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Isolation and Identification of Hsc70 Conjugates in RAW264.7 Murine Macrophage-Like Cells

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ISOLATION AND IDENTIFICATION OF HSC70 CONJUGATES IN RAW264.7 MURINE MACROPHAGE-LIKE CELLS

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Approved by
Dr. John Rakus, Committee Chairperson
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Dr. Leslie Frost

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APPROVAL OF THESIS

We, the faculty supervising the work of Michael Parsons, affirm that the thesis, *Identification and Isolation of Hsc70 Conjugates in RAW264.7 Macrophage-Like Cells*, meets the high academic standards for original scholarship and creative work established by the Master's in Chemistry and the College of Science. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

John Rakus — Dec. 13, 2019

Dr. John Rakus, Department of Chemistry — Committee Chairperson — Date

Dec 17, 2019

Dr. Menashi Cohenford, Department of Forensic Sciences — Committee Member — Date

Leslie Frost — 12/13/2019

Dr. Leslie Frost, Department of Chemistry — Committee Member — Date

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ABSTRACT

IL12R- β2 is a Type I cytokine receptor and contains the WXXW (WSNWS) sequence that often predicates the post-translational addition of mannose to a tryptophan residue via a carbon-carbon bond. This study will stimulate expression of IL12R- β2 in RAW 264.7 Macrophage-Like Cells by shutting down the extracellular signal kinase (ERK) pathway and introducing inflammatory agents lipopolysaccharide and CpG DNA in order to collect a concentrated sample of IL12R- β2. These samples will be analyzed for the presence of *C*-mannosyltryptophan residues.

CHAPTER 1

SIGNIFICANCE

The study of Hsc70 conjugates is hopeful to provide insights into the number, identity, and structure of *C*-glycosylated proteins. Identifying these proteins is key to learning the function of the modification as well as the mechanism by which it is placed.

Some evidence does suggest that *C*-glycosylated proteins could affect the signaling pathways of the innate immune system's LPS response in conjunction with Hsc70. The LPS response cascade is responsible for sepsis, and therapeutic interventions aimed at modulating that pathway are desirable. Understanding the role that Hsc70 and/or *C*-glycosylated proteins play in this signaling cascade could lead to future work aimed at creating treatments for diseases such as sepsis.

CHAPTER 2

BACKGROUND

Glycosylation

Glycosylation consists of the addition of a carbohydrate molecule to a protein during or after the process of translation has been completed. This post-translational modification serves to protect or enhance the function of a protein.

Glycosylation is an enzymatic process and can occur at various residues within a peptide or protein. Two of the better understood forms of glycosylation are referred to as *N*-linked and *O*-linked glycosylation (Figure 1).[1] Carbohydrates are attached to proteins through a carbon-nitrogen or carbon-oxygen bond respectively.

N-linked glycosylation occurs in the lumen of the endoplasmic reticulum (ER). A family of enzymes called oligosaccharyltransferases (OSTs) is responsible for carrying out the process. The process transfers an oligosaccharide from dolichyl pyrophosphate (Dol-PP) to an asparagine residue within the NXS/T sequence where "N" is asparagine, "X" is any amino acid except proline, "S" is serine, and "T" is threonine.[2]

There are many types of *O*-linked glycosylation, and a comprehensive discussion of these is beyond the scope of this project.[3] These forms of glycosylation depend on a family of transferases called the UDP-GalNAc:polypeptide*N*-acetylgalactosaminyltransferase (ppGaNTase) family.[4] The most well-defined type of *O*-linked glycosylation is the mucin-type glycosylation.[5] This enzyme transfers GalNAc from the substrate UDP-*N*-acetylgalactosamine to the hydroxyl group of a serine or threonine residue. Though some forms of *O*-linked glycosylation are sequence dependent, no consensus has been found for the mucin type

modification. The *N*-linked and *C*-linked modifications, however, are sequence dependent, and this difference could be one reason that mucin-type glycosylation is more prominently expressed.[5]

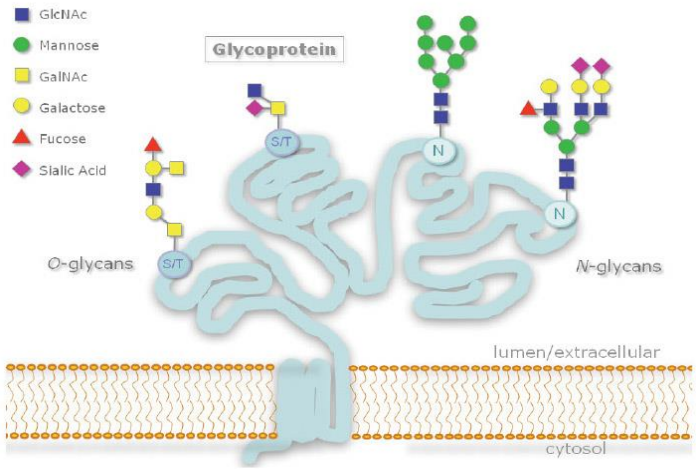


Figure 1. Diagram of N-linked and O-linked Glycosylation Sites. Above is a representation of common types of N-linked and O-linked protein glycosylation. The sites marked "S/T" denote a serine or threonine residue bound to a carbohydrate complex with an oxygen linkage. Sites marked "N" represent a residue with an amine group that is bound to a carbohydrate complex via a nitrogen bond. [1]

C-Mannosyltryptophan

More recent research found that there is at least one other type of glycosylation – *C*-linked. In this modification, a carbon-carbon bond is formed linking a single mannopyranosyl molecule to a tryptophan (W) residue.[6] The function of this modification is yet unknown, but there is evidence to suggest that it may assist in moving proteins to or through the cellular membrane.[7] It is also suggested that this modification plays a role in regulating the innate immune response.[8]

C-linked glycosylation is an enzymatic process dependent on the *C*-mannosyltransferase DPY-19. In humans, there are four DPY-19 homologues, and they share homology to the

oligosaccharyltransferase (OST) family that is participates in *N*-linked glycosylation.[9] The reaction of *N*-linked mannosyltransferases utilizes the same substrate as DPY-19 – dolichyl phosphate mannose (Dol-P-Man).[10] The process transfers a mannose group from guanosine diphosphate mannose (GDP-Man) to dolichyl phosphate – making dolichyl phosphate mannose (Dol-P-Man) – and then the mannose is transferred from Dol-P-Man to the second carbon of the tryptophan residue's indole ring (Figure 2).[11,12] Since the *C*-linked glycosylation is Dol-P-Man dependent, it is believed to occur in the lumen of the endoplasmic reticulum (ER).[13]

In 1994, the first observation of a glycosylated tryptophan was recorded in human RNase2.[14] A year later, the attached sugar was identified as a mannopyranosyl residue.[6] *C*-linked glycosylation occurs at the first tryptophan in a WXXW/C motif where W is a tryptophan residue, C is a cysteine residue, and X is any amino acid.[15]

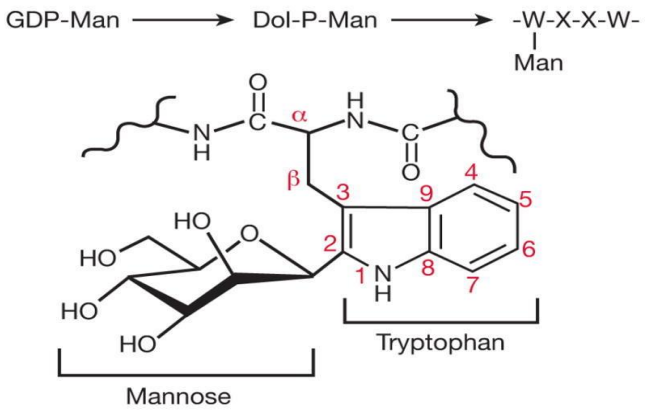


Figure 2. **Diagram of C-linked Glycosylation.** The figure above summarizes the synthesis of C-mannosyltryptophan residues. In this case, it is assumed that the residue exists within the WXXW motif. The figure also illustrates the location of the carbon-carbon bond after formation.[12]

Stimulation of the Innate Immune Response

The innate immune system is the first line of defense against microbial infections. This system recognizes many molecules present on a variety of microbes. These molecular patterns allow the innate immune system to react quickly when an infectious agent enters the body.[16]

One molecule that sets off an innate immune reaction is lipopolysaccharide (LPS). LPS is a large molecule composed of a lipid covalently bonded to a carbohydrate polymer. It is prevalent on the surface of gram-negative bacteria, and thus can be recognized readily by the innate immune system.[17]

In the following experiments, two different antigens have been used to stimulate an innate immune response –LPS and CpG DNA. LPS and CpG DNA will each be added into the media of cultured RAW 264.7 murine macrophage-like cells to stimulate an immune response. This procedure will allow for the collection and analysis of proteins involved in the innate immune response of RAW 264.7 cells.

LPS and CpG DNA

CpG DNA is defined as a short region of unmethylated, synthetic DNA that is engineered to mimic microbial DNA. The strand includes cytosine (C) and guanine (G) residues linked by phosphodiester (p) bonds. These CpG sequences – referred to as CpG islands – are not transcribed. This sequence is less common in vertebrate genomes, and it is usually in a methylated form when it is present in vertebrates. Therefore, vertebrate immune systems can identify microbial DNA by the CpG patterns present.[18]

Microbial DNA has been shown to produce an immune response in vertebrates that is entirely dependent on the CpG domains.[19] Studies have reported that CpG DNA is an effective

adjuvant in some vaccines and suggest that it could hold therapeutic potential for cancer and allergic diseases.[20] It was discovered that toll-like receptor 9 (TLR9) is the receptor molecule for CpG DNA.[21] When activated, TLR9 signals to an innate immune response that releases cytokines and other proinflammatory molecules through the MyD88 pathway.[22]

Toll-Like Receptor 4 and LPS

The toll-like receptor 4 (TLR4) pathway is a part of the innate immune response. This pathway is activated by the LPS molecule on the surface of gram-negative bacteria and is thought to be a major contributor to sepsis.[23] The pathway is activated when LPS is broken into monomers by the cluster of differentiation protein-14 (CD14). These monomers are delivered by CD14 to TLR4. TLR4 stimulation sets off a cascade that releases proinflammatory cytokines such as interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α).[24] Though TLR4 is in the same family of proteins as TLR9, it should be noted that TLR4 is not activated by CpG DNA and TLR9 is not activated by LPS.

Heat Shock Cognate 70 (Hsc70)

Hsc70 is a chaperone protein. It is responsible for identifying misfolded or denatured proteins within the cell. Once the target protein is identified, Hsc70 either helps the protein to fold correctly, or moves it to the lysosome for degradation.

Hsc70 has also been shown to play a role in the innate immune response via the TLR4 pathway. The TLR4 pathway has been shown to be activated by extracellular Hsc70, with the binding domain of Hsc70 being critical to the stimulation of this pathway. Once stimulation occurs, cytokines are released resulting in an innate immune cascade. LPS can be used as an antigen to produce this response.[25]

In 2007, Muroi et al. showed that peptides derived from the thrombospondin type 1 repeat (TSR) family with a mannosylated tryptophan in the WXXW region (peptides used in that study contained WSPW) enhanced LPS signaling in RAW 264.7 murine macrophage-like cells. The signaling increase was measured as an increase in TNF-α in cells treated with LPS plus *C*-man-WSPW verses cells treated with either LPS or LPS plus WSPW.[8]

Hsc70 has been shown to have an affinity to peptides with *C*-linked modification. Hsc70 has a higher affinity in solution for TSR derived peptides with a *C*-man-WSPW domain than peptides with an unmodified WSPW domain. Furthermore, RAW 264.7 cells treated with Hsc70 and *C*-mannosylated peptides showed higher TNF-α production than cells treated with either Hsc70 and unmodified peptides or Hsc70 alone.[26]

CHAPTER 3

PRELIMINARY DATA

Identification of *C*-linked Conjugates in Hsc70 Immunoprecipitations

Prior to these experiments, the Rakus Lab performed an immunoprecipitation of Hsc70. RAW 264.7 cells were grown as described above. Cells were exposed to 1 μg/mL LPS and collected at specific timepoints – 0, 1, 2, 6, and 24 hours. Control samples (without LPS exposure) were also collected for each timepoint. The cells were then lysed with 1% Triton X-1000 in Tris buffered saline (TBS), and the lysates were purified by precipitating proteins with acetone (80% of total volume) at -20° C for 2 hours. The pellets were resuspended in phosphate buffered saline (PBS).

Hsc70 antibody 1B5 (Abcam, Inc.) was bound to a magnetic Dynabead column and used to bind Hsc70 in the RAW 264.7 lysate samples. The co-precipitates of this experiment were assumed to interact with Hsc70 and were considered potential candidates for *C*-mannosyltryptophan residues.

Conjugate proteins from the Hsc70 immunoprecipitation were separated by size using SDS PAGE electrophoresis. Once separated, the gel was silver stained, and the bands were excised (Figure 3).

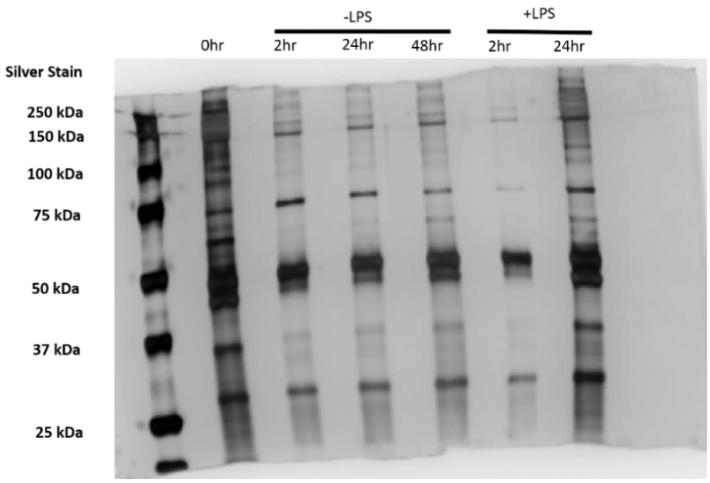


Figure 3. SDS PAGE Gel of Hsc70 Immunoprecipitation. SDS-PAGE gel treated with silver stain to reveal conjugates of Hsc70 that were co-precipitated during the pull down. These bands were excised to be analyzed by mass spectrometry.

Each band was digested with trypsin and chymotrypsin, and the peptides were sequenced using High Energy Collision Induced Dissociation (HECID) mass spectrometry at the University of Georgia to identify individual proteins and probe for modifications. While the Hsc70 conjugates provided several possible candidates for C-mannosyltryptophan residues, the sample quality was not adequate to fully verify or discount the existence of the *C*-linked modification on any novel proteins. In order to verify such a modification, the peptide(s) would have to break in such a way to produce an ion with a mass of +348 Da indicating a single tryptophan residue with an attached mannose.

The mass spectrometry data identified one conjugate as interleukin-12 receptor β2 (IL12R- β2). IL12R- β2 is a Type I cytokine receptor and contains the WXXW (WSNWS) sequence that often predicates a *C*-linked modification from residues 320-324 (Figure 4). However, the mass spectrum showed a mass shift of 477 Da between the b2 and b4 ion in a peptide that does not contain the WXXW motif (Ile-470 through Lys-483). This difference suggests that the differing residues between these two ions were glutamate (129 Da) and *C*-mannosyltryptophan (348 Da). Thus, it is believed that a *C*-mannosyltryptophan residue exists outside of the consensus sequence, on Trp-473 (Figure 5).

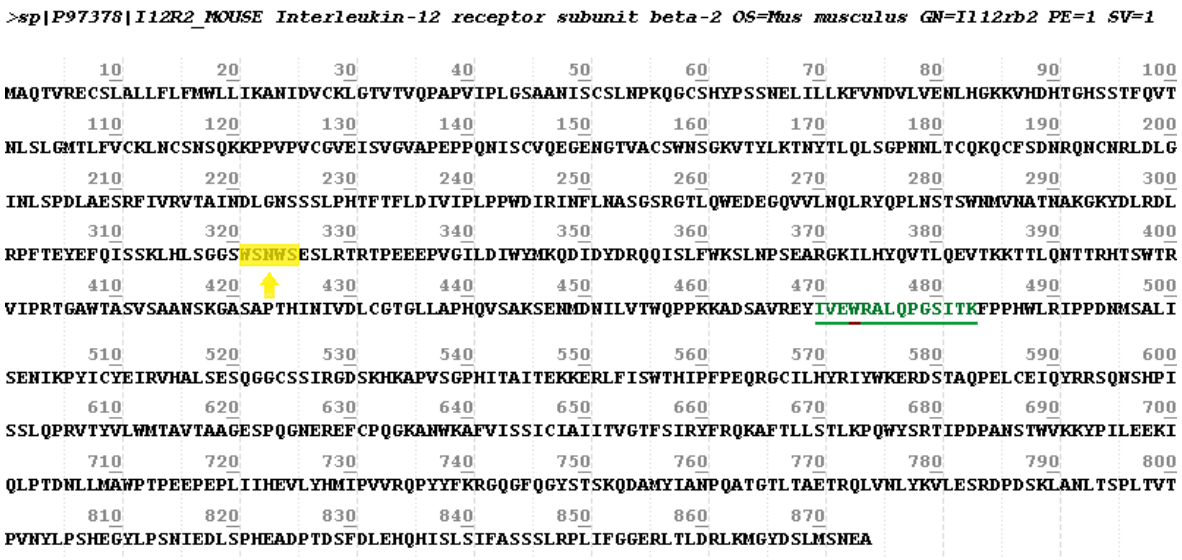


Figure 4. Sequence of IL12R-β2. Pictured above is the amino acid sequence of IL12R-β2. Note that the WSNWS sequence is found from amino acids 320-324, but the region highlighted in green is the peptide believed to contain a C-mannosyltryptophan residue.

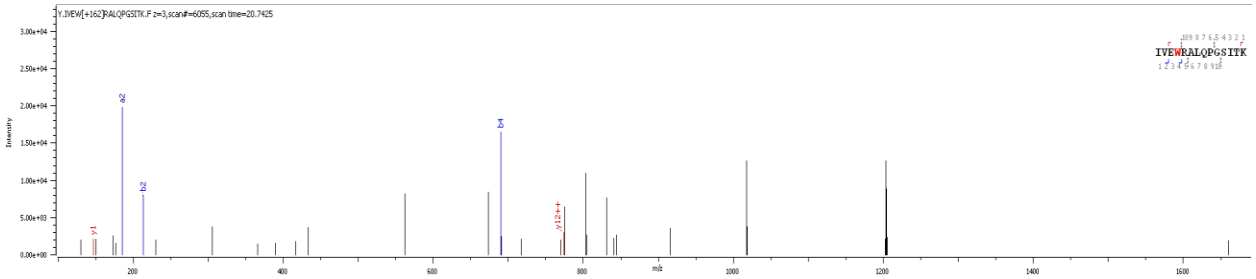


Figure 5. Mass Spectrum of IL12R-β2 Peptide. Mass spectrum taken by the Rakus Lab believed to be a peptide sequence (top right corner) from IL12R-β2 after digestion with trypsin and chymotrypsin. This peptide sequence shows a mass shift of 162 for the b4 ion, suggesting a C-mannosyltryptophan residue.

These results provide the basis for a project aimed at enriching the IL12R-β2 sample to obtain sufficient immunoblotting and mass spectrometry data to evaluate the presence or absence of *C*-linked modifications. The goal of the project will be to isolate the IL12R-β2 protein and obtain a mass spectrum that includes all b-ions in the range of b1-b4. The b3 ion can then be analyzed independently and compared to the expected mass of *C*-mannosyltryptophan – 348 Da.

CHAPTER 4

BIOCHEMICAL TECHNIQUES

Immunoprecipitation

Immunoprecipitation uses a specific antibody-antigen interaction to isolate one protein from a mixture of proteins such as a cell lysate sample. Because of the specificity of an antibody for a single antigen, this technique can be done to isolate very small amounts of protein.

An immunoprecipitation protocol generally consists of utilizing a solid support to immobilize the antibody-antigen complex. In the case of a column, the antibody is attached to the column and the lysate is passed through several times. Any antigen present will, theoretically, bind to the antibody on the column and allow the rest of the lysate to pass through. The column can then be eluted with high salt, low pH glycine, or an SDS elution buffer.

When using beads, the antibody will be bound to the beads. After incubating the beads with the sample for an appropriate amount of time, they can be spun down (or for magnetic beads, placed on a magnetic stand). The supernatant can be removed from the beads, and any antigen in the sample should remain bound to the antibody on the surface of the beads. It can then be eluted as with a column (Figure 6).[27]

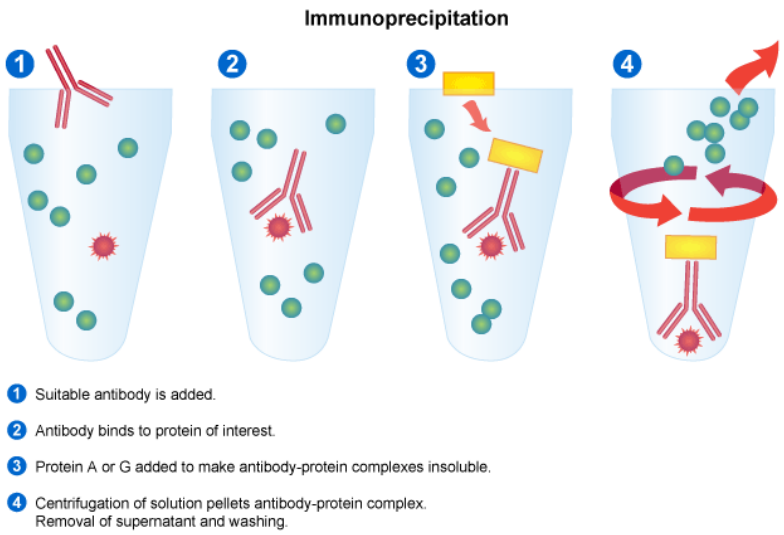


Figure 6: Diagram of Immunoprecipitation. This figure shows the general concept of immunoprecipation protocols. [27]

One notable modification that was used in this experiment is the crosslinking of the IL12R-β2 antibody to magnetic beads. Typically, the primary antibody (anti-IL12R – β2) would associate with the column (in this case protein A conjugated beads) *via* an ionic interaction. However, in this experiment disuccinimidyl suberate (DSS) crosslinking agent was used to form a covalent bond between the two.

The DSS crosslinking ensures that the primary antibody present in the immunoprecipitation does not elute out with the target protein. Thus, DSS crosslinking leads to less contamination in the immunoprecipitated sample, the final SDS gel, and/or Western blot. Minimal contamination is particularly important for Western blotting, as residual primary antibody could later bind to detection antibodies and lead to misinterpretations in the data.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique that is used to separate proteins by molecular weight. It works by coating the proteins with SDS to create a uniform charge. Once a uniform charge is achieved, the proteins migrate through a polyacrylamide gel by creating a voltage differential from the top of the gel to the bottom (this technique is known as electrophoresis). While traveling through the gel, proteins collide with the polyacrylamide polymers. Larger proteins have more collisions, and those move through the gel more slowly than smaller proteins. As a result, the proteins in a sample are separated and form "bands" at different molecular weights (Figure 7).[28]

The proteins in an SDS-PAGE gel are often invisible to the naked eye. Because of this, they are stained with either Coomassie Blue or silver chloride. Coomassie Blue is typically faster, however it is less sensitive than silver staining. Once the bands are visible, they can be excised with a razor or scalpel and analyzed by mass spectrometry.

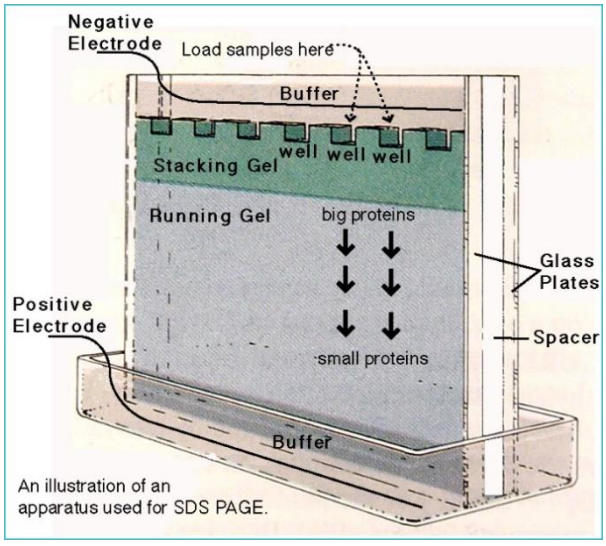


Figure 7: Diagram of SDS-PAGE. This illustration shows the general concept of an SDS-PAGE procedure.[28]

Western Blots

Before a Western blot procedure can be done, an SDS-PAGE gel must be made. The proteins in that gel can then be transferred onto a "blot" (a piece of nitrocellulose paper). As in SDS-PAGE, electrophoresis is used to push the proteins out of the gel (horizontally) and imbed them into the nitrocellulose paper.

Once the proteins are imbedded into the nitrocellulose paper, the Western blot utilizes antibody-antigen interactions in much the same way as an immunoprecipitation. First, the blot is blocked with blocking buffer (5% milk in TBS-T [Tris-Buffered Saline 0.1% Tween-20], w/v). Blocking prevents non-specific binding of the antibody to the nitrocellulose paper. Then, the blot is incubated overnight at 4°C with either blocking buffer or TBS-T containing a primary antibody specific for the protein of interest.

After incubation with the primary antibody, the blot is thoroughly washed to eliminate any nonspecific binding of the antibody. The blot is then incubated with a secondary antibody that has a high specificity for the primary antibody. The secondary antibody is conjugated with horse radish peroxidase (HRP) so that chemiluminescence can be observed upon the addition of a 1:1 luminol and peroxide solution (Figure 8).[29]

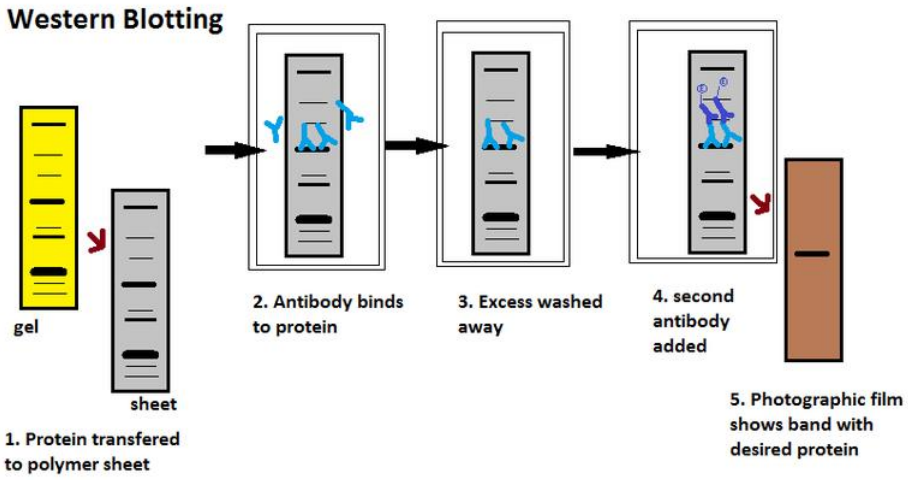


Figure 8: Diagram of Western Blot. This illustration shows the concept behind Western blot procedures.[29]

Mass Spectrometry

Mass spectrometry is a method of analyzing a chemical compound's mass to charge ratio. The basic design consists of an ionization source that forces a charge onto a molecule (often +1 for the simplicity of analysis), a mass analyzer that subjects the ions to a magnetic field, and a detector that amplifies the signal of ions hitting it. Though the applications of mass spectrometry are broad, this paper will focus only on the analysis of proteins and peptides.

Because proteins are typically very large molecules, they must first be broken into peptides by digesting the proteins with a protease. Typically, trypsin and/or chymotrypsin are used for protein digestion in mass spectrometry. After digestion, peptides are purified using Ziptip.

Because modifying a protein produces a shift from the anticipated mass, post-translational modifications have been identified deliberately and accidentally through mass

spectrometry. The indication of a post-translational modification is a mass shift between the expected mass of a peptide or residue and the observed mass.

MS/MS techniques are utilized to fragment peptides to look for post-translational modifications. In the case of looking for a hexose linked to a tryptophan residue, you would expect to see the weight of each peptide containing the tryptophan of interest increased by 162 Daltons (Da).

MALDI

Matrix Assisted Laser Desorption Ionization is a technique used to transfer a charge to peptides. The peptides are immobilized on matrix. A laser is used to impart energy into the matrix at a given spot. The matrix transfers a proton to the peptide/s present, and the energy imparted moves the peptide into the gas phase. The advantage of MALDI is that it typically provides the molecule with a charge of +1, simplifying identification of peptides.

Ion Trap

Once a peptide of interest is identified, a second protocol known as MS/MS can be done. This technique requires a type of mass analyzer called a trap. In this procedure, ionized peptides are trapped in the mass analyzer and collided with an inert gas (helium or argon). The collisions fragment the peptides, creating a pattern of ions that can be analyzed to identify the peptide sequence. An ion trap has the advantage of being able to scan a single mass range, looking for the fragments of specific peptides.

CHAPTER 5

METHODS

Selecting Target Proteins

The goal of this project was to identify conjugates of the Hsc70 protein and investigate them for the presence of *C*-linked glycosylation. Target proteins were identified by analyzing the proteins found in an Hsc70 immunoprecipitation. The premise of this technique is that any protein that was pulled down along with Hsc70 must be interacting with, that is bound to, Hsc70. Considering Hsc70's affinity for *C*-linked proteins, the likelihood of one or more of these conjugates containing the modification would be greater than analyzing proteins from a whole cell lysate.[26]

Cell Cultures

For these experiments, RAW 264.7 murine macrophage-like cells were fed in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal bovine serum (FBS) and penicillin (10 units) and streptomycin (0.10 mg/mL) from Sigma Aldrich. The cells were grown in culture flasks at 37° C in 5% CO_2 conditions.

Cell Stimulation

Cells were either unstimulated, stimulated with LPS alone, pretreated with U0126 and then stimulated with LPS, or stimulated with CpG DNA alone. These conditions are specified where appropriate.

Immunoprecipitations

Immunoprecipitation of Hsc70

RAW 264.7 macrophage-like cells were retrieved from the freezer. Samples came from cultures either stimulated with LPS or not stimulated with LPS at various points after the stimulation (0 hour, 1 hour +/- LPS, 2 hours +/- LPS, 6 hours +/- LPS, 24 hours +/- LPS). There were nine conditions total.

Two protease inhibitor cocktails were added to each cell sample. Each sample also received 3 mL of lysis buffer. To avoid cell aggregation, the samples were each split evenly into three 1.5 mL Eppendorf tubes. The samples were then chilled on ice for 30 minutes with vortexing every 10 minutes.

The samples were spun down at 12,000 rpm for 10 minutes. The supernatant was collected in clean 1.5 mL Eppendorf tubes. Each tube received 300 µL of one condition for a total of 9 tubes per condition (1.5 mL tubes were used in place of 15 mL tubes so that they could be spun down faster in following steps).

Each sample received 900 µL of cold acetone, making an 80% acetone solution. An acetone precipitation was done by putting these solutions into the freezer (-20° C) for two hours. After two hours, the samples were spun at 15,000 rpm for 10 minutes.

The supernatant of each sample was discarded. The pellets were dried in the Frost Lab using a speed vac. Pellets were then combined into one tube for each condition to be resuspended. Pellets were first subjected to pipetting up and down with plastic pipette tips. When basic pipetting failed to promote resuspension, the pellets were pipetted up and down with a 16-gauge serological needle followed by a 22-gauge serological needle.

After passing through the needles several times, the samples were sonicated to further resuspend. When the samples appeared to be resuspended, the solution was pipetted up and down with a p1000 pipet to ensure that no small pellets remained.

Magnetic Dynabeads were vortexed and 50 µl pulled out and placed in a 1.5 mL Eppendorf tube to create a bead column. Nine columns were created (one for each sample). The beads were put on a magnetic stand for one minute, and the supernatant was discarded. The beads were washed twice with PBS-T (0.02% Tween-20).

Each bead column received 200 µL of primary antibody prepared as a 1:200 dilution of anti-Hsc70 1B5 (TF MAI-36078) and PBS-T (0.02% Tween-20). The beads and antibody solutions were rotated at room temperature for 30 minutes before being placed on a magnetic stand. After one minute, the supernatant was discarded. Each bead column was washed twice with PBS-T for 5 minutes at room temperature.

The beads were covalently linked to the antibody with the use of a DSS Crosslink protocol (Pages 17-18).

Each bead column received 1 mL of lysate from a different sample and was labeled according to the sample introduced. The columns were rocked for two hours at room temperature. After two hours, the columns were placed on a magnetic stand for one minute, and the supernatant was discarded. The columns were washed three times with PBS-T.

The columns were eluted twice at 70° C with amine-based elution buffer. The elutions were combined and stored at -20° C.

IL12R-β2

The initial mass spectrometry data collected in the Haltiwanger lab implied the presence of a *C*-linked modification. However, the data was inconclusive. In order to enrich the concentration of IL12R- β2 and reduce noise, an immunoprecipitation was implemented. By binding IL12R-β2 to a stationary column, the rest of the lysate can be washed away. When IL12R-β2 is eluted from the column after the precipitation, it is more concentrated and fewer contaminating proteins are present. The more concentrated sample allows for a more accurate analysis of IL12R-β2.

A rabbit anti-mouse IL12R- β2 (Bioss Antibodies, Woburn, MA) was used as a primary antibody for the immunoprecipitation of IL12R- β2 from RAW 264.7 cells. The antibody was crosslinked to Protein A conjugated magnetic beads *via* DSS. The crosslink procedure is as follows:

The bead slurry was removed from the storage (4° C) and vortexed before transferring 100 µL into a 1.5 mL Eppendorf tube. The tube was placed on a magnetic stand for one minute, and the supernatant was pipetted off the beads. Anti-mouse IL12R- β2 was added to 400 µL of PBS-T (0.02% Tween-20) in a separate tube. The antibody solution was then added to the bead column and mixed by pipetting up and down, then rotated for 40 minutes.

The column was placed on a magnetic stand for 2 minutes and the supernatant was pipetted off. The column was washed with 400 µL of PBS-T (0.02% Tween-20) one time.

A DSS crosslinking solution was made with 432 µL of N,N-dimethylformamide and 2 mg of DSS for a final concentration of 12.5 mM. The bead column received 8 µL of DSS solution and the mixture was rotated as above.

The crosslink reaction was quenched with 100 µL of 1 M Tris-HCl (pH 7.5). The quenching solution was added to the column and rocked for 15 minutes. The solution was placed on a magnetic stand for 2 minutes and the supernatant was removed.

Next, 500 µL of cell lysate was added to the column and mixed by pipetting. The column was split into two 250 µL portions and separated into two tubes. One tube incubated at room temperature for 2 hours and the other incubated at room temperature for 6 hours.

Meanwhile, another column was prepared using the same procedure as above, with volumes cut in half. The final column of 250 µL of cell lysate and beads was not divided. This column was incubated at 4° C overnight.

After incubation, each column was washed and eluted using the same procedure. The column was placed on a bead stand for two minutes and the supernatant was removed. The column was then washed three times with 200 µL of PBS -T (0.02% Tween-20). The washes were collected and combined.

The column was then eluted with 50 µL of SDS sample buffer at 70° C for 7 minutes. Each column was eluted twice and the collections were combined.

IL12R-β1

IL12R-β2 forms a heterodimer with another receptor – IL12R-β1. These receptors are both type 1 cytokine receptors.[30] As such, IL12R-β1 was also considered a possible candidate to exhibit *C*-linked glycosylation. Immunoprecipitation was used to enrich the concentration of the IL12R-β1 protein. Santa Cruz anti-mouse IL12R-β1 was used to capture any present IL12R-β1 in the RAW 264.7 cell lysates. Magnetic beads conjugated with Protein A were used to immobilize the primary antibody. After washing, the proteins of interest were eluted with

SDS buffer at 70 °C for 7 minutes. The elution process was repeated, and the samples were combined.

Immunoblots

IL12R-β2

After the immunoprecipitation, the proteins were separated using SDS-PAGE. Once separated, the proteins were transferred to nitrocellulose paper using a current of 500 mA for 1 hour. The nitrocellulose paper was then blocked with 5% milk in TBS-T (0.1% Tween-20).

Once blocked, the blot was incubated overnight at 4° C in a 1:1000 solution of anti-IL12R-β2 antibody and blocking buffer. After incubation, the blot was washed 1 time for 15 minutes and 3 times for 5 minutes in TBS-T. HRP-conjugated goat anti-rabbit IgG secondary antibody (Abcam, Inc.) was introduced in a 1:10000 solution of antibody to TBS-T. The blot was incubated for one hour at room temperature and then washed according to the procedure above.

Once the blot was properly blocked and exposed to primary and secondary antibodies, it was incubated for 5 minutes in a 1:1 luminol and peroxide solution. The blot was imaged on the BIO RAD ChemiDoc™ XRS+ imaging system provided by the Spitzer Lab.

IL12R-β1

After the immunoprecipitation, the proteins were separated by SDS-PAGE. Proteins were transferred from the gel to nitrocellulose paper. The blot was tested using rabbit anti-mouse IL12R-β1 primary antibody and an anti-rabbit IgG secondary antibody.

Mass Spectrometry

HECID MS was used to analyze samples obtained from cell cultures.

IL12 Stimulation

Failure to identify IL12R-β2 *via* mass spectrometry led to additional literature review. Upon further analysis of the literature, it was found that LPS upregulates the ERK pathway in RAW 264.7 macrophage-like cells. Furthermore, it was found that this pathway inhibits the IL12 pathway and production.[31]

Stimulating the IL12 cytokine is necessary to upregulate the expression of its receptors, IL12R-β1 and IL12R-β2. With this in mind, a new experiment was designed to stimulate the IL12 pathway selectively. Three 75 mL flasks of cells were raised. The first flask was untreated and used as a control. The second flask was pretreated with UO126 for 30 minutes to inhibit the ERK pathway, and then LPS overnight to stimulate the IL12 pathway. The third flask was treated with CpG DNA. CpG DNA is shown to stimulate the IL12.[32] However, CpG DNA has not been shown to stimulate the ERK pathway.

The samples were sent to the Haltiwanger Lab at University of Georgia. IL12R-β2 was not detected in any samples *via* HECID MS.

Using the same lysates, levels of the IL12 p-40 domain were analyzed using enzyme-linked immunosorbent assay (ELISA). A 96 well plate was incubated with anti-p40 capture antibody in sodium bicarbonate buffer overnight at 4° C. The plate was covered with a plastic film to prevent contamination. The wells were then washed with PBS and blocked with 5% milk in PBS, covered with a plastic film, and incubated overnight at 4°C. After blocking, the wells were washed and standards and samples were added to the plate in quadruplets (Table 1), covered with a plastic film, and allowed to incubate at 37° C for 90 minutes. The wells were again washed, and biotinylated p40 detection antibody was added. The plate was covered with a plastic film and incubated for 2 hours at room temperature. A secondary antibody incubation was

done for 1 hour at room temperature using Avidin-HRP secondary antibody. Next, the plate was incubated for 15 minutes with TMB substrate and detected at 495 nm using a BioRad iMark plate reader (courtesy of Dr. Holly Cyphert).

A silver stained SDS PAGE gel and a Western blot were also made using these samples per the protocols mentioned earlier in the chapter.

CHAPTER 6

RESULTS

IL12R-β2

A mass of approximately 116 kilo Daltons was observed in the ERK shut down experiment. It is present in all three elution lanes, but more prominent in the stimulated lanes (Figure 9). The presence of the 116 kD bands strongly suggests the presence of IL12R-β2 under each condition (expected mass 112-130 kDa). The band was not seen in an SDS-PAGE gel made with the same samples after silver stain (Figure 10).

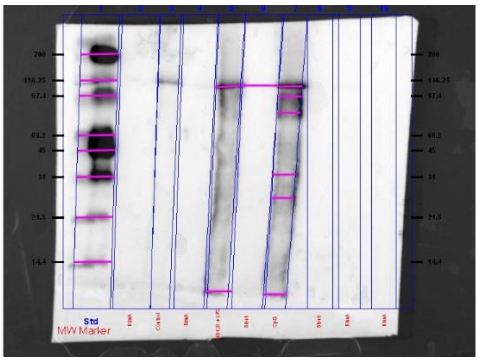


Figure 9: Western Blot of ERK Shutdown Cell Lysates. Western blot showing the presence of a band corresponding to the molecular weight of IL12R-β2 in the elutions of unstimulated RAW 264.7 cells.

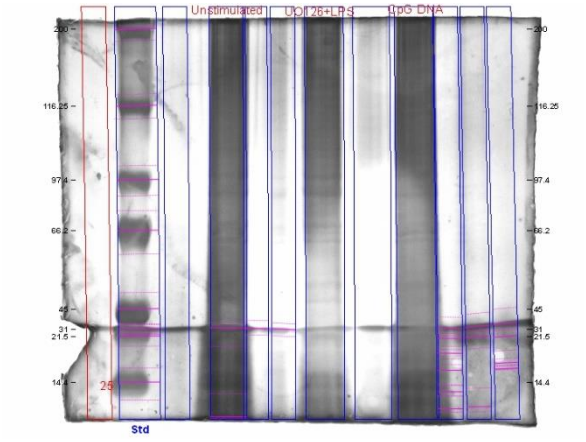


Figure 10: Silver Stain Gel of ERK Shutdown Cell Lysates. Silver stain gel does not have clear bands at the molecular weight of IL12R-β2 in the elutions of unstimulated RAW 264.7 cells.

To investigate the possibility of *C*-linked modification (whether on IL12R-β2 or not), a biotin conjugated Western blot was done using biotinylated *Galanthus nivalis* lectin (GNL) – a lectin shown to bind to terminal mannose on glycosylated amino acids. Peptide – N – Glycosidase F (PNGase F) was applied directly to the surface of the blot to cleave any *N*-linked mannose before GNL was applied. The GNL blot showed no bands (Figure 11). It is suspected that the sample proteins degraded and could not bind GNL due to several weeks of storage at 4° C prior to detection. Another GNL blot was not done due to constraints on time and materials.

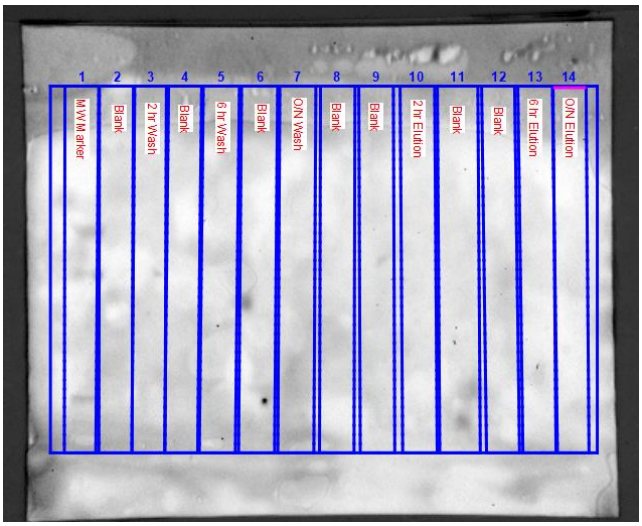


Figure 11: Western Blot Probed with Biotin Conjugated GNL. GNL bound proteins were not detected on this blot.

After immunoblotting, a second SDS PAGE gel was run from IP elutions. Instead of a transfer, the gel was stained with Coomassie Brilliant Blue to reveal protein bands. No bands corresponding to IL12R-β2 were observed. After destaining, the gel was stained again with a more sensitive silver stain technique. Bands were excised and prepared for mass spectrometry analysis. The bands were analyzed either in the Frost Lab at Marshall University or the Haltiwanger Lab at University of Georgia.

IL12R-β1

The absence of IL12R-β1 bands in the western blot was reason to discontinue this piece of the experiment.

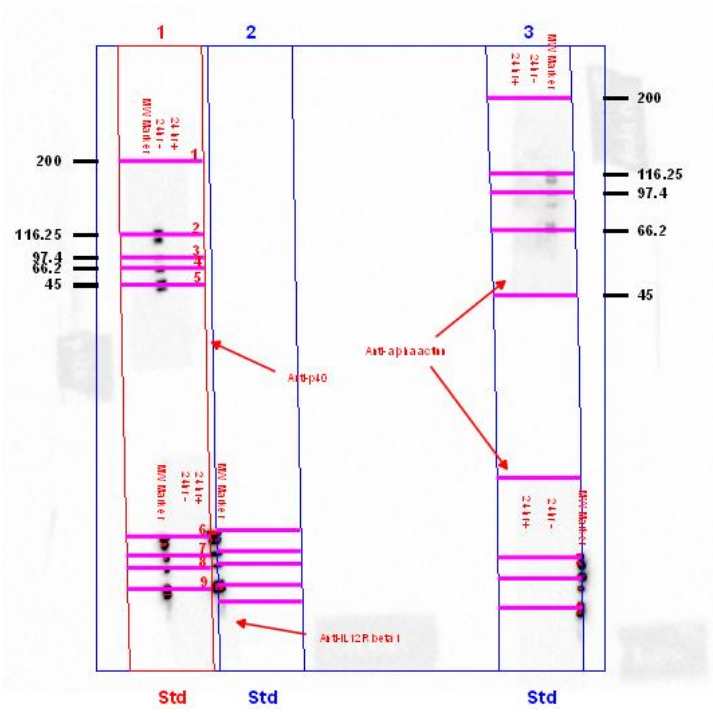


Figure 12: Western Blot of IL12R-β1. Western Blot of IL12R-β1 with p-40 and α-actin controls. The blot was cut into four strips for imaging. Each strip contained its own molecular weight marker.

IL12 Stimulation

IL12 was stimulated using various conditions. The conditions used were recorded as followed and used for analysis downstream (Table 1).

Table 1: 96 Well Elisa Sample Grid. Table of samples loaded into each well of a 96 well ELISA plate. Each sample was analyzed in quadruplets.		
Wells A1 – A4: IL-1β Standard 0 pg/mL	Wells A5 – A8: Control 1:10 Dilution	Wells A9 – A12: Control 1:100 Dilution
Wells B1 – B4: IL-1β Standard 1000 pg/mL	Wells B5 – B8: LPS + U0126 1:10 Dilution	Wells B9 – B12: LPS + U0126 1:100 Dilution
Wells C1 – C4: IL-1β Standard 500 pg/mL	Wells C5 – C8: CpG 1:10 Dilution	Wells C9 – C 12: CpG 1:100 Dilution
Wells D1 – D4: IL-1β Standard 250 pg/mL	Wells D5 – D8: RAW 0 Hour	Wells D9 – D12: Background
Wells E1 – E4: IL-1β Standard 125 pg/mL	Wells E5 – E8: RAW 1 Hour (-LPS)	Wells E9 – E12: RAW 1 Hour (+LPS)
Wells F1 – F4: IL-1β Standard 62.5 pg/mL	Wells F5 – F8: RAW 2 Hour (-LPS)	Wells F9 – F12: RAW 2 Hour (+LPS)
Wells G1 – G4: IL-1β Standard 31.3 pg/mL	Wells G5 – G8: RAW 6 Hour (-LPS)	Wells G9 – G12: RAW 6 Hour (+LPS)
Wells H1 – H4: IL-1β Standard 15.6 pg/mL	Wells H5 – H8: RAW 12 Hour (-LPS)	Wells H9 – H12: RAW 12 Hour (+LPS)

The absorbance readings of each quadruplet were averaged, and the standards were used to plot a standard curve (Table 2). The equation of the resulting line is shown in the figure as is the R^2 value.

The standard curve was used to calculate the concentration of IL12 p40 in each sample condition from the average absorbance (Table 3)**.**

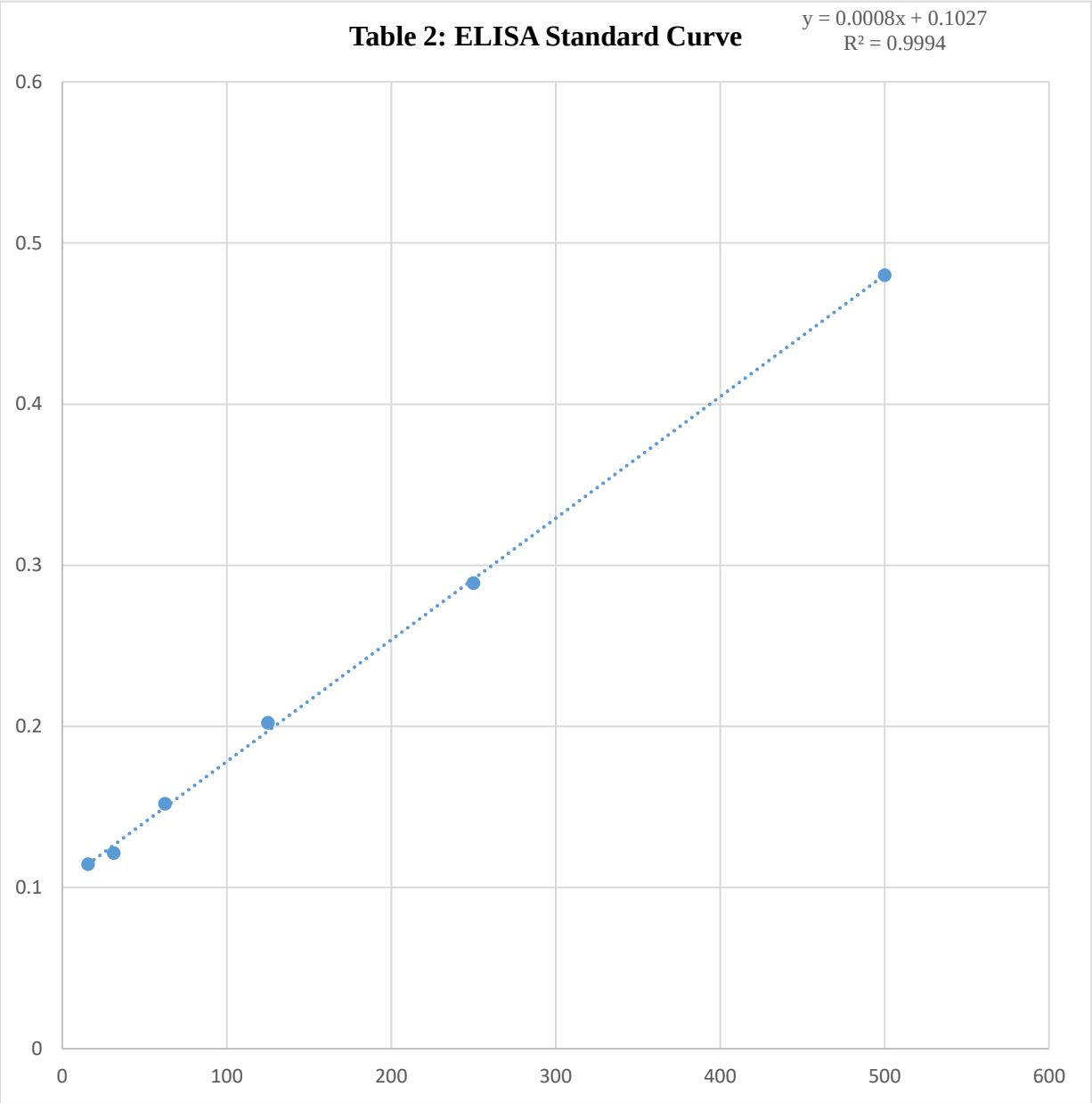
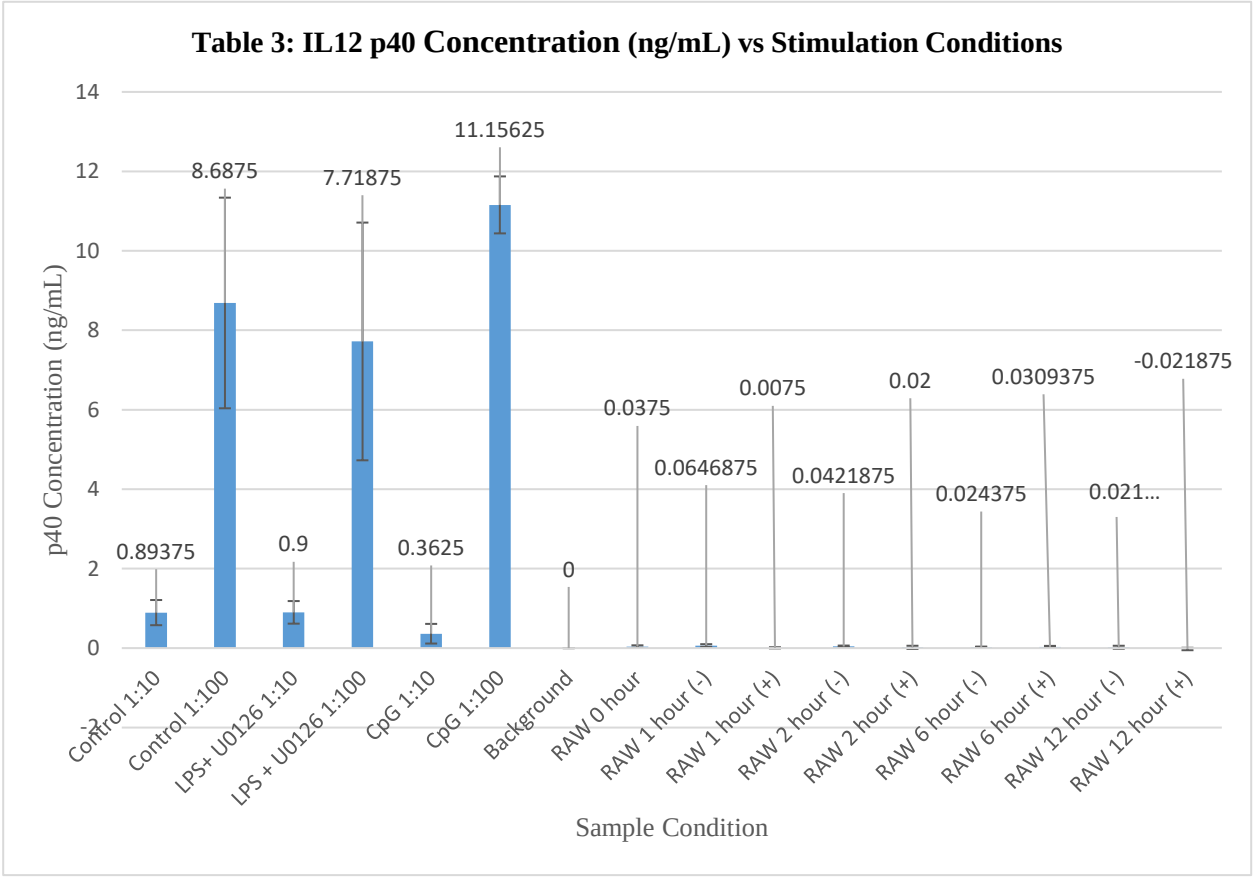


Table 3: IL12 p40 Concentration (ng/mL) vs Stimulation Conditions



REFERENCES

1. New England Biolabs 2019. https://international.neb.com/tools-and-resources/feature-articles/the-structure-function-and-importance-of-carbohydrates

2. Stanley, P., Taniguchi, N., and Aebi, M. 2017. N-Glycans. In: Varki, A., Cummings, R.D., Esko J.D., et al. editors. *Essentials of Glycobiology* [Internet]. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2015-2017. Chapter 9. Available from: https://www.ncbi.nlm.nih.gov/books/NBK453020/ doi: 10.1101/glycobiology.3e.009

3. Spiro, R.G. 2002. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds, *Glycobiology*, Volume 12, Issue 4, 1 April, Pages 43R–56R, https://doi.org/10.1093/glycob/12.4.43R

4. Ten Hagen, K.G., Bedi, G.S., Tetaert, D., Kingsley, P.D., Hagen, F.K., Balys, M.M., Beres, T.M., Degand, P., and Tabak, L.A. 2001. Cloning and characterization of a ninth member of the UPD-GalNAc:polypeptide N-acetylgalactosaminyltransferase family, ppGalNTase-T9. *J. Biol. Chem*. 276, 17395–17404.

5. Clausen, H., and Bennett, E.P. 1996. A family of UDP-GalNAc:polypeptide N-acetylgalactosaminyl-transferases control the initiation of mucin-type O-glycosylation. *Glycobiology*, 6, 635–646.

6. de Beer, T., Vliegenthart, J.F., Löffler, A., and Hofsteenge, J. 1995. The hexapyranosyl residue that is C-glycosidically linked to the side chain of tryptophan-7 in human RNase Us, is α-mannopyranose. *Biochemistry*, 34, 11785–11789.

7. Shcherbakova, A., Tiemann, B., Buettner, F., and Bakker, H. 2017. Distinct mammalian C-mannosyltransferases, *Proceedings of the National Academy of Sciences* March, 114 (10) 2574-2579; DOI: 10.1073/pnas.1613165114.

8. Muroi, E., Manabe, S., Ikezaki, M., Urata, Y., Sato, S., Kondo, T., Ito, Y., and Ihara, Y. 2007. C-Mannosylated peptides derived from the thrombospondin type 1 repeat enhance lipopolysaccharide-induced signaling in macrophage-like RAW264.7 cells, *Glycobiology*, Volume 17, Issue 9, September, Pages 1015–1028, https://doi.org/10.1093/glycob/cwm071

9. Buettner, F., Ashikov, A., Tiemann, B., Lehle, L., and Bakker, H. 2013. C. elegans DPY-19 Is a C-Mannosyltransferase Glycosylating Thrombospondin Repeats, *Mol. Biol. Cell,* April 04, *DOI: https://doi.org/10.1016/j.molcel.2013.03.003*

10. Doucey, M.-A., Hess, D., Cacan, R., and Hofsteenge, J. 1998. Protein C-mannosylation is enzyme-catalysed and uses dolichyl-phosphate-mannose as a precursor. *Mol. Biol. Cell*, 9, 291–300.

11. Krieg, J., Vicentini, A., Gläsner, W., Doucey, M.-A., Löffler, A., Hess, D., and Hofsteenge, J. 1997. C-mannosylation of human RNase 2 is an intracellular process performed by a variety of cultured cells. *J. Biol. Chem*. 272, 26687–26692.

12. *Essentials of Glycobiology* 2nd edition 2009: https://www.ncbi.nlm.nih.gov/books/NBK1947/

13. Anand, M., Rush, J.S., Ray, S., Doucey, M.-A., Weik, J., Ware, F.E., Hofsteenge, J., Waechter, C.J., and Lehrman, M.A. 2001. Requirement of the Lec35 gene for all known classes of monosaccharide-P-dolichyl-dependent glycosyltransferase reactions in mammals. *Mol. Biol. Cell*, 12, 487–501.

14. Hofsteenge, J., Müller, D.R., de Beer, T., Löffler, A., Richter, W.J., and Vliegenthart, J.F. 1994. New type of linkage between a carbohydrate and a protein: C-glycosylation of a specific tryptophan residue in human RNase Us. *Biochemistry*. November 22;33(46):13524-30

15. Krieg, J., Hartmann, S., Vicentini, A., Gläsner, W., Hess, D., and Hofsteenge, J. 1998. Recognition signal for C-mannosylation of Trp-7 in RNase 2 consists of the sequence Trp-x-x-Trp. *Mol. Biol. Cell* , 9, 301–309.

16. Espinosa, V., and Rivera, A. 2016. First Line of Defense: Innate Cell-Mediated Control of Pulmonary Aspergillosis. *Frontiers in microbiology*, 7, 272. Doi: 10.3389/fmicb.2016.00272

17. Alexander, C., and Rietschel, E.T. 2001. Invited review: Bacterial lipopolysaccharides and innate immunity. *Journal of Endotoxin Research*, 7(3), 167–202. https://doi.org/10.1177/09680519010070030101

18. Häcker, G., Redecke, V., and Häcker, H. 2002. Activation of the immune system by bacterial CpG-DNA. *Immunology*. 105(3):245–251. doi:10.1046/j.0019-2805.2001.01350.

19. Tokunaga, T., Yamamoto, T., and Yamamoto, S. 1999. How BCG led to the discovery of immunostimulatory DNA. *Jpn J Infect Dis*. February; 52(1):1-11

20. Krieg, A.M. 2000. The role of CpG motifs in innate immunity, *Current Opinion in Immunology*, Volume 12, Issue 1, p. 35-43, https://doi.org/10.1016/S0952-7915(99)00048-5.

21. Hemmi, H., Takeuchi, O., Kawai, T., et al. A Toll-like receptor recognizes bacterial DNA. *Nature* **408,** 740–745 (2000) doi:10.1038/35047123

22. Huang, X., and Yang, Y. 2010. Targeting the TLR9-MyD88 pathway in the regulation of adaptive immune responses. *Expert Opin Ther Targets*. 14(8):787–796. doi:10.1517/14728222.2010.501333

23. Kuzmich, N.N., Sivak, K.V., Chubarev, V.N., Porozov, Y.B., Savateeva-Lyubimova, T.N., and Peri, F. 2017. TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. *Vaccines (Basel)*. 5(4):34. Oct 4. doi:10.3390/vaccines5040034

24. Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J., and Gusovsky, F. 1999. Toll-like Receptor-4 Mediates Lipopolysaccharide-induced Signal Transduction, *J. Biol. Chem*. 274: 10689-. doi:10.1074/jbc.274.16.10689

25. Zou, N., Ao, L., Cleveland, J.C. Jr., Yang, X., Su, X., Cai, G.Y., Banerjee, A., Fullerton, D.A., and Meng, X. 2008. Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion, *Am J Physiol Heart Circ Physiol*. vol. 294 p. H2805-H2813

26. Ihara, Y., Manabe, S., Ikezaki, M., Inai, Y., Matsui, I.S.L., Ohta, Y., Muroi, E., and Ito, Y. 2010. C-Mannosylated peptides derived from the thrombospondin type 1 repeat interact with Hsc70 to modulate its signaling in RAW264.7 cells, *Glycobiology*, Volume 20, Issue 10, October, p. 1298–1310, https://doi.org/10.1093/glycob/cwq096

27. http://technologyinscience.blogspot.com/2013/09/immunoprecipitation-procedure-analysis.html#.WtCEaYjwbIU

28. https://ww2.chemistry.gatech.edu/~lw26/course_Information/4581/techniques/gel_elect/page_protein.html

29. https://commons.wikimedia.org/wiki/File:Western_Blotting.png

30. Robinson, R.T. 2015. IL12Rβ1: the cytokine receptor that we used to know. *Cytokine*. 71(2):348–359. doi:10.1016/j.cyto.2014.11.018

31. Saito, S., Matsuura, M., and Hirai, Y. 2006. Regulation of lipopolysaccharide-induced interleukin-12 production by activation of repressor element GA-12 through hyperactivation of the ERK pathway. *Clin Vaccine Immunol.* 13(8):876–883. doi:10.1128/CVI.00075-06

32. Krieg, A.M., Love-Homan, L., Yi, A.K., and Harty, J.T. 1998. CpG DNA Induces Sustained IL-12 Expression In Vivo and Resistance to Listeria monocytogenes Challenge *Journal of Immunology* September 1; 161 (5) 2428-2434

APPENDIX A

ORI APPROVAL

MARSHALL UNIVERSITY
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Office of Research Integrity

December 13, 2019

Michael Parsons
Chemistry Department
Marshall University

Dear Mr. Parsons:

This letter is in response to the submitted thesis abstract entitled *"Identification and Isolation of HSC70 Conjugates from RAW264.7 Macrophage-Like Cells."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director