Investigating how the pathogenic yeast *Cryptococcus neoformans* effects gene expression in host macrophages

by
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Abstract

The pathogenic yeast *Cryptococcus neoformans (Cn)* is the cause of death of nearly 181,000 people annually. When *Cn* is inhaled, it is first detected by alveolar macrophages, innate phagocytic cells that engulf and attempt to destroy *Cn*. However, post-ingestion a range of different outcomes are possible. For an example, the macrophage may kill the ingested yeast, the yeast may replicate within and eventually escape from the macrophage, or the yeast may persist within the macrophage in a dormant state. To investigate how *Cn* infection may affect the fate of host macrophages, our lab uses RNAseq-based transcriptome profiling to determine how phagosomal *Cn* influences gene expression in host cells. These experiments utilize *in vitro* cultures of *Cn* infected murine macrophages. Our preliminary data has suggested that the metabolic activity of the *Cn* may compromise our transcriptome data by artificially altering the expression of glucose-regulated genes, such as thioredoxin interacting protein (TXNIP), which may indirectly impact the activity of important transcriptional regulators including p53. To circumvent this problem, I used a nutrient replenishment strategy, replacing the growth media in the macrophage: *Cn* cultures at experimentally determined time intervals. The effectiveness of this strategy was determined by measuring the protein products of glucose-responsive genes by western blotting. Glucose concentration was measured via glucose oxidase assays by withdrawing medium from samples at our experimentally determined time intervals. This work revealed that the depletion of glucose in *Cn*:macrophage cultures could be circumvented by replenishing the media at 6-hour intervals for the duration of 24 hours. We assert that this modified *Cn*:macrophage culture system is suitable for use in future

transcriptome profiling experiments that will help us to accurately determine how *Cn*-infection alters gene expression in host macrophages.

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Definitions

Apoptosis: A type of programed cell death that occurs in metazoans.

Alveolar: Relating to alveoli, the tiny air sacs found in the lungs.

***Cryptococcus neoformans*:** Pathogenic fungus that is virulent in immunosuppressed people and can cause cryptococcosis and cryptococcal meningitis.

Cryptococcosis: Uncontrolled *Cryptococcus neoformans* infection with dissemination of the yeast to the brain, leading to cryptococcal meningitis.

Macrophage: Innate immune cell whose function is to phagocytize pathogens and cell debris. It is involved in inflammation and inflammatory signaling as well as tissue repair.

Tp53: Tumor suppressor gene encoding p53 transcription factor, which regulates the expression of cell cycle inhibitor proteins and pro-apoptotic regulators.

Pathogen: Microorganism that can cause disease.

Phagocytosis: The process by which macrophages or other phagocytes engulf and internalize extracellular material, including microbes.

Phagolysosome: Fusion of a lysosome with a phagosome. Phagolysosome formation is important for the degradation of phagosome contents through the delivery of lysosomal hydrolytic enzymes.

Phagosome: A vesicle found within the cytosol of macrophages or other phagocytic cells, which contains phagocytosed material.

Transcription: The production of a RNA copy of a DNA sequence, requiring the activity of an RNA polymerase.

***Txnip*:** The gene, thioredoxin-interacting protein, is involved in regulating the cellular response to nitrosative stress, amongst other functions, and is responsive to changes in cellular glucose concentrations.

Abbreviations

AIDS- Acquired Immune Deficiency Syndrome

BCA- bicinchoninic acid

Cn- Cryptococcus neoformans

diH_20- deionized water

DMEM- Dulbecco's Modified Eagle Medium

DMSO- dimethyl sulfoxide

ECL- enhanced chemiluminescence

EDTA- ethylenediaminetetraacetic acid

FBS- fetal bovine serum

GO- glucose oxidase assay

GXM- glucuronoxylomannan

HIV- Human Immunodeficiency Virus

IFNγ- interferon-γ

LPS- liposaccharide

MOI- multiplicity of infection

PBS- phosphate buffered saline

PMSF- phenylmethylsulfonyl fluoride

RIPA- radioimmunoprecipitation assay buffer

SDS- sodium dodecyl sulfate

YPD- yeast extract peptone dextrose

Introduction

Cryptococcus neoformans and macrophage interaction

Cryptococcus neoformans (*Cn)* is a pathogenic yeast that is the cause of death of nearly 181,000 people per year (Rajasingham *et al*., 2017). Although *Cn* is thought to infect the majority of individuals in urban environments before the age of 5 years old, it typically only causes cryptococcosis or fungal meningitis in the immunocompromised (Goldman *et al*., 2001). Consistent with this, *Cn* is a major cause of death in HIV/AIDS patients (Goldman *et al*., 2001).

C. neoformans is found worldwide and can persist in the environment, where it can be found in soil, trees, and bird guano (Coelho and Casadevall, 2014). It appears to be equally at home within human hosts and is capable of replicating at 37°C and expresses a range of different virulence factors. One of them is the polysaccharide capsule, which helps *Cn* to avoid destruction by host phagocytes (Fan *et al*., 2005). For example, the polysaccharide capsule produced by *Cn* allows the yeast to initially escape ingestion by alveolar macrophages in the lungs immediately after initial exposure (Bulmer and Sans, 1967). However, opsonization by host immunoglobulin or complement will eventually allow for phagocytosis of *Cn* by these cells.

After *Cn* is ingested by host macrophages, a range of different outcomes are possible; the macrophage may kill the ingested yeast, the yeast may replicate within and eventually escape from the macrophage (which may or may not kill the host cell depending on the method of egress), or the yeast may persist within the macrophage for days, months, or even years in a dormant state (Ben-Abdallah *et al*., 2012). However, the

factors that control these possible outcomes and the course of a *Cn* infection itself are poorly understood. Furthermore, it has also been shown that intracellular *Cn* growth induces a range of different cellular stresses, including mitochondrial dysfunction in host macrophages and yet causes surprisingly little apoptotic cell death (Coelho *et al*., 2015). We will use RNA-sequencing (RNAseq)-based transcriptome analysis to determine how *Cn* infection impacts gene expression in host macrophages in order to obtain new insights into how intracellular *Cn* affects the function and survival of host macrophages. Unfortunately, earlier attempts by our lab to conduct this study were confounded by glucose depletion in our *in vitro* culture model.

Glucose depletion in an *in vitro* macrophage: *C. neoformans* infection model

Previous transcriptome profiling experiments preformed in our lab showed that infection of RAW264.7 murine macrophages with *Cn* downregulated expression of the gene *txnip,* which encodes the thioredoxin interacting protein (TXNIP), and altered the expression of a range of genes regulated by p53. Downregulation of *txnip* transcripts and loss of p53 protein expression in infected macrophages was somewhat predictable as *txnip* is known to be downregulated in response to nitric oxide in macrophages and regulates the cellular response to nitrosative stress in these cells (Forrester *et al*., 2009). It might be expected that nitric oxide levels would be higher in M1 activated, *Cn* infected macrophages. The transcription factor, p53, is a tumor suppressor that is responsive to DNA damage and cellular stress (Pflaum *et al*., 2014). As such, we would have predicted that this protein would be up- rather than downregulated in *Cn*-infected cells.

It was later determined that these changes in TXNIP and p53 levels might have been stimulated by glucose depletion in *Cn* containing cultures rather than by intracellular *Cn* growth. This was determined by using glucose oxidase assays, showing that glucose levels dropped by >50% within 6 hours after *Cn* infection. Similar decreases were not observed in uninfected control macrophage cultures. Furthermore, in control experiments expression of *txnip* in RAW cells in the absence of *Cn* were found to be sensitive to glucose levels in culture medium and the concomitant loss of TXNIP protein levels was responsible for the changes in the expression of p53-regulated genes as TXNIP is a positive regulator of p53. More precisely, when expressed at high levels, TXNIP can directly associate with p53 proteins, blocking polyubiquitination by Mdm2, a negative regulator of p53, thereby promoting the stabilization and nuclear accumulation of p53 proteins (Suh *et al*., 2013; Jung *et al*., 2013).

As changes in the expression of *txnip* and a range of p53-regulated genes were likely an artifact of culture conditions in the RNAseq screen, it is also possible that a range of other genes were similarly affected. As a consequence, genuine changes in gene expression driven by intracellular growth of *Cn* in host macrophages were possibly masked by changes induced by the depletion of nutrients in our *in vitro* infection model and it is difficult if not impossible to disentangle these in the current data set.

Our prior transcriptome profiling experiments involved the harvesting of mRNA 24-hour post-infection. As *Cn* can be extruded from infected macrophages and doubles every 3 hours, we suspect that these extracellular *Cn* are largely responsible for the depletion of glucose in our macrophage cultures. Therefore, a simple method to abrogate glucose depletion would be the regular removal of extracellular *Cn* and replenishment of

growth medium during experiments. The success of this method could be ascertained by regular monitoring of glucose levels in our cultures and measuring TXNIP levels in infected macrophages. The successful completion of these experiments will enable us to repeat our transcriptome profiling experiments and identify a genuine transcriptional signature of intracellular *Cn* growth in macrophages which will help us better understand how macrophages respond to *Cn* infection. Due to the time required for processing of mRNA and cDNA libraries, other ways of attaining data that was relevant to our research was pertinent. Similar to RNAseq, microarray data was used to find upregulated genes in macrophages infected with *Cn* (Coelho *et al*., 2015). Existing, publicly available microarray data sets could be used to identify candidate genes that are affected by intracellular *Cn* growth, providing new insights into this host pathogen interaction.

1.2 Rationale

The overarching goal of this thesis research is to understand how *Cn* affects gene expression in host macrophages. As our preliminary experiments indicate that it is important to prevent glucose depletion in our *in vitro* infection models prior to the harvesting of mRNA from host macrophages, we developed an adjusted version of this infection model. We projected that this problem could be mitigated by replacing the culture media at regular intervals in order to remove extruded *Cn* and replenish glucose levels. The strategy used to avoid excessive glucose depletion in cultured macrophages infected with *Cn* was attempted by infecting RAW 264.7 murine macrophages with *Cn* at an multiplicity of infection of 3:1 (*Cn:* macrophages) for a period of 2 hours before removing the culture medium containing extracellular *Cn* that were not ingested by the macrophages and replacing with fresh culture medium. Over the subsequent 24 hours,

before we typically harvested our macrophages to isolate mRNA, intracellular *Cn* may be extruded from host macrophages and continue to grow and replicate, depleting glucose in the culture medium.

2

MATERIALS AND METHODS

1. Thawing of mammalian cell stocks

Frozen stock of RAW 264.7 (ATCC® TIB71™) murine macrophage-like cells were thawed immediately after taken out of a liquid nitrogen cryostore or -80°C freezer and collected via serological pipette then placed in a 15 mL conical containing 9 mL of growth medium. The cells were centrifuged at 300 x g for 10 minutes and the supernatant was discarded to remove the DMSO. The pellet was resuspended in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose, sodium pyruvate, and 25 mM HEPES, supplemented with 10% FBS, 5 mL 100x Penicillin streptomycin, and 1 µg/mL gentamicin antibiotic which was then seeded into a 100 mm tissue culture dish.

2. Tissue culture techniques

RAW 264.7 cells were cultured in DMEM. The cells were maintained in a 37°C incubator with 5% CO_2 and 95% humidity and grown to 80% - 100% confluency prior to passage. Regular maintenance was carried out in a class 2 biosafety cabinet (BSC) using sterile serological pipettes. Once confluent, the plates were taken out of the incubator and the DMEM media was discarded. Then the cells were rinsed with 5 mL of phosphate buffered saline (PBS) and dissociated from the surface of the plate by scraping with a cell scraper into 10 mL of warm DMEM. One tenth of the resultant cell suspension was reseeded into new tissue culture dishes (1:10 split ratio) with 9 mL of fresh culture medium.

3. Yeast culture

H99S (Janbon *et al*., 2014), a GXM-positive Serotype A *Cn* strain, was stored at -80°C to prevent microevolution. These stocks were used to inoculate 15 mL of yeast peptone dextrose (YPD) media. All yeast culture procedures were carried out in a BSC to prevent contamination. Using serological pipettes, 15 mL of YPD was transferred to two 20 mL flasks. Immediately after being taken out of the -80°C freezer, the stock of *Cn* was inoculated by sterile wooden stick to collect a small quantity of the frozen stock, which was then deposited inside the flask with YPD. This was done for every flask. Aluminum foil was placed on top of the flasks, which were then incubated at 37°C with shaking (150 RPM) for 24-30 hours before they were used to infect macrophage cultures.

4. *Cn* Infection

Day 1

Prior to infection, macrophages that were cultivated in the tissue culture lab that had reached a confluency of 80 % to 100 % were dissociated from the bottom of the plate using a cell scraper and centrifuged for 5 minutes at 300 x g. The media was decanted, and pellet was resuspended in 10 mL of fresh media and counted using a hemocytometer whilst diluting with tryptophan blue (TB) to label dead cells.

2 mL of cultured macrophages were seeded into each well of 6 well tissue culture plates at a density of 7.5×10^5 cells/well. After the cells adhered to the surface of the plate (occurs within ~2 hours post-plating), the macrophages that needed to be prepared for *Cn* infection or mock infection were treated with 1 μg/mL of LPS and 200 units/mL interferon-γ (IFNγ) to induce M1 polarization so that the macrophages will phagocytose the yeast, becoming infected. If the cultures were used for glucose oxidase assays, phenol

red free medium was used instead of standard phenol red-containing DMEM as phenol red will interfere with the colorimetric assay. The cells were incubated at 37°C overnight.

Day 2

The following day, 1.5 mL of *Cn* culture from each flask was transferred into 2 mL microcentrifuge tube. The *Cn* cultures were centrifuged at 693 x g for 5 minutes and the YPD was decanted. The cells were then washed with 1 mL of phosphate buffered saline (PBS) three times and pelleted between washes by centrifugation at 693 x g for 5 minutes spin, decanting the PBS after each wash and breaking up the pellet each time before the next wash. The *Cn* was resuspended in 300-500 μL of PBS depending on the concentration of *Cn* and counted using a hemocytometer, diluting with PBS. An ideal count would be ~50-200 *Cn* per quadrant. The calculations for the *Cn*/mL in the stock solution after counting are similar to those described above for macrophages. The *Cn* needed for a MOI of 3:1 (*Cn*: macrophage) in a 6 well plate with 7.5×10^5 macrophages is 2.25×10^6. Extra *Cn* was opsonized because of the amount of *Cn* lost during after opsonization during washing. The *Cn* was opsonized with 20 μg/ mL of 18B7 anti-capsular GXM antibody and 20% goat serum in PBS to a total volume of 1 mL to enhance phagocytosis by the macrophages. After opsonization, *Cn* cells were pelleted by centrifuging at 693 x g for 5 minutes, the supernatant was discarded, and the *Cn* washed with 2 mL of PBS, then pelleted at 693 x g for 5 minutes. Finally, the pellet was resuspended in 300 μL of PBS and the opsonized *Cn* were counted again so that the correct volume of cells could be calculated to deliver a sufficient MOI of 3:1, 2.25×10^6. The *Cn* was added to appropriate wells together with LPS (1 μg/mL) and 200 units/mL

interferon-γ in order to maintain M1 polarization of the macrophages. The plates were then spinoculated by centrifugation at 300 x g for 1 minute, bringing *Cn* into close proximity with the macrophages on the bottom of the culture dish wells. After 2 hours, extracellular *Cn* were removed by washing three times with 37°C PBS and warm, phenol red free growth medium was added back into the wells. The *Cn*-infected and mock-infected macrophage cultures were then incubated for up to 24 hours (with or without medium replacement at indicated intervals) prior to harvest of culture medium for glucose oxidase assay or cells for western blot analysis.

5. Glucose oxidase assay

To determine the concentration of glucose in cell culture medium samples at specific intervals post-infection, we harvested the medium and performed a colorimetric glucose oxidase assay using the Glucose (GO) Assay Kit (GAGO-20; Sigma-Aldrich, USA). We centrifuged 1 mL of each sample of the growth medium at 1000 x g for 5 minutes in a benchtop centrifuge to remove any extracellular *Cn* and all cell debris. We diluted our sample so that the expected glucose concentration in each was no greater than 1 μg/mL so that all readings fell within the linear range of the assay. Four glucose standards prepared in deionized water (diH_20) and an additional blank were generated to make a standard curve. We then added the assay reagent to all samples and standards, waiting 30 seconds between adding it to consecutive samples one at a time, and incubated these in a 37°C water bath to allow the glucose oxidase reaction to occur. After 30 minutes, the reaction was halted by adding 12 N H_2SO_4 to each tube. Using a spectrometer, the absorbance of each sample at 540 nm was determined. The glucose concentration of each medium sample was then calculated from the standard curve.

6. Western Blot

Cells were lysed at the indicated times post-treatment or infection in 1x RIPA buffer (1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Sodium deoxycholate, 1 mM EDTA). Prior to utilization, 1.5 µL PMSF, and 3 µL of 100x protease inhibitor cocktail (Sigma, USA) was added and cells were dissociated from the tissue culture plate using cell scrapers. The samples were vortexed for 30 seconds and put on ice for 5 minutes. This was repeated four times then the samples were centrifuged at 4°C for 15 minutes at 15781 x g to pellet cell debris, then the supernatant was collected. A bicinchoninic acid assay (BCA) was performed to determine the protein concentration in each sample. Once the concentrations were calculated, samples within the same set were normalized to the same concentration by diluting with RIPA buffer and then 6X Laemmli buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, Glycerol, 0.1% bromophenol blue, 2.5-5% β-metacaptoethanol) was added to each sample to a final concentration of 1x and heated at 95°C for 10 minutes to denature the proteins. The samples were then separated on a 10% SDS-PAGE gel, electrophoresed at 150 mV. The separated proteins were transferred to a nitrocellulose membrane for 45 minutes using a semi-dry blotting tank (Bio-Rad, USA) then blocked with 5 % milk/PBS/T (1x PBS/T with 5% nonfat dry milk) for one hour to prevent nonspecific binding. The blot was washed three times with PBS for 5 minutes then soaked in primary antibody for 16 hours at 4°C. The blot was then washed three times with PBS/T for five minutes then incubated in secondary antibody on a rocker for an hour at room temperature. The blot was then washed with PBS/T again three times for five minutes. The blot was then analyzed using a BioRad Gel imager with ChemiDoc Software and placing 1 mL of ECL 1 (250 nM Luminol stock in DMSO, 90

mM pCoumaric Acid Stock, 1 M Tris (pH 8.5), dilute solution with diH_20 to a volume of 100 mL) and ECL 2 (30 % H_20_2, 1 M Tris (pH 8.5), dilute solution with diH_20 to a volume of 100 mL) substrate in a 1:1 ratio.

The following primary antibodies were used: TXNIP/VDUP1(H-12) (1:500 dilution, mouse clone, Santa Cruz Biotechnology), β-actin; PA1-16889(1:1000 dilution, rabbit clone, Thermofisher Scientific), p53(1C12) (1:1000 dilution, mouse clone, Cell Signaling). Secondary antibody m-IgGκ BP-HRP:sc-516102 (Santa Cruz Biotechnology) was used in place of a secondary antibody for blots probed with mouse monoclonal antibodies and mouse anti-rabbit IgG-HRP:sc-2357 (Santa Cruz Biotechnology) was used in this study.

7. mRNA extraction and cDNA library production

Prior to RNA extraction, macrophages were seeded, M1 polarized, infected (or mock infected), and incubated as described in "*Cn* infection". At the indicated time post-infection, the *Cn*-infected macrophages were dissociated from the tissue culture plates using cell scrapers and washed with PBS three times. The cells were then counted to ensure there were enough cells necessary to complete the protocol. Each RNA extraction required a minimum of 5×10^8 cells. After checking cell number, total RNA was extracted from the cell samples using a RNeasy kit (Qiagen, USA) in accordance with the manufacturer's guideline. The extracted total RNA was converted to cDNA and prepared for RNAseq analysis using NEBNext® Ultra RNA Library Prep Kit for Illumina (New England Biolabs, USA).

RESULTS

Preliminary experiments indicate Cn-infected macrophage cultures deplete glucose at an accelerated rate.

Preliminary experiments were performed in the Nelson lab by L. Sircy to examine the effect of *Cn* infection on gene expression in host macrophages. To do this, macrophages were infected with *Cn* and RNA sequencing was done to assess any transcriptome changes 24 hours post-infection. In the previous attempts to perform these measurements, the metabolic activity of *Cn* in our *in vitro Cn*: macrophage cultures may have compromised our transcriptome data by altering the expression of glucose-regulated genes, such as thioredoxin interacting protein (TXNIP), seeing that TXNIP was in the top 10 downregulated genes in the RNAseq screen, which may have also impacted the expression and activity of genes and proteins downstream of TXNIP including the important transcriptional regulator p53.

To investigate this possibility, experiments were performed by Linda Sircy (Nelson Lab) to measure loss of glucose in *Cn*: macrophage cultures over the course of 24 hours using glucose oxidase assays. This experiment indicated a small but detectable reduction of glucose in *Cn*: macrophage cultures 4 hours post infection. The rate of glucose loss increased over the following 20 hours and was less than 10 % of initial levels by 24 hours **(Figure 1)**. Overall, these experiments showed that *Cn* infected macrophage culture at a MOI of 3:1 (*Cn*: macrophages) in 60 mm dishes could reduce glucose concentration from ~ 3.9 g/L to < 0.5 g/L in 24 hours **(Figure 2)**. Control

samples of mock-infected macrophages and M1 polarized with LPS displayed deceased levels as well but not as severe as macrophages infected with *Cn*.

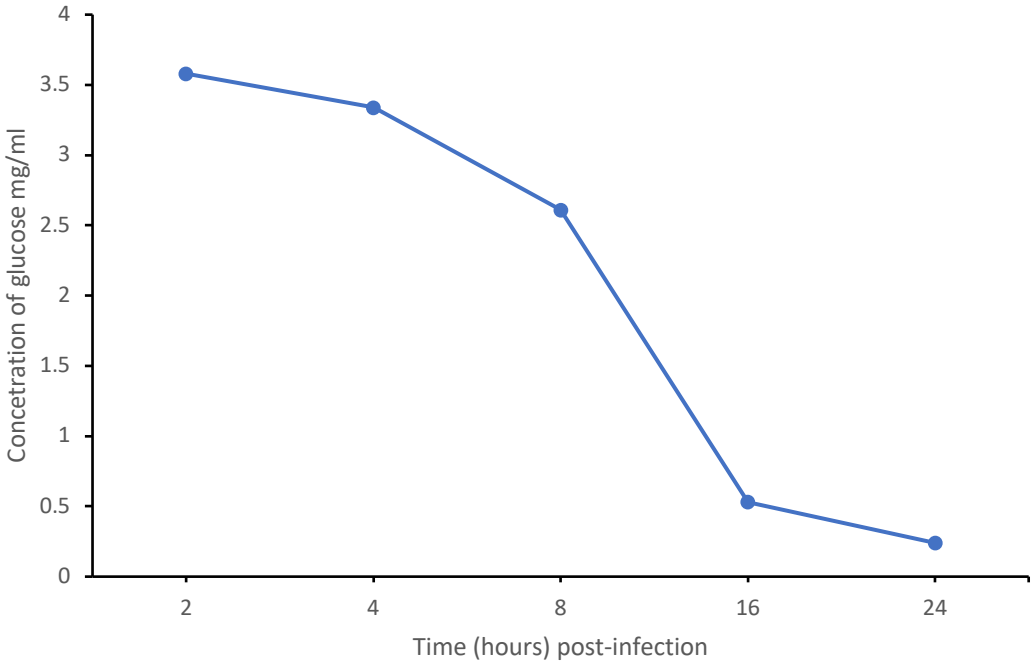
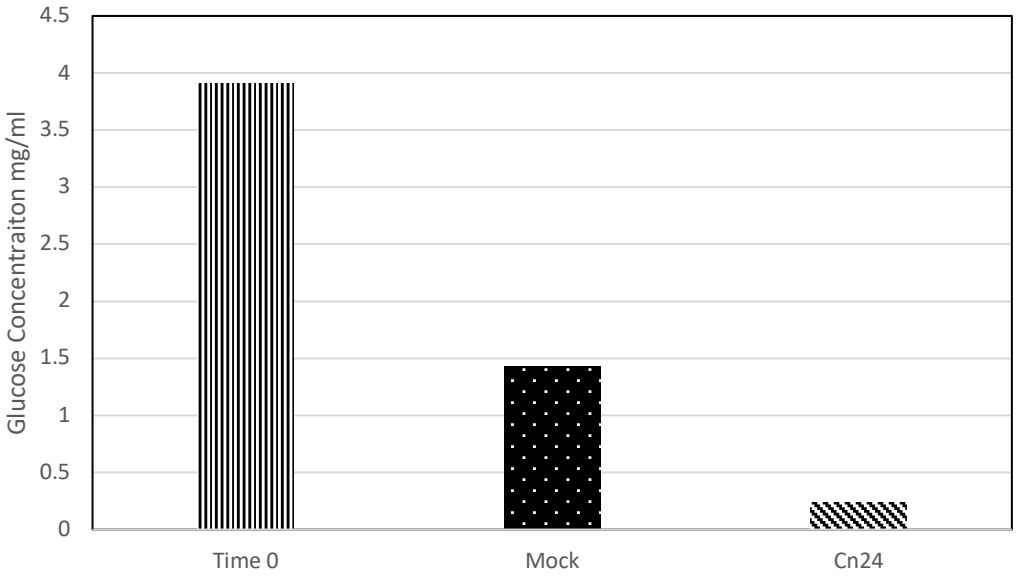


Figure 1. Reduction of glucose in *Cn*: macrophage culture in 24 hours. Macrophages infected with *Cn* were incubated 2 hours post-infection until 24 hours post-infection. Media was extracted at indicated time intervals and measured using glucose oxidase assay. Raw data generated by Linda Sircy.



Time post-infection (hours)	[Sample] (mg/ml of glucose)	[Actual glucose] (mg/ml of glucose)	Absorbance (540nm)
0	0.0695	3. 91	0.442
24 (Mock)	0.0255	1.43	0.162
2	0.0637	3.58	0.405
4	0. 0594	3.34	0.378
8	0.0464	2.61	0.295
16	0.0093	0.53	0.06
24	0.00424	0.24	0.027

Figure 2. *Cn*:macrophage culture glucose concentration diminished after 24 hours. Glucose concentration in macrophage culture medium was measured at regular intervals post *Cn* infection using a glucose oxidase assay. "Mock" indicates samples that were mock infected. Raw data generated by Linda Sircy.

Reduction in glucose concentrations stimulate decreased expression of TXNIP and p53 in macrophages

To determine whether glucose depletion and not *Cn* infection was responsible for the changes in TXNIP and p53 detected by previous RNAseq analysis, we incubated RAW 264.7 macrophages in medium supplemented with different glucose concentrations and western blotted for TXNIP and p53 **(Figure 3)**. Macrophages were cultured in six different conditions which included treatment with and without LPS (to induce M1 polarization) in high glucose (~4.5 g/L), low glucose (1 g/L), and DMEM with no glucose. Expression of TXNIP and p53 proteins were detected in lysates from cells incubated with high glucose concentrations both with and without LPS treatment. p53 was also detected in macrophages in low glucose conditions that were not activated with LPS and showed reduced levels in media with no glucose.

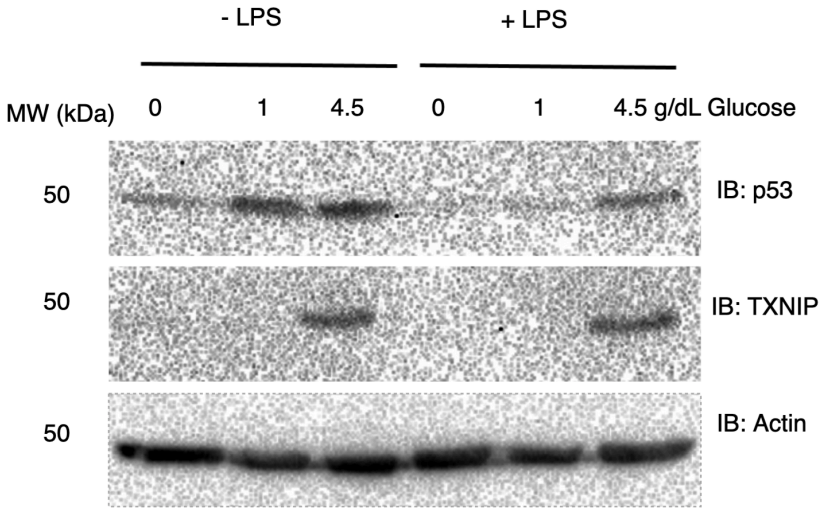
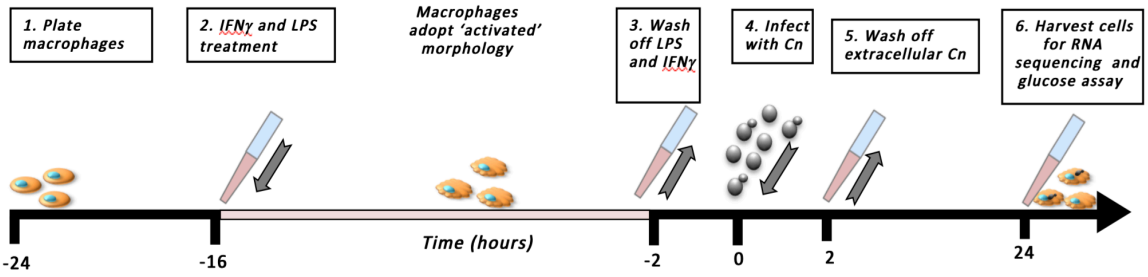


Figure 3. Glucose affects the expression of TXNIP and p*53* proteins in murine macrophages. Western blot for TXNIP, p53, and actin proteins in lysates from RAW 264.7 macrophages maintained for 24 hours in growth medium containing the indicated glucose concentrations.

Enhanced design used for Cn: macrophage infection

As the experiment shown in **Figure 1** was only preformed once by L. Sircy, I attempted to confirm the result by repeating the experiment while also testing whether replacing the growth medium at regular intervals could prevent excessive glucose depletion **(Figure 3)** and consequent changes in the expression of glucose–regulated genes. In the previous experiment, the *Cn*: macrophage culture post-infection experienced constant depletion of glucose over the course of 24 hours without media changes whereas mock-infected and untreated macrophages (M0) retained relatively high glucose concentrations compared to the initial RAW media levels by itself. The current model measures *Cn* infected macrophage glucose depletion over the course of 24 hours while trying to circumvent the glucose reduction. To do this, we replenished the media every 6 hours for 24 hours post-infection **(Figure 4)**.

A



B

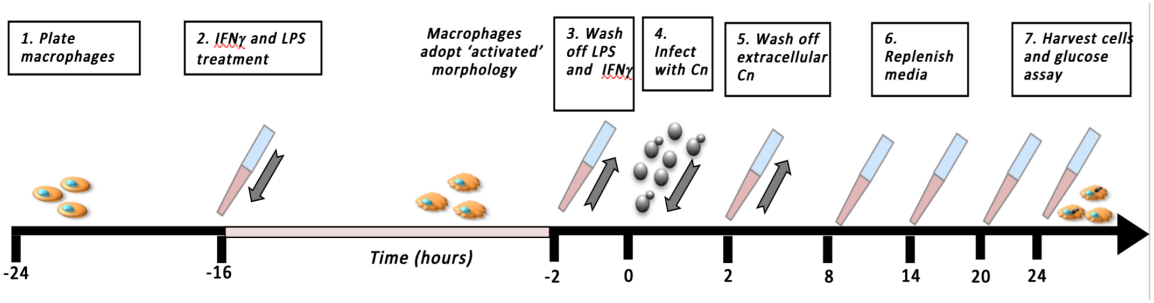


Figure 4. Visual representation of design used for *Cn*: macrophage infection. (A) original experiment design to measure effects of *Cn* infection on macrophage gene expression and **(B)** altered design used to circumvent glucose depletion post-*Cn* infection.

Regular replenishment of glucose prevents glucose depletion in Cn: macrophage cultures

Performing our alternate design, which involved replenishing the media every 6 hours over 24 hours to avoid glucose depletion in *Cn*: macrophage culture gave insight to how to avoid glucose depletion. The data suggest the replenishment of the media helped to avoid glucose depletion over the duration of 24 hours **(Figure 5)**, as little change was observed in glucose concertation in these samples while glucose decreased by more than 50 % in the infected samples without media replenishment. However, while the overall trends were consistent with expectations, there were several flaws with the data. Firstly, the time 0-hour concentrations across the three conditions, which should have been identical, were different. Secondly, reported concentration were several orders of magnitude lower than expected at time 0. The three samples had different glucose concentrations even though they were all replenished with fresh media 2 hours post infection at the time which the samples were taken. After modifying the protocol by adding a control, M0, we performed the experiment again in triplicate.

The 'No Change' cultures displayed a reduction of glucose by 24 hours that was less drastic than observed in the previous iteration of the experiment but still notable in that it showed the greatest decline compared to the other variables **(Figure 6)**. Surprisingly, M0 cultures, which are untreated macrophages cultures, showed a greater reduction of glucose concentration than mock macrophages. Mock macrophage culture exhibited similar results to the previous glucose assay data and maintained a moderately high glucose concentration compared to initial media concentration.

Media samples and cell lysates were collected simultaneously 24 hours post infection. Western blots were performed using the lysates to test for detection of other proteins besides TXNIP and p53 but was unsuccessful as there was no signal. CD137 and RIG-1 were tested because they showed one of the largest fold changes in microarray data (Coelho *et al*., 2015). These genes were of interest to us because they were both shown to be involved in the innate immune response (Wang *et al*., 2013; Dharmadhikari *et al*., 2015).

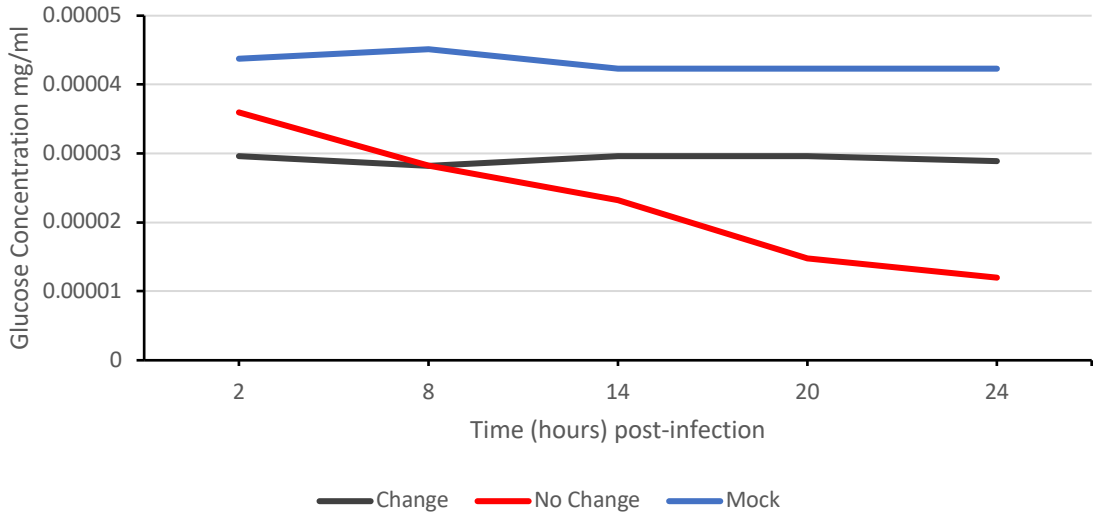
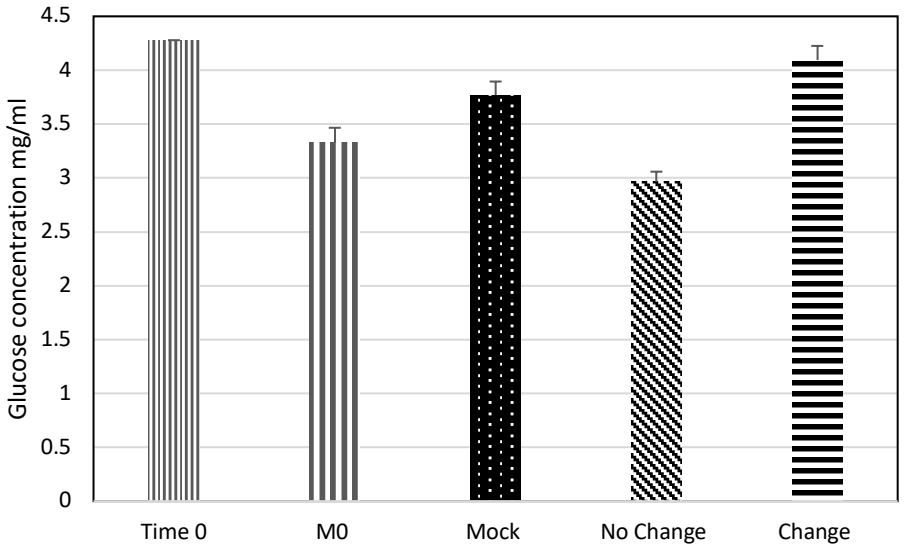


Figure 5. Regular medium replacement prevents glucose depletion in *Cn*-infected macrophage cultures. 'Change', 'No Change', and mock-infected cultures over the course of 24 hours with the Change media being replenished every 6 hours for 24 hours. Glucose concentration was measured every 6 hours using glucose oxidase assay.



Time post-infection (hours)	[Sample] (mg/ml of glucose)	[Actual glucose] (mg/ml of glucose)	Absorbance (540nm)
0	0.07606588	4.27870595	0.494
24 (M0)	0.05912691	3.41736516	0.379
24 (Mock)	0.06699096	3.76824198	0.427
24 (No change)	0.05269928	3.00054205	0.34
24 (Change)	0.07278132	4.00634669	0.462

Figure 6. Glucose replenishment circumvents glucose depletion. Glucose assay of Mock, M0, *Cn:* macrophage cultures ('Change' and 'No Change') at the 24-hour point compared to its starting point at time 0.

4

DISCUISSION

The macrophage: *Cn* host pathogen interaction is necessary for the successful clearance of the yeast from infected individuals, but it also provides an avenue for long term persistence of *Cn*. In order for us to develop strategies that assist the former and prevent the latter of these two scenarios, we need to understand how intracellular residence affects gene expression in host macrophages. To study the macrophage: *Cn* relationship, previous experiments were done in lab to investigate the macrophage transcriptome 24 hours post-infection with *Cn*. Over the 24-hour duration of the experiment, macrophages proliferate at a slow rate, especially if infected and metabolize relatively little of the nutrients in the medium. However, *Cn*, even if intracellular, can double in number every 3 hours. If not killed by the macrophages, they will replicate logarithmically, depleting glucose and other nutrients as they do so. This has the potential to have profound effects on gene expression in the macrophages and could confound efforts to identity changes in gene expression caused only by intracellular residence of *Cn* independent growth conditions.

When analyzing transcriptome data, it was observed that *txnip* was in the top 10 downregulated genes in RNAseq screen. *Txnip* is a glucose responsive gene that can increase the stability of the transcription factor p53 by preventing its ubiquitination by Mdm2 at subsequent degradation, which we believe may have indirectly impacted the expression of multiple genes in the host cell associated with cell cycle, apoptosis, and polarization state.

The purpose of this thesis research project was to confirm that glucose depletion occurred in our *Cn*-infection model and determine whether glucose depletion alone was sufficient to explain a subset of the gene expression changes seen in our transcriptome profiling experiments. In collaboration with other members of the lab, we devised a simple method to avoid glucose depletion in our *Cn*-infected macrophage cultures so that future transcriptome profiling experiments would not be affected by this.

My data showed decreased levels of glucose concentration in cell cultures containing macrophages infected with *Cn.* We were unsure at the time if it was *Cn* downregulating *txnip* in macrophages directly or if *Cn* was depleting glucose and therefore altering macrophage transcriptome so further research had to be done. To test this, a western blot was performed as shown in **Figure 3** which verified that the reduction in glucose concentration was the cause of the down regulation of TXNIP. To circumvent this problem, an alternate design was created in which macrophages were infected with *Cn* and incubated for 24 hours like the previous model, however the enhanced model required the replenishment of fresh media every 6 hours for the duration of 24 hours in an effort of decreasing the amount of glucose reduction seen in the *Cn*: macrophage cultures.

As hypothesized, the depletion of glucose in *Cn*: macrophage cultures was circumvented by replenishing the media at 6-hour intervals for the duration of 24 hours. As expected, 'No Change' displayed the greatest decrease in glucose concentration unlike 'Change', which showed little decrease compared to the initial glucose concentration of ~4.3 g/L. *Cn*-infected macrophages and mock macrophages in the preliminary data presented a more profound depletion of glucose over the course of 24 hours. This may be

due to a number of reasons, which include design errors equivalent to *Cn*-infected cultures being treated with LPS but not IFNγ which also activates macrophages, therefore lowering the amount of phagocytosis.

We propose that the loss of p53 expression stimulated by glucose depletion can cause macrophages to adopt an M2 polarized state. In this state, macrophages are less able to destroy ingested microbes, allowing *Cn* to persist within host macrophages (Li *et al*., 2014). We intend to utilize our enhanced *Cn*: macrophage culture model, which mitigates glucose depletion, to more accurately determine how *Cn*-infection alters gene expression in host macrophages.

Also, having adjusted our cell culture methods to avoid glucose depletion, we will perform transcriptome profiling on *Cn*-infected macrophages. This will be achieved by harvesting mRNA and creating cDNA libraries from *Cn*-infected and uninfected macrophage cultures 24 h post-infection (or mock infection). We predicted that the expression of TXNIP and p53 will be unchanged between these two conditions, as changes in glucose levels in the culture will be minimized, and that observed changes in gene expression would genuinely reflect a transcriptional response of the host cells to intracellular *Cn* growth rather than nutrient depletion in the cultures.

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