IDENTIFYING THE PROTEIN INTERACTORS OF CHOLESTEROL IN PHAGOSOMES

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

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April, 2024

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Certificate

This is to certify that this dissertation entitled "**Identifying the protein interactors of cholesterol in phagosomes**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Sreedev H at Indian Institute of Science Education and Research under the supervision of Dr Siddhesh S Kamat, Associate Professor, Department of Biology, during the academic year 2023-2024.

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Committee:

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THOMAS PUCADYIL

This thesis is dedicated to my parents, my little sister, friends and the Kamat lab group.

Declaration

I hereby declare that the matter embodied in the report entitled **Identifying the protein interactors of cholesterol during phagocytosis** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of **Dr Siddhesh. S. Kamat** and the same has not been submitted elsewhere for any other degree. Whenever others contribute, every effort is made to indicate this clearly, with due references to the literature and acknowledgment of collaborative research and discussion.

SREEDEV H

15.03.2024

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Abstract

Phagocytosis is an evolutionarily conserved pathway to degrade foreign pathogens and apoptotic cells. This process is highly driven by the lipid metabolism where cholesterol plays an important role. Although the role of cholesterol in the transport of late phagosomes towards lysosomes are known to an extent, there are still areas on how this is executed and the survival of pathogens inside the phagosome by manipulating cholesterol metabolism that remain as a black box. Hence identifying the proteins involved in the cholesterol interplay in phagosomes would provide a better insight to these areas and uncover new pathways that will be of therapeutic importance. Towards this goal, we standardized a technique to pick up the proteins that interact with cholesterol and found the proteins that are potential interactors of cholesterol in macrophages.

Acknowledgments

I would like to start with expressing my sincere gratitude to my supervisor Dr Siddhesh. S. Kamat for guiding me throughout the course of my project, providing necessary resources. I am also thankful to Prof. Thomas Pucadyil, my TAC expert, for his valuable inputs and suggestion in troubleshooting.

I am deeply indebted to Ojal Saharan for her undivided attention during my project, which involved conducting experiments, teaching the techniques, and writing. I want to thank her for all the support and backup she gave me during the project. I am grateful to Aakash for guiding me in learning some techniques and helping me with my doubts, even during his holidays. I want to thank Karthik, Sonali, Arnab, Kaveri, Chaitanya, Archit, Kundan, Maya, and Neeraj for the help, support, and fun during this period. I want to thank the KAMAT LAB for its excellent working atmosphere and the long, thought-provoking, enlightening, and mostly funny ‘Chai Sessions’ we had together.

Then, I would like to thank all my friends for their support and for making life at IISER Pune a memorable one. I am grateful to the IISM Kho-Kho team for giving me a different sports experience and for the good memories at IISER Trivandrum.

Finally, and most importantly, I would like to thank my parents for the love, care, and support they gave and the belief they had in me. I want to not miss out on this person, my sister, for both stress-bursting and making conversations and interactions (‘fights’ to be precise) and being the most entertaining person in my life to date.

Chapter 1 Introduction

1.1 PHAGOCYTOSIS

The immune system plays and important role in the discrimination of self and non-self entities to clear the foreign particles from the body, facilitated by various cells and protein complexes. One such group of cells are called macrophages, whose role is to engulf the foreign particles and dead cells and clear them from the body.

Phagocytosis is the process by which cells like macrophages, the primary phagocytes of the immune system, engulf the foreign particles or apoptotic cells and degrade them with the help of cellular machinery (Saharan et al., 2022). This process is carried out via five steps:

1) Particle ingestion
2) Phagosome formation
3) Phagosomal maturation
4) Phagolysosome formation
5) Particle digestion

When the cell encounters a foreign particle, the membrane invaginates and surrounds the particle, forming a vesicle with the particle inside it. This vesicle then buds out from the membrane to the cytoplasm and fuses with the early endosomes to form early phagosomes (EPs). These early phagosomes have specific protein markers like Early Endosome Antigen 1 (EEA1) and Rab5. They undergo fusion with late endosomes to form late phagosomes with specific markers like Lysosomal-Associated Membrane Protein 1 (LAMP1) and Rab7. This process by which the early phagosome turns into late phagosome is called phagosomal maturation, and this process is associated with a reduction in pH from around 6.1-6.5 to around 5.5-6. The late phagosome then fuses with lysosomes that contain the enzymes that degrade the foreign particles to form phagolysosomes and eventually digest and clear the particle (Rosales and Uribe-Querol, 2017).

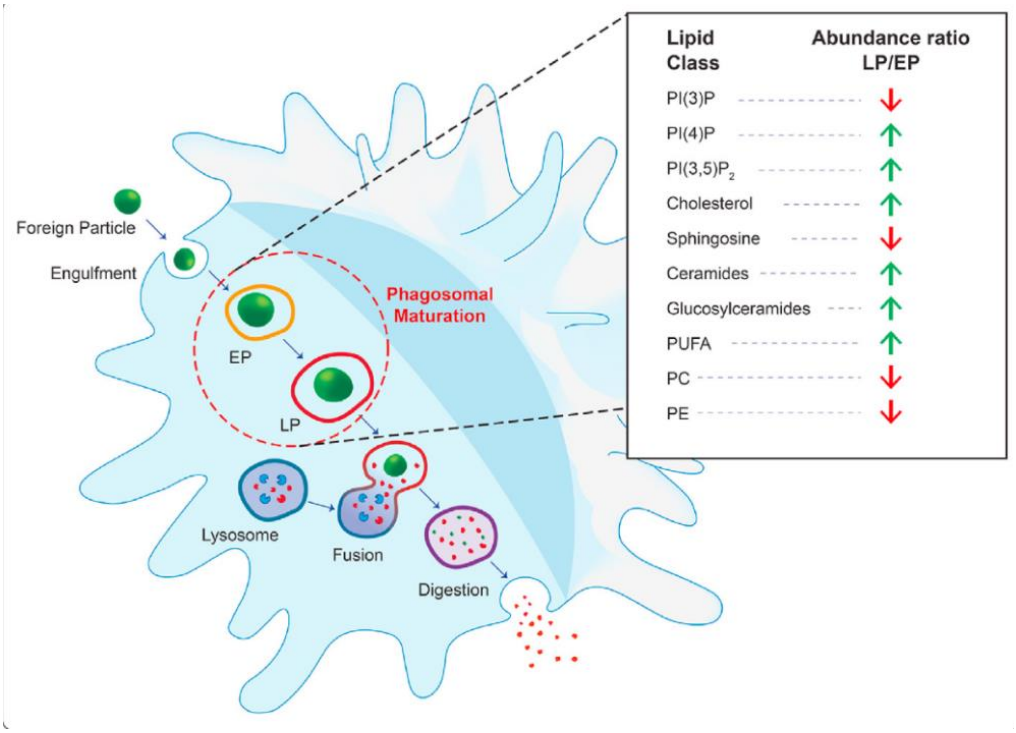

Figure 1: General scheme of phagocytosis *(Saharan et al., 2022)*.

Here, the focus will be on the process of phagosomal maturation and the players involved in the process. It has been observed that the process of phagosomal maturation is associated with changes in the lipid and protein composition of the phagosomal membrane. Most of the proteomic changes are studied, but the lipidomic changes are least known.

1.2. ROLE OF LIPIDS DURING PHAGOCYTOSIS

Previous lipidomic analysis of various lipids on EPs and LPs showed that there are considerable changes in the amounts of some of the lipids during phagosomal maturation. While looking at the variation in the lipids, it was seen that sphingosines, ceramides, and cholesterol showed significant changes. (Pathak et al., 2018)

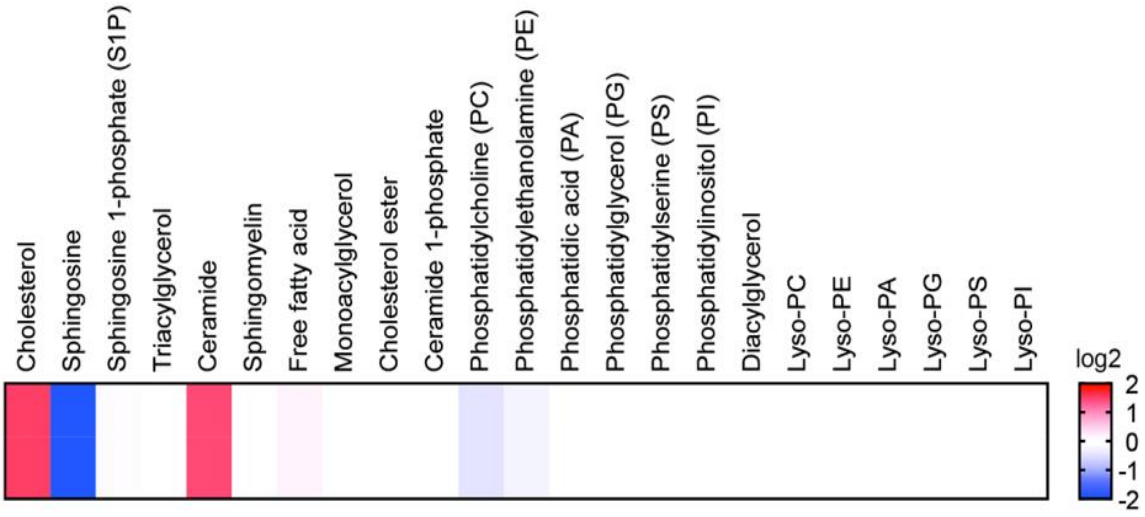

Figure 2: Heat map showing the fold changes in lipid composition during phagosomal maturation. (Pathak et al., 2018)

Lipidomic analysis suggests that the levels of sphingosine are higher in the EPs, and that of ceramides and glucosylceramides are higher in LPs. The same study also focused on the enzyme ceramide synthase since the enzyme is responsible for the biosynthesis of ceramides from sphingosine. Western blot analysis of EPs and LPs suggested that ceramide synthase also exists in phagosomes with a three-fold enrichment in EPs compared to LPs, contrary to the lipidomic result where the ceramides are more in LPs. Pharmacological blockade of this enzyme also reduces the levels of ceramides in the LPs. It is also reported that this blockade also affects the maturation of early phagosomes to late phagosomes as they observed the appearance of the protein markers of EPs on LPs after the preps and also the absence of the protein markers of LPs on it (Pathak et al., 2018) (Mehendale et al., 2021).

1.2 CHOLESTEROL: KNOWN AND UNKNOWN

Cholesterol is one of the sterols discovered to have an important role in storing energy in the form of fat. It is an essential structural component of the cell membrane as it is crucial for the maintenance of the fluidity of the membrane. Along with these functions, cholesterol also acts as a precursor for synthesizing various bile acids. Although these functions of cholesterol are understood to a greater extent, less is

known about the role of cholesterol as a signaling lipid. Here, we explore this area in terms of phagocytosis and especially phagosomal maturation.

Studies have shown that cholesterol rich lipid microdomains in the plasma membrane helps in membrane curvature and formation of phagocytic cup for the internalization of the particle. It is also observed that (Pathak and Mallik, 2017). Lipidomic studies done on phagosomes shows that there is an increase in the levels of cholesterol during phagosomal maturation.

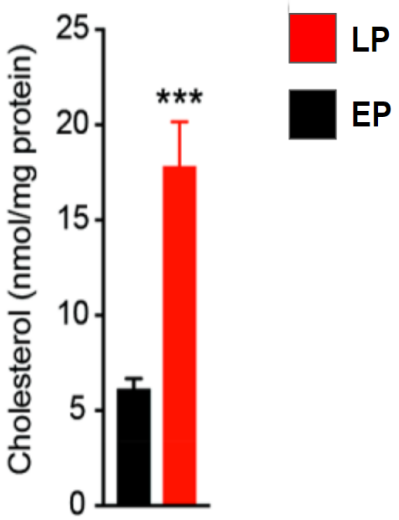

Figure 3: Cholesterol levels in EPs and LPs. (Pathak et al. 2018)

This suggested that cholesterol has some role to play in the phagosomes. It is seen that cholesterol forms rigid lipid microdomains which are stabilized by ceramides and glucosylceramides in the late phagosome. These lipid-rich microdomains are essential for the anchorage of dynein motor proteins so that the late phagosomes can be transported towards the lysosomes to form phagolysosomes. Depletion of cholesterol from the late phagosomes resulted in a significant reduction in the motion of LPs (Rai et al., 2016).

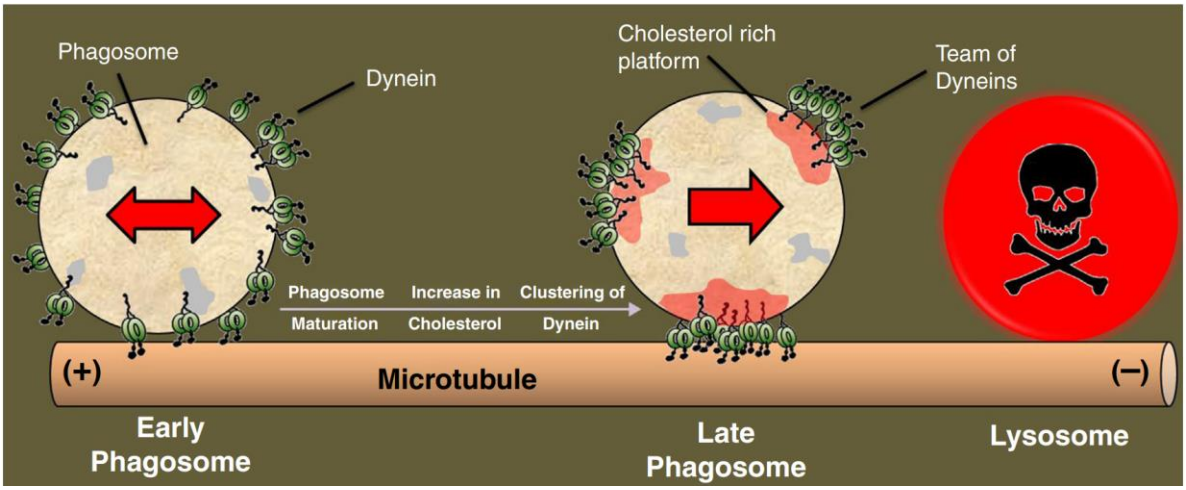

Figure 4: Cholesterol clustering and dynein assembly during phagosomal maturation. (Pathak and Mallik., 2016)

Considering that there are many cellular vesicles and each has its associated cargoes, there should be reliable mechanisms to ensure the recognition of cargoes by the motor or vice-versa. This can be possible only if a wide range of proteins are matched to a broader range of lipids. This raises the question of whether the interaction between the dynein motors and the cholesterol-rich lipid microdomains is a direct interaction with the lipids or an interaction mediated by any adapter proteins.

1.3. Pathogen and host lipid metabolism

Phagocytosis is a typical example for a cellular process that is organized by lipid metabolism. Lipids play a major role in signal generation and transduction, cytoskeletal remodeling, and the fission and fusion events during phagocytosis. The ability of lipids to modulate cell signaling, membrane trafficking, organization of membrane microdomains, and dynamics makes them an attractive target for microorganisms and pathogens to modulate the normal processes in the cells to ensure their survival in the host. Bacteria like *Salmonella enterica* secrete an enzyme SigD. This phosphoinositide phosphatase degrades the PIPs to PIs, which in turn affects the cytoskeletal dynamics of the cells and helps the pathogen survive inside the host (Steinberg and Grinstein, 2008).

Mycobacteria is another genus of bacteria that utilizes the loopholes in the lipid metabolism to survive inside the phagosome. It is seen that cholesterol is essential

for the mycobacterial uptake by the macrophages, and there is an accumulation of cholesterol at the site of entry of mycobacterium. They recruit the Tryptophan aspartate-containing coat protein (TACO) on the phagosome and prevent the fusion of phagosomes with a lysosome. It has been observed that cholesterol is essential for the recruitment and retention of this protein, as TACO has not been observed in cholesterol-depleted cells. Thus, cholesterol has a role in ensuring the survival of mycobacteria inside the phagosome and identifying the mechanisms underlying this could be of therapeutic importance in this direction. (Gatfield and Pieters., 2000).

CHAPTER 2 Materials and Methods

2.1. LIGAND AFFINITY BASED PROTEIN PROFILING (LABP)

In order to investigate the interacting proteins of cholesterol, we use the technique called Ligand Affinity Based Protein profiling. This technique makes use of a lipid probe that structurally mimics the lipid, which in our case, cholesterol and has a photoactivable group (here, a diazirine ring) and an affinity group (here, an alkyne handle).

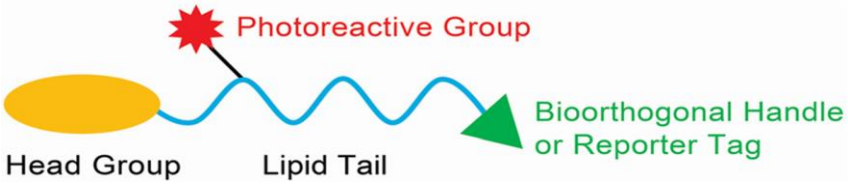

Figure 1. Schematic of a lipid probe. (K. Shanbhag et al. 2023)

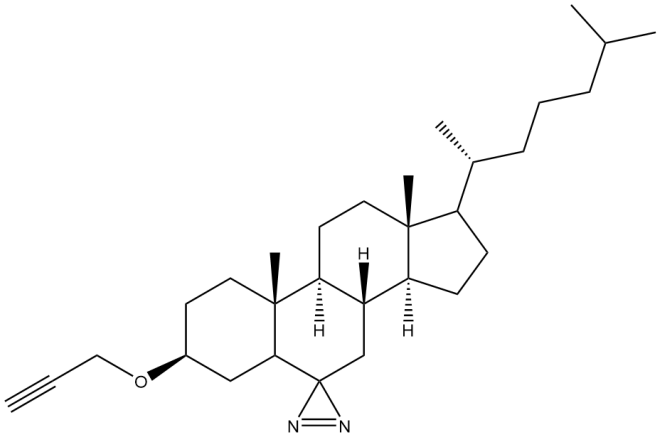

Figure 2: Structure of the cholesterol probe used in my work. (Synthesized by Dr. Kavita Sharma)

This probe, when treated to the cells or any lysates would bind to the active site of the proteins that the parent lipid is supposed to interact with. When the cells or lysates are exposed to UV, the photoactivable diazirine ring gets activated and forms a reactive carbene. This results in the lipid-binding covalently to the protein. Once

the probe is covalently crosslinked to the protein, we attach a reporter tag, which can be a fluorescent rag (rhodamine azide) or an affinity tag (biotin azide), via the alkyne handle using copper-catalyzed azide-alkyne cycloaddition reaction. This can be further visualized in gel or detected using mass spectrometry. (K. Shanbhag et al. 2023)

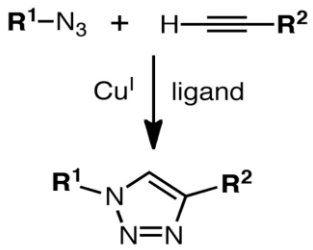

Figure 3: General copper-catalyzed azide-alkyne cycloaddition *(Presolski et al., 2011)*.

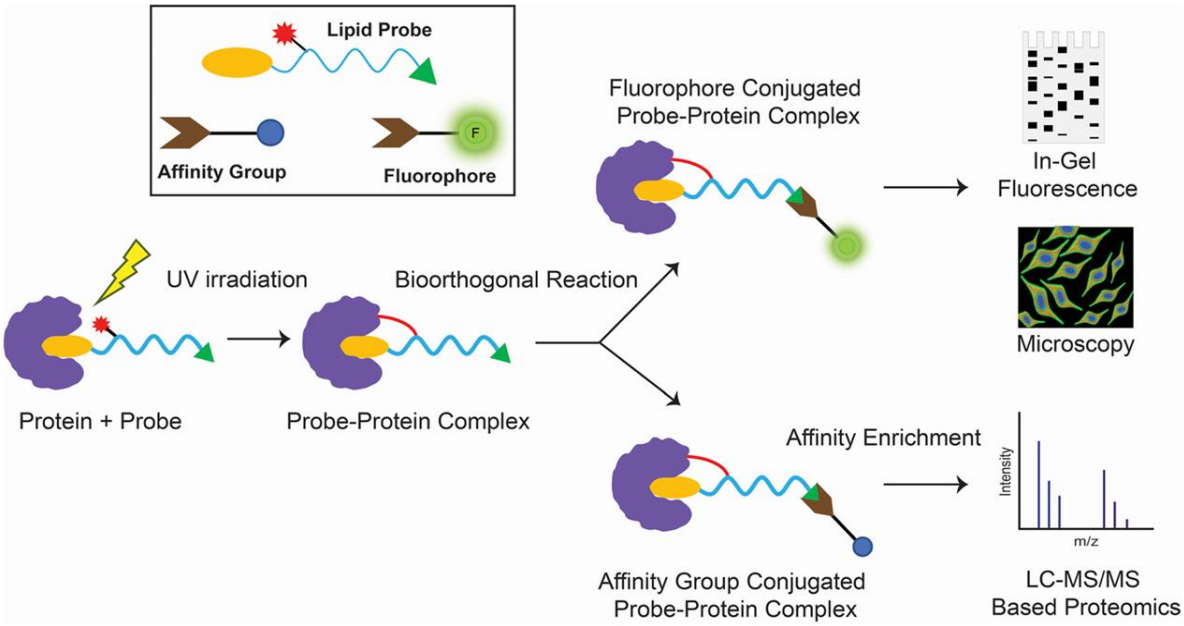

Figure 4: Workflow of LABP *(Shanbhag et al., 2023)*.

2.2. PHAGOSOME PREPARATIONS

Phagosomes are prepared and purified from cultured macrophage cells, RAW264.7 a cell line derived from mice.

2.2.1. EARLY PHAGOSOME PREPARATION

RAW264.7 cells were cultured in Dulbecco's modified eagle medium purchased from Himedia, supplemented with 10% Fetal Bovine Serum, which is heat-inactivated in a water bath at 60 degrees for an hour with shaking for a few seconds each 15 minutes, both 10cm and 15cm in diameter, and grown at 37 degree Celsius and at 5% CO_2 atmosphere.

An average of ten 15cm plates, at least 70% confluent, were used for the preps.

The cells were incubated with silica beads for five minutes at 4 degrees.

These cells were then incubated at 37 degrees for 15 minutes to form early phagosomes.

Once these incubations are done, the cells are washed thrice with PBS, and the cells are scrapped in 5 ml PBS per plate and collected in a falcon.

The cells are then passed through membrane filters of 5 microns diameter in order to lyse the membranes, and the filtrate is collected in a separate falcon.

The filtrate is centrifuged at 200g for 7 minutes so that only the phagosomes get settled down.

These pellets can be flash-frozen in liquid nitrogen and stored at -80 degrees till the experiment.

2.2.2. LATE PHAGOSOME PREPARATION

The media, culture dishes, and beads remain the same as those of early phagosomes.

The cells are incubated with the beads at 4 degrees for five minutes and 15 minutes at 37 degrees for the formation of early phagosomes.

Once this incubation is completed, the media is aspirated out, and fresh media is added to the plates. This ensures no more cells take up the beads after forming early phagosomes.

The cells are then again incubated at 37 degrees for four hours.

The cells are washed thrice using 10 ml PBS, scrapped in 5 ml PBS per plate, and transferred to a falcon.

The cells are then passed through similar membrane filters used for EP preps.

The filtrate is centrifuged at 200g for 7 minutes, and the pellet is flash-frozen and stored at -80 degrees till the experiments.

2.3. SDS PAGE: GEL-BASED ABPP

2.3.1. Gel-based ABPP is used for testing the probe's activity in cell lysates.

Cell pellets stored in -80 degrees were resuspended in 300µl of PBS and sonicated using a probe sonicator at a pulse of 2 seconds for 15 seconds with an interval of 3 seconds at an amplitude of 60%.

Protein concentrations of the lysates were estimated using the Bradford assay.

Protein concentrations in each sample were made up to 2 mg/ml.

Each sample was incubated with the probe for 30 minutes in a thermomixer with rotation (800 rpm) at 37 degrees.

The samples were then transferred to a 96-well plate and were exposed to UV light for 10 minutes for the probe to get covalently linked to the protein.

Meanwhile, we prepare the click mixture for the click reaction, which is a solution of 6µL of 1.5M TBTA, 2µL of 50mM $CuSO_4$, 2µL of 50mM TCEP (Tris(2-carboxyethyl)phosphine) and 1µL of 50µM Rhodamine azide per sample for a 100µL reaction volume.

The samples are then incubated with 11µL of the click mixture with rotation at 800 rpm at 25 degrees for 1 hour in a thermomixer.

The above steps should be done in the absence of light

4X SDS loading dye is added to the samples such that the final concentration of the dye in the sample is 1X.

The samples are then loaded on a 10% SDS PAGE gel and imaged using an iBright gel imager.

The gel is then stained using Coomassie brilliant blue to check whether the protein concentrations in each well are the same.

2.3.1. Gel-based ABPP is used to test the probe's activity in live cells.

Cells were seeded in a 6cm culture plate.

Once the cells became more than 70-75% confluent, they were incubated for different time points with a 50μM concentration of the probe after adding the fresh media to the cells.

The cells are washed using 1.5ml of PBS. The test sample (The cells that should be cross-linked with UV) was subjected to UV exposure for 10 minutes. During this time, the control (-UV) is kept at 4 degrees, covered in an aluminium foil.

The cells are scrapped in 1ml PBS and transferred to an Eppendorf tube and centrifuged at 500g for 5 minutes.

The supernatant is removed and the pellet is resuspended in 250μL of fresh PBS.

The cells are then lysed using a probe sonicator (Pulse: 2 sec ON, 3 sec OFF, Amplitude: 60% for 15 seconds).

The protein concentrations were estimated using the Bradford Assay, and the samples were made up to the concentrations of the sample that has the lowest concentrations of the protein.

The samples are then subjected to a click reaction similar to that of the lysates.

SDS loading dye is added to the samples and run on a 10% SDS gel.

It is then visualized and stained using Coomassie Brilliant Blue.

2.4. PROTEOMICS: MS-BASED IDENTIFICATION OF PROTEIN INTERACTORS

The cells were seeded in culture plates of a diameter of 10cm.

The control and test samples had three replicates each; hence, six plates (3 for +UV and 3 for -UV) were used for the experiment.

The cells were incubated at 37 degrees with a 50μM concentration of the probe.

The cells are then washed, UV treated, and lysed, similar to Gel-based ABPP's case.

Protein concentrations were estimated using Bradford Assay, and the samples were prepared with 1mg/ml of protein concentration.

Click mixture is made by substituting rhodamine azide with biotin azide and rest of the compounds remaining the same.

The volume of each reagent is ten times as that compared to the Gel based ABPP as the reaction volume used here is 1ml.

Each of the samples are then mixed with 110μL of the click mixture and incubated at 25 degrees for 1 hour with rotation at 800 rpm in a thermomixer.

These biotin labelled cell lysates are then transferred to 15ml falcon and are mixed with 2ml of ice-cold methanol, 0.5ml ice cold chloroform and 1ml cold PBS and vortexed

They are then centrifuged at 3500g for 15 minutes for the formation of a protein disc between the chloroform and methanol layer.

Aspirate the top and bottom layer carefully using a vacuum aspirator, leaving the disc intact.

The disc is then washed thrice with 1ml of 1:1 methanol-chloroform mix. Aspirate the supernatant each time carefully without letting the protein disc dry out.

The disc is then solubilized in 500μL of 6M Urea in PBS, which helps to denature the tertiary structure of the proteins by disrupting the disulphide bonds, 20μL of 10% SDS in a water bath sonicator till the solution becomes clear. 50μL of premixed TCEP (200mM) and K_2CO_3 (600mM) is also added to the solution. This step helps in reducing the disulphide bonds.

70µL of freshly prepared 55µM IAA (Iodoacetamide) is added to the solution and incubated for 30 minutes in darkness.

Avidin-agarose beads (100µL per sample) stored in glycerol is washed thrice using 0.2% SDS in PBS by centrifuging at 500g for 2 minutes each

Beads are then resuspended in 1mL of 0.2% SDS in PBS and added to the samples after the incubation along with 4.5ml of PBS and 120µL of 10% SDS

The samples are then incubated for 1.5 hours with rotating. This facilitates the interaction of avidin with biotin tag.

The unbound beads are then washed thrice with 10mL of 0.2%SDS in PBS by centrifuging at 200g for 1 minute each, thrice with 10mL PBS by centrifuging at 500g for 1 minute each, twice with 10mL Milli Q water by centrifuging at 800g for 2 minutes each and finally once with 10ml Milli Q for 5 minutes at 1250g.

Aspirate the supernatant and transfer the beads to Protein LoBind Eppendorf tubes using 1mL of 100mM TEAB

This step is followed by trypsin digestion

Trypsin is stored in a vial of 20µg on to which 2600µL of 2M urea in TEAB is added using a syringe. 200µL of the resuspended trypsin is added to each sample and incubated overnight in a thermomixer with shaking at 800 rpm at 37 degrees.

REDUCTIVE DIMETHYLATION LABELLING (ReDiMe Labelling)

This technique is used to differentiate the control and test samples

It utilized labelling of the peptide fragments with different isotopes of the same atom and later used for detection.

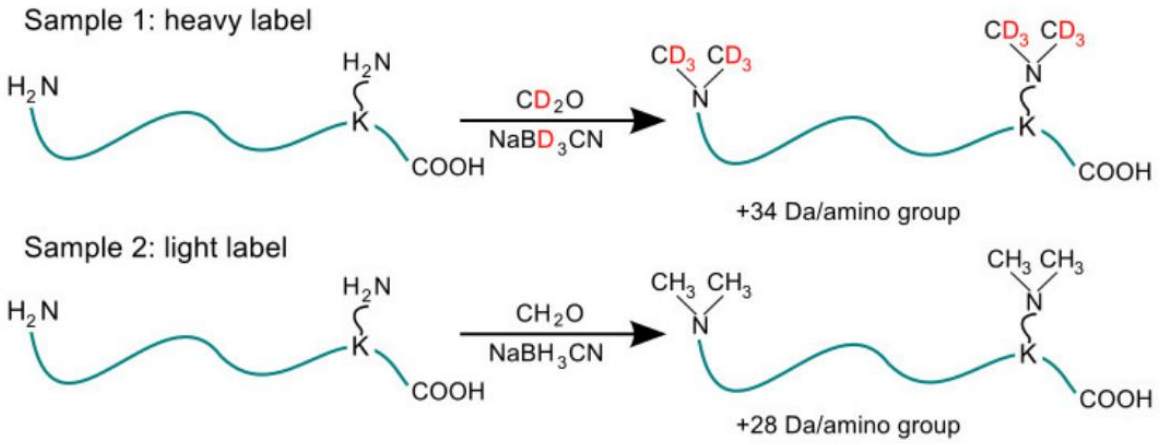

Figure 5: Schematic showing ReDiMe labelling (Tolonen and Haas, 2014)

8µL of 8% light labeled formaldehyde (CH_2O) were added to the control samples and 8µL of 8% heavy labeled formaldehyde (CD_2O) were added to the test samples

8µL of 1.2M Sodium Cyanoborohydride ($NaCNBH_3$) were added immediately to the solution and vortexed to mix it briefly. The samples are then incubated at room temperature for an hour with constant shaking (800rpm).

The reactions are then quenched by adding 8µL of 8% NH_4OH, vortexed, and incubated for 5 minutes.

The samples are then centrifuged at 21000g for 5 minutes after adding 8µL of 20% TFA to allow the beads to settle and to release the peptides from the beads to the supernatant.

The supernatant is then carefully collected in separate Eppendorf tubes. A set of control and test samples are mixed together. The samples are then desalted and sent for mass spec analysis.

DESALTING

This is done to remove the salts present in the samples after these steps by utilizing the affinity of the peptides to the C18 column.

C18 disc is inserted in a 200µL microtip and activated by passing 50µL of 100% ACN (Acetonitrile)

The disc is then equilibrated with the solution by adding 60µL of 0.1% (v/v) trifluoroacetic acid (TFA) and eluting the solvent, leaving a little behind so that the disc doesn't get dried.

We load the peptide samples to the tip and elute the solvent similarly so the disc doesn't dry.

Wash the samples with 120µL of 0.1% TFA and elute the solvent.

The peptides that are stuck to the C18 disc are then eluted out in an Eppendorf tube using a mix of 50µL of 60%ACN and 40% of 0.1% TFA.

The samples are then dried in a centrivap at 40 degrees with rotation.

Once the samples are fully dried, they are subjected to MS analysis.

The results are then analysed using the protein pilot where it gives the names of the proteins detected.

From the list of the proteins we get, we filter those proteins that have an H/L ratio greater than 2 and there should be more than 3 peptides of the protein in at least two of the three replicates.

Those proteins that pass these filtering criteria is considered as a potential hit.

2.5. LIPID EXTRACTION

The cells are seeded in cell culture plates of 10cm diameter. A total of 6 such plates were used (3 for treated and 3 for control)

The cells were incubated with a 50µM concentration of the probe (in the test group) and DMSO (in the control group) at 37 degrees overnight when they became more than 70% confluent.

The cells were then washed with 3mL of PBS, scrapped in 1mL of PBS, and transferred to Eppendorf tubes. They are centrifuged at 500g for 5 minutes.

The supernatant is removed, and the cells are resuspended in 1mL PBS and transferred to glass vials.

3ml of 2:1 mixture of chloroform (mixed with 2µL of an internal standard – Cholesterol D7) and methanol is added to the solution and vortexed.

The samples are then centrifuged at 3000rpm for 15 minutes, forming two discrete layers of chloroform and methanol with the protein disc in between the layers.

The bottom layer is carefully collected and transferred to a new glass vial.

50µL of formic acid is added to the samples to along with 2mL of chloroform and centrifuged again at 3000rpm for 15 minutes for the extraction of phospholipids that might remain in the aqueous layer.

The lower layer is then separated and pooled with the previously extracted fraction.

The samples are then dried under a stream of nitrogen gas

The dried samples are then resuspended in 1mL chloroform and transferred to a new glass vial to remove any aqueous contaminants present in the samples.

This is again dried under pure nitrogen gas and subjected to mass spec analysis.

ANALYSIS

Since we look for the presence of a particular known lipid, we search for that particular lipid in the software, Mass Hunter. Then we integrate the area under the peak of the lipid and that of the internal standard. We then normalize the area of the lipid with that of internal standard after doing a blank subtraction. The area we get will be the quantification of the lipid present in our sample.

Table 1 Constituents of culture media

Reagent	Source	Identifier
Dulbecco's Modified Eagle Medium (DMEM)	HIMEDIA	AL139A
Foetal Bovine Serum (FBS)	HIMEDIA	RM1112
Penicillin-Streptomycin	MP Biomedicals	1670249

Table 2: Probe delivery vehicles

Reagent	Source	Identifier
Dimethyl sulfoxide (DMSO)	Merck	5.43900.1000
Methyl-beta-cyclodextrin	Sigma	C4555

Table 3: Reagents for protein estimation

Reagents	Source	Identifier
Bovine Serum Albumin (BSA)	Sigma	A2153
Bradford reagent	Sigma	B6916

Table 4: Reagents for click reaction

Reagent	Source	Identifier
TBTA	Sigma	678935
$CuSO_4.5H_2O$	Merck	MD6M51035
TCEP	Sigma	C4706
Rhodamine azide	Sigma	760765
Biotin azide	Sigma	762024

Table 5: Reagents for SDS PAGE

Reagent	Source	Identifier
Tris-base	HIMESIA	TC072
SDS	HIMEDIA	MB010
Acrylamide	HIMEDIA	MB068
Bis-acrylamide	HIMEDIA	MB005
Ammonium persulfate	Sigma	A3678
TEMED	Sigma	T9281
Glycine	Qualigens	Q24755
Propan-2-ol	Qualigens	Q13825

Table 6: Reagents for proteomics

Reagent	Source	Identifier
Methanol	JT Baker	BAKR9830-03
Chloroform	Sigma	650498
Urea	Sigma	U5128
K_2CO_3	Sigma	243559
Iodoacetamide (IAA)	Sigma	I1149
Avidin-agarose beads	Sigma	A9207
Trypsin	Promega	V5111
Triethylammonium Bicarbonate buffer (TEAB buffer)	Sigma	90360
Formaldehyde (CH_2O)	Sigma	252549
Heavy formaldehyde (CD_2O)	Cambridge Isotope Laboratories, Inc.	DLM-805-20
Sodium Cyanoborohydride	Sigma	156159
Ammonium Hydroxide (28%)	Sigma	338818
Trifluoroacetic acid	Sigma	302031
C-18 Disc	Supelco	66883-U

CHAPTER 3 Results

Major objectives of the projects are:

- To test the working of the cholesterol probe in cell lysates
- Standardization of the probe concentration of the cholesterol probe in the phagosomal lysates
- To check the activity of the probe and standardization of the incubation time of the probe in the cells
- Check the activity of the probe in the phagosomes post-treatment on the cells
- Identify the protein interactors of the cholesterol probe using proteomics

1) Treatment of the cholesterol probe on RAW 264.7 cell lysates

Given that the amount of protein is similar in the lysates, there should enough substrate, which in our case is the probe, to interact with all the potential interactors present in the sample. For this there is an optimum concentration of the probe below which it won't bind to all the protein and hence some of them would be missed out and above which the addition of further probe doesn't yield any new protein interactors as all the potential interactors are already bound to the probe. Hence we need to standardize the concentration at which the probe binds to all its interactors.

Cell lysates enriched with the probe solubilized in DMSO were ran on a 10% SDS PAGE gel to find whether the probe binds to any proteins. This But the gel didn't show any difference between the control and test samples for the lower concentrations of the probe.

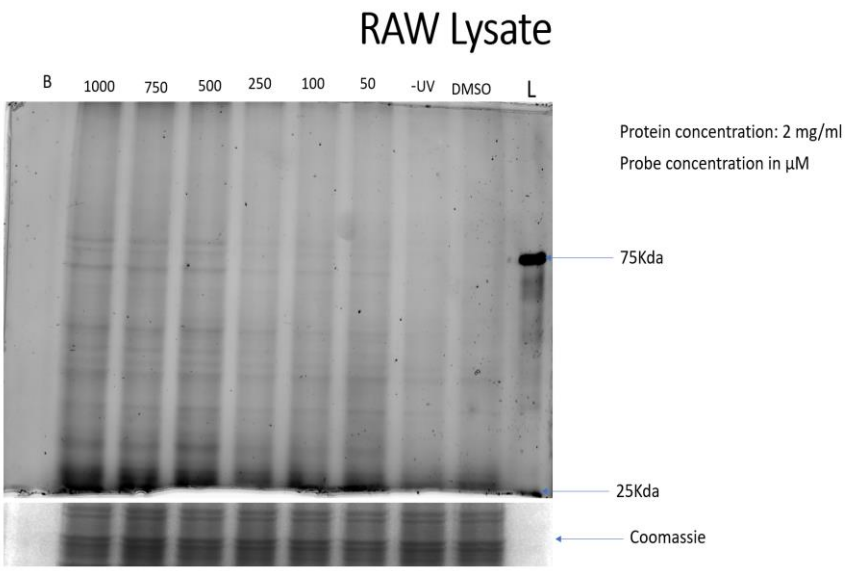

Figure 6: Gel image of probe treated on cell lysate

It can be seen that the probe doesn't get enriched in the cell lysates when treated in lower concentrations. At this point we couldn't arrive at any conclusion. We hypothesized that this could be a result of the probe not being appropriately delivered to bind to the protein.

Hence, we decided to treat the live cells with the probe at a fixed concentration, as too much DMSO could harm the cells. This enables the cells to take up the probe through endocytosis and would deliver the probe to its specific locations and interact with the proteins.

2) Treatment of the probe on the live cells

Since the probe-protein interaction is a dynamic process, we needed to find an optimum duration for which the probe has to be incubated with the cells such that it interacts with the maximum number of proteins below which it won't have the time to encounter all those proteins and above which it could either be degraded or be released from the active site of the protein.

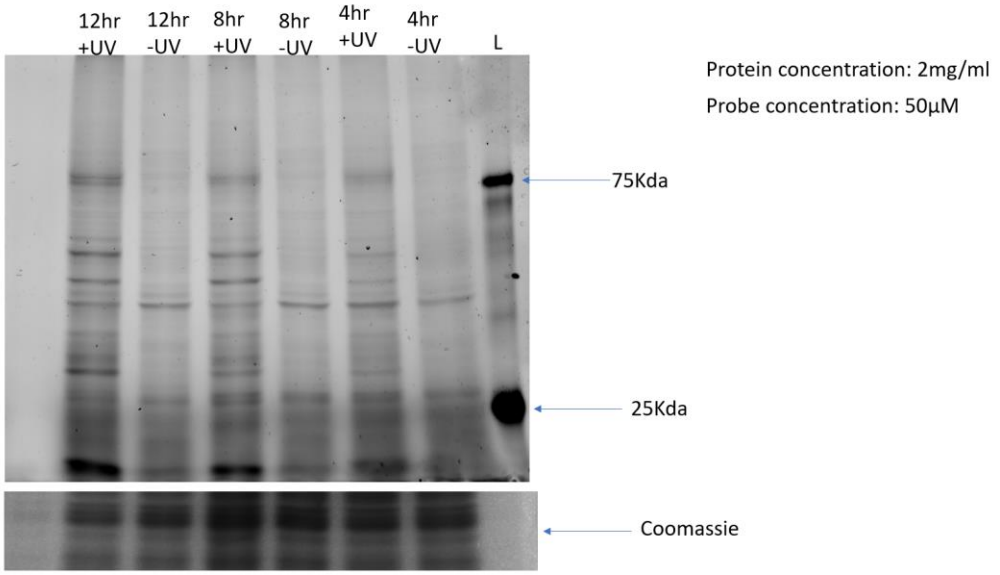

Figure 7: Gel image of probe treated on cells

We looked at different time points of incubation of the probe in the cells and checked for the band patterns in a 10% SDS PAGE gel. It turns out that the intensity of the bands is increasing with time and were seen to decrease above 12hrs or incubation. This made us choose 12 hours as a standard time for incubation of the probe so that the maximum amount of the probe is taken up by the cells and can be distributed to the desired location, if any, during the time of phagosome formation.

3) Quantification of the amount of probe being taken up by the cells

Since we found that the probe is enriching the proteins in the cells, we decided to quantify the amount of the probe being taken up by the cells so that we could know how much of the supplied probe is taken up by the cells. We would also get a rough idea on whether this is sufficient to get enriched in the phagosomes.

We did a lipid extraction on RAW 264.7 cells post treatment of the probe for 12 hours and checked for the presence of the cholesterol probe on it.

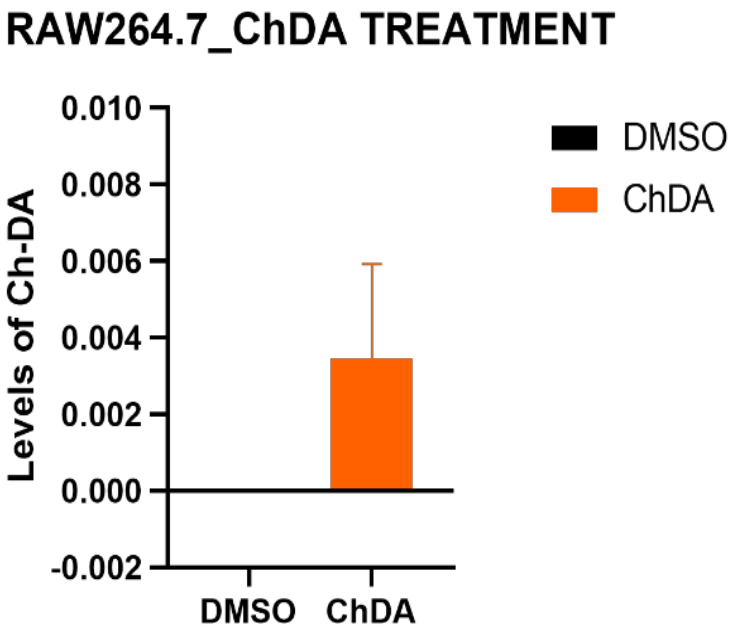

Figure 8: Graph showing the levels of cholesterol probe (AUC) in cells treated with DMSO and Ch-DA

No: of moles of probes used = $50*10^{-6}$M $*4*10^{-3}$L

= 200 nmol

No: of moles of the probe entering the cell=0.0035 nmol

Percentage of probe entering the cells = 0.00175%

4) Enrichment of the cholesterol probe on phagosome

We could see that only a small portion of the total probe supplied to the cells is taken up by them. We needed to know whether any of these are getting enriched in the phagosomes during the process of phagocytosis. So, we decided to incubate the probe for 12 hours and prepare phagosomes and perform a gel-based assay and find out whether there are any proteins getting picked up.

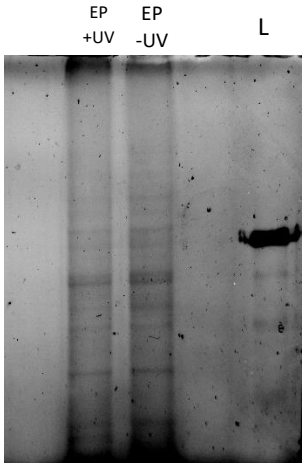

Figure 9:Gel profile of early phagosome formed after treating RAW cells with Ch-DA for 12 hours

It is seen that both the control and test samples show similar band patterns suggesting that the probe didn't pick up any interactor proteins.

We also did a lipidomic analysis to confirm this and not very surprisingly, there wasn't any probe that got enriched in the phagosomes.

5) Use of methyl-β-cyclodextrin as a delivery vehicle for the probe in cell lysates

Use of Methyl-β-cyclodextrin (MBCD) to remove and supply cholesterol to the cells have been described in previous studies (Pucadyil and Chattopadhyay, 2004) (Christian et al., 1997).

Cyclodextrins could stabilize the interactions better with Hydrogen bonding and Van der Waals forces (Dai et al., 2022) and hence we assume it solubilize the probe better compared to DMSO. So we decided to use MBCD as a delivery vehicle for the probe.

The probe was solubilized in methyl-β-cyclodextrin and treated with RAW 264.7 cell lysates to check whether it is delivered efficiently and is bound to any of the proteins.

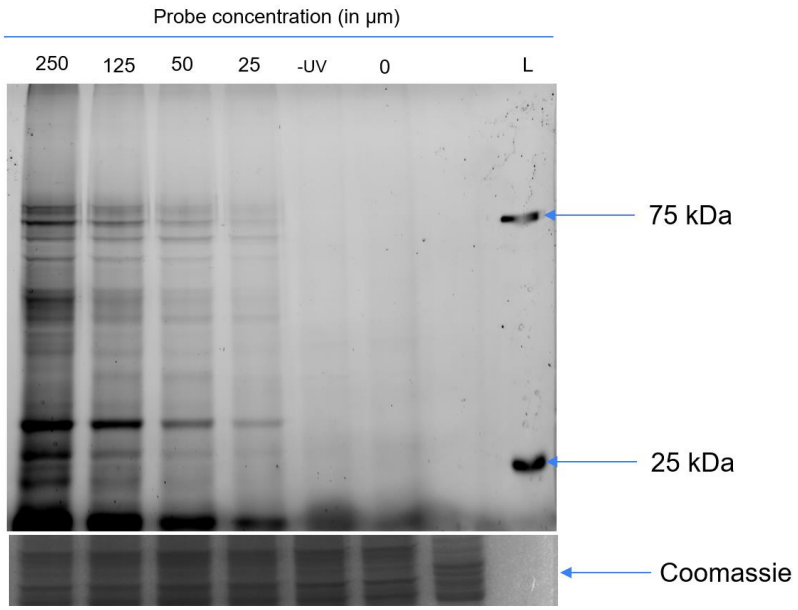

Figure 10: Gel based analysis of probe enrichment at varying concentrations

We could observe a concentration-based increase in the band number and the intensity. Thus we concluded that the probe is working efficiently in the lysates when dissolved in methyl-β-cyclodextrin when compared to that when dissolved in DMSO.

6) Treatment of EP and LP lysates with the probe dissolved in cyclodextrin

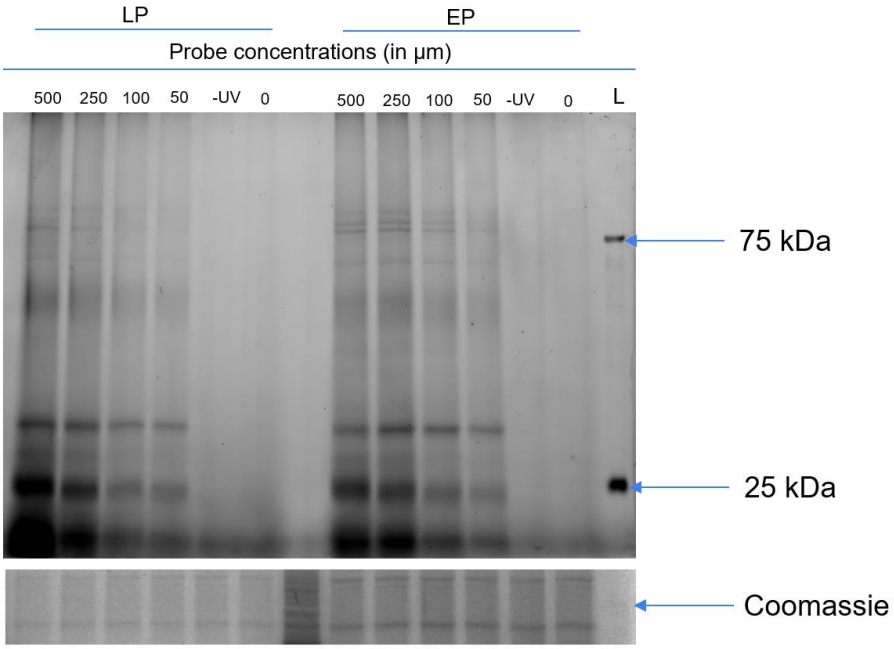

Figure 11: ABPP Gel image of EP and LP treated with different concentrations of the probe

7) Proteomics on RAW264.7 cell lysates with the cholesterol probe dissolved in cyclodextrin

Proteomic analysis of RAW264.7 cell lysates with the probe were done on 3 replicates and yielded around 600 different proteins on each replicate.

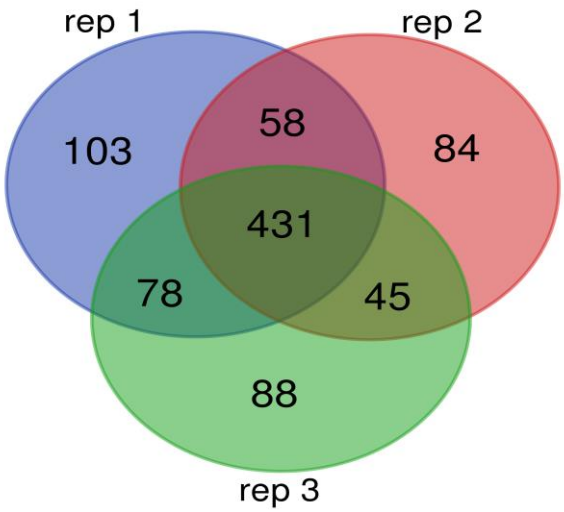

Figure 12: Venn diagram representing the number of proteins in each replicate and the number of proteins that are common between them

Of these, 114 proteins pass the filtering criteria are assumed to be of significantly enriched by the probe.

Table 7: List of proteins picked up in the proteomics on RAW 264.7 cell lysates

PROTEIN	AVERAGE H/L RATIO
ATP synthase subunit g, mitochondrial	26.64197246
sodium/potassium-transporting ATPase subunit beta-3 isoform 1	19.38658853
polypyrimidine tract-binding protein 1 isoform 2	15.16743906
glycerol-3-phosphate dehydrogenase, mitochondrial precursor	14.50631797
calnexin precursor	14.46671915
14-3-3 protein theta	14.28039217

voltage-dependent anion-selective channel protein 1 isoform 2	14.20220184
CD44 antigen isoform a precursor	13.67542203
core histone macro-H2A.1 isoform X2	13.39670006
matrin-3	13.35658916
formin-like protein 1 isoform X10	12.85387786
coiled-coil domain-containing protein 47 isoform X1	12.30593264
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	11.79339984
MICOS complex subunit Mic60 isoform 1	11.76399833
BRI3-binding protein isoform 1 precursor	7.54386584
endonuclease domain-containing 1 protein precursor	7.073102156
importin subunit alpha-1	6.337570111
translocon-associated protein subunit alpha isoform 1 precursor	6.220166604
cytochrome c oxidase subunit II (mitochondrion)	5.987428347
choline-phosphate cytidylyltransferase A	5.655307452
histone H3.2	5.488362312
voltage-dependent anion-selective channel protein 2	5.368106047
histone H1.4	5.267334223
inactive hydroxysteroid dehydrogenase-like protein 1 isoform X1	5.186450481
histone H4	5.138835907
heterogeneous nuclear ribonucleoprotein A3 isoform a	5.115072191
retinol dehydrogenase 11 isoform 1 precursor	5.017941674
serine palmitoyltransferase 1	4.644991875
H2b histone family, member A isoform 2	4.614576022
proteasome activator complex subunit 2 isoform 2	4.583018621
FAST kinase domain-containing protein 4 isoform X1	4.450492541
lamina-associated polypeptide 2 isoform delta	4.328813632
eukaryotic translation initiation factor 4 gamma 2 isoform 1	4.249057372
histone H1.2	4.247678359
60S ribosomal protein L11 isoform X1	3.951825698
transmembrane protein 199	3.93665576

high affinity immunoglobulin epsilon receptor subunit gamma precursor	3.920961062
nucleophosmin isoform 1	3.551891287
annexin A5	3.518858274
iron-sulfur clusters transporter ABCB7, mitochondrial	3.385100285
adenylyl cyclase-associated protein 1	3.343433698
26S proteasome non-ATPase regulatory subunit 12	3.341028372
eukaryotic translation initiation factor 4 gamma 1 isoform X5	3.310338736
CYFIP-related Rac1 interactor B isoform X5	3.267239094
ubiquitin-60S ribosomal protein L40 precursor	3.208501339
heterogeneous nuclear ribonucleoprotein U isoform X2	3.201315324
programmed cell death 6-interacting protein isoform X1	3.16785566
monofunctional C1-tetrahydrofolate synthase, mitochondrial precursor	3.152057787
signal recognition particle receptor subunit beta	3.142779986
monocarboxylate transporter 1	3.142380277
prosaposin isoform F	3.117827614
N-alpha-acetyltransferase 15, NatA auxiliary subunit	3.108634114
26S proteasome non-ATPase regulatory subunit 11	2.991622686
uncharacterized protein LOC105943584 isoform 1	2.908459584
cytochrome c oxidase assembly protein COX15 homolog	2.904956659
proteasome activator complex subunit 1	2.89806064
proline-, glutamic acid- and leucine-rich protein 1	2.863841613
basic leucine zipper and W2 domain-containing protein 1	2.823296984
mitochondrial 2-oxoglutarate/malate carrier protein isoform X1	2.747670333
very-long-chain 3-oxoacyl-CoA reductase	2.710855285
40S ribosomal protein SA	2.703776638
C-1-tetrahydrofolate synthase, cytoplasmic	2.688517054
basic leucine zipper and W2 domain-containing protein 2	2.686272979
poly(rC)-binding protein 2 isoform X15	2.66210103
voltage-dependent anion-selective channel protein 3 isoform 2	2.646078984
60 kDa heat shock protein, mitochondrial	2.599053383

60S acidic ribosomal protein P0	2.486445665
lactadherin isoform 1 precursor	2.481329997
dual specificity mitogen-activated protein kinase kinase 1	2.47590061
ubiquitin-conjugating enzyme E2 N isoform X1	2.455822706
heterogeneous nuclear ribonucleoprotein M isoform a	2.449239055
phosphatidylinositol transfer protein beta isoform isoform 2	2.437893033
immunity-related GTPase family M protein 1 isoform X1	2.415858666
interferon-induced transmembrane protein 3	2.350708723
ras-related protein Rab-7a	2.34549125
40S ribosomal protein S3	2.341048002
poly(rC)-binding protein 1	2.330576658
elongation factor 1-alpha 1	2.289710999
60S ribosomal protein L35	2.288634062
phosphoglycerate kinase 1	2.282212337
phosphate carrier protein, mitochondrial precursor	2.27779603
SUMO-activating enzyme subunit 2	2.259133657
neutrophil cytosol factor 2	2.227934361
signal transducer and activator of transcription 1 isoform 2	2.208251595
elongation factor Tu, mitochondrial isoform 1	2.169234673
T-complex protein 1 subunit delta	2.146755377
glyceraldehyde-3-phosphate dehydrogenase isoform 2	2.119626681
rRNA 2'-O-methyltransferase fibrillarin	2.102125963
heme oxygenase 2 isoform 1	2.095663667
surfeit locus protein 4	2.084334334
nucleoporin NDC1	2.073474328
ATP synthase subunit alpha, mitochondrial precursor	2.062098026
electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial precursor	2.054960648
ADP-dependent glucokinase isoform 1 precursor	2.005572995
receptor-interacting serine/threonine-protein kinase 3 isoform 1	24.50001597
interleukin enhancer-binding factor 2	22.22582746
14-3-3 protein beta/alpha isoform X1	19.27589035
signal recognition particle receptor subunit alpha	4.373523891

heterogeneous nuclear ribonucleoprotein K isoform 4	3.385870337
sorting nexin-5	2.080916524
translin	37.51805496
reticulon-4 isoform A	24.84822655
ruvB-like 1	16.86603877
serum paraoxonase/lactonase 3 isoform 1 precursor	4.169614017
ras-related protein Rab-1B	4.061974049
U2 snRNP-associated SURP motif-containing protein isoform 1	3.756093025
annexin A1	2.974180877
cathepsin B preproprotein	2.651533604
tubulin alpha-1C chain	2.428536057
eukaryotic translation initiation factor 5A-1 isoform X1	2.418888032
histone H2A type 2-C	2.149825573
dynamin-1-like protein isoform a	18.06655878
sorting and assembly machinery component 50 homolog	3.198427975
cytochrome b-245 light chain isoform 1	2.901356936
protein FAM162A	2.04964149

Then we looked at whether any sterol-binding proteins were picked up in the list, and we found a few previously picked up as cholesterol binders. One such protein was Inactive hydroxysteroid dehydrogenase-like protein 1, a mitochondrial protein belonging to the short-chain reductase protein family. It is seen to exhibits steroid dehydrogenase activity (Meier et al., 2009).

Apart from this protein we also picked up three proteins that were found to be interactors of cholesterol by Benjamin. F. Cravatt and co. (Hulce et al., 2013) which are:

- Calnexin precursor
- Ras-related protein Rab 1B
- Surfeit locus protein

CHAPTER 4 Discussion

The major objective of my project was to identify the role of cholesterol in phagosomal maturation and phagocytosis in general. Towards achieving this goal, our main aim was to identify the proteins that are interacting with cholesterol in phagosomes. The role of cholesterol in the uptake of microorganisms by the cells has been studied previously by many groups. The next aim was to identify the proteins to which cholesterol interacts.

Chemical probes that resemble the parent lipids are used to achieve this aim. This method could miss out on some of the interacting proteins of the lipid due to modifications like the diazirine ring or the attached alkyne group. This can be better resolved by appending the modifications to a different location in the compound.

The first step is to treat the probe to lysates, test the probe's working, and standardize the probe's working concentration in the lysate. The SDS PAGE gels showed that the probe is not working efficiently in lysates. This could be due to the defective delivery mode. Hence, we decided to change the mode of delivery of the probe from directly treating them to lysates to feeding them to growing cells, allowing them to take up the probe by cellular mechanisms like endocytosis. Incubating the probe at a fixed concentration and for various durations, we concluded that the time for which the probe should be incubated is 12 hours before performing any assays as it showed the maximum intensity of the bands in the SDS gel.

But the phagosomes purified from the cells treated with the probe for 12 hours doesn’t enrich the probe in it. This was confirmed using lipidomic assays in which the probe was not detected in phagosomes and in the cells, only a very few percentages of the treated probe were detected. This brought us to the thought that there could be a problem either with the delivery vehicle or with the ionization of the probe in the mass spec. Changing the delivery vehicle from DMSO to methyl-β-cyclodextrin provided much better results in the case of cell lysates in just 30 minutes of incubation. This is because the cyclodextrins are fairly larger compounds and have a hydrophilic outer surface and a cavity inside which is hydrophobic. This could better encapsulate the cholesterol probe with the help of Van der Waal’s interactions in a much better way compared to DMSO. Then, we see a concentration-based increase

in the band intensity and number to 250μM of the probe in RAW264.7 cell lysates. Similar observations are seen in phagosomal lysates as well. So we concluded that the probe would bind to the proteins efficiently at 250μM concentration. This was confirmed using Mass spec-based proteomics experiments, where we observed a few proteins getting picked up that are known to bind to cholesterol or such sterols or that were picked up in screens done previously using a similar probe (Hulce et al., 2013).

Contradicting our assumptions and conclusions from the Gel-based analyses, the MS-based proteomics didn't yield any proteins when the phagosomal lysates were treated with the probe. Since the probe has picked up the proteins in the SDS PAGE assay, it is unlikely that it didn't interact with any of the proteins in the phagosomal lysates. This could be a one-time incident where there could be an issue with the instrument, or the beads might not have efficiently bound to the proteins, and hence, no proteins would have been pulled down during the assay. More evidence needed to be corroborated to conclude at which step the error occurred.

Once this is resolved, we could find the potential protein interactors of cholesterol and pick up the proteins that actually are the interactors of cholesterol using competitive studies where we could preincubate the phagosomal lysates with cholesterol, then treat the probe and do a mass spec-based analysis to identify and eliminate those proteins that are showing up in the results. This could be a subset of the proteins that were picked up in the proteomics of phagosomes and hence narrow down the list to more specific binders of cholesterol.

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