treated with this agent at a concentration of 500 μM for the times indicated at 37°C.

Amiloride (Torcris), a sodium channel inhibitor which disrupts macropinocytosis as well as endocytosis, was dissolved in IMDM medium (*153*). Cells cultured in medium supplemented with BSA or as a control cultured with serum, were treated with 3 mM amiloride for 12 h at 37°C prior to flow analysis of surface levels of the BCR.

While serum deprivation is known to induce MA, pharmaceuticals as well as infection by certain microbes can also induce MA. To examine the effect of the induction of MA on Ag presentation, PG cells were treated with the drug Torin1. Torin1, an inhibitor of mTOR leading to the activation of MA, was dissolved in DMSO (*154*). PG cells were cultured in media containing various concentrations of Torin1 for 16 h prior to fixation for Ag presentation assays and flow cytometry.

Infection of B cells

To examine the effect of microbe induction of MA on MHC class II Ag presentation, B cells were infected with *Toxoplasma gondii*. *T. gondii* is an obligate intracellular pathogen, known to induce MA (*155*). PG cells were infected with increasing multiplicities of infection (MOIs) at a density of 5*10^5 cells per mL and incubated overnight at 37°C prior to fixation for Ag presentation assays, immunofluorescence and flow cytometry. Infection was verified using

immunofluorescence. B cells were infected with a strain of *T. gondii* expressing a FITC tagged version of the parasite membrane protein TgSAG1 and the infection was halted by fixing cells with 4% PFA prior to DAPI staining.

Macroautophagic flux

Upon maturation and acidification of autophagosomes, the LC3II protein within these vesicles is degraded by acidic cathepsin proteases. To prevent this rapid proteolysis of LC3II, cells were treated with choloroquine (CQ), which neutralizes acidic organelles and slows proteolysis in these vesicles. PG cells were cultured with or without serum, and treated with and without 50 μg/ml CQ for up to 48 h, then harvested and lysed to detect LC3II levels via immunoblot analysis. The relative increase in cellular LC3II levels with addition of CQ is a measure of MA flux. Densitometry using ImageJ (NIH) was used to quantitate cellular protein levels. In these studies MA flux was defined as the basal cellular levels of LC3II/GAPDH subtracted from LC3II/GAPDH levels accumulating with CQ treatment (*92*). This number was normalized to serum treated cells and the relative fold change in MA flux was graphed.

Protease assays

The following assay to measure cathepsin activity in cell lysates was adapted from a protocol published by Kirshke and Wiedernanders (*156*). PG cells ($2*10^7$ cells per sample) were cultured with or without serum for up to 12 h and resuspended in 0.5% Triton-X buffer pH 6.5 (20 mM Hepes, with HCL). Cells

were then lysed using the Dounce homogenizer for 15 strokes on ice. Cell homogenates were centrifuged at 1000 rpm for 5 minutes at 4°C to remove nuclei. To assess cathepsin (Cat) activity, the cleavage of specific substrates, including ZArgArgNMec for Cat B, ZPheArgNMec for Cat L, and ArgNMecHCl for Cat H (Sigma) was monitored using fluorometry. Cell homogenates were mixed with an activator buffer (specific for each cathepsin) along with the substrate (20 µM) for either Cat L, Cat B, or Cat H. These reaction mixtures were incubated at 37°C for 60 min. After the incubation, a buffer to stop the reaction (100 mM sodium monochloroacetate, 30 mM sodium acetate, 70 mM Acetic Acid, pH 4.3) was added to the mixture. Fluorometry with excitation at 360 nm and emission at 460 nm was used to monitor the relative cathepsin activity in samples. Cathepsin activity of Cont. cells cultured with serum was normalized and set to 1. The fold change in the test samples cultured without serum was determined relative to the serum treated control samples and graphed.

For real time analysis of cathepsin activity in live cells, Magic Red^{TM} Cathepsin B and L Kits (Immunochemistry Technologies) were used according to a method previously published (*157*). B cells were treated with 6 µM protease substrates during the final hour of a 6 or 12 h incubation with or without serum, or in serum free media supplemented with BSA. Cells were harvested and washed in FACS buffer prior to fixation at room temperature in 0.5% PFA for 8 min. Flow cytometric analysis was used to detect the increased fluorescence with substrate cleavage. The average mean fluorescence of samples from three independent

experiments for control cells treated with serum was determined and the fold change relative to this average was graphed.

The Proteasome-Glo^{TM} Chymotrypsin-like Cell-Based Assay (Promega) was used according to the manufacturer's instruction to assess proteasome activity in control cells cultured with serum and after 6 or 12 h of serum deprivation (*158*). The average proteasome activity from three independent experiments for control cells treated with serum was determined and the fold change for samples cultured without serum was determined relative to this average and graphed.

Endocytosis assays and endosomal acidification

Cellular uptake of 70 kDa dextran conjugated to tetramethylrhodamine (Invitrogen) was used to monitor fluid phase endocytosis (*159*). PG cells were incubated for 6 or 12 h with or without serum in the presence of 100 µg/mL dextran. Cells were harvested and fixed using 0.5% PFA prior to analysis by flow cytometry. The mean fluorescence intensity (MFI) for each sample was normalized to the serum feed control sample for each time point, allowing calculation of the fold change relative to the serum control which was graphed in this analysis.

To assess endosomal acidification, cellular uptake of FITC-rhodamine-dual conjugated dextrans (Invitrogen) was assessed. Cells were treated with the dual labeled dextran under conditions similar to the endocytosis assay. While the

rhodamine fluorophore is resistant to pH, the fluorescence of the FITC fluorophore is diminished upon reaching acidic endosomal compartments (*160*). The average ratio of the FITC:rhodamine MFI was determined by flow cytometry in three independent studies. In each analysis the fold change in the FITC:rhodamine ratio was determined for treated cells relative to that of the Cont. cells cultured with serum.

Endocytosis of cell surface proteins and receptors such as BCR, MHC class II, and TrF was monitored using a method previously published by our laboratory (*161*). Cell surface proteins were biotinylated using the reagent EZ-link Sulfo-NHS-SS-Biotin (Thermo, 21328), which modifies the lysines in proteins and can be released by disulfide reduction. Biotinylated cells were incubated for 30 min at 37°C in IMDM containing 10% FBS, in serum free media or media supplemented with 1% BSA. As a control, some biotinylated cells were incubated at 4°C during this 30 min incubation to block endocytosis. After this incubation, some cells washed in a glutathione solution (0.05 mM glutathione, .075 mM NaCl, 0.001 mM EDTA, 10% FBS and .075 NaCl) to strip any remaining surface biotinylation. Cells were washed in Hanks balanced salt solution (HBSS) to remove reductants and any unconjugated biotin. Then cells were lysed for determination of protein concentrations. To quantitate the biotin levels on specific proteins, cellular proteins were captured using a sandwich ELISA. ELISA plates were coated with anti-IgG Ab (Jackson ImmunoResearch, used at 2 µg/mL), anti-TrF B3/25 Ab or anti-MHC class II 37.1 Ab (L. Wicker, used at 1 µg/mL). Cell lysates were

incubated on these plates for Ab capture. After extensive washing and blocking, biotin-labeled proteins were detected using streptavidin-HRP and the colorimetric dye 2,2'-azino-bis (ABTS). Absorbance was normalized to protein concentrations. BCR endocytosis was graphed as a percent of the total surface BCR (a control that was not stripped).

Subcellular fractionation

To determine if serum starvation altered GAD intracellular localization, membrane and cytoplasmic fractions of cells were isolated using a method previously published (*162*). PG cells ($1*10^7$ per condition) were cultured with or without serum or in medium supplemented with 1% BSA for 12 h. These cells were washed in HBSS (Gibco BRL), and resuspended in ice cold homogenization buffer (10 mM Hepes, 250 mM sucrose with a protease inhibitor cocktail from Sigma). To disrupt the cell plasma membrane and release cytoplasm and membranes including organelles, cells were passed 15 times through the Balch homogenizer on ice. Nuclei were removed by centrifugation at 1600 rpm at 4°C for 10 min. These homogenates were further centrifuged at 100,000 rpm at 4°C for 1 h to pellet cell membranes. The supernatant from this step was collected as the cytoplasmic fraction. The membranes were solubilized in an extraction buffer (0.01 mM Hepes, 150 mM NaCl, 2% Triton X-100 with the protease inhibitor cocktail from Sigma). Samples of cell membranes and cytoplasmic proteins were then separated on SDS-PAGE gels using the western blot protocol.

Serum fractionation and macronutrient supplementation of media

Heat inactivated FCS was fractionated using Centricon concentrators with molecular mass cut-offs of 30 kDa and 10 kDa according to manufacturer's instructions (Amicon). Macromolecules retained by the 30 kDa filter (termed the >30 kDa fraction), and molecules flowing through the filters were used as the <30 kDa and <10 kDa fractions, respectively. PG cells were cultured in IMDM media supplemented at 10% with these various serum fractions for 12 h before cell harvest.

Alternatively, cells were cultured in serum-free media supplemented with defined macromolecules, including 1% (wt/vol) BSA (Sigma), ovalbumin (OVA) (Sigma), hen egg lysozyme (HEL) (Sigma), or dextrans (70 kDa, 40 kDa, or 10 kDa) (Invitrogen).

Osmolarity and viscosity

To determine whether addition of serum or macronutrients significantly affected osmolarity or viscosity of the solution, IMDM media containing 50 U/ml penicillin and 50 μg/ml streptomycin alone or supplemented with 10% FCS or 1% BSA or dextran was filtered and shipped to ACTA laboratories for analysis.

Electron microscopy

PG cells were cultured with or without serum or in media supplemented with BSA for 12 h. Cells were harvested, washed and fixed for 1 h in 2% glutaraldhyde.

Cells were washed in PBS and lightly spun to pellet at 900 rpm for 5 min. Cells were then sectioned and prepared for imaging by the Indiana University School of Medicine Microscopy core.

Co-immunoprecipitation of proteins

B cells were cultured in media with or without serum or in serum-free media supplemented with 1% BSA for 12 h. Cells were lysed in 10 mM Tris buffer pH 7.4 (150 mM NaCl, 1% *N*-octyl-β-glucopyranoside with a protease inhibitor cocktail from Sigma) for 20 min on ice. Lysates were centrifuged at 14,000 rpm for 10 min. Normal rabbit serum (1:1000) and Protein G-Sepharose (Sigma) were added to lysate at 4°C for 30 min as a preclear step followed by removal of the Sepharose by centrifugation. GAD Ab (2.5 µg/tube of GAD6, Sigma) or an isotype-matched control Ab were added and incubated overnight at 4°C to co-precipitate associated proteins. Protein G-Sepharose were added to each cell lysate at 4°C for 2 h. Centrifugation at 2000 rpm for 2 min was used to pull down Sepharose-Ab-Ag complexes. These complexes were washed five times in cold PBS (Gibco). Reducing sample buffer (40 µl) containing β-mercaptoethanol was added to each sample prior to boiling samples in water for 10 min to release proteins from the Ab and beads. Samples were then resolved on 10% SDS-PAGE and analyzed by immunoblotting for molecules such as HSC70. The ratio of HSC70/GAD co-immunoprecipitating in cells was set to 1 for control serum treated cells and the fold change relative to this ratio was graphed for each experimental condition.

CMA peptide translocation assay

To assess the relative rate of CMA in treated cells, a biotin-labeled peptide is electroporated into the cytoplasm of cells. Incubation of these cells at $37^{\circ}C$ allows transport of this peptide from the cytoplasm into the lysosomal network by CMA. In the lysosomal network this peptide can associate with MHC class II molecules. Detection of biotin-peptide:MHC class II complexes allows a relative assessment of the rate of CMA. To assess the affect of serum deprivation on CMA, PG cells ($1*10^7$ per condition) were washed three times in ice cold PBS (Gibco) prior to addition of a C-terminal biotin-labeled $GAD_{273-285}$ peptide (20 mM). The peptide was delivered into the cytoplasm of the cell using electroporation (270V, 125mF, pulse twice) using the BIO-RAD gene pulser II. To determine the non-specific association of the peptide with the surface of cells, control cells were incubated with peptides but not subjected to electroporation. During the assay, low levels of peptide can bind surface MHC class II molecules. Given our goal to detect only intracellular MHC class II peptide complexes formed as a result of CMA, cells were treated to remove surface MHC class II peptides. To remove any peptide bound to cell surface MHC class II complexes, cells treated with or without electroporation were washed twice in ice cold 160 mM NaCl then resuspended in 160 mM NaCl/Citric acid, pH 4.0 and incubated 30 min on ice. This low pH removed only peptides from exposed cell surface MHC class II complexes (*11*). Cells were then neutralized in ice cold serum-free media and washed another two times in ice cold media containing serum. Cells were subsequently cultured in media with or without serum at $37^{\circ}C$ for 16h. Cells

were then harvested for immunoblot analysis to detect biotin-GAD$_{273-285}$/MHC class II complexes.

Statistics

Statistics were determined using GraphPad Prism 6.0 (GraphPad Software, Inc.). Data depict mean from 3 or more independent experiments, +/- SEM unless otherwise noted. P values less than 0.05 were considered statistically significant with * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001. The statistical method is listed in each figure. When multiple test were used to assess a figure, the ANOVA was depicted by * and the Student's t test was depicted by #, with # p < 0.05 and ## p < 0.01.

Results

Chapter 1.

Serum deprivation altered MHC class II presentation of endogenous antigens

Induction of high-affinity, long-lasting humoral immunity depends on B and T cell interactions. Intracellular trafficking pathways play a key role in promoting B and T cell contact necessary for adaptive immune responses. Ags are delivered into the endosomal network by various transport routes including receptor-mediated uptake, macroautophagy (MA), and chaperone-mediated autophagy (CMA) (*9, 145*). In response to stress, cells can upregulate or downregulate these transport pathways to maintain homeostasis. During nutrient stress, cells frequently upregulate MA or CMA to promote cell survival (*106, 163*). Whether fluctuations in the activity of these pathways during nutritional stress influence immune recognition had not been previously explored.

To elucidate the effect of nutrient stress or deprivation on MHC class II Ag presentation, studies were carried out using an EBV transformed human B lymphoblast PriessGAD (PG) cell line as an APC. Use of this cell line and model Ags offered the opportunity to examine how nutrient deprivation alters MHC class II Ag presentation. To monitor MHC class II presentation through the classical endocytic network, presentation of the endogenously expressed membrane BCR

Ag and the exogenous Ag human serum albumin (HSA) were monitored in PG cells. The BCR alone or complexed with Ag is constitutively internalized into endosomes by receptor-mediated endocytosis. Here, cathepsins digest the BCR and Ags yielding peptides for presentation by MHC class II molecules (*62, 63*). To examine CMA-dependent Ag presentation, PG cells were retrovirally transduced to express the cytosolic Ag glutamic-acid decarboxylase (GAD) (*11*). Our laboratory has shown GAD accesses the CMA pathway, requiring HSC70 as well as LAMP2A to translocate into lysosomes where cathepsins further process this Ag. The resulting GAD epitopes can then associate with MHC class II molecules within the endosomal network (*78*). To monitor MHC class II presentation through the MA pathway, PG cells were transduced to express the influenza A matrix protein 1 (MP1) that is targeted to autophagosomes by linkage to LC3. This MP1-LC3 undergoes lipid conjugation promoting its association with autophagosome membranes leading to MP1-LC3 uptake and degradation within these vesicles (*164*). Using these different model Ags, the effects of nutrient deprivation on MHC class II Ag presentation was investigated in B cells cultured in the absence of serum to mimic nutrient deprivation.

Serum deprivation altered MHC class II presentation of the cytoplasmic GAD antigen

The effect of nutrient deprivation on MHC class II Ag presentation was assessed by culturing PG cells in serum-free media rich in amino acids, simple carbon sources and vitamins but lacking the complex macromolecules found in serum.

PG cells were cultured in the presence or absence of serum for 6, 12, 24 or 48 h

prior to fixation, which halts Ag processing and subsequent incubation with T

cells that recognize the specific GAD $_{273\text{-}285}$ epitope displayed in the context of

HLA-DR4 on PG cells. Compared to cells cultured with serum, serum deprivation

reduced MHC class II presentation of GAD epitopes by approximately 50%

(**Figure 6 A**). Serum deprivation did not alter cell viability as determined by

trypan blue staining (data not shown).
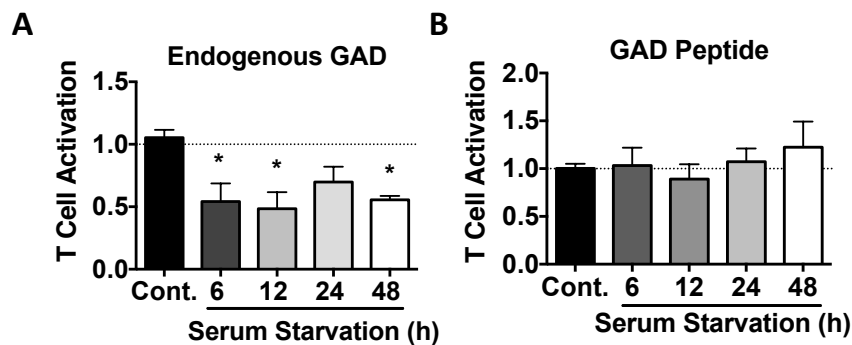
**Figure 6. Serum deprivation impaired MHC class II presentation of the
endogenous GAD antigen.** (A) MHC class II presentation of endogenous GAD in
PG cells cultured with (Cont.) or without serum for up to 48 h prior to fixation and
incubation with T cells. One-way ANOVA, followed by Dunnett's multiple
comparisons correction. (B) MHC class II presentation of the synthetic GAD
peptide in PG cells cultured with or without serum for up to 48 h, fixed and pulsed
with the synthetic peptide for 6 h prior to washing and incubation with T cells. Data
depict mean from 3 or more independent experiments, ±SEM. *p<0.05

To determine if impaired GAD presentation during serum deprivation was due to

altered co-stimulatory molecule activity or MHC class II surface levels, MHC

class II presentation of a pre-processed synthetic GAD peptide was assessed.

After serum starvation and fixation, PG cells were pulsed with the synthetic

GAD$_{273\text{-}285}$ peptide prior to incubation with T cells for analysis of MHC class II

presentation. This synthetic GAD peptide will directly load surface MHC class II molecules bypassing any processing steps. While MHC class II presentation of the endogenous Ag was significantly impaired during serum deprivation, MHC class II presentation of the synthetic GAD peptide was unaltered (**Figure 6 B**).

Long periods, but not short, of serum deprivation altered expression of MHC class II pathway components

Several molecules play critical roles in the MHC class II Ag presentation pathway as demonstrated in **Figure 1**. MHC class II molecules are guided to endosomes by Ii chain, which is proteolytically cleaved by cathepsins (*12*). The molecular editor, DM, then removes the Ii chain fragment CLIP from the MHC class II binding groove, facilitating antigenic peptide binding (*19*). DM activity is regulated by DO association (*35*). The resulting MHC class II:peptide complexes and some co-stimulatory molecules recycle from endosomes to the cell surface to engage T cells.

To further elucidate the effect of serum deprivation on the MHC class II Ag presentation pathway, mRNA and protein levels of MHC class II components were assessed. PG cells cultured with (Cont.) or without serum for 6, 12, 24 or 48 h were harvested and the relative expression of *DRα* (MHC class II molecules, DR), *CD74* (Ii chain), *DMβ* (DM), and *DOβ* (DO) mRNA were assessed. While prolonged serum deprivation significantly reduced *DMβ* mRNA,

serum starvation for up to 12 h did not significantly alter mRNA expression of the

MHC class II components (**Figure 7 A-D**).
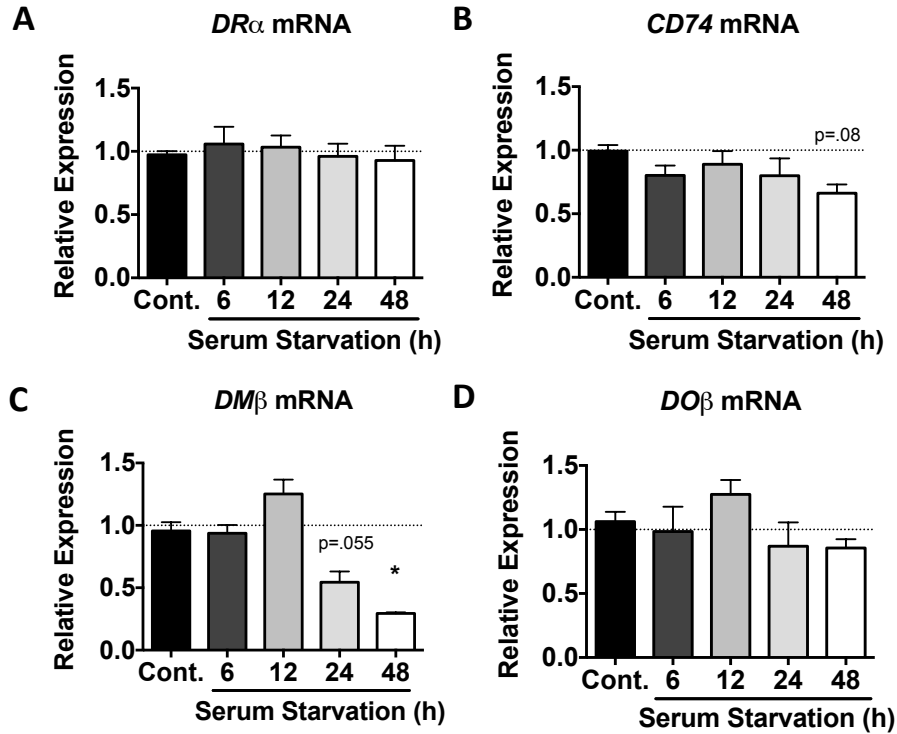
Figure 7. Prolonged, but not short, periods of serum deprivation altered mRNA expression of MHC class II pathway components. PG cells were cultured with (Cont.) or without serum for up to 48 h and mRNA expression of MHC class II components, (A) *DRα*, (B) *CD74* (Ii chain), (C) *DMβ* and (D) *DOβ* was monitored relative to GAPDH. One-way ANOVA followed by Dunnett's multiple comparisons correction. Data depict mean from 3 independent experiments, ±SEM. *p<0.05

As GAD utilizes the CMA pathway to translocate into lysosomes, mRNA

expression of the membrane channel protein LAMP2 was assessed. There are

three isoforms of LAMP2: LAMP2A which functions in CMA, LAMP2B which may

play a role in MA, and LAMP2C which has recently been shown to play a role in

RNA translocation from the cytosol to the lysosome (*78, 165, 166*). Serum

deprivation did not alter expression of LAMP2A or LAMP2B mRNA, however

prolonged serum deprivation decreased LAMP2C expression, though not

significantly (**Figure 8**).
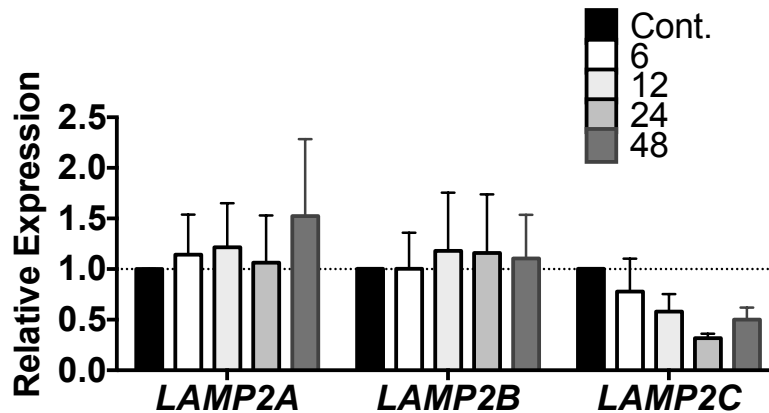
Figure 8. Effects of serum deprivation on LAMP2 mRNA expression. PG cells were cultured with (Cont.) or without serum for up to 48 h and mRNA expression of the LAMP2 isoforms was monitored relative to GAPDH. Two-way ANOVA, followed by Dunnett's multiple comparisons correction. Data depict mean from 3 independent experiments, ±SEM.

While the mRNA levels of the key co-factors for MHC class II molecules such as

Ii chain, DM and DO remained largely unchanged, protein expression and

function may be altered by serum deprivation. To assess the expression of these

proteins, PG cells were cultured with or without serum for 12 h, fixed and stained

for surface MHC class II molecules (DR) or intracellular DM and DO (**Figure 9 A,

C-E**). While MHC class II molecules and DM remained unaltered during 12 h

serum deprivation. DO expression was reduced by approximately 25%. DO binds to DM preventing its removal of CLIP from MHC class II molecules (*37*). As DM helps remove CLIP from the MHC class II binding groove, monitoring surface levels of CLIP:MHC class II complexes gives a relative readout of DM function (*19*). To assess DM function, PG cells were cultured with or without serum for 12 h and stained for surface CLIP:MHC class II complexes. Serum deprivation did not alter surface levels of CLIP:MHC class II complexes, indicating the reduction in DO protein levels during serum deprivation did not alter DM function (**Figure 9 B and E**).
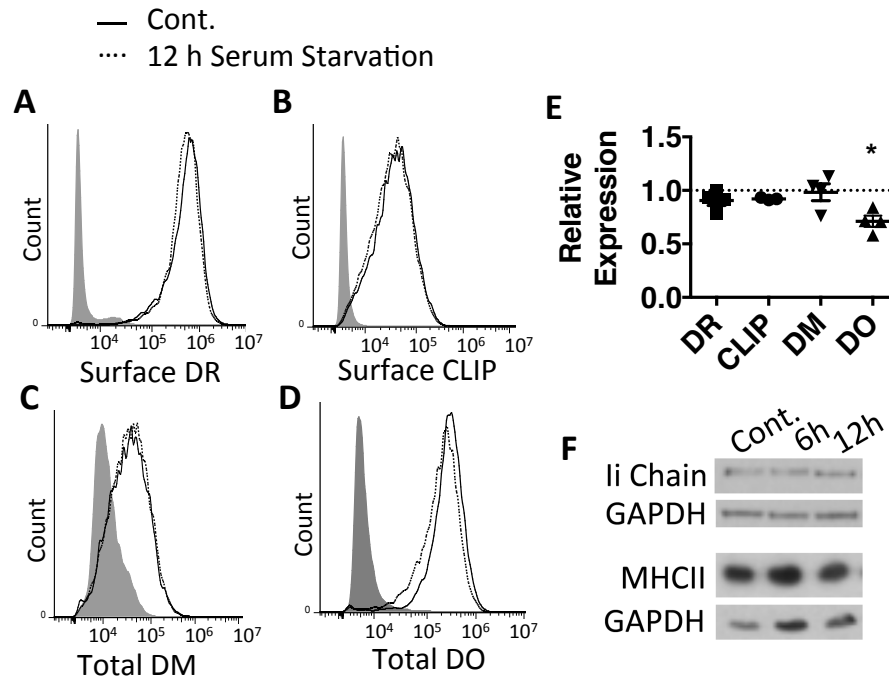
Figure 9. Serum deprivation for up to 12 h did not alter MHC class II protein levels. PG cells were cultured for 12 h with (Cont.) or without serum, fixed, and stained for flow cytometry analysis of surface expression of (A) MHC class II (DR) and (B) CLIP bound to MHC class II (CLIP) as a read out of DM activity. For intracellular detection of proteins, fixed cells were permeabilized prior to staining for (C) total DM expression and (D) total DO expression. Isotype matched control Ab staining is shown as grey shaded region. (E) Quantitation of relative protein in PG cells cultured in serum free-media for 12 h as compared to Cont. cell MFI (normalized to 1 and depicted as dotted line). Multiple T-Test to Cont. (normalized to 1), Holm-Sidak multiple comparisons correction. Data depict mean from 3 independent experiments, ±SEM. *$p < 0.05$. (F) PG cells were cultured for 6 or 12 h with or without serum and lysed for immunoblot analysis of Ii chain and MHC class II (DRα). GAPDH was used as a loading control. Data representative of 3 or more experiments.

To further assess the expression of MHC class II components, cellular levels of MHC class II molecules and Ii chain were assessed by immunoblot. MHC class II and Ii chain protein levels were overall unchanged by serum deprivation after 12 h (**Figure 9 F**). These data reveal, while serum deprivation for 6 or 12 h impaired

MHC class II presentation of GAD, the protein levels of MHC class II components appear unaltered during this time frame.

While short periods (up to 12 h) of serum deprivation did not alter MHC class II components, extending serum deprivation for 48 h significantly altered expression of MHC class II components (**Figure 10**). Significant reductions were observed in DM protein levels as well as CLIP:MHC class II complexes on the cell surface (**Figure 10 B, C, and E**). While not statistically significant, DR protein levels were reduced at 48 h in all three independent trials (**Figure 10 A and E**). Altered DM activity as well as reduced surface expression of DR, would contribute to impaired GAD presentation at these prolonged periods of nutrient stress (>24 h). Thus, the reduction in expression and function of MHC class II components likely contributes to impaired MHC class II presentation of GAD during prolonged serum deprivation.
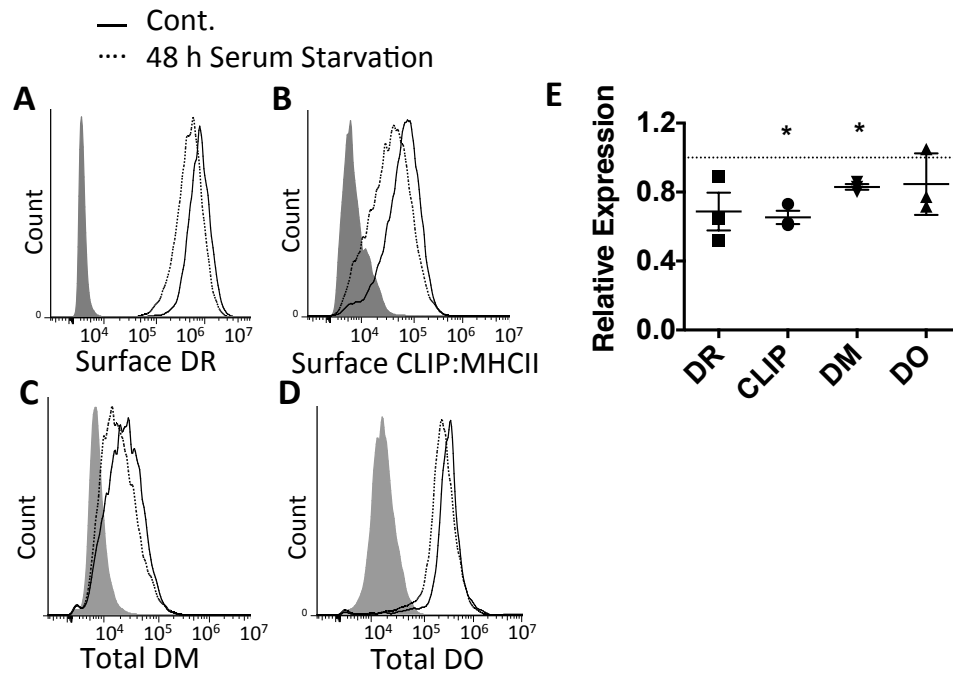
Figure 10. Prolonged serum starvation reduced protein levels and function of MHC class II pathway components. PG cells were cultured for 48 h with (Cont.) or without serum, fixed, and stained for flow cytometry analysis of surface expression of (A) MHC class II (DR) and (B) CLIP bound to MHC class II (CLIP). For intracellular protein detection, fixed cells were permeabilized prior to staining for (C) total DM expression and (D) total DO expression. Isotype matched control Ab staining is shown as grey shaded region. Data are representative of three or more independent experiments. (E) Quantitation of relative protein in PG cells cultured in serum free-media for 12 h as compared to Cont. cell MFI (normalized to 1 and depicted as dotted line). Multiple T-Test, Holm-Sidak multiple comparisons correction. Data depict mean from 3 independent experiments, ±SEM. *p<0.05

Mechanisms used to induce macroautophagy, distinct from serum

deprivation, altered MHC class II components

In vivo and in vitro, cellular deprivation of serum proteins is known to induce MA,

a process by which LC3II decorated autophagosomes engulf cytoplasmic

molecules for degradation to favor, in some cases, immune recognition (*146*).

Upon maturation and acidification of autophagosomes, LC3II protein within these vesicles is degraded. Addition of chloroquine (CQ) impairs acidification of autophagosomes, blocking autophagosomal degradation of LC3II. Cellular levels of MA or the MA flux can be monitored by tracking LC3II protein expression in response to nutrient stress (*92*). As expected, MA flux was amplified in cells cultured without serum as compared to control serum treated cells between 6-24 h. By 48 h cultivation in the absence of serum, the induction of MA had subsided (**Figure 11**).
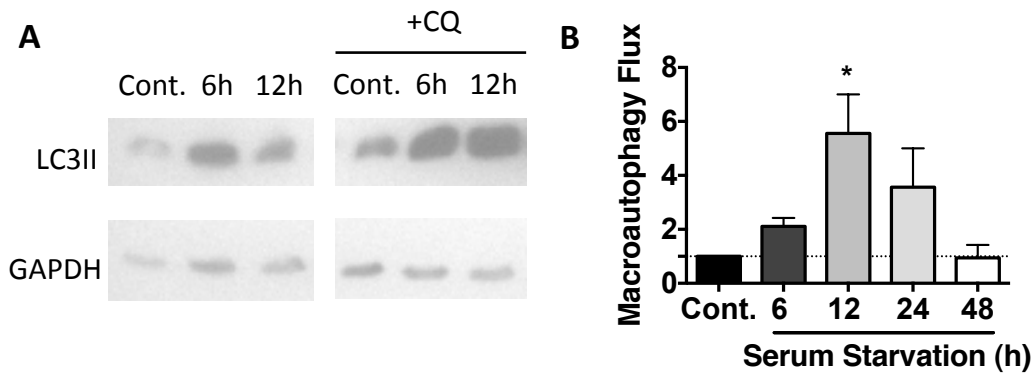
Figure 11. Serum deprivation induced macroautophagy. (A) PG cells were cultured with (Cont.) or without serum for 6 or 12 h in the presence or absence of CQ to block lysosomal acidification and LC3II degradation. Cells were harvested, and lysed for immunoblot analysis of LC3II expression. LC3II expression was compared to Cont. cells and GAPDH was used as a loading control. (B) To quantitate MA flux, LC3II levels were normalized to the loading control GAPDH and LC3II:GAPDH basal levels were subtracted from the induced CQ treated levels. MA flux in Cont. cells was normalized to 1 and fold change relative to this control graphed. One-way ANOVA, followed by Dunnett's multiple comparisons correction. Data depict MA flux from 3 independent experiments, ±SEM. *$p < 0.05$

To examine whether the induction of MA was responsible for the observed alterations in GAD presentation during serum deprivation, PG cells were treated

with Torin1. Torin1 is an inhibitor of mTOR, a key inhibitor of MA (*167*). PG cells

treated with up to 600 nM Torin1 for 16 h demonstrated reduced levels of

endogenous GAD presentation similar to those observed with serum deprivation

(**Figure 12 A**). Treatment at these concentrations of Torin1 did not affect cell

viability as assessed by trypan blue staining (data not shown). This initial result

suggested the induction of MA may be responsible for altered GAD presentation.

However, analysis of the MHC class II pathway components revealed that while

MHC class II surface expression was unaltered by Torin1 treatment, DM function

was significantly enhanced, as assessed by surface levels of the CLIP:MHC

class II complexes (**Figure 12 B, C**). Studies from our lab have shown GAD

epitope presentation is sensitive to DM function, potentially explaining the

reduced levels of GAD presentation in cells treated with Torin1 (*28*). Though

treatment with the MA inducer Torin1 resulted in reduced MHC class II

presentation of GAD, the mechanism here appears different than that of serum

deprivation, as serum deprived cells did not experience altered DM activity.
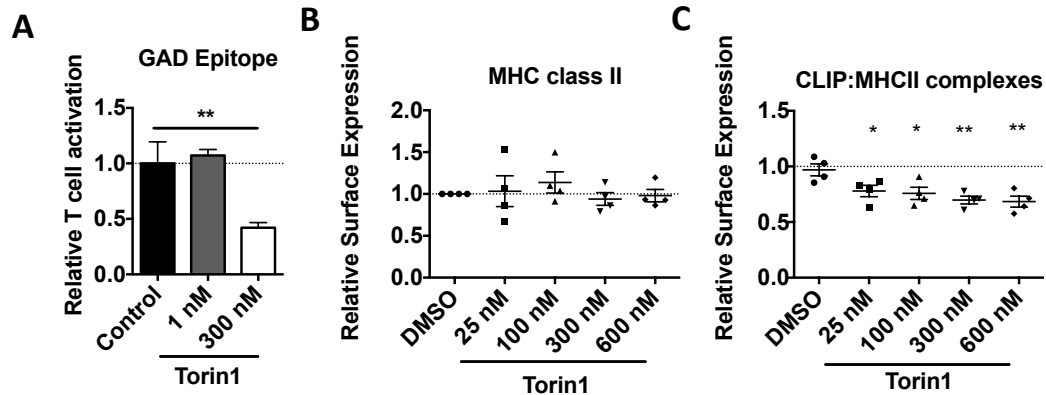
Figure 12. Torin1 treatment altered DM function. (A) PG cells cultured in the presence of the vehicle (Control) DMSO or various concentrations of Torin1 for 16 h were fixed and incubated with T cells to monitor MHC class II presentation of GAD or fixed and stained with Abs to assess surface (B) MHC class II and (C) CLIP:MHC class II complexes as an indicator of DM function. *p<0.05, **p<0.01, One-way ANOVA, Dunnett's multiple comparisons correction. Data representative of two or more independent experiments, ±SEM.

Intracellular pathogens have adapted multiple mechanisms to escape host cell detection. Several pathogens have developed the means to alter the endocytic/lysosomal pathway, altering autophagy and endocytic trafficking. These mechanisms not only protect the pathogen from cell autonomous elimination, but also may prevent the activation of the immune system by altering MHC class II presentation (*168, 169*). Several pathogens including herpesvirus, influenza, *Listeria* and *Toxoplasma* have been shown to manipulate MA (*155, 170-172*). *Toxoplasma gondii* induces host cell autophagy by secretion of the hormone abscisic acid (ABA) and manipulates vacuolar trafficking to acquire nutrients (*155, 173*). To assess whether alterations in MA during *T. gondii* infection affect MHC class II Ag presentation, PG cells were infected with *T. gondii* for 16 h. As these were the first studies to assess *T. gondii* infectivity of B

cells, PG cells were harvested and immunofluorescence was used to assess intracellular localization of *T. gondii* (**Figure 13 A**). Infection of B cells with *T. gondii* at these MOIs and time points did not significantly affect cell viability as assessed by trypan blue staining (data not shown). Infection with *T. gondii* significantly reduced MHC class II presentation of GAD epitopes, though this is likely due to significant reductions in MHC class II surface levels (**Figure 13 B and C**). These studies indicate *T. gondii* infection may globally alter MHC class II Ag presentation, as infection reduced surface expression of MHC class II in B cells. While infection with *T. gondii* impaired GAD presentation by MHC class II molecules, the reduction in MHC class II observed during infection differs from the serum starvation and Torin1 experiments. The results suggest this model using *T. gondii* may disrupt Ag presentation by a different mechanism, perturbing MHC class II gene expression.
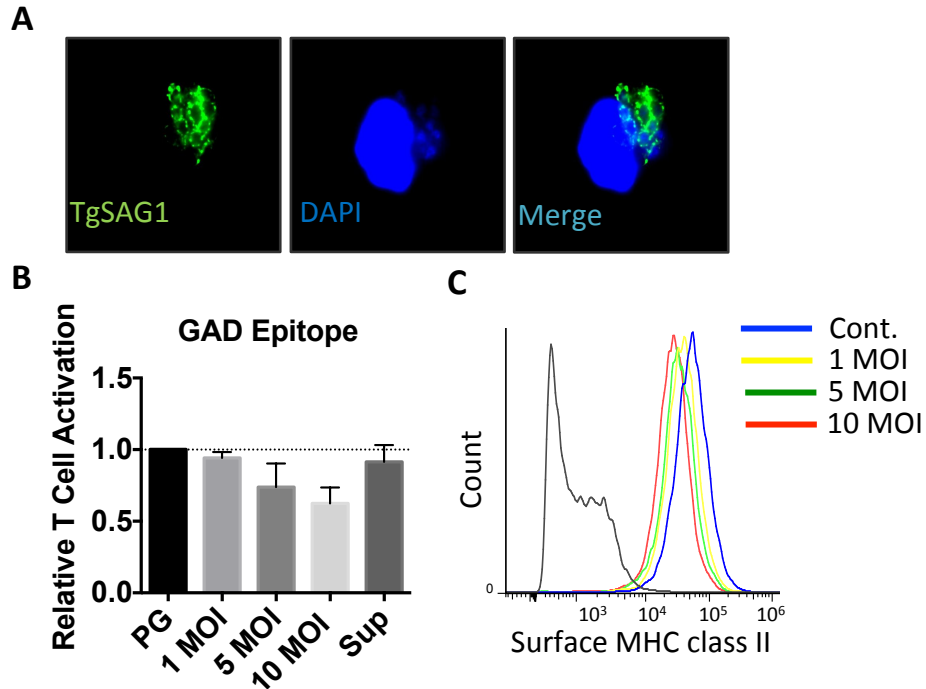
Figure 13. *Toxoplasma gondii* **infection of B cells reduced MHC class II surface expression.** (A) PG cells were infected with *T. gondii* (MOI 5) for 16 h, fixed and immunofluorescence was used to detect nuclei (DAPI, blue) and a *T. gondii* surface protein (TgSAG1, green). (B) PG cells were infected with *T. gondii* (MOI 1, 5 and 10) for 16 h, fixed and MHC class II presentation of GAD was monitored. As a control, supernatant from *T. gondii* growth culture was collected, filtered (0.2 µm) and added to PG for 16 h prior to fixation of these cells and T cell co-culture to monitor MHC class II presentation. (C) PG cells were infected with *T. gondii* for 16 h, fixed and MHC class II (DR) surface expression assessed by flow cytometry. Data representative of 2 or more independent experiments, ±SD.

Serum deprivation enhanced MHC class II presentation of antigens through

the macroautophagy pathway but impaired presentation of antigens

through other pathways

To further elucidate the mechanisms by which short periods (up to 12 h) of serum

deprivation altered MHC class II presentation, the presentation of epitopes

derived from several model Ags delivered into the endosomal network via distinct routes was monitored. To monitor presentation of an Ag which requires the MA pathway, studies were carried out in PG transduced with the MP1-LC3 fusion protein (77). MHC class II presentation of epitopes derived from this cytoplasmic Ag, MP1-LC3, was increased in cells grown in serum-free media compared to serum treated cells (**Figure 14 A**). As MA was increased in these B cells, upregulation of MHC class II presentation of epitopes derived from MP1 was not unexpected (**Figure 11**). Interestingly, MHC class II presentation of epitopes derived from the exogenous HSA Ag and epitopes derived from the membrane B cell receptor (BCR) Ag was impaired, both of these Ags traffic by endocytosis for processing and to gain access to MHC class II molecules (**Figure 14 B, C**). MHC class II presentation of the synthetic BCR peptide (Igκ I $_{188-203}$) was not altered by serum deprivation, further suggesting alteration in MHC class II presentation were not caused by altered co-stimulatory signaling or MHC class II surface expression (**Figure 14 D**).
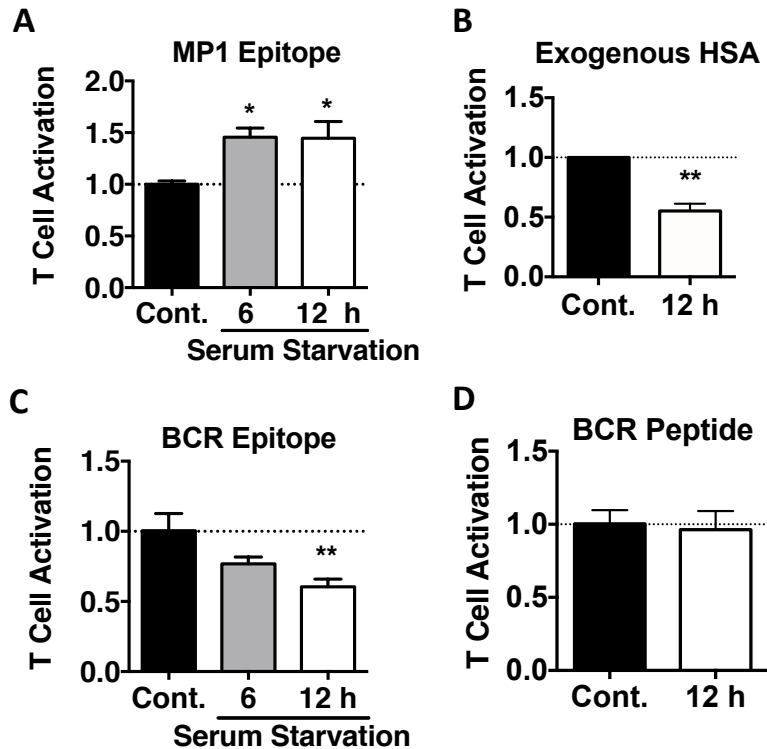
Figure 14. Serum deprivation altered MHC class II presentation of select antigens in PG cells. (A) MHC class II presentation of an epitope derived from the MA targeted MP1 Ag in PG cells cultured with (Cont.) or without serum for 6 or 12 h. One-way ANOVA followed by Dunnett's multiple comparison. (B) PG cells were cultured with or without serum in the presence of 20 uM exogenous HSA Ag for 12 h prior to fixation and detection of MHC class II presentation of an epitope derived from HSA. Student's T-Test. (C) MHC class II presentation of an epitope derived from the membrane Ag BCR in PG cells cultured with or without serum for 6 or 12 h. One-way ANOVA followed by Dunnett's multiple comparisons correction. (D) After culture of PG cells with or without serum for 12 h, cells were fixed and incubated with the synthetic BCR peptide for 6 h prior to incubation with T cells to monitor MHC class II presentation of the BCR peptide. $*p<0.05$, $**p<0.01$. Data depict means from three or more independent experiments, ±SEM.

Together the data presented in this chapter indicate that serum deprivation alters

MHC class II presentation of specific Ags and that these disruptions are

dependent on the processing route. MHC class II presentation of the MA-

targeted Ag MP1 was significantly enhanced by serum deprivation, while presentation of GAD, a CMA-targeted Ag, and the BCR as well as HSA, endocytic Ags, was impaired. While MHC class II presentation of several Ags was altered, the classical MHC class II components including MHC class II molecules, DM, DO and Ii chain remained largely unchanged during short (up to 12 h) periods of serum starvation. However, during extended periods of serum deprivation, expression of several MHC class II components was significantly impaired, likely contributing to altered Ag presentation. Furthermore, serum deprivation significantly induced MA flux. While the use of chemical regulators or infection to mimic MA induction disrupted GAD Ag presentation, these treatments also altered the expression of key components of the MHC class II Ag presentation pathways, potentially contributing to altered Ag presentation.

Chapter 2.

Serum deprivation altered trafficking of endogenous antigens

Whereas endocytosis of exogenous Ags promotes the classical MHCII Ag
presentation pathway, MA, bulk autophagy, and CMA, a selective form of
autophagy dependent on HSC70, play critical roles in MHC class II presentation
of intracellular Ags (*9*). While MHC class II components were unaltered by serum
deprivation, MHC class II presentation of Ags that utilize both the endocytic and
CMA routes of Ag delivery was impaired, though presentation of Ags through MA
was enhanced. During nutrient stress, cells upregulate MA to promote survival
and to salvage critical building blocks (*106, 163*). To further elucidate the effect of
serum deprivation on MHC class II Ag presentation, Ag degradation and
trafficking were assessed. The BCR, alone or complexed with Ag, accesses the
classical endocytic pathway to transit from the cell surface into the endosomal
network. The cytoplasmic Ag GAD utilizes CMA to reach the endo/lysosomal
network. Within endosomes and lysosomes, cathepsins digest the BCR and GAD
yielding peptides for presentation by MHC class II molecules (*62, 63*). Alterations
in the expression, degradation or trafficking of these Ags may alter the availability
of epitopes for MHC class II presentation.

Serum deprivation altered antigen degradation

To elucidate the mechanism by which serum deprivation altered epitope
selection for MHC class II presentation, MP1, GAD and BCR protein expression

was assessed in B cells. While cellular MP1 protein levels dropped with serum deprivation (**Figure 15 A**), BCR and GAD protein levels increased (**Figure 15 B and C**). To determine whether these alterations were due to changes in Ag synthesis or degradation, PG cells were treated with a protein synthesis inhibitor, cycloheximide. MP1 protein levels decreased quickly with serum deprivation regardless of cycloheximide treatment, consistent with MA-induced degradation of this Ag (**Figure 15 A**). Conversely, BCR and GAD protein levels rose irrespective of cycloheximide, indicating altered Ag levels were not due to protein synthesis but rather to impaired degradation of these Ags (**Figure 15 B and C**). Furthermore, transcripts for BCR and GAD were unchanged by serum deprivation, again pointing to perturbations in cellular protein degradation pathways (**Figure 16 A and B**).
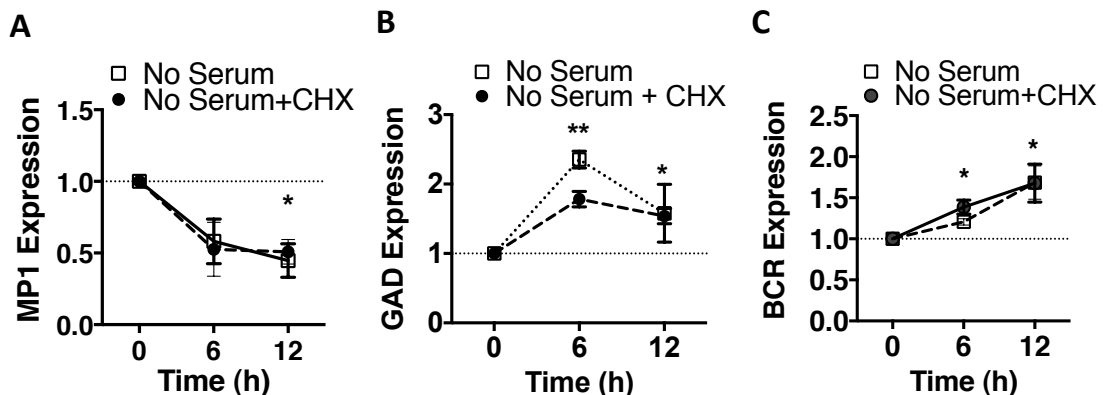
Figure 15. Serum deprivation in PG cells altered expression levels of antigens. PG cells were cultured with (Cont.) or without serum for 6 or 12 h in the presence or absence of cycloheximide (CHX) prior to lysis and immunoblot analysis of (A) MP1, (B) GAD and (C) BCR. GAPDH was used as a loading control for these studies. Ag expression was normalized to GAPDH expression. The fold change of Ag:GAPDH for serum deprived cells was determined compared with the Cont. serum-treated cells. *p<0.05, **p<0.01, Multiple T-Test, Holm-Sidak multiple comparisons correction. Data depict results from three or more independent experiments, ±SEM.

A

B
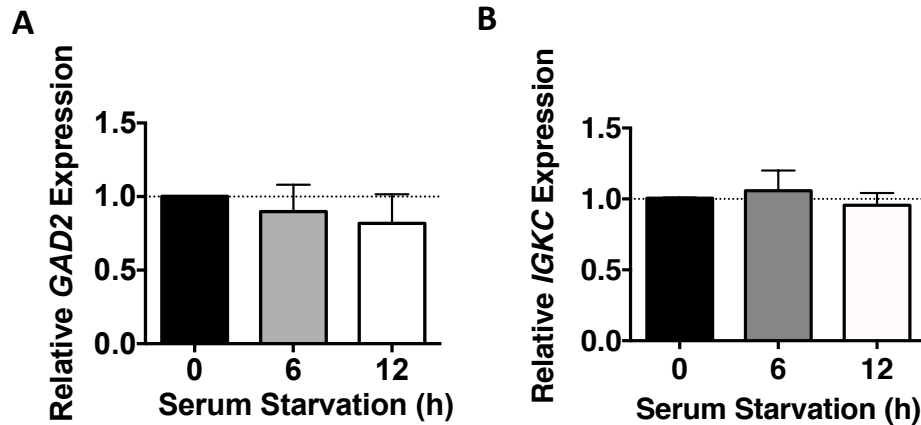
Figure 16. Serum deprivation did not alter expression of antigen mRNA. PG cells were cultured with (Cont.) or without serum for 6 or 12 h. Cells were harvested for qRT-PCR analysis. Relative expression of (A) *GAD2* (GAD) and (B) *IGKC* (BCR) was determined using GAPDH levels. Data depict means from three independent experiments, ±SEM.

Serum deprivation induced protease activity

To determine if the observed disruptions of BCR and GAD degradation were due to alterations in endosomal and lysosomal protease levels, mRNA levels of several acidic proteases were assessed. Serum deprivation did not significantly alter AEP, or cathepsin A, B or S mRNA levels as assessed by qRT-PCR (**Figure 17**). Transcription Factor EB (TFEB) is a master regulator of lysosomal biogenesis and autophagy (*174*). Analysis by qRT-PCR revealed TFEB expression was significantly reduced by serum deprivation (**Figure 18**). During starvation, phosphorylation of TFEB is prevented leading to its translocation from the cytoplasm to the nucleus where it acts as a transcription factor (*175*). While TFEB mRNA levels appear to drop with starvation, TFEB activity may still be upregulated in these cells depending on the phosphorylation of this protein.

These data show mRNA expression of lysosomal proteases was unaltered by serum deprivation, though expression of mRNA encoding the master regulator of lysosomes and autophagy biogenesis was reduced during prolonged deprivation.
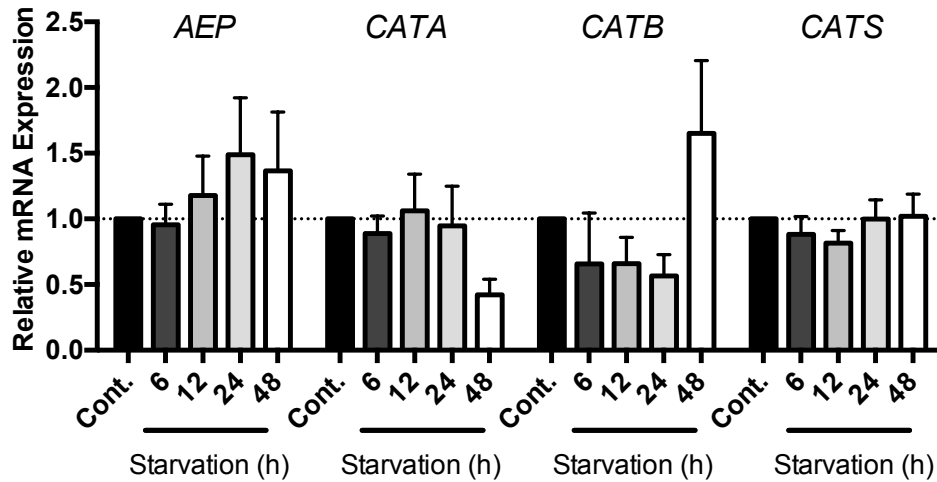
Figure 17. Serum deprivation did not significantly alter protease mRNA expression. PG cells were cultured with (Cont.) or without serum for up to 48 h. Cells were harvested for qRT-PCR analysis of AEP, and cathepsins (CAT) A, B, and S. mRNA expression was determined relative to GAPDH mRNA levels. Data depict the means from three independent experiments, ±SEM.
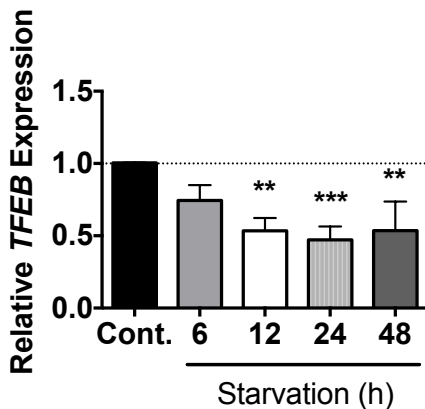
Figure 18. Serum deprivation altered TFEB mRNA expression. PG cells were cultured with (Cont.) or without serum for for up to 48 h. Cells were harvested for qRT-PCR analysis of the lysosomal gene master regulator TFEB. mRNA expression was determined relative to GAPDH mRNA levels. **p<0.01, ***p<0.001. One-way ANOVA, Dunnett's multiple comparisons correction. Data depict the means from three independent experiments, ±SEM.

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While expression of mRNAs encoding several lysosomal proteases were

unaltered by serum deprivation, the activity of these proteases could be impaired,

accounting for altered degradation of the BCR and GAD during serum

deprivation. Acidification of the endosomal/lysosomal compartments is key for

the function of multiple proteases including cathepsin B, D, S and L (*176, 177*).

PG cells were cultured with or without serum for up to 12 h in the presence of

dual-labeled FITC:rhodamine tagged dextrans. While FITC is pH sensitive and

dims upon reaching acidic compartments, the rhodamine label is pH insensitive

(*160*). This dual fluorescent-tagged probe can be used for assessing relative

rates of endocytosis as well as endosomal/lysosomal acidification. The ratio of

FITC:rhodamine can be used to assess acidification independent of endocytosis

rates as FITC dims with decreasing pH. Serum deprivation significantly reduced

the relative FITC fluorescence, indicative of lower or more acidic pH in the

endosomal and lysosomal compartments of these cells (**Figure 19**).
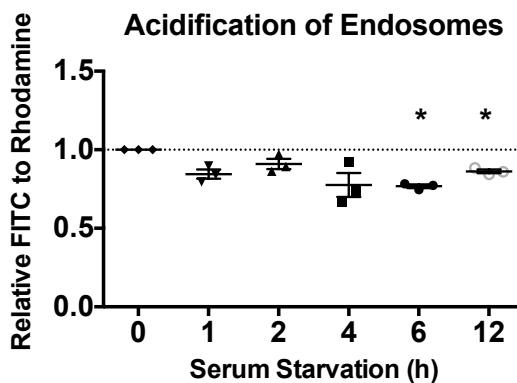
Figure 19. Serum deprivation lowered the pH of endosomes and lysosomes. PG cells were cultured with or without serum and treated with dual-fluorescent labeled dextran for up to 12 h. Relative loss of FITC fluorescence, a pH sensitive fluorochrome, was assessed relative to rhodamine, a pH insensitive fluorochrome. * $p < 0.05$, One-way ANOVA, Dunnett's multiple comparisons correction. Data depict means from three independent experiments, ±SEM.

Acidification of the endosomal/lysosomal vesicles indicates enhanced protease

activity. Leupeptin is a protease inhibitor known to disrupt cathepsin S and L

degradation of Ii chain, allowing detection of the Ii chain fragments SLIP and LIP

(*16*). To assess protease activity, Ii chain degradation was monitored in PG cells

cultured with or without serum in the presence of leupeptin. Immunoblot analysis

of Ii chain fragment LIP, revealed serum deprivation reduced the build up of LIP

during leupeptin treatment (**Figure 20**). These data together with the acidification

of endosome/lysosomes suggest greater acidic protease activity during serum

deprivation.
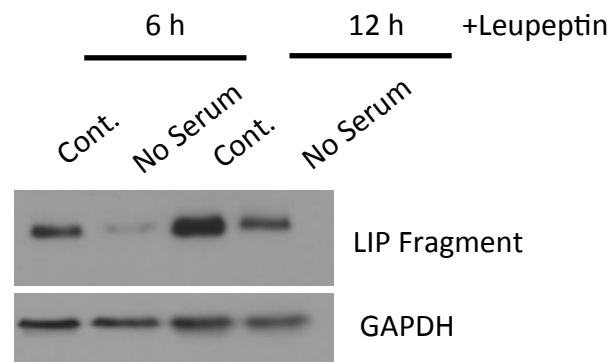
Figure 20. Serum deprivation altered invariant chain degradation. PG cells were cultured with (Cont.) or without serum for 6 or 12 h in the presence of the protease inhibitor leupeptin. Cells were harvested and lysed for immunoblot analysis of the LIP fragment of Ii chain and GAPDH. Data representative of three experiments.

To further assess protease activity cathepsin B, L and H activities were

determined in lysates from PG cells cultured with or without serum. Serum

deprivation led to a significant increase in cathepsin B and L activity, though

cathepsin H remained unchanged (**Figure 21 A**). To confirm enhanced cathepsin

activity in serum deprived cells, a real-time flow cytometric analysis of cathepsin

proteolysis was used. PG cells were cultured with or without serum in the

presence of membrane soluble cathepsin substrates that fluoresce upon

cleavage. This analysis revealed increased cathepsin B and L activity in

response to serum deprivation (**Figure 21 B and C**).
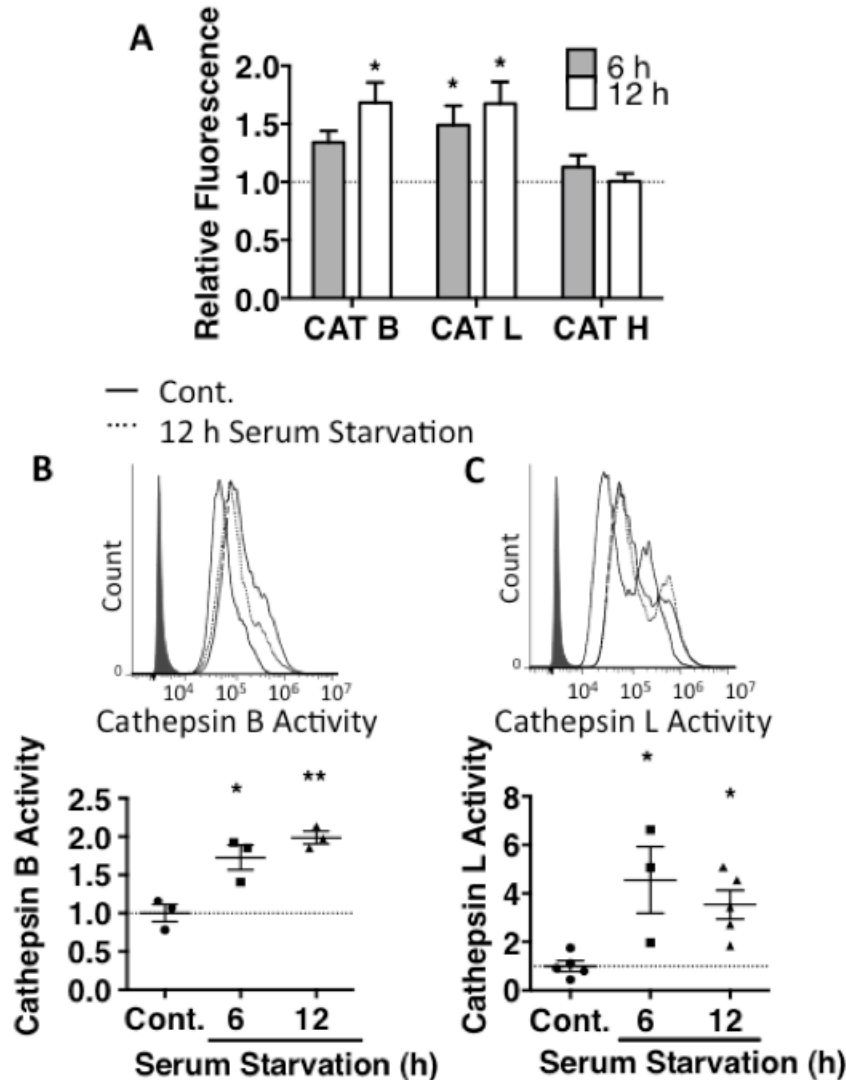
Figure 21. Serum deprivation induced lysosomal protease activity.
(A) PG cells were cultured for up to 12 h with (Cont.) or without serum, harvested, lysed and cathepsin (CAT) B, L and H activity monitored using fluorescent substrates. For each cathepsin, Cont. cell enzyme activity was normalized and set to 1 (dotted line). Relative cathepsin activity for each cell treatment was graphed for three independent experiments, ±SEM. Two-way ANOVA, Dunnett's multiple comparisons correction. (B and C) Real-time analysis of cathepsin activity using membrane soluble substrates in PG cells cultured with or without serum for up to 12 h. Cell profile for no substrate treated cells is shown (gray shaded). *p<0.05, **p<0.01, One-way ANOVA, Dunnett's multiple comparisons correction. Data depict averages from three independent experiments, ±SEM.

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To further assess the effect of serum deprivation on cathepsin activity, human B cells were isolated from peripheral blood and cultured with or without serum for 12 h in the presence of membrane permeable cathepsin substrates. Serum deprivation increased cathepsin B and L activity in these freshly isolated human peripheral blood B cells (**Figure 22 A and B**).
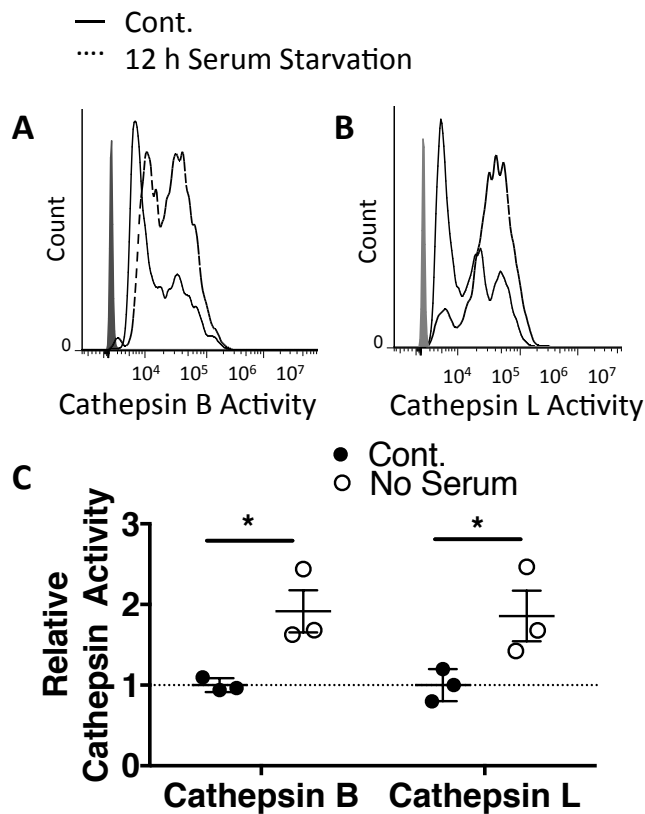
Figure 22. Serum deprivation induced lysosomal protease activity in human peripheral blood B cells. Real-time analysis of cathepsin B (A) and L (B) activity in peripheral blood B cells cultured with (Cont.) serum or without serum for 12 h. Cell profile for no substrate treated cells is shown (gray shaded). (C) Quantitation of relative cathepsin activity. *p<0.05, Two-way ANOVA, Dunnett's multiple comparisons correction. Data depict averages from three independent experiments, ±SEM.

GAD is processed not only in endo/lysosomes but also within the cytoplasm.

Processing of cytosolic GAD by the proteasome is required for MHC class II

presentation (*11*). To further assess protease activity in serum-deprived cells,

proteasome activity was monitored using a chymotrypsin substrate that

luminesces upon cleavage. Analysis revealed that, similar to cathepsin activity,

proteasome activity was significantly enhanced during serum deprivation (**Figure

23**). Together, these data indicate serum deprivation enhances cellular protease

activity, though this does not explain the reduced degradation of the GAD and

BCR Ags during serum deprivation.
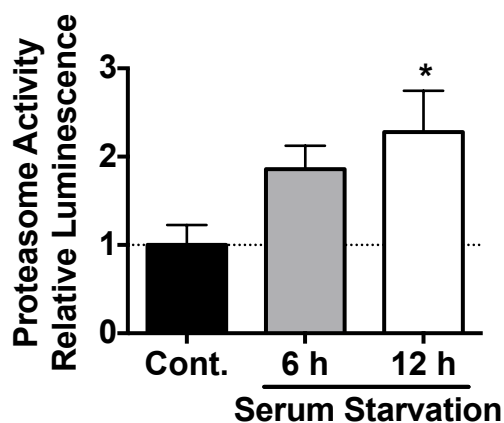
Figure 23. Proteasome activity was enhanced during serum deprivation. The chymotrypsin-like proteasome activity was determined in PG cells cultured with and without serum for up to 12 h using a luminescent substrate. * $p<0.05$ One-way ANOVA followed by Dunnett's multiple comparisons correction. Data depict means from three independent experiments, ±SEM.

Serum deprivation impaired trafficking of the BCR and GAD antigens

The reduced turnover of the BCR and GAD in the presence of increased

protease activity in these analyses point to possible alterations in the trafficking

of these Ags during serum macronutrient depletion. The BCR is a surface protein

complex that is constitutively internalized by endocytosis (*67*). To assess BCR

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trafficking during serum deprivation, surface and total levels of the BCR were

monitored by flow cytometry. Analysis of BCR trafficking revealed a 2-fold

increase in BCR surface levels in B cells grown without serum (**Figure 24 A**).

Total BCR expression increased with serum deprivation, along with the ratio of

surface:total BCR expression (**Figure 24 B**), consistent with an accumulation the

BCR on the cell surface.
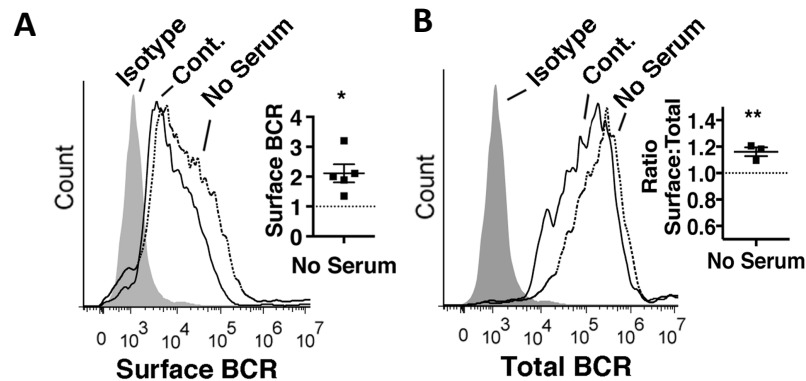
Figure 24. Surface expression of the BCR increased during serum deprivation. PG cells were cultured with or without serum for 12 h and fixed for flow analysis of (A) BCR surface levels (insert: relative surface levels of BCR for PG cells cultured without serum vs. Cont. cells indicated by the dotted line) or (B) total BCR levels (insert: relative ratio of surface:total BCR for cells treated with no serum vs. Cont. cells indicated by the dotted line). *$p<0.05$, Student's T-Test. Data representative of three or more independent experiments (inserts are averages from three or more independent experiments, ±SEM).

To further monitor the effect of serum deprivation on BCR trafficking, B cells were

isolated from human peripheral blood and incubated with or without serum.

Surface BCR distribution increased even more dramatically in peripheral blood B

cells incubated without serum (**Figure 25 A and B**). These data suggest

alterations in BCR trafficking during serum deprivation.
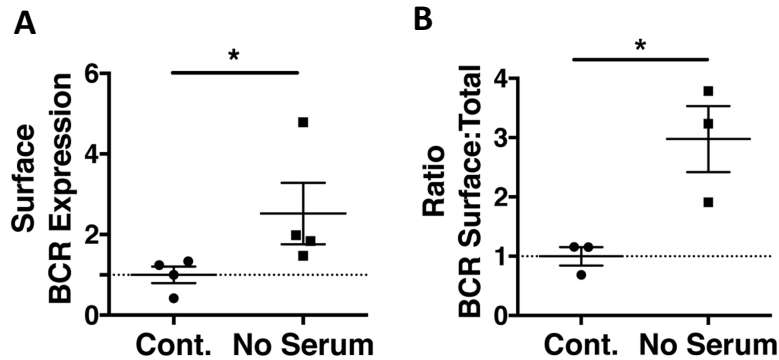
Figure 25. Surface expression of the BCR increased during serum deprivation of human primary B cells. Human primary B cells were isolated from peripheral blood and cultured with or without serum for 12 h. Cells were then fixed for flow analysis of (A) BCR surface levels or permeablized for analysis of total BCR levels and assessment of the (B) ratio of surface:total BCR expression. BCR levels in Cont. serum-feed cells were normalized to 1 and the fold change in BCR expression relative to these control cells was graphed. * $p < 0.05$, Student's T-Test. Data depict averages from three independent experiments, ±SEM.

BCR build up on the cell surface could occur due to increased expression of the

BCR or decreased internalization. Assessment of BCR turnover indicated that

synthesis of new BCR is not responsible for increased levels (**Figure 15 C**). To

monitor fluid-phase endocytosis, PG cells were cultured with or without serum

and treated with fluorescent labeled dextran. Flow analysis revealed that serum

deprivation impaired internalization of dextrans, suggestive of diminished fluid-

phase endocytosis (**Figure 26**). The BCR is internalized via receptor-mediated

endocytosis. While reduction in fluid-phase endocytosis is suggestive of alterations in the endocytic pathway, monitoring the specific internalization of the BCR gives a more complete understanding of any alterations in BCR trafficking.
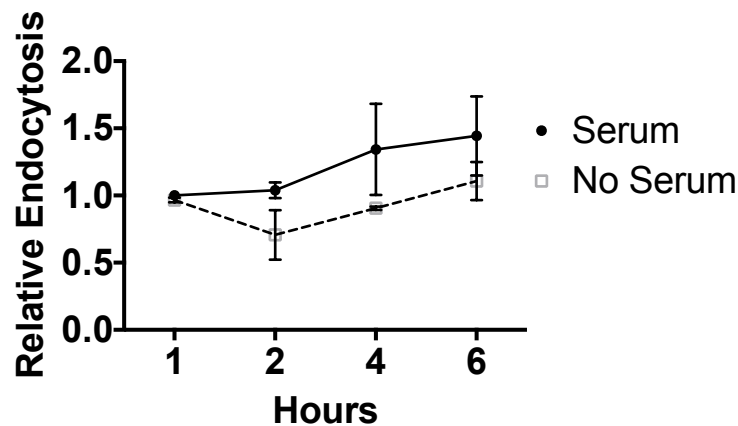
Figure 26. Fluid-phase endocytosis was impaired during serum deprivation. PG cells were cultured with or without serum in the presence of rhodamine-labeled dextrans for 1, 2, 4 or 6 h prior to flow analysis. The relative rhodamine-dextran uptake was graphed. Data depict averages from four independent experiments, ±SEM.

To assess BCR internalization, surface proteins were biotinylated and internalization of biotin-labeled surface proteins was assessed (**Figure 27 A**). BCR internalization or endocytosis was significantly impaired in response to serum deprivation of PG cells (**Figure 27 B**). To further assess the effects of serum deprivation on endocytosis, internalization of MHC class II molecules was monitored. PG cells cultured without serum had reduced internalization of MHC class II molecules, suggestive of a more global affect of serum deprivation on endocytosis (**Figure 27 C**). Furthermore, analysis of human primary peripheral blood B cells revealed a similar disruption in the endocytic pathway in cells

cultured without serum; with reductions in internalization of the BCR, a similar

though not statistically different trend was observed when monitoring the

endocytosis of MHC class II molecules and transferrin receptor (TfR) (**Figure

28**). These studies suggest a previously unrecognized role for serum nutrients in

regulating BCR trafficking and global endocytosis.
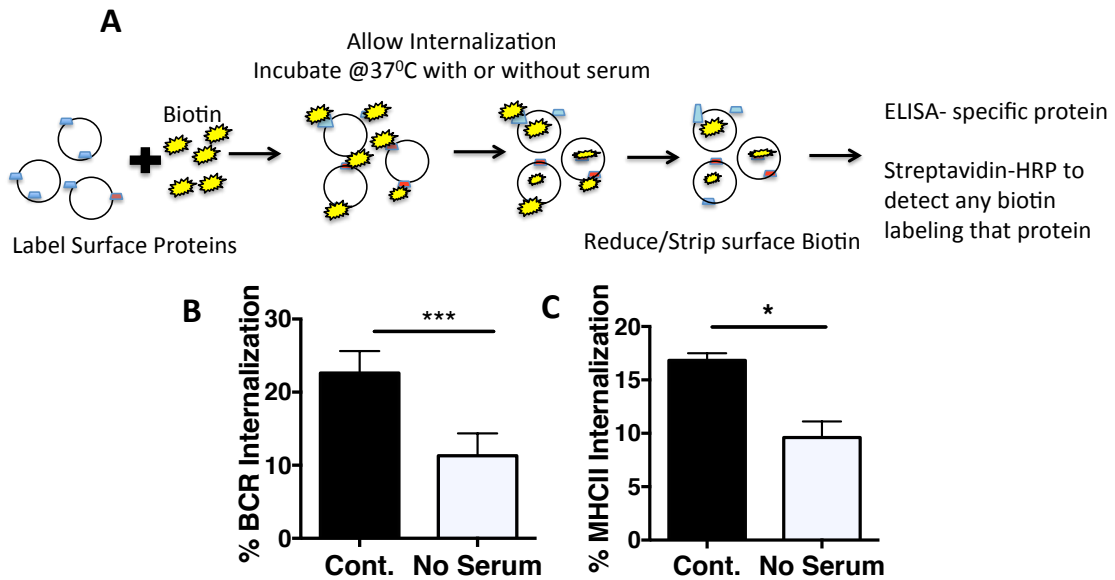
Figure 27. Internalization of the BCR and MHC class II molecules was impaired during serum deprivation. (A) PG surface proteins were biotinylated prior to incubation with or without serum to allow protein internalization. After incubation, remaining surface biotin was removed. Cells were then lysed for ELISA analysis of biotinylation of the (B) BCR and (C) MHC class II (MHCII). * p<0.05, *** p<0.001, Student's T Test. Data depict averages from three or more independent experiments, ±SEM.

Figure 28. Internalization of the BCR was impaired during serum deprivation of primary B cells. Human primary B cell surface proteins were biotinylated prior to incubation with or without serum to allow protein internalization. After incubation, remaining surface biotin was removed. Cells were then lysed for ELISA analysis of biotinylation of the BCR, MHC class II (MHCII) and transferrin receptor (TfR). *** $p < 0.001$, Two-way ANOVA followed by Dunnett's multiple comparisons correction. Data depict averages from three independent experiments, ±SEM.

To further assess the effect of serum deprivation on trafficking of surface proteins, surface expression of the TfR and another membrane protein CD45R (also known as B220) were assessed. Serum deprivation led to a significant increase in TfR surface expression, similar to the BCR (**Figure 29 A**). This is suggestive of a global defect in receptor-mediated internalization. However, surface expression of CD45R was unaltered by serum deprivation (**Figure 29 B**)**.** Unlike TfR and the BCR, CD45R does not localize within lipid rafts (*178, 179*). Together, these data indicate serum deprivation may alter lipid raft composition or internalization.
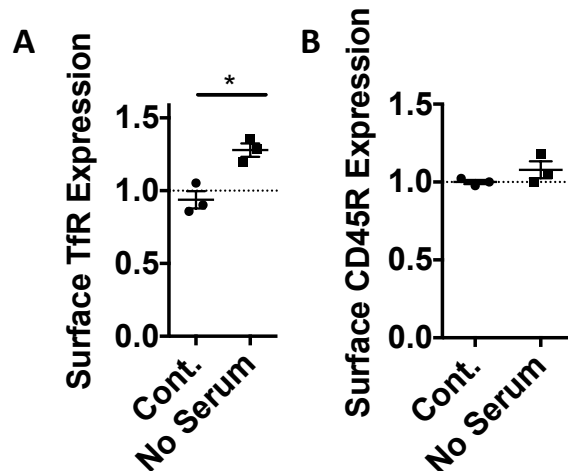
Figure 29. Transferrin receptor surface expression increased during serum deprivation. PG cells were cultured with or without serum for 12 h prior to fixation for flow analysis of (A) surface transferrin receptor (TfR) and (B) surface CD45R. Student's T-Test. * $p<0.05$. Data representative of 3 independent experiments, ±SEM.

GAD is a cytoplasmic protein that can be found free within the cytoplasm or can be palmitoylated, leading to association with the cytoplasmic of membrane (*180*). To assess whether serum deprivation altered GAD trafficking and distribution, PG cells were cultured with and without serum and lysed into cytoplasmic and membrane fractions using ultracentrifugation. Immunoblot analysis of these fractions revealed GAD distribution was altered during serum deprivation. Serum deprived cells had reduced levels of cytoplasmic GAD and increased levels of membrane associated GAD (**Figure 30 A and B**). Together these data show serum deprivation alters the trafficking and subcellular distribution of endogenous Ags.
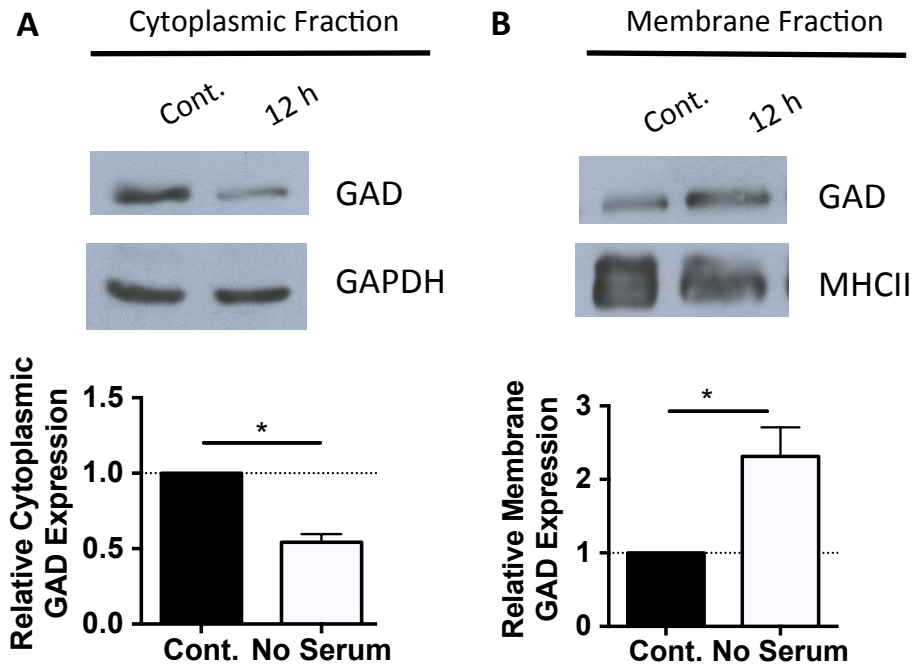
Figure 30. Serum deprivation altered GAD subcellular localization. PG cells were cultured with or without serum for 12 h, harvested and lysed into (A) cytoplasmic and (B) membrane fractions using differential ultracentrifugation. Lysates were resolved by SDS-PAGE and immunoblotted for GAD and as a loading control GAPDH (cytoplasmic) or MHC class II molecules (MHCII) (membrane). *p<0.05, Student's T-Test. Data depict results from three independent experiments, ±SEM.

To determine whether alterations in GAD distribution were the mechanism by which MHC class II presentation of GAD epitopes was disrupted by serum deprivation, Priess cells were transduced with a mutated form of GAD that is not capable of binding membranes (CytoPG) (*181*). CytoPG and PG cells were cultured with or without serum for 12 h and MHC class II presentation of an epitope derived from GAD was monitored. GAD presentation in CytoPG cells was significantly disrupted by serum deprivation, similar to wild-type GAD in PG cells (**Figure 31**). Furthermore, MHC class II presentation of a synthetic peptide

derived from GAD was unchanged by serum deprivation in both PG and CytoPG

cells (**Figure 31**). These data indicate disruptions in GAD presentation during

serum deprivation were not due to membrane attachment of GAD, but rather

perhaps GAD association with membranes is a byproduct of disruptions in GAD

degradation.
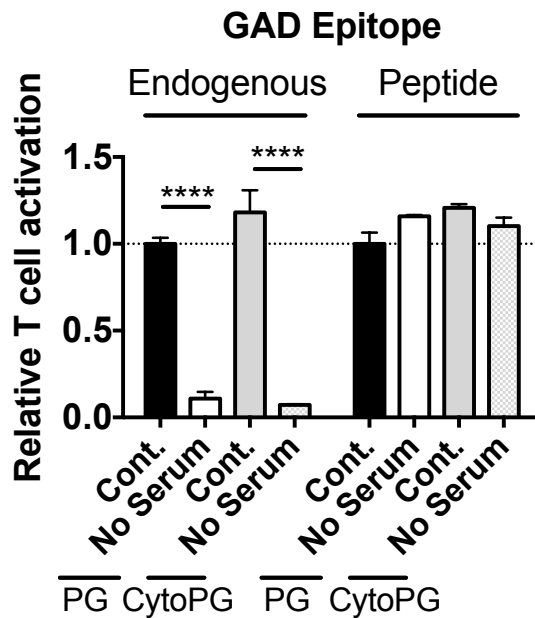
Figure 31. Serum deprivation disrupted GAD presentation regardless of subcellular distribution. PG cells or priess cells transfected to express a mutated GAD that does not associate with membranes (CytoPG) were cultured with or without serum for 12 h, harvested, fixed and MHC class II presentation of endogenous GAD or the synthetic GAD peptide was monitored. **** $p < 0.0001$, Two-way ANOVA followed by Dunnett's multiple comparisons correction. Data represents triplicates from one experiment, ±SD.

Together these data show serum deprivation alters the degradation of

endogenous Ags. While intracellular protease activity is enhanced, degradation

of specific Ags is impaired. This is likely due to altered trafficking of these Ags.

Reduced cellular endocytosis leads to disruptions in the internalization of the

BCR, preventing its degradation. Furthermore, GAD subcellular distribution is

altered during serum deprivation. While association with membranes is likely not

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the cause of disruptions in GAD presentation, altered subcellular distribution

during serum deprivation indicates disruptions in the trafficking of this Ag.

Chapter 3.

Exogenous macromolecules restored intracellular antigen trafficking and presentation during serum deprivation

Serum is macronutrient rich, containing proteins, lipids and complex carbohydrates. Some proteins such as growth factors and cytokines may have potent biological activity, which could impact B cell function. Studies have shown that loss of specific micronutrients such as zinc can affect global immune responses (*182*). The contribution of the different components of serum to the growth of cells has not been well established. While serum deprivation altered the trafficking of Ags in B cells, studies here establish whether these alterations are due to reduced levels of macronutrients in general or attributed to the availability of a specific growth factor, protein or carbohydrate.

Macronutrients restored BCR surface expression in a size dependent manner

To determine the components of serum responsible for affecting B cell Ag trafficking, serum components were fractionated using filters with molecular mass cut-offs of 30 kDa and 10 kDa. PG cells were cultured with serum, without serum or in media supplemented with <10 kDa, <30 kDa, or the >30 kDa serum fractions for 12 h prior to flow cytometric analysis of surface BCR. Internalization of BCR was reduced with serum deprivation or upon cultivation of PG cells with serum components smaller than 10 kDa. However, serum fractions with higher

molecular mass components (>10kDa) were effective at restoring surface BCR

levels (**Figure 32**). Though, studies assessing the protein concentration of these

fractions found the >30 kDa fraction contained the majority of the protein with

more then 30% (wt/vol) protein within the media whereas the smaller fractions

<10 kDa and <30 kDa were between 4-6% protein as monitored by the biorad

protein assay. This suggests a component or multiple components found within

the high molecular mass serum fraction is capable of modulating BCR trafficking.
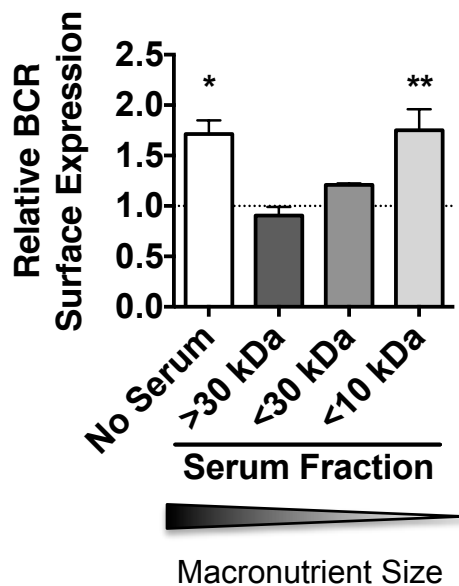
Figure 32. Supplementation with high molecular mass macronutrients prevented disruptions in BCR trafficking. (A) Serum was fractioned into >30 kDa, <30 kDa, and <10 kDa. PG was cultured for 12 h in media with or without serum or in media supplemented with these fractions. Cells were harvested and stained for flow cytometry analysis of surface BCR levels. The level of surface BCR on control cells cultured with media containing serum was normalized and set to 1 (depicted as dotted line). * $p < 0.05$ and **$p < 0.01$, One-way ANOVA followed by Dunnett's multiple comparisons correction. Data depict averages from three independent experiments, ±SEM.

To further elucidate the serum components responsible for altering BCR

trafficking, the effects of supplementing serum-free media with a single protein or

carbohydrate was assessed. Supplementation of culture media with protein BSA

(68 kDa) or with the complex carbohydrate dextran (70 kDa) at 1% (wt/vol) was

sufficient to normalize BCR surface levels (**Figure 33**). The restoration of BCR

trafficking by either BSA or dextran suggests a general role for high molecular

mass macronutrients in regulating BCR trafficking rather than a specific nutrient.

Furthermore, here media was supplemented at 1% (wt/vol) protein, which is less

then the any of the serum fractions. This suggests the lower protein

concentrations found in the smaller (<10 kDa and <30 kDa) fractions were not

contributing factors to the alterations in BCR trafficking, further suggesting a role

for high molecular mass macronutrients in regulating BCR trafficking.
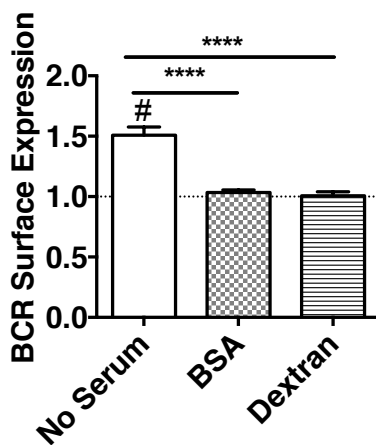
Figure 33. Exogenous macronutrients restored trafficking of the BCR. PG cells were cultured for 12 h with or without serum or in media supplemented with 1% (wt/vol) exogenous BSA or 70 kDa dextran. Cells were harvested and stained for flow cytometry analysis of surface BCR levels. The level of surface BCR on control cells cultured with media containing serum was normalized and set to 1 (depicted as dotted line) and the fold change relative to this control graphed.
**** $p < 0.0001$, One-way ANOVA followed by Dunnett's multiple comparisons correction. #$p < 0.05$, Student's T-Test to Cont. cells. Data depict averages from three independent experiments, ±SEM.

To elucidate if fatty-acids associated with BSA or if protein conformation was

responsible for preventing disruptions in BCR trafficking, PG cells were cultured

with serum, without serum or in serum-free media supplemented with denatured

(boiled) or fatty-acid free BSA. Denatured or fatty acid-free BSA also restored

surface BCR levels, suggesting protein conformation or bound fatty acids were

not critical (**Figure 34**). These data further suggest BCR trafficking is regulated

by high molecular mass macronutrients and that the biological function and

conformation of these nutrients does not matter.
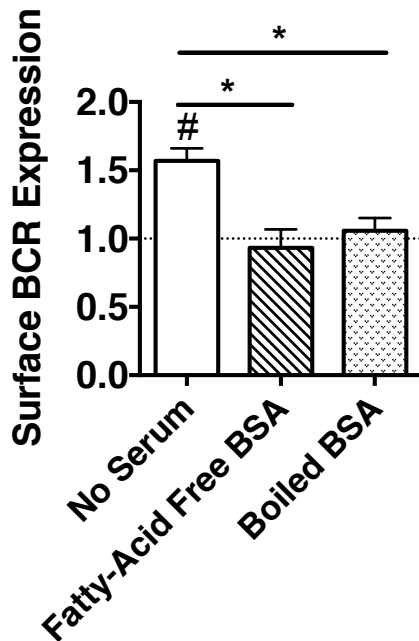
Figure 34. Fatty-acid free and boiled BSA prevented alterations in BCR trafficking. PG cells were cultured for 12 h with or without serum or in media supplemented with 1% (wt/vol) exogenous fatty-acid free or boiled BSA. Cells were harvested and stained for flow cytometry analysis of surface BCR levels. The level of surface BCR on control cells cultured with media containing serum was normalized and set to 1 (depicted as dotted line) and the fold change relative to this control was graphed. * $p<0.05$, One-way ANOVA followed by Dunnett's multiple comparisons correction. #$p<0.05$, Student's T-Test to Cont. cells. Data depict averages from three independent experiments, ±SEM.

Consistent with the serum fractionation, larger macromolecules or macronutrients

were more effective at restoring surface BCR levels as shown by supplementing

media with different sized proteins: BSA, OVA (45 kDa) or HEL (14 kDa) or

various sized dextrans (**Figures 35**). This further confirms larger macronutrients,

but not small molecular mass molecules, direct BCR trafficking. These data

suggest B cells are capable of sensing and distinguishing extracellular

macronutrients based on size and alter endocytic trafficking pathways based on

macronutrient size.
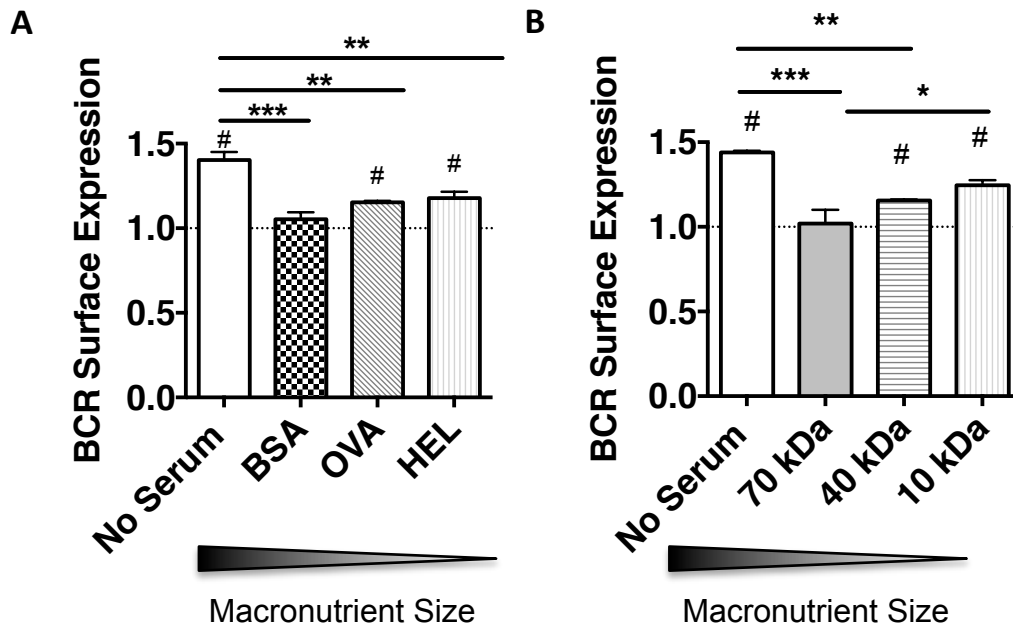
Figure 35. Macromolecular size influenced BCR trafficking. PG cells were cultured for 12 h with or without serum or in media supplemented with 1% (A) BSA (68 kDa), ovalbumin (OVA, 45 kDa), hen-egg lysozyme (HEL, 14 kDa) or (B) various sized dextrans (70, 40 or 10 kDa). The level of surface BCR on control cells cultured with media containing serum was normalized and set to 1 (depicted as dotted line) and the fold change relative to this control was graphed. *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, One-way ANOVA followed by Dunnett's multiple comparisons correction. #$p < 0.05$, Student's T-Test to Cont. cells. Data depict averages from three independent experiments, ±SEM.

To determine if restoration of BCR trafficking was dependent on endocytosis or macropinocytosis, cells were treated with amiloride, a sodium channel inhibitor which disrupts these pathways (*183*). Macropinocytosis engulfs extracellular macromolecules and membrane proteins independent of clathrin (*184*). In APCs, the resulting macropinosomes ultimately fuse with mature endosomal/lysosomal vesicles rich in MHC class II molecules (*185*). Amiloride addition abrogated the effects of BSA in restoring BCR surface expression, suggesting macronutrient

normalization of BCR trafficking was dependent on macropinocytosis or

endocytosis (**Figure 36**). Thus, B cell sensing of exogenous macronutrients

effectively altered surface BCR expression by modulating endocytic pathways.
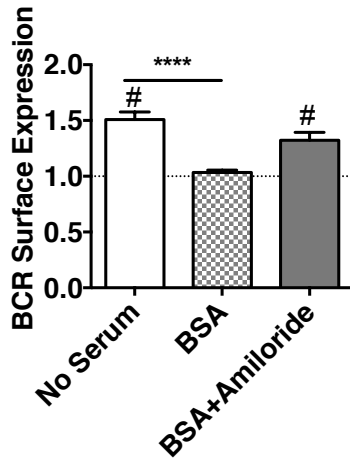
Figure 36. Amiloride blocked macronutrient restoration of BCR trafficking. PG cells were cultured with or without serum or in media supplemented with BSA for 12 h. Cells were also treated with or without amiloride, an inhibitor of macropinocytosis or endocytosis. Cells were fixed for flow analysis of BCR surface levels. The level of surface BCR in Cont. serum-fed cells was normalized and set to 1. BCR surface levels relative to this control were graphed. ****p0<.0001, One-way ANOVA followed by Dunnett's multiple comparisons correction. #p<0.05, Student's T-Test. Data depicts averages of three or more independent experiments, ±SEM.

Furthermore, BSA supplementation restored global defects in clathrin-mediated

endocytosis as trafficking of TfR was restored (**Figure 37 A**). In contrast, BSA did

not alter CD45R surface levels, a surface protein that is not internalized via

endocytosis (**Figure 37 B**). This suggests that B cell sensing of exogenous high

molecular mass macronutrients such as protein affects trafficking within the cell,

specifically trafficking through the clathrin-mediated endocytic network.
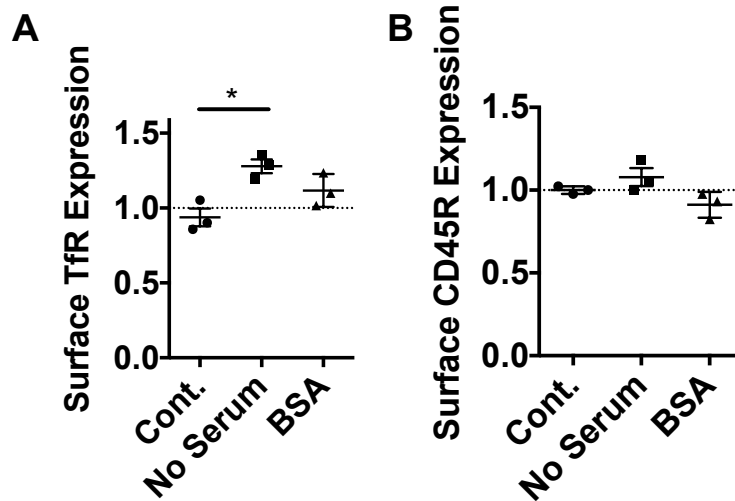
Figure 37. Macronutrients prevented disruptions in transferrin receptor trafficking. PG cells were cultured for 12 h with or without serum or in media supplemented with 1% BSA (68 kDa). PG cells were harvested and fixed for flow analysis of surface (A) TfR and (B) CD45R (B220). The level of surface TfR or CD45R on control cells cultured with media containing serum was normalized and set to 1 (depicted as dotted line) and the fold change relative to this control was graphed. * $p < 0.05$, One-way ANOVA followed by Dunnett's multiple comparisons correction. Data depict averages from three independent experiments, ±SEM.

High molecular mass macronutrients restored antigen trafficking and

presentation

To further assess the effect of macronutrient supplementation on Ag subcellular

distribution, trafficking of the BCR and GAD were monitored. Supplementation of

media with BSA was sufficient to prevent disruptions in BCR internalization

during serum deprivation (**Figure 38 A**). Furthermore, BSA supplementation

prevented disruptions in MHC class II internalization (**Figure 38 B**).
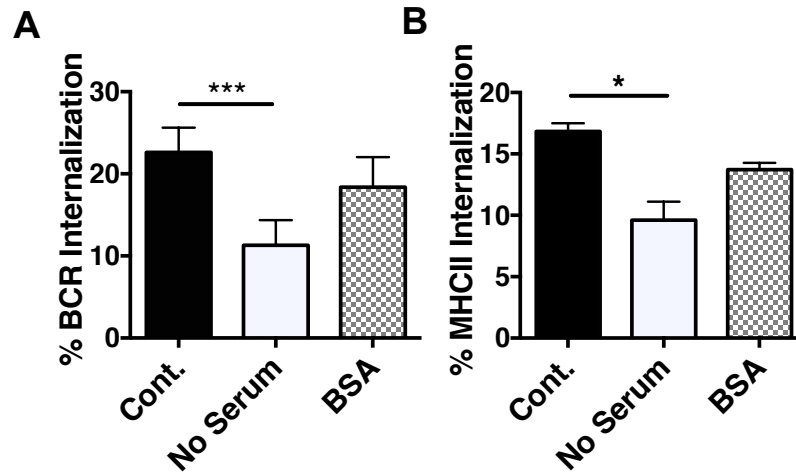
Figure 38. Macronutrient supplementation prevented disruptions in endocytosis. PG surface proteins were biotinylated prior to incubation with or without serum or in media supplemented with 1% BSA to allow protein internalization. After incubation, remaining surface biotin was removed. Cells were then lysed for ELISA analysis of biotinylation of the (A) BCR and (B) MHC class II (MHCII). * $p<0.05$ and *** $p<0.001$, One-way ANOVA followed by Dunnett's multiple comparisons correction. Data depict averages from three or more independent experiments, ±SEM.

These data indicate that exogenous macronutrient sensing regulates the endocytic network. To determine if alterations in subcellular distribution of GAD were caused by macronutrient sensing as well, PG cells were cultured with or without serum or in serum-free media supplemented with BSA for 12 h. Cells were lysed and cytoplasmic proteins were isolated from membrane associated proteins by differential ultracentrifugation. Immunoblot analysis revealed BSA supplementation prevented disruptions in GAD subcellular localization (**Figure 39**). Together these data show macronutrient regulation of Ag trafficking within B cells.
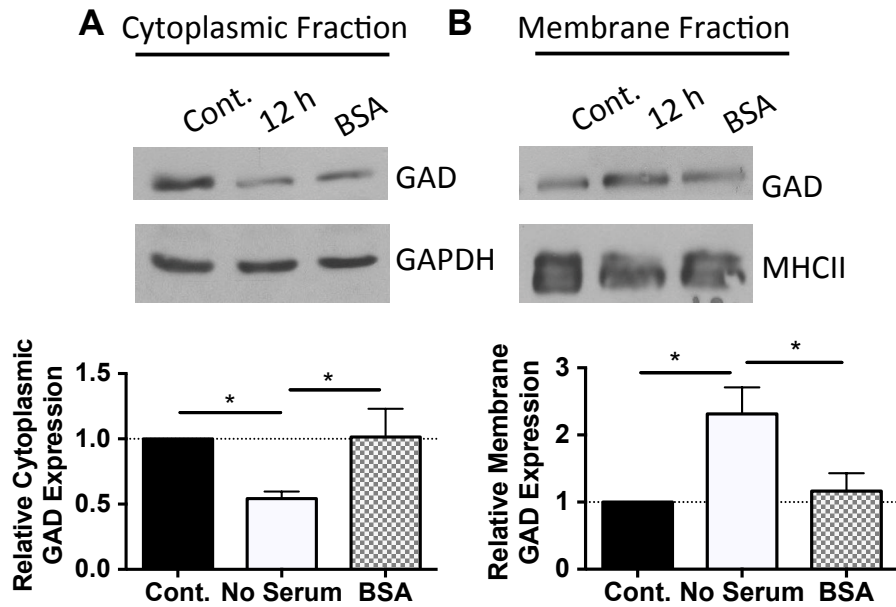
Figure 39. Macronutrient supplementation restored GAD subcellular localization during serum deprivation. PG cells were cultured with or without serum or in serum-free media supplemented with BSA for 12 h, harvested and lysed into (A) cytoplasmic and (B) membrane fractions using differential ultracentrifugation. Lysates were resolved by SDS-PAGE and immunoblotted for GAD and as a loading control GAPDH (cytoplasmic) or MHCII (membrane). *$p<0.05$, One-way ANOVA followed by Dunnett's multiple comparisons correction. Data is representative of three independent experiments, ±SEM.

To assess the effect of macronutrients on Ag presentation, MHC class II presentation of epitopes derived from GAD and BCR was monitored in PG cells cultured with or without serum or in media supplemented with BSA. Disruptions in MHC class II presentation of an epitope derived from GAD during serum deprivation were prevented by supplementing media with BSA (**Figure 40 A**). Furthermore, BSA conformation did not contribute to prevention of disruptions in GAD presentation, as boiled BSA was as effective at preventing these disruptions as native BSA (**Figure 40 A**). BSA supplementation was also effective at preventing disruptions in MHC class II presentation of an epitope

derived from the BCR (**Figure 40 B**). Supplementation of media with BSA was

successful at preventing disruptions in MHC class II presentation, though

supplementation with this macronutrient did not alter MHC class II surface

expression (**Figure 41**).
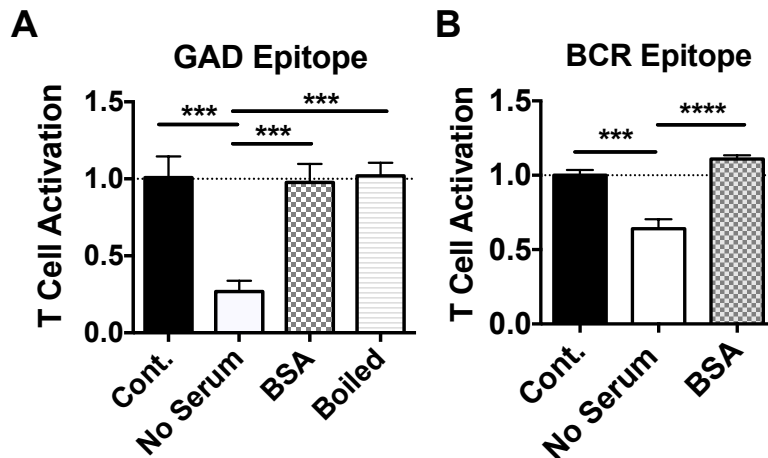
Figure 40. Macronutrients prevented disruptions in MHC class II antigen presentation during serum deprivation. PG cells were cultured for 12 h with or without serum or in media supplemented with 1% BSA (68 kDa) or boiled BSA. MHC class II presentation of an epitope derived from (A) GAD and (B) BCR were monitored. ***p<0.001 and ****p<0.0001, One-way ANOVA followed by Sidak's multiple comparisons correction. Data depict means from three or more independent experiments, ±SEM.
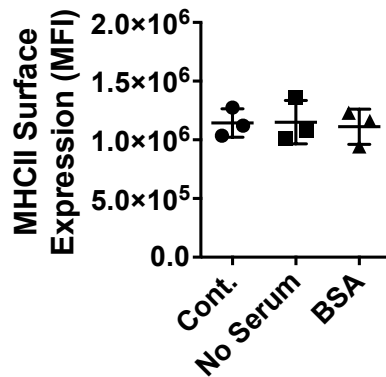
Figure 41. Macronutrient supplementation did not alter MHC class II surface levels. PG cells cultured with or without serum or in media supplemented with 1% BSA for 12 h. Cells were harvested and stained for flow cytometry analysis of surface MHC class II molecules. One-way ANOVA followed by Dunnett's multiple comparisons. Data depict MFI from three independent experiments, ±SEM.

To determine the concentration of BSA necessary for restoring MHC class II

presentation, PG cells were cultured with various concentrations of BSA and

MHC class II presentation of epitopes derived from the GAD Ag was assessed

(**Figure 42**). Here, BSA supplementation at levels as low as 0.01% were capable

of preventing disruptions in GAD presentation. This suggests low levels of

macronutrients restore Ag trafficking through this pathway.
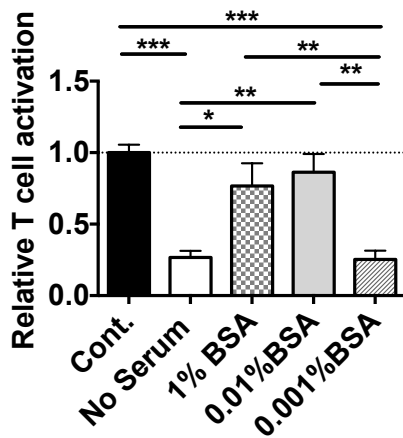
Figure 42. Low concentrations of macronutrients prevented disruptions in MHC class II antigen presentation during serum deprivation. PG cells were cultured for 12 h with or without serum or in media supplemented with various concentrations of BSA (68 kDa) and MHC class II presentation of an epitope derived from GAD was monitored. *$p<0.05$ **$p<0.01$ and ***$p<0.001$, One-way ANOVA followed by Tukey's multiple comparisons correction. Data is representative of 3 independent experiments, ±SD.

To examine if macronutrient deprivation globally regulates Ag endocytosis, MHC

class II presentation of another membrane Ag that utilizes clathrin-mediated

endocytosis was assessed. Similar to the BCR, MHC class II presentation of an

epitope derived from the constitutively expressed MHC class I membrane protein

was significantly impaired by serum deprivation and BSA supplementation

partially prevented this disruption (**Figure 43 A**). Interestingly, surface expression

of MHC class I was unaltered by serum deprivation (**Figure 43 B**). While serum

deprivation enhanced surface expression of the BCR, MHC class I and MHC

class II surface expression were unaltered (**Figure 9**). Studies assessing the

specific internalization of MHC class II did reveal reduced endocytosis of this

protein during serum deprivation (**Figure 27 and 28**), suggesting altered

trafficking of this protein even though surface expression remains unaltered.
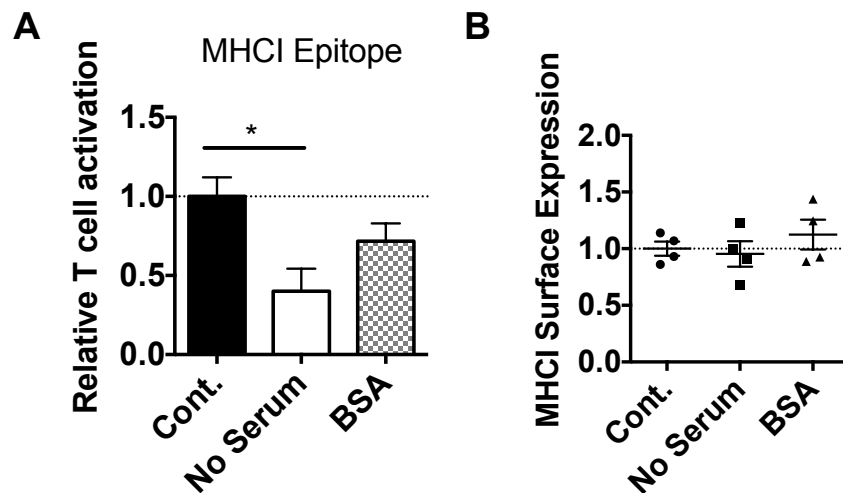
Figure 43. Macronutrient deprivation disrupted MHC class II presentation of an epitope derived from the MHC class I membrane antigen. PG cells were cultured for 12 h with or without serum or in media supplemented with 1% BSA (68 kDa). (A) MHC class II presentation of an epitope derived from MHC class I (MHCI) was monitored or (B) cells were fixed for flow analysis of surface MHCI and the relative MFI graphed. *$p < 0.05$, One-way ANOVA followed by Dunnett's multiple comparisons correction. Data depict means from three or more independent experiments, ±SEM.

At this low level, macronutrient supplementation did not prevent the

induction of macroautophagy or protease activity

Macronutrient supplementation prevented disruptions in BCR and GAD Ag

trafficking. To assess whether supplementation with macronutrients also

prevented alterations in MA pathway and protease activity with serum

deprivation, PG cells were cultured with or without serum or in media

supplemented with BSA prior to analysis of MA flux or protease activity. MA flux

remained elevated in BSA treated cells, indicating macronutrient

supplementation at this concentration did not prevent induction of the MA

pathway (**Figure 44**). Consistent with MA remaining elevated with BSA

supplementation, MHC class II presentation of an epitope derived from the MA

targeted MP1 Ag remained elevated with BSA supplementation (**Figure 45**). This

suggests the MA pathway is more sensitive to macronutrient deprivation than the

endocytic and CMA pathways, as these pathways were restored at this low

concentration of BSA.
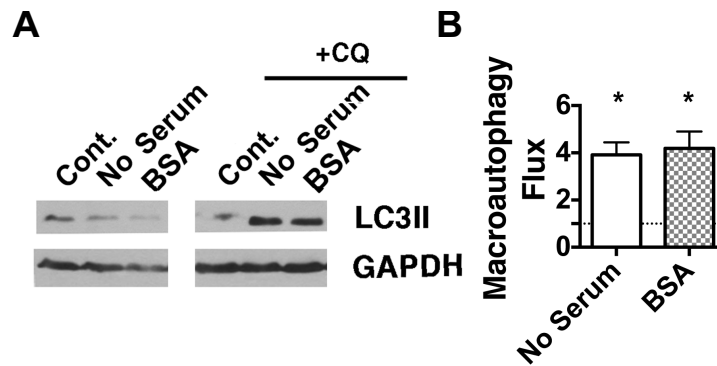
Figure 44. Macroautophagy remained elevated in macronutrient supplemented cells. PG cells were cultured with (Cont.) or without serum for 12 h in the presence or absence of chloroquine (CQ), harvested and lysed for immunoblot analysis of LC3II expression. LC3II expression was compared to Cont. cells and GAPDH was used as a loading control. (B) To quantitate macroautophagy flux, LC3II protein levels were normalized to the loading control GAPDH. LC3II:GAPDH basal levels were subtracted from the those seen in CQ treated cells for each treatment. The level of MA flux in serum-treated cells was normalized to 1 and used as a control (dotted line). Fold change relative to this control was graphed for cells cultured in serum-free media or with BSA. *$p < 0.05$, Student's T-Test to Cont. cells. Data depict MA flux from three independent experiments, ±SEM.
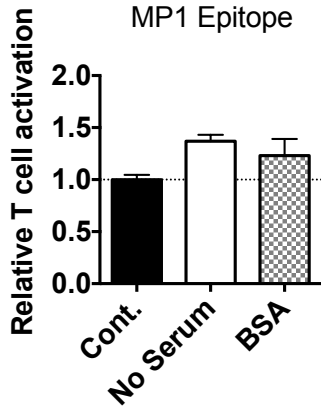
Figure 45. MP1 antigen presentation remained elevated in macronutrient supplemented cells. PG cells were cultured for 12 h with or without serum or in media supplemented with 1% BSA (68 kDa) prior to fixation and detection of MHC class II presentation of an epitope derived from the MA targeted MP1 Ag. Data depict means from three independent experiments, ±SEM.

To further assess the effect of macronutrients on the endosomal network, protease activity was monitored in cells cultured with BSA. BSA and dextran supplementation did not prevent induction of lysosomal protease activity as seen by elevated cathepsin B and L activity in PG cells cultured in media supplemented with these macromolecules (**Figure 46**).
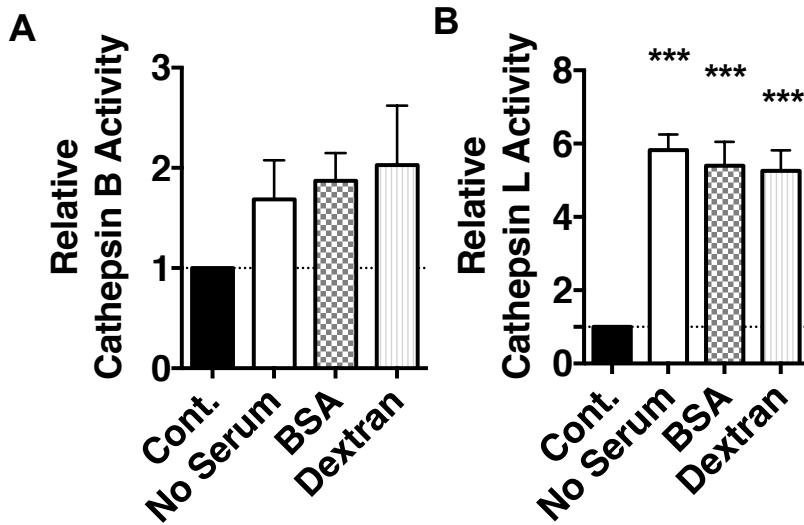
Figure 46. Cathepsin activity remained elevated during macronutrient supplementation. (A and B) Real-time analysis of cathepsin B and L activity in PG cells cultured with or without serum or in media supplemented with 1% BSA or dextran for 12 h. ***p<0.001, One-way ANOVA, Dunnett's multiple comparisons correction. Data depict averages from three independent experiments, ±SEM.

Serum deprivation appeared to alter the phenotype of B cells. During serum deprivation, the normally suspended PG cells settled onto the plate as observed by monitoring the media and light microscopy, though macronutrient supplementation did not prevent this change in phenotype (**Figure 47 A**). A similar adherence of peripheral blood B cells was observed with serum deprivation. Altering the protein and lipid composition of the media may result in changes in osmolarity and viscosity, both of which may affect cell biology. To assess this, the osmolarity and viscosity of IMDM media alone or supplemented with BSA, dextran or serum was determined (**Figure 47 B**). These data indicate serum, dextran and BSA did not significantly alter osmolarity or viscosity of IMDM media. Furthermore, the different macronutrient supplementations did not affect the pH of the media, with the pH of the control media with serum, serum-free media and BSA supplemented media ranging from 7.2 to 7.3. Furthermore, electron microscopy revealed no significant alterations in cell phenotype (**Figure 48**).

A

Cont.　　　No Serum　　　BSA
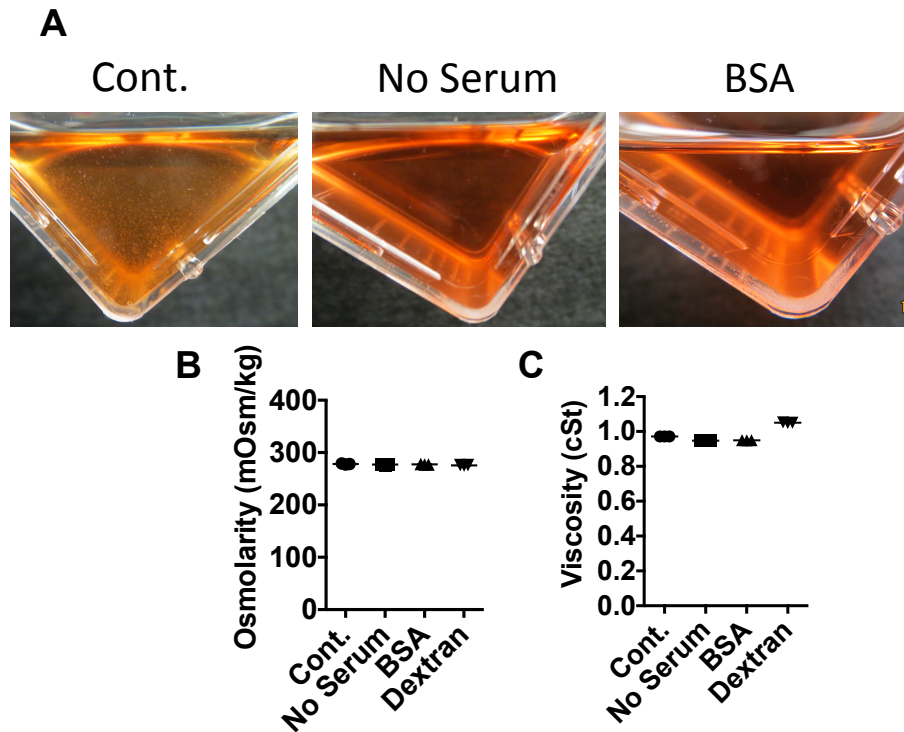
Figure 47. Serum deprivation altered the phenotype of B cells. (A) Picture of PG cells cultured with or without serum for 12 h. Cells cultured with serum grew in clusters in suspension, whereas serum-deprived and BSA-fed cells settled onto the flask surfaces. The (B) osmolarity and (C) viscosity of culture media alone or containing serum, BSA or dextran.

Cont.　　　No Serum　　　BSA
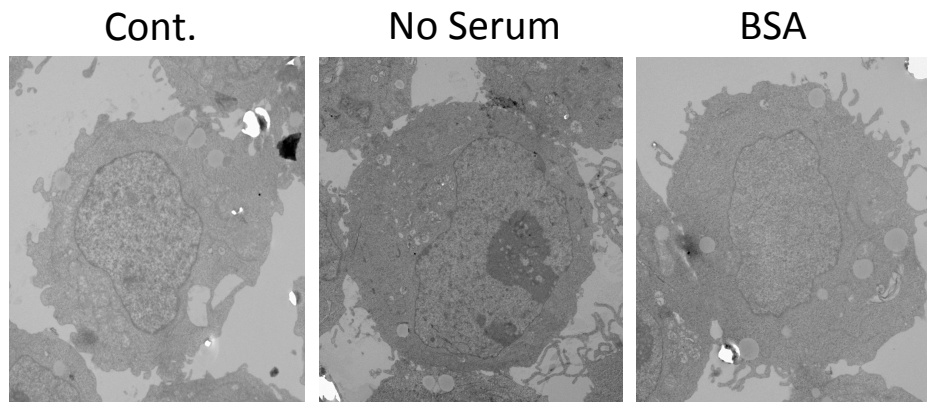
Figure 48. Electron microscopy of PG cells cultured with different media. PG cells were cultured with or without serum or in media supplemented with BSA for 12 h prior to fixation in 2% glutaraldhyde and imaging.

Together, these data indicate that B cell sensing of macronutrients regulates Ag trafficking, affecting in some cases the steady-state subcellular localization of these proteins. Furthermore, these alterations in trafficking impact the availability of epitopes derived from these Ags, altering MHC class II presentation. While supplementation with a single macronutrient such as protein was sufficient to restore Ag trafficking and MHC class II Ag presentation, this was dependent on the molecular mass of the macronutrient. Supplementation with smaller macronutrients was not able to fully restore Ag trafficking, while supplementation with larger (>70 kDa) macronutrients fully averted alterations in Ag trafficking and presentation observed in serum deprived cells. In contrast, these low levels of macronutrient supplementation did not prevent the induction of MA nor cathepsin activity suggesting these alterations are more sensitive to macronutrient deprivation.

Chapter 4.

A role for HSC70 in antigen trafficking during macronutrient deprivation

While GAD and the BCR reach the endocytic pathway by seemingly disparate pathways, macronutrient deprivation altered their trafficking, degradation and MHC class II presentation in some similar ways. These data suggest a common link between these pathways may exist which could be altered by macronutrient deprivation.

HSC70 is a conserved chaperone found in the cytoplasm as well as the lumen of lysosomes. HSC70 plays a role in several pathways linked to protein folding and transport within cells, contributing to cell homeostasis. Studies have revealed HSC70 is required for endocytosis, chaperoning clathrin and providing the energy necessary to assemble and disassemble the clathrin coat (*76, 109*). This suggests HSC70 may be required for BCR trafficking via endocytosis. Furthermore, HSC70 is known to chaperone proteins to the lysosomal membrane for translocation in the selective form of autophagy, CMA (*106*). Our laboratory has shown HSC70 is required for MHC class II presentation of the cytoplasmic GAD Ag, through the CMA pathway (*78*). Together, these studies indicate that the Ag processing pathways for the BCR and GAD may share a requirement for the chaperone HSC70. Studies in this chapter demonstrate a role for HSC70 in Ag trafficking and presentation during macronutrient deprivation.

Macronutrient deprivation did not alter HSP protein expression

HSC70 is required for both endocytosis and CMA, the pathways required for

BCR and GAD presentation by MHCII, respectively. To assess whether

macronutrient deprivation altered HSC70 expression, PG cells were cultured with

or without serum for up to 48 h. PG cells were harvested and mRNA expression

was monitored. Expression of HSC70 (*HSPA8*) mRNA was not significantly

altered during serum deprivation although an initial trend towards increase in this

mRNA was detected in the first 12 h of starvation (**Figure 49**). HSP90, another

constitutively expressed HSP found in both the cytoplasm as well as the lumen of

lysosomes, is also required for GAD presentation and can interact with HSC70 in

the cytoplasm (*97, 123*). Here, mRNA expression of the cytoplasmic HSP90

isoforms, HSP90α (*HSP90AA*) and HSP90β (*HSP90AB*), were monitored in PG

cells. Although extended periods of serum deprivation led to a significant drop in

HSP90AA, short periods (up to 12 h) did not significantly alter the expression of

these mRNAs, though there was a trend towards higher *HSP90AB* during early

time points (**Figure 49**).
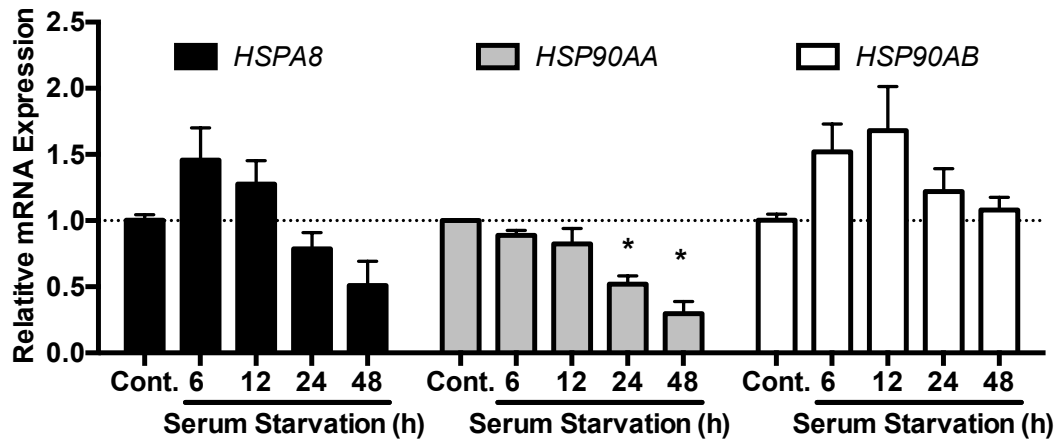
Figure 49. Macronutrient deprivation for up to 24 h did not alter HSC70 and HSP90 mRNA expression. PG cells were cultured with serum (Cont.) or without serum for 6, 12, 24 or 48 h. Cells were harvested, mRNA isolated and cDNA synthesized for qRT-PCR analysis of *HSPA8* (HSC70), *HSP90AA* (HSP90a) and *HSP90AB* (HSP90b). *$p < 0.05$, Two-way ANOVA followed by Holme's Sidak comparisons correction. Data depict averages from three independent experiments, ±SEM.

To further assess HSC70 expression during macronutrient deprivation, HSC70 protein levels were monitored in PG cells cultured with or without serum or in media supplemented with BSA for 12 h. HSC70 protein levels remained constant in these cells regardless of the availability of macronutrients (**Figure 50**). Furthermore, HSP90 protein expression was unaltered by serum deprivation (**Figure 51**).

A

Cont. No Serum BSA

HSC70

GAPDH

B
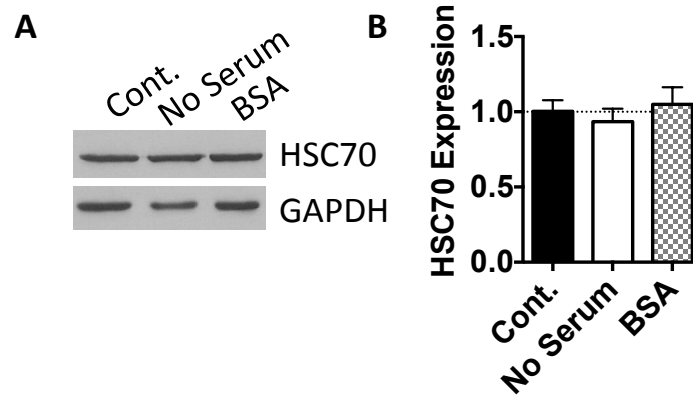
Figure 50. Macronutrient deprivation did not alter HSC70 protein expression. PG cells were cultured with serum (Cont.), without serum or in serum-free media supplemented with BSA for 12 h. (A) Cells were harvested and lysed for immunoblot analysis of HSC70. (B) Densitometry was used to quantitate relative levels of HSC70 to GAPDH. Data representative of three independent experiments, ±SEM.

Cont. No Serum
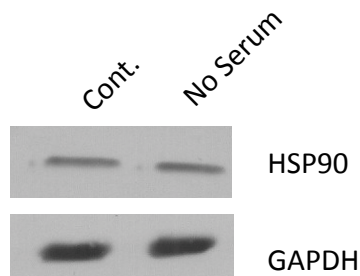
HSP90

GAPDH

Figure 51. Macronutrient deprivation did not alter HSP90 protein expression. PG cells were cultured with serum (Cont.) or without serum for 12 h. Cells were harvested and lysed for immunoblot analysis of HSP90 and GAPDH. Data representative of three independent experiments.

HSC70 is a cytosolic protein that can be found in the cytoplasm and on the

surface of membranes including autophagosomes. To assess whether

macronutrient deprivation altered the location of HSC70, PG cells were cultured

with or without serum for 12 h prior to separation of cytosolic from membrane

associated fractions. Immunoblot analysis of these different subcellular fractions

revealed HSC70 distribution on membranes was not altered by serum

deprivation (**Figure 52**).
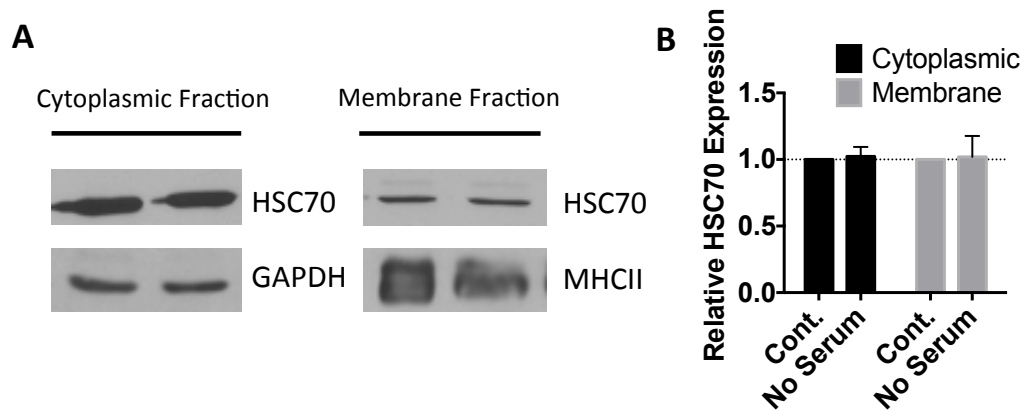
Figure 52. HSC70 subcellular distribution was unaltered by macronutrient deprivation. PG cells were cultured with or without serum for 12 h, harvested and lysed into (A) cytoplasmic and membrane fractions using differential ultracentrifugation. Lysates were resolved by SDS-PAGE and immunoblotted for HSC70 and as a loading control GAPDH (cytoplasmic) or MHC class II (MHCII) (membrane). **(**B) Densitometry was used to quantitate the ratio of HSC70 to GAPDH or MHC class II. Data depict results from three independent experiments, ±SEM.

Macronutrient deprivation impaired HSC70 association with cytoplasmic

GAD antigen

To assess whether serum deprivation altered HSC70 activity, GAD association

with HSC70 was monitored in PG cells cultured with or without serum or in media

supplemented with BSA. While GAD associated with HSC70 in control serum-

treated cells and BSA supplemented cells at similar levels, macronutrient

deprivation significantly reduced the association of GAD and HSC70 (**Figure 53**).

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These data suggest macronutrient deprivation may alter the accessibility and function of HSC70.
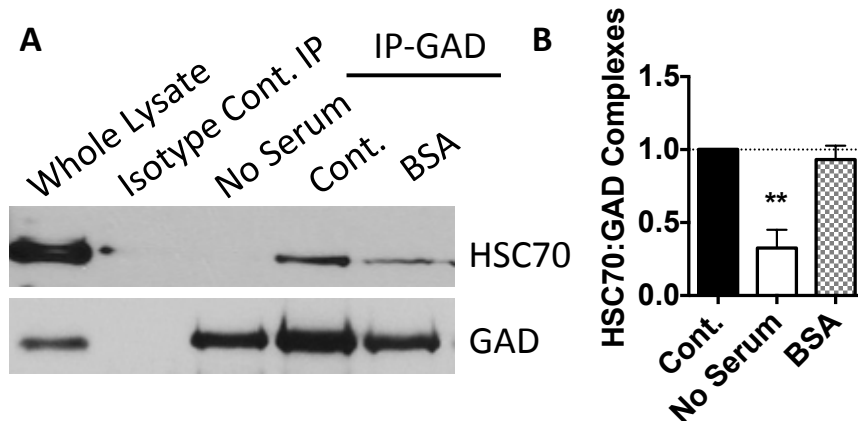
Figure 53. Macronutrient deprivation altered HSC70 association with GAD. PG cells were cultured with or without serum or in serum-free media supplemented with BSA for 12 h. Cells were harvested and lysed for immunoprecipitation of GAD. GAD and associated proteins were resolved by SDS-PAGE and (A) HSC70 or as a loading control GAD were detected by immunoblot. (B) HSC70 levels were quantitated using densitometry and relative levels of HSC70 to GAD were graphed. Data depict results from three independent experiments, ±SEM. **p<0.01, One-way ANOVA followed by Dunnett's multiple comparisons correction.

As HSC70 is vital for the CMA pathway, the functional activity of this pathway was assessed. LAMP2A is the transmembrane protein found on lysosomes that assists in the translocation of proteins during CMA. Studies have suggested LAMP2A is the limiting factor regulating CMA, with reduced levels of LAMP2A diminishing CMA activity (*186*). LAMP2 protein levels were assessed during macronutrient deprivation. LAMP2 protein levels rose during early stages of serum deprivation (**Figure 54**). Importantly, the LAMP2 Ab detects not only LAMP2A but also the LAMP2B and LAMP2C isoforms. LAMP2B is involved in

lysosome fusions and may be important for MA (*187*). The function of LAMP2C

has not been well described, though it may be involved in translocation of RNA

from the cytosol to the lysosome in a form of RNAphagy (*166*). To distinguish

changes in the expression of these different LAMP2 isoforms, the mRNA levels

of each was assessed. No significant differences were observed in the three

isoforms at these early time points of macronutrient deprivation (**Figure 8**).

Together these data suggest that the expression of LAMP2 isoforms was not

decreased or limiting and would not contribute to impaired CMA during serum

deprivation.
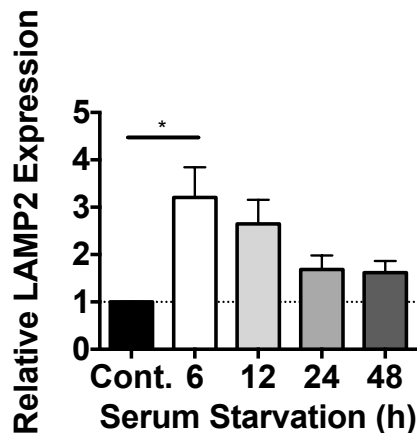
Figure 54. LAMP2 protein expression increased during serum deprivation. PG cells were cultured without serum for up to 48 h or with serum as a Cont. Cells were harvested and lysed for immunoblot analysis of LAMP2 and GAPDH protein levels. The ratio of LAMP2:GAPDH was graphed for three independent experiments, ±SEM. *p<0.05, One-way ANOVA followed by Dunnett's multiple comparisons correction.

To further monitor the effect of macronutrient deprivation on the CMA pathway,

CMA translocation of a biotin-labeled peptide was monitored. PG cells were

incubated with a biotin-labeled GAD peptide followed by electroporation to deliver the peptide into the cytosol of the cell. CMA translocation of this peptide from the cytoplasm to lysosomal and endosomal compartments was monitored by assessing MHC class II association with the electroporated peptide. Serum deprivation did not impede MHC class II association with the peptide, suggesting CMA translocation of this peptide was intact (**Figure 55**). Given this assay tracks the translocation of peptide rather than intact Ag, HSC70 may not be required. While not definitive, this study suggests the translocation steps of CMA were not perturbed by serum deprivation. This may indicate the defects in Ag trafficking by CMA are linked to the chaperone role of HSC70.

A

No electroporation   Electroporate

Lamp-2a   Lamp-2a

Wash
Acid strip to remove any surface peptide

Lamp-2a   Lamp-2a

Incubate to
allow CMA translocation of peptide

Lamp-2a   Lamp-2a

B

MHCII:GAD-B

GAPDH
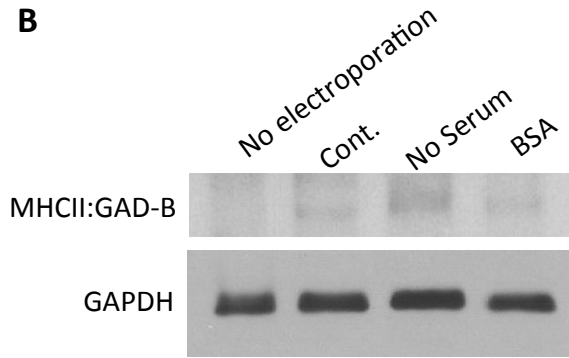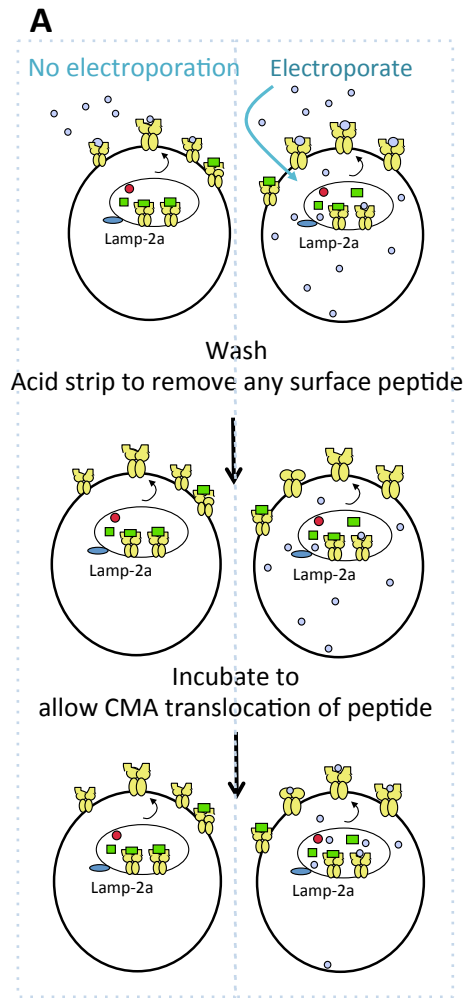
Figure 55. CMA translocation of GAD peptide was not impaired by macronutrient deprivation. (A) Diagram of the assay. PG cells were washed in ice cold HBSS and 20 uM Biotin-GAD peptide added to solution. Cells were electroporated to transfer the peptide to the cytoplasm or, as a control, not electroporated. Cells were washed and acid striped to remove any exogenous peptide or peptide bound to surface MHC class II molecules. Cells were incubated with or without serum for 16 h, harvested and lysed for (B) immunoblot analysis of biotin-GAD:MHC class II complexes. Data representative of three or more experiments.

Ectopic expression of HSC70 overcame the effects of macronutrient deprivation in B cells

To further assess the availability of HSC70 during serum deprivation, PG cells were transduced to ectopically express higher levels of cytoplasmic HSC70 (PG70). HSC70 transcript and protein levels were elevated in PG70 cells as compared to the PG parent cells (**Figure 56**).

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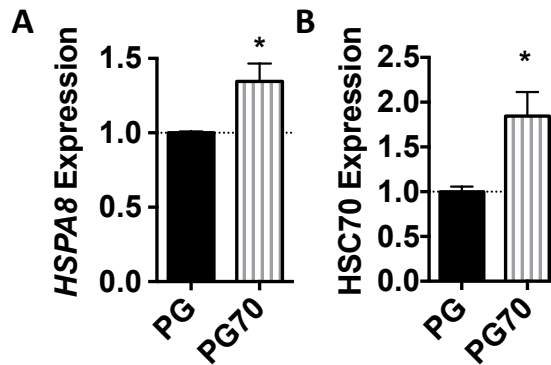
Figure 56. Ectopic expression of HSC70. PG cells were transfected with a vector encoding sense human HSC70 cDNA. PG (control) and PG70 (ectopic HSC70) cells were harvested and (A) HSC70 mRNA expression monitored by qRT-PCR (relative to GAPDH) or (B) cells were lysed for immunoblot analysis of HSC70 and GAPDH. Graphs show HSC70 relative to GAPDH from three independent experiments, ±SEM. * $p < 0.05$, Student's t-test.

As macronutrient deprivation impaired HSC70:GAD interactions (**Figure 53**), the effect of ectopic HSC70 expression on this molecular interaction was assessed during serum deprivation. PG70 cells were cultured with or without serum for 12 h and GAD protein was immunoprecipitated. Here, ectopic expression of HSC70 restored GAD association with HSC70 during macronutrient deprivation to levels seen in cells without this stress (**Figure 57**). These data suggest serum deprivation limits the availability of HSC70 to associate with GAD, as overexpression of this chaperone restored this interaction.
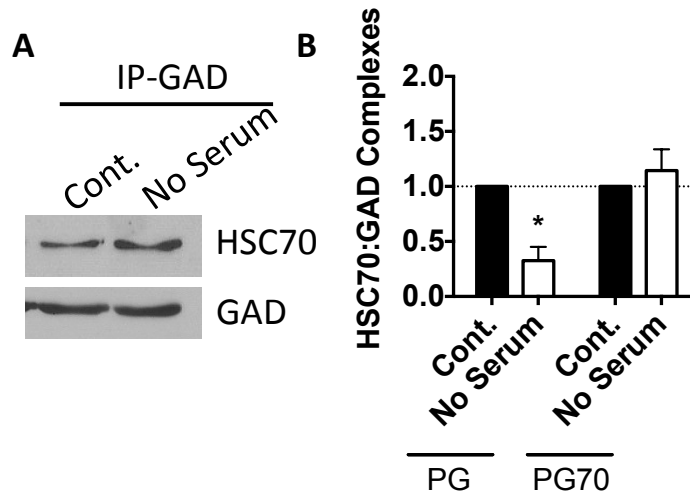
Figure 57. Ectopic expression of HSC70 overcame disruptions in HSC70:GAD association during macronutrient deprivation. PG70 or as a control the parent cell line PG were cultured with or without serum for 12 h. Cells were harvested and lysed for immunoprecipitation of GAD. GAD and associated proteins were resolved by SDS-PAGE and (A) HSC70 detected by immunoblot. (B) HSC70 levels were quantitated using densitometry and relative levels of HSC70 to GAD was graphed. *p<0.05, Student's T-Test followed by Holm-Sidak's multiple comparisons correction. Data depict results from three independent experiments, ±SEM.

To further assess the effect of HSC70 on Ag trafficking during macronutrient deprivation, GAD and BCR subcellular distribution was assessed in cells ectopically expressing HSC70. PG and PG70 cells were cultured for 12 h with or without serum prior to lysis and separation of proteins into cytoplasmic and membrane fractions. Ectopic expression of HSC70 prevented disruptions in GAD trafficking observed during serum deprivation (**Figure 58**). Furthermore, ectopic HSC70 expression restored the trafficking of BCR as seen by the normalization of surface BCR expression (**Figure 59**). Restoration of Ag trafficking with increased HSC70 expression in B cells demonstrates a key role for this chaperone in modulating cellular stress and intracellular protein transport.

A
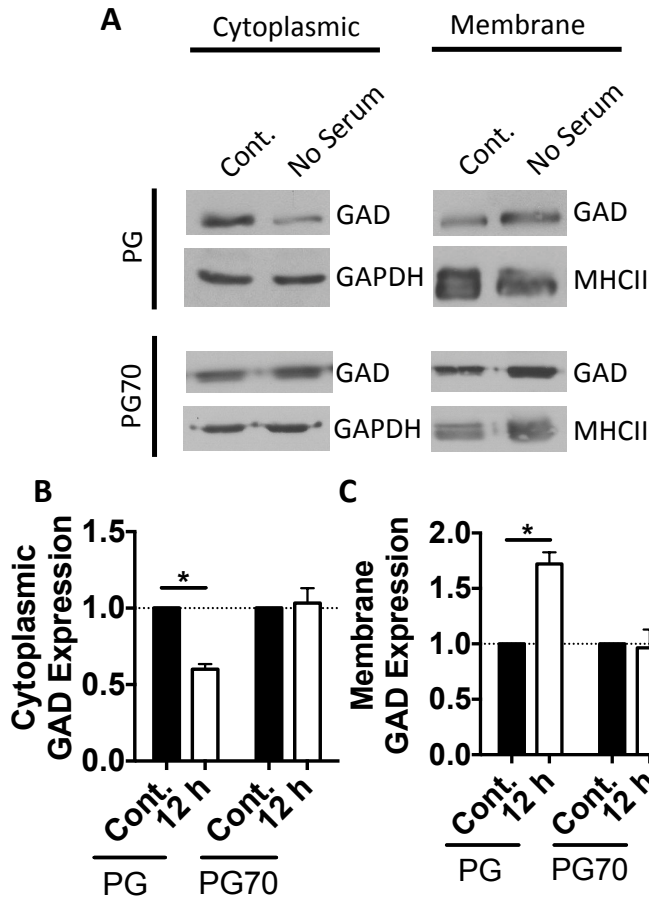
Figure 58. Ectopic expression of HSC70 prevented disruptions in GAD trafficking during macronutrient deprivation. PG and PG70 cells were cultured with or without serum for 12 h, harvested and lysed into cytoplasmic and membrane fractions using differential ultracentrifugation. (A) Lysates were resolved by SDS-PAGE and immunobloted for GAD and as a loading control GAPDH (cytoplasmic) or MHCII (membrane). Densitometric analysis of GAD protein expression relative to (B) GAPDH or (C) MHC class II (MHCII). *p<0.05, Student's T-Test followed by Holm-Sidak's multiple comparisons correction. Data are representative of three independent experiments, ±SEM.
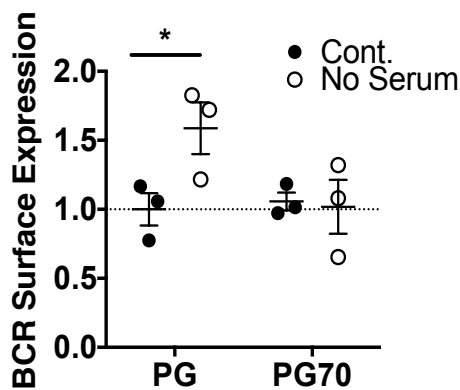
Figure 59. Ectopic expression of HSC70 prevented disruptions in BCR trafficking during macronutrient deprivation. PG and PG70 cells were cultured with or without serum for 12 h and fixed for flow analysis of the BCR surface levels. Relative surface levels of BCR for PG cells cultured with serum (Cont.) were normalized and set to one. The BCR surface expression relative to this control was graphed. *p<0.05, Two-way ANOVA followed by Sidak's multiple comparisons correction. Data representative of three independent experiments, ±SEM.

To further assess the role of ectopic expression of HSC70 on MHC class II Ag presentation, the levels of MHC class II components was monitored. Ectopic expression of HSC70 did not alter surface expression of MHC class II molecules nor surface levels of CLIP:MHC class II complexes, suggesting equivalent levels of DM activity in these cells (**Figure 60 A**). Additionally, total cellular expression of MHC class II molecules and Ii chain were unaltered by ectopic expression of HSC70 (**Figure 60 B and C**).
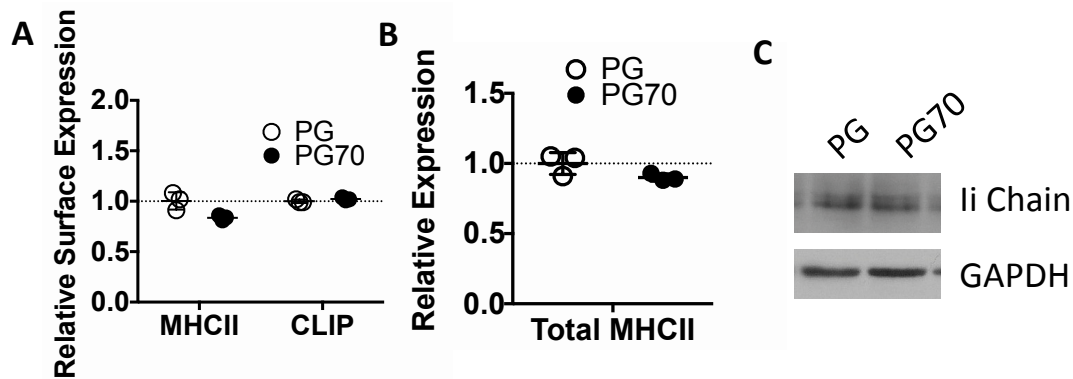
Figure 60. Ectopic expression of HSC70 did not alter MHC class II components. PG and PG70 cells were harvested and (A) surface expression of MHC class II and CLIP:MHC class II complexes were monitored or after fixation cells were permeablized for assessment of (B) total MHC class II expression by flow cytometry. (C) PG and PG70 cells were lysed for immunoblot analysis of Ii chain and GAPDH as a loading control. Data representative of 3 or more independent experiments, ±SEM.

As ectopic expression of HSC70 restored Ag trafficking, the effect of increased HSC70 on MHC class II Ag presentation was assessed. As compared to control cells, ectopic expression of HSC70 prevented disruptions in MHC class II presentation of epitopes derived from GAD, the BCR and MHC class I molecules during macronutrient deprivation (**Figure 61 A-C**). Interestingly, overexpression

of HSC70 also reversed the enhanced MHC class II presentation of epitopes derived from the MP1 Ag during macronutrient deprivation (**Figure 61 D**).
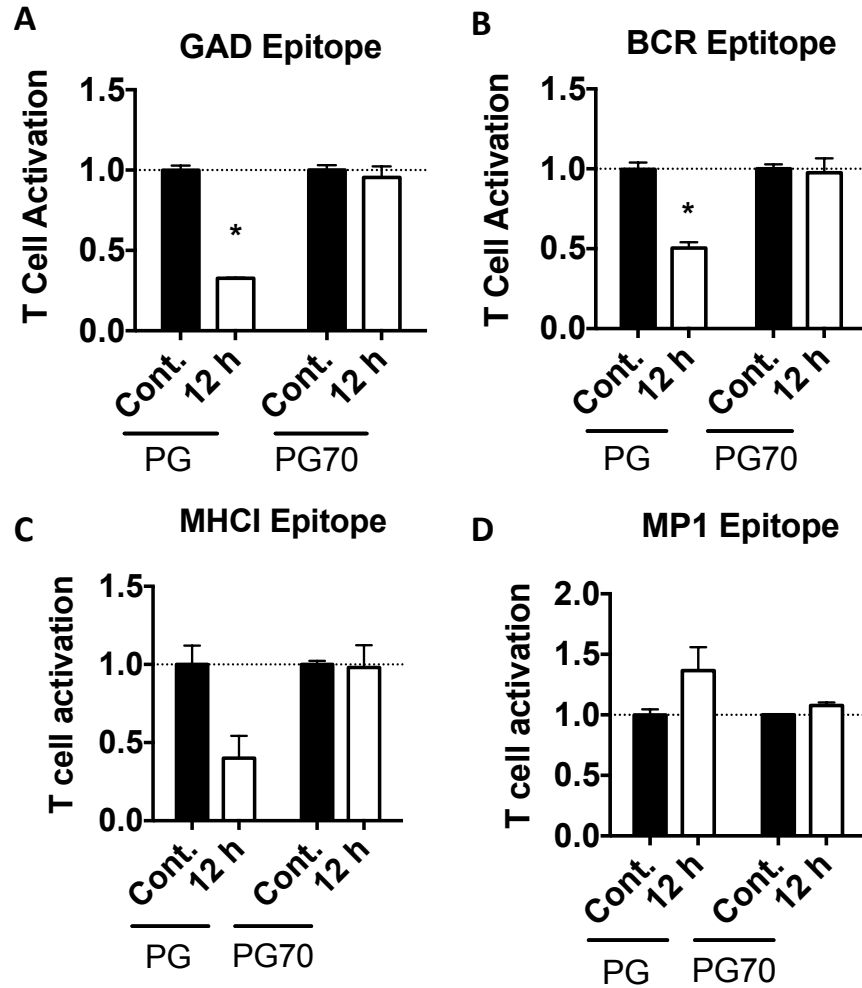
Figure 61. Ectopic expression of HSC70 prevented disruptions in MHC class II presentation of epitopes during macronutrient deprivation. PG and PG70 cells were cultured with or without serum for 12 h, harvested and fixed for analysis of MHC class II presentation of epitopes derived from (A) GAD, (B) the BCR, (C) MHC class I and (D) MP1. *p<.05, Two-way ANOVA followed by Sidak's multiple comparisons correction. Data depict means from three independent experiments, ±SEM.

As MP1 gains access to the endosomal network through MA, MA flux was monitored in these cells. Consistent with MP1 presentation, MA was induced in serum deprived PG cells and in PG cells cultured in media supplemented with BSA, though ectopic expression of HSC70 prevented this induction during serum deprivation (**Figure 62**). Together, these data suggest HSC70 availability may regulate MA during cell stress.
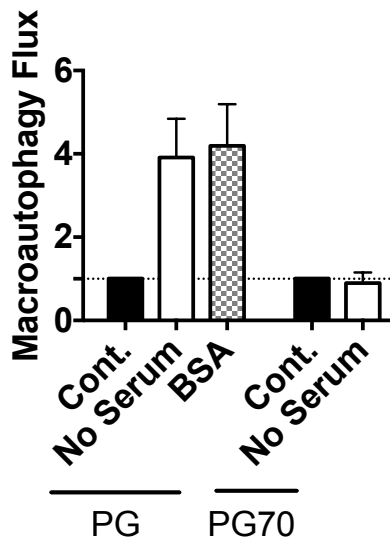
Figure 62. Ectopic expression of HSC70 prevented macroautophagy induction during serum deprivation. PG and PG70 cells were cultured with (Cont.) or without serum or in media supplemented with BSA for 12 h in the presence or absence of CQ to block lysosomal acidification and LC3II degradation. Cells were harvested, and lysed for immunoblot analysis of LC3II expression. To quantitate MA flux, LC3II levels were normalized to the loading control GAPDH and LC3II:GAPDH basal levels were subtracted from the induced CQ treated levels. MA flux in Cont. cells was normalized to 1 and fold change relative to this control graphed. Data depict MA flux from 3 independent experiments, ±SEM.

Overall, ectopic HSC70 expression in B cells prevented disruptions in Ag trafficking and presentation during macronutrient deprivation, demonstrating a key role for this chaperone in modulating cellular stress and intracellular protein transport. Here, ectopic expression of HSC70 prevented disruptions in several Ag trafficking pathways observed during macronutrient deprivation. While

macronutrient deprivation did not significantly affect total cellular HSC70 levels, the role of HSC70 in several pathways including its participation in GAD translocation via CMA were significantly disrupted. Ectopic expression to increase cellular HSC70 levels was sufficient to prevent these disruptions, suggesting the availability of this shared chaperone is limiting during nutrient deprivation.

Discussion

While it has been known for decades that protein malnourished individuals have a reduced capacity to mount immune responses and are therefore more susceptible to infection, the exact mechanisms that govern this immune deficiency are still unclear (*140*). Multiple mechanisms likely contribute to compromised adaptive immune responses in malnourished individuals (*142, 188*). For example, zinc deficiency is linked to protein malnourishment and can impair T cell responses, suggesting a role for this micronutrient in regulating host immunity (*138, 182*). The studies presented here are the first to demonstrate protein malnourishment directly impacts MHC class II Ag presentation, a pathway critical for activation of both T and B cells. This study revealed a novel link between exogenous macronutrient levels and Ag trafficking in B cells, offering a previously unrecognized explanation for reduced Ab responses and impaired T cell mediated immunity observed in protein malnourished individuals (*141*).

Macronutrient deprivation altered MHC class II antigen presentation

In the current study, macronutrient deprivation was shown to directly impact epitope selection and MHC class II Ag presentation. During prolonged periods of starvation, expression of several MHC class II pathway components was reduced, potentially contributing to altered MHC class II Ag presentation. This suggests that prolonged malnutrition may alter the immune response by disrupting expression of immune receptors. While expression of MHC class II

112

pathway components was unaltered during short periods of starvation (up to 12

h), MHC class II Ag presentation was impacted (**Figure 63**). While a study

assessing MHC class II presentation using cells cultured in buffered saline as a

model of extreme starvation has shown altered epitope selection, the current

data are the first to show altered MHC class II presentation specifically in

response to macronutrients (*145*). These data suggest that even short periods of

protein malnourishment may alter the MHC class II presentation pathway

impairing the immune response.
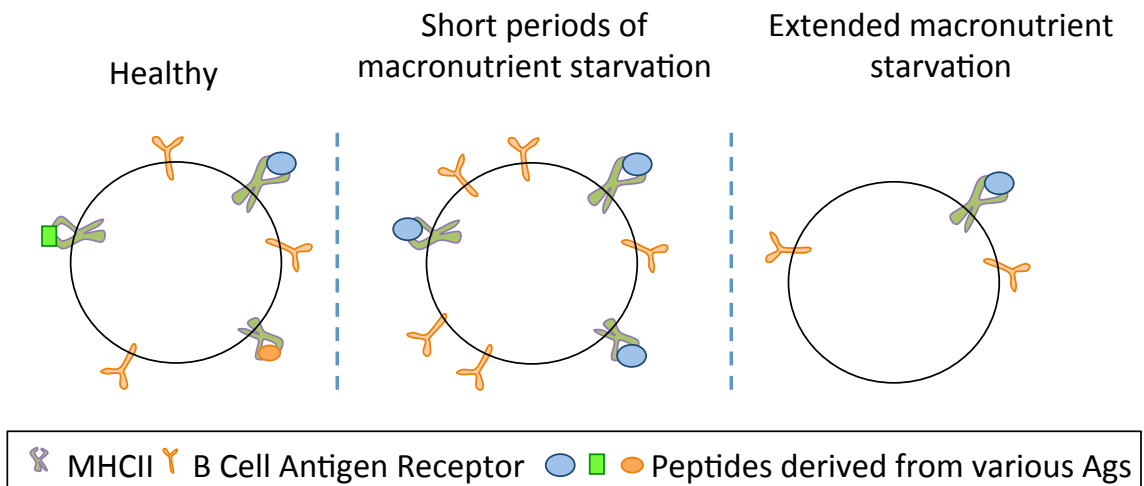
Figure 63. Extended periods of macronutrient deprivation led to a significant reduction in MHC class II pathway components. While short (up to 12 h) periods of nutrient stress did not significantly alter MHC class II pathway components, extended nutrient stress led to the downregulation of numerous MHC class II pathway components including MHC class II molecules. Short periods of nutrient stress altered Ag trafficking, affecting MHC class II presentation.

Macronutrient deprivation altered the trafficking of proteins through the endocytic network and antigen presentation

While macronutrient deprivation led to a significant increase in cellular protease activity, degradation of both the cytoplasmic Ag GAD and the BCR was impaired. Impaired degradation of these Ags was likely caused by compromised protein trafficking during macronutrient deprivation. While exogenous macronutrients were able to fully prevent these disruptions in endocytic trafficking and Ag presentation, protease activity remained elevated, further suggesting protease activity was not responsible for altered MHC class II Ag presentation. Interestingly, macronutrient deprivation significantly disrupted clathrin-mediated endocytosis, impairing internalization of not only the BCR but also MHC class II molecules and TfR. Studies have revealed that protein deprivation reduces the capacity of macrophages to phagocytose bacteria in vivo and in non-immune cells disrupts TfR endocytosis, which indicates more global affects of nutritional stress on vesicular sorting (*149, 189*).

MA and clathrin-mediated endocytosis share several key chaperones including HSC70, mAtg9, TBC1D5 and AP2, suggesting potential coordinate regulation of these intracellular transport pathways (*149, 190, 191*). Furthermore, the regulation of CMA and MA appear linked, as it is often observed that induction of one of these pathways occurs at the expense of the other (*85, 192, 193*). For example, during serum deprivation MA is induced during the initial 24 hr; then as

MA wanes, CMA is induced (*106, 163, 194*). The data presented here further suggest coordinate regulation of these Ag trafficking pathways during nutritional cell stress.

If these pathways are coordinately regulated, other mechanisms, such as pharmacological activators and inhibitors that alter one pathway may influence the efficacy of other pathways as well. Consistent with this, the pharmacological agent rapamycin, which acts to promote MA by inhibiting mTOR, has also been shown to impair receptor-mediated endocytosis and macropinocytosis in dendritic cells (*195*). The studies presented here using Torin1 and *T. gondii* to induce MA, suggest exposing B lymphocytes to these agents may also regulate other components of the MHC class II pathway. Studies here are the first to demonstrate *T. gondii* infects B cells. Consistent with the observed downregulation of MHC class II during *T. gondii* infection of B cells, studies assessing *T. gondii* infection of glioblastoma and microglia cells have shown expression of the master regulator of MHC class II components, CIITA, is disrupted (*196*). Together, these studies indicate *T. gondii* disrupts the MHC class II presentation pathway, likely to avoid detection by the immune response. Furthermore, the studies presented here indicate treatment with Torin1 reduces CLIP:MHC class II surface complexes, suggesting Torin1 affects DM function. Studies further assessing the effect of Torin1 on DM function and expression would provide more insight into the mechanisms by which Torin1 regulates MHC class II Ag presentation. Together these studies suggest mechanisms used to

modulate MA can affect not only Ag trafficking through the endocytic network but also MHC class II pathway components.

Our studies indicate B cells are capable of sensing macronutrient size, as supplementing media with small proteins or dextrans did not restore BCR trafficking, in contrast with large molecular mass macronutrients. This is the first study to suggest APCs sense nutrient size and modulate endocytic trafficking in response. In agreement with this observation, a recent publication has shown neutrophils sense the size of microbes and selectively release extracellular traps in response only to large microbes (*197*). Together these data suggest immune cells sense particulate size and modulate activity based on the molecular dimensions of the stimuli.

Accessibility of the conserved chaperone HSC70 was altered by macronutrient deprivation

The current study reveals a novel role for HSC70 in controlling trafficking pathways during macronutrient stress of B lymphocytes. HSC70 is a constitutively expressed chaperone critical for clathrin-mediated endocytosis, proteasome degradation and the selective capture of proteins for translocation into lysosomes via CMA (*108, 127*). HSC70 guided transit of proteins to the proteasome and CMA appears widely conserved in neural cells as well as immune cells (*11, 78, 85, 113*). Studies from our laboratory have previously

demonstrated HSC70 binds to select proteins such as GAD Ag, delivering them for proteolytic processing and MHC class II presentation (*11, 78*). Furthermore, in fibroblasts, HSC70 association with autophagosomes may play a role in the clearance of cytoplasmic protein aggregate complexes by MA (*198, 199*). Intracellular competition for HSC70 was recently observed, as induction of MA by protein aggregates depleted chaperone reserves impairing clathrin-mediated endocytosis (*199*). The data presented here indicate during macronutrient stress in B lymphocytes, these trafficking pathways compete for conserved chaperones such as HSC70, with upregulation of MA and proteasome activity at the expense of endocytosis and CMA (**Figure 64**). This may favor the presentation of intracellular pathogens or self Ags sequestered in autophagosomes, while limiting immune recognition of endocytosed Ags. While HSC70 accessibility is limited during macronutrient deprivation, low levels of macronutrients could prevent disruptions in both endocytosis and CMA (**Figure 64**). MA remained elevated in cells supplemented with low levels of macronutrients, suggesting MA is more sensitive to loss of macronutrients than endocytosis and CMA (**Figure 64**). Thus, HSC70 plays a key role selectively regulating endocytosis and autophagy during nutritional stress, impacting MHC class II Ag presentation and B-T cell interactions.
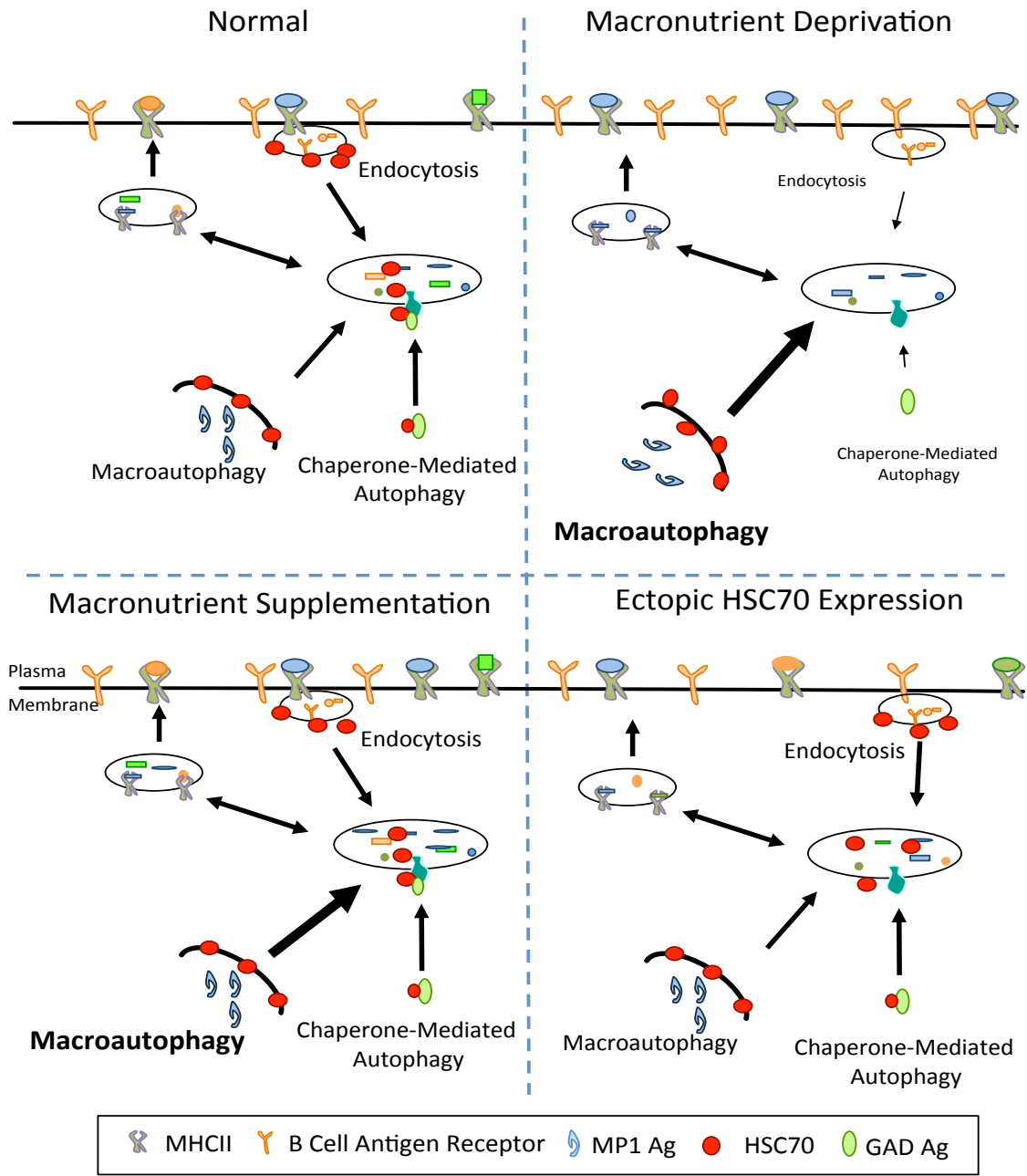
Figure 64. Limitations in HSC70 availability regulated antigen trafficking during macronutrient deprivation. HSC70 is required for several Ag presentation pathways including endocytosis, CMA and MA. Under nutrient rich conditions, HSC70 maintains basal levels of endocytosis, CMA and MA. However, during macronutrient deprivation HSC70 is limited potentially due to its involvement in MA and association with autophagosomes or perhaps its involvement with proteasomeal degradation. This limits its availability for performing its functions in endocytosis as well as CMA. Low levels of macronutrient supplementation can prevent disruptions in CMA and endocytosis, however macroautophagy remains elevated. Furthermore, ectopic expression of HSC70 prevents disruptions in these pathways during macronutrient deprivation suggesting HSC70 accessibility is limited during macronutrient deprivation.

Interestingly, cells ectopically expressing HSC70 failed to induce MA during macronutrient deprivation. While studies have linked HSC70 to autophagosomes and selective autophagy, this is the first study to suggest HSC70 accessibility may regulate MA (*198, 199*). These studies suggest during nutrient stress HSC70 sequestration may trigger MA, whereas overexpression of this protein prevents induction of this pathway.

Future Challenges

The work presented here indicates macronutrient deprivation severely disrupts trafficking pathways within B cells. While this work has shown macronutrient deprivation limits the availability of HSC70 altering Ag trafficking pathways, further studies addressing the affect of malnourishment in vivo, the crosstalk between trafficking pathways, and the mechanisms that regulate HSC70 and its role in MA are of interest.

The effect of macronutrient deprivation on APCs in vivo

Studies here point to the exquisite sensitivity of lymphocytes to nutrient stress. Studies are needed to assess the effect of protein malnutrition on APCs in vivo. While studies have shown the levels of APCs are largely unaltered in malnourished individuals, the ability of these cells to expand and mount immune responses to infection is reduced in these individuals (*142*). Here, studies

indicate macronutrient deprivation alters the ability of these cells to function, however low levels of high molecular weight protein or carbohydrate can prevent these disruptions. Blood contains numerous proteins. Even in protein malnourished individuals the protein content of blood is above 1% (wt/vol) (*200*). Our studies indicate supplementing media with 1% BSA could fully prevent alterations in MHC class II Ag presentation. Importantly, B cells and other APCs not only circulate through the blood but also through the lymphatic system where protein levels can be 1/6th that of those in plasma of healthy individuals (*201, 202*). Furthermore, studies in canines have revealed the concentration of protein in the lymph drops significantly faster than protein concentration in the plasma increasing the discrepancies in the protein concentration between these two fluids (*203*). Together these data suggest a need for studies of lymphatic protein and macronutrient levels in malnourished individuals. Our studies indicate BCR trafficking and MHC class II protein levels may be disrupted in malnourished individuals. Assessing BCR and MHC class II surface levels on APCs derived from malnourished individuals will provide insight into the effect of malnutrition on these pathways in vivo. As a preliminary study into the feasibility of this approach in human subjects, BCR surface expression was monitored on human primary peripheral blood B cells isolated from 5 different individuals (**Figure 65**). These data show all five individuals had relatively similar BCR surface expression, indicating it may be possible to detect alterations in surface expression of this molecule in malnourished individuals. While these studies will provide information on BCR trafficking in malnourished individuals, to further elucidate the effect of

protein malnourishment on Ag trafficking, development of a rodent model will be necessary. Several studies have been published assessing the effect of altering the ratio of macronutrients in a rodents diet on the development of autoimmune disorders (*204, 205*). These studies indicate autoimmune disease progression and development are dependent on diet macronutrient levels, consistent with altered immune responses in these animals. Whether altering the rodents diet affects Ag trafficking in APCs contributing to altered immune responses was not assessed. Future studies could assess the effect of protein malnutrition on APCs by feeding rodents low protein diets and isolating APCs from these mice and monitoring the characteristics of surface proteins as well as their ability to present Ags and activate T cells.
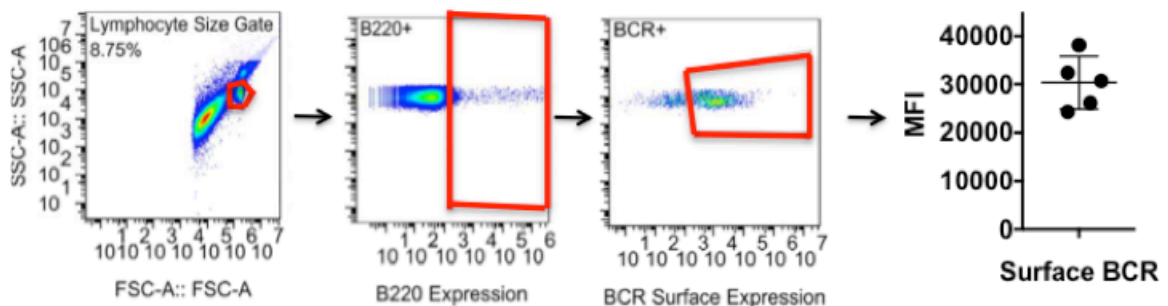
Figure 65. BCR surface levels in healthy individuals. Live peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using a ficoll gradient. Isolated PBMCs were fixed in 0.5% PFA prior to surface staining for the B cell marker B220 and for the KAPPA component of the BCR. Cells were then analyzed by flow cytometery, gating around lymphocytes, cells that were positive for B220 and cells that were positive for BCR staining. The mean fluorescent intensity was assessed in these cells.

Cross-talk among the endocytic, macroautophagy and chaperone-mediated autophagy pathways

The data presented here suggest coordinate regulation of these pathways during nutritional stress. Several studies have indicated these pathways share numerous co-regulators (*149, 190, 191*). A recent publication has suggested competition for HSC70 during MA induction results in suppression of clathrin-mediated endocytosis (*199*). Studies addressing the exact mechanisms that regulate this coordination of intracellular trafficking pathways are needed. Several stresses are known to alter autophagy and endocytic pathways including oxidative stress, hypoxia, ER stress, and DNA damage (*79-89*). Studies assessing whether upregulation of MA disrupts endocytosis with different cellular stresses could be informative as to whether this is an intrinsic property of these pathways. This is of interest as several pathogens are known to regulate MA, yet whether this regulation affects other pathways such as endocytosis or phagocytosis has not been addressed (*172, 206, 207*). Furthermore, there are several pharmaceuticals that modulate MA used as cancer therapies. If these drugs also alter other trafficking pathways within immune cells this could further affect the ability of the immune system to recognize and develop long-lived responses to eliminate cancer cells (*208*).

Regulation of HSC70

While total HSC70 protein levels remained unaltered by serum deprivation, the availability of HSC70 to perform critical functions in endocytosis and CMA was severely compromised in this analysis. While HSC70 is responsible for maintaining basal levels of several cellular pathways, the exact role or mechanisms HSC70 plays in these pathways remains unknown. HSC70 is bound by several co-factors and some studies have suggested these co-factors influence the fate of HSC70 bound client proteins (*113, 119, 120*). For example, studies have suggested when bound by the BAG-1 co-factor, HSC70 and its client protein are guided to the proteasome. Whereas when bound by BAG-3, HSC70 and its client protein are guided to the selective MA pathway (*95, 116, 117*). Studies have also linked BAG-1 to the CMA pathway and BAG-3 to the ubiquitin proteasome pathway (*98, 209*). It appears a single co-factor is not sufficient to dictate the fate of HSC70 and its associated client proteins. However, as HSC70 is bound to several co-factors at any given time, it could be the exact composition of co-factors bound to HSC70 that regulates which pathway is taken. Furthermore, it is also possible that other factors play a role in deciding the fate of HSC70 bound client proteins. Client proteins can be ubiquitinated as well as phosphorylated. These post-translational modifications may help guide HSC70 complexed with its clients. Studies addressing these questions are needed to fully understand the mechanisms by which HSC70 can affect cellular trafficking pathways.

Furthermore, studies addressing the role of HSC70 in regulating MA are needed. The current study results are the first to the indicate availability of HSC70 may regulate induction of MA during nutrient stress. Whether HSC70 regulates MA during other types of cellular stress has not been studied. Interestingly, though HSC70 does not contain a secretory signal sequence, studies have shown it can be secreted from cells and that secretion of HSC70 is enhanced during serum deprivation (*210, 211*). This suggests HSC70 may regulate MA via two distinct pathways; a direct path in the cytoplasm or by its secretion and affects in the extracellular milieu. Furthermore, the mechanisms that limit accessibility of HSC70 during nutrient stress have not been established. It is possible that autophagosomes associate preferentially with HSC70 complexed with ADP vs. ATP. Thus cellular ATP levels could reflect macronutrient availability. As the content of cellular ATP drops during serum deprivation (*212*), this may limit the abundance of HSC70:ATP complexes. Binding of ADP will alter HSC70 chaperoning function; however, whether this specifically affects the role of HSC70 in cellular trafficking pathways has not been established. Furthermore, the effect of serum deprivation on HSC70 co-factors is not known. Thus, investigations into the connections between cellular energy (ATP to ADP ratios) as well as changes in the levels of HSC70 co-chaperones, would be informative in providing more molecular insights into how cells respond to macronutrient deprivation.

Conclusion

In summary, connections between cellular endocytic and autophagic pathways shape MHC class II Ag presentation to influence adaptive immunity. Here, macronutrient availability was shown to upregulate MA flux and impair the ability of B cells to internalize cell surface Ag receptors, impacting MHC class II Ag presentation. These data revealed HSC70 as a connection between routes of Ag delivery suggesting coordinated regulation of these pathways may influence MHC class II epitope selection. Together these findings reveal a previously unknown connection between macronutrient sensing and Ag trafficking within lymphocytes, which may contribute to impaired adaptive immune response in protein malnourished individuals.

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Curriculum Vitae

Sarah N. Deffit

Education		
	Ph.D – Indiana University	2015
	Immunology, Minor Life Science	
		5-2010
	Bachelor of Science – Colorado State University-Pueblo	
	Biology major, Chemistry major, Mathematics minor	
	Suma Cum Laude	
Research Experience	**Graduate Research Assistant**	2010-
	Indiana University School of Medicine	15

The effect of cellular stress on MHC class II antigen presentation in B cells. Upon recognition of the MHC class II:antigen complex, CD4$^+$ T helper cells are activated initiating protective immunity. Several infectious agents as well as cellular stresses such as starvation have been shown to alter macroautophagy and other degradative pathways. My project has shown that protein deprivation alters trafficking and degradation of the B cell receptor and select autoantigens as well as the uptake of exogenous antigens leading to altered MHC class II presentation. Alterations in B cell receptor trafficking and degradation may alter the repertoire of peptides presented by MHC class II molecules, potentially altering susceptibility to not only infection and but also autoimmune disorders and cancer.

Undergraduate Research Assistant	2008-
Colorado State University-Pueblo	10

Use of continuous culture system for improvement of cellulose production in *Penicillim spinulosum*. With the use of a continuous culture that causes a selective pressure, the strain of *P. spinulosum* was forced

to break down cellulose at a higher rate. Cellulase activity was monitored using enzyme assays. The cellulose was purified using size-exclusion liquid chromatography. The production of a high activity cellulase may provide a break through in bio fuels, as the limiting factor in ethanol production is the break down of materials consisting of cellulose.

Research Technician   2007-
Colorado State University-Pueblo   2010

I was responsible for preparing and organizing specific laboratory experiments for both science and non-science majors enrolled in several diverse science courses. This required the preparation of solutions as well as sterile technique to prep microbiology experiments.

Teaching Experience   2013

Indiana University Graduate School
Dept. of Microbiology and Immunology
    Teaching Assistant – Nursing School Microbiology Course
- Presented lectures on microbiology, virology, immunology and microscopy to ~30 students enrolled in the nationally ranked Indiana University School of Nursing Program
- Facilitated question/answer sessions clarifying key concepts and managed course website content
- Facilitated active learning by integrating group problem sets
- Clearly integrated lecture concepts into the laboratory setting

Indiana University School of Medicine   2012-
Indiana University School of   15
Medicine BioMedical Gateway Program
    Student mentor
- Mentored undergraduate and PhD students teaching common laboratory techniques, bioassays, data analysis and interpretation and statistical methods

- Mentored PhD students providing peer tutoring sessions as well as guidance leading to higher retention rates in the program

Indiana University Graduate School
Dept. of Microbiology and Immunology   2013
Guest Lecture –Graduate Advanced Immunology
- Organized and presented advanced lecture to graduate students and post-doctoral fellows
- Facilitated group discussion on current literature

Colorado State University-Pueblo
Dept. Biology and Chemistry   2008-
Undergraduate Physics, General Chemistry   10
and Organic Chemistry Tutor
- Tutored groups as large as 10 individuals per course allowing students to recognize core concepts and apply these concepts using the incorporation of group problem sets
- Taught methods to improve study habits and practices

Teaching Workshops

Scientist Teaching Science Course   2014
New York Academy of Science
- Active Learning and Expert Learners
- Culture Awareness and Diversity
- Inquire-Based Science Education

Grants and Funding

AAI Trainee Abstract Award   2014
NIH Training Program   2011-
- **Regulation of Hematopoietic Cell**   15
  Production
Travel Fellowship- Indiana School of Medicine   2012
Travel Stipend- Tri Beta National Meeting   2009

Publications

Deffit, SN. and Blum, JS. (2015). Macronutrient deprivation modulates antigen trafficking and immune recognition through HSC70 accessibility. **J. Immunol.**

Gardiner, GJ., Deffit, SN., McLetchie, S., Pérez, L., Walline, CC., and Blum, JS. (2013). A role for NADPH oxidase in antigen presentation. *Front. Immunol.* **4**:295.

Walline, CC.*, **Deffit, SN.***, Wang, N.*, Guindon, LM., Crotzer, VL., Liu, J., Hollister, H., Eisenlohr, L., Brutkiewicz, RR., Kaplan, MH., and Blum, JS. (2014). Virus Encoded Ectopic CD74 Enhances Poxvirus Vaccine Efficacy. *Immunology*. (*Co-first authors)

Presentations & Abstracts

Deffit, S, Blum, J. (2014) A role for HSC70 in Ag trafficking during macronutrient deprivation. Poster. American Society of Cell Biology, PA, December 2014

Deffit, S, Blum, J. (2014) Protein Deprivation alters trafficking of the B cell receptor and endogenous antigens in B cells. Poster. Cancer Research Day, IN, May 2014

Deffit, S, Blum, J. (2014) Protein Deprivation alters trafficking of the B cell receptor and endogenous antigens in B cells. **Oral presentation**. American Association of Immunologist Meeting, Pittsburgh, PA, May 2014

Deffit, S, Blum, J. (2012) The function of HSP90 in MHC class II presentation. Selected for poster. American Association of Immunologist Meeting, Boston, MA, May 2012

Deffit, S., Bonetti, S., Caprioglio D. (2010) The Optimization of Purification of Cellulase from Fungus *P. fellutanum* and *P. spinulosum*. **Oral presentation**. Tri Beta National Meeting.

Deffit, S., Bonetti, S., Caprioglio D. (2009) The Optimization of Purification of Cellulase from Fungus *P. fellutanum* and *P. spinulosum*. **Oral presentation**. Tri Beta Regional Meeting. 1st place.

Professional Development	The Ins and Outs of applying for NSF funding	2-2014
	Preparing Future Faculty and Professionals Pathways Conference, Indiana University Purdue University-Indianapolis	11-2013

- Conflict Resolution
- Lecturing with the Learner in Mind
- Teaching as a Transferable Skill
- Civic Professionalism and Community Engagement

Preparing Future Faculty career development program   2013-15
- Learned the skills and critical importance of providing engaging mentorship
- Constructed a portfolio including C.V., resume, Teaching Philosophy and Research Statement

2011

Responsible Conduct of Research Course Indiana University School of Med.

Professional Memberships		
	American Society for Cell Biology	2013-15
	American Association of Immunologists	2008-15
	New York Academy of Sciences	2014
	Phi Kappa Phi	2008-10

- Vice President of Colorado State-University Chapter

	Tri-Beta	2008-10

Honors & Awards		
	AAAS/Science Program for Excellence in Science	2012
	Nominated for Threlkeld Award (Top Graduating Student)	2010
	Houston C. Simms- Top Graduating Biology Student	2010
	Legacy Bank Scholar-Athlete Award	2010
	Deans List	2007-10
	Polyed Award in Organic Chemistry-Top Organic Chemistry Student	2009

1st All Academic Rocky Mountain Athletic   2009
Conference Team