Chapter 7

Mass Spectrometric analysis of *in vitro* models of Macrophage subtypes

Mass spectrometry (MS) has emerged as a powerful analytical tool for studying macrophage subtypes, enabling detailed proteomic, lipidomic, and metabolomic profiling to distinguish between different macrophage phenotypes, such as M1 (pro-inflammatory), M2 (anti-inflammatory), and disease-associated macrophages. Several studies used mass spectrometry-based proteomics in elucidating the complex protein expression landscapes of macrophage subtypes from induced pluripotent stem cells (iPSCs) under different polarization conditions (Murugesan et al., 2022) or from macrophages differentiated from primary cutures (Oates et al., 2023). These studies only included M1 and M2 subtypes. Kratz et al., demonstrated only membrane proteomics of M1, M2 and MMe derived from primary cultures. We, therefore, ran an untargeted proteomics using MS/MS to study proteins profile of these macrophage subtypes.

7.1 Mass spectrometric analysis (Untargeted Proteomics) of *in vitro* macrophage subtypes

Adipose tissue macrophages (ATMs) exhibit distinctive features that differentiate them from conventional M1 and M2 macrophages. In our *in vitro* model of MMe, we have successfully replicated the characteristics associated with ATMs, aligning with findings from previous studies. In an effort to examine deeper into the unique attributes and distinctions among these macrophage subtypes, we conducted mass spectrometry analysis. We found 2059 proteins in THP-1, 1661 in M1, 1571 in M2 and 1833 in MMe. We used venny 2.0 to find the common and unique protein list among these cells. The results revealed a set of 421, 488, and 583 proteins that were expressed exclusively in M1, M2, and MMe, respectively (Fig 7.1).

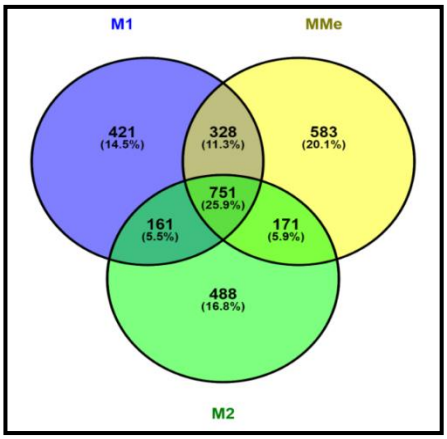

Figure 7.1 Venn diagram for overlapping and exclusive proteins among different macrophage subtypes

The analysis of unique proteins present in each subtype was conducted for further investigation. The Bioplanet database was employed to explore the pathways enriched by these distinctive proteins. This analytical approach aimed to unravel specific molecular pathways associated with each macrophage subtype, offering insights into the distinct functionalities and characteristics that define M1, M2, and MMe.

MMe Macrophages: The pathway enrichment analysis of MMe showed upregulation of several pathways, highlighting here top 10, which include Oxidative stress-induced gene expression via Nrf2, Heme degradation, Beta-oxidation of very long chain fatty acids, omega3 and omega6 acid metabolism, etc (Fig 7.2). Obesity is known to be associated with oxidative stress, and Nrf2 serves as a transcription factor that plays a crucial role in regulating the cellular response to oxidative stress while inhibiting the expression of inflammatory cytokines (Kobayashi et al., 2016). The adaptive pathway to oxidative stress via Nrf2 is significantly upregulated in MMe. Within this pathway, key components such as Hmox1, MafF, and MafG are found to be upregulated in MMe (Fig 7.3). Maf proteins are essential for maintaining redox homeostasis by forming heterodimers with Nrf2 (Itoh et al., 1997). This heterodimeric complex activates antioxidant genes, including Hmox1 (Hirotsu et al., 2012). Hmox1, in turn, contributes to heme degradation, leading to the generation of antioxidant molecules and exerting anti-inflammatory effects (Loboda et al., 2016). The enrichment of the heme degradation pathway further supports the involvement of Hmox1 in the oxidative stress response within the context of MMe. This indicates that MMe adapts to oxidative stress and potentially modulates inflammation in the context of obesity. Additionally, the analysis revealed enrichment in peroxisomal lipid metabolism. This aligns with previous findings mentioned earlier, where MMe demonstrated an upregulation of Acox1, indicating an active involvement of peroxisomes in lipid metabolism (Ch 5, fig 5.1 H, I). Acox1 is also involved in omega 3 and omega 6 metabolism. Omega 3 is inversely linked to CRP and reduces the risk of CVD, perhaps by maintaining anti-inflammatory state (Micallef et al., 2009). A balanced proportion of omega 3 and omega 6 is also an important metabolic factor in obesity and its management (Simopoulos, 2016) (Fig 7.2, 7.3).

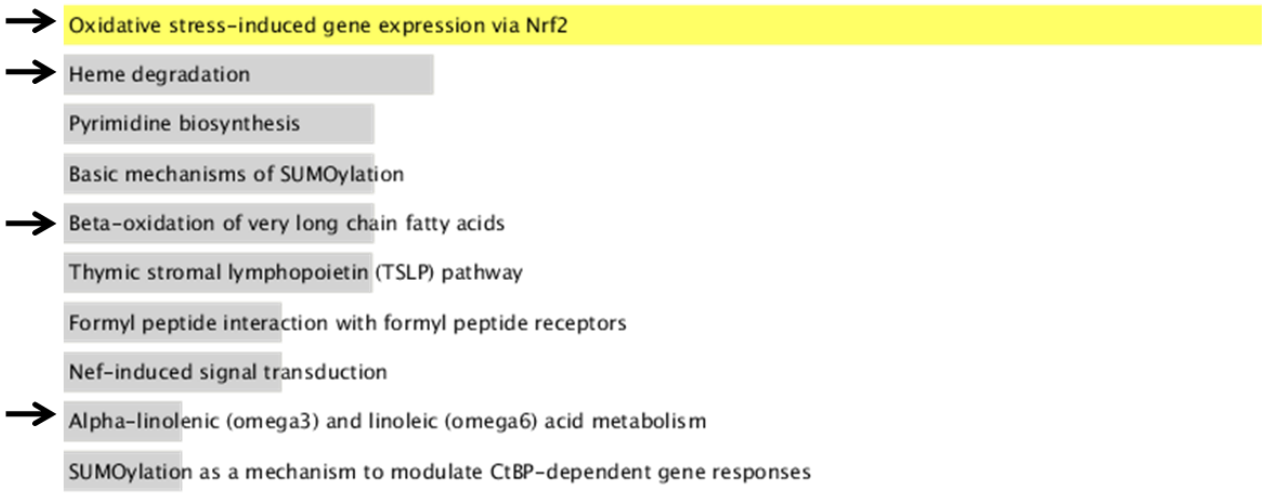

Figure 7.2 Bar graph of top pathways enriched in MMe

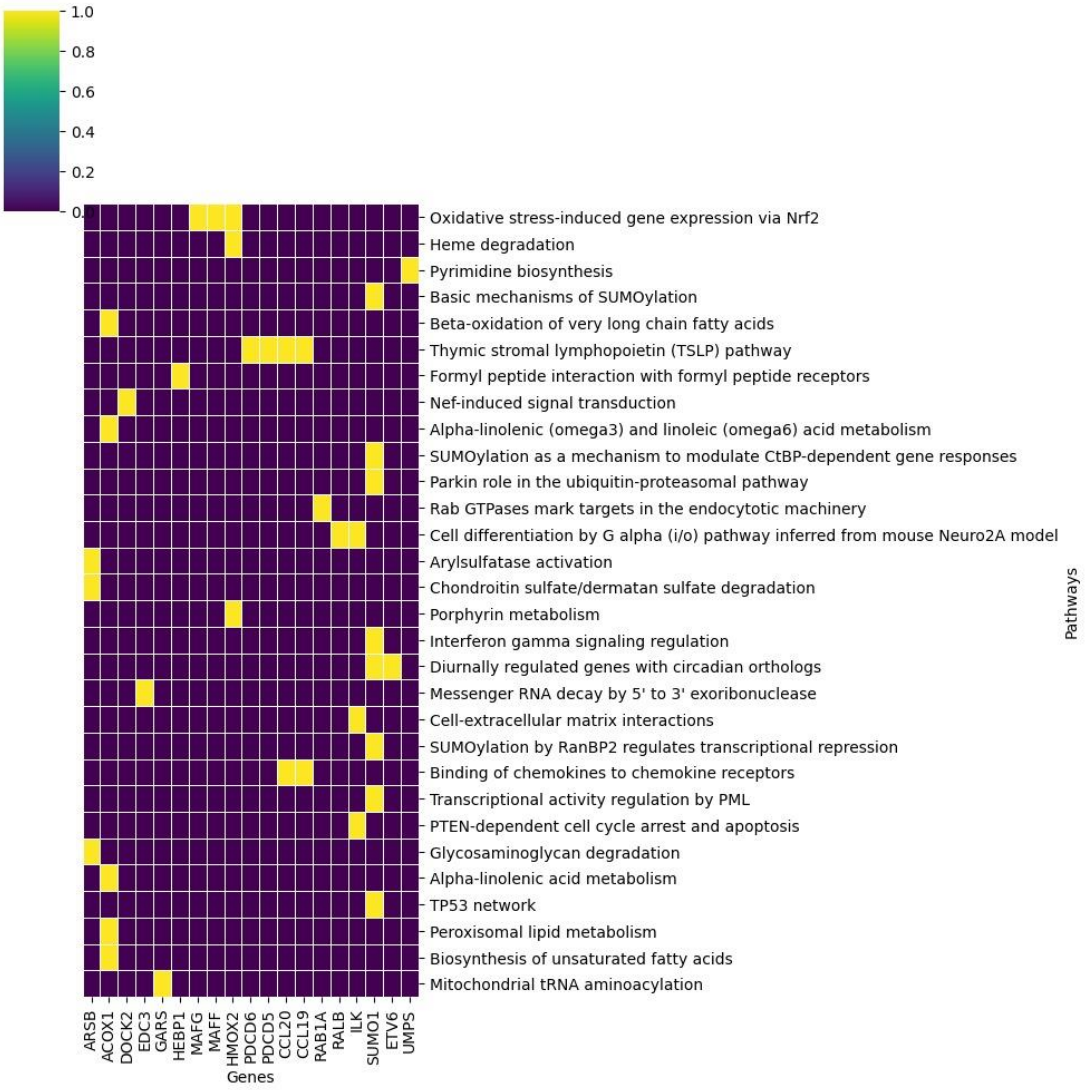

Figure 7.3 Clustergram based on input genes and enriched pathways in MMe

M1 Macrophages: Consistent with our experimental data, the enrichment analysis of M1 proteins reveals upregulation of inflammatory pathways, prominently featuring IFN signalling, IL23-mediated signalling and IL12-mediated signalling. Key proteins implicated in these pathways include IFNγ, CXCL9, STAT1, IRF1, and GBP1. IFNs, such as IFNγ, activate the transcription factor STAT1, leading to the induction of M1 marker genes (Fig 7.4, 7.5). For instance, IRF5, a regulator of IL12 expression, is influenced by STAT1 activation (Tugal et al., 2013). GBP1, strongly induced by IFNγ, plays a role in restraining cellular proliferation in inflammatory contexts (Johns & Galam, 2022). Additionally, our analysis suggests the modulation of the glucocorticoid receptor pathway through GSK3β and EGR1, with IFNγ regulating the expression of Toll-like receptor (TLR) inducible genes via GSK3 (Hu & Ivashkiv, 2009). Indeed, EGR1 (Early Growth Response 1) is a transcriptional target of STAT1 (Schiavone et al., 2011). The activation of STAT1 often leads to IFNγ signalling. These findings provide valuable insights into the intricate molecular landscape of M1 macrophages and their inflammatory responses.

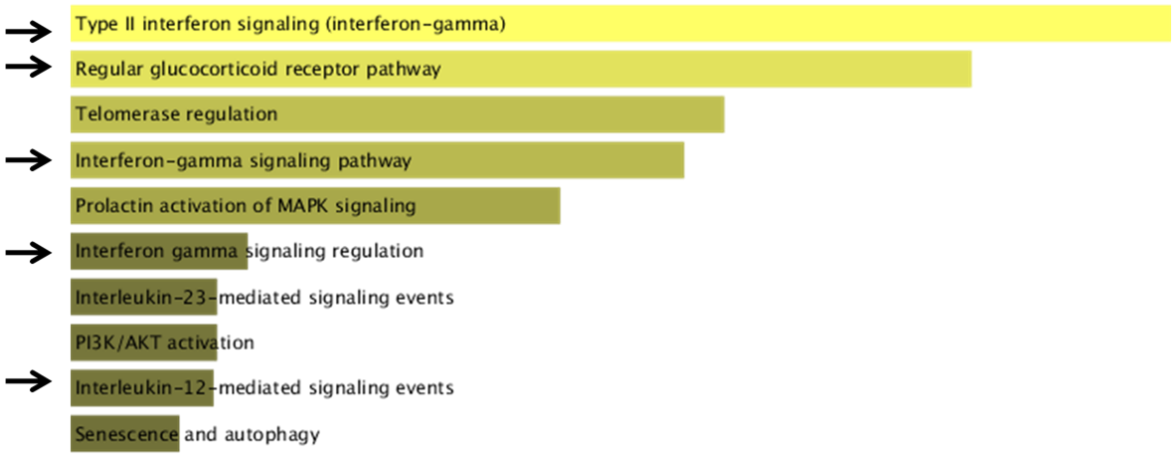

Figure 7.4 Bar graph of top pathways enriched in M1

M2 Macrophages: The pathway enrichment analysis of exclusive proteins expressed in M2 indicates its anti-inflammatory property and tissue-repairing properties. The presence of ARSA, SUMF2, ATF1, SLP1, etc are involved in different pathways like arylsulfatse activation, CREB phosphorylation, proepithilin conversion, etc, indicating M2 macrophage characteristics (Fig 7.6, 7.7). The activation of arylsulfatases, including ARSA and SUMF2, and their involvement in glycosphingolipid metabolism suggest a distinct metabolic profile in M2 macrophages. ARSA, despite being a lysosomal enzyme, exhibits hydrolytic activity

and plays a crucial role in maintaining an anti-inflammatory state (Mitsunaga-Nakatsubo et al., 2009). SUMF2, whose expression correlates with IL13, is implicated in anti-inflammatory functions (Fang et al., 2015). Additionally, the enrichment of the hypusine formation pathway in M2 macrophages is noteworthy. Hypusinated eIF5A is essential for macrophage alternative activation, promoting TCA cycle and oxidative phosphorylation (Puleston et al., 2019).

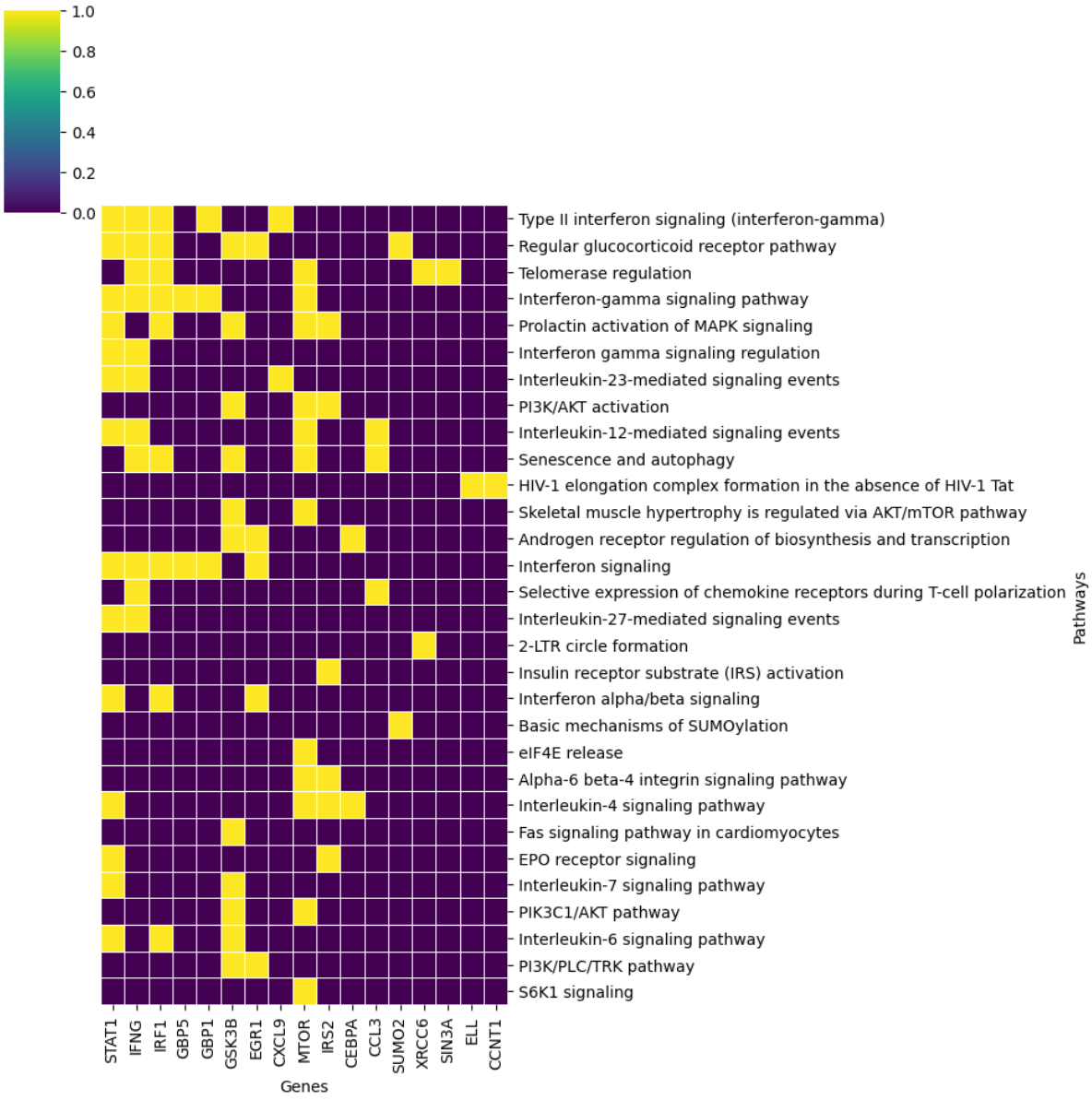

Figure 7.5 Clustergram based on input genes and enriched pathways in M1

ATF1 involvement in CREB phosphorylation is significant for tissue repair processes (Ruffell et al., 2009). The expression of SLPI (Secretory Leukocyte Protease Inhibitor) in

M2 macrophages is notable, as it forms a complex with PEPI to inhibit its conversion, thereby suppressing neutrophil and macrophage activation and promoting wound healing (Zhu et al., 2002). These pathways and molecules collectively contribute to the anti-inflammatory and tissue repair functions associated with M2 macrophages.

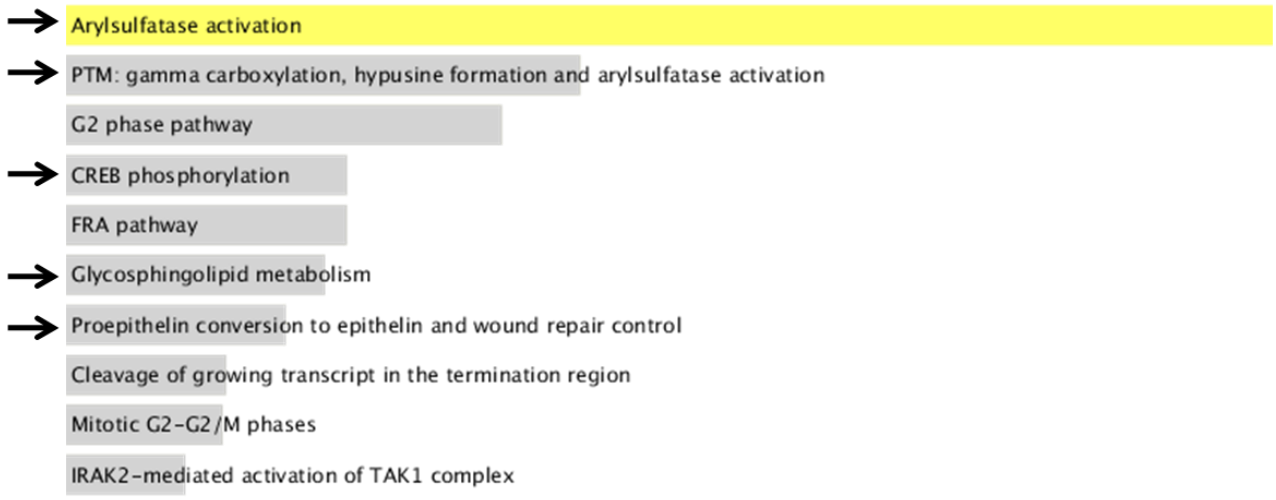

Figure 7.6 Bar graph of top pathways enriched in M2

7.2 Protein-protein interaction network analysis

The protein-protein interaction network analysis is performed to investigate the functional relationships among proteins of interest. Here, we use a STRING database for a comprehensive view of how proteins interact within these macrophage subtypes. Functional enrichment analysis of these networks can provide insights into biological pathways and disease mechanisms.

MMe Macrophages: The top protein-protein interactions with the highest combined scores (close to 1.0, indicating high confidence) include (Fig 7.8):

VPS4A and VTA1: Involved in endosomal sorting and membrane trafficking.

LSM8 and PRPF31: Associated with RNA splicing and mRNA processing.

POMP and PSMB3: Related to proteasome assembly and protein degradation.

GART and MTHFD1: Key players in folate metabolism and nucleotide synthesis.

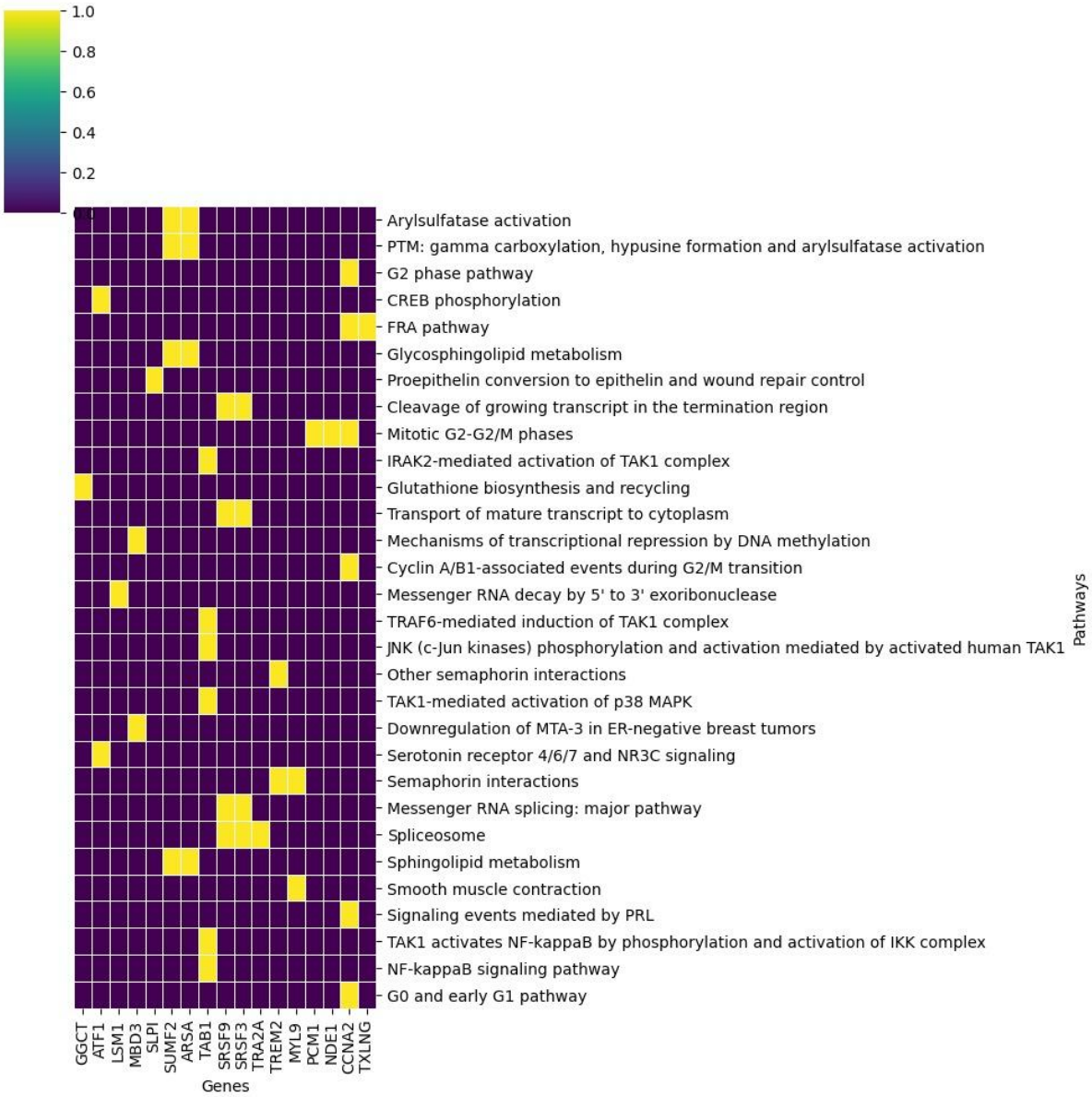

Figure 7.7 Clustergram based on input genes and enriched pathways in M2

BUD31 and SNRPA1: Involved in the regulation of pre-mRNA splicing.

Next, mapping these proteins to their respective molecular pathways, associated with the top proteins:

VPS4A and VTA1: Involved in endosomal sorting and membrane trafficking.

LSM8 and PRPF31: Associated with RNA splicing and mRNA processing.

POMP, PSMB3, PSMB2, and PSMA4: Participate in proteasome assembly and protein degradation.

GART and MTHFD1: Play a role in folate metabolism and nucleotide synthesis.

BUD31 and SNRPA1: Regulate pre-mRNA splicing.

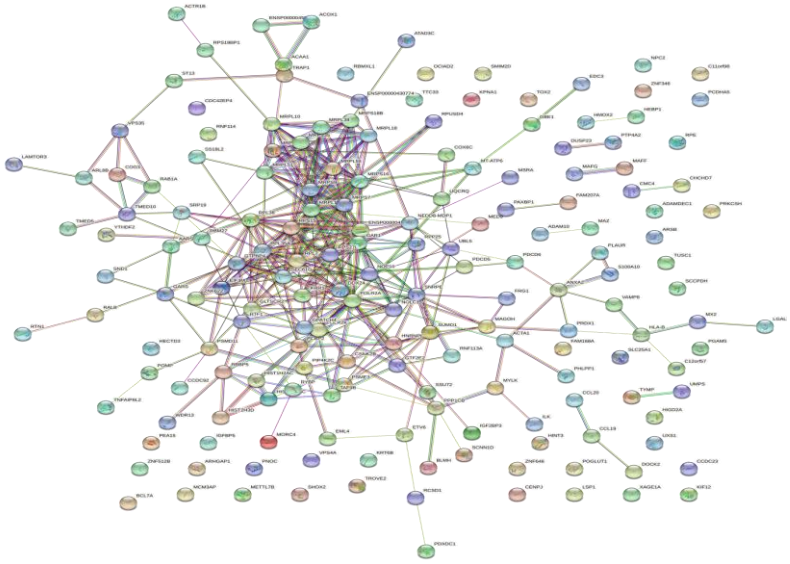

Figure 7.8 PPI network analysis of MMe

M1 Macrophages: The top 10 proteins with the most interactions in the network are (Fig 7.9):

STAT1 - 19 interactions

RPS16 - 14 interactions

RPS2 - 14 interactions

IRF1 - 11 interactions

ISG15 - 11 interactions

CD74 - 11 interactions

IFNG - 11 interactions

RPL35A - 9 interactions

MRPL13 - 9 interactions

FOS - 9 interactions

Mapping these proteins to their respective molecular pathways using established pathway annotations

The top proteins are involved in the following molecular pathways:

STAT1: JAK-STAT signaling pathway, Interferon gamma signaling

RPS16: Ribosome biogenesis, Translation

RPS2: Ribosome biogenesis, Translation

IRF1: Cytokine signalling, Interferon response

ISG15: Ubiquitin-like modification, Antiviral response

CD74: Antigen processing and presentation

IFNG: Interferon gamma signalling, Immune response

RPL35A: Translation, Ribosomal function

MRPL13: Mitochondrial translation

FOS: MAPK signalling pathway, Cell proliferation

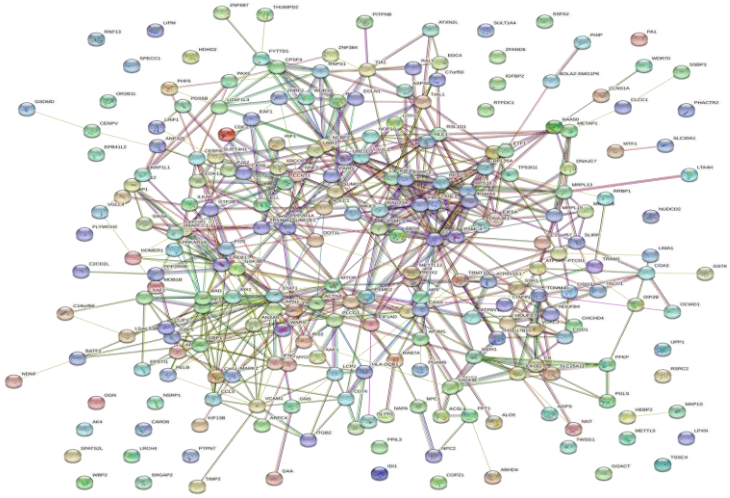

Figure 7.9 PPI network analysis of M1

M2 Macrophages: The top 10 proteins with the most interactions in the network are (Fig 7.10):

HSPA8 - 38 interactions

RPS3 - 37 interactions

RPS20 - 35 interactions

PABPC1 - 35 interactions

RACK1 - 33 interactions

RBM39 - 31 interactions

RPL5 - 30 interactions

RPS13 - 28 interactions

RPS15A - 27 interactions

RPL18 - 25 interactions

However, these proteins play crucial roles in various cellular processes:

HSPA8: A member of the heat shock protein family, involved in protein folding and protection against stress.

PABPC1: Binds to the poly(A) tail of mRNA, influencing mRNA stability and translation.

RACK1: Functions as a scaffold protein, participating in various signaling pathways.

RBM39: Involved in RNA splicing and gene expression regulation.

RPL5, **RPS20**, and **RPS15A**: Components of ribosomal subunits, essential for protein synthesis.

Discussion:

The data strongly suggests that MMe exhibits distinct characteristics compared to M1 and M2 macrophages, although further experimental validation is required. The pathogen-killing

activity of M1 macrophages is prominently mediated by IFN signalling through the JAK-STAT pathway and activation of STAT1 (Hu & Ivashkiv, 2009). GBP1 (Guanylate-binding protein 1) is indeed highly expressed in several cell types, including macrophages, endothelial cells, and epithelial cells. Its induction is facilitated by IFNγ, a cytokine associated with immune responses. In the context of macrophages, GBP1 plays a crucial role in the cellular response to infections. It has been observed that GBP1 can bind to LPS, a component of the outer membrane of certain bacteria, and promotes the recruitment of other guanylate-binding proteins such as GBP2, GBP3, and GBP4. This recruitment of GBP proteins, in turn, activates caspase-4, ultimately leading to inflammation and pyroptosis (Johns & Galam, 2022).

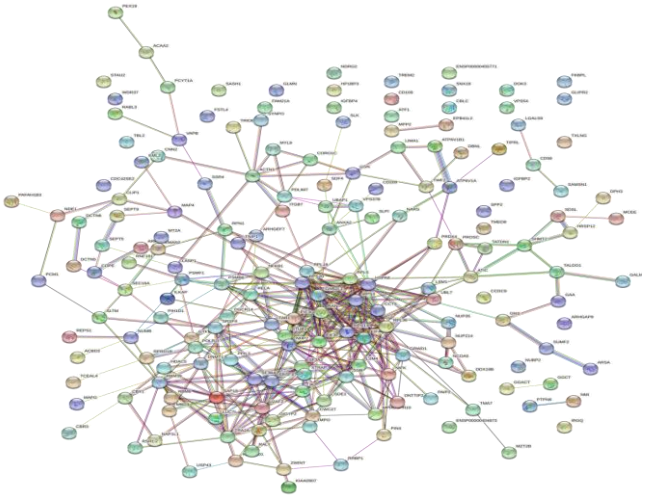

Figure 7.10 PPI network analysis of M2

IFNγ, a crucial mediator of immunity and inflammation, utilizes GSK3β to regulate TLR-inducible genes while suppressing PI3K/Akt signalling. GSK3α/β, known for its role in glycogen synthesis/metabolism, also attenuates the expression of genes governing lipid and cholesterol biosynthesis. Additionally, GSK3β in ER stress signalling, dependent on the PERK branch of UPR, modulates downstream pathways involved in apoptosis and lipid accumulation (Patel & Werstuck, 2021). Indeed, our experimental data aligns well with these bioinformatics predictions. Additionally, GSK3β in ER stress signalling, dependent on the PERK branch of UPR, modulates downstream pathways involved in apoptosis and lipid accumulation (Patel & Werstuck, 2021). Indeed, our experimental data aligns well with these bioinformatics predictions. We find that M1 macrophages are classically activated and known for their pro-inflammatory phenotype. They secrete inflammatory cytokines, such as IFNγ, and exhibit upregulated glycolysis as part of their metabolic profile. This upregulates

ER stress specifically PERK pathway. This highlights how these genes not only activate inflammatory pathways but also modulate metabolism to support an inflammatory state.

On the other hand, analysis indicates the unique metabolic and anti-inflammatory characteristics observed in MMe. The upregulation of Nrf2 activity in MMe aligns with its role in combating oxidative stress and inflammation (Kobayashi et al., 2016). The association between Nrf2 deficiency and exacerbated oxidative stress in obesity is well-established (Tarantini et al., 2018). Nrf2 opposes transcriptional regulation of pro-inflammatory cytokine genes via redox dependent and independent pathways. We have also observed that MMe express less cytokines than M1 (Kobayashi et al., 2016). This indicates that enhanced Nrf2 activity in MMe a potential adaptive response to counteract the oxidative stress and inflammation associated with obesity.

Nrf2 accumulates in nuclei upon exposure to oxidative stress along with small Maf proteins and activate the transcription of stress target genes through ARE. MafG itself is an ARE-dependent gene controlled by Nrf2 (Katsuoka et al., 2005). An Nrf2/small Maf heterodimer mediated induction of antioxidant genes via ARE that includes GCL, Txnrd1, NQO1 and Hmox1 (Loboda et al., 2016). Genetic polymorphism of Nrf2 and its downstream targets NQO1, Hmox1 and NT have been associated with coronary artery and risk factor for CVD (Sarutipaiboon et al., 2020). Hmox1 is also upregulated by heme, NO, heavy metals, growth factors, cytokines and modified lipids (Loboda et al., 2016). Hmox-1 is a marker of iron excess induced AT dysfunction (Jais et al., 2014). Impaired iron handling has seen observed in obese mice ATMs (Orr et al., 2014). Increased Hmox1 in obese individuals and positively associated with obesity metabolic disturbances while negatively associated with insulin signalling and adipogenesis (Moreno-Navarrete et al., 2017). HO1 is evident in inflammatory macrophage crown like structure. Macrophage Hmox-1 KO mice resist metabolic disease. Hmox-1 loss decreased metainflammtion and improved AT architecture and increased adiponectin while blunted NF-κB (Jais et al., 2014).

The involvement of peroxisomal lipid metabolism, particularly the upregulation of Acox1, adds another layer to the metabolic complexity of MMe. Further, involvement of Acox1 in omega 3 and omega 6 metabolism also indicates unique metabolic features of MMe. Omega3 is known to have anti-inflammatory properties and has hypoglycemic and insulin sensitizing effect (Egalini et al., 2023). Thus, MMe display unique characters of inflammation and metabolism, which important in adipose tissue dysfunction. The

maintenance of an anti-inflammatory state by M2 macrophages is a crucial aspect of their functional role. The involvement of ARSA (Arylsulfatase A) and SUMF2 (Sulfatase Modifying Factor 2) proteins in arylsulfatase activation, hypusine formation, and glycosphingolipid metabolism highlights their versatile roles in cellular processes. ARSA is known to be present on the surface of sinusoidal endothelial cells, hepatocytes, and macrophages (Mitsunaga-Nakatsubo et al., 2009). Arylsulfatase A is involved in the hydrolysis of sulfate esters from sulfatides (Hess et al., 1996). SUMF2, on the other hand, plays a role in modifying sulfatases, including ARSA. This modification is crucial for the proper functioning of sulfatases involved in glycosaminoglycan degradation (Fang et al., 2015) implicating its role in the extracellular matrix modulation. Additionally, SUMF2's involvement in hypusine formation highlights its contribution to protein synthesis and cellular processes. The presence and activities of ARSA and SUMF2 in M2 macrophages align with their anti-inflammatory phenotype (Fang et al., 2015; Mitsunaga-Nakatsubo et al., 2009). These proteins not only contribute to the regulation of lipid metabolism but also participate in processes essential for maintaining cellular homeostasis. CREB phosphorylation plays a crucial role in shaping the M2 macrophage phenotype by inhibiting NF-κB-p65 nuclear localization, thereby restricting inflammation (Leopold Wager et al., 2023; Ruffell et al., 2009). This regulatory mechanism is vital for maintaining an anti-inflammatory state in M2 macrophages. In the absence of CREB binding sites or when CREB phosphorylation is disrupted, the normal activation and induction of M2-specific genes are compromised. Thus, CREB phosphorylation plays a crucial regulatory role in influencing macrophage behaviour and immune responses (Ruffell et al., 2009).

The involvement of M2 macrophages in wound healing is further exemplified by the significant roles of SLPI. Administration of recombinant SLPI (rSLPI) has been shown to increase the expression of CD163 and FGF2 while concurrently decreasing the expression of pro-inflammatory cytokines such as IL-1 and IL-6 (Munadziroh et al., 2022). This modulation accelerates wound healing in animal models, as observed in Wistar mice (Munadziroh et al., 2022). Moreover, SLPI exerts anti-inflammatory actions by suppressing inducible nitric oxide (NO) and TNFα production (Aihao Ding et al., 1999). Studies with SLPI knockout mice demonstrated increased susceptibility to endotoxin shock induced by LPS (Nakamura et al., 2003). SLPI has also been implicated in attenuating inflammation during the clearance of apoptotic cells, where it suppresses TNFα production (Odaka et al., 2003). These findings highlight the anti-inflammatory properties of SLPI in M2

macrophages, emphasizing their contribution to wound healing and immune homeostasis. This PPI network provides a view of protein interactions, shedding light on the molecular mechanisms underlying inflammation, metabolism, and cellular stress responses.